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RESEARCH ADVANCES IN THE STUDY OF CAMPYLOBACTER, HELICOBACTER & RELATED ORGANISMS

Topic Editors
D Scott Merrell and Alain Stintzi

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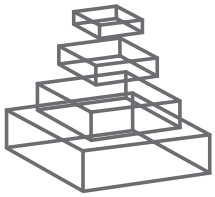
CELLULAR AND INFECTION MICROBIOLOGY



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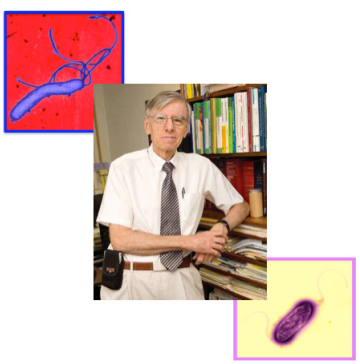
RESEARCH ADVANCES IN THE STUDY OF CAMPYLOBACTER, HELICOBACTER & RELATED ORGANISMS

Topic Editors:
D Scott Merrell, Uniformed Services University, Dept. of Microbiology and Immunology, USA
Alain Stintzi, Ottawa Institute of Systems Biology, Canada



Falsely colored images of *Helicobacter pylori* and *Campylobacter jejuni* were kindly provided by Drs. Lucinda Thompson and Patricia Guerry, respectively. *H. pylori* is shown in the upper left hand corner and *C. jejuni* on the bottom right corner. Dr. Andre Dubois, to whom this E-book is dedicated, is pictured in the center.

Campylobacter spp and Helicobacter spp are gastrointestinal pathogens that remain a major cause of acute gastroenteritis and gastric disease, respectively. The 16th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO) will be held in Vancouver, British Columbia Canada from August 28-September 1, 2011 and will highlight recent advances in our understanding of the epidemiology, survival mechanisms, host response and pathogenesis of these important species. This Research Topic issue will highlight each of these topics and will attempt to shed insight into our growing understanding of the process of host-pathogen interactions as it relates to Campylobacter and Helicobacter.

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EDITORIAL
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Research advances in the study of Campylobacter, Helicobacter, and Related Organisms

D. Scott Merrell*[1]* *and Alain Stintzi*[2]

[1] *Department of Microbiology and Immunology, Uniformed Services University, Bethesda, MD, USA*
[2] *Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada*
**Correspondence: douglas.merrell@usuhs.edu; astintzi@uottawa.ca*

Edited by:
Yousef Abu Kwaik, University of Louisville School of Medicine, USA

Reviewed by:
Yousef Abu Kwaik, University of Louisville School of Medicine, USA

Campylobacter spp. and Helicobacter spp. are gastrointestinal pathogens that remain a major cause of acute gastroenteritis and gastric disease, respectively. The 16th International Workshop on Campylobacter, Helicobacter and Related Organisms (CHRO) was organized by Erin Gaynor and Christine Szymanski and was held in Vancouver, BC, Canada from August 28 to September 1, 2011. This meeting highlighted recent advances in our understanding of the epidemiology, survival mechanisms, host response, and pathogenesis of these important species. This Research Topic issue highlights each of these topics and attempts to shed insight into our growing understanding of the process of host-pathogen interactions as it relates to Campylobacter and Helicobacter. We wish to dedicate this Research Topic to Dr. Andre Dubois, who passed away unexpectedly on June 30, 2012. We are honored that one of his research articles appears in the issue and note that his loss is deeply felt by our community.

A substantive overview of the CHRO meeting is provided by Drs. Gaynor and Szymanski (2012) and the articles within this Research Topic are broadly divided into those dealing with Campylobacter and then Helicobacter. Those articles related to Helicobacter can broadly be divided into three major topics: those that address *H. pylori* lifestyle and biological processes, those that address *H. pylori* virulence factors, and those that address interaction of *H. pylori* with the host.

Within the *H. pylori* lifestyle and biological processes, studies from the Dubois lab investigate the mechanism by which *H. pylori* enters host cells (Liu et al., 2012). Though primarily considered an extracellular pathogen, it is clear that a fraction of *H. pylori* cells enter and survive within the host cell. The Dubois team presents evidence that NudA is important in this process. Next, Liechti and Goldberg review the process of membrane biogenesis in *H. pylori* and compare it to the processes that have been elucidated in *Escherichia coli* and *Neisseria meningitidis*; not surprisingly, *H. pylori* often does not follow the paradigms established in these model systems (Liechti and Goldberg, 2012). Finally, Pernitzsch and Sharma discuss transcriptome complexity and riboregulation in *H. pylori* (Pernitzsch and Sharma, 2012). It has only recently become evident that *H. pylori* employs post-transcriptional regulation and small regulatory RNAs (sRNAs) as a mechanism of gene regulation. This fact directly conflicts the previous dogma that life within the singular niche of the host stomach has led to *H. pylori*'s loss of complex gene regulation.

Among *H. pylori* virulence factors, CagA, and VacA are undoubtedly the best studied. As such, a number of articles are devoted to these important factors. The Solnick group presents evidence that expression of genes on the *H. pylori cag* pathogenicity island, which encodes for CagA, varies significantly, and that the organization of the genes into transcriptional units is conserved among several *H. pylori* strains (Ta et al., 2012). In terms of CagA delivery, the Backert group investigates sequences/domains within the CagL protein that are important for interaction with integrins and subsequent injection of CagA into host cells (Conradi et al., 2012). Next, the Guillemin group describes the utilization of a novel Drosophila system to identify host components that affect CagA activity within host cells (Reid et al., 2012). Finally, Kim and Blanke review the role of the VacA toxin in modulation of the gastric epithelium and discuss the understudied area of VacA and CagA interaction (Kim and Blanke, 2012).

The final major topic addressed among the *H. pylori* manuscripts is the consequences of *H. pylori*-host cell interaction. From the bacterial perspective, the Cover group specifically discuss what we currently know about how host cell contact alters *H. pylori* cells (Johnson et al., 2012). Conversely, Noto and Peek discuss our current understanding of the role of microRNAs on the process of *H. pylori* pathogenesis and gastric carcinogenesis (Noto and Peek, 2012). Finally, the Müller group tackles an intriguing question that is coming to the forefront of the *H. pylori* field; does colonization with *H. pylori* actually provide any benefits to the host? Recent studies suggest that this is indeed the case, and Müller et al. review how *H. pylori* immunomodulation can confer protection against allergic and chronic inflammatory disorders (Arnold et al., 2012).

Those articles related to Campylobacter can be divided into five major topics: the function of glycans and capsule polysaccharides in Campylobacter virulence and biology, the mechanism of cell invasion, Campylobacter antimicrobial resistance, molecular typing methods, and general Campylobacter metabolism and biology.

Capsular polysaccharides (CPS) protect microbes from environmental insults and host immune defenses. Guerry et al. review our current knowledge on the role of CPS in Campylobacter virulence and provide an interesting perspective on the potential of CPS as a conjugate vaccine (Guerry et al., 2012). In an original research article, Sorensen et al. demonstrate that the *O*-methyl phosphoramidate (MeOPN) moiety of the *C. jejuni*

CPS is recognized as a receptor by several different phages (Sorensen et al., 2012). Interestingly, they observed *in vivo* phase variation of the capsular structure leading to phage resistance and suggesting phage-host co-evolution. Day et al. highlight the key role of *C. jejuni* surface glycans in its interaction with the host and the function of the host glycoconjugates in the defense against *C. jejuni* infection (Day et al., 2012).

Epithelial cell invasion is thought to be a major determinant of *C. jejuni* virulence. One review article and two original research papers focus on this important virulence trait (Boehm et al., 2011; Croinin and Backert, 2012; Neal-McKinney and Konkel, 2012). Boehm et al. used knockout cell lines to characterize the host signaling cascades involved in the process of *C. jejuni* invasion (Boehm et al., 2011). This study provides clear evidence of a role for the fibronectin, integrin beta1, FAK, and DOCK180/Tiam-1 signaling cascade and for Rac1 GTPase activation in *C. jejuni* epithelial cell invasion. Neal-McKinney and Konkel demonstrate that the flagellum secreted protein CiaC is delivered into the cytosol of the host cells and interact with the cell signaling pathways involved in *C. jejuni* cell invasion (Neal-McKinney and Konkel, 2012), possibly the signaling cascade identified by Boehm et al.

Colonization of the host gastrointestinal tract is intimately linked to the ability to survive host defenses. In this regard Hoang et al. describe the identification of genetic loci enabling *C. jejuni* to resist fowlicidin-1 exposure, a potent chicken antimicrobial peptide (Hoang et al., 2012). Intriguingly, fluoroquinolone-resistant Campylobacter have been previously shown to exhibit enhanced *in vivo* fitness. In an original research article, Han and collaborators now confirm the role of the Thr-86-Ile mutation in *gyrA* in conferring fluoroquinolone-resistance and demonstrate that this mutation modulates fitness *in vivo* and DNA supercoiling homeostasis in Campylobacter (Han et al., 2012). Given the importance of DNA supercoiling in gene expression, Han and collaborators argue that the altered DNA supercoiling observed in the fluoroquinolone-resistant Campylobacter might be directly linked to its increased fitness. Clearly, the current increase in the prevalence of fluoroquinolone-resistant Campylobacter threatens clinical treatments. This concern prompts Dufour et al. to assess the potential of phytochemicals as preservative to reduce *Campylobacter* load in food (Dufour et al., 2012). This work reports the antimicrobial properties of isothiocyanates (ITC) against 24 isolates of *C. jejuni* demonstrating their bactericidal effects and highlights the role of the γ-glutamyl-transpeptidase gene in Campylobacter resistance to ITC.

The detection of Campylobacter outbreaks is hampered by the lack of defined and standardized methods to unambiguously detect and track sources of Campylobacter. Two research articles tackle this important epidemiological issue. Through the use of publically available whole genome sequences of *C. jejuni* and *C. coli*, Carrillo and collaborators describe the development of a framework to assess the performance of the existing and emerging molecular typing methods (Carrillo et al., 2012). In terms of the multilocus sequence typing methods (MLST), Miller and collaborators describe and assess the performance of four MLST methods for differentiating strains of the emerging Campylobacter species *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus*, and *C. curvus* (Miller et al., 2012).

Finally, one original research article and four reviews highlight the recent developments in our understanding of Campylobacter physiology and biology. Haddad et al. describe the pleiotropic role of the polynucleotide phosphorylase (PNPase) in *C. jejuni* biology which affects motility, cell invasion and adherence, and chick gut colonization (Haddad et al., 2012; Matos et al., 2012). Plummer reviews our current knowledge on quorum-sensing in Campylobacter and its role in pathogenesis (Plummer, 2012). Alemka and collaborators describe Campylobacter physiology at the mucosa-luminal interface and specifically the complex interplay between *C. jejuni* and mucus (Alemka et al., 2012). Stahl et al. summarize Campylobacter ability to acquire and metabolize nutrients including a discussion of L-fucose metabolism in *C. jejuni* (Stahl et al., 2012). And lastly, Kaakoush and Mitchell highlight the emerging role of *Campylobacter concisus* as a new player of acute and chronic intestinal disease (Kaakoush and Mitchell, 2012).

Clearly we have learned a significant amount about Campylobacter and Helicobacter over the course of the last decades. However, as stated by Gaynor and Szymanski "both pioneers and new investigators in the CHRO research field continue to obtain 'unexpected results' demonstrating that Campylobacters and Helicobacters do not follow classic paradigms of other well-characterized gastrointestinal pathogens and we are learning that there is a plethora of interesting related organisms beyond *C. jejuni* and *H. pylori*." Therefore, though we have learnt much, it is clear that the coming years offer many places to advance our understanding of these important pathogens.

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REVIEW ARTICLE
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The 30th anniversary of Campylobacter, Helicobacter, and Related Organisms workshops—what have we learned in three decades?

Erin C. Gaynor[1]* and Christine M. Szymanski[2]*

[1] *Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada*
[2] *Alberta Glycomics Centre and Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada
D. Scott Merrell, Uniformed Services University, USA

****Correspondence:***
Erin C. Gaynor, Department of Microbiology and Immunology, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.
e-mail: egaynor@mail.ubc.ca

Christine M. Szymanski, Department of Biological Sciences, Centennial Centre for Interdisciplinary Science, University of Alberta, Edmonton, AB T6G 2E9, Canada.
e-mail: cszymans@ualberta.ca

As we commemorate the 30th anniversary of the Campylobacter, Helicobacter, and Related Organisms (CHRO) workshops with this special Frontiers edition, we look back upon three decades of research and provide some highlights from the 16th International CHRO meeting. Although Theodor Escherich himself provided drawings of campylobacters back in the 1880s, *Campylobacter jejuni* was not identified until the 1950s. *Helicobacter pylori* was first described to be the causative agent of stomach ulcers at a CHRO meeting by Barry Marshall and Robin Warren—who later received the Nobel Prize for their findings that bacteria could cause diseases previously believed to be caused by human factors. Now, several genome sequences for campylobacters, helicobacters, and related organisms are available and we have moved into an era examining the intersection between host microbial ecology and pathogen infection. Both pioneers and new investigators in the CHRO research field continue to obtain "unexpected results" demonstrating that campylobacters and helicobacters do not follow classic paradigms of other well-characterized gastrointestinal pathogens and we are learning that there is a plethora of interesting related organisms beyond *C. jejuni* and *H. pylori*. This review summarizes recent discoveries in CHRO research and the exciting directions ahead.

Keywords: campylobacter, helicobacter, related organisms, genome diversity, control measures, fundamental biology, host responses, pathogenesis

INTRODUCTION

Campylobacter, Helicobacter, and Related Organisms (CHRO) researchers from around the world gathered in Vancouver, Canada in August, 2011 to share their history and exciting new findings on this unique group of microorganisms. Martin Skirrow and Martin Blaser regaled the audience during the opening session with historical stories of CHRO research, while Roger Feldman and James Fox provided perspectives on the future of CHRO research at the gala closing banquet. Three CHRO field leaders co-chaired the Young Investigator Award session—Skirrow, Hubert Endtz, and Thomas Meyer—providing inspiration to the future generations of CHRO researchers. And there were many highly interactive sessions describing new discoveries in between.

The calibre of science presented at CHRO 2011, in oral and poster presentations, was exceptional. For space purposes, this review covers an overview of select oral presentations that both reflect emerging general themes and provide additional details on certain focal points. Also, for confidentiality purposes, only published data and unpublished data for which we have received permission from the authors are described in detail, while general topics are noted for other areas of interest.

GENOMES AND EMERGING SPECIES

William Miller, an expert in CHRO sequencing, provided a comparative summary of 30 fully sequenced *Campylobacter* taxa—and this number will soon reach 40—in addition to describing nine sequenced Arcobacters (also described by Sarah De Smet) and *Sulfurosprillum delayianum*. It is becoming more and more apparent that other members of this group of epsilon-proteobacteria are capable of causing disease; most have just not been accounted for due to culturing limitations. But, for *Campylobacter* species, Albert Lastovica's Cape Town protocol is becoming more and more widespread and demonstrating that organisms such as *C. concisus* (discussed by Nadeem Kaakoush and Hans Nielsen) and *C. upsaliensis* (pathogenesis described by Shauna Crowley/Christine Szymanski) are emerging species. CHRO researchers are also using non-culture-based methodologies such as PCR to detect these organisms in food and fecal samples, so the epidemiology, host range, and virulence properties of many *Campylobacter* species is yet to be determined. For example, Olivier Vandenberg described the use of 16S-PCR-DGGE to determine the role of epsilon-bacteria in children with abdominal complaints. Linda Mansfield provided a comprehensive overview of emerging *Campylobacter* species, while Lori Graham and Samuel Sheppard described new methods to differentiate and study *C. fetus* and *C. coli*, pathogens that are typically isolated from farm animals. There are also greater than 10 *Helicobacter* species sequenced. Bram Flahou described the non-*H. pylori* helicobacters (NHPH), such as *H. suis* that are also found in the stomach of both humans and pigs, and cause disease

despite lacking *H. pylori* virulence factors such as the vacuolating toxin VacA and the *cag* pathogenicity island (*cag*PAI) that encodes the type IV secretion pathway.

GENOMIC DIVERSITY

Both campylobacters and helicobacters are capable of varying the on/off status of several proteins allowing for the best adapted phenotype to predominate under a given selection pressure. One common mechanism of phase variation is through slipped-strand mispairing, where repeats of DNA (in the case of helicobacters and campylobacters, typically homopolymeric tracts of Gs or Cs) are either shortened or lengthened during DNA replication or repair resulting in premature stop codons in the translated protein. Genomic sequencing has identified many phase-variable genes in both genera—and there have been several reports in the literature about the consequences of varying bacterial surface structures. This is especially evident for *C. jejuni* where individual stocks of strain 11168 from different labs display different capsular polysaccharide (CPS) structures, and as such Brendan Wren appropriately called this organism "a moving target." Generally speaking, there are also multiple gene products that influence motility in *C. jejuni*, thus demonstrating whether a mutation affects motility requires vigorous analysis since the random rate of this event is high—similarly, the motility of the population needs to be confirmed before doing any adherence/invasion or colonization studies since motility has been repeatedly shown to influence these phenotypes. For *H. pylori*, there are 46 genes prone to slipped-strand mispairing and many elegant studies have demonstrated the variation in O-antigen Lewis structures in the lipopolysaccharides (LPS), for instance, has a role in both host immune responses and bacterial colonization ability.

H. pylori faces multiple conditions in the gut such as penetrating the host mucous and tolerating high acid levels. The organism must also confront host inflammatory mediators and has a limited number of two-component signal transduction systems that commonly allow bacteria like *E. coli* to move between niches. Instead, evidence suggests that *H. pylori* uses genetic diversity for adaptation. Jay Solnick described how *H. pylori* can "tune" the host inflammatory response and adapt to the varying conditions in this environment in order to establish a chronic infection. Solnick talked about different mechanisms of genetic diversity and variation of BabA (ABO blood group adhesion) and other virulence factors. Sebastian Suerbaum further discussed mechanisms for *H. pylori* diversity including mutation-generating single nucleotide polymorphisms (SNPs) and recombination. Remarkably, *H. pylori* genomes are "sequence mosaics" and Suerbaum suggested they may actually be used as a method to trace human migrations. Suerbaum then demonstrated that DNA imports showed significantly increased frequency in the members of the protein family which includes the Bab proteins.

Since *C. jejuni* is naturally competent similar to *H. pylori*, Eduardo Taboada pointed out that there are multiple mechanisms for strain variation including exchange of genes and entire clusters by horizontal gene transfer, gene duplication, deletion, fusion, and contingency gene variation, while Christopher Bayliss described exciting new means to detect phase-variable genes. Similarly, Lone Brondsted in collaboration with Szymanski described a study, published in this issue, examining the role of bacteriophage selection on *C. jejuni* CPS structure. Chickens were infected with a *C. jejuni* strain expressing phosphoramidate (MeOPN) on its CPS with or without a *C. jejuni* phage recognizing MeOPN. Six days later, pooled populations of *C. jejuni* from phage uninfected birds still expressed the MeOPN, were phage sensitive, and the MeOPN transferase gene showed 9 Gs ("ON"). In contrast, *C. jejuni* isolated from the phage-infected birds lacked MeOPN (in all cases except one where another phase variant was isolated), were phage resistant, and sequencing showed 8 or 10 Gs ("OFF"). Remarkably, the same level of *C. jejuni* colonization was observed in all chickens demonstrating that phage resistance does not always lead to an attenuated phenotype.

CONTROL MEASURES FOR *Campylobacter jejuni*

Qijing Zhang, Albert Lastovica, Hubert Endtz, and Patrick Kwan all presented results demonstrating that antibiotic resistance is increasing for *C. jejuni* and Francis Megraud showed similar trends for *H. pylori*. However, research is on-going to determine the molecular basis for *C. jejuni* resistance as Monika Keelan and Declan Bolton described. And scientists are using different approaches for antimicrobial development against *C. jejuni* including using CmeABC efflux pump inhibitors (Zhang), systems biology approaches (Mark Reuter and Arnoud van Vliet), and components of bacteriophages (Muhammad Javed/Szymanski). Birthe Hald presented a 4-year study on the effects of fly screens in poultry houses in Denmark and showed that screens significantly reduced flock prevalence of *C. jejuni*. Nigel French also demonstrated that a significant reduction in human cases was observed after introducing poultry interventions. Interestingly, although the majority of urban cases are associated with poultry, rural cases appear to be correlated with cattle and other environmental sources. Trudy Wassenaar emphasized that more work is needed to determine the numbers of infectious campylobacters in the environment and their source of contamination. Clarence Tam presented data from the infectious intestinal disease (IID) 2 study showing that the incidence of campylobacter in the UK has not changed in the last 15 years, while Jaap Wagenaar showed data from the European BIOHAZ group, and Birgitte Borck summarized the European CamCon project. In addition to providing a bigger-picture perspective, Julian Davies, an expert in the field of antibiotics and resistance, likewise echoed many of these sentiments in his talk and proposed novel, small molecule-based antimicrobial strategies in the future.

GLYCOBIOLOGY

Brendan Wren started this session with a prediction that *C. jejuni* will become better known for the birth of bacterial glycoengineering than as a notorious foodborne pathogen. His comments are based on the findings that *C. jejuni* was the first bacterium shown to possess an *N*-linked protein glycosylation pathway and that this pathway can be functionally transferred into *E. coli*. This resulted in the emergence of GlycoVaxyn, founded in 2004, and based on using the *C. jejuni N*-glycosylation machinery to

create novel bacterial glycoconjugate vaccines. Two talks in this session described the characterization of *N*-glycan pathways in other *Campylobacter* species (Harald Nothaft/Szymanski) and in related organisms such as *Wolinella succinogenes* (Jonathan Butler/Dennis Linton). Jos van Putten showed that *C. jejuni* *N*-glycans and some forms of lipooligosaccharides (LOS) are recognized by carbohydrate receptors on dendritic cells and may be able to modulate the host immune response. Then, Susan Logan described the process for *O*-linked protein glycosylation in both *C. jejuni* and *H. pylori* and the need for these glycan structures for proper flagellar filament assembly and subsequent motility. Due to the importance of flagella for both organisms, it is not surprising that small molecule inhibitors of the *O*-glycan pathway are being pursued. Interestingly, bacterial flagella are common pathogen-associated molecular patterns (PAMPs) that are recognized by Toll-like receptor (TLR)-5, but both *H. pylori* and *C. jejuni* evade this response. van Putten demonstrated that modification of *C. jejuni* flagella with *O*-linked glycans is not the mechanism for evasion, instead they identified a new β-hairpin structure involved in recognition. Stephen Trent described a new modification associated with the flagellar apparatus. Phosphoethanolamine is typically transferred to the LOS/LPS core of many bacteria including *C. jejuni* and *H. pylori*. The Trent group demonstrated that the *C. jejuni* phosphoethanolamine transferase not only modifies the LOS, but also the flagellar rod protein, FlgG. Mutation of the transferase results in abnormal flagellar filament assembly and reduced antimicrobial peptide resistance. Other forms of phosphorylated modifications were identified on the surface of *C. upsaliensis*: phosphoramidate on the CPS and phosphocholine on the LOS (Shauna Crowley/Szymanski), and data were presented demonstrating that both these modifications could contribute to bacterial survival and pathogenesis. Craig Parker described draft genomes of 56 *C. jejuni* and *C. coli* isolates and compared the LOS and CPS loci noting all the genetic mechanisms for diversity and some commonalities among the strains. Although *H. pylori* does not have an *N*-glycan pathway like *C. jejuni*, Mario Feldman showed that the *H. pylori* flippase was evolutionarily connected to the *C. jejuni* *N*-glycan pathway and is used for flipping LPS O-antigens into the periplasm. Feldman also demonstrated that using the *C. jejuni* *N*-glycan enzymes and the *H. pylori* fucosyltransferase together with enzymes from *Haemophilus influenzae*, he could engineer *H. pylori* Lewis antigens for the treatment of specific autoimmune diseases. Eleonora Altman, on the other hand, is interested in creating a vaccine against *H. pylori* and wants to avoid the Lewis antigens which mimic human Lewis structures. She has discovered a common α-1,6-glucan chain in clinical isolates of *H. pylori* which is now being explored. Jonathan Lane provided a different perspective to the glycobiology session when he discussed the use of sugars to inhibit bacterial binding. This would indeed be an inexpensive therapeutic, but various *C. jejuni* strains would need to be compared since it is known that *C. jejuni* infection in developing countries results in different disease symptoms. Also, Hubert Endtz reported that in their rural Bangladesh studies, breastfeeding (breast milk contains large amounts of potentially anti-infective oligosaccharides) did not prevent disease in children in the 0–6 month range.

Helicobacter pylori: CagA, VacA, AND HOST RESPONSES

CHRO 2011 saw a resurgence of presentations on Helicobacter biology and pathogenesis. In the areas of *H. pylori* pathogenesis and host interactions, a number of key advances on the Cag pathogenicity island (PAI) encoding a type IV secretion system were described. As Masanori Hatakeyama introduced, the CagA effector is injected into host cells, becomes tyrosine-phosphorylated by host cell oncoproteins, and mimics a host cell factor to activate or inactivate specific intracellular signaling pathways. In his presentation, Hatakeyama described the identification of several mammalian proteins containing CagA's critical EPIYA phosphorylation site motif, one of which was competitively inhibited by CagA for downstream events. This suggested that *H. pylori* acquired CagA to subvert one or more endogenous eukaryotic EPIYA-containing proteins to establish successful infection. Additional expert perspective on the impact of the *cag*PAI on host responses was provided by Thomas Meyer. Furthermore, three new protein components of the Cag type IV pilus that do not have homologs in other bacteria but are nonetheless required for CagA translocation into host cells and downstream events, such as IL-8 induction, were described by Carrie Shaffer/Timothy Cover, lending further insight into novel means by which *H. pylori* interacts with host cells.

A number of talks also addressed the intersection of CagA and iron. Manuel Amieva used live-cell microscopy data and other techniques to demonstrate that one of CagA's functions is to aid in iron acquisition from the host, and that this function is required both for microcolony formation on intercellular junctions and for colonization of gerbils. The model of iron-replete vs. iron-deplete gerbils described by Amieva was also used by his collaborators Jennifer Noto/Richard Peek, who showed that iron depletion significantly increased the frequency and severity of *H. pylori*-induced disease, including carcinoma, in a CagA-dependent manner. Noto and Peek's work also suggested that regulation of *H. pylori* virulence factors by iron availability contributes to these findings, a theme mirrored by D. Scott Merrell's presentation showing connections between CagA and the ferric uptake regulator Fur. Merrell's group found that Fur positively regulates CagA expression via a Fur box in the *cagA* promoter and that Fur is most important in early stages of infection. All three of these presentations clearly show that iron availability and acquisition play a critical role in *H. pylori* disease etiology, mediated at least in part by CagA.

Advances in understanding roles for the vacuolating cytotoxin VacA in pathogenesis were also presented. In addition to his CagA work, Manuel Amieva described how VacA influences the pathogen-host iron homeostasis via inducing mislocalization of the host epithelial cell transferrin receptor. VacA's striking effect on mitochondrial morphology was addressed by Steven Blanke, who presented work demonstrating VacA's activation of a host cell dynamin-related protein (Drp1) and the relationship of this activation to uncoupling of mitochondrial fission and fusion and induction of apoptosis. Blanke further hypothesized that these events aid *H. pylori* colonization by crippling cells at the epithelial barrier, rendering them less fit to respond to *H. pylori* infection.

Additional insight into mechanisms underlying host responses to *H. pylori* infection was provided by three different presentations. In one talk, James Neal/Karen Guillemin showed that transgenic zebrafish expressing CagA displayed early intestinal proliferation, adult intestinal hyperplasia, and activation of the Wnt pathway, further indicative of a key role for CagA in *H. pylori*-induced carcinogenesis. A connection between *H. pylori* chemotaxis and immune responses was demonstrated by Annah Rolig/Karen Ottemann, who showed that chemotaxis mutants were defective for early gastric recruitment of CD4+ T cells and induction of a Th17 immune response component implicated in apoptosis. As this is the first study showing an effect of bacterial chemotaxis on apoptosis, these findings will likely have relevance for other systems as well. Finally, Anne Mueller's talk addressed an intriguing paradox: namely, that persistent infection with *H. pylori* is linked to protection from allergic, chronic inflammatory, and autoimmune disease. Using an asthma model, Mueller showed an interconnection between *H. pylori* infection, immune cell responses, and reprogramming of dendritic cells toward a tolerance-promoting state. Mueller further hypothesized that this beneficial aspect of *H. pylori* infection may contribute to persistence of *H. pylori* among the human population.

Campylobacter jejuni: NEW ANIMAL MODELS PROVIDE INSIGHT INTO HOST AND BACTERIAL FACTORS MEDIATING DISEASE

A number of diverse topics were covered in sessions pertaining to *C. jejuni* pathogenesis, host responses, and host cell interactions. In addition to Patricia Guerry's overview of the contribution of CPS to virulence, various aspects of the *C. jejuni*-host cell interaction were described by Dennis Kopecko, Nicole Iovine, Dominic Mills/Nick Dorrell, and Lieneke Bouwman/Jos van Putten. Furthermore, a number of new connections between the LOS and/or sialylation and Guillain-Barré Syndrome were drawn by Ruth Huizinga, Arnoud van Vliet, and Astrid Heikema/Janneke Samsom.

An emerging area of interest, covered by two talks, is the utility of new or recently developed small animal models of disease in exploring *C. jejuni* disease markers and identifying new host and bacterial factors important for disease etiology. Christian Jobin used IL-$10^{-/-}$ mice expressing GFP from the NF-κB promoter, which develop invasive, campylobacteriosis-like colitis, to explore host signaling pathways involved in *C. jejuni*-induced inflammation. Daily injections with rapamycin allowed *C. jejuni* to colonize the intestine but prevented extra-intestinal dissemination and inflammation, implicating the mammalian mTOR pathway specifically in disease. Additional experiments suggested these effects were independent of T-cell activation, suggesting mTOR signaling components as possible targets for new treatments. Markus Heimesaat described the development, along with co-author Stefan Bereswill, of a new gnotobiotic mouse model harboring "humanized" gut flora. *C. jejuni* stably colonized these mice and elicited a pro-inflammatory immune response that was dependent on the host innate immune receptors TLR4 and TLR9, and on *C. jejuni* formate dehydrogenase utilization genes.

IN THE NICHE: INTERACTION OF CHRO WITH MICROBIAL COMMUNITIES AND SURVIVAL STRATEGIES IN DIVERSE ENVIRONMENTS

An emerging theme in CHRO research, and indeed in bacterial pathogenesis, is the interaction of CHRO and other "infecting" bacteria with resident microbial communities. This was introduced at CHRO 2011 by Brett Finlay, who described research in his laboratory dissecting the interplay between *E. coli* and *Samonella* with gut microflora, and is also of relevance to the findings described above by Heimesaat and colleagues regarding their humanized gut flora mouse model of *C. jejuni*-mediated inflammation. Two *H. pylori* talks also addressed this topic. Richard Peek discussed this from the perspective of microbial and host diversity, and the contribution of each to stomach cancers. Peek noted that in addition to contributions of *H. pylori* intra- and intergenomic diversity, *H. pylori* exist in a distinct gastric microbial ecosystem which may provide an additional genetic pool allowing *H. pylori* to develop traits further influencing its propensity to cause gastric cancers. Also noted were findings showing that host factors modulating immunity, and thus presumably also the microflora composition, likewise impact gastric cancer risks. Anica Wandler/Guillemin presented new results from a transgenic Drosophila model showing that intestinal expression of CagA alters the composition of the gut microbiome, further implicating CagA in new areas of *H. pylori*-host biology.

Survival strategies, primarily in the form of stress responses, were also discussed in the context of life "in the niche." Arnoud van Vliet, in a talk entitled "Try not to breathe . . .," provided an overview of how the microaerophilic *C. jejuni* counters various oxygen concentrations ranging from virtually anaerobic to atmospheric (aerobic) as it moves from niche to niche, as well as conditions caused by reactive oxygen species. van Vliet also noted that *C. jejuni* forms enhanced biofilms in aerobic conditions. Andrew Cameron/Erin Gaynor presented the first global characterization of the response of *C. jejuni* to hyper-osmotic conditions, some of which are encountered during host colonization. Finally, Alain Stintzi discussed the PerR regulon as a means by which *C. jejuni* counters oxidative stress, as well as how *C. jejuni* acquires iron, the importance of iron-related genes in colonization, and regulation of those genes by the regulator Fur.

FUNDAMENTAL BIOLOGY OF CHRO: BACTERIAL CELL SHAPE, METABOLISM, AND CHEMOTAXIS

Also new to the CHRO workshop was a session dedicated to research on basic bacterial processes. Well-suited to this theme were two talks exploring means by which *H. pylori* and *C. jejuni* derive their helical shape, and the ramification of shape/peptidoglycan alterations on other important processes. Nina Salama described the first characterization of shape-related genes in *H. pylori*. Deletion mutants displayed a variety of shape abnormalities ranging from "s" and "c" shapes with exaggerated curvature to straight rods. Some of the proteins involved, termed "Csd" for "cell shape determinant," are novel peptidoglycan peptidases, while others perform other cellular functions. Salama proposed two independent peptidoglycan modification networks, demonstrated roles for each of the *csd* genes in a mouse model of colonization, and described models for how each shape

change affects *H. pylori* motility. An independent screen in *C. jejuni,* described by Emilisa Frirdich/Gaynor, also identified a peptidoglycan peptidase ("Pgp") as the first factor to impact *C. jejuni* shape. Deletion of this gene resulted in a straight rod morphology, modest motility and biofilm defects, peptidoglycan that hyper-activated the intracellular innate immune receptor Nod1, and a defect in chick colonization. This gene was a homolog of an unpublished *H. pylori csd* described by Salama, deletion of which also caused a straight rod phenotype, and the specific enzymatic activity of each protein on peptidoglycan was determined. The work by Frirdich/Gaynor also provided the first muropeptide map for *C. jejuni.*

Dave Kelly presented a comprehensive talk on numerous aspects of *C. jejuni* metabolism. One was the *C. jejuni* strain-dependent utilization of select amino acids as growth substrates, and his group's recent observation that exogenous peptides can provide a source of amino acids. Kelly also described the highly complex and branched respiratory chains in *C. jejuni,* that new types of electron donors and acceptors distinct from those in other bacteria have recently been identified, and the dependence on the twin-arginine transporter (TAT) system for delivery of many important respiratory enzymes to the periplasm (and thereby dependence on the TAT system for the ability of *C. jejuni* to respire on substances requiring those enzymes). Hilde De Reuse presented data on the role of the pleiotropic *H. pylori* nickel-dependent regulator NikR to activate or repress important target genes such as *ureA* and *hydA*, which in turn encode urease and hydrogenase for which nickel is a co-factor and which are essential for *H. pylori* colonization. De Reuse showed that NikR-dependent repression occurred at higher nickel concentrations than activation, suggesting a chronological hierarchy and elaborate mechanisms for NikR target discrimination.

Chemotaxis in both *C. jejuni* and *H. pylori* was also discussed. Victoria Korolik presented findings on the first *C. jejuni* sensory receptor, Tlp1, re-named CcaA for its role as an aspartate receptor, and its role in motility, colonization, and cell invasion. As Kelly noted, aspartate is a major amino acid used as a *C. jejuni* carbon source, emphasizing the importance of CcaA to *C. jejuni* biology. Korolik further showed that for CcaA, signal transduction occurs via binding to CheV and not CheW, suggesting differences in this pathway from other enteric pathogens. Karen Ottemann described the chemotactic program in *H. pylori*, the phosphorylation-based signal transduction cascade, how the *H. pylori* system differs from that of *E. coli* and *Bacillus subtilis*, and other findings on newer components involved in *H. pylori* chemotaxis.

FUTURE PERSPECTIVES

These are exciting times in CHRO research—in the past, we have looked toward *E. coli* and Salmonella for understanding bacterial physiology—now campylobacter and helicobacter have become model systems for understanding bacterial glycobiology, survival in mucin, metabolism of microaerophiles, and the importance of cell shape and motility. There is also a growing appreciation for the importance of CHRO other than *C. jejuni* and *H. pylori*, and as such it is anticipated that we will learn much more about these organisms and their possible roles in human/animal health and disease. Although we were unable to highlight all of the oral talks or the outstanding poster presentations, we look forward to the next few years of CHRO publications. There is a bright future ahead for CHRO research, and we would particularly like to highlight the Young Investigator Award winners: Cody Buchanan, Jonathan Butler, Ilana Cohen, Shauna Crowley, Rajinder Dubb, David Hermans, Laura MacRitchie, Ana Martins, Dominic Mills, James Neal, Jennifer Noto, and Christian Penny. We look forward to many CHRO meetings to come.

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Mechanism of *H. pylori* intracellular entry: an *in vitro* study

***H. Liu*[1,2†], *C. Semino-Mora*[1,2†] *and Andre Dubois*[1,2*]**

[1] *Laboratory of Gastrointestinal and Liver Studies, Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA*
[2] *United States Military Cancer Institute, Bethesda, MD, USA*

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Richard Peek, Vanderbilt University Medical Center, USA
Karen Guillemin, University of Oregon, USA
Yoshio Yamaoka, Baylor College of Medicine, USA

****Correspondence:***
Andre Dubois, Laboratory of Gastrointestinal and Liver Diseases, Department of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA.
e-mail: adubois@usuhs.mil

†H. Liu and C. Semino-Mora have contributed equally to this work.

The majority of *Helicobacter pylori* reside on gastric epithelial cell surfaces and in the overlying mucus, but a small fraction of *H. pylori* enter host epithelial and immune cells. To explore the role of the *nudA* invasin in host cell entry, a Δ*nudA* deletion derivative of strain J99 was constructed and transformants were verified by PCR and by fluorescence *in situ* hybridization. AGS cells were inoculated with either wild type (WT) strain J99 or its Δ*nudA* mutant to determine the fraction of bacteria that were bound to the cells and were present inside these cells using the gentamicin protection assay. We observed no significant difference between either the density of *H. pylori* bound to AGS cell membranes or the density of intracellular *H. pylori*. To further explore this finding, separate chambers of each culture were fixed in glutaraldehyde for transmission electron microscopy (TEM) and immunogold TEM. This addition to the "classical" gentamicin assay demonstrated that there were significantly more intracellular, and fewer membrane-bound, *H. pylori* in WT-infected AGS cells than in Δ*nudA* allele infected cells. Thus, the sum of intracellular *and* membrane-bound *H. pylori* was similar in the two groups. Since no other similar TEM study has been performed, it is at present unknown whether our observations can be reproduced by others Taken together however, our observations suggest that the "classical" gentamicin protection assay is not sufficiently sensitive to analyze *H. pylori* cell entry and that the addition of TEM to the test demonstrates that *nudA* plays a role in *H. pylori* entry into AGS cells *in vitro*. In addition, deletion of the invasin gene appears to limit *H. pylori* to the AGS cell surface, where it may be partly protected against gentamicin. In contrast, this specific environment may render *H. pylori* more vulnerable to host defense and therapeutic intervention, and less prone to trigger normal immune, carcinogenic, and other developmental response pathways.

Keywords: *H. pylori*, intracellular, adhesion, adhesin, *nudA*, electron microscopy

INTRODUCTION

The pathogenicity of many bacteria colonizing the gastrointestinal tract often depends on their ability to gain access to cells that are normally non-phagocytic. *Helicobacter pylori* colonizes the stomach of over half the world population and is the main cause of peptic ulcer disease and gastric cancer. It often is considered to be a non-invasive pathogen present only in the lumen of the stomach and attached to gastric epithelial cells although a number of *in vivo* and *in vitro* studies have demonstrated that *H. pylori* is in fact invasive (Dubois and Berg, 1997; Engstrand et al., 1997; Amieva et al., 2002; Semino-Mora et al., 2003; Necchi et al., 2007). In addition, *H. pylori* can repopulate the extracellular environment after elimination of extracellular bacteria with gentamicin, suggesting it may be considered a facultative intracellular bacterium (Amieva et al., 2002). Finally, *H. pylori* may be present inside metaplastic, dysplastic, and neoplastic epithelial cells (Semino-Mora et al., 2003). The multiplicity of these observations and the fact that various methods were used to reach the same conclusions strongly support their validity.

Bacterial invasion of eukaryotic cells appears to be mediated by Nudix enzymes, initially called MutT because *E. coli* MutT was the first Nudix hydrolase to be described (Maki and Sekiguchi, 1992). The *Bartonella bacilliformis* Nudix hydrolase, encoded by the *ialA* gene, was shown to be associated with the ability to invade human erythrocytes using the gentamicin assay complemented by transmission electron microscopy (TEM; Mitchell and Minnick, 1995). Similarly, invasion of human brain microvascular endothelial cells by *E. coli* is accompanied by increased expression of the K1 ortholog *ygdP* and the early stages of infection of infection by *Rickettsia prowazekii* ortholog is temporarily accompanied by an increased transcription of the *invA* gene (Lundin et al., 2003). Finally, the *invA* gene was highly conserved in protein sequence and present in all tested members of the pathogenic *Leptospira* species (Luo et al., 2011).

Helicobacter pylori appears to be a suitable system to study the biological role of Nudix hydrolases since the NudA protein is the only dinucleoside polyphosphate hydrolase homolog present in the two first strains that were sequenced (Tomb et al., 1997; Alm et al., 1999). J99 *H. pylori* harbors one Nudix hydrolase ortholog, *Nud*ix hydrolase *A*, *nudA*, with the gene numbers JHP1149 (Alm et al., 1999), 26,695 strain harbors HP1228 (named *invA*; Tomb et al., 1997), and a GenBank search showed that a *nudA* gene is present in 30 additional completely sequenced *H. pylori* strains.

Due to the functional heterogeneity within this group of proteins, *H. pylori* NudA may be involved in (1) DNA repair, (2) oxidative stress and/or heat shock response, or (3) bacterial invasion of epithelial cells through degradation of toxic substances induced during invasion (Lundin et al., 2003). These authors describe the enzymatic function of the Nudix hydrolase NudA in *H. pylori* and they constructed a *nudA* insertion mutant to determine the biological role of this protein. Using the classical gentamicin protection assay (Kwok et al., 2002), the authors found no quantifiable differences in invasion frequency by the *NudA H. pylori* J99 strain mutant compared to WT but they did not show the data (Lundin et al., 2003). Their conclusion was that they found no supportive evidence for a role for the NudA protein in *H. pylori* invasion of AGS cells, although they warned that this could be due to the fact that complete eradication of extracellular bacteria is rarely obtained in the gentamicin protection assay (Amieva et al., 2002). They also concluded that the gentamicin assay of AGS cells invasion by *H. pylori* lacked the sensitivity needed to demonstrate differences in their experimental setup.

In the present study, we examined the role of *H. pylori* invasin J99 NudA in *H. pylori* entry into gastric epithelial cells. To this effect, we generated a Δ*nudA* allele of strain J99 in which *nudA* was replaced by a chloramphenicol resistance gene (CAM). Absence of the *nudA* gene was verified and the effect of this deletion on colonization was studied *in vitro* using the classical gentamicin assay aided by ultrastructural studies as used by others (Mitchell and Minnick, 1995).

MATERIALS AND METHODS

CONSTRUCTION OF A Δ*nud*a ALLELE MUTANT

A Δ*nudA* allele in which *nudA* was replaced by the chloramphenicol resistance cassette (CamR) was constructed from a low-pass isolate of the J99 strain (kindly provided by Dr. R. Peek) and using a PCR method as reported earlier (Chalker et al., 2001; Tan and Berg, 2004). Culture of single colony isolates in the presence of chloramphenicol was then used to select for Δ*nudA* alleles carrying CAM. Genomic DNA was extracted from fresh *H. pylori* isolates using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) and this DNA was used for confirmation of the mutant. Presence of CAM and absence of *nudA* in single colony isolates was then verified using PCR (**Figure 1**) and fluorescence *in situ* hybridization (**Figure 2**). The CamR and *nudA* sequences were confirmed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.).

GENTAMICIN PROTECTION VIABILITY ASSAY EXPERIMENTAL DESIGN

Human gastric adenocarcinoma cell line AGS was purchased from ATCC and maintained in 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (FBS, Gibco/BRL) for 2 days in 10-cm dishes for culture and then in 12-well plates (5×10^4/well) for gentamicin protection assay and four- or eight-chamber slides (Nunc Lab-Tek II Chamber Slide System)[1] for light and TEM.

[1] www.thermofisher.com

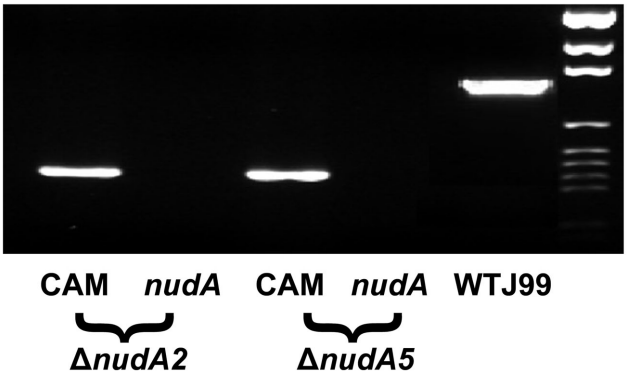

FIGURE 1 | Verification of post-transformation bacterial DNA in the Δ*nudA* allele PCR products on agarose gel. Two pairs of primers were designed for verification of post-transformation of bacterial transformed with Δ*nudA* allele PCR products. One pair was specific for *nudA* and the other pair was specific for the CamR gene. The wild type strain J99 PCR product was used as positive control. The figure illustrates that the *nudA* gene was present only in wild type strain J99 but absent in *nudA* mutant strains while the CamR gene was present in *nudA* mutant strains but not in the wild type.

AGS cells were grown to monolayer in two groups of four plates or chambers and a similar number of *H. pylori* J99 WT and of the Δ*nudA* mutant was inoculated into each group of plates/chambers (4×10^3 *H. pylori*/cell). Two plates and chambers were cultured for 6 h, used as negative control, and infected with *H. pylori* plus 200 μg/ml gentamicin (**Figure 3**; controls, left side) for 12 h. One of the two plates or wells was treated with 0.1% saponin for 15 min and then cultured for *H. pylori*. Saponin was used to permeabilize AGS cell membranes by penetrating the cholesterol monolayer and forming holes or pits (7–9 nm) that is believed to allow *H. pylori* to exit from AGS cells and be cultured (Bangham et al., 1962; Kwok et al., 2002). The remaining two plates and chambers were used to study *H. pylori* binding and cell invasion (**Figure 3**; right side) and were infected with *H. pylori* for 6 h. One of the two plates or chambers was treated with gentamicin, cultured for 12 h, treated with saponin for 15 min, and then plated on BAP for colony counting or fixed with formaldehyde or glutaraldehyde (see below).

CALCULATION AND NORMALIZATION OF *H. pylori* ENTERING INTO, OR BINDING TO, AGS CELLS

Counting of *H. pylori* can be done via OD measurement, but this method is not precise or very accurate. Instead, we counted the number of colonies used for plates and well inoculation by plating and culturing the *H. pylori* suspension for 3 days. Colony counting after treatment with and without gentamicin and saponin was then determined by culturing *H. pylori* on plates and then expressing colonization in each compartment as the percentage of *H. pylori* inoculated onto the plates.

MORPHOLOGY AND MORPHOMETRY USING FLUORESCENCE *IN SITU* HYBRIDIZATION AND TRANSMISSION ELECTRON MICROSCOPY

Tissue culture chamber glass slides from each of the four groups were treated using one of the following methods.

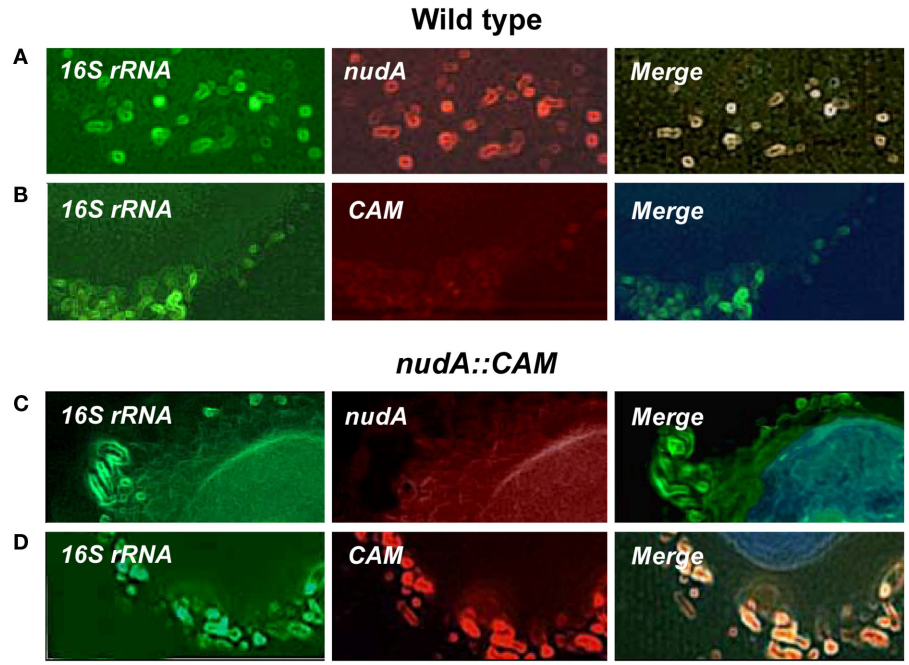

FIGURE 2 | Illustration of the expression of *H. pylori 16S rRNA, nudA,* and chloramphenicol resistance cassette (CAM) in AGS cells infected with *H. pylori* J99 WT (A,B) and ΔnudA allele (C,D). (A) *16S rRNA* and *nudA* expression and complete merge of the two genes (green, red, and yellow, respectively). **(B)** *16S rRNA* expression, no expression of CAM, and no merge. **(C)** *16S rRNA* expression, no expression of *nudA,* and no merge. **(D)** *16S rRNA* and CAM expression and complete merge of the two genes (green, red, and yellow, respectively). Original magnification: 1,000×.

For *H. pylori* dual fluorescence *in situ* hybridization (FISH), glass chambers were immediately fixed in 4% formaldehyde for 24 h and then processed using probes designed specifically for *H. pylori 16S rRNA* (digoxigenin–FITC) and *nud*A (Biotin–Texas red) or *16S rRNA* (digoxigenin–FITC) and CAM (biotin–Texas Red) were designed and synthesized as reported (Liu et al., 2008). Control of method was performed as described (Semino-Mora et al., 2003). Chambers were observed using a Nikon Eclipse 80i microscope and pictures were taken using a DS-Qi1MC Nikon camera (**Figure 2**).

For TEM, plastic chamber slides with four wells were immediately fixed in 2.5% glutaraldehyde for 24 h, the AGS cells were scraped and transferred to Eppendorf tubes, post-fixed in 1% osmium tetroxide at 4°C, and processed as reported until embedding in Spurr Low Viscosity Kit[2] to obtain epoxy blocks at 70°C (Semino-Mora et al., 2003). Semi-thin (0.5 μm) sections were stained with toluidine blue and observed by light microscopy using an Eclipse E800 Nikon interfaced with a QCapture digital camera (Micropublisher 5.0, Burnaby, BC, Canada). Grids with thin sections (500 A) were prepared, stained with uranyl acetate and lead citrate, and then observed using a Philips CM100 transmission electron microscope at 80 kV (Biomedical Instrumentation Center, USUHS).

For TEM immunohistochemistry with pre-embedding, plastic chamber slides with eight chambers each were processed inside each chamber slide (*in situ* method; Tanner et al., 1996). Immunogold was performed using rabbit anti-*H. pylori* (NeoMarkers, Fremont, CA, USA) and immunogold-labeled secondary antibodies (18 nm colloidal gold, goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA, USA) and after intermediate steps were embedded in Spurr embedding. In other experiments, dual immonogold pre-embedding was performed using both rabbit anti-*H. pylori* and mouse anti-VacA antigen IgG anti-VacA (Secondary 18 nm colloidal gold, goat anti-rabbit IgG, and 11 nm colloidal gold, respectively, Jackson, InmunoResearch, West Grove, PA, USA). After polymerization, the flat resin blocks were removed by peeling them away from each chamber, mounted in blank mold, and cut in ultramicrotomy. Sections were stained only with uranyl acetate. Control of method was performed in AGS cells infected with WT or Δ*nudA*, were treated with PBS instead of the anti-*H. pylori* first antibody, and were processed as described above for pre-embedding immunogold method. Control of *H. pylori* detection with immunogold with pre-embedding was performed using chamber slides with non-infected AGS cells treated as described above. Importantly, all positive and negative controls were positive and negative, respectively.

A Philips CM100 transmission electron microscope was used at 80 kV for the analysis (Biomedical Instrumentation Center, USUHS) and negatives and photographs were processed. Visibility of colloidal gold particles in the pictures was enhanced using color burn option (Adobe Photoshop 7.0.1) that examines the color information in each channel and darkens the base color to reflect the blend color by increasing the contrast between the two). Nickel

[2]www.polysciences.com

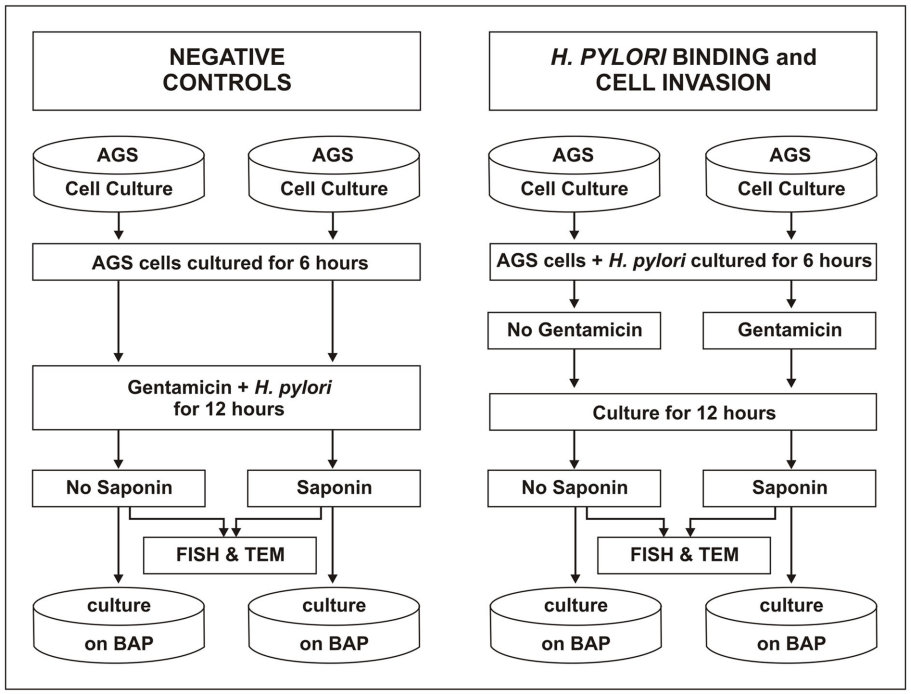

FIGURE 3 | Experimental design of the gentamicin protection assay. AGS cells were prepared to grow to monolayer in 8 wells in a 12-well plate (four wells for WT strain J99 and four wells for Δ*nudA* mutant. Each strain was inoculated into the four AGS cell types. Two wells (on the left) were designed as negative control of gentamicin treatment as it was added to *H. pylori* suspension when inoculated to the cells, and then treated with or without saponin after 12 h inoculation. A third well was used for measurement of *H. pylori* bound to cell membranes in the conditions without gentamicin and saponin. The last well was designed for measurement of *H. pylori* invasion into the cell with gentamicin and saponin added. The chamber slides prepared for morphologic observation were exactly the same as above.

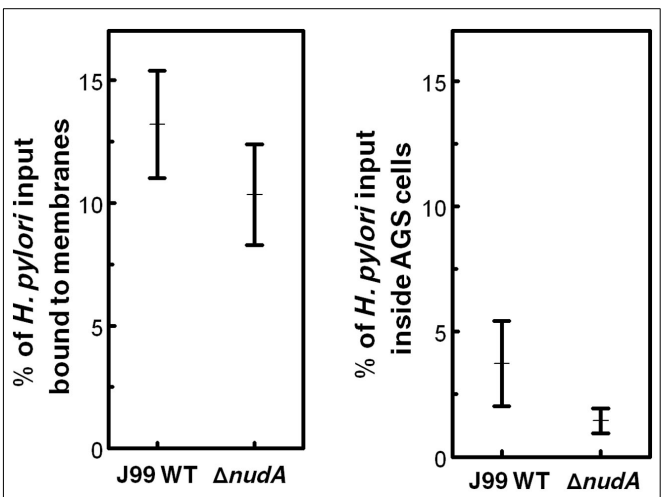

FIGURE 4 | *Helicobacter pylori* NudA invasin and AGS cell entry determined using the "classical" gentamicin protection assay. Values are Means ± SEM. Left panel shows that the density of *H. pylori* bound to AGS cell membranes and expressed as the percentage of bacteria inoculated into the AGS monolayer was not significantly different in WT J99 and the Δ*nudA* mutant ($p = 0.35$). Right panel shows that the density of intracellular *H. pylori* expressed as the percentage of bacteria inoculated into the AGS monolayer was not significantly different in WT J99 and the Δ*nudA* mutant after saponin treatment ($p = 0.23$).

grids with five sections were counted at 10,500× in a selection of three mesh areas at a low magnification (2,600×). The number of cells was around 25–30 cells/mesh and two fields were counted in each mesh.

Morphometry

Qualitative fluorescence light microscopy ranged from 0 (negative reaction) to 4 (maximum red, green and yellow). Quantitative bright light microscopy of semi-thin sections stained with toluidine blue was used to count the density of *H. pylori* located in the extracellular or intracellular compartments, or attached to the cellular membrane of AGS cells using an intraocular grid (Semino-Mora et al., 2003). Three random fields were counted with 1,000× magnification (cell range 77–115/field, mean 96 ± 3/field; total cells counted 300 cells). Elongated cells with hummingbird phenotype were counted as hallmark of infection and compared to normal round cells in uninfected controls (no infection; Schneider et al., 2008).

Quantitative pre-embedding immunogold TEM was performed by counting electron dense gold particles specifically tagging *H. pylori* located in the extracellular, intracellular, and within cell membrane compartments (Tanner et al., 1996). Three grids of each experiment were analyzed as follows: five sections mounted in the nickel grid were counted at 14,000×. Three meshes were selected at low magnification (2,600×) and two fields were counted in each mesh (number of cells counted

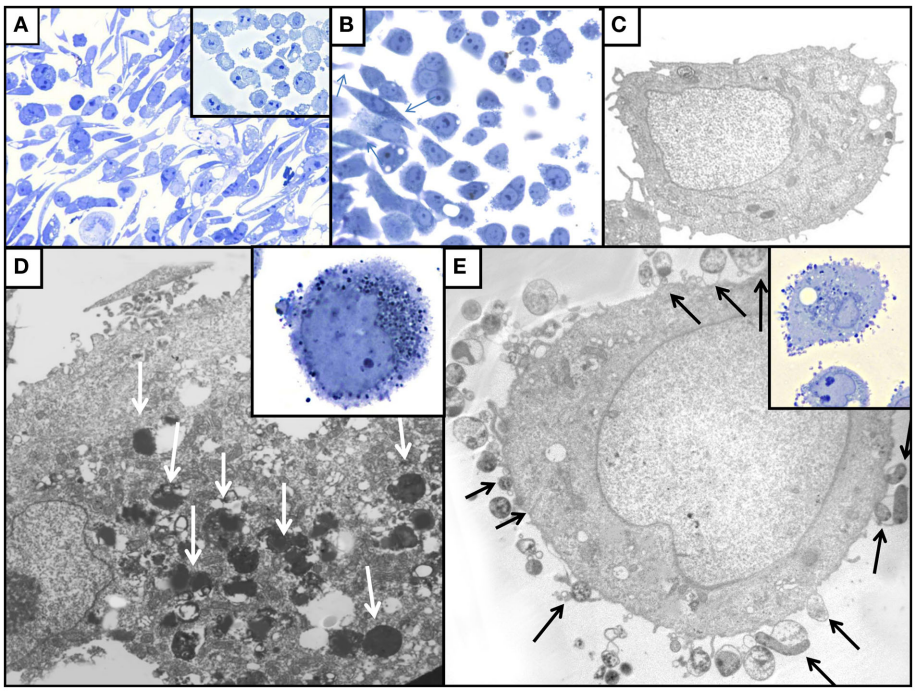

FIGURE 5 | Illustration of the effect of *H. pylori* colonization on AGS cell monolayers in toluidine blue stained sections [(A,B) inserts of (A,D,E)] and by TEM (C,D,E). (A) WT-infected AGS cells; note the presence of numerous elongated hummingbird cells that are absent among control uninfected AGS cells (insert). **(B)** AGS cells infected with Δ*nudA* allele, illustrating that fewer elongated AGS cells (arrows) are present that in WT-infected cells. **(C)** TEM of uninfected AGS cell with normal ultrastructural aspect. **(D)** TEM of AGS cell infected with J99 WT *H. pylori*; note intracellular *H. pylori* (white arrows). **(E)** TEM of AGS cell infected with Δ*nudA* allele; note *H. pylori* attached to AGS cell (arrows); insert shows the numerous Δ*nudA H. pylori* attached to AGS cell and relatively few intracellular bacteria. Original magnification of toluidine blue stained pictures **(A)**: 400×; **(B)** and insert of **(A)**: 1,000×; inserts of **(D,E)**: 1,000×. Original magnification of TEM pictures: **(C)**: 9,800×; **(D)**: 32,500×; **(E)**: 26,000×.

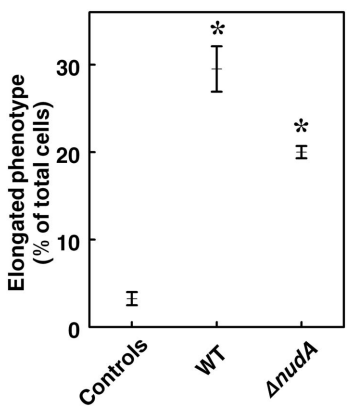

FIGURE 6 | Percentage of AGS cells with elongated ("hummingbird") phenotype in cells infected with WT J99 and Δ*nudA H. pylori* compared to control uninfected cells. Values are Means ± SEM. The percentage of cells with abnormal phenotype is higher in both types of infection (*$p < 0.001$), and also significantly higher in infection with WT *H. pylori* than with the ΔnudA mutant ($p < 0.01$).

∼25–30 cells/mesh) at higher magnification. A 17.5 cm × 23 cm transparent sheet grid with vertical and horizontal lines 1-cm apart with 374 intersections was placed on each micrograph printed on multi contrast Agfa paper. The point counting method was used to calculate the number of immunogold-tagged *H. pylori* as coincidences between bacterial electron dense gold particles and point grid intersections and expressed as a fraction of the total points intersections (Olivero et al., 1990).

STATISTICAL ANALYSIS

Data are reported as mean ± SEM. Comparisons were performed using one way analysis of variance and subsequent *t*-tests.

RESULTS

In a "classical" gentamicin protection assay, the density of *H. pylori* bound to AGS cell membranes and expressed as the percentage of bacteria inoculated into the AGS monolayer was not significantly different in WT J99 and the Δ*nudA* mutant (**Figure 4**, left panel). Similarly, the density of intracellular *H. pylori* expressed as the percentage of bacteria inoculated into the AGS monolayer was not significantly different in WT J99 and the Δ*nudA* mutant after saponin treatment (**Figure 4**, right panel).

The addition of FISH and ultrastructural techniques to the "classical" gentamicin protection assay used in our study allowed a detailed analysis of the precise location of *H. pylori* in relation to the AGS cells, as was observed in the case of invasion of erythrocytes by *B. bacilliformis* (Mitchell and Minnick, 1995). Light microscopy performed on sections stained with toluidine

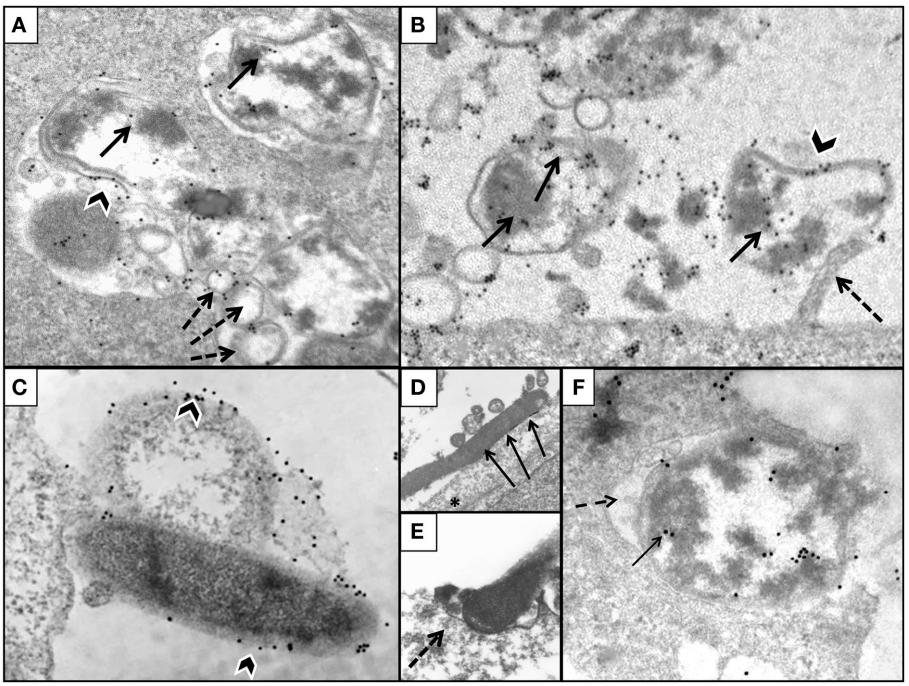

FIGURE 7 | Illustration of AGS cells with pre-embedded *H. pylori* immunogold. (A) AGS cell infected with *H. pylori* WT demonstrating intracellular presence of bacteria located inside vacuoles (gold marked bacterial wall, black arrowhead); note gold particles tagging *H. pylori* cytoplasm (arrow) and spherical membrane vesicles (dashed arrows). **(B)** Δ*nudA* allele with numerous bacteria labeled with gold (arrows) in close association with cell membrane; one of the bacteria is attached to cell pedestal (dashed arrow). **(C)** Mature and coccoid Δ*nudA* allele in close association with AGS cell membrane; note bacterial membrane is tagged with gold (arrowheads). **(D)** Δ*nudA* allele in early stage of adhesion to AGS cell; note multiple fusions between membranes of bacterium and AGS cell (arrows). **(E)** Higher magnification of "zipper-like" Δ*nudA* allele allele entry into AGS cell; note "cup formation" (dashed arrows) identifying *H. pylori* and AGS cell membranes fusing together during internalization. **(F)** Δ*nudA* allele almost completely englobed within invagination of AGS cell membrane; note that this fusion is accompanied by the presence of filamentous strands (fibrils), dense round spheres, vesicles, and amorphous deposits in the space between the membranes of the bacterium and AGS cell (dashed arrow). Immunogold-tagged bacterium (solid arrow) Original magnification **(A–C)**: 41,000×; **(D)**: 24,500×; **(E)**: 32,500×; **(F)**: 41,000×.

blue demonstrated that elongated cells ("hummingbird" phenotype) were more frequently observed in AGS cells infected with WT J99 strain (**Figures 4**, left panel and **5**) than in control uninfected cells ($p < 0.001$; **Figure 5A** insert and **Figure 6**). In Δ*nudA* allele infected cells, most *H. pylori* are associated with the membrane area and cell invasion is minimal but there still are significantly more elongated cells that in the absence of infection ($p < 0.01$) but less than with J99 WT (**Figures 5B** and **6**; $p < 0.01$). Remaining cells are round or square. These results suggest that intracellular *H. pylori* are responsible for the hummingbird transformation of AGS cell in WT-infected cells compared to control uninfected cells, but that membrane associated *H. pylori* may also play a role in the formation of elongated cells.

Transmission electron microscopy indicated that more bacteria were present inside J99 WT-infected AGS cells (**Figure 5D**) than in Δ*nudA* allele infected cells and that more bacteria were closely associated with cell membranes in the latter cells (**Figure 5E**). After infection with J99 WT isolates, more bacteria were observed inside AGS cells (**Figure 5D**) than with the Δ*nudA* allele, but more bacteria were observed within the external surface of AGS cell membranes with the Δ*nudA* allele (**Figure 5E**). More intracellular *H. pylori* were observed inside AGS cell vacuoles than in direct contact with the cytosol, free in the cytoplasm (66.7 vs. 16.7%), the remaining *H. pylori* were partly in a vacuole and partly free in the cytoplasm.

Morphometry of TEM thin sections confirmed these findings, demonstrating that the number of AGS cells infected with J99 WT was significantly greater than with Δ*nudA* infection (39.8 ± 2.8 vs. 12.3 ± 2.2%, respectively; $p < 0.001$). In addition, there were significantly more intracellular *H. pylori* in WT-infected AGS cells than in Δ*nudA* allele infected cells (15.4 ± 2.1 vs. 6.9 ± 1.9; $p < 0.01$; **Figure 5D**), and more *H. pylori* were attached to AGS cells infected with Δ*nudA* allele (8.1 ± 2.6 vs. 0.3 ± 0.2, respectively; $p < 0.01$; **Figure 5E**). Importantly, the number of extracellular *H. pylori* not attached to the plasma membrane was similar in those two groups (4.2 ± 0.9 vs. 5.3 ± 2.0 respectively).

The TEM pre-embedding immunogold method with pre-embedding of *H. pylori* infected AGS cells was more effective to detect hidden epitopes than post-embedding method as reported (Tanner et al., 1996). The use of this method confirmed observations obtained with light microscopy that more WT *H. pylori* were intracellular than in Δ*nudA* allele infection and that more Δ*nudA* allele were attached to AGS cell membranes than in WT infection. In addition, intracellular *H. pylori* WT located inside vacuoles had gold-tagged bacterial walls, cytoplasm (**Figure 7A**), and outer membrane vesicles containing *H. pylori* VacA (**Figure 8**;

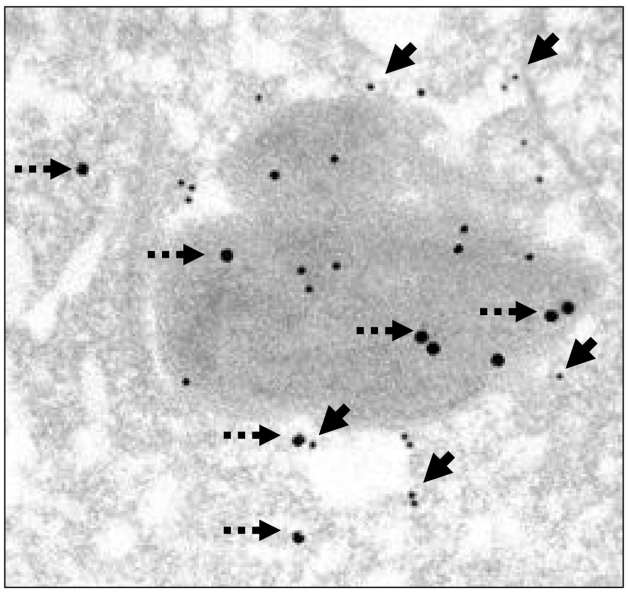

FIGURE 8 | Illustration of AGS cells with intracellular *H. pylori* WT with *H. pylori* and VacA immunogold. AGS cell infected using a dual pre-embedding immunogold (*H. pylori* gold 18 nm and VacA-gold 11 nm) demonstrating the presence of 18 nm *H. pylori* gold particles (dashed arrow) and 11 nm VacA-gold particles in cytoplasm, in the cell membrane, in membrane vesicles, and in the AGS cell. Original magnification 71,000×.

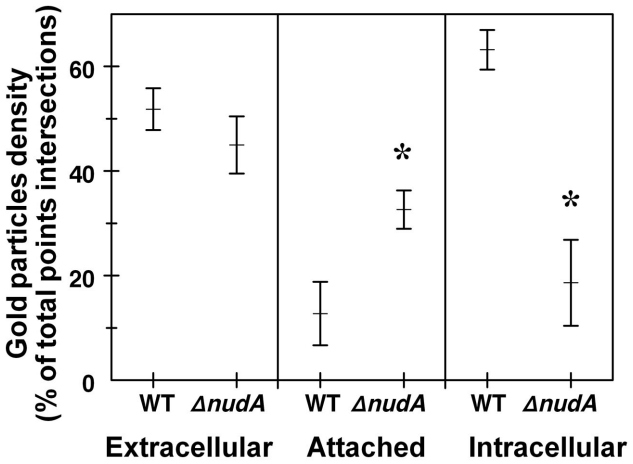

FIGURE 9 | Role of *H. pylori* NudA invasin in AGS cell entry determined using the gentamicin protection assay aided by TEM and anti-*H. pylori* antibody immunogold. *H. pylori* density is expressed as the percentage of total points intersections that were immunogold-positive (see Materials and Methods). Values are Means ± SEM. Left panel shows that the extracellular density of *H. pylori* WT and Δ*nudA* were not significantly different. In contrast, the middle and right panels show that the density of WT *H. pylori* attached to AGS cell membranes was significantly lower than that of the Δ*nudA* mutant (*$p < 0.01$) and the opposite relation for the density of intracellular *H. pylori* (*$p < 0.01$).

Beveridge, 1999; Fiocca et al., 1999; Viala et al., 2004). Similarly, immunogold-labeled Δ*nudA* allele *H. pylori* were closely associated with AGS cell membranes, either attached to cell pedestals (**Figure 7B**) or to AGS cell membranes (**Figure 7C**) or undergoing diverse stages of "zipper-like" entry into AGS cell (**Figures 7D–F**; Noach et al., 1994; Papadogiannakis et al., 2000) Morphometric quantification of these observation demonstrated that most WT *H. pylori* and very few Δ*nudA* allele *H. pylori* were present inside the AGS cells and that the opposite was observed regarding attachment to AGS cell membranes (**Figure 9**; $p < 0.01$).

DISCUSSION

A major finding of the present study was that *H. pylori* invasin appears to play a complex role in the entry of the bacterium into AGS cells *in vitro* if the "classical" gentamicin protection assay is complemented by morphology and morphometry. We first confirmed the previous report that no quantifiable differences were found when using the gentamicin protection assay to compare the invasion frequency of *H. pylori* wild type (WT) with that of a Δ*nudA* mutant in AGS cells allele (Lundin et al., 2003). The authors of the report attributed their observation to the fact that the sensitivity of the "classical" assay may not be sufficient to demonstrate differences in invasion capacity, and this lack of sensitivity may be due to the fact that complete killing of extracellular bacteria is rarely obtained (Amieva et al., 2002). In the present study, light and TEM morphology and morphometry of AGS cells demonstrated that a majority of *H. pylori* were closely associated with cell membranes after infection with the Δ*nudA* allele whereas most WT J99 bacteria were inside the cells. The TEM immunogold observations confirm this observation (**Figures 7** and **9**; $p < 0.01$) and strongly indicate that this difference demonstrates that NudA plays a biologically significant role in *H. pylori* entry into host cells.

The present study also provides precise information on *H. pylori* invasion and bacterial cell entry, demonstrating that *H. pylori* Δ*nudA* allele appears to be unable to proceed beyond the initial attachment to cell membranes that characterize the zipper-like mechanism of *H. pylori* cell entry (Griffin Jr. et al., 1975; Kwok et al., 2002). In contrast, J99 WT *H. pylori* rapidly proceeded from attachment to engulfment and internalization where it was found mostly inside vacuoles formed within AGS cells and, to a lesser extent, free in the cytoplasm. Our observations that gold-tagged outer membrane vesicles containing VacA were closely associated with intracellular *H. pylori* (**Figure 8**) demonstrates that *H. pylori* virulence factors and peptidoglycan may be delivered directly into the AGS cell cytoplasm (Blanke and Ye, 2001; Kaparakis et al., 2010) and suggests an important pathogenic role for cell invasion. This finding is similar to the observation that *H. pylori* releases small vesicles from its outer membrane by a process similar to the release of membrane vesicles by many other bacterial pathogens (Fiocca et al., 1999). The production of these vesicles may represent an important mechanism for bacterial pathogens to modulate their environment within the host and perhaps cause diseases. Finally, separate experiments showed that the *H. pylori nudA* (invasin homolog) gene is well expressed only when *H. pylori* is attached to or inside host cells and that it was closely associated with β1-integrin (Semino-Mora, unpublished).

An additional study of this highly relevant question demonstrated that the extent of adherence and internalization of *H. pylori* by AGS cells increased continuously for at least 12 h, and that a similar number of *H. pylori* were internalized by, and adherent to, AGS cells (Kwok et al., 2002) suggesting again that the "classical" gentamicin assay determines the sum of internalized and adherent *H. pylori*. However, the construction of a Δ*nudA* mutant performed in this study may have modified the expression of neighboring genes that might affect cell invasion. Since genetic complementation of the mutation and restoration of the WT phenotype was not performed as a control, our observations do not exclude the possibility that the internalization defect we observed is related to factors other than the *nudA* gene.

Helicobacter pylori invasiveness is important because intracellular bacteria are more resistant to antibiotic treatment and to immune attack by humoral antibodies. In addition, invasive organisms frequently cause common and severe diseases (Isberg et al., 1987) through mechanisms that are presently unknown (Dubois and Boren, 2007). Importantly, it is now recognized that bacteria invading host cells can induce formation of autophagic vacuoles within macrophages and dendritic cells where they can multiply, interfere with MHC class II surface expression, impair antigen presentation, and immunological defenses (Wang et al., 2009). In turn, these mechanisms would explain persistence of the infection and the various diseases caused by *H. pylori*.

In conclusion, our observations strongly indicate that deletion of the invasin gene limits *H. pylori* to the AGS cell surface, where it appears to be partially protected against gentamicin (Corthesy-Theulaz et al., 1996). In this site, however, it may be more vulnerable to host defense or therapeutic intervention than if intracellular, and less prone to trigger normal immune, carcinogenic, or other developmental response pathways. These data suggest that *H. pylori* invasin gene is important for the rare, but we propose potentially biologically significant, uptake of *H. pylori* by host cells.

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Outer membrane biogenesis in *Escherichia coli*, *Neisseria meningitidis*, and *Helicobacter pylori*: paradigm deviations in *H. pylori*

George Liechti and Joanna B. Goldberg*

Department of Microbiology, Immunology, and Cancer Biology, University of Virginia Health System, Charlottesville, VA, USA

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
David J. McGee, LSU Health Sciences Center - Shreveport, USA
M. Stephen Trent, The University of Texas at Austin, USA
Marcin Grabowicz, Princeton University, USA

****Correspondence:***
Joanna B. Goldberg, Department of Microbiology, Immunology, and Cancer Biology, University of Virginia Health System, 1300 Jefferson Park Avenue, 7230 Jordan Hall, Charlottesville, VA 22908-0734, USA.
e-mail: jbg2b@virginia.edu

The bacterial pathogen *Helicobacter pylori* is capable of colonizing the gastric mucosa of the human stomach using a variety of factors associated with or secreted from its outer membrane (OM). Lipopolysaccharide (LPS) and numerous OM proteins have been shown to be involved in adhesion and immune stimulation/evasion. Many of these factors are essential for colonization and/or pathogenesis in a variety of animal models. Despite this wide array of potential targets present on the bacterial surface, the ability of *H. pylori* to vary its OM profile limits the effectiveness of vaccines or therapeutics that target any single one of these components. However, it has become evident that the proteins comprising the complexes that transport the majority of these molecules to the OM are highly conserved and often essential. The field of membrane biogenesis has progressed remarkably in the last few years, and the possibility now exists for targeting the mechanisms by which β-barrel proteins, lipoproteins, and LPS are transported to the OM, resulting in loss of bacterial fitness and significant altering of membrane permeability. In this review, the OM transport machinery for LPS, lipoproteins, and outer membrane proteins (OMPs) are discussed. While the principal investigations of these transport mechanisms have been conducted in *Escherichia coli* and *Neisseria meningitidis*, here these systems will be presented in the genetic context of ε proteobacteria. Bioinformatic analysis reveals that minimalist genomes, such as that of *Helicobacter pylori*, offer insight into the smallest number of components required for these essential pathways to function. Interestingly, in the majority of ε proteobacteria, while the inner and OM associated apparatus of LPS, lipoprotein, and OMP transport pathways appear to all be intact, most of the components associated with the periplasmic compartment are either missing or are almost unrecognizable when compared to their *E. coli* counterparts. Eventual targeting of these pathways would have the net effect of severely limiting the delivery/transport of components to the OM and preventing the bacterium's ability to infect its human host.

Keywords: membrane biogenesis, *H. pylori*, Bam complex, Lol complex, Lpt complex

INTRODUCTION

Membrane permeability has long been understood as a critical factor of bacterial survival (Hall et al., 1980; Ruiz et al., 2005). A growing field of knowledge regarding the trafficking and insertion of bacterial membrane components has succeeded in characterizing the key factors involved in membrane biogenesis in a variety of model systems. The eventual targeting of these essential barriers by molecular inhibitors or using them as vaccine candidates has the potential for inducing either bactericidal effects outright, or increased susceptibility to more established antibiotics (Chiu et al., 2007, 2009; Ghanei et al., 2007). Enzymes/proteins found in the cytoplasm, periplasmic space, and outside the bacterial membrane have all evolved under the confines and conditions of each of these unique microenvironments. While localization of these proteins to their ultimate destination within the cell is often essential, the controlled interaction and movement of signaling molecules/nutrients/metabolites between these regions and spaces is also critical for basic microbial metabolism. More recently, studies characterizing bacterial mutants with membrane permeability defects have begun to establish that nearly all permeability deficiencies arise from mutations in the protein machinery that make up the transport apparatus for the basic components of bacterial membranes: lipoproteins, lipopolysaccharide, and outer membrane proteins (OMPs). These fundamental transport pathways are for the most part well-conserved throughout Gram-negative bacteria (Nikaido, 2003; Ruiz et al., 2006). One notable exception to this exists in the bacterial class of ε proteobacteria. Consisting of genera spanning digestive tract colonizing symbionts/pathogens (Marshall and Warren, 1984) to deep-sea thermal vent chemolithotrophs (Takai et al., 2005), this bacterial class contains numerous extremophiles for whom membrane barrier function should arguably play an indispensible role. However, upon closer bioinformatic examination, the transport pathways for each of the three major outer membrane (OM) components in

ε proteobacteria appear to have significantly diverged when compared with most other Gram-negative bacteria (Cummings et al., 2002).

One member of this class of ε proteobacteria with significant medical relevance is the gastric pathogen *Helicobacter pylori*. Able to survive and colonize the mucosal layer in close proximity to the human gastric epithelium (Blaser, 1992), *H. pylori* is capable of inhabiting an environment with an acidic pH. While adaptations to this gastric environment include motility/chemotaxis (Foynes et al., 2000; Ottemann and Lowenthal, 2002; Croxen et al., 2006) and the production of urease, which can substantially buffer the pH around the bacterium (Scott et al., 2000; Weeks et al., 2000), maintaining a level of barrier function from the OM remains an essential element in allowing this chronic colonizer to survive in this extreme environment. In addition, each of the previously mentioned transport pathways play key roles in permitting *H. pylori* to chronically colonize its human host.

This review highlights what is known of the three pathways involved in membrane biogenesis, specifically the transport of three major components/transport systems of the OM in the majority of Gram-negative bacteria: lipoprotein, LPS, and OMPs. The importance of each of these three membrane components in *H. pylori* will be discussed in terms of relevance to infection. Each transport pathway will be examined bioinformatically and the implications for potential targets for future small molecule inhibitors and candidates for vaccine development analyzed. Special attention will be given to the identification of the periplasmic components for these transport pathways, as it appears that many are significantly divergent from those found in other model bacteria or perhaps are even absent in *H. pylori* entirely (**Figure 1**). Given the continued prominence of this bacterium in the developing world (Frenck and Clemens, 2003), the current state of vaccine development for this pathogen (Czinn and Blanchard, 2011), and an unsettling rise in the number of reports of antibiotic resistant strains (Boyanova and Mitov, 2010; De Francesco et al., 2010), identifying novel targets for future antimicrobials is of paramount importance.

LIPOPROTEINS

ROLE IN INFECTION AND PATHOGENESIS

A large number of putative lipoproteins have been found in the *H. pylori* genome (Tomb et al., 1997). Their abundance and

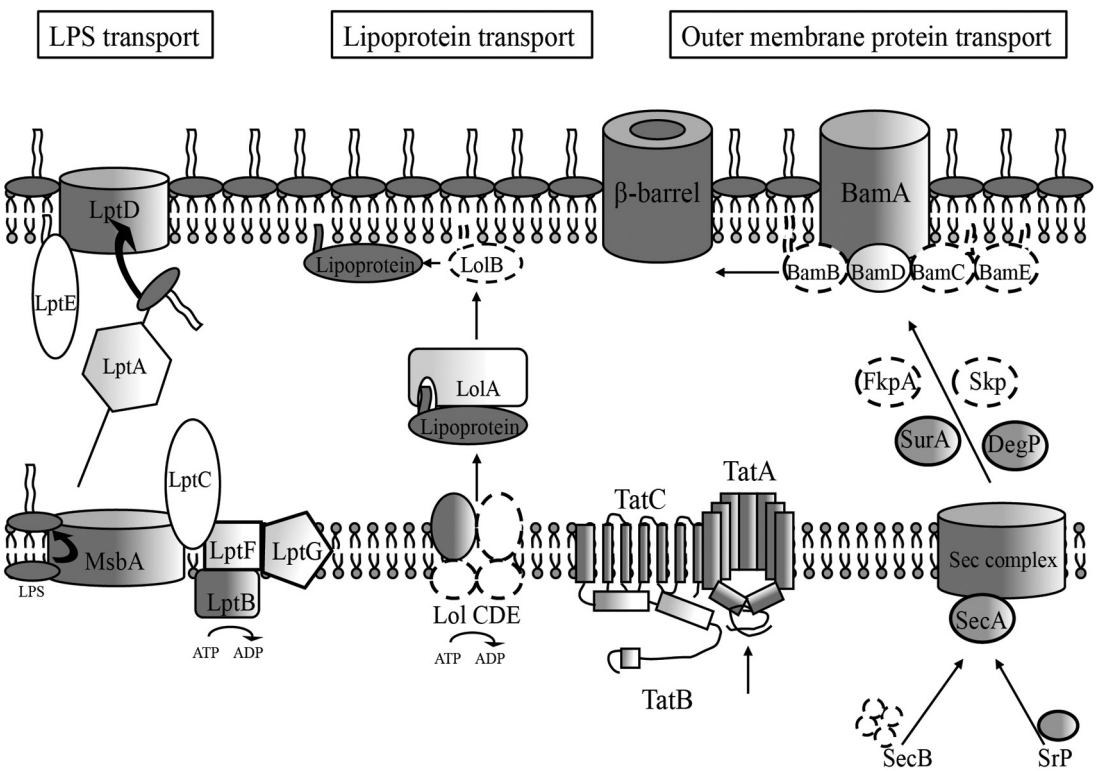

FIGURE 1 | Membrane biogenesis pathway conserved features in *H. pylori*. Dark features indicate high similarity scores ($\leq e^4$) for *H. pylori* genes compared to *E. coli* outer membrane biogenesis pathway components, which generally consist of the IM and OM spanning portions of the pathway. Lighter shaded components indicate lower similarity scores requiring comparison of numerous bacterial homologs before attaining a significant similarity score ($\leq e^4$) for an *H. pylori* locus. An absence of shading indicates no homolog for that component was found through sequence similarity scores, but the existence of a potentially functional protein exists, given the presence of a conserved genetic orientation at the particular locus and/or structural similarity as determined by crystal structure analysis software (RaptorX). Dotted lines indicate a complete genetic absence of homology of a pathway component. Those proteins with low sequence similarity, low structural similarity, and apparent absence of sequence and structural similarity make up most of the components found in the periplasmic regions for each of the three OM transport pathways.

potential for facilitating extensive linkages between inner and OMs has been hypothesized to explain the difficulty reported in experimentally separating inner and OM layers in most *Helicobacter* and *Campylobacter* species (O'Toole and Clyne, 2001). Few lipoproteins have been well-characterized in *H. pylori*, but those that have been studied play important roles in infection and the host immune response. One such lipoprotein is the highly conserved *H. pylori* adhesin A (HpaA), which was originally characterized as a neuraminyllactose-binding hemagglutinin (Evans et al., 1988, 1993), and was shown later to be a lipoprotein (O'Toole et al., 1995). HpaA has since been studied in depth, due to its conserved nature in most strains and its role in bacterial adhesion. Localization of HpaA has been highly disputed in the field, with some studies pointing to flagellar sheath localization (Luke and Penn, 1995), others showing cytoplasmic localization (O'Toole et al., 1995), and others simply showing membrane association (Bolin et al., 1995; Lundstrom et al., 2001). Due to its putative adhesin classification and putative membrane association, HpaA has been examined for use as a potential vaccine candidate (Flach et al., 2011). Similarly, another membrane-associated lipoprotein in *H. pylori* is Lpp20, first characterized as an 18-kilodalton OM antigen (Keenan et al., 2000), has been shown to be immunodominant in several studies (Keenan et al., 2002; Bakos et al., 2003). While these two lipoproteins are currently the only well-examined examples in *H. pylori* to date, the presence of several other recognized lipoproteins involved in the transport pathways of both LPS and OMPs (which will be discussed subsequently) adds additional importance to this class of membrane components.

CURRENT MODEL FOR LIPOPROTEIN MEMBRANE INTEGRATION

The mechanism for lipoprotein trafficking has been well-characterized in *E. coli* (Matsuyama et al., 1995, 1997; Yakushi et al., 2000). In this system, lipoproteins are transported to the inner membrane via the Sec pathway, embedded into the membrane, and are subsequently sorted according to the presence or absence of an inner membrane (IM) retention signal (Masuda et al., 2002). Lipoproteins lacking the signal are transported to the OM. Early lipoprotein transport studies utilized spheroplasts that were generated by EDTA-lysozyme treatment, making them devoid of periplasmic proteins (Matsuyama et al., 1995). While these spheroplasts continued to secrete proteins from the cytoplasm that are normally destined for the periplasm and OM, the major OM lipoprotein (Lpp) was not secreted and remained in the IM, which suggested that a periplasmic factor was required for OM lipoprotein release from the IM (Matsuyama et al., 1995). When periplasmic-derived materials were added externally to the spheroplasts, Lpp was released from the IM, and purification of the periplasmic components identified the periplasmic chaperone LolA (Masuda et al., 2002). Further research involving the generation of various proteoliposomes went on to characterize the OM component of the trafficking pathway, LolB (Matsuyama et al., 1997), whose role is the insertion of lipoproteins into the OM. Further analysis identified integral membrane proteins LolC and LolE, as well as a soluble, cytoplasmic ATP-binding protein (LolD) that is recruited by the membrane-associated heterodimer LolCE to form a complex that acts as an ATP-binding cassette (ABC) transporter in the IM (Yakushi et al., 2000; Ruiz et al., 2006). Lipoproteins are taken up by this complex and their lipid moiety bound to LolA, which is believed to protect their fatty-acid chains from the aqueous environment of the periplasm. LolA then transports them to an OM-associated lipoprotein, LolB, which stabilizes their internalization into the OM. Exactly how LolB inserts lipoproteins into the OM has yet to be elucidated. LolA and LolB share similarity of structure, both possessing hydrophobic cavities believed to be the binding sites for the lipid moieties of lipoproteins (Takeda et al., 2003). The cavities are comprised of unclosed β-barrels with α helical lid domains thought to close when not in complex with any lipoprotein (Takeda et al., 2003). It is worth noting that LolC and LolE are highly similar in sequence, and that unlike the Lol complex found in *E. coli*, the integral membrane portion of the complex in *N. meningitidis* appears to consists of only a single LolCE homolog (encoded by NMB1235) instead of two. This can potentially be explained in that the single *N. meningitidis* protein homolog may simply form a homodimer to supplement for the missing protein component (Bos et al., 2007). Given the essential nature of most of these Lol complex components in *E. coli* (Narita et al., 2002) and the large number of lipoproteins encoded in the *H. pylori* genome previously mentioned (Alm and Trust, 1999; Baltrus et al., 2009), their use as potential antimicrobial targets is promising.

BIOINFORMATICS OF PUTATIVE *H. PYLORI* LIPOPROTEIN TRANSPORT SYSTEM

A single, IM component of the Lol complex from *E. coli* appears to be conserved in *H. pylori* (HP0787; **Table 1**), with highest similarity to LolE. As mentioned previously, LolE and LolC are highly similar in *E. coli* and it has been postulated that possessing only one of these proteins may be adequate for Lol complex formation (Bos et al., 2007), as in the *N. meningitidis* model. Insertion mutations in HP0787 exist (Salama et al., 2004), however this result indicating a potential lack of essentiality of this gene should be interpreted with caution, as the generation of deletion mutants for this gene have not been possible to date (Liechti and Goldberg, unpublished results). A few genome sequencing projects have assigned *lolD* designations to a putative *H. pylori* homolog (strain B8, HPB8_1387/HP0179) (Farnbacher et al., 2010), however, this characterization is based solely on the ABC transporter domain, and upon closer inspection, the N-terminus of the LolD protein (approximately 100 amino acids) is missing from this *H. pylori* gene (similar dubious gene designations have been assigned in *C. jejuni*). While no definitive LolD homolog appears to exist in *H. pylori* as such, ABC transport domains are notoriously divergent in this bacteria compared with other microbes (Tomb et al., 1997), and dozens of potential candidates with similar domain structure exist in the genome whose functions have not yet been identified (Tomb et al., 1997; Marais et al., 1999). In addition, LolB (the lipoprotein involved in inserting new lipoproteins into the OM in *E. coli* and *N. meningitidis*) appears to be absent in *H. pylori,* and in fact is missing in all ε proteobacteria sequenced to date. A protein with poor genetic similarity to LolA, the periplasmic chaperone component, does exist in

Table 1 | *H. pylori* putative outer membrane biogenesis complex components.

H. pylori locus*	*E. coli* homolog	*E* value	% id./% sim.**	Function
Lol COMPLEX				
HP0785	*lolA*	***	***	Periplasmic chaperone
HP0787	*lolC*	$5e^{-24}$	23%/48%	Integral membrane protein
N/A	*lolB*	–	–	OM associated lipoprotein
N/A	*lolD*	–	–	IM ABC transporter domain
(HP0787)	*lolE*	$4e^{-25}$	24%/49%	Integral membrane protein
Lpt COMPLEX				
HP1568	*lptA*	***	***	Periplasmic chaperone
HP0715	*lptB*	$3e^{-90}$	55%/75%	IM ABC transporter domain
HP1569	*lptC*	ns.	ns.	IM associated lipoprotein
HP1216	*lptD*	$2e^{-08}$	26%/49%	OM LPS transport protein
HP1546	*lptE*	ns.	ns.	OM associated lipoprotein
HP0362	*lptF*	***	***	Integral membrane protein
HP1498	*lptG*	***	***	Integral membrane protein
HP1082	*msbA*	$2e^{-79}$	31%/57%	IM LPS flippase
BamA COMPLEX				
HP0655	*bamA*	$5e^{-24}$	24%/44%	OM β-barrel assembly component
N/A	*bamB*	–	–	OM associated lipoprotein
N/A	*bamC*	–	–	OM associated lipoprotein
HP1378	*bamD*	***	***	OM associated lipoprotein
N/A	*bamE*	–	–	OM associated lipoprotein
Sec PATHWAY				
HP0786	*secA*	0.0	49%/64%	Preprotein translocase subunit
N/A	*secB*	–	–	Protein export chaperone
HP1300	*secY*	$7e^{-105}$	42%/67%	Preprotein translocase subunit
HP1203a	*secE*	***	***	Preprotein translocase subunit
HP1255	*secG*	0.001	35%/61%	Preprotein translocase subunit
HP1550	*secD*	$2e^{-86}$	37%/57%	Preprotein translocase subunit
HP1549	*secF*	$2e^{-51}$	35%/62%	Preprotein translocase subunit
HP1551	*yajC*	$3e^{-17}$	44%/76%	Preprotein translocase subunit
HP1450	*yidC*	$1e^{-73}$	37%/62%	IM protein translocase component
HP1152	*ffh*	$1e^{-102}$	40%/64%	Signal recognition particle (SRP)
HP0763	*fts*Y	$2e^{-62}$	50%/70%	SRP receptor
Tat PATHWAY				
HP0320	*tatA*	$8e^{-06}$	52%/71%	Sec-independent translocase
HP1060	*tatB*	0.036	32%/63%	Sec-independent translocase
HP1061	*tatC*	$4e^{-39}$	35%/57%	Sec-independent translocase
PERIPLASMIC CHAPERONES				
HP0175	*surA*	$5e^{-08}$	32%/46%	Peptidyl-prolyl *cis*-trans isomerase
HP1019	*degP*	$9e^{-83}$	42%/62%	Serine protease
N/A	*skp*	–	–	Periplasmic chaperone
N/A	*fkpA*	–	–	Peptidyl-prolyl *cis*-trans isomerase

Locus defined using H. pylori strain 26695 designations.

*** % Identity and % similarity scores were based off of the regions of similarity as determined by NCBI's BLAST software, and as such represent %s only of the designated regions being aligned and not the entire proteins being examined.*

**** Indicates E. coli first required confirmation in other ε proteobacteria (Campylobacer jejuni, etc.) prior to identifying the H. pylori homolog.*

Abbreviations: N/A, indicates no homolog or putative homolog found in any sequenced H. pylori strain found in the NCBI database; OM, outer membrane; IM, inner membrane; ABC, ATP-binding cassette; ns., no similarity found at the sequence level (annotation based on gene localization alone).

H. pylori (HP0785, *E* value = 0.03) and has been shown to be non-essential (Chalker et al., 2001). Whether this protein acts similarly to the LolA characterized in *E. coli* has yet to be discerned, however amino acid similarity alignments between HP0785 and LolA show significant similarity for the core functional regions of the chaperone, with greatest similarity existing on one of the two sides of the open β-barrel domain (**Figure 2**). When examining the *H. pylori* amino acid sequence for the less well-conserved side

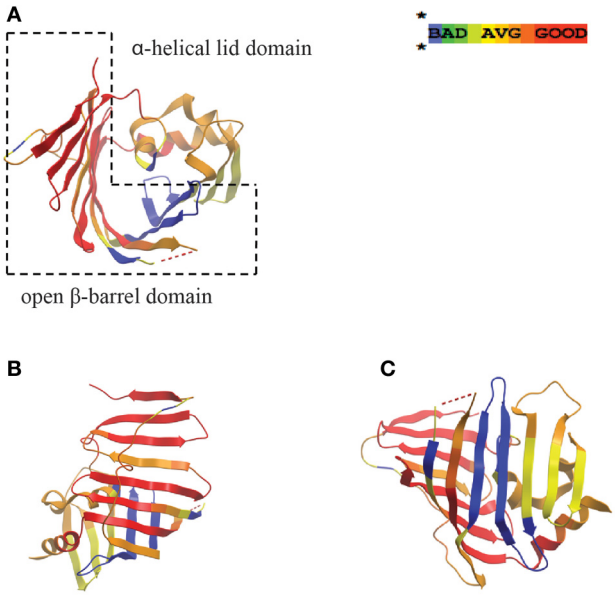

FIGURE 2 | LolA periplasmic lipoprotein chaperone from *E. coli* and *H. pylori* display structural homology at N-terminal region. Similarity protein alignments were constructed between *E. coli* and *H. pylori* LolA (HP0785) amino acid sequences and a "heat map" color scheme of protein homology based on similarity scores was generated. Crystal structures of *E. coli* LolA (PDB ID: 3KSN) were then used as a template for mapping the similarity scores. Red indicates very high confidence whereas green/blue indicate lower confidence in similarity comparisons. Views presented are an overhead of the putative, lipoprotein binding domain consisting of the open β-barrel with α helix lid **(A)**, the well-conserved, N-terminal β-sheet region **(B)**, and the less conserved, C-terminal β-sheet region **(C)** (the two β-strands missing in *H. pylori* LolA are indicated in blue). Protein sequence alignments and similarity scores were generated using T-Coffee analysis software (Poirot et al., 2003), the "heat map" CORE index for the alignment color gradient used algorithms described in the literature (Notredame and Abergel, 2003), and crystal structures were obtained from the Protein Data Bank (PDB) and viewed using ICM Brower Pro (MolSoft). The dotted red line in model represents an region in the amino acid protein sequence not present in the solved crystal structure.

of the β-barrel domain, amino acids that make up two of the β strands in this region in the *E. coli* structure are absent in the *H. pylori* sequence (**Figure 2**, shown in blue) potentially indicating a smaller, more tightly packed β sheet on that side of the open β-barrel domain.

The genetic orientation of the Lol complex-associated genes in ε proteobacteria is, in a word, perplexing. In *E. coli*, *lolCDE* (and in *Neisseria*, *lolCD*) are found in a single operon (**Figure 3A**), with *lolB* and *lolA* each existing separately, elsewhere in the genome (Yakushi et al., 2000). In most ε proteobacteria however, the single conserved homolog of *lolC* appears in an operon with *secA*, the cytoplasmic-localized membrane translocase. While this proximity to an essential gene may explain problems in targeting *lolC* for deletion in *H. pylori*, an added caveat exists. The *H. pylori lolA* homolog is, coincidentally, also located proximal to *secA*, albeit transcribed in the opposite direction (**Figure 3B**), potentially hinting at some selective element as of yet undetermined in either protein or lipoprotein trafficking in ε proteobacteria. Given the apparent absence of a *lolD* homolog at this locus combined with the presence of the membrane protein translocation ATPase (SecA) in its place, overlap in the Sec export pathway and the lipoprotein transport pathway in ε proteobacteria may be a distinct possibility.

It has previously been shown that a lipoprotein signal peptidase gene (HP0074) is essential in *H. pylori*, indicating that lipoproteins in general are important for *H. pylori* survival (Chalker et al., 2001). The presence of a potentially essential *lolE* homolog in the genome would support this hypothesis. The apparent absence of a *lolB* homolog in addition to *lolA* proving to not be essential however, indicates that if this transport pathway is still functional in *H. pylori*, that it may possess redundant/alternate protein components for lipoprotein periplasmic transport and OM insertion. Taken together, despite initial observations indicating a defunct pathway, the lipoprotein transport pathway in *H. pylori* is potentially viable, and given the prominence of lipoproteins encoded in the genome, this pathway represents a potential molecular target worthy of full characterization and further pursuit.

LIPOPOLYSACCHARIDE

ROLE IN INFECTION AND PATHOGENESIS

In most Gram-negative bacteria, the major target for the immune system during a bacterial infection is lipopolysaccharide (LPS) (Chow et al., 1999; Miyake, 2004). LPS is a key component of a Gram-negative OM, contributing to the structural integrity of the bacteria as well as providing a barrier against the host immune system. A number of cellular receptors recognize bacterial LPS and upon binding they induce an innate immune response (Caroff et al., 2002). The primary activator of the host response to the lipid A portion of LPS from *E. coli* is Toll-like receptor 4 (TLR4) (Chow et al., 1999). In many cases, the immune response resulting from the presence of LPS (also referred to as endotoxin) is so severe, that a dysregulated immune response to infection develops, known as sepsis, which can be life threatening. Studies are currently underway to develop molecular inhibitors of LPS, specifically targeting its ability to induce monocyte activation (Bosshart and Heinzelmann, 2007).

In examining the two predominant model microbes in the field of OM biogenesis, it is worth noting that differences in their LPS exist at a structural basis. While *E. coli* possesses true LPS, *Neiserria* expresses lipooligosaccharides (LOS) lacking the repeating O-antigen of most enteric bacteria; this distinction is thought to account for many of the dissimilarities in the immune response to the different bacteria (Zhang et al., 2007). *H. pylori* does produce a defined length O-chain attached to its core region whose length is variable between strains. Similar to LPS biogenesis in other bacteria, the O-chain of *H. pylori* is constructed in the cytoplasm and flipped to the periplasm where it is transferred to the core oligosaccharide by the O-antigen ligase (WaaL). Because the host's immune response to LPS is so robust, many pathogenic bacteria express an LPS structure that avoids detection. *H. pylori* is a well-studied example, as it can express the Lewis Blood Group (LBG) antigens and present them on the O side chain of its LPS (Appelmelk et al., 1996; Monteiro, 2000). LBG antigens are commonly associated with host monocytes, macrophages, granulocytes, and gastric epithelial cells. They are

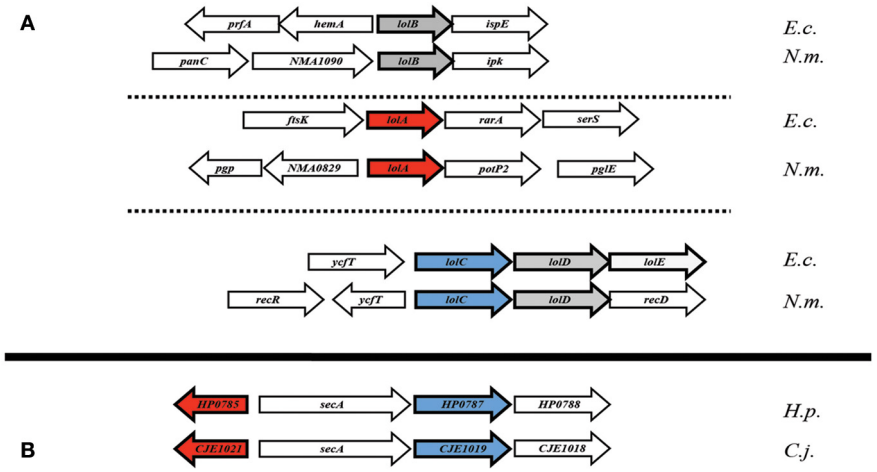

FIGURE 3 | Genetic orientation of Lol complex-homologs. (A) Lol complex associated loci (*lolA* and *lolB*, specifically) from *E. coli* and *N. meningitidis* appear spread throughout the genome **(B)** while complex-homologs from *H. pylori* and *C. jejuni* appear to be clustered around the cytoplasmic chaperone encoding gene, *secA*. Abbreviations: *E.c.*, *Escherichia coli* (K12 substr. W3110); *N.m.*, *Neisseria meningitidis* (strain Z2491); *H.p.*, *Helicobacter pylori* (strain 26695); *C.j.*, *Campylobacter jejuni* (strain RM1221).

important for low-affinity adhesion to host gastric epithelial cells as well as in phagocytosis (Kirkland et al., 1997; Lepper et al., 2005) and it has been widely speculated that *H. pylori* uses this key feature to evade the host immune system (Appelmelk et al., 1996; Moran et al., 1999). The genes responsible for the creation of these bacterial LBG antigens are present throughout the genome, and many of them undergo phase variation (Wang et al., 2000). The variation generated in the bacterial population, whether due to phase variation or other mechanisms, results in the production of a wide array of differing LPS profiles within a given population. This is believed to be a "fine-tuning" of the bacterium's defenses, further enhancing the evasion of the immune response (Wang et al., 2000; Khamri et al., 2005; Salaun et al., 2005; Salaun and Saunders, 2006)

In addition, *H. pylori* modifies the lipid A portion of its LPS. When first characterized, the lipid A from *H. pylori* was found to be unique and distinguishable from that of enterobacteria in that there is an absence of a phosphoryl group at the 4′-position and fatty acyl groups at the 3- and 3′-position, as well as the presence of 2-aminoethyl phosphate at the 1-position (Suda et al., 1997). Further studies went on to show that *H. pylori* actually synthesizes two lipid A species, a minor one resembling that of *E. coli* (bis-phosphorylated and hexa-acylated) and a major species that contains fewer acyl chains, fewer phosphate groups, and a single 2-keto-3-deoxyoctonate (Kdo) sugar attached to the disaccharide backbone (Moran et al., 1997; Tran et al., 2005). This led to the discovery that *H. pylori* modifies its lipid A and that the result is a molecule with much lower immunobiological activity (Tran et al., 2005). Additional studies identified that while the lipid A from *H. pylori* is initially synthesized in this hexa-acylated form, a series of modifications after synthesis can result in a tetra-acylated molecule (Stead et al., 2005, 2008).

Lipid A is the "hydrophobic anchor" of the LPS, and is the biological initiator of septic shock (Raetz and Whitfield, 2002). Modifications of the lipid A portion of *H. pylori* LPS results in reduced immuno-reactivity by as much as 1000 times compared to the lipid A of other bacterial species (Ogawa et al., 1997, 2003a,b; Stead et al., 2005). The exact mechanism by which *H. pylori* LPS interacts with the immune system is currently in dispute. While it was initially characterized that the host receptor for *H. pylori* LPS was TLR4 (Kawahara et al., 2001) subsequent papers have shown that *H. pylori* interacts through TLR2 and not TLR4 (Smith et al., 2003). This controversial finding is apparently not limited to *H. pylori*, and the same phenomenon has been shown for other Helicobacters as well (Mandell et al., 2004). While another study exists, showing that chemically synthesized lipid A from *H. pylori* signaled via TLR4 (Ogawa et al., 2003a,b), highly purified *H. pylori* LPS in an alternate study showed activation only through the TLR2 complex (Yokota et al., 2007). TLR4 expression in gastric cell lines has also been used as evidence of *H. pylori* LPS activation (Kawahara et al., 2001; Su et al., 2003; Schmausser et al., 2004), however in a recent paper, it was shown that *H. pylori* LPS first signals via TLR2, which then acts through nuclear factor Y (NF-Y) to activate the transcription of TLR4 (Yokota et al., 2010). Whatever the mechanism, the presentation of LPS on the OM of *H. pylori* is essential for colonization and pathogenesis partially for to its role in membrane barrier function, but also for its unique ability to mask this pathogen from the host immune system.

CURRENT MODEL FOR LPS INTEGRATION WITH THE OUTER MEMBRANE

The majority of the work on the LPS transport (LPT) pathway has been conducted in *E. coli* and *N. meningitidis*, and the pathways are summarized visually in **Figure 1**. Each model bacterial system provides differing tools that allow the system to be analyzed in a slightly different way. For example, LPS is not essential for *N. meningitidis*, allowing for comprehensive mutant studies to be conducted, while the *E. coli* system provides a high degree of technical genetic manipulation to artificially deplete components and

analyze the effects. Both systems have shown that the transport pathway consists of two major membrane proteins, the flippase MsbA in the IM and the β-barrel IMP/LptD in the OM. MsbA allows for LPS in the cytoplasmic IM leaflet to be "flipped" onto the periplasmic leaflet. Once there, LPS is extracted from the IM in a process believed to be powered by an ABC transporter consisting of two integral membrane proteins (LptF and LptG) as well as an additional IM protein located solely on the cytoplasmic face of the membrane (LptB). LPS moves from the IM LptBCFG complex to the OM LptDE complex, traversing the periplasm via LptA (Ma et al., 2008; Tran et al., 2008). LptA recognizes the lipid moiety of LPS and is thought to protect the highly hydrophobic lipid element from the aqueous periplasm during transport. The energy source that powers this movement of LPS across the periplasm has yet to be elucidated.

MsbA

MsbA was initially identified as a multi-copy suppressor of *lpxL* (*htrB*) null mutants (Karow and Georgopoulos, 1993), originally characterized as having growth and viability defects due to the accumulation of phospholipids (Karow et al., 1991). Both *htrB* null mutants and temperature sensitive *msbA* conditional mutants accumulated a precursor for LPS (*N*-acetyl [^{3}H]-glucosamine) in the IM (Polissi and Georgopoulos, 1996). Upon subsequent analysis, *msbA* showed high homology to various ABC transporters; one study using a temperature-sensitive *msbA* allelle constructed in a polymyxin-resistant background strain of *E. coli* showed that LPS recovered from the inner membrane did not contain modifications to the lipid A required for polymyxin resistance (Doerrler et al., 2004). These modifications (the covalent modification of lipid A with cationic substituents 4-amino-4-deoxy-L-arabinose and phosphoethanolamine) are believed to occur at the periplasmic leaflet of the IM, and their absence strongly indicated that the LPS in these mutants was, therefore, localized in the cytoplasmic-facing, lipid leaflet (Zhou et al., 1998; Doerrler, 2006).

Attempted mutant studies determined that *msbA* is essential for in *E. coli* (Zhou et al., 1998), and this was independently confirmed in *Pseudomonas aeruginosa* (Ghanei et al., 2007). Similar studies were conducted in *N. meningitidis*, with somewhat differing results: total LPS levels for the mutant *msbA* strain were shown to be lower in the parental strain, indicating the potential presence of a feedback inhibition pathway not seen in *E. coli* (Tefsen et al., 2005). It has been speculated that because the LPS appears to localize on the cytoplasmic side of the IM in *E. coli*, that MsbA plays a role in the flipping of the lipid A-core oligosaccharide onto the periplasmic side of the membrane. This flippase activity has since been confirmed in a reconstituted system (Eckford and Sharom, 2010). It is believed that MsbA forms a homodimer with a chamber oriented toward the cytoplasm capable of accommodating lipid molecules, a finding supported by crystal structure analysis (Ghanei et al., 2007; Ward et al., 2007; Eckford and Sharom, 2008).

LptD

The identification of the OM protein LptD (Imp/OstA) was revealed through screening *E. coli* mutant libraries for strains with alterations in their membrane permeability (Sampson et al., 1989; Aono et al., 1994). *lptD* mutants resulted in an increased permeability of OMs to maltodextrins, dyes, as well as antibiotics (Sampson et al., 1989). In addition, it has been established that *lptD* is regulated by sigma factor E, a stress response regulator that reacts to disruptions of LPS structure and found to up-regulate various proteins involved in membrane biogenesis, such as periplasmic and cytoplasmic chaperones (Dartigalongue et al., 2001). Overexpression of LptD was only possible when LptE was also overexpressed simultaneously, and the two proteins were found to form a stable, heterodimeric complex (Chng et al., 2010). In *N. meningitidis*, inactivating *lptD* gave results similar to those found in the *E. coli msbA* conditional mutants: significantly reduced total cellular LPS, further confirming the linkage between LPS synthesis and transport in this model system (Braun and Silhavy, 2002; Bos et al., 2004). By using a PagL induction system (PagL acts by removing an acyl chain on the LPS after it is incorporated into the OM) researchers were able to show that the LPS isolated from *N. meningitidis* strains with an inactivated *lptD* showed no signs of this modification, indicating that the LPS was not transported to the outer leaflet of the OM (Bos et al., 2004). Studies in *E. coli* identified a complex partner of LptD, a lipoprotein originally named RlpB and now denoted as LptE. Conditional mutants for LptE were similar to LptD mutants in that they resulted in an increase in total cellular LPS (Wu et al., 2006).

LptA and LptB

Screening for essential genes in *E. coli* lead to the classification of other proteins involved in envelope biogenesis. The proteins LptA and LptB were identified, and when these proteins were down regulated, phenotypes similar to *lptD* and *msbA* mutants were observed. In addition, the genes encoding these two proteins were found together in an operon (Sperandeo et al., 2007). In contrast with *E. coli*, in the *N. meningitidis* model, these genes are not essential, and when deletion mutants were generated, they were found to have severely reduced LPS production, similar to *lptD* and *msbA* mutants. Upon sequence and structural analysis, LptA was found to have no membrane spanning domains while also sharing a conserved N-terminal domain with Imp, indicating a common but as yet un-described function. The operon partner (*lptB*) encodes a protein that possesses the general features of a typical ABC transporter but also lacks any membrane spanning regions (Sperandeo et al., 2007). LptB exists as a 27-kDa protein that is found in a 140-kDa complex (Stenberg et al., 2005) and biochemical evidence of this LptB-containing complex has also been reported (Narita and Tokuda, 2009). LptB works in conjunction with LptC (previously YrbK) in the complex with LptBFG, an ABC transporter involved in the transport of LPS. LptA is believed to act as a periplasmic chaperone, delivering LPS to the OM, where LptD acts to orient it (Sperandeo et al., 2007; Tran et al., 2008).

While the components of the transport system are characterized, the exact mechanism of transport through the periplasm is still in dispute. The two competing hypotheses differ not in what proteins are involved but rather their orientation to each other during transport. The chaperone hypothesis proposes that

LptA acts similarly to LolA of the lipoprotein transport pathway or Skp, DegP, and SurA of the membrane protein transport pathway (Sperandeo et al., 2007). The competing hypothesis is the "periplasmic bridge" model (Bayer, 1991; Tefsen et al., 2005; Sperandeo et al., 2007) in which all components together make up a protein bridge that effectively connect both membranes and simply pass LPS between themselves until it arrives in the OM. Characterized crystal structures of long, fiber-like structures formed by multiple LptA molecules arranged in head-to-tail fashion while in the presence of LPS (Suits et al., 2008; Merten et al., 2012) seem to support the periplasmic bridge model.

A number of other studies seem to favor this "periplasmic bridge" or "trans-envelope" model. Lpt proteins have been shown to colocalize and can be found in both OM and IM preparations (Chng et al., 2010). LptA was first believed to be soluble and localized only in the periplasm (Sperandeo et al., 2009) however, subsequent experimentation has shown that LptA preferentially interacts with membranes and that polyhistidine tags that were used to purify the protein are potentially culprits in effecting the localization that was observed in previous studies (Chng et al., 2010). Subsequent experimentation has also shown that defects in components of the Lpt pathway in either the OM or IM result in LptA degradation, potentially indicating that LptA levels can act as a marker for proper formation of the transmembrane bridge (Sperandeo et al., 2011). The evidence of the existence of these bridges appears definitive, however, whether these bridges are transient or always present remains to be elucidated.

LptC and LptE

The roles of the stabilizing proteins LptC and LptE have only more recently been clarified. Studies have found that while LptC interacts directly with LptA (Sperandeo et al., 2011; Bowyer et al., 2011), LptE does not (Bowyer et al., 2011). The structure of LptC is highly similar to that of LptA, and it has been shown that like LptA, LptC binds LPS *in vitro* (Tran et al., 2010). Additionally, LptA can displace LPS from LptC *in vitro* but LptC cannot displace LPS from LptA, indicating a unidirectional export pathway (Tran et al., 2010). LptE has been shown in *E. coli* to interact directly with LPS (Chng et al., 2010) and it is theorized to act as a substrate recognition site at the OM. However, when LptE was deleted from *N. meningitidis*, LPS transport to the cell surface was not noticeably affected and only levels of LptD were altered (Bos and Tommassen, 2011) indicating a potential role in trafficking of LptD to the membrane. One study has shown that several residues of LptE interact directly with LptD *in vivo*, specifically in a predicted extracellular loop structure, and a "plug-and-barrel" architecture in which LptE resides within the LptD β-barrel has been postulated (Freinkman et al., 2011). The interaction between LptD and LptE has also been shown to occur while LptD is being assembled into the membrane by the Bam complex (Chimalakonda et al., 2011).

It has been proposed that LPS itself may play a role in membrane biogenesis, specifically in OMP transport (Bulieris et al., 2003; Qu et al., 2009). This appears to be species-specific however, as in *N. meningitidis*, OMP biogenesis is independent of LPS. Predicted binding domains for LPS appear to exist on the periplasmic chaperone Skp in both *E. coli* (Walton and Sousa, 2004) and *N. meningitidis*, indicating the potential for at least some overlap between the LPS and OMP transport pathways, however Skp-LPS interactions have to date only been detected *in vitro* (de Cock et al., 1999). A less direct and more plausible effect of LPS on OMP biogenesis has been described, in which LPS acts merely to stabilize membrane proteins that have already been transported to the membrane via mediating rearrangements of the proteins' surface-exposed loops (de Cock and Tommassen, 1996). Porin trimers have been shown to be stabilized in this fashion (de Cock and Tommassen, 1996).

BIOINFORMATICS OF PUTATIVE *H. PYLORI* LPS TRANSPORT SYSTEM

From a genetic standpoint, the LPS transport pathway in *H. pylori* appears to be conserved in regard to the integral membrane proteins MsbA, LptB, and LptD (**Table 1**). The encoding genes (HP1082, HP0715, and HP1215/1216, respectively) are present in all sequenced *H. pylori* isolates available (strains 26695, J99, HPAG1) as well as *Helicobacter acinonychis* and *Campylobacter jejuni* and are highly conserved when compared with their *E. coli* homologs. Transposon mutagenesis studies have generated mutations in all three genes (Salama et al., 2004), however, whether the proteins are still functional in these mutants is not known.

Both *msbA* and *lptD* were found to be significantly upregulated when *H. pylori* strain NTUH-C1 was treated with glutaraldehyde (Chiu et al., 2009) and Δ*lptD* and Δ*msbA* mutants have been characterized in the same *H. pylori* strain (Chiu et al., 2007, 2009). These researchers determined that their mutants, derived from the clinical isolate NTUH-C1, were more susceptible to both n-hexane as well as several antibiotics (novobiocin, erythromycin, tetracycline, and penicillin) when compared to the parental strain, suggesting that MsbA and LptD play an important role in maintaining membrane integrity in *H. pylori*. Silver staining of proteinase-K digested whole cell lysates indicate lower amounts of total LPS present in Δ*lptD*, Δ*msbA*, and Δ*lptD*Δ*msbA* mutants. Immunoblots using antibodies for Lewis antigens A and B on *H. pylori* LPS preparations were used to show the amounts of O-antigen in the preparations, and both antibodies showed significantly reduced amounts of O-antigen in all three mutants (Chiu et al., 2009). While these studies raise the potential that *H. pylori* can exist without LPS, as of yet, no examination of the lipid A of these mutants has been conducted. LptA and LptD from *E. coli* have been shown to interact with the lipid A moiety of LPS, indicating that it is lipid A that is actually the key component that is being transported by the Lpt transport pathway. There are examples in the literature of alterations in *H. pylori* lipid A resulting in a "truncation of the core oligosaccharide and loss of the attachment of the O sidechain containing Lewis X and Y" (Moran et al., 2004) and the expression of Lewis antigens in a cholesterol-dependent manner (Hildebrandt and McGee, 2009), however, no studies to date have shown that *H. pylori* can exist without lipid A in its OM, or have shown an alternate pathway for lipid A integration into the membrane. Strain specificity potentially plays a role with regard to the generation of membrane biogenesis mutants in *H. pylori*, as at present, generating directed insertion or deletion or mutants in *lptD*, *msbA*, or any of the other components of the Lpt complex in strains 26695, G27,

1061, or SS1 have proven unsuccessful (Liechti and Goldberg, unpublished results).

In searching for possible homologs for all the components of the LPS trafficking from *E. coli* in *H. pylori*, an interesting phenomenon found in all of *H. pylori* membrane trafficking pathways again presents itself. While the membrane spanning pathway components appear quite similar to their *E. coli* counterparts (**Table 1**), the components localizing to the periplasmic region of the pathway appear barely conserved and potentially non-existent in any recognizable form. Putative *H. pylori* homologs to LptF and LptG (HP0362 and HP1498, respectively) were only found after identifying homologs with either *E. coli* or *N. meningitidis* proteins in *Helicobacter hepaticus* (HH0602 and HH1496, respectively). The purported periplasmic LPS chaperone, LptA, found in both *E. coli* and *N. meningitidis* is present in *H. pylori*, albeit at a very low similarity, whereas the membrane-associated ABC transporter component LptB is highly conserved. Analysis of the putative *H. pylori* LptA shows high structural similarity at the central region of the protein, and interestingly the majority of divergence from the *E. coli* homolog exists at both the N- and C-termini (**Figure 4**). These regions of lower similarity comprise the putative points of interaction between LptA molecules when they form polymers, resulting in a twisted, β-jellyroll structure, in the presence of LPS (Suits et al., 2008). No obvious homologs exist for either LptC and LptE in *H. pylori* or any ε proteobacteria at the level of amino acid identity/similarity, however one report from the literature indicates that *lptE* is always present in a common locus in most bacterial genomes, and when it does not appear to exist on the level of genetic similarity, a putative lipoprotein can be found in its same genetic locale (Bos and Tommassen, 2011). In the case of *H. pylori*, a putative lipoprotein that shares common domain characteristics of LptE orthologs exists at this locus, directly downstream of *leuS* (**Figure 5**) and this appears to be the case for all other ε proteobacteria as well (Bos and Tommassen, 2011). When protein structural analysis software [RaptorX, (Peng and Xu, 2011)] is used to compare the putative *H. pylori* protein at the structural level to all solved crystal structures in the Protein Data Bank, the two most structurally similar proteins are characterized as "rare lipoprotein B" (early nomenclature for LptE) from *Nitrosomonas europaea* and *Shewanella oneidensis* (PBD designations 2JXP and 2R76), respectively. Similarly, a conserved protein in ε proteobacteria appears to exist in the same genetic locus as *lptC* (directly upstream of the *lptA* homolog) (**Figure 6**). Similarly, the *H. pylori* homolog to *lptB* (HP0715) is found directly upstream of *rpoN*, similar to its genetic localization in *E. coli* and *N. meningitidis*. Amino acid alignments show well-conserved residues in the N-terminus of the putative *H. pylori* LptC and poorer similarity past ∼ residue 110. The LptC periplasmic domain has been described as a "twisted boat" structure comprised of two β-sheets in opposition to each other (Tran et al., 2010) and the *H. pylori* putative protein possesses amino acid similarity through the first five β-strands (**Figure 7**). Unlike LptE, LptC is believed to be the crucial link between LptA and the ABC transporter (LptBFG) and as all localization and protein interaction data to date supports this hypothesis, the presence of a potential LptC-like protein in ε proteobacteria is encouraging.

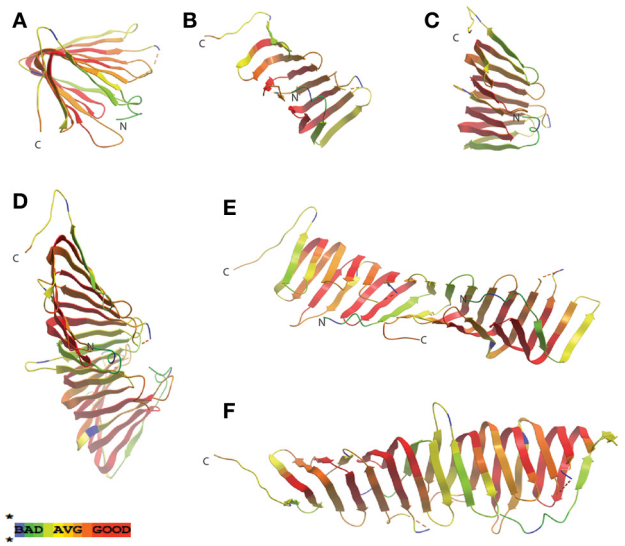

FIGURE 4 | LptA periplasmic LPS chaperone displays greatest structural homology at the central region. Similarity protein alignments were generated between LptA amino acid sequences from *E. coli* and *H. pylori* (HP1568) and a color scheme based on similarity scores was generated, described previously in **Figure 2**. Crystal structures of LptA from *E. coli* [PDB ID: 2R19, (Suits et al., 2008)] were then used as a template for mapping the similarity scores. Monomeric **(A–C)**, as well as the dimeric **(D–F)** "head-to-tail" conformation (in which the N-terminal β-strand from each molecule interacts with the C-terminal β-strand of the adjacent LptA molecule) of protein structures are shown. Greatest similarity is found in the central portions of the protein (shown in red) whereas areas believed to be involved in monomer stacking to form filamentous structures, believed to potentially span the periplasm, give significantly lower similarity scores (shown in yellow and green). The dotted yellow and red lines in the model represents regions in the amino acid protein sequence not present in the solved crystal structure.

OUTER MEMBRANE PROTEINS

ROLE IN INFECTION AND PATHOGENESIS

H. pylori contains an OMP family consisting of approximately 33 genes. This family is divided into two subfamilies comprising of the *H. pylori* outer membrane protein (Hop) and the Hop-related (Hor) families (Alm and Trust, 1999). The vast majority of *H. pylori* OMPs belong to the Hop sub-family, including almost all known adhesion proteins that allow for the binding of *H. pylori* to numerous substrates from fucosylated histo-blood group antigens (Ilver et al., 1998) to host laminin (Senkovich et al., 2011). The protein family also contains a substantial number of porins (Exner et al., 1995; Doig et al., 1995; Peck et al., 2001) thought to play roles in nutrient uptake, though to date, no study has closely examined their function with regard to specificity of substrates. It is also worth noting that the flagella apparatus (Eaton et al., 1996) and chemotactic machinery (Foynes et al., 2000; Croxen et al., 2006) of *H. pylori* are both essential for colonization and both possess OM and IM protein components, as do the transport pathways for LPS and lipoproteins, not to mention the Cag and Com type IV secretion systems, required for injecting the CagA protein into host cells (Censini et al., 1996)

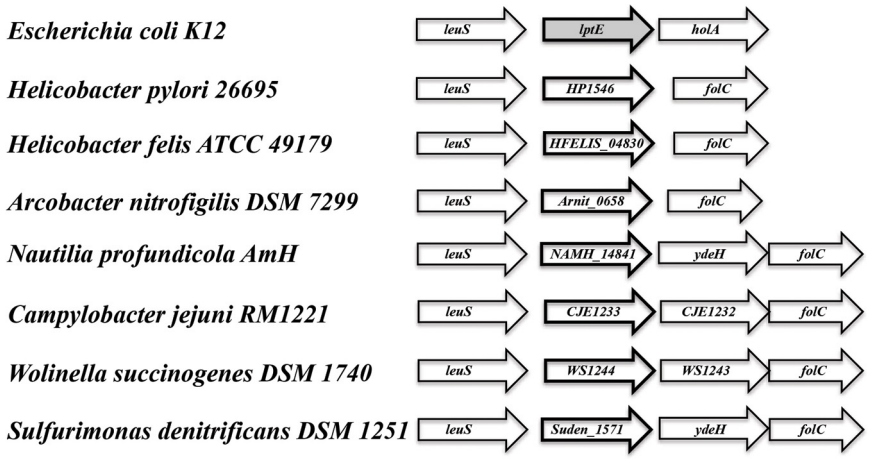

FIGURE 5 | Genes encoding putative lipoproteins of unknown function are found adjacent to *leuS* (leucyl-tRNA synthetase) in ε proteobacteria. While having limited sequence similarity to *lptE*, the conserved orientation of the lipoprotein encoding genes downstream of *leuS*, in addition a high similarity score at the putative protein structural level to LptE from *Nitrosomonas europaea* and *Shewanella oneidensis* [Protein DataBase #s 2JXP and 2R76, respectively: analysis conducted utilizing the 3D modeling software RaptorX: (Di Tommaso et al., 2011)] indicates the potential of a functionally equivalent protein in all ε proteobacteria species observed.

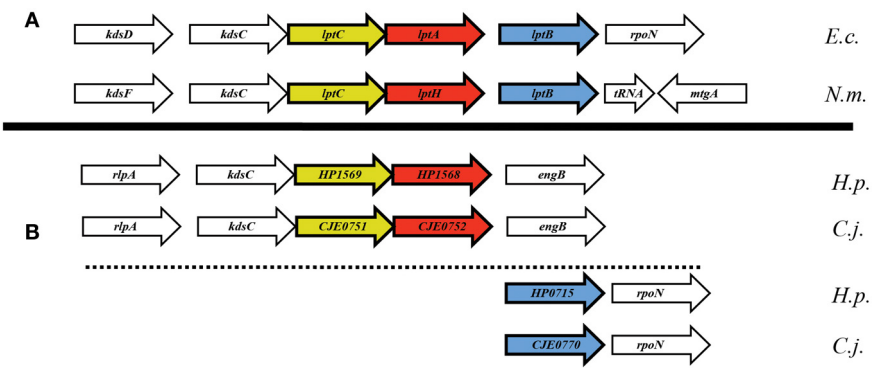

FIGURE 6 | Apparent genetic reorientation of Lpt operon in ε proteobacteria. While the orientation of *lptA*, *lptB*, and *lptC* in most Gram-negative bacteria is that of a single putative operon, the locus in ε proteobacteria appears to have dissociated while still maintaining many of the genes proximal to the Lpt components, namely *rpoN* and *kdsC*.

and taking up DNA for homologous recombination (Stingl et al., 2010), respectively. It is quite extraordinary how almost everything required for the survival and pathogenesis of *H. pylori* (DNA and nutrient uptake, adherence, motility, chemotaxis, immune stimulation/evasion, VacA secretion, iron acquisition, etc.) requires β-barrel proteins in the OM; these proteins may all depend on a single mechanism by which they are inserted into the OM.

CURRENT MODEL FOR MEMBRANE PROTEIN INTEGRATION

In attempting to use *E. coli* as a model to discern proteins involved in OM biogenesis, researchers needed to designate set criteria to judge whether a given protein was likely involved in the transport pathway. They reasoned that defects in membrane transport would result in a loss of barrier function and membrane permeability would significantly increase as a result (Braun and Silhavy, 2002; Ruiz et al., 2006). Therefore, alterations in membrane permeability became a key element to examine when considering potential candidate proteins for OMP transport. It was also shown early on that mis-folding of OMPs resulted in an envelope stress response, in which various chaperones involved in the protein transport pathway were up-regulated (Cosma et al., 1995; Raivio and Silhavy, 1997). Genes involved in the OM transport pathways were also assumed to exist in operons with other transport pathway genes in addition to being essential in *E. coli* and well-conserved in other bacteria. The ultimate test for proteins playing roles in OM biogenesis was depletion experimentation using either temperature-sensitive mutants or inducer-dependent expression systems. In both cases, levels of OMPs could be examined and locations within the cell detected and compared with wild-type bacteria. It was also shown that, in many cases, problems in OMP transport resulted in a down-regulation of

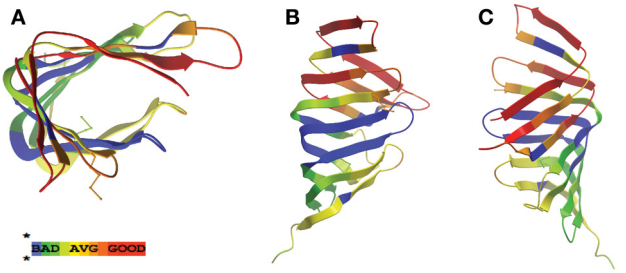

FIGURE 7 | LptC displays greatest structural homology at the N-terminal region. Ribbon structure of LptC protein from *E. coli* [PDB ID: 3MY2, (Tran et al., 2010)] with areas of similarity to the *H. pylori* homolog (HP1569) displayed in color code described previously in **Figure 2**. Views presented are overhead **(A)** and side **(B–C)** profiles.

membrane proteins, preventing the accumulation of mis-folded proteins at various stages of the transport process (Werner and Misra, 2005; Ruiz et al., 2006; Bos et al., 2007).

This basic understanding of membrane biogenesis became much less universal once similar studies were conducted in the bacterium *N. meningitidis*. In stark contrast to *E. coli*, *N. meningitidis* proved a more complicated model for recognizing new transport pathway components for a number of reasons. For example, *N. meningitidis* appears to completely lack the majority of proteins involved and essential for a periplasmic stress response (Bos et al., 2007), as characterized by the *E. coli* system. In addition, numerous genes identified as essential components of the OM biogenesis apparatus in *E. coli* are not essential in *N. meningitidis* (Bos et al., 2007). This does allow for the creation of mutants in this model system however, enabling a more straightforward, reductionist approach.

Sec-DEPENDENT AND -INDEPENDENT TRANSPORT

The *sec* pathway is a well-established system for trafficking proteins from the cytoplasm through the IM to the periplasm. In this transport pathway, chaperones SecA and SecB sequester proteins bound for the IM transport pathway by recognizing leader sequences on the designated protein (Kumamoto and Francetic, 1993; Valent et al., 1998). These chaperones then bring the newly formed protein to the membrane-localized portion of the Sec complex (SecDEFGY) (Van den Berg et al., 2004), which acts to move the protein through the IM. SecA then acts as the ATPase motor for this protein transport channel, providing energy for the mechanical "pushing" of the preproteins through the SecYEG complex (Tomkiewicz et al., 2006) and in so doing changes localization from cytoplasmic to peripheral IM and finally to integral IM. The leader sequence on the preprotein is removed by a leader peptidase, and the protein is then generally recognized by various periplasmic chaperones. In addition to the SecB-dependent transport of preproteins to the SecYEG complex, an alternate means of cytoplasmic transport exists (Koch et al., 1999) in the signal recognition particle (SRP) that recognizes either signal recognition peptides or the first transmembrane segment of proteins targeted for the IM. Once bound to the preprotein, the SRP complex migrates to the membrane, where a SRP receptor catalyzes the release of the preprotein and its insertion into the SecYEG complex. While initially it was thought that the majority of OMPs were SecB-dependent (Kumamoto and Beckwith, 1985), it has also been shown that OMPs misdirected to the SRP-dependent pathway can be secreted across the IM and are correctly assembled in the OM (Lee and Bernstein, 2001; Bowers et al., 2003).

Another IM transport pathway for proteins has also been characterized. Known as the Twin-Arginine Translocation (Tat) complex, this system involves three IM proteins (TatA, TatB, and TatC) that allow for protein transport independent of the Sec pathway (Lee et al., 2006). The general model for this complex is that TatA, with its multiple membrane spanning domains, forms a pore complex, while TatB and TatC stabilize the complex and allow for interactions with incoming cytoplasmic proteins. The three genes encoding these proteins are found in a single operon in *E. coli* and mutants have shown defects in IM trafficking for certain proteins (Lee et al., 2006). It has been postulated that the Tat pathway generally is utilized for proteins that undergo the majority of their conformational changes in the cytoplasm, as the transport pathway is relatively fast as compared with the Sec pathway. Studies have shown that mutations of genes encoding proteins involved in this pathway in various bacteria can result in a loss of infectivity resulting from the mislocalization of virulence determinants, which are normally moved through the periplasm by this mechanism (Voulhoux et al., 2001; Lee et al., 2006).

Bam (β-BARREL ASSEMBLY MACHINERY) COMPLEX

Recent *E. coli* studies have led to the discovery of an OM component of the OMP trafficking pathway, the Bam (YaeT) complex (Ruiz et al., 2006). Researchers, in an attempt to identify proteins potentially involved in membrane biogenesis, created an *E. coli* strain with a defective *lptD* allele. Barrier function of the OM in wild-type *E. coli* generally results in resistance to a number of antibiotics and various toxic compounds. However, *E. coli* strains containing mutations in key OM biogenesis genes, such as *lptD*, display a "leaky phenotype" in that their membranes are more permeable and as a consequence these mutants show increased susceptibility to toxins and antibiotics. By using the *lptD* mutant as a starter strain, and then selecting for spontaneous suppressor mutations that result in a return to wild-type levels of antibiotic resistance, researchers were able to characterize other proteins involved in the transport pathway (Ruiz et al., 2005). Among the mutations identified was the *yfgL* (*bamB*) gene, encoding an OM-associated lipoprotein. Co-immunoprecipitation studies revealed that BamB existed in a complex in the OM with three other lipoproteins; YfiO (BamD), NlpB (BamC), and SmpA (BamE), as well as the β-barrel protein BamA (Wu et al., 2005).

The homolog of BamA in *N. meningitidis* (Omp85) had previously been linked to OM biogenesis, and it was initially uncertain whether it also had an additional role in transport of LPS (Voulhoux et al., 2003; Voulhoux and Tommassen, 2004). Subsequent experimentation was able to show that LPS transport is affected when the overall levels of BamA are reduced. This effect was later found to be indirect, as the alterations in

LPS transport were the result of the OMP of the LPS transport pathway (LptD) not being inserted correctly into the membrane (Bos et al., 2004). In both bacterial systems, *bamA* is an essential gene and is involved in the assembly of β-barrel proteins. In examining its genetic orientation, it was discovered that *bamA* is flanked by two other genes involved in membrane trafficking: the periplasmic chaperone *skp* as well as *yaeL*, a protease involved in the envelope stress response described earlier (Bos et al., 2007). Lipoprotein BamD was also shown to be essential for viability in *E. coli* and *N. meningitidis*, while mutations in the other three lipoproteins result in only mild alterations in membrane permeability (Onufryk et al., 2005; Wu et al., 2005). However, upon comparison of the pathway mechanics of both microbes, significant differences emerged. Depletion studies for BamA in *E. coli* showed no alteration in LPS trafficking, indicating no role for BamA involving LPS in *E. coli* (Doerrler and Raetz, 2005). It was also shown that depletion for BamA in *N. meningitidis* led to the accumulation of unfolded OMPs in the IM (Voulhoux et al., 2003) while in *E. coli*, depletion resulted in significantly reduced amounts of all OMPs (Wu et al., 2005). It is believed this is due to the absence of the envelope stress response in *N. meningitidis*, thereby preventing the down-regulation of OMP synthesis (Bos et al., 2007). Subsequent studies have also shown that BamA also acts as the receptor for contact-dependent growth inhibition (CDI) in *E. coli*, and that antibodies specific for extracellular epitopes on BamA block inhibitor cell binding (Aoki et al., 2008). The potential for utilization of CDI to target bacterial growth is currently being examined (Aoki et al., 2011).

PERIPLASMIC CHAPERONES

The four major periplasmic proteins involved in OMP transport in *E. coli* are SurA (Rouviere and Gross, 1996), DegP (Misra et al., 1991; Krojer et al., 2002), FkpA (Dartigalongue et al., 2001), and Skp (Harms et al., 2001; Bulieris et al., 2003; Walton and Sousa, 2004). Unlike various other members of the OM biogenesis pathway, Skp as well as other periplasmic chaperones, have been shown to be non-singularly essential, and mutants in *E. coli* show only modest defects in membrane biogenesis (Rizzitello et al., 2001). This has led to the belief that potentially multiple mechanisms exist for periplasmic trafficking, a view supported by greater OM defects seen in sequential knockout studies for other established periplasmic chaperones (Rizzitello et al., 2001), indicating the presence of parallel pathways. To date, SurA is recognized as the main periplasmic chaperone, with Skp/DegP acting as a back-up pathway (Sklar et al., 2007). Evidence does however exist in the literature that some proteins appear to be preferentially transported by SurA (Vertommen et al., 2009). LptD is currently believed to be transported through the periplasm by the SurA chaperone with cooperation from periplasmic disulfide isomerase (DsbC) and thiol oxidase (DsbA) (Denoncin et al., 2010). Further analysis revealed that LptD contains two disulfide bonds that are essential for function that act to stabilize the LptD protein by correctly positioning the N- and C-terminal domains of the protein in the periplasm (Ruiz et al., 2010) and that oxidation is carried out by DsbA. However, this apparent preference for periplasmic chaperones is potentially species-specific, as Δ*surA* mutants generated in *N. meningitidis* did not appear to affect membrane biogenesis, while a Δ*skp* mutant resulted in significantly lower levels of porins PorA and PorB (Volokhina et al., 2011).

BIOINFORMATICS OF PUTATIVE *H. PYLORI* OMP TRANSPORT SYSTEM

A large number of the membrane-associated proteins found on the surface of *H. pylori* consist of β-barrels, indicating their transport through the membranes from the cytoplasm may be through the Sec and BamA complex (**Table 1**). The importance of the Sec pathway in *H. pylori* was made readily apparent via bioinformatic analysis of the *H. pylori* strain 26695, where it was found that 517 of the 1,590 open reading frames possessed putative signal sequences (Alm et al., 1999). The Sec pathway for IM transport has already been well-characterized in *H. pylori* and transposon mutagenesis studies have shown that many of the genes encoding components of the Sec-pathway are capable of being mutated (albeit definite conclusions based on these results should be tempered with the knowledge that often insertions in a gene do not always lead to a complete inactivation of that gene), with the exception of *secY* (Salama et al., 2004). *H. pylori* does seem to lack the cytoplasmic chaperone SecB, but this is apparently common and has been characterized in other bacterial species (Yamane et al., 2004), indicating SecB acts to supplement the effectiveness of SecA. This may also hint at a more pronounced role for the SRP-dependent pathway in OMP trafficking to the Sec complex in *H. pylori*, however, to date no studies have tested this potential hypothesis. *E. coli secD* and *secG* temperature-sensitive mutants when grown at the non-permissive temperature result in the accumulation of OMPs in the cytoplasm (Fitchen et al., 2003). However, when complementation studies were preformed using plasmids expressing the *H. pylori* homologues in these mutants, cytoplasmic OMP levels returned to their wild-type levels, indicating a conservation in function for these genes in *H. pylori* (Fitchen et al., 2003).

Similarly, the Sec-independent IM transport system, the Tat complex, appears to be well-conserved in *H. pylori*. *tatA* (HP0320), *tatB* (HP1060), and *tatC* (HP1061) are all present (**Table 1**), with the only difference being the apparent removal of *tatA* from the original operon and insertion elsewhere in the genome, whereas in *E. coli* the three genes comprise a single operon (Lee et al., 2006). No characterized mutants for *tatB* or *tatC* have been described while a single transposon insertion mutant exists for *tatA* (Salama et al., 2004) and the low coverage of transposon mutants in these genes suggests that all three maintain an essential function in *H. pylori*. This would seem to differ with previous studies in *E. coli* where the Tat system has been shown to be dispensable under laboratory conditions, however, there are examples of bacteria that rely heavily on the Tat system for almost all protein secretion (Dilks et al., 2005).

The periplasmic chaperones DegP and SurA appear to be conserved in *H. pylori*, while the chaperone Skp does not appear to be well-conserved. *N. meningitidis* is similar to *H. pylori* in this respect, as it posseses only *surA* and *skp* homologs and is missing a functional *degP* homolog (Volokhina et al., 2011). The synthetic lethal phenotypes characterized in *E. coli*

double mutants (*surA*/*skp* and *surA*/*degP*) indicate that SurA and Skp/DegP exist in parallel periplasmic chaperone pathways, and the absence of a Skp homolog in *H. pylori* is possible due to the presence of SurA. No mutants in *degP* or *surA* have been characterized to date in *H. pylori* however, and insertional mutants in these genes were not generated in the construction of a transposon library in *H. pylori* of ∼10,000 clones (Salama et al., 2004), indicating that they may be essential.

The integral OMP component of the OMP trafficking pathway, BamA, is well-conserved in *H. pylori* (**Table 1**) and all ε proteobacteria. What is striking is that in *E. coli* and *N. meningitidis,* this protein is part of a complex along with at least four other conserved lipoproteins (BamB, BamC, BamD, and BamE) while in *H. pylori* only BamD (ComL) appears to be present. It is worth noting that BamA and BamD appear to form the core of the OMP export machinery, as they are the only two essential components in both *E. coli* and *N. meningitidis*. In *E. coli* BamE has been recently shown to modulate the conformation of BamA, likely through its interactions with BamD, and when Δ*bamE* mutant cells are treated with proteinase K, BamA is degraded beyond recognition (Rigel et al., 2012). No studies on the Bam complex have been reported in *H. pylori* to date.

PERSPECTIVES

There is currently a relatively large amount of circumstantial genetic evidence suggesting that the OM biogenesis pathways established in *E. coli* and *N. meningitidis* are present in some form or derivation in *H. pylori* and in fact all ε proteobacteria. The OM- and IM-protein components of all of these pathways appear to be conserved (**Table 1**), but what is striking is that, in these initial genetic and bioinformatic observations, nearly all of the periplasmic components from each of these characterized pathways from *E. coli* and *N. meningitidis* are missing or severely altered in all ε proteobacteria (**Figure 1**). An examination of the divergent residues of putative *H. pylori* homlogs for LolA, LptA, LptC, and LptE all indicate that in general, the N-terminal domains are conserved, while the more C-terminal domains are not. Divergence at the N- and C-terminal domains of the *H. pylori* LptA homolog are particularly interesting, as these residues are potentially important for the formation of the long, end-to-end, filamentous structures formed in the presence of LPS. Regions of interaction between filament monomers are generally well-conserved between bacterial species, given the importance of monomer-monomer interaction (Samatey et al., 2001), however, instances of variability at these binding domains are present in the *H. pylori* literature (Andersen-Nissen et al., 2005). Given that all of the membrane-associated components from each transport system are present and given that in every case, the periplasmic-associated components are highly divergent or potentially missing, there appears to be some strong, and as of yet uncharacterized selection affecting the periplasmic components of all three of the discussed transport pathways in *H. pylori* and other ε proteobacteria.

Little is known about the conditions inside the periplasm of *H. pylori* and the field of periplasmic transport has only just begun to expand. Recent studies have examined the transport of urea via UreI for use in buffering the periplasm in *H. pylori*, maintaining a pH of ∼6.1, compared to a pH as low as ∼2 outside of the OM and pH greater than 7 in the cytoplasm (Scott et al., 2000; Weeks et al., 2000; Krulwich et al., 2011). In this respect "the key to gastric colonization by *H. pylori* is periplasmic pH homeostasis" (Krulwich et al., 2011). The possibility exists that using the periplasmic space as a pH buffer may be driving this apparent selection affecting the composition of periplasmic-associated lipoproteins as well as potential periplasmic chaperones in all three, membrane biogenesis transport systems. Divergence in sequence perhaps reflects amino acid domains important for survival in harsh environments. However, UreI is found in only a handful of *Helicobacters* while the divergence in periplasmic-associated lipoproteins is found throughout all ε proteobacteria. Though different from *E. coli* and *N. meningitidis*, those lipoprotein components found in *H. pylori* are highly conserved within ε proteobacteria, indicating the selective element is affecting the entire bacterial family, or at least an early common ancestor. Given the apparent absence of LolB and LolDE, lipoprotein transport in ε proteobacteria may be severely altered and the effect on lipoprotein transport may explain the divergence in lipoprotein components of both the Bam and Lpt transport pathways. Another possibility may be an alteration in membrane lipid composition in ε proteobacteria compared with other bacterial families. This explanation may explain the apparent inability to identify many of the lipoprotein components of all three membrane biogenesis transport systems, as potentially unique alterations in the lipid moiety of these components may be required for insertion into membranes of ε proteobacteria. The fact that the vast majority of sequence divergence in lipoprotein components of trafficking pathways in *H. pylori* were in domains not involved with interacting with the actual LPS, lipoproteins, or OM proteins indicates that the membrane lipid composition or lipoprotein localization may significantly differ in ε proteobacteria. Phospholipids are known to make up the majority of lipids in *H. pylori* (Hirai et al., 1995) and cholesterol glucosides, lipids rarely found in animals or bacteria, account for roughly 25% of the lipids in *H. pylori* (Haque et al., 1996; O'Toole and Clyne, 2001), suggesting an altered membrane composition. As stated previously, inner and OMs in *Helicobacters* and *Campylobacters* are notoriously hard to separate, and the alterations seen in both lipid composition and the lipoproteins found in each of these three systems when compared with other bacterial families may offer clues as to the nature of this phenomena.

The field of membrane biogenesis in ε proteobacteria to date is remarkably unexplored. The recent studies undertaken by Chiu et al. have directly shown the importance of the LPS transport pathway components in membrane permeability and that a decrease in membrane integrity is achievable by removing the function of two of these components, resulting in an increased susceptibility of *H. pylori* to antibiotics (Chiu et al., 2007, 2009). In addition, targeting these pathways not only has the potential to affect membrane permeability, but also prevent virulence determinants essential for the survival of *H. pylori* from being expressed on the bacterial surface. Despite having shown this correlation between OM biogenesis and membrane integrity, no studies to date have examined the effects

of alterations in these pathways on pH resistance, adherence, or colonization in *H. pylori*. The argument could be made that, given the lack of an effective *H. pylori* vaccine and the emergence of antibiotic resistant organisms, the time has come to begin exploring therapeutics that target these three essential mechanisms by which all known colonization/virulence determinants are expressed on or secreted from the bacterial surface.

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Transcriptome complexity and riboregulation in the human pathogen *Helicobacter pylori*

Sandy R. Pernitzsch and Cynthia M. Sharma*

Research Center for Infectious Diseases, University of Würzburg, Würzburg, Germany

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
D. Scott Merrell, Uniformed Services University, USA
Gisela Storz, National Institutes of Health, USA
David Scott, David Geffen School of Medicine at UCLA, USA

****Correspondence:***
Cynthia M. Sharma, Research Center for Infectious Diseases (ZINF), University of Würzburg, Josef-Schneider-Str. 2/Bau D15, 97080 Würzburg, Germany.
e-mail: cynthia.sharma@uni-wuerzburg.de

The Gram-negative Epsilonproteobacterium *Helicobacter pylori* is considered as one of the major human pathogens and many studies have focused on its virulence mechanisms as well as genomic diversity. In contrast, only very little is known about post-transcriptional regulation and small regulatory RNAs (sRNAs) in this spiral-shaped microaerophilic bacterium. Considering the absence of the common RNA chaperone Hfq, which is a key-player in post-transcriptional regulation in enterobacteria, *H. pylori* was even regarded as an organism without riboregulation. However, analysis of the *H. pylori* primary transcriptome using RNA-seq revealed a very complex transcriptional output from its small genome. Furthermore, the identification of a wealth of sRNAs as well as massive antisense transcription indicates that *H. pylori* uses riboregulation for its gene expression control. The ongoing functional characterization of sRNAs along with the identification of associated RNA binding proteins will help to understand their potential roles in *Helicobacter* virulence and stress response. Moreover, research on riboregulation in *H. pylori* will provide new insights into its virulence mechanisms and will also help to shed light on post-transcriptional regulation in other Epsilonproteobacteria, including widespread and emerging pathogens such as *Campylobacter*.

Keywords: RNA-seq, sRNA, *Helicobacter pylori*, post-transcriptional regulation, transcriptome analysis

INTRODUCTION

For a successful infection of the host or survival in the environment, bacteria have to rapidly adapt their gene expression in response to changing conditions. Besides regulation at the transcriptional level, post-transcriptional regulation is an important layer of gene expression control in both pro- and eukaryotes. Together with several RNA binding proteins which influence RNA structure and stability, the 50- to 400-nt long bacterial small RNAs (sRNAs) act as post-transcriptional regulators under various stress and growth conditions or during virulence (Waters and Storz, 2009; Papenfort and Vogel, 2010; Storz et al., 2011). For example, RyhB and OxyS sRNAs from *E. coli* are involved in iron metabolism and the oxidative stress response, respectively (Altuvia et al., 1997; Masse and Gottesman, 2002).

Although a small number of sRNAs can directly bind and interfere with protein activity, the majority of functionally characterized sRNAs act as antisense RNAs by base-pairing with target mRNAs (Waters and Storz, 2009). *Cis*-encoded sRNAs originate from the opposite DNA strand relative to the target and share fully complementary regions with their target genes. In contrast, *trans*-encoded sRNAs are encoded elsewhere in the genome and interact with their target mRNAs by short and imperfect base-pairing. Most of the *trans*-encoded sRNAs compete with ribosome binding and, thus, lead to translation repression, which is often coupled to mRNA degradation. Furthermore, an increasing number of sRNAs that activate target-gene expression by various mechanisms have been described (Fröhlich and Vogel, 2009). Similar to transcription factors, sRNAs can modulate the expression of multiple target genes, thereby functioning as key regulators in metabolic pathways or during stress responses (Papenfort and Vogel, 2009; Beisel and Storz, 2010). Besides sRNAs, the RNA chaperone Hfq is a key-player in sRNA-mediated regulation in many bacteria and is required for the stability and activity of sRNAs (Vogel and Luisi, 2011).

A variety of experimental and biocomputational approaches have been developed to identify sRNAs (Altuvia, 2007; Sharma and Vogel, 2009; Backofen and Hess, 2010). Genome-wide computational approaches in particular have predicted hundreds of sRNA candidates in diverse bacteria, which now await experimental validation and functional characterization. Furthermore, massively parallel cDNA-sequencing (RNA-seq) based on next-generation sequencing methods has been revolutionizing transcriptome studies in prokaryotes, including several important pathogens (Wang et al., 2009; Croucher and Thomson, 2010; van Vliet, 2010).

Helicobacter pylori is a Gram-negative, microaerophilic Epsilonproteobacterium which colonizes the stomach of about half of the world's population, where it can lead to peptic ulcer disease, chronic gastritis, or gastric cancer (Cover and Blaser, 2009). Surprisingly, only a few transcriptional regulators have been discovered in the small 1.67 Mbp *H. pylori* genome, including only three sigma factors, namely RpoD, FliA, and RpoN (Tomb et al., 1997). In contrast to enterobacteria, almost nothing is known about post-transcriptional regulation in *Helicobacter*. The systematic identification and subsequent functional characterization of sRNAs will help us to understand the role of

riboregulation in *H. pylori* and provide new insights into its virulence mechanisms.

RNA-seq REVEALS A COMPLEX TRANSCRIPTOME STRUCTURE AND FIRST sRNAs IN *H. pylori*

None of the enterobacterial sRNAs, except for the housekeeping RNAs, tmRNA, SRP RNA, and M1 RNA, are conserved in *H. pylori* (Sharma et al., 2010). Until recently, *Helicobacter* was even regarded as an organism that lacks riboregulation and that carries only basic regulatory circuits, due to its small genome size, a limited number of transcriptional regulators, and the apparent absence of the RNA chaperone Hfq (Mitarai et al., 2007). In addition, based on biocomputational predictions and a small scale cDNA cloning approach only a few natural antisense transcripts and sRNAs were predicted in *Helicobacter* (Livny et al., 2006; Xiao et al., 2009a,b). However, a novel differential RNA sequencing approach (dRNA-seq) selective for the 5′ end of primary transcripts allowed for the definition of a global map of transcriptional start sites and operons in *H. pylori* strain 26695, revealing a very complex and compact transcriptome structure (Sharma et al., 2010). Moreover, it led to the discovery of massive antisense transcription as well as an unexpected high number of more than 60 small RNAs including potential regulators of *cis*- and *trans*-encoded mRNA targets, indicating that *H. pylori* uses riboregulation for its gene expression control (**Figure 1**).

POTENTIAL FUNCTIONS OF sRNAs IN *H. pylori*

Many different *in vitro* and *in vivo* approaches have been established to characterize sRNAs and to validate their targets (Vogel and Wagner, 2007; Sharma and Vogel, 2009). Since sRNAs were discovered only recently in *Helicobacter* our knowledge about their mechanisms, target genes, and their own transcriptional regulators is still limited. The only sRNA that has so far been shown to be essential and required for stress response in *H. pylori* is tmRNA, which is involved in rescue of stalled ribosomes (Thibonnier et al., 2008).

CIS-ENCODED sRNAs

Cis-encoded antisense RNAs can overlap the 5′- or 3′-end, the middle, or entire genes and influence expression of their target genes, e.g., by translation inhibition, transcription interference and attenuation, transcript stabilization, or degradation (Thomason and Storz, 2010). Using dRNA-seq, more than 900 *cis*-encoded antisense RNAs have been identified in *H. pylori*, including at least one antisense transcriptional start site for almost half of all ORFs (Sharma et al., 2010). Similarly, an increasing number of antisense transcripts have been observed in many other prokaryotes (Thomason and Storz, 2010; Georg and Hess, 2011). Whether all of them are functional or rather represent spurious transcription still needs to be clarified. Obviously, these antisense RNAs can in principle harbor a regulatory function, since expression of an artificial

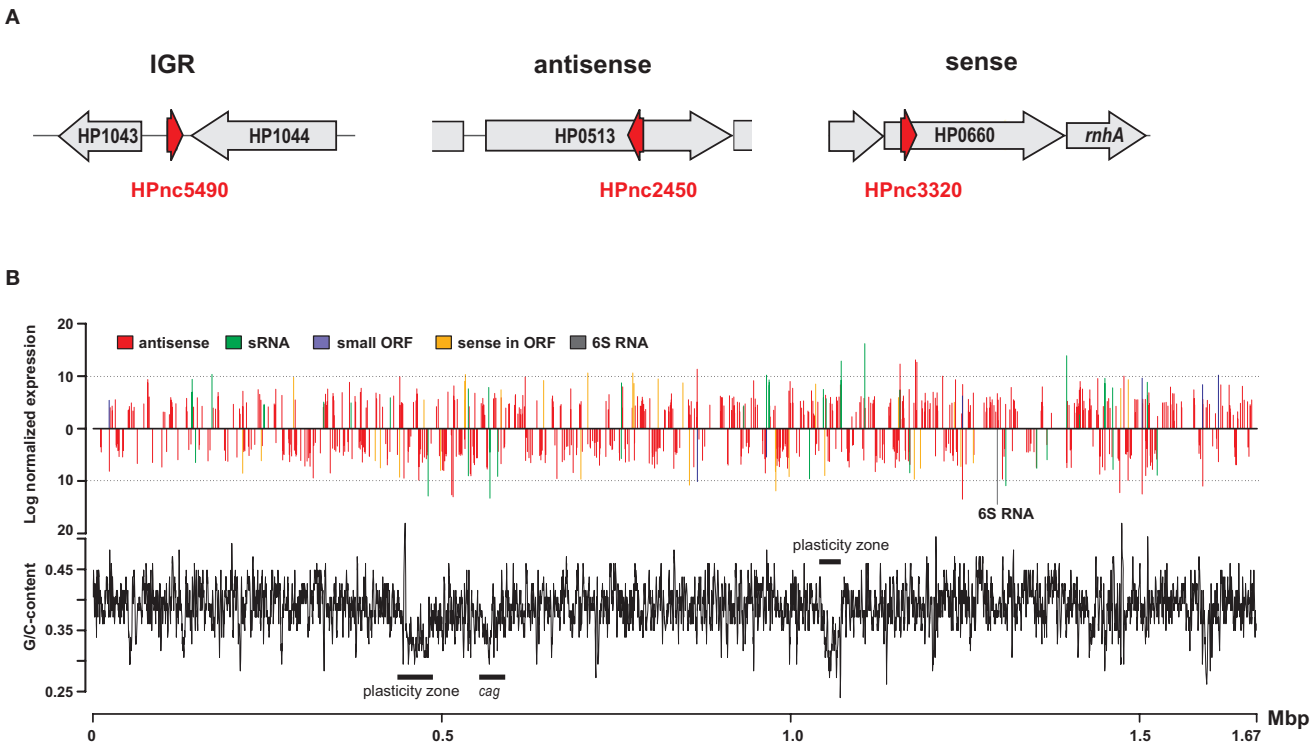

FIGURE 1 | *Helicobacter pylori* sRNA candidates identified by dRNA-seq. **(A)** A differential RNA-seq analysis of the primary transcriptome of *H. pylori* strain 26695 revealed hundreds of novel sRNA candidates (denoted as HPnc). These are located either in intergenic regions (left), antisense to ORFs (middle), or sense to ORFs (right). **(B)** Relative expression of the different types of sRNA candidates is given as log2 values of read numbers of all sequenced libraries (upper part) and plotted together with local G/C-content (lower part) across the *H. pylori* strain 26695 genome. One of the most abundant sRNAs is the previously missed 6S RNA homolog from the Epsilon subdivision (gray bar). The Figure was adapted from Sharma et al. (2010).

antisense RNA has been successfully used to repress the essential *ahpC* gene, encoding for alkyl hydroperoxide reductase (Croxen et al., 2007). Since *H. pylori* lacks homologs of the endonucleolytic RNases E/G (Tomb et al., 1997; Parkhill et al., 2000), antisense-mediated processing by the double-strand specific ribonuclease RNase III could be a major layer of gene regulation in these bacteria, as was recently suggested for *Staphylococcus aureus* (Lasa et al., 2011).

The characterization of a naturally occurring 292-nt long *cis*-encoded antisense sRNA from the opposite strand of the urease operon in *H. pylori* strain 43504 further demonstrated the functionality of antisense RNAs in *Helicobacter* (Wen et al., 2010). *In-vitro* and *in-vivo* approaches demonstrated that 5′*ureB*–sRNA is induced under neutral pH conditions by the unphosphorylated ArsR response regulator of the acid-responsive ArsRS two-component-system. In its phosphorylated form, ArsR activates transcription of the urease operon in response to low pH (Pflock et al., 2005). The 5′*ureB*–sRNA shows full complementarity to the 5′ end of *ureB* mRNA and represses urease activity by mediating cleavage of the *ureAB* mRNA (**Figure 2A**). Whether the truncated *ureAB* transcript is degraded afterwards or whether the reduced amount of UreB leads to lower urease activity is still unclear.

Besides sRNAs, the dRNA-seq study revealed several new small hydrophobic proteins (<50 aa), some of which are associated with *cis*-encoded antisense RNAs (Sharma et al., 2010). For example, six structurally related ∼80 nt sRNAs, IsoA1–6 (RNA-inhibitor of small-ORF family A), are expressed antisense to the small ORFs, AapA1–6 (antisense-RNA-associated peptide family A), representing homologous 22–30 aa long peptides. Some of these small ORFs resemble antimicrobial peptides or small toxic peptides from bacteria. The latter are often part of so-called class I toxin–antitoxin loci in which an unstable RNA antitoxin represses expression of a stable peptide toxin. Overexpression of the AapA peptides in *H. pylori* leads to cell death but not to cell lysis (Darfeuille, personal communication) and *in-vitro* translation assays indicated that protein synthesis of the peptides is specifically inhibited by the cognate antisense RNAs (Sharma et al., 2010). Thus, the *aapA–isoA* loci might represent the first examples of class I toxin–antitoxin systems in *H. pylori*.

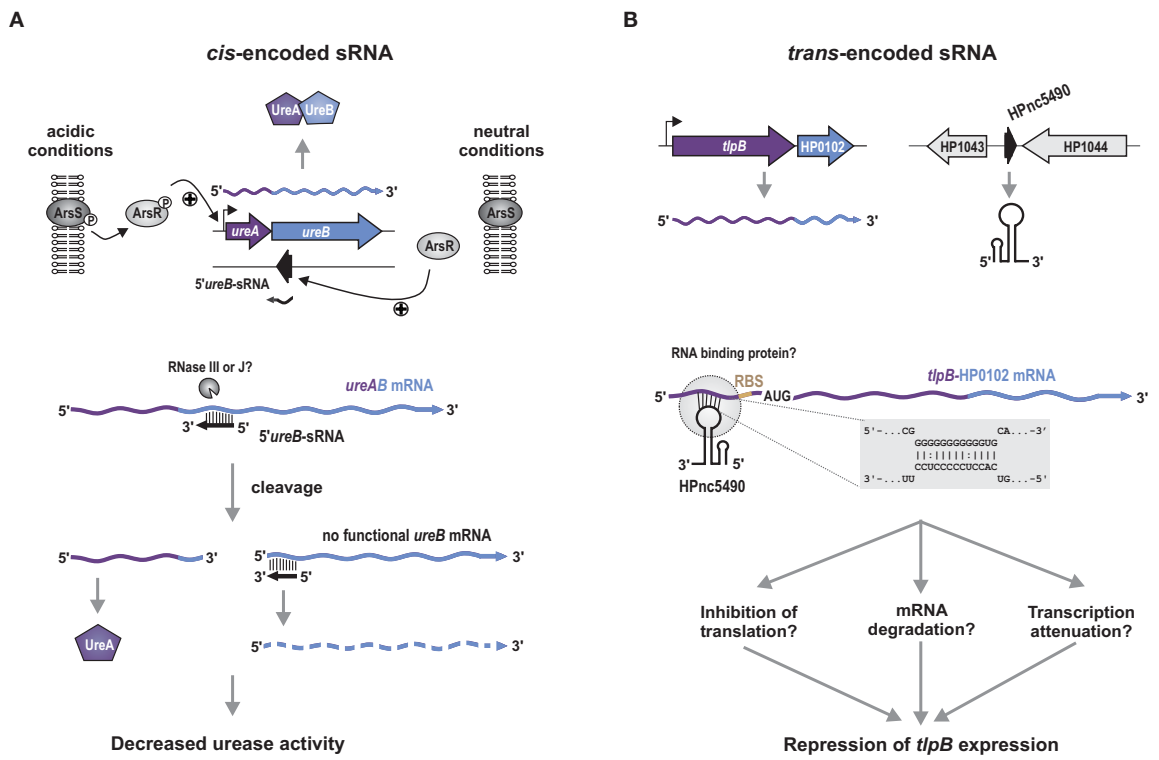

FIGURE 2 | First examples for *cis*- and *trans*-encoded antisense RNAs in *H. pylori*. (A) Regulation of urease by the *cis*-encoded antisense 5′*ureB*–sRNA. Expression of the *ureAB* operon, which encodes for the urease apo-enzyme, is induced under acidic conditions by the phosphorylated ArsR response regulator of the acid-responsive ArsRS two-component-system. In contrast, expression of the *cis*-encoded antisense transcript, 5′*ureB*–sRNA, is induced by unphosphorylated ArsR under neutral conditions. An interaction between 5′*ureB*–sRNA and the 5′ region of *ureB* leads to cleavage of *ureAB* mRNA, possible mediated by RNase III or RNase J. Truncation of *ureB* mRNA prevents its translation, resulting in a reduced amount of UreB and an overall decrease of urease activity. **(B)** Repression of the chemotaxis receptor TlpB by HPnc5490 sRNA. The 87-nt long HPnc5490 sRNA is encoded in the intergenic region next to the orphan response regulator HP1043. HPnc5490 binds to a G-stretch upstream of the RBS in the 5′ UTR of *tlpB*–HP0102 mRNA by short and imperfect base-pairing (gray panel), which could be facilitated by a so far unknown RNA binding protein. The exact mechanism of how HPnc5490 mediates repression of *tlpB* is still unknown but could be mediated either by inhibition of translation, mRNA destabilization by recruitment of RNases, or transcription attenuation.

TRANS-ENCODED sRNAs

One of the most abundant transcripts that was identified in the *H. pylori* dRNA-seq study is the previously missed homolog of 6S RNA (**Figure 1B**), a ubiquitous riboregulator, which mimics an open promoter complex and thereby sequesters RNA polymerase (Wassarman and Storz, 2000; Barrick et al., 2005; Sharma et al., 2010). Despite only little sequence conservation to *E. coli* 6S RNA, the 180-nt long RNA from *H. pylori* can fold into the characteristic long hairpin structure of 6S RNA (Trotochaud and Wassarman, 2005). The dRNA-seq data also revealed 12 to 15–nt long product RNAs, which are transcribed using 6S RNA as a template (Wassarman and Saecker, 2006), providing further evidence that this RNA is a functional 6S homolog in *H. pylori*. Deletion of 6S RNA results in no obvious phenotype during exponential growth but altered cell survival during stationary phase and under extreme stress conditions in *E. coli* (Wassarman, 2007). Whether 6S RNA has a role during stress response or stationary phase growth in *H. pylori* or, like in *Legionella* (Faucher et al., 2010), impacts on its virulence still needs to be investigated.

Several of the newly identified sRNAs in *Helicobacter* are potential candidates for *trans*-encoded antisense RNAs. For example, the abundant 87-nt long HPnc5490 sRNA, was predicted to interact by a C/U rich stretch with a G-repeat in the 5′ UTR of *tlpB* mRNA encoding for one of the four chemotaxis receptors in *H. pylori* (**Figure 2B**; Sharma et al., 2010). Comparison of *tlpB* expression in the wild-type and a HPnc5490 deletion strain confirmed down-regulation of the *tlpB* mRNA as well as TlpB protein levels by HPnc5490. It has been suggested that TlpB senses protons and diverse studies have demonstrated its potential role in pH-taxis, quorum sensing as well as colonization, and inflammation of the gastric mucosa (McGee et al., 2005; Croxen et al., 2006; Williams et al., 2007; Rader et al., 2011). Therefore, HPnc5490, and probably additional *H. pylori* sRNAs, could play important roles during stress responses or infection, as described for other bacterial pathogens (Papenfort and Vogel, 2010).

PROTEIN FACTORS INVOLVED IN POST-TRANSCRIPTIONAL REGULATION

Besides sRNAs, RNA binding proteins and RNases are involved in post-transcriptional regulation in bacteria (Pichon and Felden, 2007). In *H. pylori*, analysis of the urease operon transcript revealed different mRNA stabilities dependent on the pH value and processing into multiple species, indicating an extensive post-transcriptional regulation of the urease mRNA (Akada et al., 2000). Moreover, it has been shown that a gene of previously unknown function, HP0958, is essential for flagellum biogenesis and encodes for a post-transcriptional regulator of flagellin mRNA by modulating the amount of *flaA* mRNA available for translation (Douillard et al., 2008). Together with the recent identification of sRNAs it is now clear that *H. pylori* uses post-transcriptional regulation to control its gene expression and future identification of the protein factors involved will help to understand the underlying mechanisms in these bacteria.

RNA BINDING PROTEINS

The Sm-like RNA chaperone Hfq is required for the stabilization of sRNAs and facilitates the base-pairing of *trans*-encoded sRNA with their target mRNAs (Vogel and Luisi, 2011). Deletion of *hfq* leads to pleiotropic phenotypes including reduced fitness and virulence in several bacterial pathogens (Chao and Vogel, 2010). However, as is the case for 50% of all bacterial genomes, *hfq* appears to be absent in Epsilonproteobacteria. Whether a different RNA binding protein replaces the function of Hfq or whether sRNAs act without a chaperone in these bacteria is still an open question.

Besides Hfq, several other RNA binding proteins, such as the carbon storage regulator CsrA/RsmA family or the Crc protein involved in catabolite repression participate in post-transcriptional regulation in bacteria (Sonnleitner et al., 2009; Timmermans and Van Melderen, 2010). The CsrA protein acts as a translation regulator and its own activity is regulated by two sRNAs, CsrB/C, which mimic the RNA substrate of CsrA (Babitzke and Romeo, 2007). The CsrA homolog in *Helicobacter* is required for full motility, survival under oxidative stress, and infection of mice (Barnard et al., 2004). However, neither the target genes of CsrA nor the associated CsrA-regulating sRNAs, CsrB/C, have been identified in *H. pylori*, yet. The recent findings that additional proteins with so far unknown functions or functions unrelated to RNA metabolism, are involved in post-transcriptional control of mRNAs are just one indication that we are far away from knowing all RNA binding proteins and their roles in bacteria (Mitobe et al., 2011; Pandey et al., 2011).

RIBONUCLEASES

Although the exact mechanism of repression is still unclear, both functionally characterized antisense RNAs in *Helicobacter* (**Figure 2**) cause reduced protein levels as well as reduced target-mRNA levels, most likely through transcript destabilization or active recruitment of RNases. In enterobacteria, sRNA-mediated target-mRNA decay mainly depends on the RNA degradosome, a protein complex composed of the endoribonuclease RNase E, polynucleotide phosphorylase PNPase, and the RNA helicase RhlB (Morita et al., 2005; Caron et al., 2010). In *H. pylori*, several RNases are annotated (RNase H, RNase H-II, RNase J, RNase N, RNase P, RNase R, and RNase III) but like all Epsilonproteobacteria it appears to lack a homolog of RNase E. Recent studies on *Bacillus subtilis* and *S. aureus* identified RNase J1 and RNAse J2 as the functional orthologs in the RNA degradosome of Gram-positive bacteria (Mathy et al., 2010; Roux et al., 2011). Furthermore, in *S. aureus* the double-strand specific RNase III degrades, mainly in concert with the sRNA RNAIII, several mRNAs encoding virulence factors (Huntzinger et al., 2005; Boisset et al., 2007; Chevalier et al., 2008). Therefore, RNase J and RNase III could be potential RNases participating in sRNA-mediated transcript destabilization in *H. pylori*. Moreover, the 3′–5′ exoribonuclease RNase R has been shown to post-transcriptionally down-regulate six virulence related genes (Tsao et al., 2009), but its potential role in sRNA-mediated regulation still needs to be investigated.

TRANSCRIPTIONAL REGULATION OF SMALL RNAs IN *H. pylori*

In enterobacteria, several of the functionally characterized sRNAs have been shown be key regulators of larger regulatory networks (Papenfort and Vogel, 2009; Beisel and Storz, 2010). Moreover, some sRNAs are controlled by key transcriptional regulators of

certain metabolic pathways and several transcriptional regulators have been shown to be themselves regulated at the post-transcriptional level (Storz et al., 2011). Thus, besides the identification of their target genes, the investigation of transcriptional regulators of sRNAs and antisense RNAs will help provide insights in the conditions in which their expression is required and what are their physiological roles. As the *H. pylori* genome contains only a few transcriptional regulators, it is likely that some of the sRNAs are regulated by one or more of these transcription factors and *vice versa*.

PERSPECTIVE

The discovery of an unexpected wealth of sRNAs in *Helicobacter* opens an additional area of gene expression control in this pathogenic Epsilonproteobacterium. The characterization of selected *cis*- and *trans*-encoded sRNAs will uncover their potential regulatory functions in virulence and stress response of *H. pylori*. Moreover, these studies could reveal novel mechanisms of post-transcriptional regulation independent of the RNA chaperone Hfq. The identification of novel ribonucleoprotein complexes will expand our knowledge about cellular regulators and could provide new targets for antimicrobial therapies against emerging pathogens.

Besides mechanistic aspects of riboregulation, research on sRNAs in *H. pylori* could also help to understand phenotypes observed in previous genetic screens. Global transposon screens failed to hit around 340 genes indicating their potential essentiality and revealed about 220 candidate mutants with a colonization defect in mice (Salama et al., 2004; Baldwin et al., 2007). Since at least 60 sRNAs have been validated in *H. pylori* and considering the high fraction of *cis*-encoded antisense RNAs, several of the virulence associated genes could be subjected to post-transcriptional regulation. For example, the above mentioned first examples of functional sRNAs in *H. pylori* prove post-transcriptional regulation of the virulence factors, urease, and TlpB in *H. pylori* (Sharma et al., 2010; Wen et al., 2010). In addition, a transposon screen for virulence and colonization factors in *H. pylori* revealed several mutants with a transposon hit at the end of genes or in intergenic regions, indicating that there might be additional sRNAs with a role in virulence (Baldwin et al., 2007).

Next-generation sequencing methods have turned out to be an effective tool to uncover the transcriptome structure and identify novel transcripts in prokaryotes. RNA-seq based expression profiling of bacteria grown under different conditions or in comparison to mutant strains will help to identify sRNAs, which are induced under certain stress or virulence conditions. Moreover, the full understanding of infection processes requires the parallel investigation of gene expression in both, pathogen and host. Microarray-based techniques are limited by the requirement of relatively high amounts of starting material as well as cross-hybridization problems of host and pathogen RNA. RNA-seq methods could overcome those limitations by parallel sequencing of host and pathogen RNA without any prior separation steps followed by mapping of the cDNA reads to the host and pathogen genomes. Furthermore, the investigation of single cell transcriptomes (Tang et al., 2009; Kang et al., 2011) as well as the study of expression profiles of several strains by comparative RNA-seq approaches could lead to new insights into gene expression in bacterial sub-populations or differences between pathogenic and non-pathogenic species.

The study of riboregulation in *H. pylori* will also help to understand post-transcriptional regulation in other Epsilonproteobacteria, such as the emerging pathogen *Campylobacter jejuni*, which is considered as the leading cause of bacterial gastroenteritis (Young et al., 2007). Recently, first sRNA candidates have also been identified in *C. jejuni* NCTC11168 based on conventional RNA-seq combined with predictions of conserved RNA structures (Chaudhuri et al., 2011). A dRNA-seq analysis of the same strain revealed more than 20 candidate sRNAs as well as a complex and compact transcriptional output (Porcelli and van Vliet, personal communication). A comparative dRNA-seq analysis of multiple *C. jejuni* strains indicates that many of the sRNAs are conserved among *C. jejuni* strains and show similar expression profiles (Dugar and Sharma, unpublished). However, some conserved sRNAs show distinct expression patterns in certain strains indicating that they could have varying function. Overall, *Helicobacter* and *Campylobacter* seem to have evolved their own specific sRNA repertoires, but with respect to their smaller genome sizes compared to *E. coli* express a comparable number of sRNAs. Research on sRNAs in *Helicobacter* and *Campylobacter* as well as parallel transcriptome analysis of host and pathogen during the time-course of infection will help to shed light on post-transcriptional regulation and virulence mechanisms not only in Epsilonproteobacteria, but also other bacterial pathogens, including species that lack Hfq.

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Conserved transcriptional unit organization of the cag pathogenicity island among *Helicobacter pylori* strains

Linda H. Ta[1], Lori M. Hansen[1], William E. Sause[2], Olga Shiva[3], Aram Millstein[1], Karen M. Ottemann[2], Andrea R. Castillo[2]* and Jay V. Solnick[1]*

[1] *Departments of Medicine and Microbiology & Immunology, Center for Comparative Medicine, University of California, Davis, Davis, CA, USA*
[2] *Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, Santa Cruz, CA, USA*
[3] *Department of Biology, Eastern Washington University, Cheney, WA, USA*

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Jeong-Heon Cha, Yonsei University, South Korea
Hilde De Reuse, Institut Pasteur, France

****Correspondence:***
Andrea R. Castillo, Department of Biology, Eastern Washington University, Cheney, WA 99004, USA.
e-mail: acastillo@ewu.edu;
Jay V. Solnick, Center for Comparative Medicine, University of California Davis, Davis, CA 95616, USA.
e-mail: jvsolnick@ucdavis.edu

The *Helicobacter pylori cag* pathogenicity island (*cag* PAI) encodes a type IV secretion system that is more commonly found in strains isolated from patients with gastroduodenal disease than from those with asymptomatic gastritis. Genome-wide organization of the transcriptional units in *H. pylori* strain 26695 was recently established using RNA sequence analysis (Sharma et al., 2010). Here we used quantitative reverse-transcription polymerase chain reaction of open reading frames and intergenic regions to identify putative *cag* PAI operons in *H. pylori*; these operons were analyzed further by transcript profiling after deletion of selected promoter regions. Additionally, we used a promoter-trap system to identify functional *cag* PAI promoters. The results demonstrated that expression of genes on the *H. pylori cag* PAI varies by nearly five orders of magnitude and that the organization of *cag* PAI genes into transcriptional units is conserved among several *H. pylori* strains, including, 26695, J99, G27, and J166. We found evidence for 20 transcripts within the *cag* PAI, many of which likely overlap. Our data suggests that there are at least 11 operons: *cag1-4*, *cag3-4*, *cag10-9*, *cag8-7*, *cag6-5*, *cag11-12*, *cag16-17*, *cag19-18*, *cag21-20*, *cag23-22*, and *cag25-24*, as well as five monocistronic genes (*cag4*, *cag13*, *cag14*, *cag15*, and *cag26*). Additionally, the location of four of our functionally identified promoters suggests they are directing expression of, in one case, a truncated version of *cag26* and in the other three, transcripts that are antisense to *cag7*, *cag17*, and *cag23*. We verified expression of two of these antisense transcripts, those antisense to *cag17* and *cag23*, by reverse-transcription polymerase chain reaction. Taken together, our results suggest that the *cag* PAI transcriptional profile is generally conserved among *H. pylori* strains, 26695, J99, G27, and J166, and is likely complex.

Keywords: *cag* PAI, operon structure, expression

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterium that infects the stomachs of approximately half the human population. Although infection is typically asymptomatic throughout the lifetime of the host, it causes peptic ulcer disease in about 10% of those infected and gastric adenocarcinoma in about 1–3% (Kusters et al., 2006). The best-studied bacterial factor associated with clinical sequelae of *H. pylori* infection is the cytotoxin associated gene pathogenicity island (*cag* PAI). Patients infected with *H. pylori* strains that contain the *cag* PAI are at increased risk for both peptic ulcer and gastric cancer (Kusters et al., 2006). Experimental studies in gerbils (Rieder et al., 2005), mice (Arnold et al., 2011), and rhesus macaques (Hornsby et al., 2008) have also demonstrated the pro-inflammatory effects of the *cag* PAI.

The 40-kb *cag* PAI contains on average 27 genes, several of which encode a type IV secretion apparatus that is required for translocation of the effector molecules CagA (*cag26*) and peptidoglycan into host epithelial cells (Segal et al., 1997; Odenbreit et al., 2000; Rohde et al., 2003; Viala et al., 2004). Of the 27 genes on the *cag* PAI, 18 are required for the translocation of CagA into host cells and 15 are required to induce transcription of the pro-inflammatory cytokine IL-8 (Fischer et al., 2001; Shaffer et al., 2011). CagA is reliant on the secretion chaperone protein CagF (*cag22*) for recruitment to the type IV translocation channel (Pattis et al., 2007). Upon translocation into the cell, CagA is phosphorylated at C-terminal tyrosine residues by c-Src and other kinases, which results in the activation of receptor tyrosine kinase (RTK)-like signaling pathways (Segal et al., 1997; Selbach et al., 2002). Both phosphorylated and unphosphorylated CagA contribute to *H. pylori* pathogenesis via multiple mechanisms, including the disruption of the cytoskeleton, interruption of cellular signaling, and interference with adhesion between adjacent cells (Backert and Selbach, 2008).

Several studies have provided a glimpse of the *cag* PAI transcriptional unit organization. One initial study employed a urease transcription fusion to check for promoters in nine *cag* PAI DNA regions that were upstream of groups of co-directional genes (Joyce et al., 2001). This analysis determined that there were at least

five promoters on the *cag* PAI. Another early study identified the promoters responsible for regulating *cagA* and *cagB* (Spohn et al., 1997). A more recent genome-wide transcriptional unit analysis that used RNA sequencing identified 14 transcriptional units within the *cag* PAI. Additionally, they found many potential small regulatory RNAs (Sharma et al., 2010). Other studies have suggested that several *cag* PAI genes are differentially regulated *in vivo* compared to *in vitro* (Joyce et al., 2001; Boonjakuakul et al., 2005; Castillo et al., 2008b). In one such study, an *in vivo* induced promoter called P*ivi66*, was internal to the *cag7* gene (Castillo et al., 2008b), which suggested that promoters may not always be within intergenic regions.

Here we sought to determine the conservation of operon structure in the *cag* PAI among *H. pylori* strains, and to identify promoters responsible for the transcription of *cag* PAI genes in strains 26695, J99, and G27, whose genomes are sequenced (Tomb et al., 1997; Alm et al., 1999), and in strain J166 that we and others have used to infect rhesus macaques (Hornsby et al., 2008). Operon structure was first predicted by a gene expression analysis that used quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for both open reading frames (ORFs) and intergenic regions. The predicted putative operons were further defined by qRT-PCR after deletion of selected promoter regions. Since our transcription analyses suggested a potentially complex operon structure, we augmented these studies with a non-biased promoter-trap study that identified *cag* PAI promoters as DNA regions capable of directing expression of a heterologous reporter. Our results demonstrate that there is remarkable consistency across strains in the expression of genes in the *cag* PAI, which is organized into at least 20 transcriptional units.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE

Helicobacter pylori strains 26695 (Tomb et al., 1997), J99 (Alm et al., 1999), J166 (Hornsby et al., 2008), and ACHP17 (mG27 *HP0294/295::res1-aphA3-res1*; Castillo et al., 2008a) were used for these studies. DNA and RNA for qRT-PCR were prepared from strains cultured on *Brucella* agar (Difco Laboratories, Detroit, MI, USA) containing 5% bovine calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 5 μg/mL trimethoprim, 10 μg/mL vancomycin, 2.5 IU/mL polymixin B, 2.5 μg/mL amphotericin B (TVPA, all from Sigma, St. Louis, MO, USA) and incubated at 37˚C with an atmosphere that contained 5% CO_2. Plate grown bacteria were then transferred to *Brucella* broth containing bovine calf serum with TVPA and incubated at 37˚C in 5% CO_2 with gentle rotation at 60 rpm. The OD_{600} was determined for each culture 18–24 h after inoculation. The promoter reporter *H. pylori* strain ACHP17 and strain G27 from which RNA was isolated for RT-PCR were grown under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37˚C on columbia blood agar plates with 4% (w/v) columbia agar base, 5% (w/v) defibrinated horse blood (Hemostat labs), 0.2% (w/v) β-cyclodextrin, 10 μg/mL vancomycin, 50 μg/mL cycloheximide, 5 μg/mL cefsulodin, 8 μg/mL amphotericin B, 2.5 IU/mL polymyxin and 5 μg/mL trimethoprim. *H. pylori* strains were stored at −80˚C in brain heart infusion media supplemented with 10% fetal bovine serum, 1% (w/v) β-cyclodextrin, 25% glycerol, and 5% dimethyl sulfoxide.

Escherichia coli strain DH10B (Grant et al., 1990) was grown at 37˚C in Luria–Bertani (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl), with 100 μg/mL ampicillin. *E. coli* was also grown on solid LB media consisting of LB broth with 1.5% (w/v) agar. All antibiotics were purchased from Sigma-Aldrich, Fisher, or ISC BioExpress. All culture media were purchased from Remel, Fisher, or Difco unless otherwise indicated.

RNA AND DNA EXTRACTION

At OD_{600} 0.4–0.5 (early exponential growth phase) 2 mL aliquots were taken from *H. pylori* liquid cultures and centrifuged at 16,000 × *g* for 30 s at room temperature. Supernatants were removed and 1 mL of TriZol (Invitrogen) was immediately added. Samples were vortexed and RNA was extracted according to the manufacturer's directions. RNA was treated with DNase I (Roche Applied Sciences, Mannheim, Germany), purified using an RNeasy clean up kit (QIAGEN, Inc., Valencia, CA, USA), and suspended in ultra pure water (Invitrogen) at a concentration of 20 ng/μL.

DNA was extracted from plate grown bacteria using a DNeasy Tissue Kit (QIAGEN). DNA samples were diluted in ultra pure water to a concentration of 5 ng/μL and stored at −20˚C.

RT-PCR TO DETECT PROMOTERS, PIII, PIX, AND PXII

Reverse-transcriptase polymerase chain reactions were carried out using the Super Script One-step RT-PCR kit with Platinum Taq (Invitrogen). One hundred or 250 ng of RNA was used as a template for each RT-PCR reaction. For the reverse-transcription step (55˚C for 30 min), only the oligonucleotide that was complementary to the putative transcript was included in the reaction, PIIIR (5′-cctagcgaccaaaagcgatgaa-3′), PIXR (5′-gaaactgctaagaatatcagtg-3′), and PXIIR (5′-cgtcattaatcaaatagaacaaagc-3′). The reverse-transcriptase in these reactions was then inactivated by incubation at 94˚C for 5 min. Prior to starting the PCR program (35 cycles, 94˚C/30 s, 55˚C/30 s, 72˚C/30 s) the reactions were briefly incubated on ice (∼1 min) while the second oligonucleotides, PIIIF (5′-cattgtggtctttcccgaaagc-3′), PIXF (5′-cactcttgcctataaaggcc-3′), and PXIIF (5′-ctgagacgacaagctatgatttc-3′) were added. Oligonucleotides for our positive control were HP188F (5′-ccactataaaagagatctttcaagcggaagg-3′) and HP187R (5′-gcttgccctcggtgtctgcatc-3′); HP187R was present in the RT reaction and both HP187R and HP188F were present in the PCR reaction. As a control for amplification, each set of oligonucleotides was used in a PCR reaction with DNA as the template. Additionally, each set of oligonucleotides was used in an RT-PCR reaction with the RNA template and Platinum Taq only. This control was done to verify our RNA samples were DNA free.

qRT-PCR AND AGAROSE GEL ELECTROPHORESIS

Quantitative real time RT-PCR was performed with primer pairs specific for each *cag* gene (**Table A1** in Appendix) and for each intergenic region (**Table A2** in Appendix), using methods essentially as described (Boonjakuakul et al., 2004, 2005). In brief, RT and PCR were performed in a single 20 μL reaction mixture using the thermostable recombinant *Tth* (r*Tth*) DNA polymerase (Applied Biosystems) with 100 ng RNA extracted as described above. In the presence of $Mn(OAc)_2$, r*Tth* has reverse-transcriptase activity and DNA polymerase activity. Two-step amplification was

performed with 45 cycles at 95°C for 20 s followed by 59.5°C for 1 min. Accumulation of PCR product was detected during each cycle by excitation of SYBR green at 490 nm. Relative fluorescence was characterized by a cycle threshold (*C*t) value, which was defined as the crossover point of the kinetic curve with an arbitrary fluorescence level set at 150 relative fluorescence units. The absence of contaminating DNA was examined by performing the RT-PCR with $MgCl_2$, in which r*Tth* has DNA polymerase but no RT activity. All qRT-PCR products were electrophoresed on a 2% agarose (Invitrogen) gel to verify correct product size. Transcript abundance was calculated only if the observed *C*t with RNA template was less than that of the no-template control, and there was a band of the appropriate size on an agarose gel. Otherwise, transcript was considered absent. All transcript copy numbers were normalized to 16S RNA and the data presented represents the average of duplicate wells.

CONSTRUCTION OF *cag* PAI PROMOTER DELETION MUTANTS

The chloramphenicol resistance conferring *cat* gene from plasmid pNR9589 (Wang and Taylor, 1990) and 1–2 kb DNA fragments of the genes directly flanking the region targeted for deletion were PCR amplified (oligonucleotides in **Table A3** in Appendix) with compatible restriction sites. All three fragments were digested with the appropriate enzymes and ligated with compatibly digested pBluescript SK− (Stratagene, La Jolla, CA, USA) to generate a shuttle plasmid with fragments of the *cag* PAI flanking the *cat* gene. The shuttle plasmid was amplified in *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA), sequence verified, and then used to transform *H. pylori* strain J166 by a standard natural transformation procedure (Salama et al., 2001). *H. pylori* transformants were selected on *Brucella* agar plates with TVPA and 4 μg/mL chloramphenicol. Correct replacement of *cag* PAI DNA regions with the *cat* gene was verified using PCR and DNA sequence analyses.

GENERATING THE *H. PYLORI cag* PAI LIBRARY OF PUTATIVE PROMOTERS

Genomic DNA was isolated from *H. pylori* J166 and mG27 (Wizard genomic prep kit, Promega). The DNA region representing the *cag* PAI was amplified from each strain as a set of 13 PCR products of ∼2.5 kb in length with 600 bp of overlap between adjacent PCR products (oligonucleotides in **Table A4** in Appendix). For each strain, the PCR products were pooled, partially digested with *Sau3A*, and ligated to *Bgl*II digested p*cat-T*-tnpR (Castillo et al., 2008a) to generate recombinant plasmids, p*cat-T-caglibmG27-tnpR* and p*cat-T-caglibJ166-tnpR*. After ligation, the recombinant plasmids were transformed into *E. coli* DH10B and the *E. coli* were plated on LB agar with ampicillin. For these strains, ∼2193 (p*cat-T-caglibmG27-tnpR*) or 5000 (p*cat-T-caglibmJ166-tnpR*) individual ampicillin resistant (Amp^R) colonies were pooled, grown overnight, and treated (Qiagen miniprep extraction kit, Qiagen) to extract the recombinant plasmids. For a subset of colonies from each library, individual recombinant plasmids were analyzed for the presence and size of a *H. pylori* cag PAI insert. All recombinant plasmids analyzed contained inserts and had an average insert size of 469 bp for p*cat-T-caglibmG27-tnpR* and 96 bp for p*cat-T-caglibmJ166-tnpR*.

To isolate putative promoters, *H. pylori* strain *ACHP17* was transformed using natural transformation (Salama et al., 2001) with either p*cat-T-caglibmG27-tnpR* or p*cat-T-caglibJ166-tnpR*, and transformants were selected based on their resistance to chloramphenicol (Cm) on CBA plus 13 μg/mL Cm. Cm resistant (Cm^R) transformants were passed twice on Cm prior to being analyzed for kanamycin sensitivity (Km^S) on CBA plus 15 μg/mL kanamycin.

To examine the diversity of the *cag* PAI library clones in *H. pylori*, 10–30 Cm^R clones were selected from each library and the region upstream of *tnpR* was sequenced using primers rrnB1 and tnpRbk75 (Castillo et al., 2008a). The average insert size was 232 bp for p*cat-T-caglibmG27-tnpR* and 100 bp for p*cat-T-caglibJ166-tnpR*. PCR amplicons were sequenced and compared to the 26695 and G27 genomes to assess randomness of the cloned regions. The number of transformants needed to obtain 100% coverage of the *cag* PAI for each library was determined using the formula $N = \ln[1 - P/\ln(1 - I/G)]$ (N = number of independent clones, I = size of averaged cloned fragment, G = size of target genome, and P = probability). These calculations suggested that 791 p*cat-T-caglibmG27-tnpR* and 1840 p*cat-T-caglibJ166-tnpR* transformants would be required for complete coverage of the *cag* PAI.

RESULTS

We used both transcription profiling and a functional genetic approach to define *cag* PAI operons and the putative promoters that regulate them. First, we performed qRT-PCR to determine the mRNA copy number within each ORF and each intergenic region on the *cag* PAI. Our assumption was that contiguous genes transcribed in the same direction, with the presence of intergenic message and similar mRNA copy number, would likely form an operon. Selected putative operons were then further analyzed by deletion of the promoter region and reanalysis of mRNA copy number of downstream genes. We then augmented these analyses by using a non-biased promoter-trap system to find active promoters within the *cag* PAI.

CO-EXPRESSION OF *cag* PAI GENES BASED ON GENE AND INTERGENIC TRANSCRIPT COPY NUMBER

We first calculated the transcript copy number for each gene and intergenic region within the *cag* PAI of *H. pylori* J99, 26695, and J166, using methods described previously (Boonjakuakul et al., 2004, 2005). Briefly, three factors were used to calculate copies per cell: (a) a 10-fold change in starting template concentration corresponds to a 3.3-cycles change in *C*t ($2^{3.3} = 10$); (b) 100 ng of RNA equals 10^6 *H. pylori* cells, and (c) the empirically derived observation that a *C*t of 19 corresponds to 1×10^5 copies of starting DNA template (assuming 1 copy per bacterial chromosome). We have previously shown that calculation of mRNA copies/cell using *C*t corrected for primer efficiency yields values that are essentially identical to those obtained by the more conventional method using standard curves (Boonjakuakul et al., 2004).

Transcript levels for all genes on the *cag* PAI for each *H. pylori* strain are shown in **Figure 1**. For clarity, intergenic transcript is shown only as present (adjacent bars representing gene transcript levels are shaded identically) or absent (adjacent bars are shaded differently). For example, intergenic transcript was

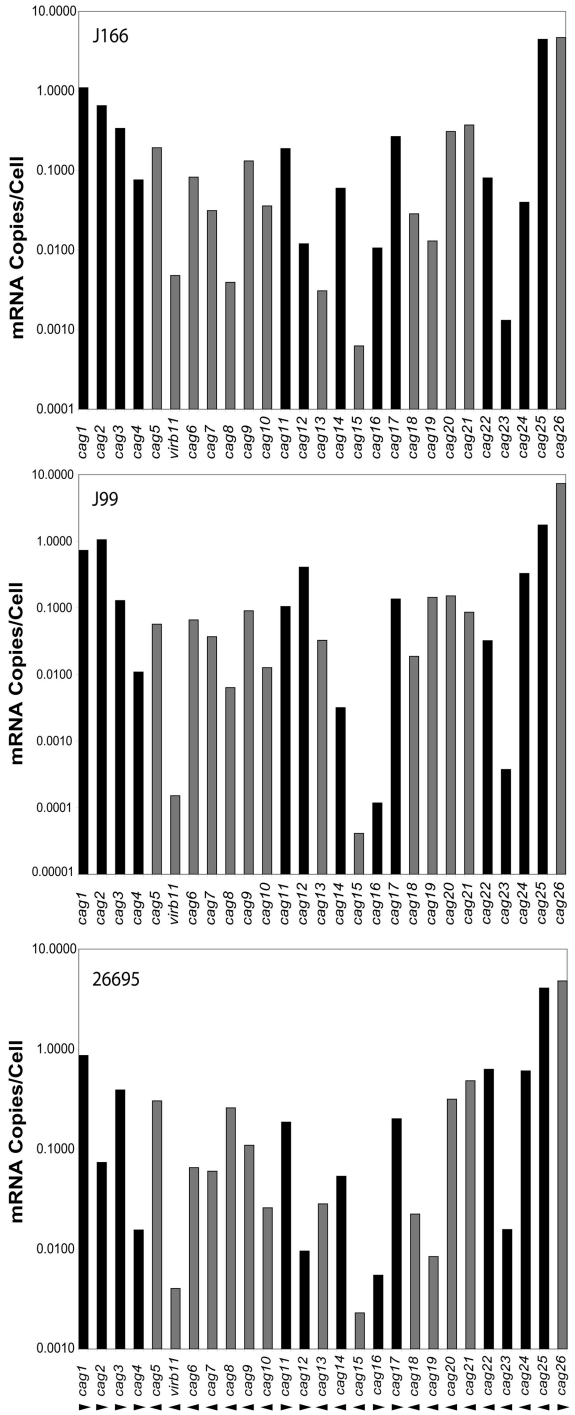

FIGURE 1 | Expression level of each gene on the *cag* PAI (mRNA copies/cell; normalized to 16S rRNA) for *H. pylori* strains J166 (top panel), J99 (middle panel), and 26695 (bottom panel). Our data represents the average of duplicate reactions. Adjacent genes for which intergenic transcript was detected are indicated with the same shading (black or gray). Direction of transcription is shown by arrowheads below the bottom panel. Since *cag26* (*cagA*) is not contiguous with the PAI in *H. pylori* J166, the *cag25-26* intergenic message was not measured, but presumed to be absent because *cag25* and *cag26* transcription is in opposite directions.

detected between *cag1* and *cag2* (both black bars) and between *cag10* and *cag9* (both gray bars), but not between *cag15* (gray bar) and *cag14* (black bar). Transcript levels varied within each strain by as much as five orders of magnitude, ranging from about 10 copies/cell to as low as 1 copy per 10,000 cells. These estimates are consistent with our previous studies (Boonjakuakul et al., 2004, 2005) and with estimates of gene expression levels in *Saccharomyces cerevisiae* (Kang et al., 2000) and *E. coli* (Young and Bremer, 1975). The highest transcript abundance was found for *cag26* and for *cag25*. Since *cag26* encodes an effector protein, CagA, secreted via the type IV secretion system, and *cag25* encodes a virB2 ortholog that is thought to encode a pilin protein that forms a multimeric structure (Andrzejewska et al., 2006), it is not surprising that these genes are highly expressed. Although in general, the expression level of genes on the *cag* PAI was similar across the three strains analyzed, there is some variation that appears to occur within the operons predicted by these experiments (**Figure 2**).

We reasoned that adjacent genes transcribed with ORFs in the same direction, with the presence of intergenic transcript, might represent a single transcriptional unit, particularly if the transcript abundance was similar across genes. Therefore, we initially considered the possibility that the following may represent *cag* PAI operons (numbered in the direction of transcription): *cag1-4*, *cag10-5*, *cag11-12*, *cag16-17*, *cag21-18*, and *cag25-22* (**Figure 1**). However, there were sometimes marked differences in transcript abundance of genes within these putative operons (e.g., *cag25-22*, **Figures 1** and **2**). This might occur due to differential decay of the transcript or possibly because the gene is part of more than one transcriptional unit. To address these possibilities, we deleted the genomic region immediately upstream of the translational start of the first gene in each of six putative operons in *H. pylori* strain J166, a region likely to contain the promoter, and then measured *cag* PAI gene transcript abundance. We reasoned that deletion of this region should decrease the expression level of all genes in the transcriptional unit, and leave others unchanged.

Deletion of the putative promoter regions upstream of *cag1*, *cag10*, *cag11*, *cag16*, *cag21*, and *cag25* had differential effects on the expression of downstream genes when compared to the isogenic wild type *H. pylori* J166 strain (**Figure 3**). Deletion of the region upstream of *cag1* reduced expression of *cag1-3* by three orders of magnitude and *cag4* by only 1.5 orders of magnitude. By contrast, expression of *cag5*, a gene transcribed in the opposite direction of this putative operon, remained essentially unchanged. Deletion of the region upstream of *cag10* reduced expression of both *cag10* and *cag9* by similar levels and had no effect on expression of *cag8-7*. Deletion of the putative promoters upstream of *cag11*, *cag16*, and *cag21* reduced expression of the downstream genes, *cag11-12*, *cag16-17*, and *cag21-18*, but in each case to different levels, ranging from 1 to 3 orders of magnitude (**Figure 3**). Finally, deletion of the region upstream of *cag25* reduced expression of the downstream genes *cag25-23* to different levels and had no effect on the expression of *cag22*. In some cases, these results make clear predictions about operon structure. For example, our original prediction of *cag10-5* and *cag25-22* as operons was incorrect, since in each case one or more downstream genes did not

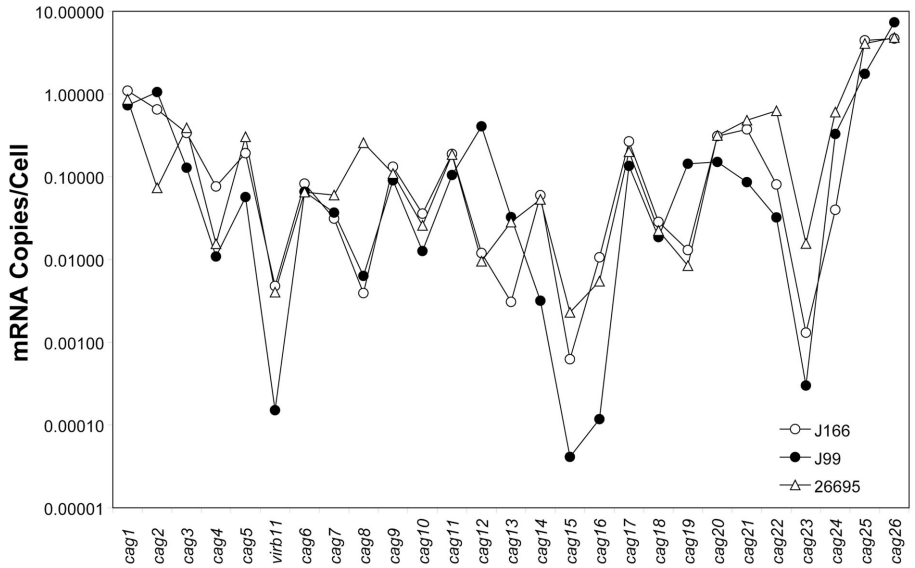

FIGURE 2 | Composite gene expression (mRNA copies/cell, normalized to 16S rRNA) for each gene on the *cag* PAI of *H. pylori* strains J166 (open circles), J99 (closed circles), and 26695 (open triangles). Our data represents the average of duplicate reactions.

change appreciably in the promoter knockouts. Thus, *cag10-5* consists of at least two operons, *cag10-9* and *cag8-7*, which also may be organized into one or more transcriptional units. Similarly, *cag25-22* appears to have only *cag25-24* on one transcriptional unit, with *cag22* and perhaps *cag23* on separate transcripts. The variable change we observed in *cag* PAI gene expression after deletion of the predicted upstream promoter again suggests that either the transcripts are being degraded or that there are additional promoters controlling expression of these genes. To identify additional promoters that may contribute to the more complex expression pattern we observed here, we undertook a non-biased promoter-trap approach.

NON-BIASED PROMOTER-TRAP IDENTIFIES ADDITIONAL *cag* PAI PROMOTERS

We next employed a functional identification of *cag* PAI promoters strategy based on the ability of short cloned regions of the *cag* PAI to direct expression of a heterologous promoter. We used a *tnpR* transcriptional reporter developed for *Vibrio cholerae* (Camilli et al., 1994) that had been previously modified to function in *H. pylori* (Camilli et al., 1994; Castillo et al., 2008b). We constructed libraries of putative *cag* PAI promoters using both *H. pylori* strains J166 and G27 as template for PCR; we cloned the Sau3A-digested fragments upstream of the promoterless *tnpR* gene in p*CT-tnpR*. If the cloned *cag* PAI region contained a promoter, we predicted it would direct *tnpR* expression and the creation of the TnpR protein. TnpR in turn would catalyze the removal of an unlinked kanamycin resistance (Km^R) cassette and convert the *H. pylori* reporter strain ACHP17 from Km^R to Km^S.

For promoter identification, *H. pylori* strain ACHP17 bearing the *res1-kan-res1* marker was transformed with p*cat-T-caglibmG27-tnpR* or p*cat-T-caglibJ166-tnpR* to Cm^R, followed by screening for retention or loss of the *res1-kan-res1* cassette. We screened 1060 *H. pylori* p*cat-T-caglibmG27-tnpR* transformants and 1274 *H. pylori* p*cat-T-caglibmJ166-tnpR* transformants, representing 100 and 71% coverage, respectively. This analysis identified 34 and 27 transformants, respectively, that were sensitive to kanamycin and thus had expressed *tnpR*. After removing redundant clones, we determined that the DNA sequences upstream of *tnpR* in these Km^S transformants correspond to 14 unique loci (**Table 1**). Eleven and four promoters were identified through the screening of our p*cat-T-caglibmG27-tnpR* and p*cat-T-caglibmJ166-tnpR* libraries, respectively; one promoter, PIII (**Table 1**; **Figure 4**), was isolated from both libraries.

PROMOTER-TRAP-IDENTIFIED PROMOTERS

We next mapped our promoter-trap-identified promoters onto the *cag* PAI map and compared these promoters to those found in by our initial qRT-PCR analysis and also to the work of others. Several of the promoter-trap-identified promoters were located in *cag* PAI regions that were either predicted by the qRT-PCR or promoter deletion analyses (**Table 1**). These include the promoters upstream of *cag10* (PVIII), *cag11* (PII), *cag 21* (PI), and *cag25* (PV; **Figures 3** and **4**; **Table 1**). The promoter-trap approach also identified several possible promoters that were located within operons that might account for the variable gene expression observed after deleting the main promoter (**Figures 3** and **4**). These include PIV, PX, and PXIII that are located in genomic positions to suggest they contribute to the expression of *cag4*, *cag8-7*, and *cag23-22* (**Figures 3** and **4**).

The other putative promoters identified in our promoter-trap study are either consistent with *cag* PAI transcripts predicted by other groups, or as of yet, unique. We identified a promoter that is upstream of *cag26* (PXI) and one that is within, and in the same direction as, *cag26* (PXIV). The promoter upstream of *cag26* was identified in work done by Spohn et al. (1997) and more recently

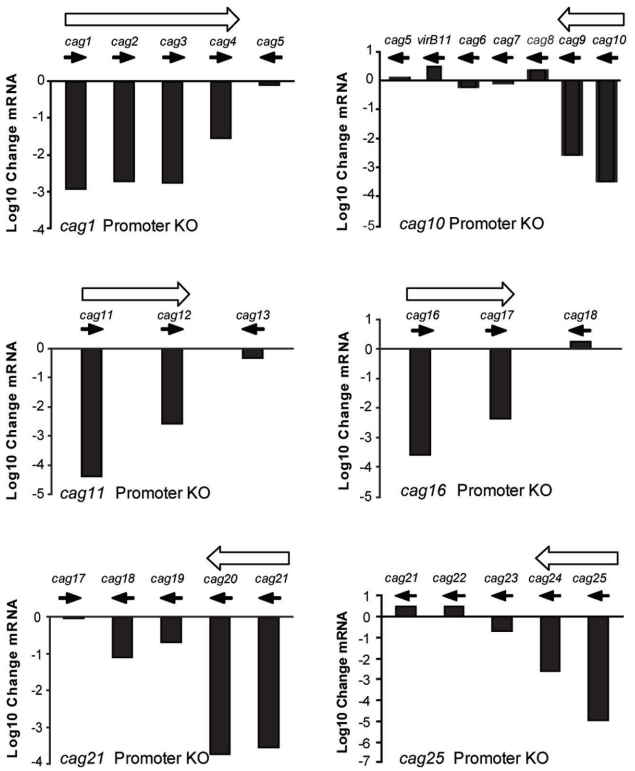

FIGURE 3 | Change in *cag* PAI gene expression (log 10 change mRNA) between wild type *H. pylori* J166 and isogenic deletions of DNA upstream of the translational start codon in *cag1* (416 bp, top left), *cag10* (498 bp, top right), *cag11* (498 bp, middle left), *cag16* (532 bp, middle right), *cag21* (615 bp, bottom left), and *cag25* (706 bp, bottom right). Filled arrows indicate direction of transcription and open arrows indicate putative operons.

by Sharma et al. (2010) as a promoter that drives expression of *cag26* (**Figure 4**; Spohn et al., 1997; Sharma et al., 2010). We also identified a unique putative promoter (PVI) that overlaps *cag3* and the adjacent upstream region and is in the correct direction to promote expression of a polycistronic mRNA including *cag3* and *cag4* (**Figure 4**). Two of our putative promoters were located within *cag7*, one in the same direction (PVII) and one antisense (PIX) to *cag7* (**Figure 4**). We hypothesize the promoter located within *cag7* contributes to expression of *cag6-5* and the promoter that is antisense to *cag7* may direct expression of a regulatory sRNA. Neither of these promoters has been identified by other studies. Finally, the last two putative promoters we identified, PIII and PXII, were within and antisense to *cag23* and in the 3′ end of *cag18* and may direct expression of sRNAs that are antisense to *cag23* and *cag17*, respectively. These promoters are also unique to this study.

Although our transcription, promoter deletion and promoter-trap analyses do not completely overlap, they show reasonable agreement in predicting transcripts and operon structure and are generally consistent with operon structure predicted by others (**Table 1**, discussion). Taken together our data suggests the existence of at least 20 *cag* PAI transcripts (**Figure 4**).

PIII AND PXII DIRECT EXPRESSION OF ANTISENSE TRANSCRIPTS

To determine if the promoters PIII, PIX, and PXII direct expression of transcripts that are antisense to *cag23*, *cag7*, and *cag17*, respectively, we carried out additional RT-PCR reactions on RNA isolated from *H. pylori* strain G27. The oligonucleotides (PIIIR, PIXR, and PXIIR) used in the reverse-transcription reactions were located ∼100–150 nt downstream of PIII, PIX, and PXII and were antisense to the putative transcripts. For the subsequent PCR reactions in which reverse-transcriptase had been inactivated, the sense oligonucleotides, PIIIF, PIXF, and PXII were added. Amplicons were detected downstream of PIII and PXII in the RT-PCR reactions and were absent in the corresponding polymerase only controls, suggesting that these promoters do in fact direct expression of transcripts (**Figure 5**). We did not detect a transcript downstream of PIX in our experiments; while it is possible that PIX is not a promoter, it is more likely that the transcript is regulated or is in very low abundance. The promoter-trap system by which PIX was identified was designed to capture low abundant and transient expression events.

DISCUSSION

In this study we used transcript profiling coupled with putative promoter deletion and a non-biased promoter-trap system to analyze expression of *cag* PAI genes and their organization into transcriptional units across several *H. pylori* strains. We found that *cag* PAI gene expression varies by nearly five orders of magnitude across the *cag* PAI, and that expression of *cag* PAI genes is similar across strains 26695, J99, and J166. Based on transcript profiling of *cag* PAI ORFs and intergenic regions, we initially placed *cag* PAI genes into six polycistrons and four monocistrons. However, subsequent promoter deletions coupled with transcript profiling and promoter-trap promoter identification studies suggested *cag* PAI operon structure was much more complex. Our data suggests that there are at least 11 operons: *cag1-4*, *cag3-4*, *cag10-9*, *cag8-7*, *cag6-5*, *cag11-12*, *cag16-17*, *cag19-18*, *cag21-20*, *cag23-22*, and *cag25-24*, as well as five monocistronic genes (*cag4*, *cag13*, *cag14*, *cag15*, *cag26*). Additionally, the location of four of our promoter-trap-identified promoters suggests they direct expression of, in one case, a truncated version of *cag26* and in the other three, transcripts that are antisense to *cag7*, *cag17*, and *cag23*. Using RT-PCR we verified the presence of transcripts that are antisense to *cag17* and *cag23*.

CONSERVATION OF *cag* PAI GENE EXPRESSION AMONG *H. PYLORI* STRAINS

Our transcript profiling of *cag* PAI ORFs and intergenic regions of three *H. pylori* strains, 26695, J99, and J166, suggested that *cag* PAI expression is generally conserved among strains. There were some genes, however, whose expression showed appreciable differences across strains. Potential reasons for these differences may be attributed to one or a combination of the following: (1) difficulty in accurate quantitation of low abundance transcripts, (2) differential stability of the transcripts, and (3) differential strength of the promoters. We suspect that the differences in *cag15* expression between strains may be due to its very low expression *in vitro* (Joyce et al., 2001). The reduced expression of *cag12*, *cag13*, and *cag19* in *H. pylori* strain 26695 compared to that of

Table 1 | Chromosomal location of putative *cag* PAI promoters.

Putative promoter (length; nt) ± strand	Cag PAI library, genome source	G27 genome position	26695 genome position	Sharma et al. (2010)[a], transcription start site (TSS)	Joyce et al. (2001)[b]	This study[c]
I (244)−	G27	542756-542513	574379-574136	TSS575200−	Y	Y (P)
II (38)+	G27	530249-530286	563751-563788	TSS564140+ TSS564347+	Y	Y (P)
III (91)+	G27 and J166	544137-544227	575760-575850	None	N	N
IV (104)+	G27	515270-515371	548689-548790	TSS549427+	N	Y (T)
V (71)−	G27	547735-547665	579357-579287	TSS579114−		Y (P)
VI (314)+	G27	514575-514888	547996-548307	None	N	N
VII (234)−	G27	525984-525661	558996-558763	None	N	N
VIII (635)−	G27	530667-530918	564409-563785	TSS564078− TSS564329−	Y	Y (P)
IX (132)+	G27	521375-521506	554800-554931	None	N	N
X (269)−	G27	528142-527874	561644-561372	TSS561595−	N	Y (T)
XI (419)+	G27	547665-548083	579287-579744	TSS579817+	N	N
XII (54)−	J166	539314-539259	570937-570882	None	N	N
XIII (68)−	J166	546797-546736	578420-578359	None	N	Y (T)
XIV (70)+	J166	548866-548927	580539-580604	None	ND	N

[a]*Transcription start sites (TSS) predicted by Sharma et al. (2010) that are downstream of, or within, the promoter-trap-identified promoter. The 26695 genomic position and DNA strand (+ or −) of the TSS are indicated. None, no TSS near the functionally identified promoter.* [b]*Y, promoter was previously identified by Joyce et al. (2001). N, promoter was not previously identified by Joyce et al. (2001).* [c]*P, the promoter-trap-identified promoter was within the genomic region deleted in our promoter deletion analysis; T, it is in a location that is consistent with the changes we observe in cag gene expression; N, it was not within a genomic region deleted in our promoter deletion analysis or indicated by changes in cag PAI gene expression.*

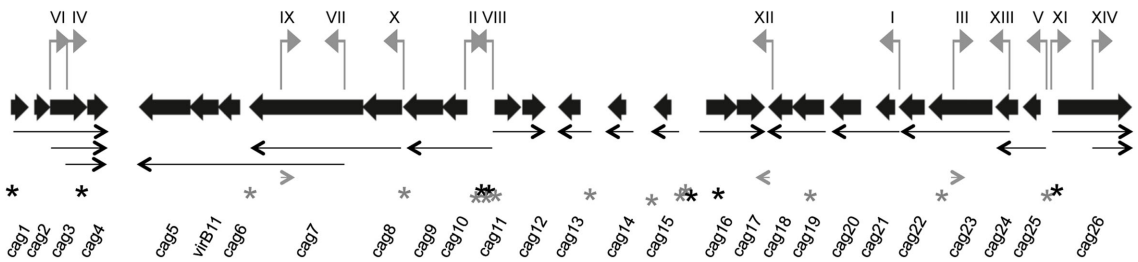

FIGURE 4 | Promoter-trap-identified promoters and proposed transcript map on the *H. pylori cag* PAI. Each gene on the *cag* PAI is represented by a thick black arrow, oriented in the direction of transcription, whose length and spacing are approximately proportional to the annotated gene length and intergenic spacing. DNA segments represented in the promoter library that contained functional promoters (numbered I–XIV) to correspond with **Table 1** and sequences in **Table A5** in Appendix) are shown as small gray flags pointing in the direction they direct transcription and positioned in their *cag* PAI location. Thin black arrows represent 17 of the 20 proposed transcripts and gray arrowheads represent the three potential antisense transcript start points. All arrows point in the direction of transcription. Transcription start sites identified in Sharma et al. (2010) are indicated by an asterisk (∗); black asterisks indicate the transcription start site is on the plus strand and gray asterisks indicate the minus strand.

J99 and J166 is more likely attributed to transcript instability and differences in promoter strength. Our expression findings should allow researchers to more confidently apply our and other *cag* PAI expression data to unique clinically isolated *H. pylori* strains.

DIFFERENT STUDIES PREDICT SIMILAR *cag* PAI OPERON STRUCTURE

Our findings are generally consistent with previous predictions of *cag* PAI promoters, expression and operon structure. First, our promoter-trap and promoter deletion studies identified four of the five *cag* PAI promoters, upstream of *cag1*, *cag10*, *cag21*, and *cag25* (not *cag15*), that were predicted by Joyce et al. (2001) in the *H. pylori* Alston strain. However our transcript profiling of *cag* PAI ORFs and intergenic regions did predict the promoter upstream of the *cag15* (**Figure 1**). The failure of our promoter-trap to identify the promoter upstream of *cag15* was not surprising, as Joyce et al. (2001) found that this promoter was only induced in co-culture with epithelial cells or in mice. A similar profile of promoters between the clinically isolated Alston strain and 26695, J99, J166, and G27 again supports conservation of *cag* PAI operon structure and expression between *H. pylori* strains.

Our promoter analyses also identified promoters upstream of *cag25* (*cagB*) and *cag26* (*cagA*) that were in positions similar to

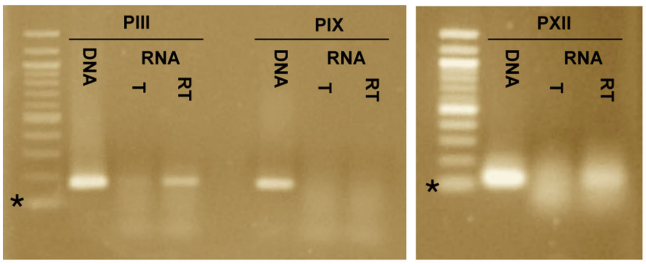

FIGURE 5 | Reverse-transcriptase polymerase chain reaction (RT-PCR) identifies transcripts downstream of promoter-trap-identified promoters PIII and PXII. RT-PCR was carried out for the three promoters that potentially directed expression of antisense transcripts, PIII, PIX, and PXII. Three reactions were included for each promoter, a DNA template + DNA polymerase (DNA), an RNA template + DNA polymerase (T) and an RNA template + reverse-transcriptase and DNA polymerase (RT). Amplicons of the correct size were detected for PIII and PXII in the RT reactions but not in the T reactions. An amplicon was not detected for PIX in the RT or T reactions. This supports expression of transcripts downstream of PIII and PXII, but not PIX. The * indicates the 100-bp marker of the 100-bp ladder.

those previously reported by Spohn et al. (1997) for *H. pylori* strain G27, and by Sharma et al. (2010) for *H. pylori* strain 26695. Spohn et al. (1997) identified two transcription start points upstream of *cag25* that are ∼200 bp upstream of what we and Sharma et al. (2010) found for *cag25*. All three studies predicted the same start point that is upstream of *cag26*, but we found an additional promoter that is located within *cag26*. The significance of multiple start sites upstream of *cag25* and within *cag26* are, as of yet, unclear. However, a recent study suggests discreet roles for the amino- and carboxy-terminus of Cag26 (CagA) and it is interesting to speculate this promoter could separate Cag26 function by creating a truncated protein (Pelz et al., 2011).

Our transcript profiles obtained from our work were also consistent with many of the 14 *cag* PAI operons identified in the *H. pylori* genome-wide transcript analysis conducted by Sharma et al. (2010). In common, we predicted five polycistrons: *cag1-4*, *cag6-5*, *cag8-7*, *cag11-12*, and *cag16-17*, and the three monocistrons: *cag4*, *cag13*, and *cag26*. Our promoter locations are consistent with their transcripts that start at *cag10*, *cag14*, and *cag25*, but our data did not predict that the transcripts extended to *cag7*, *cag13*, and *cag18*, respectively. We also did not find functional promoters upstream of *cag17* and *cag18* that would suggest they were also expressed as monocistrons. However, in addition to the truncated *cag26* transcript mentioned above, we also identified the following set of transcripts that were not identified by Sharma et al. (2010), including the polycistrons *cag3-4*, *cag21-20*, *cag19-18* and three transcripts that were antisense to *cag7*, *cag17*, and *cag23*. A transcript for *cag15* was also not identified by Sharma et al. (2010), likely due to its very low abundance *in vitro* (Joyce et al., 2001). We speculate that these discrepancies are due to potential issues with transcript abundance and stability here and in Sharma et al. (2010) and incomplete screening of our *cag* PAI promoter libraries.

INCOMPLETE SCREENING OF *cag* PAI PROMOTER LIBRARIES

Outstanding observations in our screening of the p*cat-T-caglibJ166-tnpR* and p*cat-T-caglibG27-tnpR* libraries in *H. pylori* ACHP17 were that we only identified four promoters grouped at the 3′-end of the *cag* PAI from p*cat-T-caglibJ166-tnpR* and that we did not identify promoters from the central region of the *cag* PAI from p*cat-T-caglibG27-tnpR*. We hypothesize that this was due to a combination of two things: (1) incomplete representation of the *cag* PAI region in both of our libraries and then (2) restriction modification system differences that were apparent in transforming our G27 based reporter strain ACHP17 with J166 *cag* PAI DNA. Although our library screening calculations (see Materials and Methods) suggested that we had screened 100% of the *H. pylori* G27 *cag* PAI and 71% of the *H. pylori* J166 *cag* PAI, our control experiments with 10 or 30 randomly selected *H. pylori* transformants, respectively, suggested that our libraries were biased; the *H. pylori caglibG27-tnpR* library was biased toward the left and right ends of the *cag* PAI and the *H. pylori caglibJ166* library was biased toward the right side of the *cag* PAI. Nonetheless, this methodology was very effective at identifying promoters in positions where we observed slight differences in expression of adjacent genes. Specific amplification of *cag* PAI regions (e.g., *cag12-17*) that were underrepresented in our *cag* PAI libraries will ensure better representation of the G27 *cag* PAI region in our library for future *in vivo* analyses.

COMPARING *cag* PAI EXPRESSION *IN VITRO* AND *IN VIVO*

This and previous studies have contributed to building a more complete expression profile of the clinically important *cag* PAI of *H. pylori* grown *in vitro* (Spohn et al., 1997; Joyce et al., 2001; Sharma et al., 2010). The promoters identified by these *in vitro* studies can now be analyzed for their potential regulation during *H. pylori* infection of a host. It is clear in at least two cases that *in vitro* predicted promoters, those upstream of *cag15* and *cag21*, are expressed at higher levels when co-cultured with an epithelial cell monolayer and in mice (Joyce et al., 2001). While we anticipate that a subset of our *in vitro* identified promoters will be regulated *in vivo* and may contribute to virulence, other studies suggest that there is a set of promoters or transcripts uniquely expressed *in vivo* (Scott et al., 2007; Castillo et al., 2008b); analysis of *H. pylori* transcripts isolated from gerbil stomachs predicted that *cag25* is expressed as a monocistron *in vivo* (Scott et al., 2007) and a promoter-trap study identified a unique promoter, P*ivi66*, within *cag7* (Castillo et al., 2008b). Analysis of our *H. pylori cat-T-caglibmG27-tnpR* library in rodents has the potential to identify additional *in vivo* induced *cag* PAI promoters.

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APPENDIX

Table A1 | Open reading frame primer pairs selected for real time RT-PCR.

Primer	Strain[a]	Forward primer (5′-3′)	Reverse primer (5′-3′)
Cag1	U	GCTATGGGGATTGTTGGGATAA	GCTTCAGTTGGTTCGTTGGTAA
Cag2	A	TGTAAGGGCGTTTTACGAGAA	TTTGGGATATTTAGGATTTTGTGAA
Cag2	B	ACTGTAAGGGCGTTTTACGAGAA	GGGCGTGTTTTCACAATGTAA
Cag2	C	GAATTTGTCCAATAGGGGATTTTTA	AACAGAGAGATTGCCTTTTTTTGTA
Cag3	A, C	GACACCTTGAATGTGAATGACAAA	GTTGTAATACCCATTGACTTGCTCTAA
Cag3	B	AAACAAGAGCGATGGGAACTTA	TAGGGGCGAACACACTTCA
Cag4	A, B	GCGAGAAAAATCCCTTAAAGACA	GTGTTTCATTCCCCCATTGTAA
Cag4	C	CCATCACTTTCAAGCAATACGA	GAGCGTTTTAGAATAGGTAGGGTAGAA
Cag5	A, C	CGGACTAGAGATATAGGAGCGAATAA	GCCACTGCCTGCCTACAA
Cag5	B	TAGTAGGAGCAATCAAGCCAATAAA	TAGGGACATAGGAGCGAATAAAAA
virB11	U	CCTCTAAGGCATGCTACTGAAGAA	TCGCTAAATTGCTGCTCAAAA
Cag6	U	GAAAGCACGTATCAAAAATGAACTAA	CAGATAAGAAGCCACTAGGTCTGAA
Cag7	U	AAGTCAGAAGAAATAACTGACGACTCTAA	TCACGATAAGAACAGCGACTACAA
Cag8	U	AGCAATGAACAGATTATCAACAAAGA	GTAGTAGTTGTAGTTTCTAGGCACGG
Cag9	U	TCTCATTGTTCCTAATTGGTTGAAA	CTTGTGGCTAATGGTGTGCTAA
Cag10	U	AACGAAGAAGTCTTGATTGATGAAA	CTGTGTATCGATCAATGCCATAA
Cag11	U	CACCTAGCAACTCACAGAGCAA	CCCACCCATACACAATCCTAA
Cag12	A, C	TGTTTTAATCGGTGCGACAA	GAGCGTAATCTTTTTAGAATGGTGAA
Cag12	B	CAAACTCAAAGACACACCATTCA	TTGTTGTTGGGATTATCATTGTAGTTA
Cag13	U	AATAACATGCGAAAACTCTTCTCAA	CTCCATAGTCTCACTCTCAAGCAA
Cag14	U	ACGCATTAGAGATCCGAACAA	CCATTCTTCAACACTTCTGCCATAA
Cag15	U	CAGGGGTGATTTTAGTTTATCCAA	TATGCTGAGAGAAACGAGTAGCAA
Cag16	U	GAAGAAGTGGCTGCAAAAGAA	CATAGGCATAAGGGTTAGGAAGAA
Cag17	A, C	TCAAAGACATGACGACGAAGAA	GCTCTTGCCCTATCATTTCGTAA
Cag17	B	TCGCTCTTTATTCTTTGGTTGTTTA	TGCTCTACTCTCGCCCTATCA
Cag18	U	CCAACCAACAAGTGCTCAAAAA	TCAATAACGCTAAATCTCCTCTCAA
Cag19	A, C	GACTTTTTGTGGTTTGTCTCTGAA	CGCCAAGCAAGATGTCTGAA
Cag19	B	GAATGGCTTTTTCTTTGGCA	TTTTTGTGGCTTGTCCTTGAA
Cag20	U	GCTGCTAACCAACAATACAACCAA	CTAAGATACCGCTCATCATTTCAA
Cag21	U	GGGGCTTGTTTCTAGAGATCAACTAA	GAAAGGATTGTTTGGACCGTAA
Cag22	A	CTTGCCCATCGTTTATTTCCTTA	ACCTTACCGCTCTTTATGATTTTTCTA
Cag22	B, C	TTTATGTTTATGCTTACTTCATGCTAGAA	CGCTCATATCAATCTGAATCCAA
Cag23	U	GCTAGTCATAGAGCAAGAGGTTCAAAA	CACAATAACAATCGCTACAATCAAA
Cag24	U	GTATGGGTTAGCAAATGACGATAAA	TTAAGGACTCTATTGACAATCACGAA
Cag25	U	CAAGAATCACTGACAGCTACAAGAA	ATACCGCCTGCCACCGCTAA
Cag26	U	ATAAAGCGATCAAAAATCCTACCAA	GGGGGTTGTATGATATTTTCCATAA

[a] *Strain specificity: A-26695, B-J99, C-J166, U-universal.*

Table A2 | Intergenic primer pairs selected for real time RT-PCR.

Primer pair[a]	Strain[b]	Forward primer (5′-3′)	Reverse primer (5′-3′)
Cag1/2	A	CGTTACCAACGAACCAACTGA	TCATTGAGCCATTTATTTCTCGTAA
Cag2/3	A	GTGTAAATGGACTGCTAGTGTGGA	AGCGATACAGCGGTTGCTA
Cag2/3	B	ATGTCCCAAATGCAACAGATTAA	CGGCTTCACTTATTTCTTTAGCATAA
Cag2/3	C	TTCACTCCCAATGAGTTTTTTACA	CGGCTTCACTTATTTCTTTAGCATA
Cag3/4	A	GTGTGGTTATGAGAGCGTTCAA	ATTTTTCTCGCCTGTTGTTCAA
Cag3/4	B	AACTGCTTTCACCACTAAGGGA	GGATTTTTCTCGCTTGTTGTTCA
Cag3/4	C	TGTGTGGTTATGAGAGCGTTCA	GGTGAGATTTTCGTATTGCTTGA
Cag4/5	A	TTCTCAAGTGCGATATAACGAGTAGA	TCTTTAGTGCCTGTGGGTTCAA
Cag4/5	B	CTAAGAGCGATGGTTGGCAA	AAAATATGATCTTTGTCTTGCATGAA
Cag4/5	C	GGGGAATGAAACACAACCCTAA	GAAGGCAAAAAGCCTATTCCAA
Cag5/virB1 1	U	ACCCTTTCTTTCAGCCCATCTATAA	TGTCCATATCAACCACCACAA
virB11/Cag 6	U	AGGATTTAATGCCGCTTCTTTTAA	CAATGAGATGGTCCAAGATATAGGGA
Cag6/7	U	GTTCCATTGCTGTTTCCTTTCA	AATCACCACAAGCCCCAAA
Cag7/8	A, B	GGTGAATCTTGTTGGGCTTTTTTA	AGGATTGAGATGGTATAGAGTTAATGAAA
Cag7/8	C	TTCAAGTTTATCGTTTTCTTCATTCA	ATGGTATAGAGTTAATGAAATTGCAGAA
Cag8/9	U	AATAACCAAGACAGAAACAGCCAA	GATGGTAGCAGAATGGATAGAGAAA
Cag9/10	U	AATAGCTTTCAACCAATTAGGAACAA	AACTCTTCTCAAGAAAATCTTATCATCAA
Cag10/11	U	CCCAACCAAATTTTCATCAATCA	GTTTGAAGCAATCCGCTACTTACA
Cag11/12	A, B	AAACAAGGCGGTGCAGAA	GGTGTGTCTTTGAGTTTGTCATTTAA
Cag11/12	C	GCTTCATAGGTATGGGCTATTTGA	TGTTTCACCACTTTTTTCGCATA
Cag12/13	U	AAATCAGAAGTTTGCTCAGTGGTAA	CGCTAATCTAAAAACCATTGAACAA
Cag13/14	U	AGCGGTCATAATTCAAAGAGCAA	CAACAAGGCAATAGATTACTAGCT GAA
Cag14/15	U	ACCAATCGCAAACAAATCAAA	GATATGGTGGTGGTTTTCCAA
Cag15/16	A, C	AATACCAACAAGCCGCATACAA	CTCCAAACGCAACCAATGA
Cag15/16	B	AATACCAACAAGCCGCATACAA	TGGATCAGATTAGGGATTATTGGAA
Cag16/17	U	CGATCCTATGATGAGCGACAA	GATAGCGTTTAAGCCCCCATAA
Cag17/18	U	GCTCTAAATCTGAACTGCCCAATA	GAACAAAGTAAGCGACAATACCTACA
Cag18/19	U	GGCTAGTGGTTGAAAAAATCTCATCTA	AAAGAGAAACGACAGCAAGAAACA
Cag19/20	U	ATAACGCCATTAGCCCCTTTTAA	GGGTGCAAACTAAAATAATCGTGAA
Cag20/21	A, B	GATTAGTAAATCCCACAACAATAGGAATAA	TTTTTACCACCGATCTTAGGGTATTAA
Cag20/21	C	GCGGTCATTTGCGGATTAGTA	GGCGATTACGGTCCAAACA
Cag21/22	U	AGCGTTAAACATGCCAATGATAA	GATATGAGCGTTGAAGCTAAAAAGAA
Cag22/23	U	TCACCTTCCATTTCTTCTTCTATGAA	GTTTTAATTTGAGGGGCATTCCTA
Cag23/24	U	CGCTTTTGAACCTCTTGCTCTA	TCAGCACGACCAACAAACAA
Cag24/25	U	CATACGAACTGAAAACAACGAGACTTA	ATATTGGCGAGAGTGGAGGA

[a] *One primer was in the intergenic space and the other was sometimes located in an adjacent gene.* [b] *Strain specificity: A-26695, B-J99, C-J166, U-universal.*

Table A3 | Primer pairs used to construct promoter knockouts.

Primer pair	Forward primer (5′-3′)	Reverse primer (5′-3′)
Cag1-U	TATGCGGCCGCCGCTTTCACTAACGCTTCCACTA	GCACTGCAGGCGCAAGAATACAGCATTGGGC
Cag1-D	AGTGGATCCATGGCTGACACAATCAATACAACTG	CTGCTCGAGCTTCATCATCCACATTCTTGTTGAAG
Cag10-U	ACACTCGAGTCCACTCACATCATAGCCATGCA	ACACTGCAGCCTAGCAACTCACAGAGCAATGA
Cag10-D	ACAGGATCCGCCCTTGATAGATTGGCTAAACTCA	ACAGCGGCCGCCGACAAAAGCAAGCATGGCTGTA
Cag11-U	ACAGCGGCCGCCGACAAAAGCAAGCATGGCTGTA	ACAGGATCCGCCCTTGATAGATTGGCTAAACTCA
Cag11-D	ACACTGCAGCCTAGCAACTCACAGAGCAATGA	ACACTCGAGTCCACTCACATCATAGCCATGCA
Cag16-U	AACCTCGAGGAGTCTTACTTGTGGGACACTC	AACGGATCCATAGGCTGTTCAATATCAGCTCTATC
Cag16-D	AACCTGCAGAGCTCATTGGTTGCGTTTGGAG	AACGCGGCCGCTTCTCTCAAAGCGTTAGTGGCG
Cag21-U	AACGCGGCCGCAACCTTATCACAGGAGATATGAACC	AACGAGCTCTAGCATTGAGACTATCTATGAGACC
Cag21-D	AACGGATCCGCTTGGTGTCTTATCATTGGCATG	AACCTCGAGGATGTAATCAAGGTAAGTCAAATGCG
Cag25-U	AGTGCGGCCGCCTTGTCTAAAGCCAAATTCATGCC	AGTCTGCAGCCTTCCAATACAGCTTGATTGTCA
Cag25-D	AGTGGATCCCGCACAAGAATCACTGACAGCTACAAGA	AGTCTCGAGGAGAATAGTTGTTAGTAAGGATCAC
CAT-1[a]	AACGGATCCGCGGACAACGAGTAAAAGAG	AACCTGCAGGCAGGACGCACTACTCTCG
CAT-2[b]	AACGGATCCGCGGACAACGAGTAAAAGAG	AACGAGCTCGCAGGACGCACTACTCTCG
CAT-3[c]	AACCTGCAGGCGGACAACGAGTAAAAGAG	AACGGATCCGCAGGACGCACTACTCTCG

U, upstream arm; D, downstream arm.

[a,b,c] Knockouts were constructed with amplification of the CAT gene using primer pairs CAT-1 (cag1, cag10, cag25), CAT-2 (cag21), or CAT-3 (cag11, cag16), which differed only in the 5′ restriction sites (underlined).

Table A4 | Primer pairs used to generate amplicons for *cag* PAI libraries.

Oligo upstream	Sequence	Oligo downstream	Sequence
J166		**J166**	
MWG268	cgctcaaacctgaaagatcaa	MWG653	taggggcgaacacacttca
MWG607	ctaaagagaccaagaaagaggctaaa	MWG18571	cactatggagacttgcggaaa
MWG406	ctaagagcgatggttggcaa	MWG463	cctctaaggcatgctactgaagaa
MWG103	tggacaatcatatcaatcaaatcttta	MWG1198	gtttgagcgatgaagagaagc
MWG988	acaagagggagctttttaatcaca	MWG19145	tctcattgttcctaattggttgaaaa
MWG1008	aacgagctccatagaatctttgaaccaatctag aacga	MWG028	tgcggttttgtttttggattagaa
MWG410	aatgtaagtagcggattgcttcaa	MWG19155	acgcattagagatccgaacaa
MWG416	agcggtcataattcaaagagcaaa	MWG657	tgctctactctcgccctatca
MWG063	cagcttcaattttgatacccaatc	MWG475	gacttttttgttgtttgtctctgaa
MWG476	cgccaagcaagatgtctgaa	MWG436	gttttaatttgaggggcattccta
MWG18880	ctagcatgaagtaagcataaacataaactaa	MWG17626	gctaatgacatccacttaaatccaaa
MWG734	aacctcgagaccttgagatacaagtcttttctgttg	MWG807	gacagattttcaaagacagcttca
MWG17643	aacctcgaggctttactttatggtgagccataac	MWG9165	ttagaataatcaacaaacatcacgccat
G27		**G27**	
A-cag	gcgagcggcgatgtgatctggc	B-cag	ggaacgccaccgttggttataaagac
C-cag	gattggatcgtaatgcttcaaatcc	MWG9165	ttagaataatcaacaaacatcacgccat
D-cag	cctgtatctgtccctagctc	MWG807	gacagattttcaaagacagcttca
F-cag	ggactccattgttcctaatgg	E-cag	gatgatggggtgatccttactaacaac
Q-cag	gacccgttagggaattataatc	R-cag	cctatcaataacaacataagcgag
O-cag	cagagcagtcataattcaaagagc	P-cag	tgctctgctctcgccctatca
M-cag	gtaaggtagcggattgcttcaaacaag	N-cag	cacgcattagagatccgaacaagc
L-cag	gctttaagactctctttagcttc	I-cag	gatgggaaattgagcatgactg
J-cag	gaagctaaagagagtcttaaagc	K-cag	gtaaagaaccgagtttggtaaac
G-cag	ccttagcaccattcctgccataacc	H-cag	gaaggaagctcaatgagattgtc

Several additional oligonucleotide pairs were required to amplify the entire H. pylori G27 strain cag PAI region, due to poor or no amplicon presence in some PCR reactions.

Table A5 | Putative promoter sequences that direct expression of the reporter *tnpR*.

Promoter	Length (nt)	Sequence
I	244	GATCAAAAAAATCAAAACAAAAATAACGATTGAGTGGCGTTAATGCGCTAGAATAGTGCTAAAAATAAGAATAAAGGAATCAAAA GTATGAAAACGAATTTTTATAAAATTAAATTACTATTTGCTTGGTGTCTTATCATTGGCATGTTTAACGCTCCGCTTAACGCTGACC AAAACACTGATATAAAAGATATTAGTCCTGAAGATATGGCACTAAATAGCGTGGGGCTTGTTTCTAGAGATC
II	38	GATCGTTTGACAATTTTAAATTCTCCTGTGTATCGATC
III	91	GATCTGTTGCTTTATTGTCAAAAAGCCATTGAAATTCACCATTGGTTGATTTGCAAAAA GGCGCTAATCGCGCGACAAGCCCAT TAGGATC
IV	104	GATCCCAATGCCCACACGCTTGATAAGGGAGCGTCAATTGATGAGAACAAGCTTTTT GAACAACAAAAACGCGCGTATTTCAA CTACGCCAACGATGTGATC
V	71	GATCAGCTTGGGTTTGTTTCTGCTTGTTTTTAGGTTTCAACCTGAGACGATTAAAAAA TACATCAAAGATC
VI	314	GATCCTAAATATCCCAAATGCAATGGATTGATGAAAAGAAAAAAGAATTTCAAAAACAATGAGTTTTTTACAGCTGCATTACTT ACCTTAAATGCAATGGAATTTTGTCTCTATATCAATTCTGAAAAAAAAGGAAACTAATGTTTAGAAAACTAGCAACCGCTGTATC GCTCATAGGCTTACTAACCTCTAACACTCTTTATGCTAAAGAAATAAGTGAAGCCGATAAGGTCATTAAGGCCACTAAAGAAACT AAAGAGACCAAGAAAGAAGTTAAACGACTCAAAAAAGAAGCTAAACAGCGCCAACAGATC
VII	234	GATCAAGAGATTATCAAAGGAAGCAAAAAAAAAATACATTATTAGTGGCATTGTAGTCGCTGCTCTTATCGTGATTATTTTATTTTCT AGAAGCATTTTTCACTACTTTATACCTTTGGAAGATAAAAGCTCTCGTTTTAGCAAAGACAGGAATCTTTATGTCAATGATGAAATC CAAATAAGGCAAGAGTATAACCGATTGCTGAAAGAACGGAATGAAAAAGGCAATATGATC
VIII	635	GATCCATGATGCTCTGTTGTATCGTTCATGAAATTCCTTTCAAGAATTAAATTGAGAAATTGTTTTGATATTATACCATTCTCTCTCT GAGTTGTGATTGTCTTATCTCTTTGAATTAGGCGCTTCTAAAATTTCATTACTGATTACGACTGCTTACTTATTGCTCTTACTTTTTG AGTTGCATCGTGTTTCATCTTGCTTCTTGTTTGAAGCAATCCGCTACCTTACATTTATTATAAGGAATCTTTGTTCAACGCCTTATCC AAAAAGGTTTTTATTAAAGGTTTTTTCAAATATATATTTTTACAGAAATTTTGCTATACTATAACTGAAATTGTTTTAAGGAGTTTTTG ATGAAAAAATTTCTCAAGTATTTCTCAAGTTTTGCAACCAACAAACCAATAACTAAACAATAAAGCTGTCGCATGTTAGGGAAAA AAAACGAGGAAGTCTTGATTGATGAAAATTTGGTTGGGGGTGTGATAGCCCTTGATAGATTGGCTAAACTCAATAAGGCCAATA GGACTTTCAAAAGGGCTTTTTATCTCTCTATGGCGCTCAATGTCGCCGCTGTAACGAGTATTGTGATGATGATGCCTTTGAAGAA AACGGATATATTTGTTTATGGCATTGATC
IX	132	GATCATAGTGCCGTTCATGTTCCATACATCTTTGGCTACAACCCCACTCACTATACCT GTGAGAGTGGCATCTACTTTAGAAGTCA-GAGTGATTTCAATAGGGGTGTATTGCGCT AAAACAAATGTGGGATC
X	269	GATCTATGTTTAAAGGCTAGCCGCTTTATTCTTGTGTTACAATTACAAATATTTTTTAAGAGGAATTGTTGATGGGGCGGGCATTG TTTAAAAAAATTGTTGGCTGTTTCTGTCTTGGTTATTTATTTTTATCTAGCGTAATAGAAGCAGCACCTGACATTAAAAATTTTAATC GTGGTAGGGTGAAAGTGGTGAATAAGAAGATTGCTTATTTGGGAGATGAAAAACCTATTACGATTTGGACTTCATTAGACAATGTT ACTGTGATC
XI	419	GATCTTTGATGTATTTTTTAATCGTCTCAGGTTGAAACCTAAAAACAAGCAGAAACAAACCCAAGCTGATCAGAGTGAGAATAAA GCTCCATTTTAAGCAACTCCATAGACCACTAAAGAAACTTTTTTTGAAGCTGTCTTTGAAAATCTGTCCTATTGATTTGTTTTCCATG TGAATCACAAACGCTTAATTGCAAATATATACTTTATGGTAAGCATGACACACAAACCAAACCATTTTTAGAACGCTTCATGCACTC ACCTTGATTCCAACTATATTTAAGCATTGCATTTGATTTATTCTTGAAGGTTCATTTCTTATTTCTTTTCTTTGTTAAAATTCGTTCATT TTAGCAAATTTTTGTTAATTGTGGGTAAAAATGTGAATCGTTCCTAGCCTTTAGACGCCTGCAACGATC
XII	54	GATCCCTAGAACAAAGTAAGCGGCAATACCTACAAGAAAGGCAATCAAGTAAGATC
XIII	68	GATCCAATCATTGAAAAAATCTTTGATGAAAAGGGTAAAGAAATGGGATTGAATGTAG AATTACGATC
XIV	70	GATCCTACTGGTGGGGATTGGTTGGATATTTTTCTCTCATTTATATTTGACAAAAAACA ATCTTCCGATC

Table A6 | Key to *cag* PAI gene names.

26695 ORF	Gene number	Gene name	T4SS homolog
HP0520	*cag1*	*cagC*	
HP0521	*cag2*/hypothetical	Hypothetical	
HP0522	*cag3*	*cagA*	
HP0523	*cag4*	*cagγ*	VirB1
HP0524	*cag5*	*cagβ*	VirD4
HP0525		*cagα*	VirB11
HP0526	*cag6*	*cagZ*	
HP0527	*cag7*	*cagY*	VirB10
HP0528	*cag8*	*cagX*	VirB9
HP0529	*cag9*	*cagV*	VirB6
HP0530	*cag10*	*cagW*	VirB8
HP0531	*cag11*	*cagU*	
HP0532	*cag12*	*cagT*	VirB7
HP0533	Hypothetical	Hypothetical	Hypothetical
HP0534	*cag13*	*cagS*	
HP0535	*cag14*	*cagQ*	
HP0536	*cag15*	*cagP*	
HP0537	*cag16*	*cagM*	
HP0538	*cag17*	*cagN*	
HP0539	*cag18*	*cagL*	VirB5
HP0540	*cag19*	*cagI*	
HP0541	*cag20*	*cagH*	
HP0542	*cag21*	*cagG*	
HP0543	*cag22*	*cagF*	
HP0544	*cag23*	*cagE*	VirB3/VirB4
HP0545	*cag24*	*cagD*	
HP0546	*cag25*	*cagC*	VirB2
HP0547	*cag26*	*cagA*	

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An RGD helper sequence in CagL of *Helicobacter pylori* assists in interactions with integrins and injection of CagA

Jens Conradi[1†], Nicole Tegtmeyer[2,3†], Marta Woźna[1], Marco Wissbrock[1], Carmela Michalek[1], Corinna Gagell[2,3], Timothy L. Cover[4,5,6], Ronald Frank[7], Norbert Sewald[1]* and Steffen Backert[2,3]*

[1] *Department of Chemistry, Bielefeld University, Bielefeld, Germany*
[2] *Department of Microbiology, Otto-von-Guericke University, Magdeburg, Germany*
[3] *School of Biomolecular and Biomedical Sciences, University College Dublin, Dublin, Ireland*
[4] *Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA*
[5] *Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA*
[6] *Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, USA*
[7] *Department of Chemical Biology, Helmholtz Center for Infection Research, Braunschweig, Germany*

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Jeong-Heon Cha, Yonsei University, South Korea
Nina Salama, Fred Hutchinson Cancer Research Center, USA
Jay V. Solnick, University of California Davis, USA

****Correspondence:***
Norbert Sewald, Department of Chemistry, Bielefeld University, PO Box 10 01 31, D-33501 Bielefeld, Germany.
e-mail: norbert.sewald@uni-bielefeld.de;
Steffen Backert, School of Biomolecular and Biomedical Sciences, Science Center West, University College Dublin, Belfield Campus, Dublin-4, Ireland.
e-mail: steffen.backert@ucd.ie

†Jens Conradi and Nicole Tegtmeyer have contributed equally to this work.

Helicobacter pylori is a specific gastric pathogen that colonizes the stomach in more than 50% of the world's human population. Infection with this bacterium can induce several types of gastric pathology, ranging from chronic gastritis to peptic ulcers and even adenocarcinoma. Virulent *H. pylori* isolates encode components of a type IV secretion system (T4SS), which form a pilus for the injection of virulence proteins such as CagA into host target cells. This is accomplished by a specialized adhesin on the pilus surface, the protein CagL, a putative VirB5 ortholog, which binds to host cell β_1 integrin, triggering subsequent delivery of CagA across the host cell membrane. Like the human extracellular matrix protein fibronectin, CagL contains an RGD (Arg-Gly-Asp) motif and is able to trigger intracellular signaling pathways by RGD-dependent binding to integrins. While CagL binding to host cells is mediated primarily by the RGD motif, we identified an auxiliary binding motif for CagL–integrin interaction. Here, we report on a surface exposed FEANE (Phe-Glu-Ala-Asn-Glu) interaction motif in spatial proximity to the RGD sequence, which enhances the interactions of CagL with integrins. It will be referred to as RGD helper sequence (RHS). Competitive cell adhesion assays with recombinant wild type CagL and point mutants, competition experiments with synthetic cyclic and linear peptides, and peptide array experiments revealed amino acids essential for the interaction of the RHS motif with integrins. Infection experiments indicate that the RHS motif plays a role in the early interaction of *H. pylori* T4SS with integrin, to trigger signaling and to inject CagA into host cells. We thus postulate that CagL is a versatile T4SS surface protein equipped with at least two motifs to promote binding to integrins, thereby causing aberrant signaling within host cells and facilitating translocation of CagA into host cells, thus contributing directly to *H. pylori* pathogenesis.

Keywords: CagL, binding motifs, cortactin, ERK kinase, integrin interaction, $\alpha_5\beta_1$

INTRODUCTION

About 50% of the world's human population is infected by *Helicobacter pylori*, a gastric pathogen causing gastritis in all infected individuals and more severe gastric disease in 10–15% of cases (Amieva and El-Omar, 2008; Atherton and Blaser, 2009; Polk and Peek, 2010). *H. pylori* can infect humans lifelong as the consequence of a highly complex host–pathogen crosstalk, and is an excellent model system to study bacterially induced epithelial cell signaling cascades which are of relevance to neoplasia. *H. pylori* strains are surprisingly diverse in both their genome sequences and resulting virulence. Multiple bacterial virulence factors such as the *cag* pathogenicity island (PAI), the protein CagA, and the vacuolating toxin VacA have been identified. Considerable research interest worldwide is currently focused on the effector protein CagA because CagA-positive but not CagA-negative *H. pylori* strains are associated with the development of severe gastric diseases. A direct causal link between CagA and carcinogenesis *in vivo* was achieved by the generation of transgenic mice expressing CagA (Ohnishi et al., 2008). The *cag* PAI has been shown to encode a type IV secretion system (T4SS) for the delivery of CagA into the cytoplasm of host target cells, where CagA is phosphorylated by host tyrosine kinases (Backert and Meyer, 2006; Mueller et al., 2012). T4SSs are typically composed of 11 VirB proteins (encoded by *virB1–11*) and the so-called coupling protein (VirD4, an NTPase). The *H. pylori cag* PAI contains up to 32 genes encoding orthologs of all 11 VirB proteins and VirD4 as well as various associated factors (Backert et al., 2002; Backert and Selbach, 2008; Fischer, 2011; Tegtmeyer et al., 2011a). Scanning electron microscopy (SEM) studies showed that the *H. pylori* T4SS is induced upon host cell contact and forms pilus-like structures protruding from the bacterial membrane (Rohde et al., 2003; Kwok et al., 2007).

Various cell surface molecules are required for T4SS function, suggesting a sophisticated control mechanism by which *H. pylori* injects CagA (Wessler and Backert, 2008). The first identified host

receptor for the T4SS was integrin $\alpha_5\beta_1$, according to a series of experiments including respective knockout cell lines, gene silencing RNAs, function-blocking antibodies, and competition studies (Kwok et al., 2007). The bacterial interaction partner was identified as CagL, a VirB5 ortholog, and specialized adhesin that is targeted to the pilus surface, where it binds to integrin $\alpha_5\beta_1$ and mediates receptor-dependent delivery of CagA into gastric epithelial cells (Backert et al., 2008, 2011). Like fibronectin, an extracellular matrix protein and natural ligand of integrin $\alpha_5\beta_1$, CagL contains an arginine–glycine–aspartate (RGD) motif that was shown to be important for interaction with integrin $\alpha_5\beta_1$ on host cells (Kwok et al., 2007). Binding of CagL during infection or by incubation with recombinant protein *in vitro* elicits downstream signaling like tyrosine kinase activation of several proteins including EGF-receptor, FAK, and Src (Kwok et al., 2007; Saha et al., 2010; Tegtmeyer et al., 2010) as well as stimulation of ERK1/2 MAP kinase (Wiedemann et al., 2012). In line with these observations, various other structural T4SS proteins have subsequently also been demonstrated to bind to β_1 integrins *in vitro*, including CagA, CagI, and CagY (Jiménez-Soto et al., 2009). In the same study, mutation of the RGD motif in CagL revealed no reduction of injected CagA during infection (Jiménez-Soto et al., 2009). Using mass spectrometry it was further reported that surface exposed CagL associates with two other *cag*PAI proteins, CagI, and CagH (Shaffer et al., 2011). All three factors are required for CagA translocation into host cells. SEM analysis revealed that these proteins are involved in the formation of T4SS pili. The *H. pylori* mutant strains Δ*cagI* and Δ*cagL* failed to form T4SS pili, while the Δ*cagH* mutant revealed a hyperpiliated phenotype and produced pili that are elongated and thickened as compared to those of the wild type (WT) bacteria (Shaffer et al., 2011). This suggests that T4SS pilus dimensions may be regulated by CagH. Taken together, the above results indicate that CagH, CagI, and CagL are components of a T4SS subassembly complex involved in the biogenesis of pili that interact with integrin $\alpha_5\beta_1$ (Kwok et al., 2007; Shaffer et al., 2011). However, the exact co-operation of the various integrin-targeting *cag*PAI proteins is not yet clear and needs to be investigated.

We recently reported on cell-based *in vitro* binding studies with WM-115 and AGS cells revealing that CagL not only interacts with integrin $\alpha_5\beta_1$, but also with $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins (Conradi et al., 2011; Wiedemann et al., 2012). Infection and *in vitro* binding studies showed that CagL induces gastrin expression via a novel integrin $\alpha_V\beta_5$-integrin linked kinase signaling complex independent of CagA injection (Wiedemann et al., 2012). CagL–integrin $\alpha_V\beta_5$ interactions were demonstrated by immunoprecipitation and Biacore binding studies. In addition, the adhesion of WM-115 cells to immobilized CagL was inhibited by cyclic RGD peptides with pre-defined conformation, where the sequence was based on the CagL sequence Ala-Leu-Arg-Gly-Asp-Leu-Ala (ALRGDLA; Conradi et al., 2011). The application of the so-called spatial screening approach had previously been applied in the screening of cyclic RGD peptides and resulted in highly active peptides that efficiently and selectively inhibit the interaction of extracellular matrix proteins like vitronectin, fibronectin, and fibrinogen with the integrins $\alpha_V\beta_3$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ in the nanomolar IC_{50} range (Gurrath et al., 1992; Pfaff et al., 1994; Haubner et al., 1996, 1997; Weide et al., 2007). The spatial screening approach makes use of incorporation of single D-amino acids into cyclic peptides in order to lock the conformation and to predictably present the amino acid side chain functional groups in a well-defined manner. D-Amino acids are known to induce and stabilize discrete turn structures in linear and, especially, cyclic peptides. Different cyclic RGD peptides with the CagL basic sequence ALRGDLA and a D-amino acid in different positions were synthesized. The variation of the amino acid stereochemistry contributes to different backbone structures, increases the spatial diversity, and provides constrained peptides with different β- and γ-turns. The structures were evaluated using NMR and molecular dynamics calculations, and activities of the different peptides were determined in cell adhesion assays. These structure–activity relationship studies revealed a β-turn around Asp-D-Leu-Ala-Arg to be favorable for integrin interactions (Conradi et al., 2011). The corresponding cyclopeptides were shown to inhibit integrin-mediated interactions of WM-115 cells with CagL. In particular, the cyclopeptide-7 [c-(-RGDlA-); **Table 3**, D-amino acids are written in small letters] displayed high activity (Conradi et al., 2011).

In comparison with purified $CagL^{WT}$, purified CagL mutants ($CagL^{RAD}$ and $CagL^{RGA}$) exhibited decreased, but not completely abolished affinity to integrin $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ *in vitro* (Kwok et al., 2007; Conradi et al., 2011; Wiedemann et al., 2012). Other recent studies indicated that the RGD motif of CagL is certainly important but not sufficient for triggering cell signaling (Tegtmeyer et al., 2010; Wiedemann et al., 2012). Taken together, these data strongly underscore the importance of the RGD motif for CagL binding (Conradi et al., 2011), but also suggested the existence of one or more yet unidentified auxiliary motifs that is/are involved in CagL–integrin interactions. Here we report that another CagL motif, a FEANE-containing sequence, in proximity to the RGD motif, participates in binding of CagL to integrins (thus referred to as RHS) and is able to induce cell binding and signaling both *in vitro* and during *H. pylori* infection of gastric epithelial cells.

MATERIALS AND METHODS

HUMAN CELL CULTURE AND *H. PYLORI* STRAINS

The human epithelial melanoma cell line WM-115 (ATCC, Wesel, Germany; CRL-1675™) is an adherent growing cell line with continuous integrin expression (mainly integrin $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$) and was cultivated in DMEM medium (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin (PAA, Pasching, Austria). The human gastric adenocarcinoma cell line AGS (ATCC CRL-1739™) was cultivated in RPMI 1640 medium, which was supplemented with 10% fetal calf serum (Gibco, Paisley, UK). Cells were cultivated at 37°C and 5.3% (v/v) CO_2 and subcultivated in a ratio of 1:3–1:5 every 2–3 days at a confluence of 70% to 90%. The *H. pylori* strains P12 WT and P12Δ*cagL* were generated and grown as described (Kwok et al., 2007). To complement the P12Δ*cagL* mutant strain, genes encoding $CagL^{WT}$ or CagL mutant proteins were introduced into the chromosomal *ureA* locus, using a pAD1-derived plasmid. CagL proteins expressed from the *ureA* promoter contain a hemagglutinin (HA) tag introduced following the signal sequence at amino acid position 22 (Shaffer et al., 2011). For infection, *H. pylori* were

grown for 2 days in thin layers and added at a multiplicity of infection (MOI) of 50 or 100 (Selbach et al., 2002; Moese et al., 2004). The number of elongated AGS cells in each experiment was quantified in 10 different 0.25 mm^2 fields (Tegtmeyer et al., 2011b). All experiments were done in triplicate.

CagL PROTEIN EXPRESSION AND PURIFICATION

$CagL^{WT}$ and mutants were expressed as C-terminal His-tag fusions in vector pET28a (Novagen®, Merck, Darmstadt, Germany) and purified as described earlier (Kwok et al., 2007). Briefly, *E. coli* ER2566 (NEB, Ipswich, USA) transformed with the plasmids were grown in 10 ml LB medium at 37˚C. After overnight incubation, 500 ml of fresh LB medium were added and shaken for another 2.5–3 h until reaching $OD_{600} = 1$. Then 300 µl of a 1-mM IPTG stock solution was added and the bacteria were grown for 1.5 h to induce CagL expression. Bacterial pellets were collected by centrifugation and then resuspended in ice-cold buffer CW (50 mM KH_2PO_4–K_2HPO_4, pH 7.5, 200 mM NaCl) supplemented with 20 µM PMSF. After lysis using a French Press, the overexpressed CagL present in the inclusion bodies was solubilized in buffer LW (50 mM KH_2PO_4–K_2HPO_4, pH 7.5, 200 mM NaCl, 6 M guanidine hydrochloride) and refolded by a rapid dilution approach in ice-cold refolding buffer (50 mM Tris–HCl, pH 8.2, 20 mM NaCl, 0.1 mM KCl, 1 mM EDTA, 2 mM reduced glutathione, 0.2 mM oxidized glutathione) using a dual-channel syringe pump (KD Scientific Inc., Holliston, USA) with a flow of 0.1 ml/min. After refolding, CagL was further purified by metal-chelate affinity chromatography through Talon® resin (BD Biosciences). Protein concentrations of the resultant samples were determined by NanoDrop 1000 (Thermo Scientific, Waltman, USA) and typically yielded a total amount of about 1.5 mg CagL in 10 ml buffer. Purification of CagL was judged to be of >95% homogeneity by SDS-PAGE/Coomassie Blue staining. The folded conformation of the purified CagL proteins was subsequently confirmed by circular dichroism (CD) as described below.

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis of CagL was performed using the corresponding pET28a or pAD1 vectors as DNA template (**Table 1**). For amplification, Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, USA) was used, followed by PCR purification (MinElute PCR Purification Kit, Qiagen, Hilden, Germany), digestion with *Dpn*I (Promega, Madison, USA), and ligation using T4 DNA Ligase (Promega). Re-sequencing and Western blotting of *E. coli* or *H. pylori* strains, respectively, verified the appropriate expression of CagL in the resulting plasmids.

CELL ADHESION ASSAYS WITH WM-115 CELLS

The cell adhesion assays to immobilized CagL proteins were performed as described previously (Conradi et al., 2011). A 96 well microtiter plate (Nunc Maxisorp™, Thermo Fisher Scientific Inc., Waltham, USA) was coated with 100 µl of protein solution ($CagL^{WT}$ or mutant; 10–20 µg/ml) per well, and immobilized for 18 h at 37˚C. The solution was aspirated and free binding sites were blocked with 100 µl of a solution, consisting of 2% (w/v) fatty acid-free BSA (PAA, Pasching, Austria) in PBS for 1 h at 37˚C. The adhesion assays were performed with WM-115 human epithelial cancer cells. This cell line was chosen for the cell adhesion assays due to a constitutive expression of integrin $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$, and was shown to display reproducible adherence to CagL and fibronectin (Conradi et al., 2011). The WM-115 cells were cultivated to a confluence of 70%, and detached with Trypsin-EDTA (0.05/0.02% in D-PBS; PAA, Pasching, Austria). After washing with DMEM medium, the cells were resuspended in DMEM medium with 1 mg/ml fluorescein diacetate (Sigma-Aldrich, St. Louis, USA), adjusted to a cell density of 1×10^5 cells/ml and incubated at 37˚C for 30 min under steady shaking. Two DMEM washing steps were performed to remove excess fluorescein diacetate. The cells were resuspended in DMEM medium containing divalent cations Ca^{2+} and Mg^{2+} (2 nM) to a cell density of 1×10^5 cells/ml and incubated in the dark on ice for 30 min. Subsequently, the cell solution was distributed into solutions of peptides ranging in concentrations of millimolar to nanomolar, and the resulting mixtures were incubated at 37˚C for 30 min. The solutions were then dispensed on the coated microtiter plate (5×10^3 cells/well) and incubated for 1 h at 37˚C. After several washing steps the fluorescence of cells, adherent to the immobilized CagL, was measured (λ_{ex} 485 nm; λ_{em} 514 nm) using an Infinite 200 Microplate Reader (Tecan, Männedorf, Switzerland). The inhibition concentration IC_{50}, confidence interval $CI_{95\%}$, and the square of the correlation coefficient R^2 values were evaluated. To test the accuracy of the fit model for the non-linear regression a "Runs test" was performed and high *P*-values were obtained for all measurements (data not shown), which support the chosen regression model. All evaluation was performed using the GraphPad Prism 4.03 Software (GraphPad, San Diego, USA).

Table 1 | Primers used for generating the different CagL mutants.

Protein		Primers
$CagL^{F86A}$	*fw*	5′-*Pho*-GCC GAA GCG AAT GAG TTA TTT T
	rv	5′-*Pho*-ATT GGC TTT CAA TAA CGC TAA ATC
$CagL^{E87A}$	*fw*	5′-*Pho*-GCG AAT GAG TTA TTT TTC ATC TCA G
	rv	5′-*Pho*-TGC AAA ATT GGC TTT CAA TAA CGC TA
$CagL^{E87A/A88E}$	*fw*	5′-*Pho*-GCG AAT GAG TTA TTT TTC ATC TCA GAA
	rv	5′-*Pho*-TC AAA ATT GGC TTT CAA TAA CGC TAA
$CagL^{N89A}$	*fw*	5′-*Pho*-GCT GAG TTA TTT TTC ATC TCA GAA
	rv	5′-*Pho*-CGC TTC AAA ATT GGC TTT CAA TAA
$CagL^{E90A}$	*fw*	5′-*Pho*-GTT ATT TTT CAT CTC AGA AGA TGT
	rv	5′-*Pho*-GCA TTC GCT TCA AAA TTG GC
$CagL^{Q40A}$	*fw*	5′-*Pho*-GTG CTC AAA AAC TTA GAT GAG ATT TTT TCA
	rv	5′-*Pho*-TGC TTG GTT GGT CTC TTG GTA G
$CagL^{A84E/E87A}$	*fw*	5′-*Pho*-TTT GCA GCG AAT GAG TTA TTT TTC
	rv	5′-*Pho*-ATT CTC TTT CAA TAA CGC TAA ATC TC
$CagL^{A88E/L91A}$	*fw*	5′-*Pho*-GAG GCA TTT TTC ATC TCA GAA GAT G
	rv	5′-*Pho*-ATT CTC TTC AAA ATT GGC TTT CAA

HELICOBACTER PYLORI BINDING TO AGS CELLS

Infection of AGS at a density of 3.2×10^5 cells/well was performed using a MOI of 50 or 100 per *H. pylori* strain (Kwok et al., 2002). After infection for 4 h, the AGS-bacterium co-cultures were washed three times with 1 ml of pre-warmed RPMI medium per

well to remove non-adherent bacteria. To determine the total number of colony forming units (CFU) corresponding to host-bound *H. pylori*, the infected monolayers were incubated with 1 ml of 0.1% saponin in PBS at 37°C for 15 min. The resulting suspensions were diluted and incubated on GC agar plates as described (Kwok et al., 2002). The total CFU of cell-bound *H. pylori* are given as CFU per well of AGS cells.

CagL PEPTIDE SYNTHESIS

All Fmoc-α-amino acids (9-fluorenylmethylcarbonyl-protected α-amino acids) were purchased from IRIS Biotech (Marktredwitz, Germany) and Advanced ChemTech (Louisville, USA). MALDI-ToF MS analyses were performed on a Voyager-DE (Perseptive Biosystems, Foster City, USA) using 2,5-dihydrobenzoic acid as the matrix. The analytical RP-HPLC was performed with UV detection at 220 nm and the following elution gradients: eluent A: 95% water, 5% acetonitrile, 0.1% TFA; Eluent B: 95% acetonitrile, 5% water, 0.1% TFA; 0.7 ml min^{-1}, 0–5 min 100% A → 100% B; 5–6 min 100% B → 100% A; 6–6.5 min 100% A (Thermo Separation Products apparatus equipped with a Hypersil Gold (3 μm, 150 mm × 2.1 mm) column (Thermo Fisher Scientific, Waltham, USA). Preparative RP-HPLC was performed on a Thermo Separation Products apparatus equipped with a Jupiter C18 (350 Å, 10 μm, 250 mm × 21.2 mm) efficiency column (Phenomenex, Torrance, USA) with water/acetonitrile gradients as the eluent and UV detection at 220 nm. Linear peptides were synthesized by solid phase peptide synthesis on a Liberty 12 channel microwave-assisted automated peptide synthesizer (CEM, Matthews, USA) according to a Fmoc-protocol with 2-chlorotrityl resin (IRIS Biotech) as solid support. The resin loading was 0.8 mmol/g. The C-terminal resin bound amino acids were Phe for cyclopeptides-2 to -5, D-Phe for cyclopeptide-1, and Val for the linear peptide 6. Peptide coupling was performed with three equivalents of Fmoc-amino acid (0.5 M in DMF), three equivalents TBTU (3-[bis-(dimethylamino)methyliumyl]-3*H*-benzotriazol-1-oxide; 0.5 M in DMF; dimethylformamide), and six equivalents DIPEA [diisopropylethylamine; 2 M in NMP (1-methyl-2-pyrrolidone)]. After washing with DMF the Fmoc group was cleaved with a solution of 20% piperidine in DMF. After synthesis of the bound linear peptides, resin cleavage was performed with 1% TFA (trifluoroacetic acid) in dichloromethane (10 times for 5 min each). The peptides one to five were cyclized under pseudo-high dilution conditions (Malesevic et al., 2004). For slow reagent addition a dual-channel syringe pump (KD Scientific Inc., Holliston, USA) was used. 200 μmol of linear precursor were dissolved in 20 ml DMF, and 600 μmol HATU (1-[bis-(dimethylamino) methyliumyl]-1*H*-1,2,3-triazolo[4,5-*b*]pyridine-3-oxide) were dissolved in the same volume of DMF. Each solution was transferred into a syringe, and both solutions simultaneously were added to a stirred solution of 1200 μmol DIPEA and 20 μmol HATU in 10 ml DMF at a rate of 1.00 ml/h (Malesevic et al., 2004). Finally, the mixture was stirred for further 15 min, and the solvent was evaporated under reduced pressure at a temperature below 30°C. Peptide purification was carried out using preparative RP-HPLC. Deprotection of the cyclic peptides took place in a mixture of TFA (95%), TIS (triisopropylsilane; 2.5%), and water (2.5%), with shaking at room temperature for 2 h. The solvent was evaporated and cold diethyl ether (30 ml) was added to the residue. After centrifugation for 1 h at 0°C and 4000 × *g*, diethyl ether was decanted and the residue was dissolved in water, lyophilized, and purified by preparative RP-HPLC. The yield and purity of the synthesized peptides are given in **Table 2**.

CagL PEPTIDE SPOT ARRAYS

The CagL peptide arrays were generated by the SPOT-synthesis technique as described earlier (Beutling et al., 2008). Briefly, the indicated peptides in **Figure 5** were synthesized on an amino-functionalized cellulose membrane using Fmoc/*tert*-butyl chemistry. The spots consist of ∼5 nmol of each peptide (Dikmans et al., 2006). For the binding assays, the peptide arrays were blocked overnight at room temperature with blocking buffer consisting of 2× blocking buffer concentrate (Sigma-Aldrich, St. Louis, MO/USA) and 5% (w/v) sucrose in TBS-T (0.02 M sodium phosphate buffer with 0.15M sodium chloride (pH 7), 0.05% Tween 20). Approximately 1 μg of purified integrins $\alpha_5\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ (Chemicon-Millipore, Billerica, MA, USA) in blocking buffer was added to CagL peptide arrays for 4 h at room temperature. Next, the arrays were washed with a 10-fold volume of TBS-T three times and then incubated with α-integrin-β_1, α-integrin-α_v, or α-integrin-β_5 antibodies (Santa Cruz, Santa Cruz, USA). Finally, a chemiluminescence reaction using the ECL Plus kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) was performed as described below for Western blotting.

CIRCULAR DICHROISM SPECTROSCOPY

Circular dichroism spectroscopy was performed on a Jasco J-810 spectrometer (Jasco, Groß-Umstadt, Germany). For the CD measurements of the CagL proteins a buffer containing 5 mM NaCl and 10 mM Na_2HPO_4 (pH 7.4) was used. The $CagL^{WT}$ and all mutants were measured in a 1-mm quartz cuvette, adjusted to a concentration of 3.9 nM. The secondary structure was evaluated using the deconvolution function of the Spectra Manager II Software (Jasco) based on CDPro structure analysis methods using Yang's references (Yang et al., 1986; Sreerama and Woody, 2004).

ANTIBODIES AND WESTERN BLOTTING

Treated/infected cells were harvested in ice-cold PBS containing 1 mM Na_3VO_4 (Sigma-Aldrich). Western blotting was done

Table 2 | Characteristics of synthesized CagL peptides.

Peptide	Sequence	Yield (%)	Purity[a] (%)
1	c-(-fEANE-)	13.6	>96
2	c-(-FeANE-)	8.0	>99
3	c-(-FEaNE-)	8.0	>95
4	c-(-FEAnE-)	4.0	>99
5	c-(-FEANe-)	16.8	>99
6	ANFEANELFFISEDV	2.1	>94

[a]*The purity of each peptide was confirmed by analytical HPLC.* D-*amino acids are given in small letters.*

as previously described (Tegtmeyer et al., 2009). Rabbit α-CagL antiserum was raised against the C-terminal peptide (C-RSLEQSKRQYLQER) of the protein and was prepared by Biogenes (Berlin, Germany). The α-HA-tag antibody (Invitrogen, Darmstadt, Germany) was also used to detect tagged CagL. The pan-α-phospho-tyrosine antibody PY-99 (Santa Cruz) and α-CagA (Austral Biologicals, San Ramon, CA, USA) were used to investigate the phosphorylation of CagA. The polyclonal α-phospho-ERK1/2-PT202/PY-204 antibody was purchased from NEB (Frankfurt, Germany). The polyclonal α-phospho-Cortactin-PS-405 antibody was described recently (Tegtmeyer et al., 2011b). The α-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz) served as loading control in each Western blot. As secondary antibodies, horseradish peroxidase conjugated α-mouse, α-rabbit, or α-goat polyvalent sheep immunoglobulin were used and antibody detection was performed with the ECL Plus chemiluminescence kit (Amersham Pharmacia Biotech). Band intensities and corresponding kinase activities were quantitated with the Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany).

STATISTICAL ANALYSIS

All data were evaluated using Student *t*-test with SigmaStat statistical software (version 2.0). Statistical significance was defined by $P \leq 0.05$ (*) and $P \leq 0.005$ (**). All error bars shown in figures and those quoted following the ± signs represent standard deviation.

RESULTS

SYNTHETIC RHS-CYCLOPEPTIDES INFLUENCE WM-115 CELL ADHESION TO IMMOBILIZED CagL MUTANTS

Previous studies indicated that the RGD sequence in CagL is important but not sufficient to trigger host cell receptor binding and signaling (Kwok et al., 2007; Tegtmeyer et al., 2010; Conradi et al., 2011; Wiedemann et al., 2012). The recently published 3D homology model of CagL (Backert et al., 2008) was screened for other surface exposed amino acid sequences to further explore the hypothesis that other CagL features besides the RGD sequence might be important for integrin interactions. Among other possible motifs that are surface exposed and with an orientation that is directed to the plausible CagL–integrin interaction site, the so-called RHS motif in proximity to the RGD motif (**Figure 1**) was identified as a candidate for host interaction. A series of five cyclopeptides comprising the RHS sequence was designed and synthesized to mimic the exposed motif in CagL (**Figure 1**). According to the spatial screening approach each of the five amino acids was mutually replaced by its D-configured counterpart to stabilize the overall conformation and increase the spatial peptide backbone diversity (**Tables 2** and **3**). None of the cyclopeptides-1 to -5 inhibited WM-115 cell adhesion to immobilized $CagL^{WT}$ ($IC_{50} > 1$ mM; **Table 3**). However, WM-115 cell adhesion to the mutant $CagL^{RAD}$ was inhibited by cyclopeptide-2 [c-(-FeANE-)] with an IC_{50} value in the range of 54–430 μM (**Table 3**). In addition, cyclopeptides-1 and -2 displayed weak inhibition of WM-115 cell adhesion to the mutant $CagL^{RGA}$ with IC_{50} values of 43–340 and 30–530 μM, respectively. The other cyclopeptides-3 to -5 did not display any significant effect on the WM-115–CagL interaction ($IC_{50} > 1$ mM; **Table 3**).

A LINEAR RHS 15-mer PEPTIDE INTERFERES WITH WM-115 ADHESION TO THE $CagL^{RAD}$ AND $CagL^{RGA}$ MUTANTS

In addition, cell adhesion assays were performed to investigate whether the linear peptide 6 (ANFEANELFFISEDV) has effects on the adhesion of WM-115 cells to immobilized $CagL^{WT}$ and its RGD mutants $CagL^{RGA}$ and $CagL^{RAD}$. Linear peptide 6 mimics the RHS motif with the Phe-Glu-Ala-Asn-Glu sequence and its adjacent amino acids within the CagL protein sequence (**Figure 1**). According to its CD spectrum, it does not adopt a discrete conformation in solution (data not shown). While linear peptide 6 did not significantly interfere with $CagL^{WT}$–WM-115 interaction in the cell adhesion assay (IC_{50}: >1 mM), it exhibited a slight inhibitory activity on the $CagL^{RAD}$–WM-115 interaction (IC_{50}: 239 μM; $CI_{95\%}$: 164–346 μM; R^2: 0.88) and the $CagL^{RGA}$–WM-115 interaction with an IC_{50} of 9 μM ($CI_{95\%}$: 6–15 μM; R^2: 0.76; **Figure 2**; **Table 3**). In comparison to the properties of the earlier mentioned c-(-RGDlA-) peptide to inhibit the WM-115 cell adhesion to $CagL^{WT}$ (IC_{50}: 2.31 μM; $CI_{95\%}$: 1.40–3.82 μM; R^2: 0.89) and its RGD mutants $CagL^{RGA}$ (IC_{50}: 1.63 μM; $CI_{95\%}$: 1.01–2.63 μM; R^2: 0.93) and $CagL^{RAD}$ (IC_{50}: 0.91 μM; $CI_{95\%}$: 0.58–1.43 μM; R^2: 0.94), the inhibition properties of the linear peptide 6 are obviously reduced (**Table 3**). Nonetheless the results support involvement of the RHS motif in CagL–WM-115 interaction.

THE RECOMBINANT CagL MUTANTS ARE CORRECTLY FOLDED

For further investigation of the RHS motif and to reveal the relevance of specific amino acids in this sequence, CagL point mutants were recombinantly produced in *E. coli*, where individual amino acids were replaced by alanine residues (**Table 4**). CD spectroscopy was used to verify the correct folding of the mutants $CagL^{F86A}$, $CagL^{E87A}$, $CagL^{E87A/A88E}$, and $CagL^{E90A}$ in comparison to $CagL^{WT}$. The secondary structure composition calculated for $CagL^{WT}$ is in good accordance with the proposed CagL homology model (Backert et al., 2008) and displays a high content of α-helices (∼35%), accompanied by β-strands (∼29%, **Figure 3A**). The mutants $CagL^{F86A}$, $CagL^{E87A}$, $CagL^{E87A/A88E}$, and $CagL^{E90A}$ CD spectra display comparable curve shapes like the $CagL^{WT}$ (**Figure 3D**). Unfortunately, the amount of $CagL^{N89A}$ protein necessary to estimate the secondary structure and to validate the correct protein folding with CD spectroscopy could not be obtained and, therefore, could not be included in the study. However, to obtain information on the structural stability and to further compare the $CagL^{E87A}$ and $CagL^{E90A}$ mutants with $CagL^{WT}$, temperature-interval dependent CD measurements were performed ranging from 0 to 60°C (**Figures 3A–C**). In general, all tested proteins behaved in similar manner, exhibiting temperature-dependent denaturation above 40°C. Below 40°C, the secondary structures are comparable, with only minor structural differences. In comparison, the $CagL^{E90A}$ CD spectrum shows maximum deviations at temperatures below 20°C (**Figure 3C**). In addition, we performed a pH screening by measuring the CD spectra of $CagL^{WT}$ over a pH range from four to nine in phosphate buffer and could show that the protein is very stable under the tested conditions (**Figure 3E**).

WM-115 CELL ADHESION STUDIES WITH CagL RHS MUTANTS

Different CagL mutants with amino acid variations in the RHS motif ($CagL^{F86A}$, $CagL^{E87A}$, and $CagL^{E90A}$) were immobilized

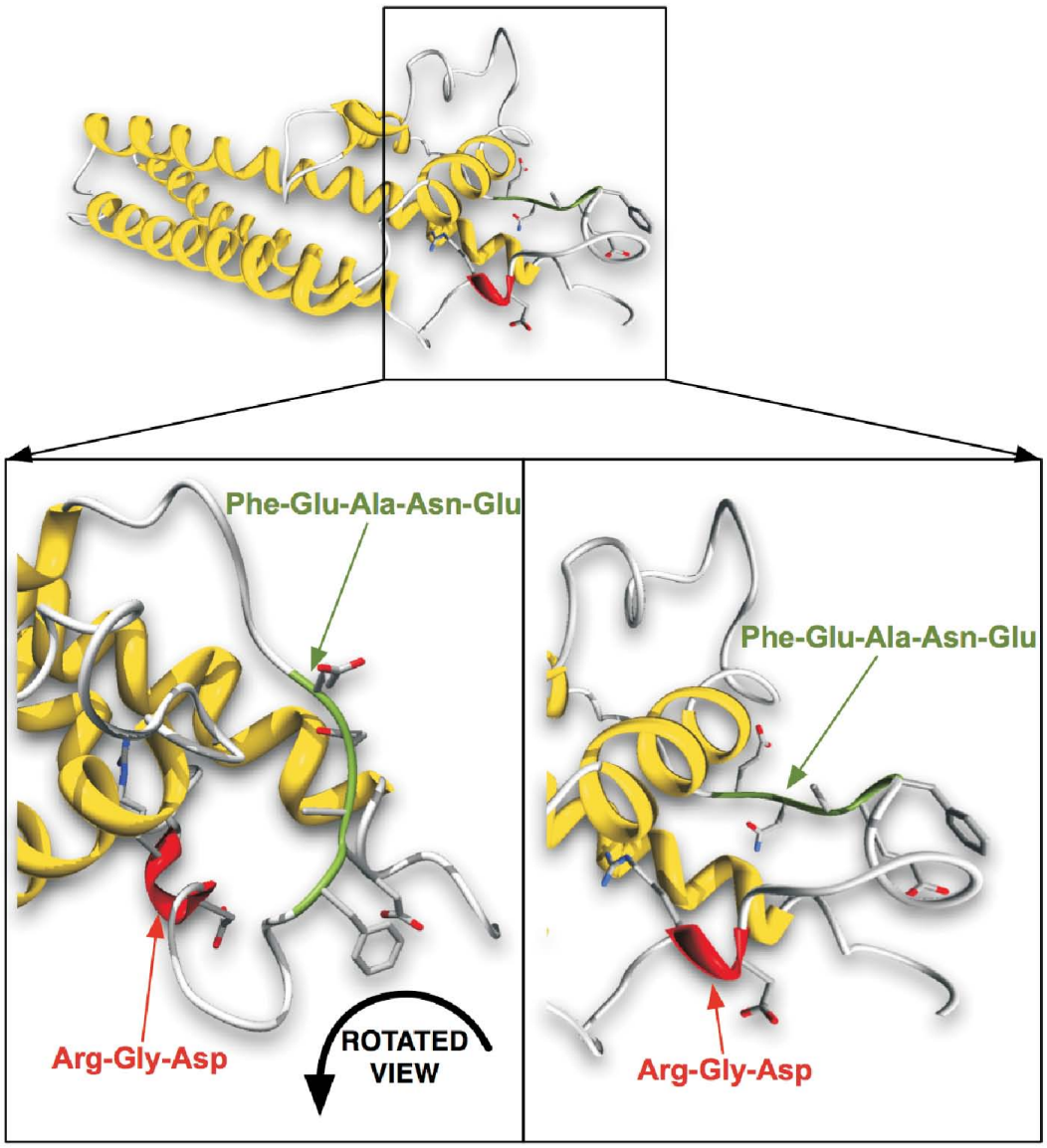


FIGURE 1 | Computational 3D-structure model of CagL^{WT} with highlighted motifs of interest. The ribbon diagram of CagL shown in this figure consists of three major helices and a globular domain with the exposed RGD motif highlighted in the front. The second highlighted FEANE sequence (here called RHS motif) is shown in a flexible loop, also exposed at the surface. The CagL homology model is derived from the VirB5 ortholog TraC protein (PDB: 1R8I) encoded in plasmid pKM101 (Yeo et al., 2003; Backert et al., 2008).

and investigated in cell adhesion assays. The WM-115 cells were pre-incubated with the previously described cyclopeptide-7 [c-(-Arg-Gly-Asp-D-Leu-Ala-)] in different concentrations from nano- to millimolar ranges to block the RGD-binding site of the integrins. The dose–response curves obtained in these assays are shown in **Figure 4**. For comparison, the cell adhesion results of the recently published CagL^{WT}, CagL^{RGA}, and CagL^{RAD} mutants are also given in **Table 4** (Conradi et al., 2011). Additionally, the CagL^{Q40A} mutant was included as a negative control to exclude the possibility that genetic mutations in the CagL sequence may

Table 3 | Inhibition of WM-115 cell adhesion to CagL by synthetic peptides[a].

	Peptide	$CagL^{WT}$ IC_{50} ($CI_{95\%}$); R^2	$CagL^{RGA}$ IC_{50} ($CI_{95\%}$); R^2	$CagL^{RAD}$ IC_{50} ($CI_{95\%}$); R^2
1	c-(-fEANE-)	>1 mM	43–340 μM	>1 mM
2	c-(-FeANE-)	>1 mM	30–530 μM	54–430 μM
3	c-(-FEaNE-)	>1 mM	>1 mM	>1 mM
4	c-(-FEAnE-)	>1 mM	>1 mM	>1 mM
5	c-(-FEANe-)	>1 mM	>1 mM	>1 mM
6	ANFEANELFFISEDV	>1 mM	9 (6–15) μM; 0.76	239 (164–346) μM; 0.88
7	c-(-RGDlA-)	2.31 (1.40–3.82) μM; 0.89	1.63 (1.01–2.63) μM; 0.93	0.91 (0.58–1.43) μM; 0.94

[a]*The IC_{50} values of cyclic peptides one to five are given in ranges, due to weak inhibition properties giving fluctuating results.* d-*Amino acids are given in small letters.* *$CI_{95\%}$, confidence interval; R^2, squared correlation coefficient.*

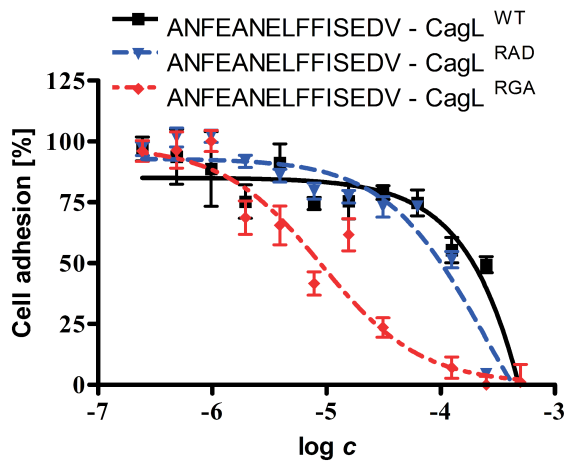


FIGURE 2 | Dose–response curve of a linear RHS motif-containing peptide, inhibiting binding of WM-115 cells to immobilized $CagL^{WT}$ and mutants. The WM-115 cells were pre-incubated in different concentrations of the linear 15-mer peptide ANFEANELFFISEDV, which mimics the exposed RHS motif of CagL. Purified $CagL^{WT}$ and the $CagL^{RAD}$ and $CagL^{RGA}$ mutants were immobilized on plastic surface followed by addition of the WM-115 cells. After 1 h of co-incubation, the amounts of attached cells were determined. The data were evaluated using non-linear fit for the evaluation of the potency of peptide ANFEANELFFISEDV to inhibit the binding of WM-115 cells to immobilized CagL.

Table 4 | Inhibition of WM-115 cell adhesion to CagL mutants by c-(-RGDlA-)[a].

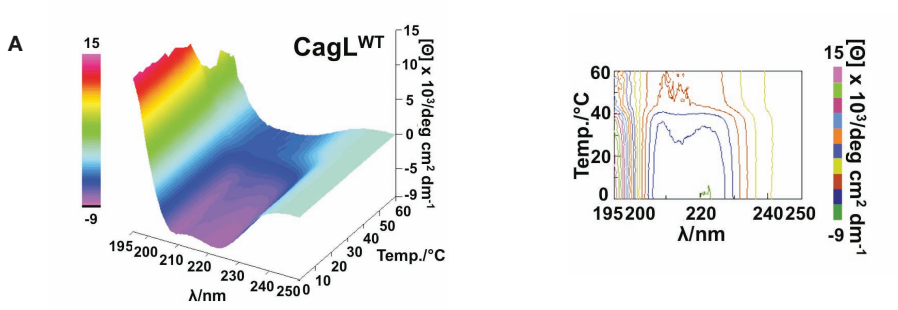
Protein[a]	c-(-Arg-Gly-Asp-d-Leu-Ala-) peptide IC50 ($CI_{95\%}$); R^2
$CagL^{WT}$	2.31 (1.40–3.82) μM; 0.89
$CagL^{RAD}$	1.63 (1.01–2.63) μM; 0.93
$CagL^{RGA}$	0.91 (0.58–1.43) μM; 0.94
$CagL^{F86A}$	2.39 (1.87–3.04) μM; 0.91
$CagL^{E87A}$	0.67 (0.38–1.18) μM; 0.72
$CagL^{E87A/A88E}$	No integrin affinity observed
$CagL^{N89A}$	No data for evaluation
$CagL^{E90A}$	0.88 (0.58–1.35) μM; 0.80
$CagL^{Q40A}$	1.65 (1.06–2.55) μM; 0.92

[a]*Measurements of peptide c-(-RGDlA-) for $CagL^{E87A}$ and $CagL^{E90A}$ proteins exhibit decreased IC_{50} values, indicating an integrin affinity loss for both CagL mutants.* d-*Amino acids are given in small letters.* *$CI_{95\%}$, confidence interval; R^2, squared correlation coefficient.*

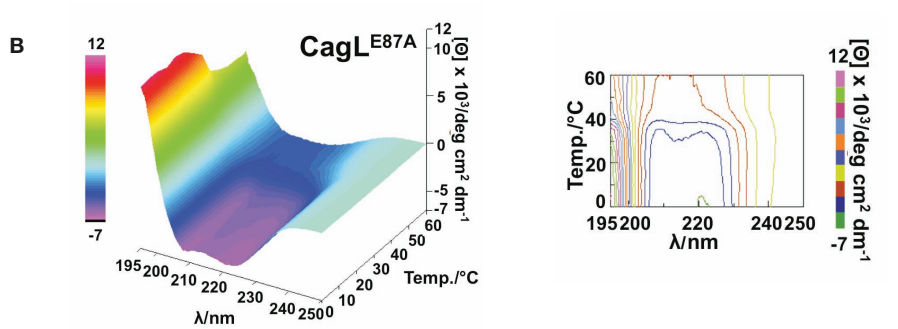
generally lead to loss of function. Cyclopeptide-7 inhibited WM-115 cell adhesion to $CagL^{Q40A}$ with an IC_{50} value of 1.65 μM ($CI_{95\%}$ = 1.06–2.55; R^2 = 0.92), while the cell adhesion of WM-115 cells to immobilized $CagL^{F86A}$ was inhibited with an IC_{50} value of 2.39 μM ($CI_{95\%}$ = 1.87–3.04 μM; R^2 = 0.91), a value that is very similar to that of $CagL^{WT}$ (IC_{50} = 2.31 μM; $CI_{95\%}$ = 1.87–3.04 μM; R^2 = 0.91). Interesting results were observed in the inhibition assays for the mutants $CagL^{E87A}$ and $CagL^{E90A}$. WM-115 cell adhesion to immobilized $CagL^{E87A}$ was inhibited by cyclopeptide-7 with an IC_{50} of 0.67 μM ($CI_{95\%}$ = 0.38–1.18 μM; R^2 = 0.72), the lowest value observed for all CagL mutants investigated (**Figure 4**; **Table 4**). The IC_{50} value of 0.88 μM ($CI_{95\%}$ = 0.58–1.35 μM; R^2 = 0.80) for the inhibition of WM-115 cell adhesion to immobilized $CagL^{E90A}$ by cyclopeptide-7 is similar, and is also comparable to the values observed for $CagL^{RGA}$ (0.91 μM; **Figure 4**; **Table 4**). In an additional experiment we tested the double mutant $CagL^{E87A/A88E}$ to reveal more details on the involvement of CagL amino acid Glu^{87} in the interaction with WM-115 cells. The double mutant $CagL^{E87A/A88E}$ formally is characterized by the shift of an acidic amino acid side chain from position 87 to position 88. The immobilized $CagL^{E87A/A88E}$ protein displayed no binding to the WM-115 cells, which may indicate a loss of integrin affinity. In conclusion, the results show remarkably reduced adhesion of WM-115 cells to immobilized $CagL^{E87A}$ and $CagL^{E90A}$ compared to the $CagL^{WT}$, which implies a participation of the glutamates in the CagL–WM-115 interaction.

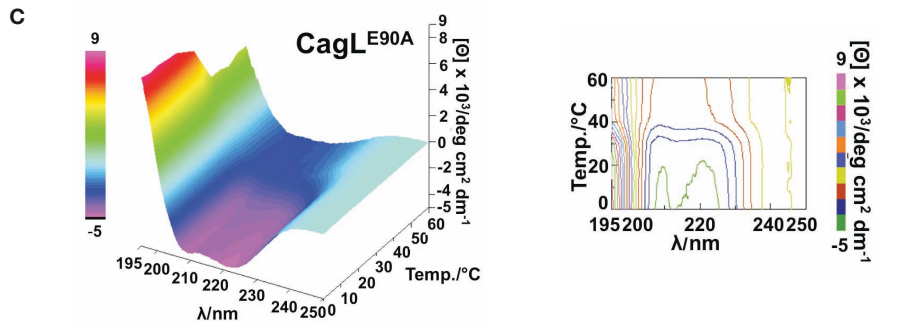
PEPTIDE ARRAY MAPPING OF THE CagL RHS MOTIF FOR INTEGRIN BINDING

The previously described SPOT technique (Frank, 2002) was applied to identify the CagL amino acid sequence responsible for binding to integrin $\alpha_5\beta_1$. Overlapping linear 15-mer peptides derived from the CagL sequence from amino acid position 60–104 were chemically synthesized on a cellulose membrane by the SPOT method (Frank, 1992). As shown in **Figure 5A**, adjacent peptides share the same sequence of 12 amino acids but differ by three amino acids at the C- or N-terminal ends, respectively.



CagL^{WT} structure estimation	
	Ratio [%]
α-helix	35.3
β-helix	29.3
β-turn	6.0
Random	29.4
Total	100.0
RMS Value	4.271





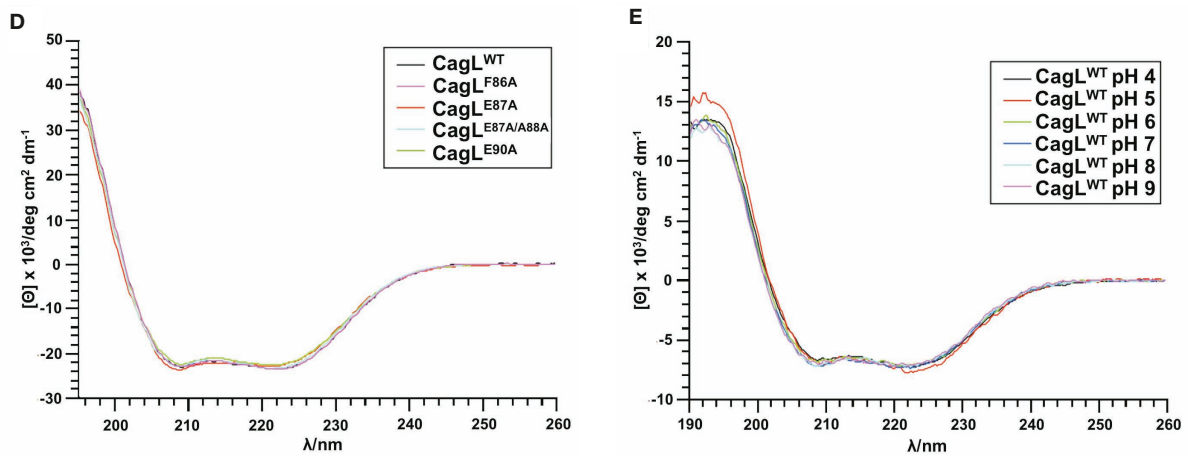


FIGURE 3 | Structural evaluation of purified CagL proteins with CD spectroscopy. (A) The CagL^{WT} secondary structure was evaluated as described previously (Yang et al., 1986). **(A–C)** The temperature-dependent structure stability of the mutants CagL^{E87A}, CagL^{E90A}, and the CagL^{WT} protein was evaluated in CD measurements. **(D)** Secondary structure of CagL^{WT} and mutants CagL^{F86A}, CagL^{E87A}, CagL$^{E87A/A88E}$, and CagL^{E90A} were evaluated to control correct folding of the proteins after expression and purification. **(E)** pH stability of CagL^{WT} in the pH range four to nine.

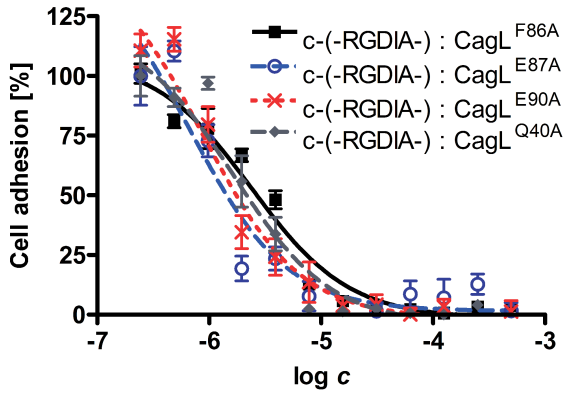


FIGURE 4 | Dose–response curves showing the inhibitory effect of cyclic RGD peptides on WM-115 cell binding to the different immobilized CagL proteins in competitive cell adhesion assays. The purified CagL^{Q40A}, CagL^{F86A}, CagL^{E87A}, and CagL^{E90A} mutant proteins were immobilized on plastic surface. WM-115 cells were pre-incubated with varying concentrations of cyclic peptide c-(-RGDIA-; d-amino acids in small letter), which is known to inhibit WM-115 cell binding to immobilized CagL in the low μM range (Conradi et al., 2011). Subsequently, the WM-115 cells were incubated with the immobilized CagL mutants. The amount of WM-115 cells bound to immobilized CagL was quantified, and the resulting dose–response curves were evaluated with a non-linear fit to determine the binding inhibition potency of peptide c-(-RGDIA-) for the interaction of WM-115 cells with immobilized CagL.

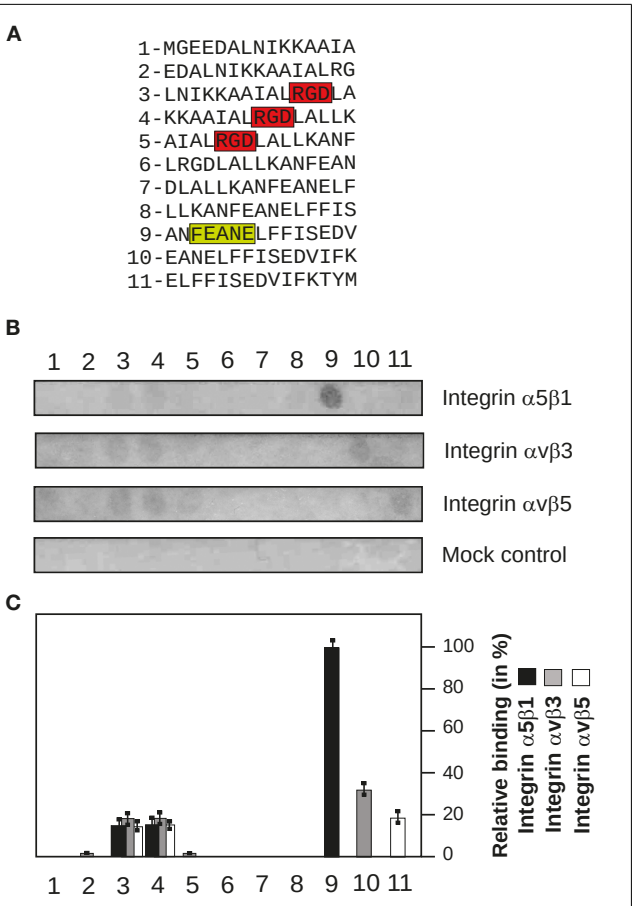


FIGURE 5 | Mapping of the RHS motif and flanking CagL sequences in binding to integrins using a peptide array. (A) The design of the array of 15-mer peptides and corresponding CagL amino acid sequences from position 60–104 is shown. The RGD and RHS motifs are highlighted with color. **(B)** Purified integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ were incubated with these membranes and bound proteins were identified using antibodies as described in Materials and Methods. As a mock control, buffer-treated membranes followed by incubation with an integrin-β_1 antibody revealed no signals, as expected. **(C)** Densitometric measurement of individual spot intensities using the Lumi-Imager F1 (Roche) revealed the relative amount of bound binding partner per given peptide in %. The strongest spot intensity seen was set as 100%.

Purified integrin $\alpha_5\beta_1$ was incubated with these membranes and the binding was assayed as described in the Section "Materials and Methods." **Figure 5B** shows that integrin $\alpha_5\beta_1$ binds to the RGD motif-containing arraypeptides-3 and -4. In addition, a very strong signal for arraypeptide-9 covering the RHS motif was recorded (**Figure 5B**, top and **Figure 5C**). Interestingly, two other FEANE sequence-containing array peptides (-7 and -8) did not bind integrin $\alpha_5\beta_1$, suggesting that the flanking C-terminal sequences are also important for this interaction. As a control, incubation of the membranes with two other integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ also revealed signals for the two RGD containing CagL peptides and some weak signals for arraypeptides-10 or -11, respectively (**Figure 5B**, middle and **Figure 5C**). The mock control blot revealed no signals as expected (**Figure 5B**, bottom).

GENERATION OF RHS POINT MUTATIONS OF CagL IN *H. PYLORI*

To investigate the importance of the RHS motif in CagL directly during infection with *H. pylori*, two CagL mutants were generated carrying the A84E/E87A and A88E/L91A point mutations. Based on the above described peptide array the two point mutants were constructed to contain one mutation in the RHS sequence and one either N- or C-terminal, to cover additional amino acids that may be relevant for host cell interactions. CagL^{WT} and both mutants were chromosomally integrated into a P12Δ*cagL* deletion mutant and expressed as HA-tag fusions in the *ureA* locus as described (Shaffer et al., 2011). The correct expression of each of these CagL variants was verified by Western blotting using an α-HA antibody (**Figure 6A**). The AGS cells were infected with each of these *H. pylori* strains for 4 h, followed by analysis of viable bacterial binding to cells, as described in Materials and methods. The results show that each of these strains bound to AGS cells with high efficiency. Some minor differences in binding were seen among the different strains but were not statistically significant (**Figure 6B**). This suggests that mutation of the RHS motif in CagL has no significant inhibitory impact on the overall capacity of the bacteria to bind to AGS host cells.

MUTATION OF THE RHS MOTIF INHIBITS CagA PHOSPHORYLATION AT EARLY TIMES OF INFECTION

AGS cells were infected with the different complemented *H. pylori* strains during a time course of 2 and 4 h, respectively, to investigate whether strains expressing CagL mutant proteins can trigger the

injection and phosphorylation of CagA. The results of the α-phospho-tyrosine and α-CagA specific Western blots show that P12Δ*cagL* re-expressing CagL^{WT} can efficiently inject and phosphorylate CagA in a time-dependent fashion (**Figures 7A,B**). Infection of AGS with P12Δ*cagL* expressing the CagL$^{A84E/E87A}$ mutant revealed a ∼46% reduced phospho-CagA signal at 2 h, while very strong phospho-CagA signals were produced at the 4-h time point with almost no difference as compared to the complemented CagL^{WT} control (**Figures 7A,B**). This suggests that mutation of A84E/E87A in CagL affects the injection and phosphorylation of CagA at very early times of infection. In contrast, infection with *H. pylori* expressing the CagL$^{A88E/L91A}$ mutant revealed a stronger reduction of phospho-CagA signals (∼69%) at 2 h as compared to the complemented CagL^{WT} control, while this low level of phospho-CagA did only slightly increase after 4 h of infection (**Figures 7A,B**). This result shows that a strain expressing the CagL$^{A88E/L91A}$ mutant protein has a significant defect in injection and phosphorylation of CagA at both time points of infection.

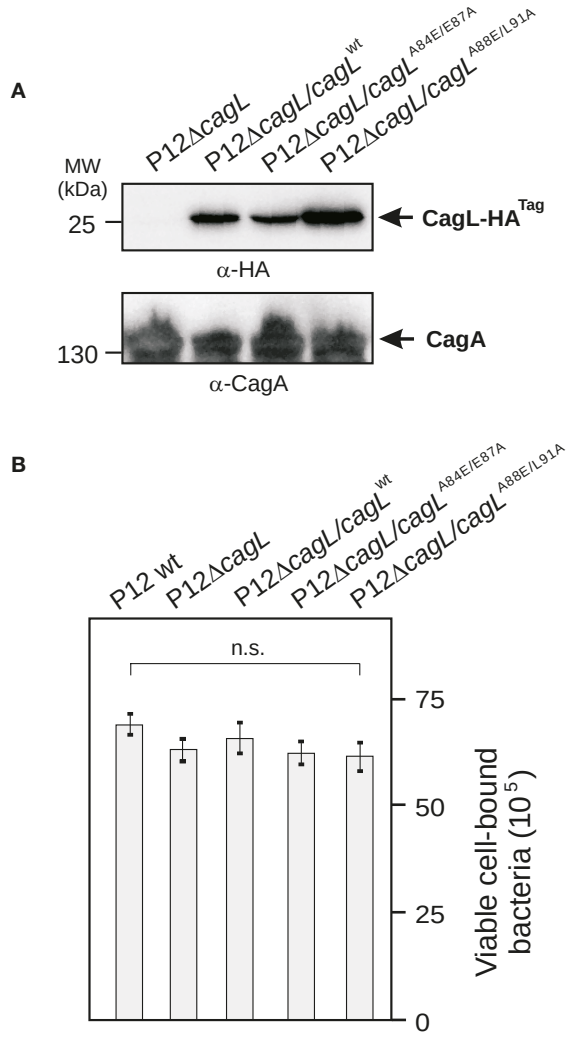


FIGURE 6 | Effect of CagL deletion or point mutations in the CagL RHS motif on binding of *H. pylori* to AGS cells. (A) A Δ*cagL* deletion mutant in strain P12 was generated by replacing the *cagL* gene with an *aphA3* cassette. CagL^{WT} and two CagL mutants carrying either the A84E/E87A or A88E/L91A point mutations were expressed as HA-tag fusion proteins from genes integrated into the *ureA* locus of the P12Δ*cagL* strain. The correct expression of each of these CagL variants was verified by Western blotting using an α-HA antibody. The α-CagA Western blot was performed as loading control. **(B)** AGS cells were infected with each of these strains for 4 h using MOI = 100, followed by determination of the amount of viable bound *H. pylori* per input strain. The quantification data indicate that each of these strains bound to AGS cells equally well with high efficiency. No significant differences in results were seen when MOI = 50 was used (data not shown).

MUTATION OF THE RHS MOTIF EXHIBITS SIGNIFICANT SIGNALING DEFECTS IN *H. PYLORI*-INDUCED ERK1/2 ACTIVATION

Finally the various RHS mutations in CagL were inspected in relation to impacts on cellular downstream signaling involved in the *H. pylori*-induced AGS cell elongation phenotype. It was recently shown that besides CagA phosphorylation, the activation of the ERK1/2 MAP kinase pathway either by *H. pylori* infection (Mimuro et al., 2002; Tegtmeyer et al., 2009) or by transfection CagA in the absence of *H. pylori* (Higashi et al., 2004) is also crucial for phenotypical outcome. Therefore, we tested whether the different CagL-expressing *H. pylori* strains induced the activation of ERK1/2, using the same conditions as described for **Figure 7**. The results of the α-phospho-ERK1/2 Western blots show that P12 WT or P12Δ*cagL* re-expressing CagL^{WT} (but not P12Δ*cagL*) can efficiently activate this MAP kinase in a time-dependent fashion (**Figures 8A,B**). Infection of AGS with *H. pylori* expressing the complemented CagL$^{A84E/E87A}$ mutant revealed a significantly (46%) reduced phospho-ERK1/2 signal at 2 h, and a similarly reduced (38%) phospho-ERK1/2 signal at 4 h time point as compared to the complemented CagL^{WT} control (**Figures 8A,B**). This suggests that mutation of A84E/E87A in CagL downregulates not only the injection and phosphorylation of CagA, but also reduced the activation of ERK1/2. In addition, infection with *H. pylori* expressing the CagL$^{A88E/L91A}$ mutant revealed an even stronger reduction of phospho-ERK1/2 signals by about 63 or 70% at the 2- or 4-h time points, respectively. This result indicates that the CagL$^{A88E/L91A}$ mutant has a significantly pronounced defect in activating ERK1/2 during infection.

MUTATION OF THE RHS MOTIF RESULTS IN REDUCED CORTACTIN SERINE PHOSPHORYLATION AND AGS CELL ELONGATION

Very recently, we demonstrated that one important downstream target of activated ERK1/2 during infection is the actin-binding protein cortactin, phosphorylated at serine residue 405 (Tegtmeyer et al., 2011b). Hence it was tested whether the various CagL-expressing strains induce the phosphorylation of cortactin at S-405 during a time course of 2 and 4 h infection. The results of the α-phospho-cortactin Western blots show that P12Δ*cagL* re-expressing CagL^{WT} but not P12Δ*cagL* can efficiently phosphorylate cortactin in a time-dependent manner (**Figures 8A,B**). Infection of AGS cells with *H. pylori* expressing either CagL$^{A84E/E87A}$ or CagL$^{A88E/L917A}$ mutants revealed the induction of phospho-cortactin signals similar to that of CagL^{WT} with no significant difference at 2 h, but a strongly reduced (about 45–46%)

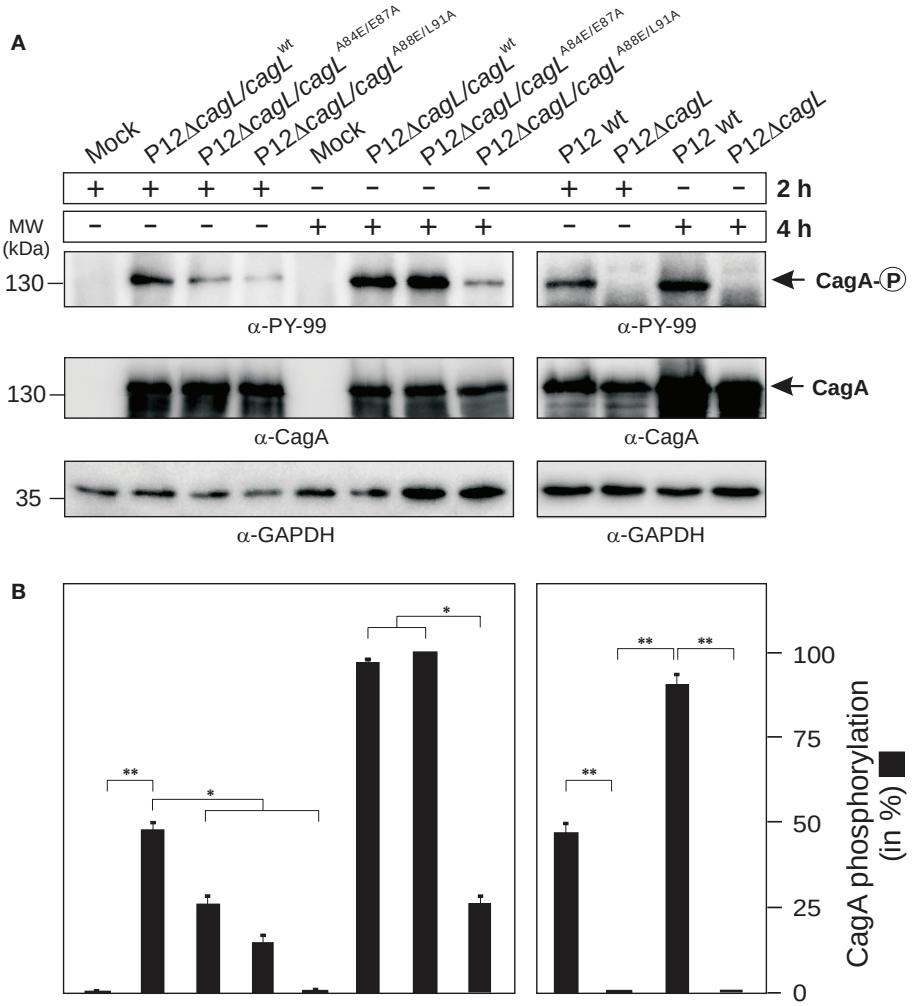


FIGURE 7 | Effects of CagL point mutations in the RHS motif on CagA injection and phosphorylation during *H. pylori* infection of AGS cells. (A) The P12Δ*cagL* deletion mutant was complemented with $CagL^{WT}$ or various CagL point mutants. AGS cells were infected with the different indicated *H. pylori* strains (MOI = 100) during a time course of 2 or 4 h, respectively. The resulting protein lysates were subjected to Western blotting using α-phospho-tyrosine (PY-99) and α-CagA antibodies. The α-GAPDH blot served as loading control in each sample. **(B)** Quantification of CagA phosphorylation. Densitometric measurement of individual band intensities of the α-CagA and α-phospho-tyrosine (PY-99) blots in **(A)** was performed with the Lumi-Imager F1 (Roche). Quantification revealed the relative amount of phospho-CagA per sample in %. Similar inhibitory activities of all mutants were seen when MOI = 50 was used (not shown).

phospho-cortactin signal at the 4-h time point (**Figures 8A,B**). This suggests that mutation of CagL in the RHS motif (either A84E/E87A or A88E/L91A mutations) downregulates cortactin's serine phosphorylation to a similar high extent at the 4-h time point, indicating that the $CagL^{A84E/E87A}$ and $CagL^{A88E/L91A}$ mutants share a significantly pronounced defect in activating cortactin during infection. Finally, the elongation phenotype was monitored in the same set of experiments. As shown in **Figure 8C**, mutation of the RHS motif at A84E/E87A or A88E/L91A inhibited the elongation phenotype by more than 50% at the 2-h time point. At the 4-h time point, the $CagL^{A84E/E87A}$ mutant exhibited a ∼20% reduction and the $CagL^{A88E/L91A}$ mutant revealed a ∼43% reduction as compared to the complemented $CagL^{WT}$ control. This set of experiments reveals that mutation of the RHS motif in *H. pylori* results in significant defects in signaling to cortactin and cell elongation, especially at very early times of infection.

DISCUSSION

Our previous studies have suggested that the RGD sequence is an important structural motif in the *H. pylori* CagL pilus protein, able to trigger T4SS-mediated host cell binding and signaling, but this sequence alone is not sufficient (Kwok et al., 2007; Tegtmeyer et al., 2010; Conradi et al., 2011; Wiedemann et al., 2012). Thus, we examined the 3D structural CagL model (Backert et al., 2008) to identify other potential surface exposed CagL–integrin interaction motifs. In this study, we report on the discovery of a novel motif in CagL, called RHS consisting of a Phe-Glu-Ala-Asn-Glu core sequence, which also plays a role in the interaction with host cell

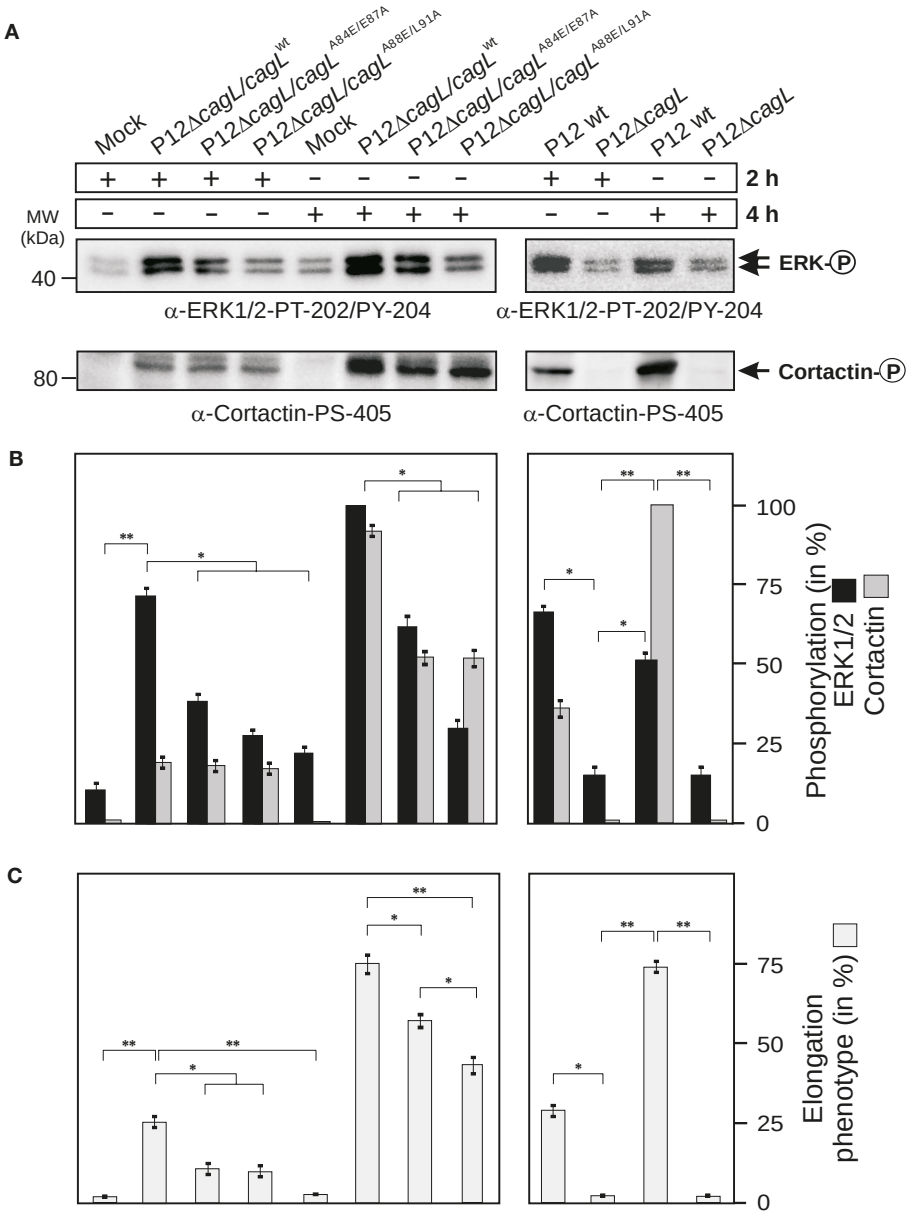


FIGURE 8 | Mutation of CagL in specific amino acids within the RHS motif results in defects in *H. pylori*-triggered ERK1/2 activation, cortactin phosphorylation, and AGS cell elongation. (A) AGS cells were infected with the different complemented *H. pylori* strains (MOI = 100) during a time course of 2 or 4 h, respectively. The same protein lysates as shown in **Figure 7** were subjected to Western blotting using the indicated α-phospho-ERK1/2 and α-phospho-cortactin antibodies. **(B)** Densitometric measurement of individual band intensities of the blots in **(A)** and comparison to total α-ERK1/2 and α-cortactin blots (not shown) was performed with the Lumi-Imager F1 (Roche). The relative amounts of phospho-ERK1/2 and phospho-cortactin per sample were quantified and given in %. **(C)** Quantification of the AGS cell elongation phenotype in the same set of experiments. Similar inhibitory activities of all mutants were seen when MOI = 50 was used (not shown).

integrins. A peptide array of overlapping 15-mer peptides derived from the CagL primary structure showed that peptides containing the RHS motif of CagL can bind particularly to integrin $\alpha_5\beta_1$, whereas they bind only weakly to other integrins, such as $\alpha_V\beta_3$ and $\alpha_V\beta_5$. Further studies, including the use of CagL-derived peptides in competitive WM-115 cell binding assays, and the analysis of recombinant CagL point mutants in cell binding *in vitro* and during infection of AGS cells with *H. pylori*, collectively indicated that the RHS sequence plays an important role in the interaction of CagL with host cells.

The spatial screening approach (Haubner et al., 1996; Weide et al., 2007) was employed with five conformationally designed synthesized cyclopeptides-1 to -5, wherein each of the five amino acids was mutually replaced by its D-configured correlate to mimic the RHS sequence of CagL. Receptor binding of such cyclopeptides is regarded to be entropically favored, if the receptor-bound

conformation is still accessible. However, neither the linear RHS-peptide 6 (ANFEANELFFISEDV) nor the RHS-cyclopeptides-1 to -5 showed a significant effect on WM-115 cell adhesion to immobilized $CagL^{WT}$. In contrast, RGD-dependent WM-115 cell adhesion could be efficiently influenced by the RGD-cyclopeptide-7. Mutations within the RGD sequence in extracellular matrix proteins such as fibronectin are usually accompanied by loss of integrin affinity (Giancotti and Ruoslahti, 1999; Takahashi et al., 2007; Leiss et al., 2008). This seems not to be fully applicable to CagL, as integrin-expressing WM-115 cells still adhere to the mutated proteins $CagL^{RAD}$ and $CagL^{RGA}$ but with reduced affinity (Conradi et al., 2011). Interestingly, both the linear RHS-peptide ANFEANELFFISEDV and the RHS-cyclopeptide-2 were able to interfere with WM-115 cell adhesion to immobilized $CagL^{RGA}$ and $CagL^{RAD}$ proteins with IC_{50} values in the micromolar range, indicating significant binding. The studies with the $CagL^{RGA}$ and $CagL^{RAD}$ mutants allowed us to distinguish RGD-dependent interaction from other types of binding. Not all RHS-cyclopeptides displayed such inhibitory biological activity, which clearly proves the influence of peptide conformation and three-dimensional structure on the anti-adhesive properties. We thus conclude that the WM-115 cell adhesion to recombinant CagL is mainly driven by the RGD recognition sequence, but is significantly assisted by the RHS sequence as a proposed auxiliary motif. There is an interesting analogy to fibronectin. Besides the RGD motif, fibronectin harbors another sequence required for maximum binding affinity to integrin $\alpha_5\beta_1$, called the synergy region, located in the ninth type III module adjacent to the RGD site (Nagai et al., 1991; Aota et al., 1994; Bowditch et al., 1994; Chada et al., 2006). Subsequent studies showed that the synergy region is not directly in contact with the integrin $\alpha_5\beta_1$ bound to fibronectin, and proposed an indirect function of the synergy region for the high affinity binding by optimally exposing the flexible RGD motif or by inducing long range electrostatic steering (Baron et al., 1992; Takagi et al., 2003). In the case of CagL, however, a FEANE-containing peptide alone can bind integrin $\alpha_5\beta_1$ as shown in peptide arrays, and the synergy region of fibronectin shares no sequence homology to CagL or the RHS domain.

The role of the RHS motif and neighboring amino acids was further investigated on the protein level with recombinant CagL proteins carrying corresponding point mutants. The secondary structure and temperature stability of all generated point mutants corresponds with that observed for $CagL^{WT}$ according to CD spectra. Given the above assumptions, pre-incubation of the WM-115 cells with the RGD-cyclopeptide-7 should provide information on the contribution of the RHS sequence to CagL binding phenomena. Strikingly, there is an effect of mutations in the RHS motif of CagL on WM-115 cell adhesion. RGD-cyclopeptide-7 was able to inhibit cell adhesion to CagL with mutations in the RHS sequence ($CagL^{F86A}$, $CagL^{E87A}$, $CagL^{E90A}$) even at lower concentrations than for $CagL^{WT}$. Granted that cell adhesion is governed by both RGD and RHS sequences, a mutation in the CagL RGD sequence ($CagL^{RGA}$, $CagL^{RAD}$) would lead to reduced cell adhesion and, hence, require a smaller concentration (reduced IC_{50}) of the RGD-peptide that competes for binding (**Table 4**; Conradi et al., 2011). Conversely, WM-115 cell adhesion to immobilized CagL with mutations in the RHS motif conferring decreased affinity could be inhibited by the RGD-peptide at a smaller concentration (reduced IC_{50}). This was indeed observed for $CagL^{E87A}$ and $CagL^{E90A}$. In conclusion, the point mutant $CagL^{F86A}$ and a control mutant $CagL^{Q40A}$ show no significant affinity loss in their binding to WM-115 cells as compared to $CagL^{WT}$, while the point mutants $CagL^{E87A}$ and $CagL^{E90A}$ display remarkably reduced affinity to WM-115 cells. The replacement of the negatively charged glutamates E87 and E90 by the hydrophobic amino acid alanine resulted in a decreased affinity toward WM-115 cells. In contrast, the exchange of the uncharged amino acids phenylalanine F86 and glutamine Q40 by alanine did not have any consequence in the same assays. These data imply that especially glutamate E87 is important for the interaction with integrin on the surface of host cells.

At first sight, the peptide array experiments in which we investigated the binding of immobilized CagL 15-mer peptides with the different purified integrins seem to implicate a stronger signal of a RHS containing peptide for bound integrin $\alpha_5\beta_1$, but much weaker signals compared to RGD containing peptides on the same membrane. Regarding the conformation of RGD containing peptides, cyclic RGD peptides with a β-turn around Asp-D-Leu-Ala-Arg display a much higher affinity to some integrins than do linear RGD peptides. The linear RGD containing peptides do not prefer this structural β-turn conformation, which could explain the fact that linear RGD peptides on the peptide array display very little affinity to integrins as compared to their cyclopeptide counterparts. While the arraypeptides-7 to -9 contain the whole FEANE amino acids of the RHS motif, only arraypeptide-9 reveals integrin β_1 interaction (**Figure 5**). In arraypeptide-10 and -11 the 15-mer peptide sequence is shifted C-terminally for three amino acids. Arraypeptide-10 therefore is lacking the phenylalanine of the RHS motif and displays a different interaction with integrin subunit β_3, while the arraypeptide-11 includes only the final glutamate of the RHS motif and displays weak interaction with the integrin β_5 subunit. In cell binding studies with the 15-mer peptide of arraypeptide-9 corresponding to linear peptide 6, a binding inhibition of WM-115 cells to immobilized $CagL^{RAD}$ and $CagL^{RGA}$ could be shown (**Tables 2** and **3**). Overall the binding potency of this linear peptide was surprisingly better than that observed for the cyclic RHS peptides. These results emphasize the possible involvement of adjacent amino acids next to the FEANE sequence for integrin interactions that probably are located in the C-terminal flanking site. The presented results allow no complete elucidation of amino acids important for integrin interaction, but combining the results of cell binding studies and the peptide array, the CagL F86 amino acid is not involved in the interaction with WM-115 cells (**Table 3**) and the interaction with purified integrins (**Figure 5**), while both glutamates E87 and E90 have an impact and are probably exposed on the CagL surface. The observed binding of three different integrins to a relatively small CagL sequence region of about 18 amino acids on peptide arrays is a very interesting finding, and is relevant to recent reports which showed that CagL interacts not only with integrin $\alpha_5\beta_1$ on cells, but also $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (Kwok et al., 2007; Conradi et al., 2011; Wiedemann et al., 2012).

Finally, it was investigated if the newly discovered RHS motif in CagL might also play a role during infections with *H. pylori*.

As discussed above, an explicit amino acid region for the so-called RHS motif could not be clearly defined. Therefore two double point mutants CagL$^{A84E/E87A}$ and CagL$^{A88E/L91A}$ were chosen, with one mutation in the RHS region and another located at the N- or C-terminus, respectively. Based on infection experiments, we could show that *H. pylori* P12 WT, deletion mutant P12Δ*cagL*, and P12Δ*cagL* re-expressing CagL^{WT} or CagL point mutants (A84E/E87A and A88E/L91A) bound equally well to cultured AGS cells. This suggests that CagL is not involved in general host cell binding during infection, in agreement with previous reports showing the overall importance of the major canonical adhesins BabA/B, SabA, AlpA/B, or OipA (for review, see Tegtmeyer et al., 2011a). However, this illustrates clearly that the observed signaling defects discussed below are not due to a more general deficiency of the bacteria to adhere to AGS cells. First, *H. pylori* expressing the generated CagL point mutants exhibited significant defects in injection and phosphorylation of CagA between 2 and 4 h of infection. Second, each of the CagL mutants in *H. pylori* had significantly pronounced defects in activating the MAP kinases ERK1/2 and serine phosphorylation of cortactin as well as the induction of the AGS cell elongation phenotype during infection. This suggests that mutation of the RHS motif in *H. pylori* results in various defects in the interaction of CagL with integrin $\alpha_5\beta_1$ with resulting inhibitory consequences for (i) the injection of CagA and (ii) for the downstream signaling to ERK1/2 and cortactin triggering cell elongation, especially at early times of infection.

Taken together, our presented data provide evidence for a novel domain in CagL playing a significant role in binding to integrin $\alpha_5\beta_1$ *in vitro* and for initiating signaling pathways through certain motifs encoded in the so-called RHS motif comprising the FEANE sequence and its flanking amino acids. We propose that the RGD motif is important for the CagL binding to integrins, while subsequent binding of other integrin-specific CagL motifs may assist in certain intracellular signaling events. Although we could demonstrate that the RHS motif and its adjacent amino acids are relevant in CagL–integrin interactions and have an impact on the resulting signaling, more studies are necessary to further define the specific amino acids relevant for integrin interaction and downstream signaling. In addition, it has been shown that besides CagL, multiple other *H. pylori* factors are also involved in integrin β_1 binding, including CagA, CagI, CagY, and possibly CagH (Kwok et al., 2007; Jiménez-Soto et al., 2009; Shaffer et al., 2011). Their individual role for integrin-targeting/binding is not yet fully clear. Future studies should investigate whether these proteins act together or at different stages of the infection process.

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Identification of genetic modifiers of CagA-induced epithelial disruption in *Drosophila*

David W. Reid*†‡, *Jonathan B. Muyskens*†, *James T. Neal*†‡, *Gino W. Gaddini*‡, *Lucy Y. Cho*‡, *Anica M. Wandler*, *Crystal M. Botham*‡ *and Karen Guillemin*

Institute of Molecular Biology, University of Oregon, Eugene, OR, USA

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Richard Peek, Vanderbilt University Medical Center, USA
Steffen Backert, Universtiy College Dublin, Ireland

***Correspondence:**
Karen Guillemin, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA. e-mail: guillemin@molbio.uoregon.edu

† *These authors contributed equally to this work.*

‡ ***Current address:***
David W. Reid, Department of Biochemistry, Duke University School of Medicine, Durham, NC, USA;
James T. Neal and Crystal M. Botham, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA;
Gino W. Gaddini, College of Public Health and Human Sciences, Oregon State University, Corvallis, OR, USA;
Lucy Y. Cho, Saint Louis University School of Medicine, St. Louis, Missouri, MO, USA.

Helicobacter pylori strains containing the CagA protein are associated with high risk of gastric diseases including atrophic gastritis, peptic ulcers, and gastric cancer. CagA is injected into host cells via a Type IV secretion system where it activates growth factor-like signaling, disrupts cell-cell junctions, and perturbs host cell polarity. Using a transgenic *Drosophila* model, we have shown that CagA expression disrupts the morphogenesis of epithelial tissues such as the adult eye. Here we describe a genetic screen to identify modifiers of CagA-induced eye defects. We determined that reducing the copy number of genes encoding components of signaling pathways known to be targeted by CagA, such as the *epidermal growth factor receptor* (EGFR), modified the CagA-induced eye phenotypes. In our screen of just over half the *Drosophila* genome, we discovered 12 genes that either suppressed or enhanced CagA's disruption of the eye epithelium. Included in this list are genes involved in epithelial integrity, intracellular trafficking, and signal transduction. We investigated the mechanism of one suppressor, encoding the epithelial polarity determinant and junction protein Coracle, which is homologous to the mammalian Protein 4.1. We found that loss of a single copy of *coracle* improved the organization and integrity of larval retinal epithelia expressing CagA, but did not alter CagA's localization to cell junctions. Loss of a single copy of the *coracle* antagonist *crumbs* enhanced CagA-associated disruption of the larval retinal epithelium, whereas overexpression of *crumbs* suppressed this phenotype. Collectively, these results point to new cellular pathways whose disruption by CagA are likely to contribute to *H. pylori*-associated disease pathology.

Keywords: CagA, *Helicobacter pylori*, *Drosophila*, genetic modifier, epithelia, *coracle*, *crumbs*

INTRODUCTION

H. pylori infects approximately 50% of the world's population and is a leading cause of ulcers and gastric cancer (Amieva and El-Omar, 2008). Strains harboring the virulence factor, CagA, are up to three times more potent in contributing to cancer progression than strains lacking this factor (Blaser et al., 1995; Huang et al., 2003; Wu et al., 2003). In cell culture experiments, CagA has been shown to interact physically with at least 20 proteins, such as SHP-2 and Par1, and to modulate the activity of many other host proteins (Hatakeyama, 2008; Backert et al., 2010). However, progress in characterizing the *in vivo* significance of these putative host effectors of CagA has been hampered by a lack of experimental models to study CagA's effects on intact tissues. We have developed a transgenic *Drosophila* model to study the expression of CagA in epithelial tissues such as the larval and adult eye (Botham et al., 2008; Muyskens and Guillemin, 2011). In this system, CagA is expressed as a full-length protein that is tyrosine phosphorylated by host kinases and localizes to cell junctions, as in mammalian cells (Botham et al., 2008).

Using this system, we showed that CagA interacts genetically with proteins identified as its physical targets in tissue culture cells. Several of CagA's physical interaction partners include members of receptor tyrosine kinase (RTK) signaling pathways that are normally scaffolded together in the cell by the adaptor protein Grb2-associated binder (Gab) (Hatakeyama, 2003). We demonstrated that expression of CagA could rescue phenotypes associated with loss of the *Drosophila* Gab, Son of sevenless, indicating that CagA functions as a Gab mimic and restores the physical interactions required for efficient RTK signaling. In these studies we also discovered that ectopic expression of CagA in the developing *Drosophila* eye, unlike over-expression of Son of sevenless, profoundly disrupted the morphogenesis of the retinal epithelium, resulting in adult eyes with a "rough" phenotype in which the crystalline array of facets is perturbed. We went on to show that CagA's disruption of the larval retinal epithelium was due to

Abbreviations: Moc, Modifier of CagA; ESEM, Environmental scanning electron microscopy.

over-activation of myosin light chain (Muyskens and Guillemin, 2011), which has been implicated in disruption of gastrointestinal epithelial barriers (Shen et al., 2009) and *H. pylori* pathogenesis (Wroblewski et al., 2009). In this study we describe a forward genetic screen to uncover additional host genes that influence CagA's activity in the retinal epithelium.

The *Drosophila* eye has been a fertile genetic system for discovering genes involved in cellular signaling pathways, including many RTK signaling pathway members (Voas and Rebay, 2004). Because of the *Drosophila* eye's repeating pattern of facets or ommatidia, even subtle perturbations in signaling pathways that regulate eye morphogenesis can be distinguished by the severity of the rough eye phenotype of the adults, making possible rapid, high throughput screens for dominant enhancer and suppressor mutations (St Johnston, 2002). These genetic screens have proven to be extremely fruitful because of the high degree of conservation in molecular signaling pathways in eukaryotic cells. For example, the important CagA interactor SHP-2 was originally identified in a genetic screen in the *Drosophila* eye (Simon et al., 1991), and subsequently identified in mammals (Freeman et al., 1992). The functional conservation between human and *Drosophila* SHP-2 is illustrated by the fact that expression of the human protein can rescue the eye defects of a *csw* mutant lacking the *Drosophila* SHP-2 (Oishi et al., 2006). The high degree of molecular conservation in cellular processes targeted by bacterial pathogens has allowed researchers to screen for host factors that interact genetically with bacterial effector proteins in genetically tractable systems such as fruit flies and yeast (Siggers and Lesser, 2008; Boyer et al., 2012).

Here we exploited the CagA-induced rough eye phenotype to identify host genes that are important for pathogenic mechanisms of CagA. We used molecularly defined chromosomal deficiencies to screen over half of the *Drosophila* genome for dominant suppressors or enhancers of CagA-induced epithelial disruption. Our deficiency screen identified 12 novel genetic interactors, capable of modulating the severity of CagA-induced disruption of the adult retinal epithelium. We refer to these genetic interactors collectively as the modifier of CagA (Moc) genes. Moc genes have been shown to function in numerous cellular pathways including those involved in maintenance of epithelial integrity, intracellular trafficking, and signal transduction. We further investigated CagA's genetic interactions with one Moc suppressor, the epithelial polarity determinant *coracle* that is the homolog of the mammalian 4.1 protein. In addition, we extended our genetic interaction network to show that other polarity determinants with antagonistic functions to *coracle* behave as dominant enhancers of CagA-associated epithelial phenotypes. The Moc genes provide new avenues of investigation toward understanding CagA's pathogenicity in humans.

MATERIALS AND METHODS

Drosophila STRAINS

All flies were raised on standard *Drosophila* media at 22°C unless otherwise noted. The *P{w[UAS-CagA]}* transgenic line was generated as described (Botham et al., 2008). Transgenes were expressed in the eye using *P{w[+mC] = GAL4-ninaE.GMR}12* [*GMR*, Bloomington Stock Center (BSC # 1104)]. Deficiency lines used for the initial identification of genomic regions were generated by Exelixis (Parks et al., 2004). The genetic null allele of *csw* (csw^{C114}) was obtained from Michael Simon (Stanford University). All other alleles used are described on FlyBase (Tweedie et al., 2009), including $EGFR^{t1}$ (FBst0002079), $par1^{k06323}$ (FBal0064446), $rho1^{72F}$ (FBst0007326), and the Moc genes listed in **Table 1**.

Moc GENETIC SCREEN

Males carrying a genetic deletion (generally spanning between 5 and 30 genes) on one chromosome and a visual marker such as *CyO* on the other were crossed to female virgins homozygous for the *GMR-GAL4* driver and *CagA*. Moc mutants were identified by comparing the overall eye roughness of adult flies carrying a deficiency to siblings that carried the visual marker by light microscopy. We screened 237 deficiency stocks covering 7451 genes, or 53% of *Drosophila* genes. We found a surprisingly high proportion—49%—of the deficiencies resulted in suppression, while only a few enhancers were identified (**Figure 1**). Particularly severe Moc mutants were chosen for further investigation in an attempt to identify a single gene responsible for the modification. Additional deficiencies overlapping the genetic region of interest were used to narrow the number of potentially responsible genes. Assuming that a single gene were responsible for the modification of the rough-eyed phenotype, genes within an overlapping deficiency that did not act as a Moc could be eliminated as candidates, while genes not included within deficiencies that acted as a Moc could also be eliminated. Once the number of candidate genes was sufficiently low, males carrying null alleles for candidate genes were crossed to *GMR-GAL4/GMR-GAL4*; *UAS-CagA/UAS-CagA* female virgins and the eyes of adult progeny were screened for modification of the rough-eyed phenotype. This method allowed for identification of a single Moc gene in 17 of the 22 initial Moc deficiencies that were chosen for analysis.

ENVIRONMENTAL SCANNING ELECTRON MICROSCOPY

To evaluate the CagA-induced eye phenotypes at higher resolution, we used environmental scanning electron microscopy (ESEM). Flies were anesthetized with FlyNap (Carolina Biological Supply Company) and imaged using an FEI Quanta 200 environmental scanning electron microscope. Images of at least 10 flies of each genotype were recorded and scored in a blinded fashion by five investigators. Scoring classes were defined as follows: (0) Geometric organization intact. (1) Loss of geometric organization, fewer than 25% of ommatidia fused or malformed. (2) Loss of geometric organization, greater than 25% of ommatidia fused or malformed. (3) Loss of geometric organization, greater than 25% of ommatidia fused, malformed, and greater than 1% but less than 25% of the eye lacks a recognizable morphology. (4) Loss of geometric organization, greater than 25% of ommatidia fused or malformed, and greater than 25% of the eye lacks a recognizable morphology. (5) Loss of geometric organization, greater than 25% of ommatidia fused and malformed, greater than 25% of the eye lacks a recognizable morphology, and pronounced invaginations on the eye surface.

IMMUNOHISTOCHEMISTRY

Eye discs were dissected from third instar larvae and fixed for 30 min (4% formaldehyde, 0.1 M PIPES (pH 6.9), 0.3% Triton

Table 1 | Moc genes identified in *Drosophila* adult eyes.

Gene[1]	Moc class[2]	Mutant allele[3]	Function[4]	Human homolog	References[5]
EPITHELIAL INTEGRITY					
Coracle	S	*cora*^{EY07598} (FBst0016848)	Septate junction polarity protein; epithelial polarity determinant	Protein 4.1	Laprise et al., 2009
Lasp	S	*Lasp*^{DG14505} (FBst0020424)	Actin binding protein; cell migration; RNA localization to cytoskeleton	Lasp1	Suyama et al., 2009
outspread	S	*osp*^{1} (FBst0001023)	Binds actin, RhoA, and myosin phosphatase	Myosin phosphatase-RhoA interacting protein	Surks et al., 2003; Mulder et al., 2004
moladietz	S	*mol*^{e02670} (FBst0018073)	Numb binding; asymmetric cell division	Numb-Interacting Protein/Dual oxidase maturation factor	Qin et al., 2004
gartenzwerg[6]	S	*garz*^{EP2028} (FBst0017017)	GEF for Arf; protein trafficking and epithelial morphogenesis	Arf1GEF	Szul et al., 2011
Epac	S	*Epac*^{f07038} (FBst0019033)	GEF for Rap1; E-cadherin mediated cell adhesion and eye development	RapGEF	Dupuy et al., 2005
INTRACELLULAR TRAFFICKING					
ranGAP	S	*RanGap*^{EP1173} (FBst0016995)	GAP for Ran; nuclear import and eye development	RanGAP1	Minakhina et al., 2005
unc104	E	*unc-104*^{R757} (FBst0024631)	Kinesin; organelle trafficking	Kinesin family member 1A	Klopfenstein et al., 2002
RECEPTOR TYROSINE KINASE SIGNALING					
disabled[7]	E	*Dab*^{EY10190} (FBst0016974)	Able kinase signaling antagonist; epithelial morphogenesis and vesicle trafficking	Disabled-1	Song et al., 2010; Kawasaki et al., 2011
Spitz	S	*spi*^{s3547} (FBst0010462)	Agonist of EGFR signaling; eye development	Neuregulin 1	Tio and Moses, 1997
NUCLEAR SIGNALING AND EYE DEVELOPMENT					
spalt major	S	*salm*^{1} (FBst0003274)	Transcription factor; eye development	Spalt-like zinc finger transcription factor	Domingos et al., 2004
CG5790	S	*CG5790*^{f04763} (FBst0018803)	Cell cycle kinase that promotes G1/S transition	CDC7 kinase	Grishina and Lattes, 2005
Rotund	S	*rn*$^{roe-1}$ (FBst0000572)	Transcription factor; eye development	Zinc finger transcription factor	St Pierre et al., 2002
String	S	*stg*^{04614b} (FBst0011382)	Cell cycle phosphatase that promoting G2/M transition; eye development	CDC25B phosphatase	Thomas et al., 1994
OTHER AND UNKNOWN FUNCTION					
chitinase-like	S	*CG30463*^{KG01703} (FBst0014380)	Putative chitinase	Chitinase 1	Zhu et al., 2004
CG13272	S	*CG13272*^{DG29412} (FBst0020510)	Unknown	None	
CG17141	S	*CG17141*^{f03838} (FBst0018700)	GTPase involved in mitochondrial translation	mitochondrial GTPase 1	Barrientos et al., 2003

[1] *Moc genes that were confirmed in the secondary ESEM screen are indicated in bold.*

[2] *Moc classes are Suppressors (S) and Enhancers (E).*

[3] *Drosophila mutant allele name (FlyBase strain number).*

[4] *Whenever possible, the biochemical and relevant cell or tissue function of the gene product are indicated.*

[5] *Most relevant references to the gene's function, and whenever possible to the gene's function in the Drosophila eye.*

[6] *Also belongs to intracellular trafficking class.*

[7] *Also belongs to epithelial integrity and intracellular trafficking classes.*

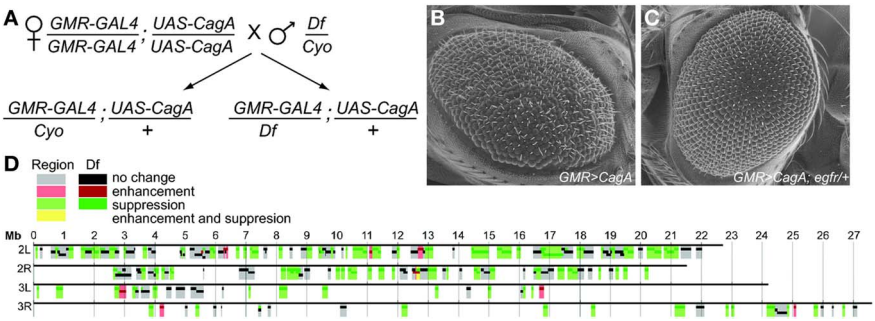

FIGURE 1 | (A) Crossing scheme for the Moc deficiency screen. Flies containing the genetic deficiency were compared to those containing a visual marker such as *CyO*. Flies expressing CagA in a wild-type, **(B)** or $egfr^{-/+}$ background, **(C)** were imaged by ESEM. **(D)** Chromosomal map of the genetic deficiency screen. The result from each deficiency (darker colors) is indicated along with the inferred functionality of each genetic region (lighter colors), where deficiencies that caused no change override those that cause enhancement or suppression.

X-100, 2 mM EGTA, 1 mM MgSO4), then washed (0.3% Triton X-100 in phosphate buffered saline, PBS) for 20 min and blocked for at least one hour in 1% bovine serum albumin and 0.3% Triton X-100 in PBS (PBSBT). Tissues were then incubated in primary antibody mouse anti-Dlg [4F3 (Developmental Studies Hybridoma Bank)], mouse anti-DCAD2 (DSHB), or mouse anti-HA (Covance) overnight at 1:100 in PBSBT. Tissues were rinsed for 1 h in PBSBT, then incubated with anti-mouse conjugated Cy3 (Jackson ImmunoResearch) at 1:200. Imaginal discs were mounted in VectaShield (Vector Laboratories) and visualized with a Nikon TE2000 U with C1 Digital Eclipse confocal microscope.

EVALUATION OF LARVAL RETINAL EPITHELIAL MORPHOLOGY

Z-stacks of eye discs stained for the septate junction component Dlg were generated using a 0.2 μm step size and compiled in ImageJ. The areas chosen for Z-stacks were ∼1 mm^2 in area and devoid of ectopic furrows. The intensity of fluorescence 4.8 microns below the peak intensity was taken to represent the relative integrity of the epithelium, with higher values representing a more disrupted tissue. Intensities were normalized to the maximum density in the Z-stack to generate the final metric.

RESULTS

A DEFICIENCY SCREEN FOR MODIFIERS OF CagA-INDUCED ADULT EYE DEFECTS

In this study, we screened transgenic *Drosophila* expressing the *H. pylori cagA* gene for dominant modifiers of CagA-induced epithelial disruption. We had shown previously that CagA expression in the developing eye results in a rough eye phenotype that is easily detected using a dissecting microscope and that is sensitive to dosage, with expression of two copies of *cagA* resulting in a much more severe disruption of the adult structure than a single copy (Botham et al., 2008). We used the Gal4 transcription factor under the GMR promoter to drive expression of a *UAS-CagA* transgene in the developing eye beginning in the larval eye imaginal disc as photoreceptors are first being specified. GMR-Gal4 expression is maintained in the eye primordia throughout subsequent development and into adulthood.

For our genetic screen, we crossed homozygous *GMR-GAL4*; *UAS-CagA* females to males carrying molecularly defined chromosomal deletions maintained over a balancer chromosome with a dominant marker, such as the *CyO* balancer on the second chromosome (**Figure 1A**). Half of the resulting progeny would contain the deficiency and could be compared to the other half with the marker to look for enhancement or suppression of the CagA-induced rough eye phenotype.

To assess the feasibility of this genetic screening strategy, we tested whether deletion of single copies of genes encoding known genetic interactors of CagA would modify the CagA-associated phenotype. CagA is a potent activator of RTK pathway signaling in tissue culture cells (Backert et al., 2010). In the *Drosophila* eye, EGF receptor is a critical RTK required for multiple steps during development (Dominguez et al., 1998). We, therefore, asked whether reducing RTK signaling by removing a single copy of the *egfr* gene would reduce the severity of the CagA-induced rough eye phenotype. As predicted, the severity of eye disruption was significantly reduced in $egfr^{-/+}$ flies expressing CagA as compared to CagA-expressing control flies (**Figures 1B,C**). This demonstrated that it is possible to genetically suppress CagA's disruption of the *Drosophila* adult eye, thus motivating us to use this system for an unbiased genetic screen for Moc genes.

We took advantage of a publicly available collection of *Drosophila* stocks containing deficiencies in defined genomic regions (Parks et al., 2004) to systematically search for chromosomal regions that modify CagA's disruption of the epithelium. Using this collection, we tested 237 stocks with genomic deletions for their ability to modify the CagA-induced rough eye phenotype. Combined, this collection covered 7451 genes, or approximately 53% of all *Drosophila* genes (**Figure 1D**).

From this initial panel of deficiency stocks, 22 chromosomal regions were identified that modified CagA's disruption of the eye epithelium with high expressivity and penetrance. To identify the individual genes responsible for the modification of CagA's activity, CagA-expressing flies were subsequently crossed to fly stocks containing smaller deficiencies located within the 22 chromosomal regions identified in the initial screen to narrow the number of candidate Moc genes. Once we whittled the number of

candidates down to 5–10 genes, we obtained all available strains with mutations in the candidate genes within the interval to test for their ability to modify the rough-eyed phenotype. This method allowed us to identify a single Moc gene in 17 of the 22 initial Moc intervals. These 17 genes are listed in **Table 1**. Moc genes fit broadly into the functional classes of epithelial integrity, intracellular trafficking, signal transduction, and nuclear signaling, with three additional genes of miscellaneous or unknown function.

A SECONDARY SCREEN FOR STRONG MODIFIERS OF THE CagA-INDUCED PHENOTYPE

To assess the degree of modification caused by the identified modifiers, we used ESEM to obtain high-resolution images of adult eyes from multiple individuals expressing each of the Moc genes in the *GMR>CagA* background. At this high resolution, we saw that CagA expression induced mispolarized and supernumerary bristles, fusion of ommatidia, and in the most severe instances, loss of apparent ommatidial patterning and the development of large invaginations in the epithelium. From our large data set of images, we were able to discern a continuum of severity and develop a scoring system that enabled us to quantify the rough eye phenotype (**Figures 2A–F**). Eyes scoring 0 resembled wild-type flies and had no apparent sign of disruption. In contrast, eyes scoring 5 were the most severely disrupted. Eyes scoring 1–4 had intermediate levels of disruption. For a complete description of the scoring system, see Materials and Methods.

For each of the Moc genes, at least 10 ESEM images of adult eyes from different flies were scored in a blinded fashion by five investigators, and the average score was tallied. From this analysis, we verified that 12 of the 17 genes were bona fide modifiers of CagA-induced eye disruption (**Figure 2G** and **Table 1**). Two of these behaved as enhancers and 10 were suppressors. The most abundant functional group among these 12 genes was the epithelial integrity class.

To calibrate the effects of the validated Moc genes, we quantified the ability of four known CagA signaling modulators to modify the CagA-induced eye phenotype: *egfr* (Keates et al., 2007; Bauer et al., 2009), *csw* (the homologue of SHP-2) (Higashi et al., 2002), *rhoA* (Muyskens and Guillemin, 2011), and *par1* (Saadat et al., 2007). Of these, *egfr* proved to be the most potent suppressor of CagA-induced disruption. Loss of single copies of both *csw* and *rhoA* caused suppression, whereas loss of a single copy of *par1* did not cause a significant modification of the CagA rough eye phenotype (**Figure 2G**). This analysis demonstrated that the 12 newly identified Moc genes had similar or stronger effects on the CagA-induced phenotype than known CagA signaling modulators.

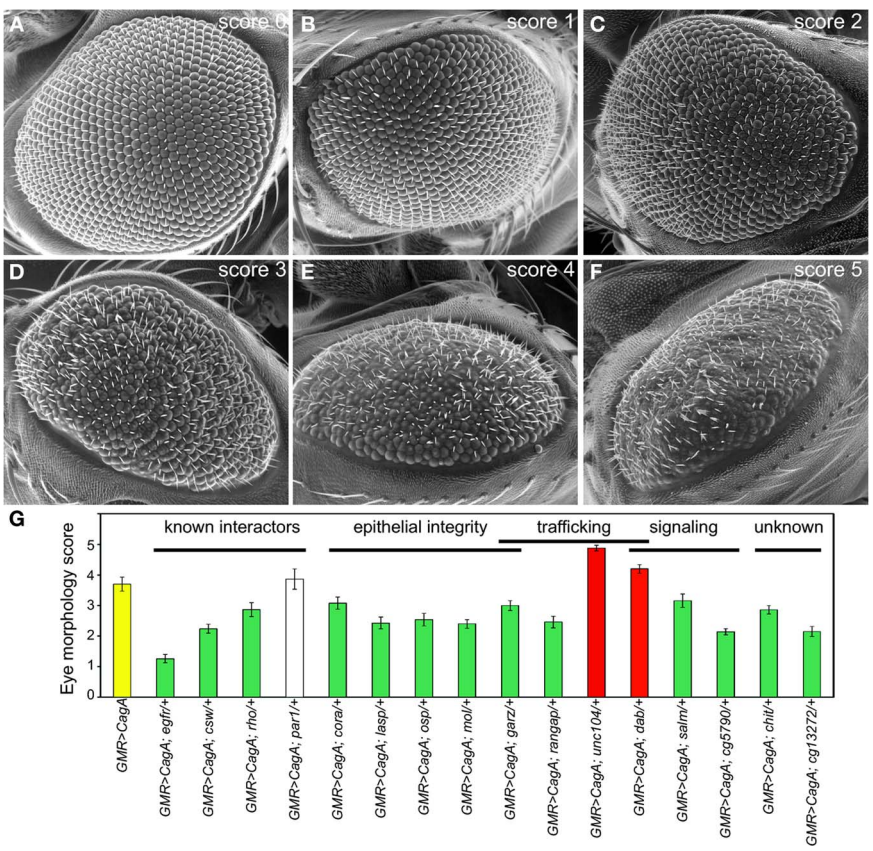

FIGURE 2 | (A–F) Representative ESEM images for each class of disruption by CagA. The scoring rubric is described in Materials and Methods. **(G)** The mean ESEM-based eye disruption for CagA, known interactors and Moc genes of different functional classes. Error bars represent standard error.

MODIFICATION OF CagA-INDUCED DISRUPTION OF THE LARVAL RETINAL EPITHELIUM

Our Moc screen identified genetic modifiers of the CagA phenotype in the adult eye. We had previously reported that CagA expression with the GMR driver induces profound disruption of the morphogenesis of the larval retinal epithelium shortly after initiation of CagA expression (Muyskens and Guillemin, 2011). We showed that by overactivating Rho and non-muscle myosin in the larval epithelium, CagA causes ectopic furrowing of the epithelial sheet. Because so many of the Moc genes were implicated in epithelial integrity, we wished to determine whether any of them might modify CagA's effects at these early stages of epithelial disruption. We decided to focus on Coracle (Cora), because it had the best characterized function as an epithelial polarity determinant (Laprise et al., 2009, 2010).

To measure the integrity of the larval retinal epithelium, we stained the tissue with an antibody against Discs large (Dlg), a component of the septate junction, the invertebrate cell junction that is structurally homologous to the chordate tight junction. We used laser scanning confocal microscopy to image from the apical to basal poles a region of epithelium devoid of obvious ectopic furrows. We quantified the intensity of the Dlg signal as a function of depth from the apical surface. Maximal Dlg signal was just below the apical epithelial surface at the septate junction. When we compared the relative intensity of Dlg signal below this maximal point, we found that the CagA-expressing discs had significantly more Dlg signal at deeper positions relative to the GMR control discs (**Figure 3A**). For our further analysis, we quantified the relative Dlg intensity 4.8 microns below the point of peak Dlg intensity (arrow in **Figure 3A**), the point at which we observed the maximum difference between CagA-expressing and control larval retinal epithelia.

We used this method to analyze the Dlg distribution in CagA-expressing larval eye discs lacking a single copy of a Moc gene. We first tested the consequence of depleting the CagA interactor and junctional protein Par1. Loss of a single copy of *par1* caused a slight expansion of Dlg staining (**Figure 3B**). We also tested the consequence of depleting Cora, which is normally localized to the septate junctions. Loss of a single copy of *cora* had no effect on the distribution of Dlg at the septate junctions (**Figure 3B**). We then analyzed the Dlg distribution when these genes were deleted in the presence of CagA. In the larval epithelium, *par1* behaved as a dominant enhancer of the CagA-associated disruption in Dlg protein distribution (**Figure 3B**), despite having no effect on the adult eye phenotype caused by CagA. In contrast, *cora* behaved as a dominant suppressor of the CagA phenotype in the larval epithelium, as it had done in the adult eye (**Figures 2G,3B**). The dominant enhancement of the CagA-induced epithelial disruption by *par1* could be explained as the further impairment of a compromised tissue through the depletion of a junctional component. Less obvious was the mechanism by which *cora* depletion suppressed the CagA phenotype, which we sought to understand with further experiments.

CORA REDUCTION SUPPRESSES CagA-INDUCED EPITHELIAL DISORGANIZATION BUT NOT CagA PROTEIN LOCALIZATION TO SEPTATE JUNCTIONS

Our finding that Dlg protein extended deeper from the apical surface in the CagA-expressing epithelium as compared to wild-type tissue could arise from multiple mechanisms. Two possible mechanisms are illustrated in **Figures 4A–C**. In the first model, CagA-expression could cause a loss of junctional integrity and expansion of Dlg protein toward the basal end of the cell (**Figure 4B**). Alternatively, CagA could cause disorganization of the epithelial sheet, resulting in a broader zone of Dlg expression when averaged across multiple cells (**Figure 4C**). To distinguish these possibilities, we examined the organization of the larval epithelium and cell junctions at high resolution. We co-stained larval retinal epithelia for both the septate junction marker Dlg and the adherens junction marker E-cadherin (E-cad). In *Drosophila* epithelia, the adherens junction is apical to the septate junction, and in the larval retinal epithelium these junctions are located at the apices of photoreceptors and supporting cells that surround the ommatidia (**Figure 4D**). In CagA-expressing eye imaginal disc epithelia, the zone of Dlg expression was frequently found to extend deeper from the apical surface. In some instances this

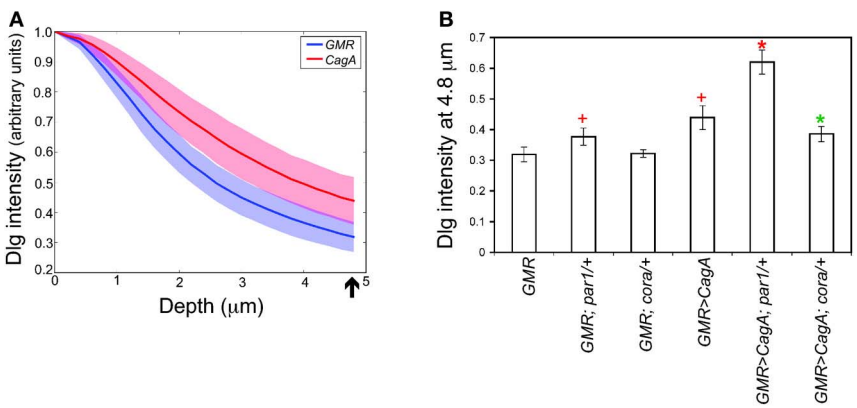

FIGURE 3 | (A) Pattern of Dlg staining in Z-stacks of larval retinal imaginal discs. CagA-expressing eye discs are compared to those expressing the GMR-Gal4 driver alone. Shaded areas represent standard error. The arrow indicates the point at 4.8 microns below the peak intensity where the distribution was evaluated. **(B)** Quantification of larval retinal epithelial morphology for *par1* and *cora* mutants, and their interactions with CagA.

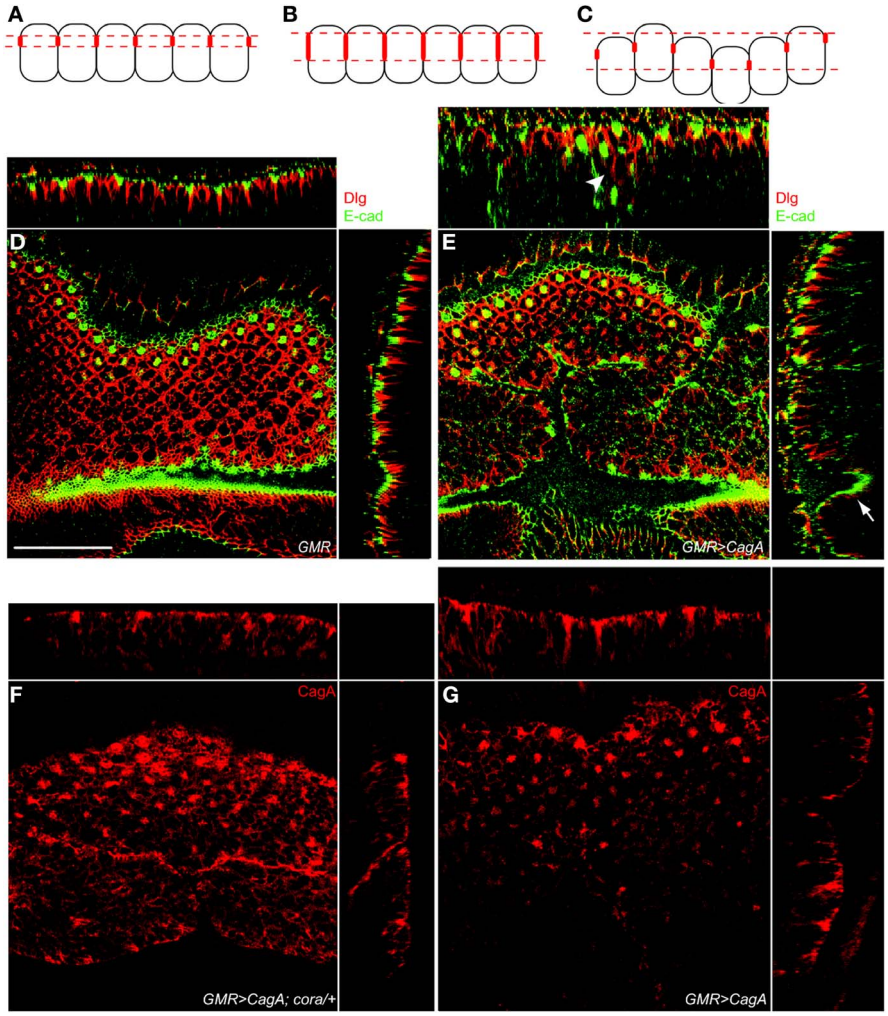

FIGURE 4 | ***cora*** **reduction suppresses CagA-induced epithelial disorganization but not CagA protein localization to septate junctions. (A–C)** Model for the basal displacement of Dlg. Panel A represents the wild-type distribution of Dlg (represented as red structures on the lateral membranes of the epithelial cells). Panel B represents basally expanded Dlg expression due to expansion within individual cells. Panel C shows how epithelial disruption can cause basal mispositioning of Dlg expression by positioning cells deeper within the epithelium. **(D)** Control larval retinal epithelium (*GMR-Gal4*) stained with Dlg (red) and E-cad (green). YZ and XZ orthogonal planes are shown on the side and top, respectively, in **D** and **E**. Scale bar is 30 microns for all panels. **(E)** CagA-expressing larval retinal epithelium (*GMR-Gal4; UAS-CagA*) also stained with Dlg (red) and E-cad (green). Arrowhead in the upper orthogonal section shows basally mispositioned Dlg staining. Arrow indicates Dlg staining that is deep within the epithelium due to irregularities in the epithelial sheet. **(F)** $cora^{+/-}$ larval retinal epithelium expressing CagA (*GMR-Gal4; UAS-CagA*) showing CagA localization as labeled with anti-HA. Apical HA puncta are present. **(G)** A larval retinal disc expressing CagA (*GMR-Gal4; UAS-CagA*) labeled with HA antibody.

appeared to be due to more disorganized junctions (arrowhead in **Figure 4E**), but frequently the integrity of the junctions looked normal and the Dlg staining was displaced deeper into the tissue due to irregularities in the epithelial sheet (arrow in **Figure 4E**). Because the integrity of Dlg and E-cad staining looked mostly normal in the CagA-expressing larval retinal epithelia, we concluded that loss of Cora suppresses CagA-associated phenotypes in this tissue by reducing the overall disorganization of the epithelial sheet.

We had previously shown that CagA is localized to the apical junctional structures in the larval retinal epithelium and that a CagA mutant that fails to localize in this manner is a less potent disruptor of epithelial integrity (Muyskens and Guillemin, 2011). We wondered if loss of a single copy of *cora* could disrupt the localization of CagA to the apical cell junctions. We visualized CagA distribution using an HA epitope tag we had engineered into the protein. As we previously reported, we found that CagA was enriched in apical foci of CagA-expressing eye discs (**Figure 4F**). We found that this expression pattern was not different in eye discs lacking a single copy of *cora*, (**Figure 4G**). Therefore, *cora*'s ability to suppress CagA-induced eye morphology does not appear to be due to failure of CagA protein to localize to apical cell junctions in the absence of one copy of *cora*.

EPITHELIAL POLARITY DETERMINANTS MODIFY CagA-INDUCED DISRUPTION OF THE LARVAL RETINAL EPITHELIUM

To further explore the basis for *cora* suppression of CagA-induced larval retinal epithelial disorganization, we tested other epithelial polarity determinants for their ability to modify the CagA-induced larval retinal epithelium phenotype. Polarity in many epithelial tissues of both *Drosophila* and mammals is established and maintained by four conserved groups of polarity determinants: the apically localized Crumbs (Crb) group and three functionally distinct basolaterally distributed groups with defining members Cora, Scribble (Scrib), and Par1 (Laprise and Tepass, 2011). In contrast to *cora*, and similar to *par1*, *crb,* and *scrib* behaved as dominant enhancers of the CagA-induced larval epithelial disruption (**Figure 5A**), whereas loss of a single copy of these genes caused no epithelial disruption on their own (data not shown). To ask whether all Cora group members behaved as suppressors of CagA, we tested another Cora group member, Na, K-ATPase (encoded by the *atpα* gene). Unlike *cora*, *atpα* behaved as a dominant enhancer of CagA in the retinal epithelium (**Figure 5A**), and had no effect when depleted in the absence of CagA (data not shown). Cora and Crb mutually inhibit each other's activities in many epithelial structures (Laprise et al., 2009, 2010; Laprise and Tepass, 2011). We therefore asked whether overexpression of Crb would have the same effect as loss of Cora. Using a *UAS-crb* construct, we were able to achieve a significant suppression of CagA-induced epithelial disorganization, as measured by the basal distribution of Dlg (**Figure 5A**).

DISCUSSION

We have demonstrated that a transgenic *Drosophila* model can be used to identify conserved genes that modulate the effects of a virulence factor from a human pathogen. We show that CagA-induced perturbation of the *Drosophila* adult eye is a sensitive read-out for identification of genes that can alter CagA's ability to disrupt this tissue. Our approach is reductionist in that it characterizes the bacterial effector in isolation from other aspects of the infection process, such as immune responses to the bacteria and cellular interaction with the type IV secretion system that normally delivers CagA. The potential utility of the screen is limited by the extent of conservation between *Drosophila* and human genes and by the functional similarity between retinal and gastric epithelia. Nonetheless, we found that when depleted by one copy, genes encoding three known effectors of CagA, EGFR, Csw (SHP-2), and RhoA, significantly suppressed the eye morphological defects caused by CagA expression. This validated our approach to screen for dominant modifiers of CagA in this tissue. In our F1 screen we surveyed over half the *Drosophila* genome and identified 17 Moc genes, 12 of which we confirmed by high resolution ESEM.

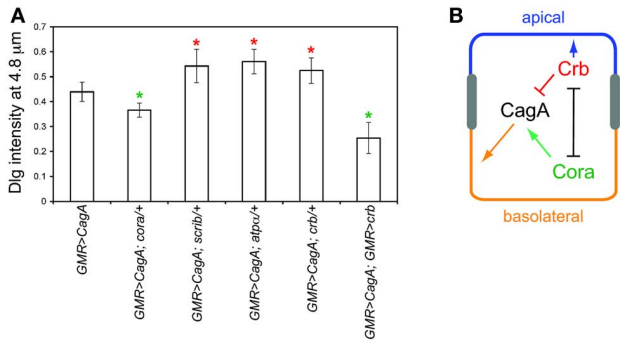

FIGURE 5 | (A) Interactions of several epithelial polarity determinants with CagA, using the Dlg distribution assay described in **Figure 3**. Error bars represent standard error. Green asterisks represent mutants that significantly suppress CagA-induced epithelial disruption; red asterisks represent mutants that significantly enhance epithelial disruption ($p < 0.05$). **(B)** Model for the interactions of CagA with epithelial polarity determinants Cora and Crb.

Across the list of Moc genes, several themes of cellular and biochemical functions emerge. Eight of the 17 genes have known roles in epithelial integrity, including interactions with polarity determinants (*coracle* and *moladietz*) and the actin cytoskeleton (*lasp* and *outspread*). Three function in protein or organelle trafficking (*gartenzwert, ranGAP*, and *unc104*) with known or suspected roles in epithelial organization. Indeed, the ArfGEF, *gartenzwerz*, which is required for normal protein trafficking and morphogenesis of the *Drosophila* salivary gland epithelium (Szul et al., 2011), exemplified a growing appreciation of the connection between epithelial polarity and intracellular trafficking (Shivas et al., 2010). A frequent biochemical function among the Moc genes is interaction with GTPases or GTPase activity (*outspread*, *gartenzwerz, epac*, *ranGAP*, *CG17141*), which is interesting in light of the fact that we have shown that CagA's disruption of the larval retinal epithelium is due in part to excessive RhoA signaling (Muyskens and Guillemin, 2011). Another theme among the Moc genes is signal transduction and nuclear signaling, including two zinc finger transcription factors (*spalt major* and *rotund*) and two cell cycle regulators (homologs of CDC25B and CDC7). An additional signaling Moc, *disabled*, is an antagonist of Abl kinase (Song et al., 2010). In gastric epithelial cells, CagA has been shown to activate Abl and subsequently be phosphorylated by this kinase, resulting in enhanced CagA-mediated signaling, including RTK-dependent cell elongation (Tammer et al., 2007). Consistent with its molecular function as an inhibitor of Abl, loss of one copy of *disabled* results in enhancement of the CagA-mediated rough eye phenotype, the opposite effect of reduction of *egfr*, or the EGFR ligand, *spitz*. *disabled* has also been shown to be required for normal epithelial morphogenesis in *Drosophila* (Song et al., 2010) and to function in vesicle trafficking (Kawasaki et al., 2011), thereby linking the functions of RTK signaling, epithelial morphogenesis, and intracellular trafficking that run throughout the Moc list.

Within this group of modifiers, we focused our attention on *cora* because of its previously characterized role in epithelial polarity and septate junction regulation. The sepatate junction and its mammalian equivalent, the tight junction, regulate paracellular flux across epithelia. In the gastrointestinal tract, tight junctions are often targeted by enteric pathogens for invasion of deeper tissues or access to nutrients (Vogelmann et al., 2004). CagA has been shown to alter the distribution of the tight junction component ZO-1 and, over extended periods of time, impair tight junction integrity in *H. pylori*-infected cultured epithelial

cells (Amieva et al., 2003). However, even under conditions when tight junctions remain intact, CagA confers on *H. pylori* the ability to replicate in the nutrient-poor environment of the epithelial apical surface (Tan et al., 2009). This CagA-mediated adaptation involves disruption of apical-basal polarity and expansion of basolateral markers to the apical surface (Tan et al., 2011).

We found that depletion of *cora* suppressed CagA-induced disruption of the larval retinal epithelium, but not by a perceptible change to the organization of the cell junctions or the localization of CagA to these structures. Intriguingly we found that overexpression of *crb* resulted in the same phenotypic suppression achieved by depletion of *cora*. Cora and Crb have mutually antagonistic activities, and in the absence of Cora, Crb will promote expansion of apical cell surfaces within the epithelium (Laprise et al., 2006, 2009). We hypothesize that this activity of Crb counteracts CagA's ability to promote more basolateral cell surface identities (**Figure 5B**). Thus, over-expressing Crb, or depleting its inhibitor, Cora, achieves a more balanced pull between apical promoting forces from Crb and basolateral promoting forces from CagA that is manifest as more normal epithelial organization in the CagA-expressing retinal epithelium in these genetic backgrounds.

In summary, our genetic screen has identified a number of host signaling pathways that modulate CagA's potency in disrupting host tissue. Further analysis of these Moc genes should lead new insights into CagA's mechanism of action in host tissue and may yield new strategies for pharmaceutical modulation of these pathways to treat *H. pylori*-associated pathologies.

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Remodeling the host environment: modulation of the gastric epithelium by the *Helicobacter pylori* vacuolating toxin (VacA)

Ik-Jung Kim and Steven R. Blanke*

Department of Microbiology, Institute for Genomic Biology, University of Illinois, Urbana, IL, USA

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Richard Peek, Vanderbilt University Medical Center, USA
Timothy Cover, Vanderbilt University School of Medicine, USA

***Correspondence:**
Steven R. Blanke, Department of Microbiology, Institute for Genomic Biology, University of Illinois, 302A Burrill Hall, 601 S. Goodwin Ave., Urbana, IL 61801, USA.
e-mail: sblanke@illinois.edu

Virulence mechanisms underlying *Helicobacter pylori* persistence and disease remain poorly understood, in part, because the factors underlying disease risk are multifactorial and complex. Among the bacterial factors that contribute to the cumulative pathophysiology associated with *H. pylori* infections, the vacuolating cytotoxin (VacA) is one of the most important. Analogous to a number of *H. pylori* genes, the *vacA* gene exhibits allelic mosaicism, and human epidemiological studies have revealed that several families of toxin alleles are predictive of more severe disease. Animal model studies suggest that VacA may contribute to pathogenesis in several ways. VacA functions as an intracellular-acting protein exotoxin. However, VacA does not fit the current prototype of AB intracellular-acting bacterial toxins, which elaborate modulatory effects through the action of an enzymatic domain translocated inside host cells. Rather, VacA may represent an alternative prototype for AB intracellular acting toxins that modulate cellular homeostasis by forming ion-conducting intracellular membrane channels. Although VacA seems to form channels in several different membranes, one of the most important target sites is the mitochondrial inner membrane. VacA apparently take advantage of an unusual intracellular trafficking pathway to mitochondria, where the toxin is imported and depolarizes the inner membrane to disrupt mitochondrial dynamics and cellular energy homeostasis as a mechanism for engaging the apoptotic machinery within host cells. VacA remodeling of the gastric environment appears to be fine-tuned through the action of the Type IV effector protein CagA which, in part, limits the cytotoxic effects of VacA in cells colonized by *H. pylori*.

Keywords: *Helicobacter pylori*, VacA, vacuolation, mitochondria, apoptosis

Helicobacter pylori AND VacA

Virulence mechanisms underlying *Helicobacter pylori*-mediated gastric maladies have remained enigmatic, in part, because disease risk is multifactorial and complex (Compare et al., 2010; Polk and Peek, 2010). However, several bacterial factors clearly contribute to the pathophysiology associated with *H. pylori* infections (Fischer et al., 2009), and one of the most important is the vacuolating cytotoxin (VacA) (Cover and Blanke, 2005).

Since the discovery of VacA nearly 25 years ago as the proteinacious factor within *H. pylori* culture filtrates that intoxicates epithelial cells and induces vacuole biogenesis (Leunk et al., 1988), the study of this toxin has been challenging in part, because the toxin possesses a number of surprising and unusual characteristics that don't fit neatly into current concepts of bacterial toxins. Nonetheless, several interesting and important properties of VacA have become apparent. First, the gene encoding VacA (*vacA*) is characterized by a high degree of genetic variation; strains with specific allelic variants of *vacA* that exhibit greater levels of VacA-mediated cytotoxic activity *in vitro* are associated with a greater risk of gastric disease in *H. pylori*-infected humans. Moreover, experimental evidence supports the idea that VacA may promote *H. pylori* colonization, persistence, and infection-associated disease pathophysiology.

STRUCTURAL PROPERTIES OF VacA

H. pylori synthesize VacA as an approximately 140 kDa pre-protoxin (**Figure 1**), which undergoes sequential proteolytic processing during Type Va secretion as an auto-transporter protein (Fischer et al., 2001). The secreted mature form of VacA is a 88 kDa monomer (Cover and Blaser, 1992), that is purified from *H. pylori* growth medium (Gonzalez-Rivera et al., 2010) as water-soluble hexameric or heptameric rings (**Figure 1**) in single or bilayered structures (**Figure 1**) (Lupetti et al., 1996; Cover et al., 1997; Lanzavecchia et al., 1998; Czajkowsky et al., 1999; Adrian et al., 2002; El-Bez et al., 2005). Acidic or alkaline pH promotes dissociation of VacA oligomeric complexes into monomers (Cover et al., 1997; Molinari et al., 1997; Yahiro et al., 1997), which is likely the form of the toxin to bind host cells during infection (Gonzalez-Rivera et al., 2010).

The mature 88 kDa form of the toxin is sometimes detected as a proteolytically-nicked protein, comprising two domains designated p33 (residues 1–311) and p55 (residues 312–821), that

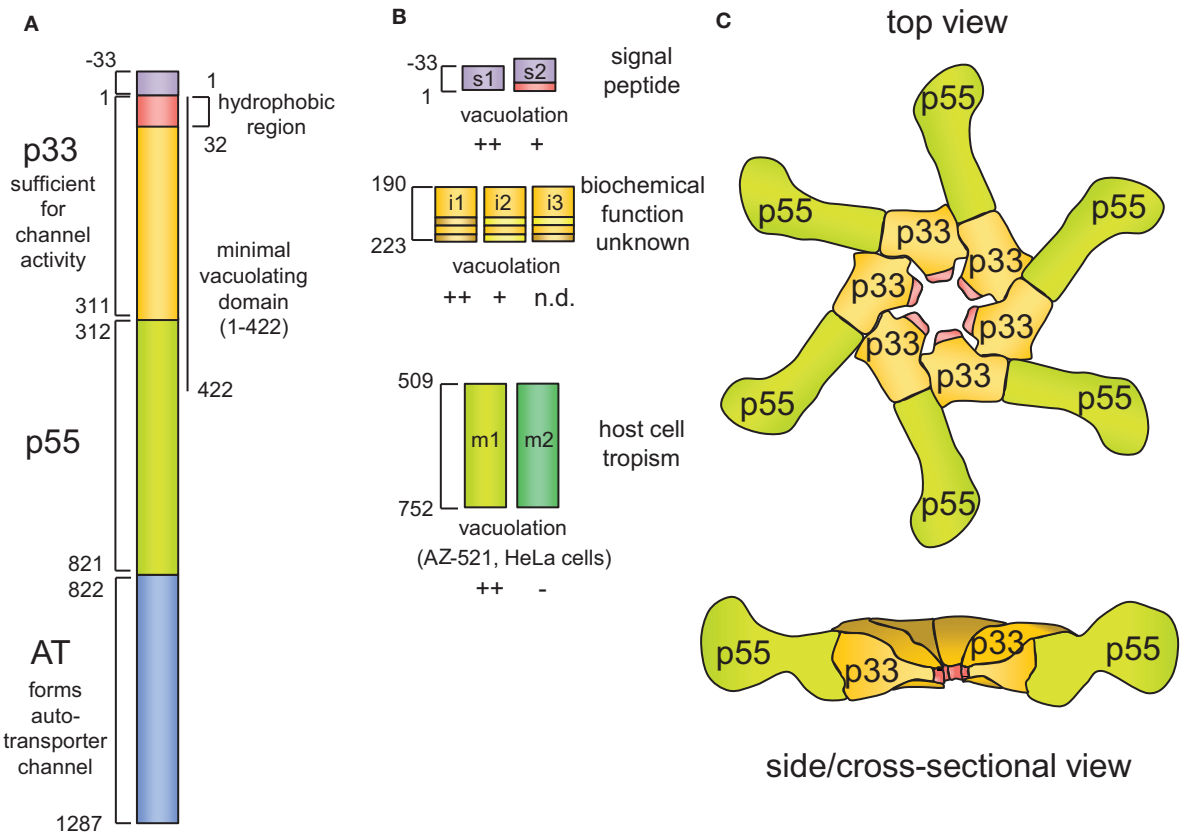

FIGURE 1 | VacA structure. (A) Schematic VacA structure. Each domain is denoted by a different color and by the first and last amino residue of that particular domain. The name of each domain is denoted in bold, and its function (if known) is described. **(B)** The polymorphic nature of the *vacA* gene is emphasized by highlighting the three major allele families, which are located in the signal region (s region), the intermediate region (i region), and the mid-region (m region). **(C)** The proposed structure of the VacA oligomeric assembly, based on the crystal structure of a portion of p55 [Gangwer et al. (2007)] and electron microscopy imaging of VacA oligomers [El-Bez et al. (2005)].

remain non-covalently associated (**Figure 1A**) (Telford et al., 1994; Cover et al., 1997; Ye et al., 1999; Nguyen et al., 2001; Willhite et al., 2002; Torres et al., 2004, 2005). Recently, a crystal has been solved for p55 (Gangwer et al., 2007), revealing a predominantly right-handed parallel beta-helix structure, which is typical for autotransporter passenger domains. High-resolution structural data for p33 are not yet available. Proteolytic cleavage into discrete functional domains is a characteristic of a number of so-called intracellular-acting AB toxins (Blanke, 2005). However, proteolytic cleavage of VacA into p33 and p55 is apparently not required for VacA activity (Burroni et al., 1998). While neither p33 nor p55 alone are sufficient to induce vacuole biogenesis, the cellular activity of VacA can be reconstituted from two separate recombinant proteins added exogenously (Gonzalez-Rivera et al., 2010) or expressed ectopically within cultured cells (Ye et al., 1999; Ye and Blanke, 2000; Willhite et al., 2002; Ye and Blanke, 2002). All of p33 and approximately the amino-terminal 100 residues of p55 are apparently required to induce vacuolation, revealed when a fragment comprising VacA residues 1–422, but not a fragment comprising residues 1–394, induced cellular vacuolation when expressed in transiently transfected cells (de Bernard et al., 1997, 1998; Ye et al., 1999).

The association of p33 or p55 with discrete toxin functions remains poorly understood. Several lines of evidence suggest that p55 possesses a domain important for binding the toxin to the cell surface (Pagliaccia et al., 1998; Reyrat et al., 1999; Wang and Wang, 2000; Wang et al., 2001). However, the binding of p55 to cells appears to be considerably weaker than that of full-length VacA. Moreover, p33 appears to also contribute to VacA cell surface binding (Torres et al., 2005; Gonzalez-Rivera et al., 2010).

The amino-terminal 34 residues of p33 domain comprise what is predicted to be a highly hydrophobic domain, which has been implicated to be critical for the capacity of VacA to form ion-conducting channels (Vinion-Dubiel et al., 1999; Ye and Blanke, 2000; McClain et al., 2003). As discussed below, VacA channels are probably the biochemical activity most critical for the cellular modulating activity of the toxin. However, deletion of part of this region (residues 1–27) resulted in a mutant form of VacA that also demonstrated some channel activity in planar lipid bilayers, albeit requiring a significantly longer time to for channel formation (Vinion-Dubiel et al., 1999), suggesting that the hydrophobic amino-terminus is not absolutely required for VacA channel activity. More recently, channel activity in planar lipid

bilayers was also demonstrated for a mutant form of p33 lacking residues 1–37 (Domanska et al., 2010). Thus, the molecular basis underlying VacA channel formation, at least in artificial lipid bilayers, remains to be clarified.

INTRACELLULAR MEMBRANE CHANNEL FORMATION BY VacA: THE TRUE BIOCHEMICAL ACTIVITY OF VacA?

Analogous to many other bacterial toxins, VacA is able to insert into membranes and form ion-conducting channels (Czajkowsky et al., 1999, 2005; Tombola et al., 1999a,b, 2001b). Characterization of VacA channels has revealed slight anion selectivity (Czajkowsky et al., 1999; Iwamoto et al., 1999; Tombola et al., 1999a,b, 2001b) and several electrophysiological properties that are similar to some anion-selective channels found in human cells (Campello et al., 2002; Czajkowsky et al., 2005). Electron microscopy images of membrane-associated VacA have revealed primarily hexagonal ring-shaped structures (Czajkowsky et al., 1999; Adrian et al., 2002; Geisse et al., 2004) that likely correspond to VacA channels or a pre-channel form of the toxin, although direct evidence is lacking for both of these possibilities. Based on studies indicating that both VacA binding to lipid membranes (Czajkowsky et al., 1999) and channel formation (Czajkowsky et al., 1999; Iwamoto et al., 1999; Geisse et al., 2004) are enhanced by acid-activation of the toxin support a model that VacA binds to the membrane surface as a monomer, and subsequently undergoes assembly into higher-ordered structures and membrane insertion.

VacA channel activity has been associated with a number of the toxin's cellular modulatory effects. The exact nature of cell modulation is apparently dependent on the site of channel formation. The release of cellular ions and small organic molecules has been attributed to VacA channel formation at the plasma membrane (Szabo et al., 1999; Tombola et al., 2001a). Cellular vacuolation is been ascribed to VacA channels within endocytic compartments (Montecucco and Rappuoli, 2001). VacA channel formation within the inner membranes of mitochondria (Galmiche et al., 2000; Willhite and Blanke, 2004; Calore et al., 2010; Domanska et al., 2010) is believed to be the biochemical activity of the toxin responsible for depolarization of the inner membrane (Kimura et al., 1999; Willhite and Blanke, 2004) and disruption of mitochondrial dynamics, ultimately responsible for inducing cell death (Jain et al., 2011).

THE RELATIONSHIP BETWEEN *vacA* GENETIC DIVERSITY AND DISEASE

Understanding the importance of VacA for *H. pylori*-mediated disease is not as straightforward as demonstrating the correlation between the presence or absence of the *vacA* gene in human isolates and the incidence of gastric disease. Indeed, the very earliest studies revealed that the *vacA* gene is present in essentially all human isolates. Further study revealed in short order that not all *vacA* genes are identical.

THE ALLELIC DIVERSITY OF *vacA*

Although essentially all *H. pylori* strains carried a *vacA* gene, the levels of vacuolating activity with bacterial culture filtrates were found to be markedly different from strain to strain (Leunk et al., 1988; Figura et al., 1989; Cover et al., 1990, 1993; Cover and Blaser, 1992). Several reasons underlying the diversity in vacuolating activity among *H. pylori* strains were discovered. *H. pylori* isolates were identified that carried non-sense mutations, internal duplications, deletions, or 1 bp insertions within the *vacA* gene (Ito et al., 1998). Differences in the transcription of *vacA* or the efficiency in VacA secretion have also been found to influence the levels of vacuolating activity with *H. pylori* culture filtrates (Forsyth et al., 1998). Perhaps the most important reason, however, for differences in vacuolating activity between differences in strains is variation discovered in the VacA amino acid sequences (Atherton et al., 1995), which subsequently have been associated with the observed divergence in cellular vacuolating activity (**Figure 1A**) (Atherton et al., 1995; Strobel et al., 1998; van Doorn et al., 1998). The *vacA* genotype is recognized as an important determinant of the toxin' cellular activity.

The maximum amount of sequence diversity is found in several defined regions of *vacA* (**Figure 1A**). The middle of *vacA*, which was named the "m (for middle) region," encodes an approximately 800 bp region in the carboxyl-terminal p55 domain of VacA. Within the m region, the two primary allelic families that are differentiated among strains are called m1 and m2 (Atherton et al., 1995, 1999; Pagliaccia et al., 1998; Ji et al., 2002). Sequence diversity within the m-alleles of *vacA* has been functionally associated with differences in cell tropism between VacA proteins (Pagliaccia et al., 1998; Ji et al., 2000; Wang et al., 2001). There is some evidence to indicate that the differences in cell type specificities demonstrated for the s1/m1 and s1/m2 forms of VacA may be due to, at least in part to distinct cell binding properties (Pagliaccia et al., 1998; Ji et al., 2000; Wang et al., 2001). The VacA determinants that influence the cell-type specificities demonstrated for the m1 and m2 forms of VacA have been mapped to a 148-residue region within p55 (Ji et al., 2000; Skibinski et al., 2006). However, the entire m-region is apparently essential for cell surface binding and vacuolating activity of the toxin (Wang and Wang, 2000; Skibinski et al., 2006).

The amino-terminal end of VacA, designated as the "s region," also demonstrates considerable sequence diversity, which extends from the signal sequence that directs secretion across the bacterial inner membrane into the amino terminus of the processed mature toxin (**Figure 1A**) (Atherton et al., 1995; Strobel et al., 1998; van Doorn et al., 1998). The two primary allelic groups that have been differentiated among strains are referred to as s1 and s2, and the s1 group further differentiates into the s1a, s1b, and s1c subtypes. Notably, sequence differences between strains carrying the s1 or s2 alleles correspond directly to functional differences, as strains carrying the s2 *vacA* allele fail to induce cellular vacuolation (Atherton et al., 1995). Analysis of s2 forms of *vacA* revealed that the signal sequences of these proteins are processed at a different cite, resulting in a mature form of VacA with a 12 amino acid extension that inactivates the toxin (Letley and Atherton, 2000; McClain et al., 2001; Letley et al., 2003).

Most recently, a third region of sequence diversity, called the "i (for intermediate) region" has been identified in the carboxyl-terminal half of p33 between the s and m regions of VacA (Rhead et al., 2007). Three primary i-region types (i1, i2, and i3) (Chung et al., 2010) have been identified and revealed to be associated

with the divergent vacuolating activities associated with different forms of the toxin. Although the structure-function relationships underlying the contributions of the i-region to the cellular activity of VacA have not been identified, i1 *vacA* strains were found to have the strongest vacuolating activity on mammalian cells. The i region has been proposed to be a more reliable predictor of severe gastric disease than the s- or m-regions of the *vacA* gene, and i1 strains are strongly associated with gastric adenocarcinoma and peptic ulcer disease (Rhead et al., 2007; Basso et al., 2008).

THE ASSOCIATION OF *vacA* GENOTYPES WITH DISEASE

Efforts to evaluate potential roles of VacA in the pathogenesis of human gastroduodenal diseases are complicated by the multifactorial nature of *H. pylori* pathophysiology in the human stomach. Nonetheless, epidemiology studies have been extremely useful for demonstrating a correlation between particular *vacA* i-, m-, and s-region allelic types and occurrence and severity of disease in *H. pylori*-infected humans. Many studies have provided evidence for a higher association of disease in individuals infected with *H. pylori* strains possessing s1 *vacA* alleles encoding toxin variants with greater cellular activity *in vitro* than strains with s2 *vacA* alleles (Atherton et al., 1995, 1997; Han et al., 1998; Rudi et al., 1998; Strobel et al., 1998; van Doorn et al., 1998, 1999; Gerhard et al., 1999; Kidd et al., 1999; de Gusmao et al., 2000). In addition, strains with s1 alleles are a higher risk for gastric carcinoma than strains harboring *vacA* s2 alleles (Miehlke et al., 2000; Ashour et al., 2002; Figueiredo et al., 2002). As far as the m region, *H. pylori* strains harboring m1 *vacA* alleles have been more highly associated with gastric carcinoma (Miehlke et al., 2000; Ashour et al., 2002; Figueiredo et al., 2002), as well as gastric alterations that normally precede the onset of gastric cancer, including epithelial damage, atrophic gastritis, and intestinal metaplasia (Atherton et al., 1997; Nogueira et al., 2001), than are those strains with m2 *vacA* alleles. Strains carrying both the s1 (Atherton et al., 1995; Rudi et al., 1998; Vinion-Dubiel et al., 1999; Ye and Blanke, 2000) and the m1 allele (Pagliaccia et al., 1998; Ji et al., 2000; Letley et al., 2003; Skibinski et al., 2006), are strongly associated with increased bacterial load and PMN infiltration within human gastric mucosa (Hofman et al., 2007), duodenal and gastric ulceration (Atherton et al., 1995, 1997; Rudi et al., 1998; Strobel et al., 1998; Figueiredo et al., 2001) and gastric cancer (Basso et al., 1998; Evans et al., 1998; Kidd et al., 1999; Miehlke et al., 2000; Figueiredo et al., 2001). As discussed above, more recent studies have suggested the possibility that the identity of the i allele may be the most reliable predictor of severity of disease, with the i1 allele predictive of the most severe disease outcome (Rhead et al., 2007).

PROPERTIES OF VacA RELATED TO *H. pylori* COLONIZATION AND DISEASE

H. pylori have co-evolved along with humans, and thus the use of existing rodent and non-human primate models for studying bacterial colonization and gastric disease would be predicted to have limitations. Nonetheless, animal studies have been valuable for revealing that VacA may contribute to *H. pylori* pathogenesis in several ways. Administration of purified toxin within a mouse model induces the degeneration of the gastric mucosa, inflammatory cell recruitment, and the formation of gastric lesions resembling the pathology observed in humans (Telford et al., 1994; Ghiara et al., 1995; Marchetti et al., 1995; Fujikawa et al., 2003). VacA has been implicated as a contributing factor within a Mongolian gerbil disease model (Ogura et al., 2000). VacA promotes *H. pylori* colonization within a mouse infection model (Salama et al., 2001). More recently, several studies suggested that VacA may contribute to *H. pylori* subversion of the host's immune response through elaboration of several immunosuppressive activities (Montecucco and de Bernard, 2003; Fischer et al., 2004). An enormous body of work supports the importance of VacA in *H. pylori*-mediated human disease. In contrast, the molecular and cellular basis by which VacA interacts with and modulates human cells remains poorly understood.

VacA AS A CELLULAR MODULATOR: THE MULTIPLE CONSEQUENCES OF VacA INTOXICATION

In an effort to understand how VacA contributes to *H. pylori* colonization of the stomach and development of gastroduodenal disease, the effects of VacA on human cells have been investigated *in vitro*. VacA is able to intoxicate a wide range of cell types, including gastric epithelial cells and several types of immune cells, resulting in an array of multiple different cellular alterations (**Figure 2**).

The published modulatory effects of VacA on epithelial and immune cells will be discussed briefly. In contrast, recent evidence implicating VacA as a mitochondrial-targeting toxin will be considered in more detail.

VacA MODULATION OF EPITHELIAL CELL FUNCTION

The first documented (Leunk et al., 1988; Smoot et al., 1996; Pagliaccia et al., 1998) cellular activity of VacA was the capacity to induce the biogenesis of large intracellular vacuoles that occupy almost the entire epinuclear region (**Figure 2B**). VacA-mediated vacuolar compartments are acidic (Cover et al., 1991), enriched in the small GTPase Rab7 as well as other late endocytic markers (Papini et al., 1994, 1996, 1997; Molinari et al., 1997; Li et al., 2004), and exclude trypan blue (Leunk et al., 1988), indicating that vacuolation per se is not a cytolethal response to VacA intoxication. Vacuolation may be due to toxin-mediated alterations in the trafficking of endocytic vesicles, as indicated by VacA-dependent inhibition of the intracellular degradation of epidermal growth factor, and inhibition of procathepsin D maturation (Satin et al., 1997).

The capacity of VacA to form anion-conducting channels is important for the biogenesis of vacuoles (Szabo et al., 1999; Tombola et al., 1999b; Vinion-Dubiel et al., 1999; Ye and Blanke, 2000; McClain et al., 2003; Genisset et al., 2007), which then swell further in the presence of membrane permeable weak bases (Czajkowsky et al., 1999; Szabo et al., 1999; Tombola et al., 1999b; Morbiato et al., 2001). The sources of membrane required for the formation of the large intracellular vacuoles remains unclear. The demonstration that the SNARE protein syntaxin 7, which is involved in intracellular membrane fusion events, is localized to the membranes of VacA-induced vacuoles supports a model that VacA-induced vacuoles may result from the fusion of multiple smaller endocytic compartments (Gauthier et al.,

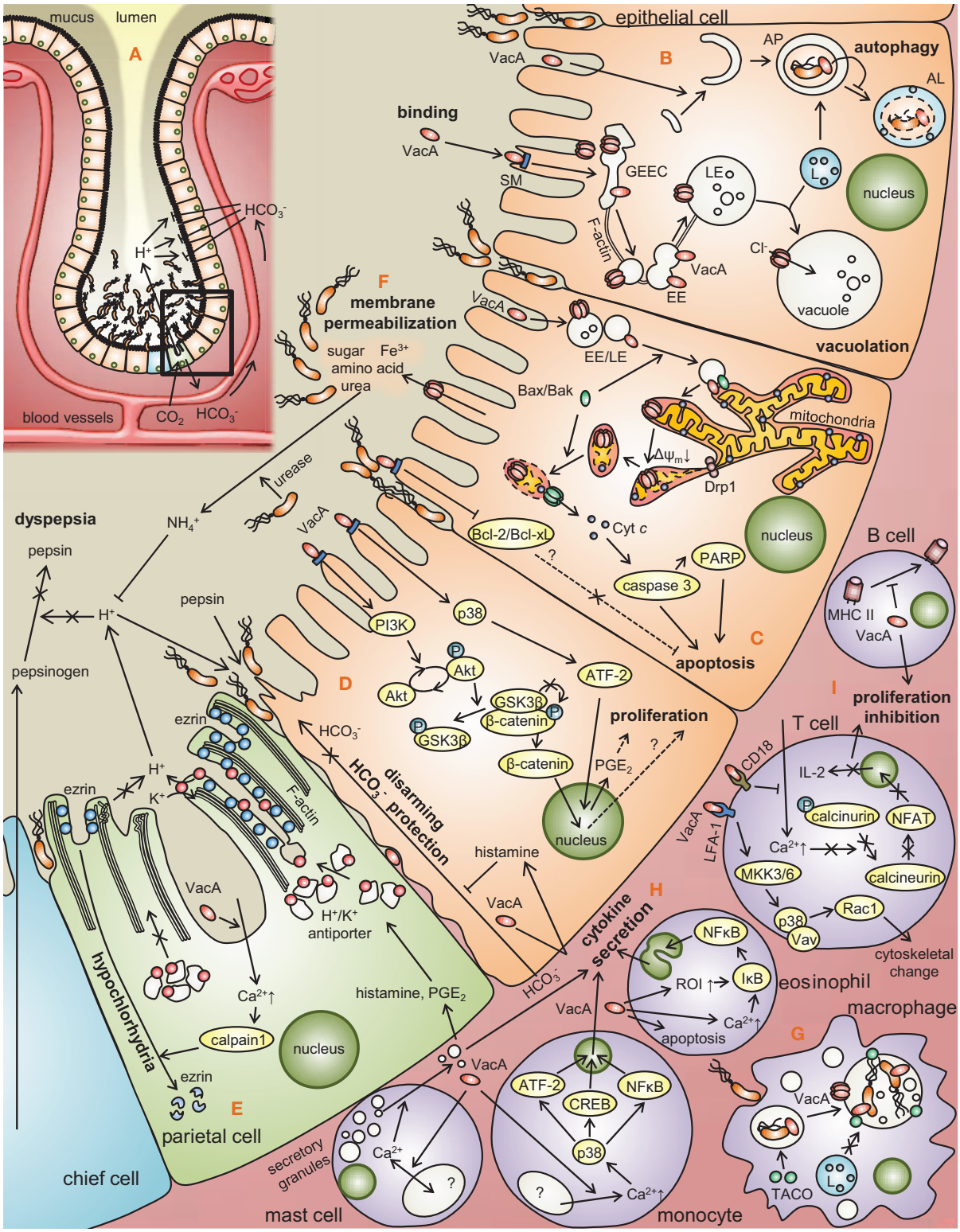

FIGURE 2 | Modulation of gastric cell functions by VacA. (A) Distribution of *H. pylori* at the gastric epithelium. Approximately 80% of *H. pylori* localize to the layer of mucus overlaying the epithelial membrane, while the remainder attach to the membrane surface as microcolonies. **(B)** VacA binds to epithelial cell surface receptors (SM, RPTP-a/β), and is taken up by a Cdc42-dependent, pinocytic-like mechanism into (GPI)-enriched early endosomal compartments (GEECs) followed by F-actin-dependent transport to early and late endosomal compartments (EE and LE). LE fusion with lysosomes (L) promotes vacuole biogenesis in a manner dependent on VacA *(Continued)*

FIGURE 2 | Continued
channel activity. VacA was recently reported to induce the formation of autophagosomes (AP), which typically mature to autophagolysosomes (AL). **(C)** A portion of VacA-containing EEs/LEs are transported to mitochondria within Bax-enriched vesicles. VacA channel formation within the mitochondrial inner membrane induces depolarization, $\Delta\Psi m$ dissipation, and disruption of mitochondrial dynamics at the level of Drp1-dependent fission, triggering Bax permeabilization of the mitochondrial outer membrane and cytochrome *c* release, resulting in apoptosis. VacA-mediated downregulation of Bcl-2/Bcl-xL [Matsumoto et al. (2011)] also promotes cell death. **(D)** VacA has been proposed to promote cellular proliferation via p38 activation, which increases ATF-2 regulated expression of prostaglandin E2 (PGE_2) [Hisatsune et al. (2007)], as well as the activation of β-catenin though the deregulation of phosphorylation of glycogen synthase kinase 3 (GSK3β) and Akt, initiated by the activation of phosphoinositide 3-kinase (PI3K) [Nakayama et al. (2009)]. Histamine secretion stimulated by VacA inhibits HCO_3^- mediated protection against acid and pepsin [Tuo et al. (2009)]. **(E)** VacA induces the influx of Ca^{2+} into parietal cells, thereby activating calpain 1, a protease that degrades ezrin, an actin binding protein that is critical for proton release [Wang et al. (2008)], thereby deregulating acid secretion machinery within parietal cells in a manner that may promote *H. pylori* persistence. **(F)** VacA channel formation in the apical membrane surface may expose *H. pylori* to diffusible nutrients such as amino acids, sugar, and metal ions [Szabo et al. (1999); Debellis et al. (2001); Montecucco and Rappuoli (2001); Tombola et al. (2001a)]. *H. pylori* urease may convert released urea into ammonia which neutralizes acid, which inhibits activation of pepsinogen to pepsin, eventually causing dyspepsia [Mobley et al. (1995); Carter et al. (2009)]. **(G)** VacA-dependent remodeling of *H. pylori*-containing vacuoles facilitates bacterial survival within macrophages [Zheng and Jones (2003)]. **(H)** VacA stimulates the release of secretory granules within mast cell by causing the oscillation of intracellular Ca^{2+} levels [de Bernard et al. (2005)]. VacA intoxication of monocytes results in Ca^{2+}-dependent p38 activation, leading to proinflammatory cytokine production via ATF-2, CREB, and NF-κB-dependent mechanisms [Hisatsune et al. (2008)]. Within eosinophils, VacA deregulates ROS intermediates (ROI) and intracellular Ca^{2+} to stimulate proinflammatory cytokine release by a NF-κB-dependent mechanism [Kim et al. (2007)]. **(I)** In T cells, VacA recognized by CD18, blocks Ca^{2+} influx, the activation of calcineurin, and nuclear factor of activated T-cells (NFAT) a transcription factor required for the expression of interleukin-2 (IL-2), ultimately suppressing T-cell proliferation [Sewald et al. (2008)]. VacA recognized by lymphocyte function-associated antigen 1 (LFA-1) receptor activates mitogen-activated protein kinase kinase 3/6 (MKK3/6), leading to the activation of p38 and Rac 1, resulting in the cytoskeletal change [Sewald et al. (2008)]. In B cells, VacA disrupts antigen presentation of MHC II.

2007). Conversely, several other studies instead support a model of swelling of the internal membranes of the late endosomal compartments (de Bernard et al., 2002; Genisset et al., 2007).

Despite intensive study into the molecular basis and mechanism underlying vacuole biogenesis in VacA intoxicated cells, it remains poorly understood whether vacuolation *per se* directly contributes to *H. pylori* colonization, persistence or disease pathophysiology. Alternatively, vacuolation may represent the general stress response of cells intoxicated with VacA.

Modulatory effects of VacA on cell viability

Sustained infection with *H. pylori* results in alterations within in the gastric mucosa that have been associated with the progression of infection to gastric disease. As mentioned above, VacA alone is sufficient to damage the gastric mucosa of mice (Telford et al., 1994; Ghiara et al., 1995; Marchetti et al., 1995; Fujikawa et al., 2003). A hallmark of *H. pylori* infection is increased level of apoptosis in human gastric mucosa (Mannick et al., 1996; Moss et al., 1996; Jones et al., 1997; Wagner et al., 1997; Fan et al., 1998), mice (Jones et al., 2002), and Mongolian gerbil models (Peek et al., 2000).

Increased cell death within the epithelial membrane may contribute to *H. pylori* disease pathogenesis in several ways (Correa and Houghton, 2007). Increased apoptosis may alter the gastric environment to promote *H. pylori* persistence (Cover and Blanke, 2005), while at the same time, contribute to gastric disease, including peptic ulcers and gastric adenocarcinoma (Cover and Blaser, 2009). Although several *H. pylori* factors (Peek et al., 1999; Kawahara et al., 2001) have been implicated as mediators of cell death, VacA is *per se* sufficient to induce cell death (**Figure 2C**) (Boquet et al., 2003; Cover et al., 2003). VacA-mediated cell death is apparently complex, involving cellular alterations that are consistent with both apoptotic (Kuck et al., 2001; Boquet et al., 2003; Cover et al., 2003; Willhite et al., 2003; Oldani et al., 2009) and necrotic (Willhite and Blanke, 2004; Radin et al., 2011) cell death programs.

Modulatory effects of VacA on signaling transduction pathways regulating cellular homeostasis

VacA-mediated cellular vacuolation is typically observed within several hours after addition of VacA to cells. However, VacA causes several cellular effects that can be detected at much earlier time points. Within 10 min after addition of VacA to a human gastric adenocarcinoma cell line (AZ-521), two classes of mitogen-activated protein (MAP) kinases (p38 and ERK1/2) and the activating transcription factor 2 (ATF-2) signaling pathway were reported to be activated (**Figure 2D**) (Nakayama et al., 2004; Hisatsune et al., 2007). An inhibitor of p38 kinase activity (SB203580) does not block VacA-induced vacuolation or VacA-induced cytochrome *c* release, which indicates that VacA-induced activation of the p38/ATF-2 signal pathway is independent of VacA effects on late endocytic compartments and mitochondria (Nakayama et al., 2004). One reported consequence of VacA-induced p38 activation is upregulation of cyclooxygenase-2 (COX-2) expression, leading to increased PGE_2 production (**Figure 2D**) (Hisatsune et al., 2007). In addition to effects of VacA on MAP kinases, VacA is reported to activate a signaling pathway involving tyrosine phosphorylation of the G protein-coupled receptor kinase interactor (Git1) (Fujikawa et al., 2003) and a signaling pathway that leads to upregulated expression of vascular endothelial growth factor (Caputo et al., 2003).

In contrast to effects of VacA on endocytic compartments and mitochondria, the relatively rapid cellular responses to VacA described above are likely to be the consequences of VacA interactions with specific cell surface components, without a requirement for internalization of the toxin. The cell surface receptors for VacA that are involved in activating MAP kinase signaling pathways have not yet been characterized. Activation of the Git1 pathway is dependent on VacA binding to RPTP-β (Fujikawa et al., 2003). VacA-induced upregulation of vascular endothelial growth factor reportedly occurs through a pathway involving activation of epidermal growth factor receptor, MAP kinases, and COX-2 (Caputo et al., 2003).

Modulatory effects of VacA on epithelial monolayer permeability/nutrient acquisition

VacA has been reported to lower the trans-epithelial electric resistance (TER) of monolayers formed by several different types of polarized epithelial cells, an effect that is attributable to increased paracellular epithelial permeability of the monolayers to low molecular mass molecules and ions (**Figure 2F**) (Papini et al., 1997). Selective permeabilization of epithelial monolayers by VacA has been proposed to support the growth of *H. pylori* in the gastric mucus layer by promoting the release of factors such as Fe^{3+}, Ni^{2+}, sugars, and amino acids (Papini et al., 1997). The mechanisms by which VacA alters paracellular permeability are not yet well understood. This activity does not require acid-activation of purified oligomeric VacA, and is not inhibited by bafilomycin A1.

VacA also been reported to increase the transepithelial flux of certain molecules, including urea and bicarbonate (**Figure 2E**) (Guarino et al., 1998; Szabo et al., 1999; Debellis et al., 2001; Tombola et al., 2001a). Transepithelial flux of these molecules is attributed to the formation of VacA channels in the plasma membrane (Szabo et al., 1999). Bicarbonate release has been suggested to promote bacterial survival within the acidic gastric mucus layer, and release of urea may optimize the enzymatic activity of *H. pylori* urease. Most recently, VacA has been demonstrated to cause mislocalization of the transferrin receptor to the apical membrane of polarized monolayers, thereby facilitating iron acquisition from interstitial holotransferrin to *H. pylori* microcolonies attached to the apical membrane (Tan et al., 2011).

Modulatory effects of VacA on the cytoskeleton

VacA-mediated cytoskeletal alterations in both actin filaments and the microtubule system have been reported (Ashorn et al., 2000; Pai et al., 2000; Tabel et al., 2003; Yuan et al., 2004; Hennig et al., 2005; Wang et al., 2005). Studies emerging from a yeast two-hybrid screen suggested that, at least in this system, VacA interacted with an intermediate filament interacting protein (de Bernard et al., 2000). Moreover, VacA may alter the expression of multiple cytoskeletal-related genes (Pai et al., 2000; Yuan et al., 2004; Wang et al., 2005), although the mechanisms underlying these cytoskeletal alterations have not been demonstrated.

More recently, VacA intoxication of parietal cells was reported to prevent the recruitment of H,K-ATPase-containing tubulovesicles to the apical membrane (**Figure 2E**), thereby disrupting apical membrane-cytoskeletal interactions (Wang et al., 2008). The disruption in the radial arrangement of actin filaments in apical microvilli was found to be due to the loss of ezrin integrity. The authors proposed that the proteolysis of ezrin in VacA-infected parietal cells represents a novel mechanism underlying *H. pylori*-induced inhibition of acid secretion, thereby resulting in hypochlorhydria.

Modulatory effects of VacA in promoting intracellular survival of H. pylori in gastric epithelial cells

The importance of an intracellular reservoir of *H. pylori* during persistent gastric infection has been debated. While there is now consensus that *H. pylori* are found primarily in the mucus layer overlying gastric epithelial cells within humans, with the remainder on the apical surface of the epithelial membrane, there is evidence that the bacteria might occasionally localize within gastric epithelial cells. Several studies have suggested that VacA might promote survival of *H. pylori* within gastric epithelial cells (Bjorkholm et al., 2000; Petersen et al., 2001). VacA was shown to promote intracellular survival of *H. pylori* via a mechanism involving VacA-dependent retention of Rab7, fusion of late endocytic compartments, and sequestration *H. pylori*-containing cell compartments away from the lysosomal degradative pathway (Terebiznik et al., 2006). In contrast, another study did not detect a role of VacA in intracellular survival of *H. pylori* (Amieva et al., 2002).

Modulatory effects of VacA resulting in the induction of autophagy

Recently, VacA was demonstrated to induce autophagic signaling within intoxicated epithelial cells (**Figure 2B**) (Terebiznik et al., 2009; Raju et al., 2012). The autophagic vesicles and vacuoles induced within VacA intoxicated cells are apparently not the same vesicles. While the role of autophagy during *H. pylori* infection is currently unclear, autophagy might function to limit the toxin-mediated cellular cytotoxicity (Terebiznik et al., 2009).

VacA AS A MODULATOR OF IMMUNE CELL FUNCTION

Beyond the well-documented effects of VacA on epithelial cells, additional roles for the toxin in modulating the properties of T lymphocytes have been demonstrated (**Figure 2H**) (Boncristiano et al., 2003; Gebert et al., 2003; Sundrud et al., 2004; Oswald-Richter et al., 2006; Algood et al., 2007). VacA inhibits Jurkat T cell production of interleukin 2 (IL-2), which promotes T cell viability and proliferation, as well as down-regulates surface expression of the IL-2 receptor (Boncristiano et al., 2003; Gebert et al., 2003; Sundrud et al., 2004), by inhibiting activation of nuclear factor of T cells (NFAT) (Boncristiano et al., 2003; Gebert et al., 2003), a transcriptional regulator required for optimal T cell activation. VacA inhibits NFAT activation by a mechanism involving the blocking of calcium influx into cells from the extracellular milieu, thereby inhibiting the activity of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin (an enzyme that dephosphorylates NFAT) (Boncristiano et al., 2003; Gebert et al., 2003).

In contrast to what was reported for Jurkat T cells VacA intoxication primary human CD4 + T cells inhibited the proliferation of activated cells, induced mitochondrial depolarization, ATP depletion, and cell cycle arrest (Sundrud et al., 2004; Oswald-Richter et al., 2006). Moreover, VacA also inhibits activation-induced proliferation of human CD8 + T cells and B cells (Torres et al., 2007). The immunomodulatory actions of VacA on T- and B-lymphocytes suggest the possibility that VacA might contribute to the ability of *H. pylori* to establish a persistent infection in the human gastric mucosa.

Interestingly, the effects of VacA on T cells may occur via more than one mechanism. While some effects are dependent on the formation of VacA channels in cell membranes (Boncristiano et al., 2003; Sundrud et al., 2004; Oswald-Richter et al., 2006), others are the result of activation of intracellular signaling in T cells, via a channel-independent mechanism (Boncristiano et al., 2003). It is hypothesized that VacA enters the lamina propria via

disruptions in the gastric epithelial layer, and thereby intoxicates T cells and various other types of immune cells. In addition, it is possible that VacA may target intraepithelial T lymphocytes. Most of the effects of VacA on T lymphocytes described above are expected to result in localized immunosuppression. VacA also stimulates expression of COX-2, a proinflammatory enzyme, in T lymphocytes, which is expected to have a proinflammatory effect (Boncristiano et al., 2003). Thus, the effects of VacA on immune cells are complex, and are characterized by both immunostimulatory and immunosuppressive actions.

MODULATORY EFFECTS OF VacA ON ANTIGEN PRESENTATION

VacA has been reported to interfere with the process of antigen presentation by B lymphocytes (**Figure 2I**). In one model system, VacA interfered with proteolytic processing of tetanus toxoid and inhibited the invariant chain (Ii)-dependent pathway of antigen presentation mediated by newly synthesized major histocompatibility complex (MHC) class II molecules (Molinari et al., 1998). This effect of VacA on antigen-presenting cells is likely due to VacA-induced effects on endocytic compartments, resulting in alterations in endocytic trafficking.

MODULATORY EFFECTS OF VacA ON ANTIGEN MACROPHAGES

Two reports provided evidence that VacA contributes to the formation of large vesicular compartments (termed megasomes) in *H. pylori*-infected macrophages by stimulating homotypic phagosome fusion (Allen et al., 2000; Zheng and Jones, 2003). VacA stimulates the recruitment and retention of the tryptophan aspartate-containing coat protein (TACO or coronin 1) to phagosomes, thereby disrupting proper vesicular maturation (Zheng and Jones, 2003), which in turn, may impair phagocytic killing of *H. pylori*. Notably, one report did not detect any effects of VacA on phagosome formation or intracellular survival of *H. pylori* in human monocytes (Rittig et al., 2003). In addition to having effects on vesicular compartments in macrophages, VacA also is reported to stimulate activation of p38 MAP kinase and cause increased expression of COX-2 in macrophages (Boncristiano et al., 2003). Finally, it has been reported that VacA may contribute to the apoptosis of macrophages infected with *H. pylori* (Menaker et al., 2004).

MODULATORY EFFECTS OF VacA ON MAST CELLS, EOSINOPHILS, AND NEUTROPHILS

Incubation of VacA with mast cells stimulates production of pro-inflammatory cytokines and induces mast cell chemotaxis (**Figure 2G**) (Supajatura et al., 2002; de Bernard et al., 2004). Binding of VacA to a mast cell line (RBL-2H3 cells) induces an oscillation in levels of cytosolic calcium and exocytosis of secretory granules (de Bernard et al., 2004). The rapid changes in calcium concentration that follow VacA interaction with mast cells may result from VacA interactions with cell surface receptors and activation of specific signal transduction pathways, without a requirement for membrane channel formation.

VacA is reported to have an effect on eosinophils, manifested as upregulated expression of chemokines (**Figure 2G**) (Kim et al., 2007). This effect is reported to occur via a pathway involving calcium influx, mitochondrial generation of reactive oxygen intermediates, and NF-κB activation (Kim et al., 2007). VacA also is reported to have effects on neutrophils, including activation of p38 MAP kinase and increased expression of COX-2 (Boncristiano et al., 2003; Brest et al., 2006).

TARGETING THE POWER SOURCE: VacA AS A MITOCHONDRIAL-ACTING TOXIN

One of the most intriguing and highly studied activities associated with VacA over the past 10 years, is the capacity of the toxin to target and modulate the properties of mitochondria within host cells (**Figure 2C**). In addition to the well-known role of mitochondria in central metabolism, this organelle has emerged as a central hub of regulation for many of the most fundamental cellular processes, including programmed cell death, progression of the cell cycle, and innate immune sensing. At the same time, it is now clear that a loss of mitochondrial function, or dysfunction, has been associated with perhaps hundreds of human diseases and disorders. However, the extent to which mitochondrial function is altered during infection of host cells with pathogenic microbes as well as the possible consequences of pathogen-mediated mitochondrial dysfunction, are both poorly understood. This is a particularly relevant issue because several dozen pathogenic bacteria and viruses have been reported to generate protein effectors that localize to mitochondria (Blanke, 2005).

MITOCHONDRIAL DYSFUNCTION IS A CHARACTERISTIC OF VacA CELLULAR INTOXICATION

VacA-mediated modulation of mitochondrial function was first reported approximately a decade after the toxin was discovered as the factor within *H. pylori* culture filtrates that induces vacuolation within cultured epithelial cell lines (Leunk et al., 1988). VacA intoxication of human gastric epithelial AZ-521 cells was reported to induce mitochondrial dysfunction (**Figure 2C**), as manifested by a drop in cellular ATP levels, mitochondrial transmembrane potential ($\Delta\Psi_m$), and oxygen consumption (Kimura et al., 1999). Since this first report, VacA-dependent alterations in mitochondria, and in particular $\Delta\Psi_m$ dissipation, have been confirmed independently by several groups (Galmiche et al., 2000; Willhite and Blanke, 2004; Yamasaki et al., 2006; Calore et al., 2010; Domanska et al., 2010; Foo et al., 2010), and are considered as one of the primary consequences of toxin activity at mitochondria (Blanke, 2005; Galmiche and Rassow, 2010; Rassow, 2011).

CONSEQUENCES OF VacA-DEPENDENT MITOCHONDRIAL DYSFUNCTION

Because of the central importance of mitochondria in cellular metabolism, it is perhaps not be surprising that VacA-mediated mitochondrial dysfunction has distinct consequence for host cells. In general, mitochondrial health is dependent on the functional integrity of the organelle's inner and outer membranes, which have distinct biochemical and functional properties. Increased permeability can result in depolarization of the mitochondrial inner membrane, leading to $\Delta\Psi_m$ dissipation and a loss of metabolism (**Figure 2C**), which as indicated above, occurs characteristically within VacA intoxicated cells (Galmiche

et al., 2000; Willhite and Blanke, 2004; Yamasaki et al., 2006; Calore et al., 2010; Domanska et al., 2010; Foo et al., 2010). Increased outer membrane permeability (MOMP) results in the release of pro-apoptotic protein effectors from the inner membrane space into the cytosol (Blanke, 2005). Several studies have reported the release of mitochondrial cytochrome *c* within cells intoxicated with VacA (Galmiche et al., 2000; Willhite et al., 2003, 2004; Yamasaki et al., 2006), resulting in apoptotic cell death, which has been demonstrated to be a consequence of VacA intoxication (Smoot et al., 1996; Kuck et al., 2001; Cover et al., 2003).

WHERE IN THE CELL DOES VacA ACT TO INDUCE MITOCHONDRIAL DYSFUNCTION?

The case for VacA as a mitochondrial-targeting toxin

VacA-mediated changes in mitochondrial properties could result as an indirect consequence of vacuole formation, or potentially from VacA action at the cell surface. However, the discovery that the amino-terminal p33 domain (but not the carboxyl-terminal p55 domain) of VacA localized to mitochondria when expressed ectopically within mammalian cells suggested a different possibility, that at least the p33 fragment might be hardwired to localize to mitochondria from the cytosol of mammalian cells (Galmiche et al., 2000).

Several groups have now demonstrated that subsequent to uptake into epithelial cells, full-length VacA toxin from *H. pylori* partially localizes to mitochondria, as shown by both fluorescence imaging and biochemical fractionation (Willhite and Blanke, 2004; Yamasaki et al., 2006; Oldani et al., 2009). The importance of VacA mitochondrial localization for toxin dependent cell death has been questioned based on the observation that a large fraction of VacA remains associated with endocytic compartments (Yamasaki et al., 2006). However, disrupting VacA intracellular trafficking with the actin-disrupting agent cytochalasin D prevented VacA localization to mitochondria, as well as toxin-dependent $\Delta\Psi_m$ dissipation and cell death (Willhite and Blanke, 2004; Oldani et al., 2009). Likewise, in cells transfected with CagA, VacA was prevented from trafficking to mitochondria, and VacA intoxicated cells were protected from toxin-dependent apoptosis, further supporting the idea that VacA exerts its biochemical activity directly at mitochondria (Willhite and Blanke, 2004; Oldani et al., 2009).

An inside job? VacA localizes to the mitochondrial inner membrane

When p33 was expressed as a fusion with GFP within transiently transfected mammalian cell lines, ultrastructural immunocytochemistry studies using transmission electron microscopy provided images showing that a substantial fraction of the fusion protein clearly localizes in the interior of mitochondria (Galmiche et al., 2000). Perhaps more convincing have been studies using isolated mitochondria, which have demonstrated that VacA is imported beyond the outer membrane, but probably not past the inner membrane (Galmiche et al., 2000; Domanska et al., 2010; Foo et al., 2010). Together, these studies support a model that the site of action of VacA is the mitochondrial inner membrane.

Turning off the power: how does VacA cause mitochondrial dysfunction?

The exact mechanism by which VacA induces mitochondrial dysfunction remains to be determined. Several studies have indicated that mutant forms of VacA with disrupted membrane channel activity are attenuated in their capacity to induce $\Delta\Psi_m$ dissipation, as well as cytochrome *c* release and other downstream changes associated with apoptosis within intact cells (**Figure 2C**) (Willhite and Blanke, 2004) or isolated mitochondria (Yamasaki et al., 2006). In studies conducted with intact cells, mutant forms of VacA with point mutations (P9A, G14A) that attenuated channel activity were taken into cells and localized to mitochondria (Willhite and Blanke, 2004). These data strongly support a model that subsequent to mitochondrial import, VacA inserts into the mitochondrial inner membrane and forms ion-conducting channels to cause membrane depolarization, resulting in $\Delta\Psi_m$ dissipation and a loss in ATP production. Many aspects of this model remain to be evaluated, including the intriguing question of how VacA channels at mitochondria alone might result in what appears to be widespread $\Delta\Psi_m$ dissipation throughout the cell. It also remains to be seen whether or not VacA recognizes and/or interacts with a mitochondrial-specific protein or lipid localized to the mitochondrial inner membrane to facilitate membrane insertion and channel formation.

Break-in: how does VacA breach the mitochondrial outer membrane

While some AB toxins possess the remarkable ability to translocate an active fragment across host cell membranes, there is no indication that VacA facilitates its own entry past the mitochondrial outer membrane and ultimately to the inner membrane. Rather, recent work (Domanska et al., 2010) suggests that VacA translocation across mitochondrial outer membranes may require toxin-exploitation of existing and highly conserved protein import machinery. Studies with isolated mitochondria revealed that the mitochondrial import receptor Tom20 is involved in the uptake of p33, potentially reflecting the capacity of Tom20 to interact with the hydrophobic residues of precursor proteins (Abe et al., 2000). Subsequent transport of p33 across the mitochondrial outer membrane was found to be mediated by the general import pore formed by Tom40.

HOW DOES INTRACELLULAR VacA TARGET MITOCHONDRIA?

Does VacA mimic endogenous proteins destined for mitochondrial import?

Mitochondria-localized proteins are generally hardwired with targeting sequences embedded within their amino-acid sequences. However, VacA does not possess an easily identifiable mitochondrial-targeting motif. Fluorescence imaging studies revealed that when expressed as GFP fusions in transiently transfected cells, p33, but not p55, localized to mitochondria (Galmiche et al., 2000). The sufficiency of the amino-terminal p33 domain for mitochondrial localization within cells has been confirmed independently (Domanska et al., 2010).

However, the molecular basis by which VacA targets mitochondria appears to be complex. One study showed that that neither

the first 100 nor 200 amino-terminal residues were sufficient to target a amino-terminally fused green fluorescence protein (GFP) expressed ectopically or microinjected into the cytosol of cells to intracellular mitochondria (Galmiche et al., 2000). On the other hand, another study showed that the amino-terminal 34 residues, comprising a highly hydrophobic sub-domain, is sufficient to promote mitochondrial localization when expressed ectopically as a fusion-protein with carboxyl-terminal GFP (Domanska et al., 2010). The reasons underlying the differences between these two studies are unclear, but may originate in the different orientation of the GFP fusion proteins between the two studies.

AN ALTERNATIVE MODEL FOR VacA LOCALIZATION TO MITOCHONDRIA

DOES VacA ENTER THE CYTOSOL DURING INTRACELLULAR TRANSPORT?

The earliest studies to define the molecular basis of VacA localization to mitochondria employed fragments or mutant forms of the toxin either microinjected or expressed within the cytosol of transiently transfected cells (Galmiche et al., 2000). These approaches are clearly useful for interrogating VacA within the cytosol. Nonetheless, there is a lack of experimental evidence for cytosolic VacA within cells exposed to exogenous toxin. One of the earliest papers to consider the intracellular trafficking of VacA (Garner and Cover, 1996) reported VacA, as detected by indirect immunofluorescence microscopy, located in a perinuclear region, but not associated with cytotoxin-induced vacuoles, although later studies using higher resolution techniques indicated that VacA is in fact detectable in vacuoles (Li et al., 2004).

Subsequent studies of intracellular trafficking revealed VacA within small punctate vesicles just after uptake from the cell surface (Gauthier et al., 2004, 2005, 2007; Gupta et al., 2008, 2010) and at later time points, localized to epinuclear structures confirmed to be mitochondria (Willhite and Blanke, 2004; Oldani et al., 2009). Although it can be perilous to base a model partially on "negative data," the lack of evidence that VacA enters the cytosolic compartment after uptake from the cell surface suggests that VacA cellular activity does not require either VacA or a fragment of VacA to be first translocated to the cytosol.

The unusual intracellular routing of VacA

If the intracellular action of VacA indeed does not require the toxin to become cytosolic, then other mechanisms must be considered to explain localization of the toxin to mitochondria subsequent to cellular uptake. One alternative mechanism could conceivably depend the transport of VacA-containing membrane-bound compartments from the cell surface to mitochondria. To our knowledge, a well-defined pathway for the transport of any component from the cell surface to mitochondria has not been defined.

Studies of VacA intracellular transport have failed thus far to produce evidence that VacA exploits either the acidic environment of late endosomal compartments or the ERAD pathway to target mitochondria. Instead, work over the past decade has indicated that VacA is taken up and trafficked by an unusual pinocytic mechanism that had not previously been described for other intracellular acting bacterial exotoxins (**Figure 2B**). Specifically, VacA is internalized into epithelial cell lines by a Cdc42-dependent pinocytic mechanism, and is trafficked to late endosomal/lysosomal compartments (Molinari et al., 1997; Papini et al., 1997; McClain et al., 2000; Gauthier et al., 2004, 2005, 2007). As the first step during cellular intoxication, VacA binds to the plasma membrane of sensitive cells (Garner and Cover, 1996), analogous to other intracellular-acting bacterial toxins (Blanke, 2005). Recently, sphingomyelin (SM) has been identified as a plasma membrane receptor that confers cellular sensitivity to VacA by binding the toxin to the cell surface (Gupta et al., 2008). Moreover, VacA-SM interactions were demonstrated to be required for Cdc42-dependent pinocytic uptake from the plasma membrane and trafficking of VacA (Gupta et al., 2010).

What is the molecular GPS for VacA localization to mitochondria?

The relationship between VacA intracellular trafficking and mitochondrial localization remains poorly understood. It is tempting to speculate that the unusual aspects of VacA uptake and transport from the cell surface specifically promote, and perhaps are even required for toxin transport to cellular mitochondria, although this idea remains to be formally tested. One recent study implicated the pro-death, multi-domain Bcl-2 proteins Bax and Bak, as essential for the intracellular positioning of VacA-containing endocytic compartments in close juxtaposition with mitochondria (**Figure 2C**) (Calore et al., 2010). In the absence of Bax/Bak, VacA-containing vesicles were not observed to localize with mitochondria. Likewise, vesicles coated with Bax or Bak were not observed to be associated with mitochondria in cells unless the cells had been intoxicated with VacA. This was an unexpected observation, as neither Bax nor Bak were previously considered to have a functional role in endocytic vesicle trafficking.

Bax/Bak was previously reported to be important for VacA-mediated cell death (Yamasaki et al., 2006), but these new findings suggest a potentially expanded and novel role for these two molecules in the targeting and/or docking of VacA-containing transport vesicles with mitochondria. The exact mechanisms by which Bax/Bak induce the spatial juxtaposition of VacA-containing endosomal compartments with mitochondria, or the possible contributions of either of these two endogenous effectors to VacA translocation to the mitochondrial inner membrane are potentially exciting areas of future enquiry. One intriguing possibility is that Bax and Bak promote membrane fusion between mitochondria and the VacA-containing vesicles, allowing VacA to localize to the inner membrane without having to dissociate from one membrane to insert into a second membrane. However, such a scenario has not yet been tested.

MITOCHONDRIAL TARGETING AND EPITHELIAL REMODELING BY VacA

The issue of *why* VacA targets mitochondria remains an intriguing question. The multiple important roles of mitochondria for cellular function suggest that VacA-dependent alterations in mitochondria would have consequences for gastric epithelial cells. As discussed above, an increase in apoptotic cells within the gastric epithelium is a hallmark of persistent *H. pylori* infection, and

VacA has been demonstrated to be essential and sufficient among *H. pylori* factors for inducing cell death. But is VacA action at mitochondria linked to cell death?

Recent studies have provided new insights into how VacA action at mitochondria might be functionally associated with cell death. VacA was demonstrated to disrupt mitochondrial dynamics (**Figure 2C**) (Jain et al., 2011), which is the cellular balance between mitochondrial fission and fusion, the two processes that together control mitochondrial morphology, quality control, and energy transduction. VacA-mediated disruption of mitochondrial dynamics required hyperactivation of dynamin-related protein 1 (Drp1), which is a master regulator of mitochondrial fission, and Drp1 activation, in turn, was essential for activation of Bax, thereby committing VacA intoxicated cells to undergoing apoptosis. Thus, the discovery of VacA-mediated activation of Drp1 provides the direct molecular link between VacA action at mitochondria and toxin-dependent cell death.

The mechanism by which VacA induces Drp1 is not clear. VacA-mediated dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) may induce Drp1 activation indirectly, as mitochondrial depolarization has previously been shown in unrelated studies to induce calcineurin-mediated dephosphorylation of Drp1 as a mechanism to drive Drp1 translocation to mitochondria (Cereghetti et al., 2008). On the other hand, the possibility that VacA may act more directly to activate Drp1 cannot currently be ruled out.

FINE-TUNING THE HOST PATHOGEN INTERFACE: SYNERGISTIC AND ANTAGONISTIC ASSOCIATIONS BETWEEN THE VacA EXOTOXIN AND CagA, THE TYPE IV EFFECTOR OF *H. pylori*

Bacterial toxins are most typically studied as purified proteins in order to best tease out their specific contributions to modulation of host cell function. While often appropriate, these approaches do not take into account possible functional interactions that may exist between virulence factors. CagA is an important *H. pylori* virulence factor, and analogous to *vacA*, specific *cagA* alleles are highly predictive for the incidence and severity of disease associated with *H. pylori* infection. Like VacA, CagA modulates host cell function in several ways, primarily by disrupting signal transduction within intoxicated cells.

In principle, VacA and CagA are fundamentally different in their contributions to modulating the host epithelium. CagA, as a Type IV effector injected directly into the eukaryotic cytosol, modulates the functional properties of only those cells with which *H. pylori* have specifically colonized. In contrast, secreted VacA can act both proximally at the site of bacterial attachment to the epithelial membrane, as well as distally upon uninfected epithelial cells after diffusion from the site of adherent bacteria. Thus, infected epithelial cells would be predicted to be subject to the modulating effects of both VacA and CagA (**Figure 3**). There is some evidence that some the cellular modulatory effects of VacA and CagA might be synergistic. For example, a recent report suggests that VacA and CagA may both facilitate iron acquisition of *H. pylori* colonizing the apical surface of polarized epithelial monolayers (Tan et al., 2011), without severely damaging the host cells.

However, most studies to date however indicate that the cellular activities of VacA and CagA are primarily antagonistic. VacA and CagA inhibit each other's effects on epithelial cells (Argent et al., 2008), with CagA down-regulating cellular vacuolation (**Figure 3**), and VacA down-regulating the hummingbird phenotype of gastric epithelial cells associated with CagA injection. Another report demonstrated that whereas CagA activates the NFAT pathway via activation of calcineurin, VacA blocks calcineurin activation through decreased calcium influx, thereby down-regulating the NFAT pathway (Yokoyama et al., 2005).

Strikingly, the capacity of VacA to induce the death of epithelial cells is blocked by CagA (**Figure 3**) (Oldani et al., 2009). The authors demonstrated phosphorylated CagA blocks the ability of VacA to traffic to intracellular compartments, whereas unphosphorylated CagA blocks apoptosis in a manner that mimics Bcl2 (an anti-apoptotic factor) overexpression. CagA further inhibits VacA-dependent apoptosis by blocking the cellular uptake of VacA from the cell surface (Akada et al., 2010).

Increasing evidence suggests that VacA and CagA can functionally interact in manner that directly affects disease outcome. This idea is supported by epidemiological studies revealing significant 3-way associations between *vacA* alleles, *cagA* alleles, and the development of gastric diseases, including cancer (van Doorn et al., 1999; Yamazaki et al., 2005; Jang et al., 2010; Jones et al., 2011).

How might VacA and CagA function together to promote colonization and disease? A recent study of the molecular evolution of *vacA* indicates that the *vacA* gene and the core *H. pylori* genome do not share the same evolutionary history (Gangwer et al., 2010). The *vacA* and *cagA* genes are separated on the *H. pylori* genome by a considerable distance, and several lines of evidence suggest that the *cag* PAI was acquired more recently than *vacA* (Gressmann et al., 2005; Olbermann et al., 2010). This type of evolutionary history might support the idea that acquisition of the genes encoding VacA and CagA established the pro-/anti-inflammatory and pro-/anti-apoptotic balance necessary to promote long-term colonization of the human gastric mucosa.

Overall, these findings are consistent with the idea that VacA and CagA most effectively promote *H. pylori* persistence by functioning together in manner that remodels the gastric niche occupied by the bacterium, but at the same time limits the degree to which the epithelial mucosa is damaged. The capacity to limit damage to host cells and tissues during infection is an increasingly recognized strategy employed by a number of pathogenic bacteria to fine tune virulence while promoting propagation and/or persistence (Shames and Finlay, 2010).

VacA AS A HOST CELL REMODELER

VacA does not fit neatly into our current notions of intracellular-acting bacterial exotoxins. Whether the p33 and p55 fragments of VacA ultimately comprise the A and B fragments corresponding to the AB architecture of most intracellular-acting toxins remains to be seen. The absence of a defined enzymatic activity, as well as the strong association of VacA membrane channel activity with most of the toxin's intracellular effects suggests that VacA does not rely on the power of catalytic turnover to modulate

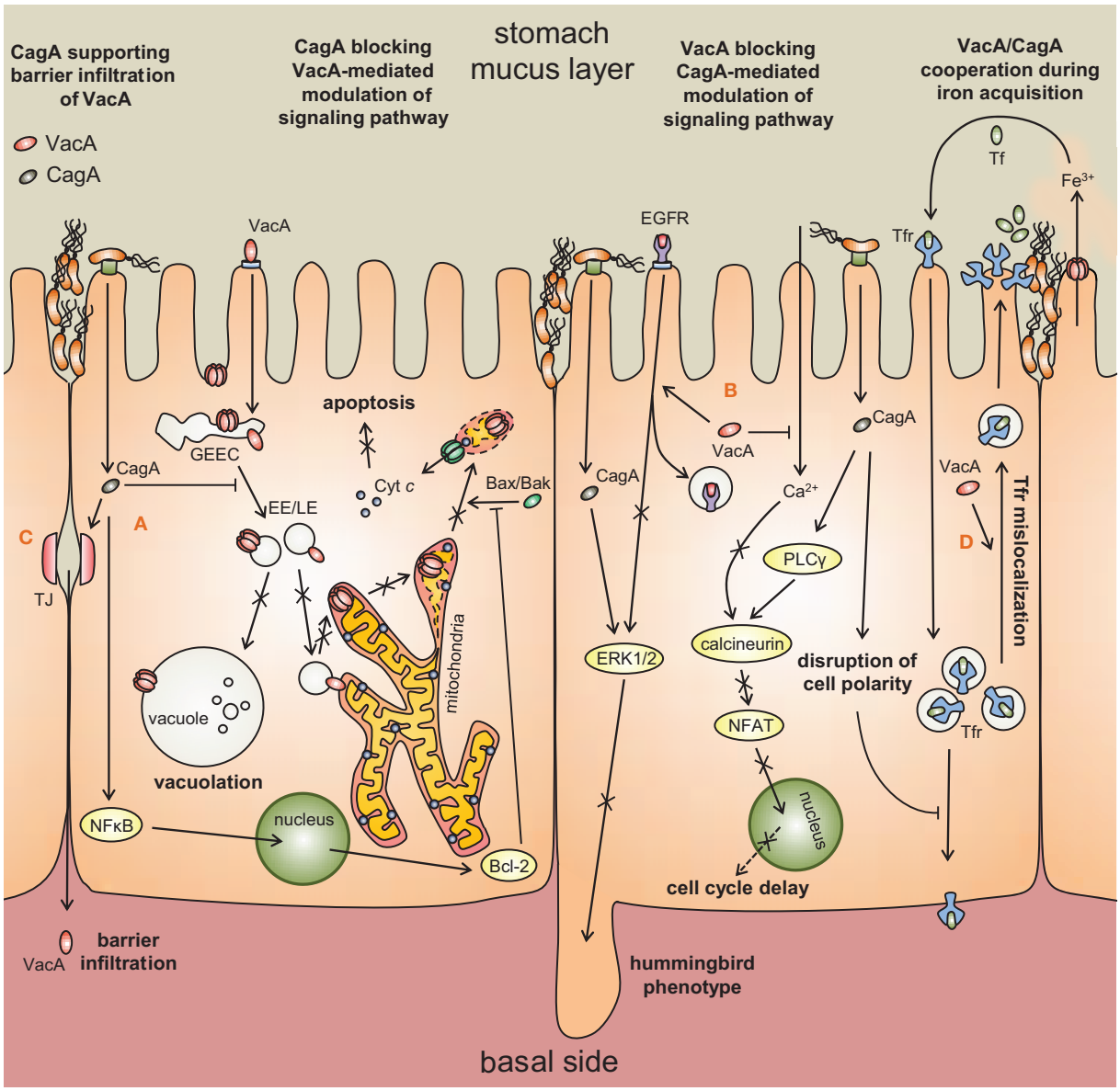

FIGURE 3 | Synergistic and antagonistic interactions between VacA and CagA. (A) CagA blocks intracellular trafficking of VacA [Oldani et al. (2009)], thereby inhibiting VacA-mediated vacuolation and apoptosis [Argent et al. (2008); Oldani et al. (2009)]. VacA-dependent apoptosis is further antagonized by CagA activation of Bcl-2 [Oldani et al. (2009)]. **(B)** VacA prevents CagA-mediated activation of ERK1/2 and hummingbird-like cellular morphological changes by misrouting the epidermal growth factor receptor (EGFR) [Tegtmeyer et al. (2009)]. VacA also counteracts the ability of CagA to activate NFAT, which has multiple roles in cell growth and differentiation [Yokoyama et al. (2005); Oldani et al. (2009)]. **(C)** CagA disruption of epithelial tight junctions (TJ) allows VacA to access underlying immune cells. **(D)** VacA and CagA collaborate to misdirect holotransferrin to *H. pylori* micro-colonies at the apical surface.

host cells. Because toxin translocation from membrane bound compartments is generally considered a highly inefficient process, an intriguing question remains how VacA intracellular activity is mediated by channel activity at mitochondria. As suggested by recent work into the mechanism underlying the uptake and transport of VacA, perhaps VacA activity does not require toxin translocation across a membrane, but perhaps, instead, delivery by a mechanism involving membrane fusion between VacA intracellular transport vesicles and mitochondria.

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Alterations in *Helicobacter pylori* triggered by contact with gastric epithelial cells

Elizabeth M. Johnson*[1†], *Jennifer A. Gaddy*[1†] *and Timothy L. Cover*[1,2,3]

[1] *Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN, USA*
[2] *Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA*
[3] *Veterans Affairs Tennessee Valley Healthcare System, Nashville, TN, USA*

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Robert Maier, University of Georgia, USA
Jay V. Solnick, University of California Davis, USA

***Correspondence:**
Timothy L. Cover, Division of Infectious Diseases, Vanderbilt University School of Medicine, A2200 Medical Center North, Nashville, TN 37232, USA.
e-mail: timothy.l.cover@vanderbilt.edu

[†] *Elizabeth M. Johnson and Jennifer A. Gaddy have contributed equally to this work.*

Helicobacter pylori lives within the mucus layer of the human stomach, in close proximity to gastric epithelial cells. While a great deal is known about the effects of *H. pylori* on human cells and the specific bacterial products that mediate these effects, relatively little work has been done to investigate alterations in *H. pylori* that may be triggered by bacterial contact with human cells. In this review, we discuss the spectrum of changes in bacterial physiology and morphology that occur when *H. pylori* is in contact with gastric epithelial cells. Several studies have reported that cell contact causes alterations in *H. pylori* gene transcription. In addition, *H. pylori* contact with gastric epithelial cells promotes the formation of pilus-like structures at the bacteria–host cell interface. The formation of these structures requires multiple genes in the *cag* pathogenicity island, and these structures are proposed to have an important role in the type IV secretion system-dependent process through which CagA enters host cells. Finally, *H. pylori* contact with epithelial cells can promote bacterial replication and the formation of microcolonies, phenomena that are facilitated by the acquisition of iron and other nutrients from infected cells. In summary, the gastric epithelial cell surface represents an important niche for *H. pylori*, and upon entry into this niche, the bacteria alter their behavior in a manner that optimizes bacterial proliferation and persistent colonization of the host.

Keywords: *Helicobacter pylori*, VacA, CagA, type IV secretion, *cag* pathogenicity island, iron, gastric cancer

INTRODUCTION

Helicobacter pylori is highly adapted for colonization of the human stomach, and is found in about half of all humans worldwide (Amieva and El-Omar, 2008; Atherton and Blaser, 2009; Cover and Blaser, 2009). *H. pylori* associates specifically with gastric mucosal tissue in the stomach or the duodenum, but not with intestinal or squamous-type epithelium (Wyatt et al., 1987, 1990; Carrick et al., 1989). The reasons for a specific association between *H. pylori* and gastric epithelium are not well understood. One possibility is that *H. pylori* utilizes specific components of gastric mucus or other factors released by gastric epithelial cells as nutritional sources. In addition, *H. pylori* may have a competitive advantage compared to other bacteria in the environment overlying gastric epithelial cells, but may lack this advantage in other sites.

Within the stomach, *H. pylori* can occupy a range of different microenvironments. The bacteria are typically most abundant within the gastric antrum, but can also be found within the corpus. *H. pylori* is found predominantly within the gastric mucus layer (Hazell et al., 1986), but occasionally can be internalized by gastric epithelial cells (Dubois and Boren, 2007); invasion beyond the epithelial layer is considered to be a rare event. Within the gastric mucus layer, the bacteria can be found relatively close to the gastric lumen or deep within gastric glands, and can be either free-swimming (Hazell et al., 1986; Schreiber et al., 2004; Celli et al., 2009) or attached to gastric epithelial cells (Hessey et al., 1990). At any given time, the proportion of adherent *H. pylori* is lower than the proportion of non-adherent organisms. Adherent bacteria localize preferentially to intercellular junctions (Hazell et al., 1986), but also can adhere to non-junctional sites. Relatively little is known about the dynamics of bacterial attachment to gastric epithelial cells. For example, it is not known whether adherent organisms remain attached for short durations and then detach, or whether adherent bacteria remain permanently attached and are eventually shed along with the gastric epithelial cells.

Because *H. pylori* is found in close proximity to gastric epithelial cells, there are numerous opportunities for the bacteria to cause alterations in gastric epithelial cell architecture and function. Many of the changes in gastric epithelial cells caused by *H. pylori* are attributable to the actions of two secreted bacterial proteins, VacA and CagA. VacA is a pore-forming toxin that is secreted by the bacteria through an autotransporter pathway. The cellular alterations caused by VacA include increased permeability of the plasma membrane, changes in endosomal structure and function, changes in mitochondrial membrane permeability, and cell death (Montecucco and de Bernard, 2003; Cover and Blanke, 2005; Rieder et al., 2005; Jones et al., 2010). CagA is an effector protein that is translocated directly from bacteria into host cells through the action of a type IV secretion system (Hatakeyama, 2004; Bourzac and Guillemin, 2005; Rieder et al., 2005; Backert et al., 2010; Fischer, 2011; Tegtmeyer et al., 2011; Terradot and Waksman, 2011). Both *cagA* and genes encoding components of this type IV secretion system are contained within a 40 kb chromosomal region known as

the *cag* pathogenicity island (PAI). There is heterogeneity among *H. pylori* strains, such that strains may contain an intact *cag* PAI, may contain fragments of this PAI, or may completely lack this region (Olbermann et al., 2010). Upon entry into gastric epithelial cells, CagA interacts with multiple intracellular target proteins and causes a wide array of alterations in cellular signaling, leading to changes in cell shape, increased cellular motility and cellular invasiveness, alterations in monolayer polarity and permeability, and increased cellular proliferation (Hatakeyama, 2004; Bourzac and Guillemin, 2005; Rieder et al., 2005; Backert et al., 2010; Tegtmeyer et al., 2011). Because CagA activates signaling pathways associated with carcinogenesis, it has been termed a "bacterial oncoprotein" (Hatakeyama, 2004). The actions of VacA and CagA on epithelial cells have been described in detail in other reviews (Montecucco and de Bernard, 2003; Hatakeyama, 2004; Bourzac and Guillemin, 2005; Cover and Blanke, 2005; Rieder et al., 2005; Backert et al., 2010; Jones et al., 2010; Tegtmeyer et al., 2011) and will not be discussed in detail here.

The effects of *H. pylori* on gastric epithelial cells and the specific bacterial factors that mediate these effects have previously been described in great detail. In contrast, relatively little work has been done to investigate alterations in *H. pylori* that may be triggered by bacterial contact with human cells. In this article, we review the multiple ways in which contact with gastric epithelial cells causes alterations in *H. pylori*.

TRANSCRIPTIONAL REGULATION OF *H. PYLORI* GENES IN RESPONSE TO BACTERIAL CONTACT WITH GASTRIC EPITHELIAL CELLS

Several studies have reported that *H. pylori* adherence to gastric epithelial cells triggers alterations in *H. pylori* gene transcription (Joyce et al., 2001; van Amsterdam et al., 2003; Kim et al., 2004; Gieseler et al., 2005). These alterations have been detected using a variety of approaches, including transcriptional reporter assays, quantitative real-time PCR (RT-PCR), and array-based hybridization methods.

To identify bacterial genes that are differentially expressed upon bacterial contact with gastric epithelial cells, one study analyzed a plasmid library derived from *H. pylori* strain 1061, containing random chromosomal fusions to a promoterless *cat* gene, which allowed chloramphenicol resistance to be used as a marker of gene expression (van Amsterdam et al., 2003). Twenty-one unique clones exhibited increased resistance to chloramphenicol in the presence of HM02 gastric epithelial cells, compared to the level of chloramphenicol resistance in the absence of these cells. Most of the clones were not characterized in detail, but one that exhibited a marked increase in chloramphenicol resistance contained a fusion to *vacA* (encoding the secreted toxin VacA; van Amsterdam et al., 2003). RT-PCR confirmed that bacterial contact with gastric epithelial cells resulted in increased levels of *vacA* transcription. These results provide evidence that *vacA* expression is upregulated upon bacterial contact with gastric epithelial cells.

Effects of cell contact on *H. pylori* gene transcription have also been assessed by using transcriptional reporter assays to monitor the expression of selected genes (Joyce et al., 2001). The transcriptional reporters were constructed by fusing nine putative promoter regions from the *cag* PAI with *ureB* (encoding the B subunit of urease). These reporters were introduced into the *hpn* locus of an *H. pylori* strain (C57) in which the endogenous *ureB* locus had been disrupted. When co-cultured with HEp-2 cells, two of the reporter strains [containing promoters upstream from *cagP* (*cag15*) and *cagG* (*cag21*)] exhibited increased UreB expression in comparison to when the bacteria were cultured in medium alone (Joyce et al., 2001). The other reporter strains (containing putative promoter regions of seven other *cag* genes) did not exhibit any significant increase in UreB expression following attachment to HEp-2 cells. A recent report, which describes the use of a high-throughput approach to analyze the transcriptome of *H. pylori*, verified that a transcriptional start site is present upstream from *cagP*, but a transcriptional start site was not identified immediately upstream from *cagG* (Sharma et al., 2010). Instead, it was reported that *cagG* is transcribed within an operon that originates further upstream (with *cagC* as the first gene), or as part of a suboperon (with *cagF* as the first gene) (Sharma et al., 2010). Multiple genes within these operons might be upregulated upon attachment of *H. pylori* to epithelial cells.

Macroarray hybridization methods also have been used to detect changes in *H. pylori* gene transcription following bacterial attachment to AGS gastric epithelial cells (Kim et al., 2004). When *H. pylori* strain 69a was co-cultured with these cells, the transcription of 22 *H. pylori* genes was upregulated and transcription of 21 genes was downregulated. Regulation of a subset of these genes was confirmed by PCR-based methods. The list of genes confirmed to be regulated included one in the *cag* PAI (*cag3*), two encoding outer membrane proteins (*omp6* and *omp11*), and genes encoding proteins involved in chemotaxis and motility (*flaA* and *flgB*), transport and binding functions (*tonB*), metabolic functions (*sodB*), and transcription- and translation-related functions (Kim et al., 2004).

Another study used quantitative RT-PCR to compare transcription levels of five *H. pylori* genes following infection of AGS cells with eight different *H. pylori* strains (Gieseler et al., 2005). Changes in gene expression in individual strains were reproducible, but many results were strain-specific. For example, upon infection of AGS cells, *cagA* mRNA was upregulated in one strain and downregulated in two other strains (Gieseler et al., 2005). Similar variation was also observed in the transcriptional regulation of *katA* (a catalase important for oxidative defense), *napA* (neutrophil activating protein), *ureA* (a urease subunit), and *vacA*. Therefore, there may be variation among *H. pylori* strains in the genes that are differentially expressed following contact with gastric epithelial cells.

Collectively, these reports provide evidence that *H. pylori* contact with gastric epithelial cells leads to alterations in bacterial gene transcription. Notably, there is very little overlap among the genes that have been identified. Some of the variability may be attributed to variation in study design. Differences in the choice of bacterial strains or cell lines also contribute to the variability. Despite the considerable variability in results, some cohesive elements can be found among these reports. For example, genes encoding virulence factors (including *vacA* and genes in the *cag* PAI) have been identified in several studies (Joyce et al., 2001; van Amsterdam et al., 2003; Boonjakuakul et al., 2004, 2005; Kim et al., 2004; Gieseler

et al., 2005; Scott et al., 2007; Castillo et al., 2008). Alterations in host cells induced by these virulence factors are predicted to alter the gastric environment in a manner that is favorable for *H. pylori*.

At present, very little is known about the mechanisms through which contact with epithelial cells leads to alterations in *H. pylori* gene transcription. One possibility is that specific factors (including ions, small molecules, or peptides) are released or secreted by the epithelial cells and sensed by adherent *H. pylori*. Conversely, if specific factors are bound, internalized, or metabolized by the epithelial cells, there may be a reduced concentration of these factors, and the reduced concentration might be sensed by adherent *H. pylori*. A second possibility is that binding of *H. pylori* to components on the surface of host cells might trigger alterations in the bacteria. A third possibility is that epithelial cells might release factors that can be used as nutrients by *H. pylori*, thereby stimulating bacterial metabolism and growth. Although multiple variables, including pH and concentrations of various metals or other ions are known to have an effect on *H. pylori* gene transcription (Merrell et al., 2003a,b; Loh et al., 2007), thus far the factors that stimulate transcriptional alterations in adherent *H. pylori* have not been identified. Similarly, the signaling pathways that may be involved in mediating cell contact-induced alterations in *H. pylori* gene transcription have yet to be identified.

There has been considerable interest in identifying genes that are upregulated during *H. pylori* colonization of mammalian hosts, compared to bacterial growth *in vitro* (Graham et al., 2002; Boonjakuakul et al., 2004, 2005; Scott et al., 2007; Castillo et al., 2008). One approach involved the use of recombination-based *in vivo* expression technology (RIVET) analysis to detect *H. pylori* gene expression in mice (Castillo et al., 2008). Among the six identified bacterial promoters that were induced in the host compared to *in vitro* conditions, three were predicted to regulate genes with potential roles in host colonization. Specifically, the promoter region upstream of *cagZ* (which likely controls expression of three genes in the *cag* PAI), the promoter predicted to regulate *mobA*, *mobB*, and *mobD* genes (which have roles in horizontal gene transfer of plasmid DNA), and the region upstream of a *vacA* paralog (HP0289, which encodes an autotransporter protein of unknown function) were all found to be upregulated in the host. Analysis of deletion mutant strains revealed that *cagZ*, *mobA*, *mobB*, and *mobD* are important for *H. pylori* colonization of mice (Castillo et al., 2008). Another study analyzed *H. pylori* gene expression during infection of rhesus macaques, and reported that the expression of seven genes, including several from an operon within the *cag* PAI, was increased early in infection of rhesus macaques in comparison to *H. pylori* growth *in vitro* at stationary phase (Boonjakuakul et al., 2005). In contrast, the transcription of many other genes, including five genes from the *cag* PAI, was decreased *in vivo* compared to stationary phase *in vitro* (Boonjakuakul et al., 2005). Although the experiments with animal models provide important insight into the differential expression of *H. pylori* genes *in vivo* and *in vitro*, it is not possible to discern whether the observed changes in gene expression *in vivo* result from bacterial contact with gastric epithelial cells or from bacterial exposure to other environmental conditions.

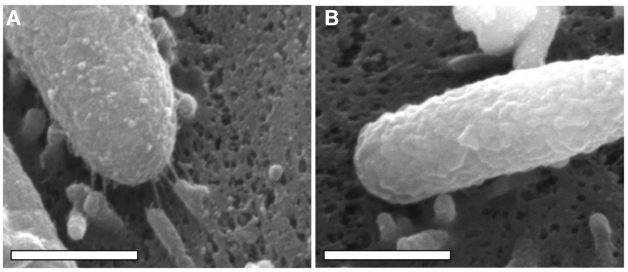

FIGURE 1 | Scanning electron microscopy analysis of bacterial cells in contact with AGS human gastric epithelial cells. *H. pylori* strain 26695 and a mutant strain of 26695 lacking the entire *cag* PAI (26695 Δ*cag* PAI) were co-cultured with AGS cells, and the cells were visualized by scanning electron microscopy, as described previously (Shaffer et al., 2011). Scanning electron micrographs of **(A)** *H. pylori* 26695 adhering to an AGS cell, **(B)** *H. pylori* 26695 Δ*cag* PAI adhering to an AGS cell. In **(A)**, pili are visible at the interface between the bacteria and the host cell. Magnification bars indicate 500 nm.

ASSEMBLY OF PILI FOLLOWING *H. PYLORI* CONTACT WITH GASTRIC EPITHELIAL CELLS

In addition to the observed alterations in *H. pylori* gene transcription that occur upon bacterial contact with gastric epithelial cells, alterations in bacterial cell morphology also occur. Specifically, extracellular structures termed "pili" are formed, and extend from the surface of the bacteria to the surface of the gastric epithelial cells (**Figure 1**). Multiple studies have reported that these structures are produced when *H. pylori* is co-cultured with gastric epithelial cells, whereas the structures are produced infrequently when *H. pylori* is cultured in the absence of epithelial cells (Rohde et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009; Shaffer et al., 2011). Initial studies noted that pili were synthesized by a wild-type *H. pylori* strain when in contact with gastric epithelial cells, but not by a mutant strain lacking the *cag* PAI (Rohde et al., 2003). Several individual genes within the *H. pylori cag* PAI have been reported to be required for pilus formation (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011). Since the *cag* PAI encodes components of a T4SS that translocates CagA into host cells (Fischer et al., 2001), these pili are considered to be bacterially encoded structures associated with the T4SS, rather than protrusions from the gastric epithelial cell.

Similar pili are features of the T4SSs of several bacterial species, including the *Agrobacterium tumefaciens* VirB/VirD4 system, T4SSs of *Legionella* and *Brucella*, and T4SSs associated with plasmid-encoded conjugation systems (Frost et al., 1986, 1994; Lai and Kado, 1998, 2000; Eisenbrandt et al., 1999; Lai et al., 2000; Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). The *Agrobacterium* VirB/VirD4 system serves as a model for understanding T4SS assembly and architecture (Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). The pili encoded by this system, known as T-pili, are about 10 nm in diameter (Lai and Kado, 2000; Lai et al., 2000; Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009), which allows them to be distinguished from other types of pili (3 nm diameter) and flagella (15 nm diameter). Plasmid-encoded conjugative pili are also 8–12 nm in diameter (Frost et al., 1986, 1994;

Eisenbrandt et al., 1999; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). *Agrobacterium* T-pili are comprised of a major component (VirB2) and a minor component (VirB5) that is localized to the tips of the pili (Aly and Baron, 2007; Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). VirB2 association with VirB5 is required for pilus production (Krall et al., 2002; Yuan et al., 2005), and VirB5 also controls the length of the pilus (Aly and Baron, 2007). In addition to having a role in DNA and protein translocation, these proteins may have a role in facilitating bacterial contact with host cells (Yeo et al., 2003; Hwang and Gelvin, 2004; Alvarez-Martinez and Christie, 2009). T-pilus biogenesis requires multiple *virB* genes, but not *virD4* (Fullner et al., 1996; Lai et al., 2000; Fronzes et al., 2009).

Several Cag proteins have been detected as constituents of *H. pylori cag* T4SS-associated pili, using immunogold labeling and electron microscopic methods. These include CagY, CagT, CagX, and CagL, as well as the effector protein CagA (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009). Immunoelectron microscopy analyses revealed that CagY is present on the sides of the pili in patches or continuous extensions, and it was proposed that CagY represents a pilus sheath (Rohde et al., 2003). CagY is characterized by numerous repeat units; variation in these repeat units may provide a means for evading host immune defenses (Liu et al., 1999; Aras et al., 2003; Delahay et al., 2008). CagT was detected at the base of the pili in one study and along the length of the pili in another study (Rohde et al., 2003; Tanaka et al., 2003). Since CagT, CagX, and CagY are homologs of T4SS components (VirB7, VirB9, and VirB10, respectively) that comprise a core complex spanning the inner and outer membranes in T4SSs of other bacterial species, it is somewhat surprising that these proteins have been localized to the pilus in *H. pylori*. Each of these proteins has been localized to a *H. pylori* membrane fraction, and there is evidence that CagT, CagX, and CagY are constituents of a protein complex (Kutter et al., 2008); these observations suggest that these proteins might comprise a T4SS core complex in *H. pylori*.

Helicobacter pylori CagC exhibits weak homology to VirB2, the major structural component of *Agrobacterium* pili, and therefore it has been proposed that CagC may serve the same function in the *H. pylori cag* T4SS (Andrzejewska et al., 2006). Although CagC was detected on the surface of *H. pylori* (Andrzejewska et al., 2006), this protein has not been definitively localized to *H. pylori* pili. CagL is proposed to have a role corresponding to the VirB5 minor pilus subunit of *Agrobacterium* T-pili (Backert et al., 2008). CagL exhibits weak sequence homology to the VirB5 ortholog of *Brucella* spp., but it lacks any substantial sequence relatedness to *Agrobacterium* VirB5 (Backert et al., 2008). CagL contains a conserved LQxR motif at the C terminus, which is similar to the motif found in VirB5 of *Brucella suis* and plasmid-encoded conjugation systems (Backert et al., 2008).

The reported dimensions of *H. pylori cag* T4SS-associated pili vary considerably among different studies. One study reported that the structures measure about 14 nm in width (Shaffer et al., 2011), which is similar to the diameters of pili in T4SSs of other bacterial species (Alvarez-Martinez and Christie, 2009; Fronzes et al., 2009). Another study reported that the structures were either 45 or 70 nm in width, depending on whether or not a sheath was present (Rohde et al., 2003). The difference in reported dimensions among studies is not attributable to a difference in *H. pylori* strains, since *H. pylori* strain 26695 was used in all of these studies. Potentially the discrepancy in dimensions is attributable to differences in electron microscopy methods. For example, in some studies, images of the pili were generated using a method in which the structures were coated with carbon, whereas in other studies, images were generated using a method in which the structures were coated with a relatively thinner layer of gold (Rohde et al., 2003; Kwok et al., 2007; Jimenez-Soto et al., 2009; Shaffer et al., 2011). Another factor that may help to account for different results among the studies is that the bacteria were not consistently cultured under the same conditions. Some studies visualized exclusively structures that were present when *H. pylori* was co-cultured with gastric epithelial cells, whereas other studies also visualized structures that were present when the bacteria were cultured in the absence of epithelial cells.

While multiple lines of evidence indicate that the assembly of *H. pylori* pili requires genes in the *cag* PAI (Rohde et al., 2003; Kwok et al., 2007; Shaffer et al., 2011), the composition of these pili remains incompletely characterized and the complete set of genes required for pilus biogenesis has not been defined. Biochemical analysis of these pili has been difficult because the assembly of these structures requires co-culture of *H. pylori* with gastric epithelial cells; pili are produced infrequently when *H. pylori* is grown in the absence of gastric epithelial cells (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011). Several individual genes within the *H. pylori cag* PAI, including *cagI*, *cagL*, *cagT*, *cagX*, and *cagY* are reported to be required for biogenesis of pili (Rohde et al., 2003; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011), and these genes are also required for CagA translocation into host cells (Fischer et al., 2001; Shaffer et al., 2011). In many cases, the evidence supporting a role of these genes in pilus production was reported as "data not shown" and complemented mutant strains were not analyzed. One study reported that *cagα/virB11* ATPase was required for pilus formation, and another study reported that it was not required (Tanaka et al., 2003; Kwok et al., 2007). Since at least fourteen genes encoded within the *cag* PAI are essential for CagA translocation (Fischer et al., 2001), it seems probable that several additional Cag proteins might be required for pilus formation. It is notable that pilus biogenesis requires at least two genes (*cagI* and *cagL*) that lack obvious homologs in T4SSs of other bacterial species (Shaffer et al., 2011), and CagL has been detected as a structural component of *H. pylori* pili (Kwok et al., 2007). This suggests that there are unique features of the *H. pylori* T4SS compared to T4SSs in other bacteria.

The dimensions of appendages on the surface of bacteria can be regulated by a variety of processes. For example, molecular rulers such as YscP of *Yersinia pestis* and FliK flagellar protein of *Salmonella* spp. control the dimensions of T3SS needles and flagella, respectively (Makishima et al., 2001; Journet et al., 2003; Mota et al., 2005). As a clue into the mechanisms by which assembly of *H. pylori cag* pili might be regulated, it is of interest that a Δ*cagH* mutant strain formed pili that were thicker and longer than the pili formed by a wild-type strain (Shaffer et al., 2011). This suggests that CagH has a role in regulating pilus dimensions. The mechanism by which CagH regulates pilus dimensions is not known, but CagH contains a flagellar hook (FlgK) domain closely related to

the corresponding domain in FlgK from *Burkholderia* spp. (Shaffer et al., 2011). Possibly CagH has a role in terminating pilus assembly, analogous to the role of FlgK in terminating FlgK assembly. Alternatively, CagH may serve as a molecular ruler, analogous to FliK and YscP, to control the dimensions of *cag* T4SS pili in *H. pylori*.

An important finding was the discovery that CagL, a component of *H. pylori* pili, binds to integrins on the host cell surface (Kwok et al., 2007; Saha et al., 2010; Tegtmeyer et al., 2010; Conradi et al., 2012). Initially it was reported that CagL binding to α5β1 integrin was mediated by an RGD motif within CagL (Kwok et al., 2007), but a subsequent report suggested that this binding may occur independently of the RGD motif (Jimenez-Soto et al., 2009). In addition to CagL, several other Cag proteins, including CagI, CagY, and CagA are capable of binding β1 integrin (Jimenez-Soto et al., 2009). Integrins are primarily localized to the basolateral surfaces of polarized epithelial cells, and therefore, it is not clear how *H. pylori* on the apical surface of an intact gastric epithelial cell monolayer would contact integrins. One possibility is that *H. pylori* may alter the localization of this receptor, resembling the process by which enteropathogenic *Escherichia coli* perturbs the basolateral localization of β1 integrin (Muza-Moons et al., 2003). Alternatively, *H. pylori* may gain access to integrins by disrupting the integrity of the gastric epithelial monolayer (Amieva et al., 2003).

Since pili are formed when *H. pylori* is in contact with gastric epithelial cells but rarely in the absence of eukaryotic cells (Rohde et al., 2003; Kwok et al., 2007; Shaffer et al., 2011), the formation of these structures must be tightly regulated and there must be a stimulus that triggers pilus formation. Similarly, assembly of the *Agrobacterium* T4SS is regulated and is stimulated by factors such as acetosyringone (Stachel et al., 1986). One hypothesis is that the interaction of *H. pylori* with host cell integrins might provide a stimulus for pilus formation. Alternatively, other components of the plasma membrane or environmental conditions found at the plasma membrane surface may be relevant. α5β1 integrin associates with cholesterol-rich microdomains or lipid rafts (Hutton et al., 2010), and depletion of host cholesterol results in an abrogation of *H. pylori cag* T4SS function (Lai et al., 2008; Hutton et al., 2010). Therefore, it is possible that multiple components of lipid rafts may promote assembly of *cag* T4SS-associated pili.

The binding of *H. pylori* adhesins to receptors on host cells may also influence assembly or action of the T4SS. For example, the activity of the T4SS is enhanced by the presence of the BabA adhesin (Ishijima et al., 2011). This suggests that adherence of *H. pylori* to gastric epithelial cells through BabA or other adhesins might be required for pilus biogenesis.

The functional role of pili associated with the *cag* T4SS is not yet fully understood. One hypothesis is that the pili are directly responsible for CagA translocation. In support of this hypothesis, CagA has been detected by immunogold labeling at the tip of the pili (Kwok et al., 2007; Jimenez-Soto et al., 2009), and several *H. pylori* mutant strains that fail to form pili lack the ability to translocate CagA into host cells (Fischer et al., 2001; Tanaka et al., 2003; Kwok et al., 2007; Shaffer et al., 2011). If pili are directly responsible for CagA translocation into cells, it is unclear whether CagA passes through a channel within the pilus or along the outside of the pilus. Another hypothesis is that CagA translocation into cells occurs through a pilus-independent process, and the pili merely serve to anchor *H. pylori* to host cells. For example, when *H. pylori* adheres to epithelial cells, host phospholipids such as phosphatidylserine are externalized to the outer leaflet of the membrane, and this mislocalization of phosphatidylserine may facilitate the delivery of CagA into host cells through a pilus-independent process (Murata-Kamiya et al., 2010).

Helicobacter pylori stimulates production of IL-8 by epithelial cells through a *cag* PAI-dependent pathway (Fischer et al., 2001), but the role of pili in this process also remains poorly defined. *H. pylori*-induced IL-8 secretion can occur through both a CagA-dependent process and a CagA-independent process, and the latter phenomenon is attributed to intracellular entry of peptidoglycan (Viala et al., 2004; Brandt et al., 2005). Since intracellular entry of peptidoglycan is dependent on an intact *cag* T4SS (Viala et al., 2004), it is possible that this process may require the presence of pili. If so, it is unclear whether peptidoglycan is translocated through the pilus, or whether the pilus serves alternate functions, such as anchoring *H. pylori* to host cells or permeabilizing the epithelial cells to allow intracellular entry of peptidoglycan.

FORMATION OF *H. PYLORI* MICROCOLONIES ON THE EPITHELIAL CELL SURFACE

Investigations of *H. pylori* and polarized epithelial cell systems have shown that bacterial contact with epithelial cells can stimulate growth of the bacteria. In these experiments, *H. pylori* preferentially attached to intercellular junctions on the apical surface of cells and then formed microcolonies at these sites (Tan et al., 2009). Time-lapse microscopy experiments indicated that the microcolonies arose as a result of binary fission (Tan et al., 2009). *H. pylori* cultured under the same conditions in the absence of epithelial cells were not capable of replicating.

Isogenic *cagA* mutants were unable to replicate on the apical surface of polarized epithelial cells, which indicates that actions of CagA are required for bacterial replication in this site (Tan et al., 2009). Interestingly, *cagA* mutant strains, while defective in ability to replicate on the apical cell surface, were able to replicate on the basolateral surface. Since CagA can cause alterations in cell polarity (Amieva et al., 2003), it was proposed that CagA-induced disruption of cell polarity contributes to *H. pylori* growth on the apical surface (Tan et al., 2009). In support of this hypothesis, disruption of cell polarity with inhibitors of atypical protein kinase C or Parb1 enabled a *cagA* mutant strain to grow on the surface of polarized cells (Tan et al., 2009). Apical co-infection of polarized epithelial cells with *cagA* mutant bacteria and wild-type bacteria did not rescue the growth defect of the *cagA* mutant bacteria, which suggests that cellular alterations induced by CagA may occur in a localized manner.

As a potential mechanism for the observed growth of *H. pylori* on the apical surface of polarized epithelial cells, it has been proposed that the bacteria can derive nutrients from the epithelial cells, and that acquisition of such nutrients permits growth of adherent bacteria. Thus far, acquisition of iron has been studied in greatest detail. One series of experiments showed that although *cagA* mutant bacteria were unable to grow on the apical surface of polarized epithelial cells, the addition of exogenous ferric chloride to the apical medium partially rescued this defect (Tan et al., 2011). Likewise, in comparison to wild-type bacteria, an isogenic *vacA* mutant strain demonstrated decreased microcolony formation on the apical surface of polarized epithelial cells, and addition of iron

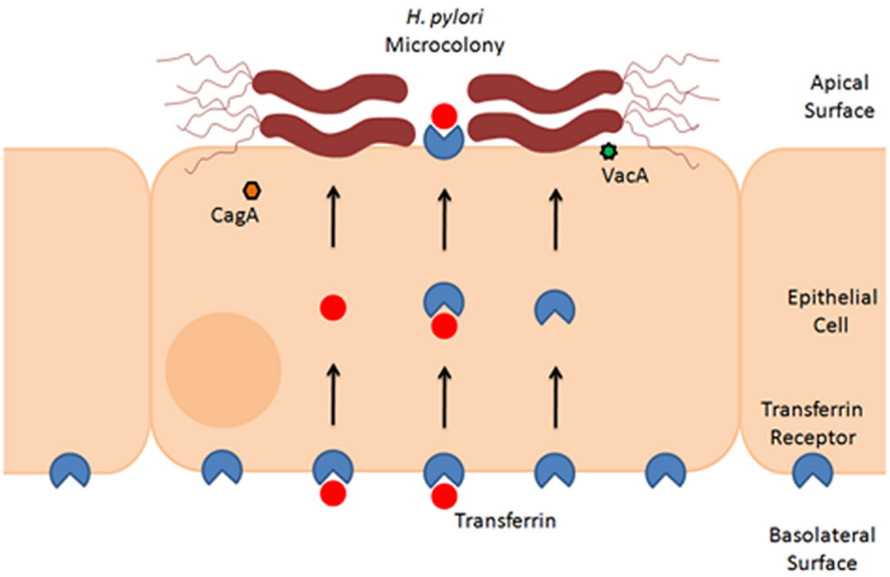

FIGURE 2 | Formation of *H. pylori* microcolonies on the surface of epithelial cells. This schematic figure illustrates that *H. pylori* can form microcolonies on the surface of polarized epithelial monolayers. Microcolony formation is dependent in part on acquisition of iron from host cells, through a process that involves actions of CagA and VacA (Tan et al., 2011). Experimental studies indicate that *H. pylori* stimulates basolateral uptake and transcytosis of transferrin (red symbols), as well as mislocalization of the transferrin receptor (blue symbols) from the basolateral to the apical surface of polarized cells (Tan et al., 2011). Therefore, a current model proposes that *H. pylori* acquires iron from cells by utilization of holotransferrin (i.e., transferrin saturated with iron) (Tan et al., 2011).

to the apical chamber rescued this defect (Tan et al., 2011). This leads to the hypothesis that CagA and VacA facilitate acquisition of iron from host cells. As a possible mechanism, it was shown that both CagA and VacA contribute to increased basolateral uptake and transcytosis of transferrin as a source of iron (Tan et al., 2011; **Figure 2**). Mislocalization of the transferrin receptor from the basolateral to the apical surface of polarized cells was also observed. Silencing of transferrin receptor expression resulted in reduced *H. pylori* growth on the apical surface (Tan et al., 2011). The ability of CagA to increase transferrin uptake was dependent on intact CagA EPIYA motifs, which are sites where CagA undergoes tyrosine phosphorylation within host cells. Although VacA is involved in apical mislocalization of the transferrin receptor to sites of bacterial attachment, the addition of exogenous VacA to cells did not stimulate transferrin receptor mislocalization. This suggests that the observed alterations in transferrin receptor localization are dependent on delivery of VacA by intact bacteria.

These studies using polarized epithelial monolayers provide important insights into the process whereby *H. pylori* can obtain iron from host cells, and reveal that CagA and VacA have important roles in this process. It is notable that the addition of iron to the apical medium only partially rescued the ability of a Δ*cagA* mutant to grow on the apical surface of polarized cells (Tan et al., 2011). This leads to the hypothesis that the bacteria may acquire a variety of other nutrients besides iron from host cells.

CONCLUSION

The surface of gastric epithelial cells represents an important niche for *H. pylori*. The literature discussed in this review, focusing on alterations in *H. pylori* that occur upon bacterial contact with these cells, provides a glimpse of the complex interactions that occur at the bacteria–host cell interface. Alterations in *H. pylori* gene transcription upon bacterial contact with gastric epithelial cells have not yet been investigated in detail, but the finding that transcription of *vacA* and several *cag* PAI genes may be altered provides an initial framework for understanding changes that the bacteria undergo in this environment (Joyce et al., 2001; van Amsterdam et al., 2003; Boonjakuakul et al., 2004, 2005; Kim et al., 2004; Gieseler et al., 2005; Scott et al., 2007; Castillo et al., 2008). Upon contact with gastric epithelial cells, the bacteria do not only change their transcriptional profile, but also form distinct pilus-like structures. Changes in bacterial transcription and the formation of these pili probably facilitate the delivery of bacterial effector molecules, such as VacA and CagA, into host cells. These effector molecules cause cellular changes that result in an increased availability of iron and other nutrients to the bacteria. Upon uptake of these nutrients, *H. pylori* alters its behavior by replicating and forming microcolonies on the apical cell surface. We speculate that specific components on the surface of gastric epithelial cells (or specific environmental conditions present at the host–pathogen interface) are sensed by *H. pylori*, and that the bacteria accordingly modulate their morphology and ultrastructure to adapt to this environment. Such alterations may optimize the ability of *H. pylori* to proliferate and may promote persistent colonization of the host.

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Exoribonucleases as modulators of virulence in pathogenic bacteria

Rute G. Matos, Cátia Bárria, Vânia Pobre, José M. Andrade and Cecília M. Arraiano*

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal
**Correspondence: cecilia@itqb.unl.pt*

A commentary on

Polynucleotide phosphorylase has an impact on cell biology of *Campylobacter jejuni*

Haddad, N., Tresse, O., Rivoal, K., Chevret, D., Nonglaton, Q., Burns, C. M., Prévost, H., and Cappelier, J. M. (2012). Front. Cell. Inf. Microbio. doi: 10.3389/fcimb.2012.00030

Pathogenic bacteria are responsible for severe diseases worldwide. RNA stability is a major player controlling the expression of virulence factors. Ribonucleases (RNases) are the enzymes responsible for the maturation and degradation of RNA molecules (Arraiano et al., 2010; Silva et al., 2011). Exoribonucleases have been implicated in virulence in an increasing number of pathogens such as *Salmonella enterica*, *Helicobacter pylori*, *Shigella flexneri*, and *Aeromonas hydrophila* (see Andrade et al., 2009; Matos et al., 2011 and references below). However, the mechanisms underlying virulence are still mostly elusive (Arraiano et al., 2010; Lawal et al., 2011).

The recently published paper by Haddad et al. (2012) adds to this list *Campylobacter jejuni*, one of the most important human foodborne pathogens. *Campylobacter* is recognized as the leading bacterial cause of gastroenteritis and even more severe clinical manifestations can arise. The present work shows that *C. jejuni* bacteria lacking an 3′–5′ exoribonuclease called polynucleotide phosphorylase (PNPase) is significantly less virulent than the wild-type strain (Haddad et al., 2012).

Different steps have been identified in the ability of different pathogenic bacteria to promote infection, namely motility, adherence, invasion, intracellular replication, or spreading to the neighboring cells. Inactivation of the *C. jejuni* PNPase is shown to affect many of these steps, with *pnp* mutants showing distinct phenotypes such as limitations in swimming, substantial delay in the colonization of the chicken gut and a decreased ability to adhere and invade cells. Defects in motility are suggested to be responsible for many of the attenuation of the virulent traits of *C. jejuni* in the mutant *pnp* strain. Interestingly, the authors suggest that PNPase may be able to affect flagella-dependent motility by modulation of the NANA synthetase (*neu*B), involved in the post-translational modification of the flagellin subunit. Furthermore, proteomic studies also showed that PNPase affects the synthesis of proteins involved in virulence, such as LuxS and PEB3. This work confirms the importance of exoribonucleases, namely PNPase, in cell biology, and virulence (Haddad et al., 2012).

Bacterial pathogens rapidly adapt to environmental challenges. Adaptation requires a rapid adjustment in RNA levels, requiring not only transcriptional regulation, but also fine-tuning control of RNA stability. Stress-resistance plays an essential role in the capacity of many pathogenic bacteria to establish and maintain long-term intracellular residence in host cells. Many ribonucleases are regulated by stress conditions, being critical enzymes involved in the adaptation of bacteria to new environmental conditions. In particular, PNPase is a cold-shock protein in *Escherichia coli* being essential for growth at low temperatures (Zangrossi et al., 2000). Haddad et al. (2009) had previously shown that PNPase was also crucial for *C. jejuni* growth under cold-shock conditions. This was a relevant discovery especially when considering that this pathogen can persist and grow at refrigerated temperatures. PNPase also seems to be involved in *C. jejuni* resistance to acidic and oxidative stresses, as the *pnp* strain shows variations in the levels of the stress-response proteins KatA, DnaK, and Hsp90 (Haddad et al., 2012). In *S. enterica*, PNPase was shown to be important for acute infection and lethality in a murine model as result of increasing expression of the pathogenicity islands (Clements et al., 2002). In *Yersinia pseudotuberculosis* and *Y. pestis* PNPase was shown to be essential for the function of the *Yersinia* type tree secretion system (TTSS), an organelle that injects effector proteins directly into host cells (Rosenzweig et al., 2007). Interestingly, PNPase has been involved in the post-transcriptional regulation of small non-coding RNAs (Andrade and Arraiano, 2008; De Lay and Gottesman, 2011; Andrade et al., 2012). In *C. jejuni*, not much is known about this class of regulatory RNAs but transcriptomic studies have identified five candidate regions for harboring sRNAs (Chaudhuri et al., 2011). It is an exciting hypothesis that PNPase is able to regulate small RNAs possibly involved in the virulence traits of *C. jejuni* although this lacks experimental evidence at the time.

Together with PNPase, RNase II, and RNase R are the major exoribonucleases involved in RNA degradation in *E. coli* (**Figure 1**). Orthologs have been described in all domains of life (Arraiano et al., 2010). RNase R, a hydrolytic exoribonuclease, is also known to be involved in the virulence of several microorganisms. Like PNPase, RNase R is a cold-shock protein essential for the survival at low temperatures of several microorganisms, such as *E. coli*, *Pseudomonas putida*, *P. syringae*, and *A. hydrophila*. In some microorganisms RNase R was shown to be necessary for the expression of several invasion factors and mutations on its gene resulted in the reduced expression of virulence phenotypes in *S. flexneri* and in enteroinvasive *E. coli* (Tobe et al., 1992). *Legionella pneumophila* is an intracellular parasite of free-living protozoa which inhabits man-made water distribution systems, and is the most frequent cause of human legionellosis, community-acquired, and nosocomial pneumonia in adults. In this microorganism, RNase R is the only hydrolytic exoribonuclease present. Its activity was shown to be essential for growth and viability at low temperatures and induces competence (Charpentier et al., 2008). Similarly to what was shown in *E. coli* (Cairrão et al., 2003),

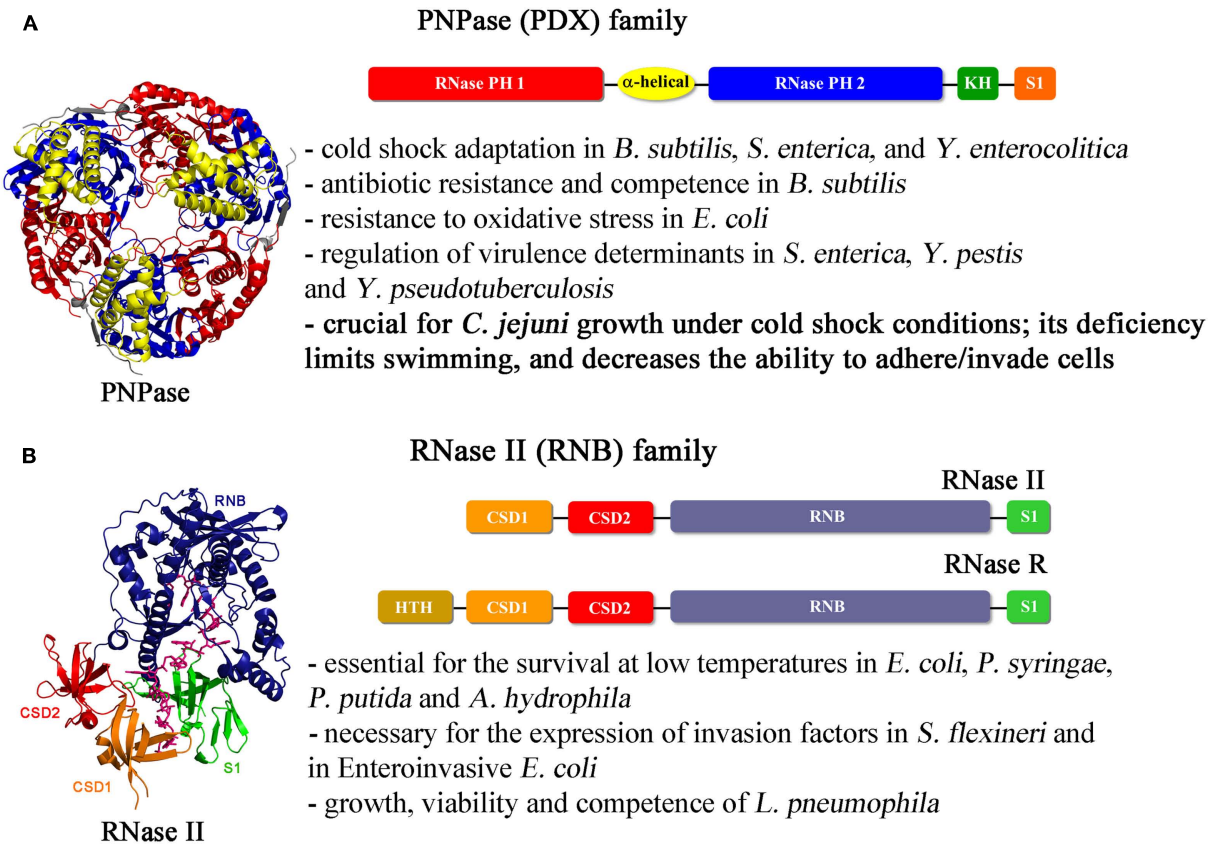

FIGURE 1 | Schematic representation of the domains found in the exoribonucleases from PNPase and RNase II families and structures of representative members. (A) Top: PNPase (PDX family) primary structure: two RNase PH catalytic domains followed by two RNA binding domains (KH and S1). PNPase is a trimer and assembles in a donut like shape (on the left; Shi et al., 2008). The S1 binding domain was shown to restore PNPase deletion effect in *Yersinia* (Rosenzweig et al., 2007). **(B)** Top: linear representation of RNase II and RNase R domains: the central catalytic RNB, and the CSD1, CSD1, and S1 RNA binding domains. Members of the family can have additional domains at the N-terminal region, namely Helix-Turn-Helix in RNase R. On the left is represented the *E. coli* RNase II–RNA complex crystal structure, showing the distinct domains of the enzyme, and the 13-mer bound RNA (Frazão et al., 2006). The RNB domain (in blue), is the responsible for the catalytic activity of the protein which, for example, is important for the development of competence in *L. pneumophila* (Charpentier et al., 2008).

RNase R is also a cold-shock protein in *A. hydrophila*. In this highly toxic microorganism, which is resistant to multiple medications, chlorine, and cold temperatures, RNase R was shown to be essential for viability at lower temperatures and its absence leads to a reduction in motility. The infection of mouse cells with *A. hydrophila rnr* mutant strains showed that their virulence was attenuated in comparison to the wild-type, which confirms the role of RNase R in pathogenesis (Erova et al., 2008).

Considering the important functions that these proteins have in the establishment of virulence, ribonucleases (namely RNase II, RNase R, and PNPase) offer a new perspective for developing efficient compounds in clinical treatments: they can be potential targets to design compounds able to kill specific microorganisms or to reduce their virulence ability. The further study of the function of exoribonucleases in the control of pathogenesis will certainly help in the comprehension of RNA-related processes involved in infection.

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The role of microRNAs in *Helicobacter pylori* pathogenesis and gastric carcinogenesis

Jennifer M. Noto*[1] *and* ***Richard M. Peek*[1,2]**

[1] *Division of Gastroenterology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA*
[2] *Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN, USA*

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Anne Müller, University of Zurich, Switzerland
Fabien Darfeuille, INSERM, France
Aime Franco, University of Arkansas for Medical Sciences, USA

***Correspondence:**
Jennifer M. Noto, Division of Gastroenterology, Department of Medicine, Vanderbilt University Medical Center, 2215 Garland Avenue, 1030C MRB IV, Nashville, TN 37232-0252, USA.
e-mail: jennifer.noto@vanderbilt.edu

Gastric carcinogenesis is a multistep process orchestrated by aberrancies in the genetic and epigenetic regulation of oncogenes and tumor suppressor genes. Chronic infection with *Helicobacter pylori* is the strongest known risk factor for the development of gastric cancer. *H. pylori* expresses a spectrum of virulence factors that dysregulate host intracellular signaling pathways that lower the threshold for neoplastic transformation. In addition to bacterial determinants, numerous host and environmental factors increase the risk of gastric carcinogenesis. Recent discoveries have shed new light on the involvement of microRNAs (miRNAs) in gastric carcinogenesis. miRNAs represent an abundant class of small, non-coding RNAs involved in global post-transcriptional regulation and, consequently, play an integral role at multiple steps in carcinogenesis, including cell cycle progression, proliferation, apoptosis, invasion, and metastasis. Expression levels of miRNAs are frequently altered in malignancies, where they function as either oncogenic miRNAs or tumor suppressor miRNAs. This review focuses on miRNAs dysregulated by *H. pylori* and potential etiologic roles they play in *H. pylori*-mediated gastric carcinogenesis.

Keywords: gastric cancer, *Helicobacter pylori*, microRNA, cell cycle, proliferation, apoptosis

INTRODUCTION

Microbial infections are among the most significant causes of cancer worldwide with nearly one in five malignancies resulting from infectious agents (Parkin, 2006). Gastrointestinal cancers represent a major global health concern and account for nearly 20% of all cancer-related deaths (Ferlay et al., 2007). Despite the decreasing incidence of gastric cancer in developed countries, it remains the second leading cause of cancer-related death throughout the world, with ∼700,000 deaths attributed to this malignancy annually (Parkin et al., 2005). The major contributing factor to the development of gastric cancer is colonization and chronic infection by the bacterial pathogen, *Helicobacter pylori*. *H. pylori* selectively colonizes the gastric epithelium of over 50% of the world's population and typically persists for the lifetime of the host. Among colonized individuals, however, only a fraction develop gastric adenocarcinoma, emphasizing the importance of understanding the pathogenic mechanisms by which *H. pylori* promotes chronic inflammation and the progression to gastric cancer.

VIRULENCE FACTORS THAT MEDIATE *HELICOBACTER PYLORI* PATHOGENESIS

Chronic gastric inflammation induced by the bacterial pathogen, *H. pylori*, is the strongest known risk factor for the development of atrophic gastritis, metaplasia, dysplasia, and ultimately gastric adenocarcinoma (**Figure 1**). *H. pylori* is a Gram-negative, helical-shaped bacterium specifically adapted to persist within the human gastric niche. *H. pylori* possesses numerous elements to successfully colonize the gastric mucosa, establish chronic infection, and induce gastric pathology. *In vivo*, approximately 20% of *H. pylori* adhere to the gastric epithelium (Hessey et al., 1990). The large repertoire of adhesins expressed by *H. pylori* likely contribute to its specific adaptation to the gastric niche, allowing flexibility to target specific host cells and to exert a dynamic range of effector functions on host cells. *H. pylori* expresses a number of adhesins that have been linked to virulence. SabA (sialic acid-binding adhesin), which binds host sialyl-Lewis^x, contributes to *H. pylori* persistence and mediates chronic gastric inflammation and injury (Mahdavi et al., 2002). The presence of blood group antigen-binding adhesin (BabA), which binds the host Lewis^b blood group antigen, increases the risk of gastric cancer in a synergistic fashion with other virulence factors, such as CagA (Ilver et al., 1998; Gerhard et al., 1999).

Following adherence and colonization of the gastric mucosa, *H. pylori* induces chronic gastritis and gastric injury, which are characterized by both neutrophilic and lymphocytic inflammation (Marshall et al., 1985; Goodwin et al., 1986). *H. pylori* expresses a number of factors capable of modulating the host immune system and eliciting proinflammatory immune responses. Some of these virulence factors include vacuolating cytotoxin (VacA) and the *cag* (cytotoxin associated gene) pathogenicity island. VacA is coded by the gene *vacA*, which is present in all strains of *H. pylori*, and which exhibits vacuolating activity (Leunk et al., 1988; Cover and Blaser, 1992; Cover et al., 1994; Phadnis et al., 1994; Schmitt and Haas, 1994; Telford et al., 1994). Additionally, VacA can induce apoptosis of host cells (Kuck et al., 2001; Xia and Talley, 2001) and suppress proliferation of T and B lymphocytes (Boncristiano et al., 2003; Gebert et al., 2003; Sundrud et al., 2004), which may contribute to the persistence of *H. pylori* through dysregulation of the host immune response. The *cag* pathogenicity island is present in

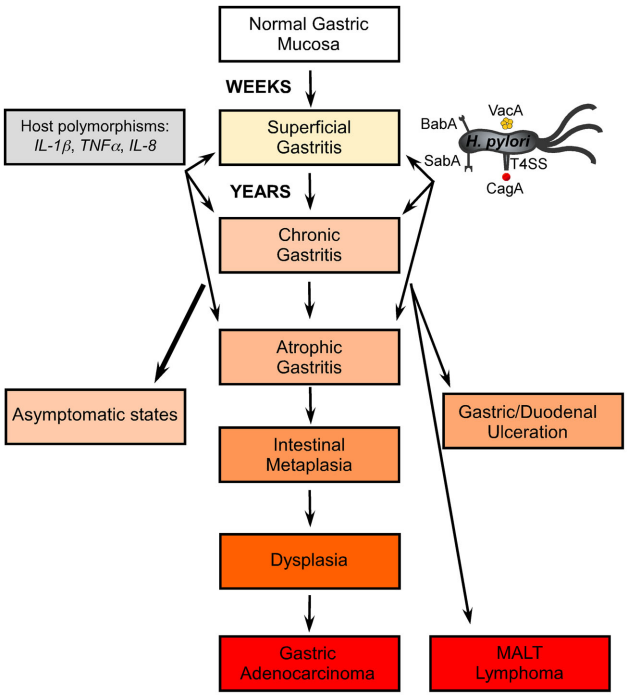

FIGURE 1 | Progression to gastric adenocarcinoma. *H. pylori* colonizes the human gastric mucosa and causes superficial gastritis and chronic inflammation over weeks to months. *H. pylori* expressing key virulence factors, such as the *cag* type 4 secretion system (T4SS), and host genetic polymorphisms in *IL-1β*, *TNFα*, and *IL-8* promoters that promote high expression of proinflammatory cytokines, augment the risk for developing atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately, gastric adenocarcinoma.

∼60% of all *H. pylori* strains and its presence is strongly associated with an increased risk of severe gastritis, ulcer disease, and gastric cancer (Censini et al., 1996; Tomb et al., 1997; Akopyants et al., 1998; Alm et al., 1999). The *cag* island encodes a type 4 secretion system (T4SS), which injects effector molecules, such as CagA, into host cells. CagA is a 120- to 140-kD protein that contains a number of tyrosine phosphorylation motifs (Covacci et al., 1993; Tummuru et al., 1993). Following its injection into host cells, CagA exerts a wide range of phosphorylation-dependent and independent effects, such as cytoskeletal rearrangements, disruption of cell polarity, and mitogenic and proinflammatory responses (Polk and Peek, 2010). Cumulatively, these bacterial factors contribute to adherence, persistence, host immune modulation, and virulence of *H. pylori* within the gastric niche, ultimately resulting in *H. pylori*-mediated chronic inflammation and a series of pathological outcomes that facilitate the development of gastric cancer.

HOST FACTORS THAT CONTRIBUTE TO GASTRIC CARCINOGENESIS

In addition to microbial factors that potentiate gastric disease, there are a number of host factors that contribute to chronic gastritis and the progression to gastric adenocarcinoma. Cyclooxygenases (COX) are key enzymes that catalyze prostaglandin synthesis. Of the three isoforms identified, COX-2 is upregulated in gastric epithelial cells upon co-culture with *H. pylori* (Romano et al., 1998; Juttner et al., 2003; Meyer et al., 2003; Wu et al., 2005) and within the gastric mucosa of *H. pylori*-infected individuals (Sawaoka et al., 1998; Fu et al., 1999; McCarthy et al., 1999). *In vivo* studies show that COX-2 is further upregulated in *H. pylori*-mediated adenocarcinoma (Ristimaki et al., 1997; Sung et al., 2000). COX-2 expression levels are considered an independent factor for poor prognosis and correlate with reduced patient survival, suggesting that *H. pylori*-induced COX-2 overexpression is a risk factor for the development of gastric cancer.

Other host factors that increase the propensity for chronic inflammation and gastric adenocarcinoma are polymorphisms within human *IL-1*β (interleukin-1), *TNF*-α (tumor necrosis factor), and *IL-8* promoters (El-Omar et al., 2000, 2003; Machado et al., 2001; Furuta et al., 2002), which lead to increased expression of the proinflammatory cytokines IL-1β, TNF-α, and IL-8 (**Figure 1**). These polymorphisms in combination with *H. pylori* virulent genotypes increase the risk of gastric cancer up to 87-fold over baseline (Figueiredo et al., 2002), emphasizing the importance of microbial–host interactions in the development of gastric cancer. Collectively, data demonstrate that *H. pylori* virulence factors, host genetics, and environmental factors interact to induce and maintain the persistent inflammatory immune response that initiates the multistep process leading to gastric cancer.

microRNAs

Recent discoveries have shed new light on the involvement of host microRNAs (miRNAs) in gastric carcinogenesis. miRNAs are small, non-coding RNAs ∼20–25 nucleotides in length, which function as critical post-transcriptional regulators of gene expression (Bartel, 2009). miRNAs were first characterized in 1993 (Lee et al., 1993), but their distinct role in transcriptional regulation was not recognized until the early 2000s. Most miRNAs are found in intergenic regions and contain their own promoter and regulatory units. Processed miRNAs function by binding to the 3′ untranslated region (3′UTR) of messenger RNAs (mRNAs), typically resulting in mRNA degradation and gene silencing or translational repression (Bartel, 2009). It is estimated that the human genome encodes thousands of miRNAs, targeting ∼30–60% of all protein-coding genes (Lewis et al., 2003). miRNAs are involved in many biological processes, including development, differentiation, angiogenesis, cell cycle progression, proliferation, apoptosis, and signal transduction pathways (Ambros, 2004). Dysregulation of miRNA expression with subsequent disruption of these biological processes can result in disease states. There is an increasing body of evidence regarding the regulatory roles of miRNAs in immune and inflammatory disorders (Wu et al., 2008; Sonkoly and Pivarcsi, 2009), and aberrant expression of miRNAs is observed in many cancers (Lu et al., 2005; Volinia et al., 2006). Thus, recent studies have begun to dissect the mechanisms by which miRNAs function as either oncogenic miRNAs (oncomiRs) or tumor suppressors to promote or prevent tumorigenesis.

DYSREGULATION OF miRNAs IN *H. PYLORI*-INDUCED GASTRIC CARCINOGENESIS

The number of studies analyzing miRNA expression profiles in gastric cancer is rapidly increasing and a comprehensive list of

miRNAs dysregulated in gastric cancer, confirmed mRNA targets, and the biological processes affected is shown in **Tables A1** and **A2** in the Appendix. The first study to address miRNA expression profiles in various cancers, including gastric cancer, was performed in 2005 (Lu et al., 2005). Subsequent studies have not only focused on miRNA expression profiles in gastric cancer, but also those that are altered in response to *H. pylori*.

Matsushima et al. (2011) conducted a study to characterize miRNA expression signatures in *H. pylori*-infected human gastric mucosa. Using high throughput profiling analysis, 31 miRNAs were identified as being differentially expressed between *H. pylori*-infected and *H. pylori*-uninfected gastric mucosa. The relationship between miRNA expression levels and *H. pylori*-induced acute inflammation, characterized by neutrophil infiltration, and chronic inflammation, characterized by mononuclear cell infiltration were also determined. Expression levels of many miRNAs correlated with either the degree of acute or chronic inflammation and in some cases both (**Tables 1** and **2**). The relationship between miRNA expression and extent of glandular atrophy, and intestinal metaplasia was also assessed, but no significant correlations were found (Matsushima et al., 2011). A comprehensive list of miRNAs dysregulated by *H. pylori*, confirmed mRNA targets, and biological processes affected is shown in **Tables 1** and **2**. These data suggest that *H. pylori* infection affects global miRNA expression in human gastric mucosa, and this effect is, in part, linked to *H. pylori*-induced host inflammatory immune responses.

miRNAs that are dysregulated in response to *H. pylori* infection may not be the same miRNAs that are dysregulated in later stages of gastric disease. A comprehensive review of the literature, however, revealed that there is a select subset of miRNAs dysregulated both following *H. pylori* infection as well asin gastric cancer. These include downregulated miRNAs, *let-7a*, *miR-31*, *miR-101*, *miR-141*, *miR-203*, *miR-210*, *miR-218*, *miR-375*, and *miR-449* as well as upregulated miRNAs, *miR-17*, *miR-20a*, *miR-21*, *miR-146a*, *miR-155*, and *miR-223*. These miRNAs may be more biologically relevant to *H. pylori*-induced gastric inflammation and carcinogenesis and represent fruitful targets for studies focused on cancer that develops in the context of *H. pylori* infection.

The next sections will discuss miRNA dysregulation in *H. pylori*-induced disease and how specific miRNAs control various biological processes related to (1) host inflammatory immune response, (2) cell cycle progression, and (3) apoptosis and proliferation.

HELICOBACTER PYLORI-INDUCED miRNA DYSREGULATION TO CONTROL HOST INFLAMMATORY RESPONSES

Host cells recognize invading pathogens and/or pathogen-associated molecular patterns (PAMPs) through membrane-associated or cytoplasmic pathogen recognition molecules known as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), respectively. PAMPs activate adaptor proteins and transcription factors that mediate host innate immunity through activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling (**Figure 2**). Gastric epithelial cells are the initial host element encountered by *H. pylori*. The innate immune response induced in epithelial cells is characterized by NOD1-dependent activation of NF-κB in response to *H. pylori* peptidoglycan (PGN), which is injected into host cells via the *cag* T4SS (Viala et al., 2004). Activation of NF-κB by *H. pylori* leads to induction of the proinflammatory cytokine IL-8 and likely contributes to carcinogenesis through activation of downstream targets that mediate inflammation, cell cycle progression, proliferation, and apoptosis. Myeloid cells constitute a second line of defense and secrete proinflammatory cytokines such as IL-6, IL-1, and TNF-α to establish T and B lymphocyte-mediated adaptive immunity.

The involvement of miRNAs in modulating both the innate and adaptive immune responses is well established (Chen et al., 2004) and *H. pylori* can dysregulate miRNA expression to evade host defenses and successfully persist in the gastric niche. *miR-146a* and *miR-155* are specifically involved in *H. pylori*-induced negative regulation of the proinflammatory immune response (**Figure 2**). Changes in *miR-146a* expression occur in the development of gastric cancer and in the negative regulation of the innate inflammatory immune response. Single-nucleotide polymorphisms (SNPs) in *miR-146a* are associated with an increased susceptibility to gastric cancer (Okubo et al., 2010) and *H. pylori* upregulates *miR-146a in vitro* and *in vivo* in a CagA-independent and an NF-κB-dependent manner (Liu et al., 2010; Li et al., 2011a). *miR-146a* targets the TLR-signaling adaptor molecules *IRAK1* (interleukin-1 receptor-associated kinase) and *TRAF6* (TNF receptor-associated factor), resulting in negative regulation of TLR and downstream proinflammatory signaling (**Figure 2**; Liu et al., 2010; Li et al., 2011a). As a result, *miR-146a* overexpression negatively regulates *H. pylori*-induced IL-8, TNF-α, IL-1β, GRO-α [CXCL1, chemokine (C-X-C motif) ligand], and MIP-3α (macrophage inflammatory protein) expression, all key components to the proinflammatory innate and adaptive immune responses (Liu et al., 2010; Li et al., 2011a).

The second miRNA involved in *H. pylori*-induced downregulation of the host inflammatory immune response, *miR-155*, plays a critical role in regulating lymphocyte homeostasis and tolerance (Thai et al., 2007). *miR-155* is increased in many malignancies of B cell or myeloid origin (Volinia et al., 2006). In transgenic murine models of *miR-155* overexpression, mice develop spontaneous B cell lymphomas (Costinean et al., 2006). *miR-155* is induced during both bacterial and viral infections in myeloid cells through activation of TLR-signaling pathways. *H. pylori* upregulates *miR-155* expression *in vitro* and *in vivo*, which occurs in an NF-κB-dependent manner, and ultimately results in decreased induction of the proinflammatory cytokines, IL-8, and GRO-α (Xiao et al., 2009b; Tang et al., 2010). *miR-155* targets *MyD88* (myeloid differentiation primary response gene), the universal adapter protein used by TLRs to activate NF-κB (**Figure 2**; Xiao et al., 2009b; Tang et al., 2010). Decreased levels of MyD88 subsequently result in decreased NF-κB activation and dampening of the host inflammatory response (Xiao et al., 2009b; Tang et al., 2010). Therefore, these data demonstrate that *H. pylori* dysregulates host miRNA expression to manipulate the host inflammatory immune response, which may promote bacterial survival and persistence within the gastric mucosa. Because these miRNAs have established roles in carcinogenesis as well as innate immunity, they could serve as an important link between *H. pylori*-induced inflammation and carcinogenesis.

Table 1 | miRNAs downregulated in response to *H. pylori*.

miRNAs	Target mRNAs	Biological process targeted	Reference
let-7a+	***RAB40C***	**Cell cycle progression**	Matsushima et al. (2011), Motoyama et al. (2008), Yang et al. (2011)
		Proliferation	
	HMGA2	**Invasion**	
*let-7b**	*HMGA2*	Invasion	Matsushima et al. (2011), Motoyama et al. (2008)
let-7d	*HMGA2*	Invasion	Matsushima et al. (2011), Motoyama et al. (2008)
let-7e	*HMGA2*	Invasion	Matsushima et al. (2011), Motoyama et al. (2008)
let-7f	*HMGA2*	Invasion	Matsushima et al. (2011), Motoyama et al. (2008)
miR-1	ND	Proliferation	Saito et al. (2011)
miR-31+	**ND**	**ND**	Matsushima et al. (2011)
miR-32	ND	ND	Matsushima et al. (2011)
miR-34b	ND	ND	Suzuki et al. (2010)
miR-34c	ND	ND	Suzuki et al. (2010)
miR-101	***COX-2***, ***FOS***	**Proliferation**	Matsushima et al. (2011), Varambally et al. (2008), Wang et al. (2010)
	MCL1	**Apoptosis**	
	EZH2	**Invasion migration**	
miR-103#	ND	ND	Matsushima et al. (2011)
miR-106b	*p21*	Cell cycle progression	Kan et al. (2009), Matsushima et al. (2011), Petrocca et al. (2008)
		Proliferation	
	BIM	Apoptosis	
miR-125a	*ERBB2*	Proliferation	Matsushima et al. (2011), Nishida et al. (2011)
miR-130a	ND	ND	Matsushima et al. (2011)
miR-133	ND	Proliferation	Saito et al. (2011)
miR-141#	***FGFR2***	**Proliferation**	Du et al. (2009), Matsushima et al. (2011)
miR-200a+	*ZEB1, ZEB2*	Epithelial to mesenchymal transition (EMT)	Ahn et al. (2011), Matsushima et al. (2011), Shinozaki et al. (2010)
miR-200b+	*BCL2, XIAP*	Apoptosis	Ahn et al. (2011), Matsushima et al. (2011), Shinozaki et al. (2010),
	ZEB1, ZEB2	EMT	Zhu et al. (2011a)
miR-200c+	*BCL2, XIAP*	Apoptosis	Matsushima et al. (2011), Shinozaki et al. (2010), Zhu et al. (2011a)
		EMT	
miR-203	***ABL1***	**Proliferation**	Craig et al. (2011b), Matsushima et al. (2011)
		Invasion	
miR-204	*EZR*	Proliferation	Lam et al. (2011), Matsushima et al. (2011)
miR-210	**ND**	**ND**	Matsushima et al. (2011)
miR-214	ND	ND	Matsushima et al. (2011)
miR-218	***ECOP***	**Proliferation**	Gao et al. (2010), Tie et al. (2010)
		Apoptosis	
	ROBO1	**Invasion and metastasis**	
miR-320+	ND	ND	Matsushima et al. (2011)
miR-372	*LATS2*	Cell cycle progression	Belair et al. (2011)
miR-373	*LATS2*	Cell cycle progression	Belair et al. (2011)
miR-375+	***PDK1***, ***14-3-3***	**Apoptosis**	Ding et al. (2010), Matsushima et al. (2011), Tsukamoto et al. (2010)
	JAK2	**Proliferation**	
miR-377	ND	ND	Matsushima et al. (2011)
miR-379	ND	ND	Matsushima et al. (2011)
miR-429+	*BCL2, XIAP*	Apoptosis	Matsushima et al. (2011), Sun et al. (2011), Zhu et al. (2011a)
	MYC	Proliferation	
miR-449	***GMNN***, ***CCNE2***	**Cell cycle progression**	Bou Kheir et al. (2011), Lize et al. (2011)
	MET, ***SIRT1***	**Proliferation**	
miR-455	ND	ND	Matsushima et al. (2011)
miR-491-5p	ND	ND	Matsushima et al. (2011)
miR-500	ND	ND	Matsushima et al. (2011)

(Continued)

Table 1 | Continued

miRNAs	Target mRNAs	Biological process targeted	Reference
miR-532#	ND	ND	Matsushima et al. (2011)
miR-652#	ND	ND	Matsushima et al. (2011)

*Target criteria included (1) reduced protein expression upon miRNA transfection in gastric cells or expression inversely correlated with miRNA in gastric tissue or (2) presence of miRNA binding site on the 3′ UTR of target mRNA confirmed by luciferase reporter assay. ND, target mRNA or biological process not determined. *, miRNA expression correlates with acute inflammation. #, miRNA expression correlates with chronic inflammation. +, miRNA expression correlates with both acute and chronic inflammation. Bold indicates miRNA also downregulated in gastric cancer.*

Table 2 | miRNAs upregulated in response to *H. pylori* infection.

miRNAs	Target mRNAs	Biological process targeted	Reference
miR-17^	***p21***	**Cell cycle progression**	Saito et al. (2010)
miR-20a^	***p21***	**Cell cycle progression**	Saito et al. (2010)
miR-21	***PDCD4***	**Proliferation**	Zhang et al. (2008)
	RECK	**Apoptosis**	
	PTEN	**Invasion**	
miR-146a	***IRAK1, TRAF6***	**Immune response**	Li et al. (2011a), Liu et al. (2010), Xiao et al. (2011)
		Proliferation	
	SMAD4	**Apoptosis**	
miR-155	***IKK-ε, SMAD2***	**Immune response**	Fassi Fehri et al. (2010), Oertli et al. (2011), Tang et al. (2010), Xiao et al. (2009b)
	FADD, PKIα	**Apoptosis**	
miR-223*	***EPB41L3***	**Invasion and metastasis**	Li et al. (2011b), Matsushima et al. (2011)

*Target criteria included (1) reduced protein expression upon miRNA transfection in gastric cells or expression inversely correlated with miRNA in gastric tissue or (2) presence of miRNA binding site on the 3′ UTR of target mRNA confirmed by luciferase reporter assay. ND, target mRNA or biological process not determined. ^, miRNA expression induced by CagA overexpression, not H. pylori infection (Saito et al., 2010). *, miRNA expression correlates with acute inflammation. Bold indicates miRNA also upregulated in gastric cancer.*

HELICOBACTER PYLORI AND miRNAS REGULATE CELL CYCLE PROGRESSION

Disruption of cell cycle progression and increased cellular proliferation are common features of malignancies. Cell cycle progression requires coordinated expression of cyclins, which results in sequential activation of cyclin-dependent kinases (CDKs). miRNA dysregulation promotes cell cycle progression by upregulating cyclin expression and/or downregulating expression of CDK inhibitors (p15, p16, p18, p19, p21, p27, p28, p57) in various cancers, including gastric cancer (**Figure 3**). *miR-449*, a miRNA downregulated in *H. pylori*-infected gastric tissue and in gastric cancer, targets *GMNN* (geminin) and *CCNE2* (cyclin E2; **Figure 3**). Both geminin and cyclin E2 are overexpressed in numerous malignancies and promote M/G1 and G1/Scell cycle progression and cell proliferation (Bou Kheir et al., 2011; Lize et al., 2011). Consequently, downregulation of *miR-449*, as occurs following *H. pylori* infection, promotes cell cycle progression and proliferation through upregulation of geminin and cyclin E2.

p42.3, a recently identified protein significantly upregulated in gastric cancer, regulates G2/M cell cycle progression and proliferation in gastric cancer cells (Xu et al., 2007). *miR-29a*, a miRNA significantly downregulated in gastric cancer, targets *p42.3* (Cui et al., 2011; **Figure 3**). Thus, the downregulation of *miR-29a* results in a reciprocal increase in p42.3 expression, promoting increased cell cycle progression and proliferation.

The retinoblastoma protein (RB1) is a tumor suppressor dysregulated in many cancers. RB1 functions to prevent excessive cell proliferation by inhibiting G1/S cell cycle progression. RB1 binds and inhibits transcription factors of the E2F family. When RB1 is bound to E2F the complex acts as a growth suppressor and prevents progression through cell cycle. A number of miRNAs target these factors. For instance, *miR-106a* is upregulated in gastric cancer and targets *RB1* (Volinia et al., 2006), while *miR-331-3p* is downregulated in gastric cancer and targets *E2F1* (Guo et al., 2010; **Figure 3**).

TGFβ suppresses gastric cancer cell proliferation via the transcriptional upregulation of the CDK inhibitor, *p21* (Yoo et al., 1999). *miR-93* and *miR-106b* directly target *p21*, resulting in its transcriptional silencing and impairment of the tumor-suppressing activity of TGFβ (Petrocca et al., 2008; Kan et al., 2009; **Figure 3**). In addition, *miR-25* targets the CDK inhibitor, *p57*, while *miR-221* and *miR-222* target the CDK inhibitors, *p27* and *p57* (Kim et al., 2009; **Figure 3**). These oncogenic miRNA clusters are also significantly upregulated in gastric cancer (Volinia et al., 2006; Petrocca et al., 2008; Guo et al., 2009; Kim et al., 2009; Yao et al., 2009). Overexpression of most of these miRNAs results in activation of CDK2, thereby promoting G1/S phase progression. Since numerous reports have described the role of *H. pylori* in the modulation of cyclins, CDKs, and CDK inhibitors and their link to gastric carcinogenesis (Shirin et al., 2001), these

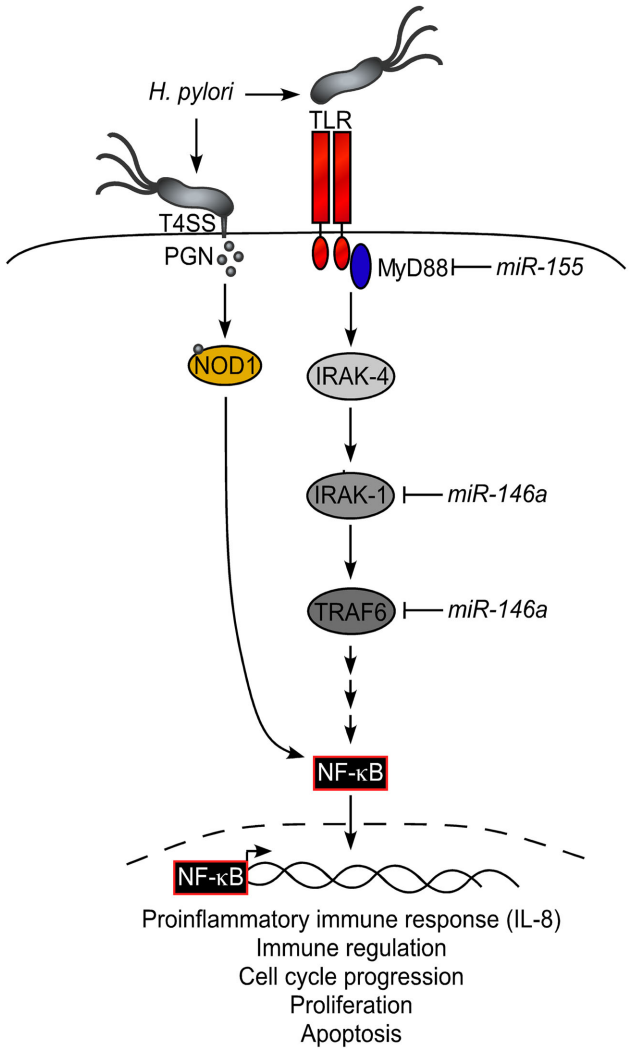

FIGURE 2 | TLR, NOD, and the NF-κB signaling pathways. Host cells recognize invading pathogens through extracellular Toll-like receptors (TLRs) and intracellular Nod-like receptors (NOD). Pathogens, such as *H. pylori*, activate TLRs and adaptor molecules that ultimately lead to NF-κB activation and a proinflammatory immune response. The key adaptor molecule responsible for signaling by TLRs is MyD88. MyD88 and other adaptor proteins (IRAK-1 and TRAF6) in this signaling cascade are targeted by miRNAs to dampen the host immune response.

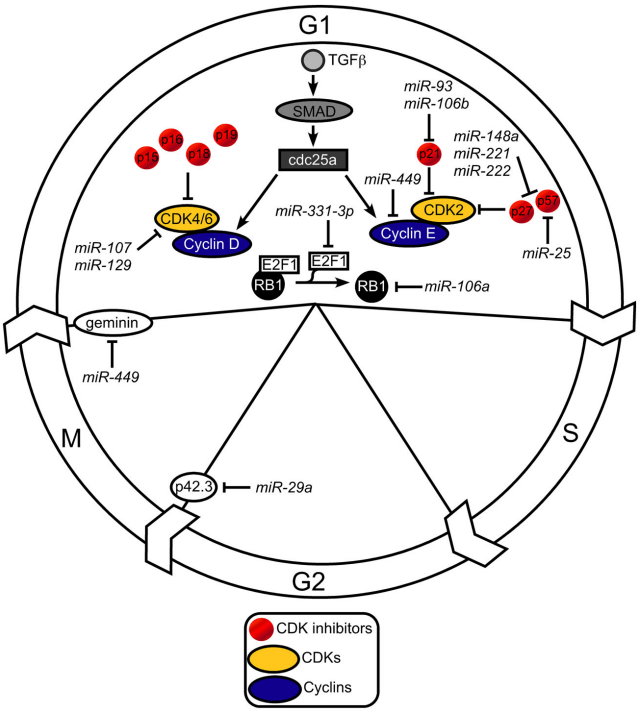

FIGURE 3 | Regulation of cell cycle progression. The cell cycle consists of four distinct phases: G1, S, G2, and M. Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), determine a cells progress through the cell cycle. CDK inhibitors prevent the progression of cell cycle and function as tumor suppressors. miRNAs target the key regulatory molecules, cyclins and CDKs, as well as CDK inhibitors to disrupt normal cell cycle progression.

data suggest that *H. pylori* modulates expression of cyclins, CDKs, and CDK inhibitors through dysregulation of host miRNAs, which may increase the propensity for gastric transformation.

HELICOBACTER PYLORI AND miRNA DYSREGULATION INHIBIT APOPTOSIS AND PROMOTE CELL SURVIVAL

Increased cellular proliferation and evasion of apoptosis are hallmarks of cellular transformation. Apoptosis can be classified as being dependent on either the intrinsic or extrinsic pathways. The intrinsic pathway is initiated within cells and hinges on the balance of activity between pro-apoptotic and anti-apoptotic members of the Bcl-2 (B cell lymphoma 2) superfamily of proteins, which act to regulate the permeability of the mitochondrial membrane. miRNAs regulate apoptosis by altering expression and balance of members of the pro-apoptotic (e.g., Bax, Bak, Bim, Bad, Bid, and BNIP3L) and anti-apoptotic (e.g., Bcl-2, Bcl-xL, and Mcl-1) Bcl-2 protein family (**Figure 4**).

Numerous miRNAs overexpressed in gastric cancer function as oncomiRs by targeting members of the pro-apoptotic Bcl-2 protein family. In addition to their role in regulating cell cycle progression, *miR-25*, *miR-93*, and *miR-106b* also inhibit apoptosis by preventing expression of the pro-apoptotic protein, Bim (Kan et al., 2009; **Figure 4**). Overexpression of *miR-130b* also contributes to suppression of Bim and apoptosis by targeting *RUNX3* (runt-related transcription factor; Lai et al., 2010), a known tumor suppressor frequently silenced in gastric cancer (Li et al., 2002). *miR-150* targets the *EGR2* (early growth response protein; Wu et al., 2010), a tumor-suppressive transcription factor that induces apoptosis by direct transactivation of pro-apoptotic factors, Bak, and BNIP3L (Unoki and Nakamura, 2003).

Numerous tumor suppressor miRNAs target members of the anti-apoptotic Bcl-2 protein family and are consequently downregulated in gastric cancer. *miR-15b*, *miR-16*, *miR-34*, *miR-181b*, *miR-181c*, and *miR-497* directly target anti-apoptotic *BCL2* (Ji et al., 2008; Xia et al., 2008; Zhu et al., 2010b, 2011b; **Figure 4**). These miRNA clusters are downregulated in gastric cancer cells

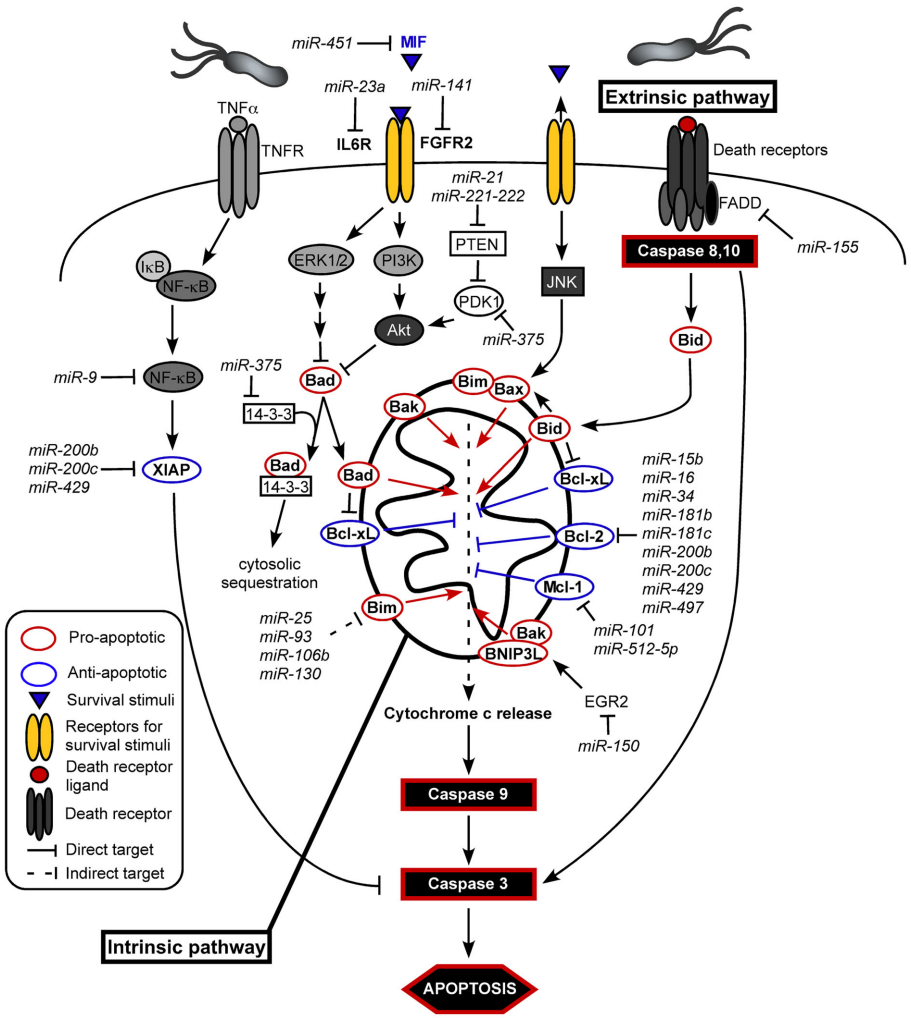

FIGURE 4 | Signaling cascades that regulate the intrinsic and extrinsic pathways of apoptosis. TNFα signaling leads to activation of NF-κB and the anti-apoptotic protein XIAP. Other receptors that detect survival factors, such as growth factors and cytokines, induce ERK1/2 and PI3K/Akt signaling cascades that ultimately result in the inhibition of the pro-apoptotic protein, Bad. In contrast, upon removal of survival factors, these receptors can signal via JNK to induce the pro-apoptotic protein, Bax. Pro-apoptotic and anti-apoptotic proteins govern the intrinsic pathway of cell death, which results in the release of cytochrome c from the mitochondria and induction of the caspase cascade. Signaling through death receptors initiate the extrinsic pathway of apoptosis, leading to the induction of caspases and cell death. There are numerous miRNAs that regulate each of these pathways and dysregulation of these miRNAs can lead to anti-apoptotic and tumorigenic responses.

(Guo et al., 2009), leading to increased expression of Bcl-2 and inhibition of apoptosis. The *miR-200bc/429* cluster is downregulated in gastric cells, and these miRNAs directly target *BCL2* and *XIAP* (x-linked inhibitor of apoptosis), leading to reduced expression and increased apoptosis (Zhu et al., 2011a; **Figure 4**). *miR-101* and *miR-512-5p* target another anti-apoptotic member of the Bcl-2 family, *MCL1* (myeloid leukemia cell differentiation protein; Saito et al., 2009; Wang et al., 2010; **Figure 4**). Both *miR-101* and *miR-512-5p* are downregulated in gastric cancer, leading to increased levels of Mcl-1 and an anti-apoptotic phenotype. In addition, *miR-101* is downregulated by *H. pylori* (Matsushima et al., 2011). *miR-449* is also likely involved in mediating the intrinsic pathway of apoptosis and has been classified as a potent inducer of cell cycle arrest and cell death. *miR-449* expression is reduced in *H. pylori*-infected gastric tissue, and its expression is lost in gastric tumors (Bou Kheir et al., 2011; Lize et al., 2011). Conversely, overexpression of *miR-449* inhibits cellular proliferation and induces significant levels of apoptosis, and since *miR-449* belongs to the family of p53-responsive miRNAs, its overexpression also results in activation of p53 and apoptosis-specific marker, caspase 3.

In contrast to the intrinsic pathway, the extrinsic pathway of apoptosis is initiated on the cell surface through the activation of specific pro-apoptotic, death receptors. Specific pro-apoptotic ligands are known to activate the extrinsic pathway of apoptosis via specific receptor binding. Ligand binding induces receptor clustering and the recruitment of the adaptor protein Fas-associated death domain (FADD), leading to induction of caspases and ultimately cell death. In addition to its role in regulating the host

immune response, *miR-155* targets *FADD* (**Figure 4**), leading to decreased expression of this key adaptor molecule (Xiao et al., 2009b). Therefore, the upregulation of *miR-155* by *H. pylori* and during carcinogenesis results in downregulation of FADD and inhibition of apoptosis.

In addition to targeting proteins directly involved in the intrinsic and extrinsic pathways of cell death, miRNAs target other factors that ultimately lead to inhibition of apoptosis and increased proliferation. *miR-375* targets 14-3-3 zeta, an anti-apoptotic protein that mediates cell survival by binding the pro-apoptotic protein Bad and sequestering it to the cytosol (Tsukamoto et al., 2010; **Figure 4**). *miR-375* also targets *PDK1* (3-phosphoinositide dependent protein kinase), a kinase that directly phosphorylates Akt, thereby regulating the PI3K/Akt signaling pathway (**Figure 4**). Overexpression of *miR-375* was shown to substantially reduce cell viability through induction of the caspase-dependent apoptotic pathway. *miR-375* is one of the most highly downregulated miRNAs in gastric cancer (Tsukamoto et al., 2010), suggesting its role as a potent tumor suppressor that contributes to the development of gastric carcinoma.

In contrast, *miR-21*, a known oncomir that targets many known tumor suppressors, is consistently upregulated in various human cancers, including gastric cancer (Volinia et al., 2006; Chan et al., 2008; Petrocca et al., 2008; Zhang et al., 2008; Guo et al., 2009), and *miR-21* expression is increased in *H. pylori*-infected gastric tissues (Zhang et al., 2008). Overexpression of *miR-21* shifts the balance between proliferation and apoptosis, increasing cellular proliferation and inhibiting apoptosis. Specifically, *miR-21* targets *PTEN* (phosphatase and tensin homolog), a tumor suppressor and negative regulator of the PI3K/Akt signaling pathway (Yamanaka et al., 2009), which is involved in both apoptotic and proliferative pathways (**Figure 4**). Mutations in *PTEN* are important in the progression of many cancers, including gastric carcinoma (Kang et al., 2002). *miR-21* likely also contributes to apoptosis by targeting *PDCD4* (programmed cell death protein 4), which is localized to the nucleus of proliferating cells; however, its direct role in apoptosis has not been elucidated (Lu et al., 2008; Motoyama et al., 2010).

Similar to PI3K/Akt signaling, the NF-κB signaling pathway is important in inhibition of apoptosis and cell survival. The NF-κB signaling cascade is activated during *H. pylori*-induced gastritis and is constitutively active in gastric cancer (Sasaki et al., 2001). *miR-218* expression is reduced in numerous cancers, including gastric cancer. *H. pylori* infection also reduces the expression of *miR-218 in vitro* and *in vivo*. *miR-218* induces apoptosis in gastric cancer cells through direct targeting of *ECOP* (epidermal growth factor receptor-co-amplified and overexpressed protein), a known positive regulator of NF-κB transcriptional activity. Downregulation of *miR-218* leads to overexpression of ECOP, inhibition of NF-κB transcriptional activation, and transcription of a downstream target COX-2, ultimately inhibiting apoptosis, and inducing cell proliferation (Gao et al., 2010). Another miRNA important in regulating NF-κB signal transduction pathways is *miR-9*, which directly targets *NF-κB1*, thereby suppressing NF-κB transcriptional activity (**Figure 4**). *miR-9* is downregulated in gastric cancer and *in vitro* studies have shown that restoration of *miR-9* expression suppresses proliferation of gastric cancer cells (Luo et al., 2009; Wan et al., 2010). Cumulatively, these studies demonstrate that aberrant activation of NF-κB signaling as a result of *H. pylori*-induced miRNA dysregulation results in inhibition of apoptosis and increased proliferation, thereby sensitizing cells for subsequent mutagenesis.

miR-451, another downregulated miRNA in *H. pylori*-infected gastric mucosa and gastric cancer, targets *MIF* (macrophage migration inhibitory factor; Bandres et al., 2009), a lymphokine involved in cell-mediated immunity that is expressed in response to *H. pylori* infection and during gastric carcinogenesis (He et al., 2006). Overexpression of *miR-451* results in targeted downregulation of *MIF*, which is accompanied by a decrease in cell proliferation and increased apoptosis (**Figure 4**). Furthermore, there is an inverse correlation between *miR-451* and MIF expression in gastric cancer, suggesting that *miR-451* functions as a tumor suppressor by silencing *MIF* expression, leading to a proliferative and anti-apoptotic phenotype (Bandres et al., 2009). *miR-141*, another miRNA significantly decreased in *H. pylori*-infected gastric tissue (Matsushima et al., 2011) as well as gastric carcinoma, targets *FGFR2* (fibroblast growth factor receptor), and overexpression of *miR-141* leads to decreased FGFR2 expression and inhibition of proliferation (Du et al., 2009; **Figure 4**). *miR-23a* functions as a growth-promoting and anti-apoptotic factor. It is significantly upregulated in gastric adenocarcinoma and targets *IL-6R* (interleukin-6 receptor), which promotes increased proliferation and decreased apoptosis in gastric adenocarcinoma cells (Zhu et al., 2010a; **Figure 4**).

HELICOBACTER PYLORI AND miRNA DYSREGULATION PROMOTES CELL INVASION AND METASTASIS

Invasion and metastasis are hallmarks of cancer cells. Several intracellular signaling pathways, such as those mediated by TGFβ and hepatocyte growth factor/Met signaling, promote metastasis. In addition to its role in regulating cell cycle progression, the *H. pylori* downregulated *miR-449* also targets *Met*, a known proto-oncogene that encodes the hepatocyte growth factor receptor. Aberrant activation of Met triggers oncogenic processes, such as proliferation, angiogenesis, invasion, and metastasis (Bou Kheir et al., 2011; Lize et al., 2011). Thus, the targeted downregulation of *miR-449* by *H. pylori* and during gastric carcinogenesis results in upregulation of Met, increased cell proliferation, and likely other oncogenic processes.

The metastatic potential of cancer cells is also regulated by mechanisms that control cell survival, cytoskeletal changes, as well as the activity of extracellular matrix-degrading proteinases (MMPs). Many miRNAs known to regulate cell cycle progression, proliferation, and apoptosis pathways are also involved in metastasis. For example, overexpression of *miR-21* has been shown to increase the invasiveness of gastric cancer cells. In addition to its known tumor suppressor targets, *miR-21* also targets *RECK* (reversion-inducing-cysteine-rich protein with kazal motifs), a tumor and metastasis suppressor that inhibits tumor metastasis and angiogenesis through modulation of matrix metalloproteinases (MMPs; Zhang et al., 2008). *H. pylori* induces expression of MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, which have been linked to *H. pylori*-induced disease and carcinogenesis (Elkington et al., 2005). These data suggest that *H. pylori* has the potential to modulate expression of MMPs through

dysregulation of host miRNAs and these disruptions may increase the propensity for gastric transformation.

miR-106a is significantly upregulated in cancer cells (Volinia et al., 2006) and is known to correlate with increased lymphatic and distant metastasis (Xiao et al., 2009a). Conversely, *miR-218*, a tumor suppressor miRNA, is downregulated in gastric cancer (Volinia et al., 2006), which correlates with increased metastasis and invasion. This is thought to occur through direct targeting of *ROBO1* (roundabout homolog), which leads to enhanced signaling through the ROBO1 receptor. The SLIT/ROBO signaling pathway has been implicated in many biological responses through regulation of cell migration (Tie et al., 2010). Thus, disruption of this signaling cascade can result in increased invasion and metastasis.

CONCLUSION

The discovery of miRNAs just over a decade ago has challenged the central dogma of genetic and epigenetic regulation. Although extensive work has been dedicated to identifying miRNAs, mRNA targets, and their contribution to accepted regulatory networks, we have only begun to scratch the surface. With thousands of miRNAs within the human genome, and the ability of each miRNA to target and regulate numerous protein-coding mRNAs, affected regulatory networks are likely to be modified by countless miRNA contributors and will continue to evolve.

Many questions arise when comparing miRNA expression profiles in different model systems *in vitro* and *in vivo* and when comparing miRNA expression profiles in *H. pylori*-infected gastric tissue and gastric cancer. For example, *miR-106b*, a known oncogenic miRNA, upregulated in various malignancies including gastric cancer, is decreased in *H. pylori*-infected gastric mucosa. Similarly, *miR-34b*, *miR-34c*, *miR-103*, *miR-200a*, *miR-200b*, *miR-214*, and *miR-372* are all overexpressed in gastric cancer, while in *H. pylori*-infected gastric mucosa these miRNAs are significantly downregulated. In contrast, *miR-146a* is significantly decreased in gastric cancer, but upregulated in *H. pylori*-infected gastric tissue. However, a recent report has shown that *miR-146a* is upregulated in a subset of gastric cancers. Although examining miRNA expression signatures in gastric cancer is clearly important in understanding the disease, development of novel therapeutics requires greater insight into the miRNA profiles in precancerous gastric tissues. Since the majority of gastric cancers arise within the context of chronic inflammation, it will be particularly important to discriminate between preneoplastic and tumor-specific miRNA expression profiles. A more comprehensive understanding of the roles of miRNAs in normal biological processes and disease is needed to fully appreciate miRNA dysregulation by pathogens, such as *H. pylori*. Furthermore, the relationship between single miRNAs and their targets are important to consider, but many of these relationships are cell and context specific. Thus, it is critically important to dissect these intricate pathways and understand how host–pathogen interactions disrupt these encompassing regulatory pathways.

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APPENDIX

Table A1 | miRNAs downregulated in gastric cancer.

miRNAs	Target mRNAs*	Biological processes targeted	Reference
let-7a	*RAB40C*	Cell cycle progression	Li et al. (2010, 2011c), Motoyama et al. (2008), Tsujiura et al. (2010), Yang et al. (2011), Zhang et al. (2007), Zhu et al. (2010c)
		Proliferation	
	HMGA2	**Invasion**	
miR-9	*CDX2*	Cell cycle progression	Luo et al. (2009), Rotkrua et al. (2011), Tsai et al. (2011a), Wan et al. (2010)
	NFκB1		
	GRB2, RAB34	Proliferation	
miR-15b	*BCL2*	Apoptosis	Xia et al. (2008)
miR-16	*BCL2*	Apoptosis	Shin et al. (2011), Xia et al. (2008)
miR-29a	*p42.3*	Cell cycle progression	Cui et al. (2011), Lang et al. (2010)
		Proliferation	
	CDC42	Invasion	
miR-29b	*CDC42*	Proliferation	Lang et al. (2010)
		Invasion	
miR-29c	*CDC42*	Proliferation	Lang et al. (2010)
		Invasion	
miR-30a	ND	ND	Li et al. (2010)
miR-30b	ND	ND	Ueda et al. (2010)
miR-30c	ND	ND	Ueda et al. (2010)
miR-31	**ND**	**ND**	Guo et al. (2009), Zhang et al. (2010b)
miR-33b	ND	ND	Volinia et al. (2006)
miR-34	*BCL2*	Apoptosis	Ji et al. (2008)
miR-96	ND	ND	Volinia et al. (2006)
miR-101	***COX-2, FOS***	**Proliferation**	Varambally et al. (2008), Wang et al. (2010)
	MCL1	**Apoptosis**	
	EZH2	**Invasion migration**	
miR-126	*CRK*	Cell cycle progression	Feng et al. (2010), Li et al. (2010, 2011c), Otsubo et al. (2011)
		Proliferation	
		Invasion and metastasis	
	SOX2	Proliferation	
miR-128b	ND	ND	Katada et al. (2009)
miR-129	*CDK6*	Cell cycle progression	Katada et al. (2009), Shen et al. (2010), Tsai et al. (2011b), Wu et al. (2010a)
		Proliferation	
	SOX4	Apoptosis	
miR-133b	ND	ND	Guo et al. (2009), Wu et al. (2011a)
miR-136	ND	ND	Ueda et al. (2010)
miR-138	ND	ND	Volinia et al. (2006)
miR-139-5p	ND	ND	Guo et al. (2009)
miR-141	***FGFR2***	**Proliferation**	Du et al. (2009)
miR-143	ND	Proliferation	Li et al. (2011a), Takagi et al. (2009), Wu et al. (2011a)
miR-145	ND	ND	Li et al. (2011a), Takagi et al. (2009), Tchernitsa et al. (2010)
miR-146a	*IRAK1, TRAF6*	Immune response	Hou et al. (2011), Kogo et al. (2011), Li et al. (2011a,d), Okubo et al. (2010), Tchernitsa et al. (2010)
		Proliferation	
		Apoptosis	
miR-148	ND	ND	Katada et al. (2009)
miR-148a	*CCKBR*	Proliferation	Chen et al. (2010), Guo et al. (2011), Zheng et al. (2011)
	p27	Cell cycle progression	
	ROCK1	Invasion and metastasis	
miR-148b	*CCKBR*	Proliferation	Song et al. (2011)
miR-152	*CCKBR*	Proliferation	Chen et al. (2010), Ueda et al. (2010)

(Continued)

Table A1 | Continued

miRNAs	Target mRNAs*	Biological processes targeted	Reference
miR-181b	*BCL2*	Apoptosis	Jiang et al. (2011), Li et al. (2011c), Zhu et al. (2010b)
miR-181c	*NOTCH4, KRAS*	Proliferation	Hashimoto et al. (2010), Zhu et al. (2010b)
	BCL2	Apoptosis	
miR-195	ND	ND	Guo et al. (2009), Wu et al. (2011a)
miR-197	ND	ND	Li et al. (2011c)
miR-203	***ABL1***	**Proliferation**	Chiang et al. (2011), Craig et al. (2011b)
		Invasion	
miR-210	**ND**	**ND**	Li et al. (2011c)
miR-212	*MECP2*	Proliferation	Volinia et al. (2006), Wada et al. (2010), Wu et al. (2011a), Xu et al. (2010)
	MYC		
miR-218	***ECOP***	**Proliferation**	Gao et al. (2010), Tie et al. (2010), Ueda et al. (2010), Volinia et al. (2006)
		Apoptosis	
	ROBO1	**Invasion and metastasis**	
miR-331-3p	*E2F1*	Cell cycle progression	Guo et al. (2010)
		Proliferation	
miR-339	*ICAM-1*	Immune response	Ueda et al. (2009)
miR-375	***PDK1, 14-3-3***	**Apoptosis**	Ding et al. (2010), Tsukamoto et al. (2010), Ueda et al. (2010), Xu et al. (2011)
	JAK2	**Proliferation**	
miR-378	ND	ND	Guo et al. (2009), Yao et al. (2009)
miR-433	*GRB2*	Proliferation	Luo et al. (2009)
miR-449	***GMNN,CCNE2***	**Cell cycle progression**	Bou Kheir et al. (2011), Lize et al. (2011)
	MET, SIRT1	**Proliferation**	
miR-451	*MIF*	Proliferation	Bandres et al. (2009)
		Apoptosis	
miR-497	*BCL2*	Apoptosis	Guo et al. (2009), Zhu et al. (2011b)
miR-512-5p	*MCL1*	Apoptosis	Saito et al. (2009)
miR-638	ND	ND	Yao et al. (2009)
miR-768-3p	ND	ND	Guo et al. (2009)

**Target criteria included (1) reduced protein expression upon miRNA transfection in gastric cells or expression inversely correlated with miRNA in gastric tissue or (2) presence of miRNA binding site on the 3′ UTR of target mRNA confirmed by luciferase reporter assay. ND, target mRNA or biological process not determined. Bold indicates miRNA also downregulated following H. pylori infection.*

Table A2 | miRNAs upregulated in gastric cancer.

miRNAs	Target mRNAs*	Biological processes targeted	Reference
miR-7	ND	ND	Volinia et al. (2006), Wu et al. (2011b)
miR-17	**ND**	**ND**	Guo et al. (2009), Volinia et al. (2006), Yao et al. (2009), Zhou et al. (2010)
miR-17-5p	ND	ND	Petrocca et al. (2008), Tsujiura et al. (2010), Ueda et al. (2010), Volinia et al. (2006)
miR-18a	ND	ND	Guo et al. (2009), Yao et al. (2009)
miR-18b	ND	ND	Guo et al. (2009)
miR-19a	ND	ND	Guo et al. (2009), Ueda et al. (2010)
miR-20a	**ND**	**ND**	Guo et al. (2009), Volinia et al. (2006)
miR-20b	ND	ND	Guo et al. (2009), Katada et al. (2009), Ueda et al. (2010)
miR-21	***PDCD4***	**Proliferation**	Chan et al. (2008), Guo et al. (2009), Li et al. (2010, 2011c), Lu et al. (2008), Motoyama et al. (2010), Petrocca et al. (2008), Shin et al. (2011),
	RECK	**Apoptosis**	Tsujiura et al. (2010), Ueda et al. (2010), Volinia et al. (2006), Zhang et al. (2008)
	PTEN	**Invasion**	
miR-23a	*IL-6R*	Proliferation Apoptosis	Li et al. (2011c), Volinia et al. (2006), Zhu et al. (2010a)
miR-23b	ND	ND	Li et al. (2011c)
miR-24	*AE1*	Proliferation Differentiation	Chan et al. (2010), Volinia et al. (2006), Wu et al. (2010b)
miR-25	*p57*	Cell cycle progression	Kan et al. (2009), Kim et al. (2009), Li et al. (2011c), Petrocca et al. (2008), Ueda et al. (2010), Volinia et al. (2006)
	BIM	Apoptosis	
miR-27	*APC*	Epithelial to mesenchymal transition (EMT)	Zhang et al. (2011)
miR-27a	*PHB*	Proliferation	Katada et al. (2009), Li et al. (2011a); Liu et al. (2009), Sun et al. (2010), Zhao et al. (2011)
	ZBTB10	Metastasis	
miR-34a	*SIRT1*	Cell cycle progression	Craig et al. (2011a), Yamakuchi and Lowenstein (2009), Yao et al. (2009)
	FOXP1	Proliferation	
miR-34b	ND	ND	Katada et al. (2009), Suzuki et al. (2010), Tsai et al. (2011b)
miR-34c	ND	ND	Katada et al. (2009), Suzuki et al. (2010)
miR-92	ND	ND	Li et al. (2011c), Petrocca et al. (2008), Ueda et al. (2010), Volinia et al. (2006)
miR-93	*p21*	Cell cycle progression	Kim et al. (2009), Petrocca et al. (2008), Ueda et al. (2010)
	BIM	Apoptosis	
miR-98	ND	ND	Yao et al. (2009)
miR-99a	ND	ND	Li et al. (2011c)
miR-99b	ND	ND	Volinia et al. (2006)
miR-103	ND	ND	Li et al. (2011c), Tchernitsa et al. (2010), Volinia et al. (2006)
miR-106a	*RB1*	Cell cycle progression Proliferation	Guo et al. (2009), Petrocca et al. (2008), Tsujiura et al. (2010), Ueda et al. (2010), Volinia et al. (2006), Xiao et al. (2009a), Yao et al. (2009)
miR-106b	*p21*	Cell cycle progression	Guo et al. (2009), Kim et al. (2009), Petrocca et al. (2008), Tsujiura et al. (2010), Ueda et al. (2010), Yao et al. (2009)
	BIM	Apoptosis	
miR-107	*CDK6*	Proliferation	Feng et al. (2011), Li et al. (2011b,c), Volinia et al. (2006)
	DICER	Invasion and metastasis	
miR-125b	ND	ND	Li et al. (2011c), Ueda et al. (2010), Volinia et al. (2006)
miR-128a	ND	ND	Katada et al. (2009)
miR-130b	*RUNX3*	Apoptosis	Li et al. (2002), Lai et al. (2010), Yao et al. (2009)
miR-135a	ND	ND	Ueda et al. (2010)
miR-138	ND	ND	Yao et al. (2009)
miR-146a	***SMAD4***	**Proliferation** **Apoptosis**	Xiao et al. (2011)
miR-147	ND	ND	Yao et al. (2009)
miR-150	*EGR2*	Apoptosis	Katada et al. (2009), Wu et al. (2010c)

(Continued)

Table A2 | Continued

miRNAs	Target mRNAs*	Biological processes targeted	Reference
miR-155	***IKK-ε, SMAD2***	**Immune response**	Fassi Fehri et al. (2010), Oertli et al. (2011), Tang et al. (2010), Thai et al. (2007), Volinia et al. (2006), Xiao et al. (2009b), Yao et al. (2011)
	FADD, PKIα	**Apoptosis**	
miR-181a-2	ND	ND	Yao et al. (2009)
miR-185	ND	ND	Yao et al. (2009)
miR-191	*NDST1*	Proliferation	Li et al. (2011c), Shi et al. (2011), Ueda et al. (2010), Volinia et al. (2006)
miR-192	*ALCAM*	Proliferation	Jin et al. (2011), Volinia et al. (2006)
miR-196a	ND	ND	Okubo et al. (2010), Yao et al. (2009)
miR-200a	*ZEB1, ZEB2*	Epithelial to mesenchymal transition (EMT)	Ahn et al. (2011)
miR-200b	*ZEB1, ZEB2*	EMT	Ahn et al. (2011), Zhu et al. (2011a)
	BCL2, XIAP	Apoptosis	
miR-214	ND	ND	Li et al. (2011c), Ueda et al. (2010), Volinia et al. (2006)
miR-215	*ALCAM*	Proliferation	Jin et al. (2011), Volinia et al. (2006)
		Apoptosis	
miR-221	*p27, p57*	Cell cycle progression	Chun-Zhi et al. (2010), Kim et al. (2009), Li et al. (2011c), Volinia et al. (2006), Yao et al. (2009)
	PTEN	Proliferation	
miR-222	*p27, p57*	Cell cycle progression	Chun-Zhi et al. (2010), Kim et al. (2009), Li et al. (2011c), Ueda et al. (2009), Volinia et al. (2006)
	PTEN	Proliferation	
	ICAM-1	Immune response	
miR-223	***EPB41L3***	**Invasion and metastasis**	Li et al. (2011c), Petrocca et al. (2008), Volinia et al. (2006), Yao et al. (2009)
miR-302f	ND	ND	Yao et al. (2009)
miR-337-3p	ND	ND	Yao et al. (2009)
miR-340	ND	ND	Guo et al. (2009), Yao et al. (2009)
miR-345	ND	ND	Ueda et al. (2010)
miR-372	*LATS2*	Cell cycle progression	Cho et al. (2009)
		Apoptosis	
miR-421	*CBX7, RBMXL1*	Proliferation	Guo et al. (2009), Jiang et al. (2010)
miR-520c-3p	ND	ND	Yao et al. (2009)
miR-575	ND	ND	Yao et al. (2009)
miR-601	ND	ND	Yao et al. (2009)
miR-616	ND	ND	Yao et al. (2009)
miR-650	*ING4*	Apoptosis	Zhang et al. (2010a)
miR-658	ND	ND	Guo et al. (2009)
miR-1259	ND	ND	Yao et al. (2009)

**Target criteria included (1) reduced protein expression upon miRNA transfection in gastric cells or expression inversely correlated with miRNA in gastric tissue or (2) presence of miRNA binding site on the 3′ UTR of target mRNA confirmed by luciferase reporter assay. ND, target mRNA or biological process not determined. Bold indicates miRNA also upregulated following H. pylori infection.*

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The immunomodulatory properties of *Helicobacter pylori* confer protection against allergic and chronic inflammatory disorders

***Isabelle C. Arnold*†, *Iris Hitzler* and *Anne Müller* ***

Institute of Molecular Cancer Research, University of Zürich, Zürich, Switzerland

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Steve Blanke, University of Illinois Urbana-Champaign, USA
Holly Algood, Vanderbilt University, USA

***Correspondence:**
Anne Müller, Institute of Molecular Cancer Research, University of Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland.
e-mail: mueller@imcr.uzh.ch

†***Present address:***
Isabelle C. Arnold, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK.

Chronic infection with the gastric bacterial pathogen *Helicobacter pylori* causes gastritis and predisposes carriers to a high risk of developing gastric and duodenal ulcers, gastric cancer, and gastric lymphoma, but has also recently been shown to protect against certain allergic and chronic inflammatory disorders. The immunomodulatory properties that allow the bacteria to persist for decades in infected individuals in the face of a vigorous, yet ultimately non-protective, innate, and adaptive immune response may at the same time confer protection against allergies, asthma, and inflammatory bowel diseases. Experimental evidence from mouse models suggests that *H. pylori* has evolved to skew the adaptive immune response toward immune tolerance rather than immunity, which promotes persistent infection on the one hand, and inhibits auto-aggressive and allergic T-cell responses on the other. Regulatory T-cells mediating peripheral immune tolerance have emerged as key cellular players in facilitating persistent infection as well as protection from allergies, in both observational studies in humans and experimental work in mice. Recent data suggest that *H. pylori* actively targets dendritic cells to promote tolerance induction. The findings discussed in this review raise the possibility of harnessing the immunomodulatory properties of *H. pylori* for the prevention and treatment of allergic and auto-immune diseases, and also provide new insights relevant for *H. pylori*-specific vaccine development.

Keywords: *Helicobacter* immunomodulation, asthma and allergies, dendritic cells and regulatory T-cells, immune tolerance

INTRODUCTION

Helicobacter pylori is the most common bacterial infection in humans worldwide. The bacteria possess the remarkable ability to persist in infected individuals for many decades and have intimately co-existed with humans at least since they first migrated out of East Africa approximately 60000 years ago (Linz et al., 2007). During this long period of co-evolution, the bacteria have acquired traits that allow them to evade and subvert both innate and adaptive branches of the immune system to ensure their persistence in the face of a vigorous, yet ultimately non-protective, local and systemic immune response (Muller et al., 2011). The evolutionary costs possibly associated with the gastric pathology induced by chronic *H. pylori* infection have been proposed to be offset by potential benefits and a selective advantage for human carriers (Blaser and Atherton, 2004). Indeed, epidemiological and experimental data now point to a strong protective effect of *H. pylori* infection on the development of many extra-gastric diseases, including gastroesophageal reflux disease and its associated sequels (Vaezi et al., 2000; Islami and Kamangar, 2008; Whiteman et al., 2010), childhood asthma and allergy (Chen and Blaser, 2008; Amberbir et al., 2011), and certain metabolic disorders (Osawa et al., 2005; Mera et al., 2006). The epidemiological and experimental evidence for possible protective properties of *H. pylori* infection, as well as the mechanistic basis underlying these effects, are the subject of this review.

H. PYLORI PROTECTS AGAINST ALLERGIC ASTHMA AND OTHER ATOPIC DISEASES

THE PATHOGENESIS OF ASTHMA

In allergic asthma, genetically susceptible individuals respond to environmental allergens with inappropriate T-cell-mediated immune responses, leading to chronic obstruction of the airways. Allergic asthma is characterized by the accumulation of inflammatory infiltrates in the lung, airway hyper-responsiveness to a variety of specific and non-specific stimuli, increased serum immunoglobulin E (IgE) levels, and mucus hypersecretion. Chronic inflammation further leads to structural changes (airway remodeling) with collagen deposits, hyperplasia, and thickening of the airway wall. In asthmatic patients, allergic episodes trigger the bronchoalveolar infiltration of various immune cell populations, mostly eosinophils, mast cells, and activated $CD4^+$ T-cells. Effector T-helper 2 (Th2) cells play a central role in orchestrating the immune response to allergens by releasing cytokines that trigger the predominant features of asthma (Robinson et al., 1992): the secretion of IL-4 and IL-13 contributes to B-cell production of IgE (Wills-Karp et al., 1998; Bacharier and Geha, 2000), the release of IL-5 drives eosinophilic inflammation (Wang et al., 1989; Rosenberg et al., 2007), and IL-9 stimulates mast cell proliferation (Renauld et al., 1995). The action of IL-4 and IL-13 on lung epithelia further induces goblet cell metaplasia, whereas IL-13 acting on smooth muscle cells promotes the development of airway

hyper-responsiveness (Wills-Karp et al., 1998). Recently, other subsets of T-helper cells have been linked to asthma pathogenesis, including Th9 (Shimbara et al., 2000; Erpenbeck et al., 2003), Th25 (Tamachi et al., 2006; Ballantyne et al., 2007), and Th22 cells (Nakagome et al., 2011). A subset of lung-infiltrating T-cells known as Th17 cells has been described to account for neutrophilic airway inflammation, but also for enhanced Th2-cell-mediated eosinophilic airway inflammation (Wakashin et al., 2008). IL-17 secretion by both Th17 cells and eosinophils was further found to be increased in asthmatic patients (Molet et al., 2001). Although asthma is generally considered to be an adaptive immune disorder, the innate arm of the immune system also contributes to the pathology through the production of pro-inflammatory mediators by bronchial epithelial cells (Kauffman et al., 2006), mast cells (Amin et al., 2005), basophils (Ono et al., 2010), natural killer T (NKT) cells (Akbari et al., 2003), and dendritic cells (DC; Lambrecht et al., 2000).

There is now ample evidence that a variety of suppressive and regulatory mechanisms are crucially involved in preventing the activation of potentially harmful effector responses in the lungs of healthy individuals (Ray et al., 2010). This task is predominantly accomplished by $CD4^+CD25^+$ regulatory T-cells (Tregs) that either develop in the thymus (natural Tregs) or are induced in the periphery in response to specific antigens (adaptive or induced Tregs). Tregs can suppress pathogenic T-cell responses through direct contact with their target cells or via the release of anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β). Both $CD25^{hi}$ (Curotto de Lafaille et al., 2008) and IL-10-producing Tregs (Akdis et al., 2004) have been implicated in preventing Th2 responses to allergens. Peripheral blood-derived $CD4^+CD25^+$ Treg subsets of healthy individuals indeed inhibit the proliferation and cytokine production of their allergen-responsive $CD4^+CD25^-$ effector counterparts *in vitro*. In contrast, this ability was reduced in Treg subsets of allergic individuals, suggesting that effective suppression of pathogenic Th2 responses might be defective or overridden in patients with allergic diseases (Robinson, 2009). In bronchoalveolar lavage fluid (BALF) from asthmatic children, both the percentage and the suppressive capacity of Tregs were reduced compared to healthy control individuals (Kay, 2001). In a mouse model of ovalbumin-induced airway inflammation, the transfer of ovalbumin-specific Tregs could prevent or reverse the development of airway hyper-responsiveness and Th2 immune responses in an IL-10-dependent manner (Boudousquie et al., 2009). Overall, these findings suggest that functional Tregs of healthy individuals shift allergen-specific immune responses toward tolerance, thereby preventing the development of asthma and other allergic disorders.

HELICOBACTER INFECTION IS INVERSELY CORRELATED WITH ALLERGIC ASTHMA IN HUMANS

Many atopic individuals never develop allergic disease manifestations in their lifetime, suggesting that the genetic background acts synergistically with environmental factors to determine individual allergy susceptibility. The influence of environmental factors is drastically illustrated by the alarming increase of asthma and associated allergic disease incidence in Western societies over the last decades, especially among children (Eder et al., 2006). A plausible explanation has been formulated in the "hygiene hypothesis," which postulates a causal inverse relationship between allergies and pediatric infectious diseases (Strachan, 1989). At the immunological level, this hypothesis proposes that early life exposure to microbial antigens is required for the normal maturation of the immune system and the generation of protective regulatory T-cell responses. This notion has recently been revisited by Blaser and Falkow (2009), who suggest that the specific loss of our ancestral indigenous microbiota due to modern hygienic practices and the widespread use of antibiotics, rather than a general decline in arbitrary childhood infections, is causally associated with the epidemic increase of asthma and other allergic diseases.

In experimental models of airway inflammation, several viral and parasitic pathogens, including influenza viruses and helminths, have been implicated in protection against asthma and allergy (Wilson et al., 2005; Kitagaki et al., 2006). In addition, the role of *H. pylori* as a protective agent against atopic disorders has been suggested by numerous cross-sectional (Matricardi et al., 2000; Kosunen et al., 2002; Linneberg et al., 2003; Jarvis et al., 2004; Radon et al., 2004; von Hertzen et al., 2006; Herbarth et al., 2007; Janson et al., 2007; Shiotani et al., 2008) and case–control studies (Bodner et al., 2000; Matricardi et al., 2000; Tsang et al., 2000; Jun et al., 2005). McCune and colleges have reported that individuals carrying *H. pylori* were 30% less likely to have concomitant allergic conditions, including asthma, eczema, and allergic rhinitis (McCune et al., 2003). Several independent studies have further suggested more pronounced protective effects of *H. pylori* in children and in individuals with early onset asthma (Chen and Blaser, 2007, 2008; Amberbir et al., 2011) and in CagA-seropositive individuals (Chen and Blaser, 2007; Reibman et al., 2008).

ASTHMA PROTECTION CONFERRED BY *H. PYLORI* IS MEDIATED BY REGULATORY T-CELLS

We have recently reported that experimental *H. pylori* infection prevents allergic asthma in a mouse model of ovalbumin- or house dust mite-induced airway inflammation (Arnold et al., 2011a). Infected mice were efficiently protected against allergen-induced airway hyper-responsiveness, tissue inflammation, and goblet cell metaplasia, and exhibited reduced pulmonary and bronchoalveolar infiltration with eosinophils, Th2 cells, and Th17 cells (Arnold et al., 2011a). The protection against asthma could be attributed to *H. pylori*-induced, highly suppressive Tregs, which accumulate in the lungs of infected mice and block pathogenic effector T-cell responses (**Figure 1**). The depletion of $CD4^+CD25^+$ Tregs by systemic administration of a CD25-neutralizing antibody led to enhanced pulmonary inflammation and abrogated the characteristic T-cell hypo-responsiveness to ovalbumin in infected mice. The adoptive transfer of Tregs purified from the mesenteric lymph nodes of infected but not naïve donors was further sufficient to transfer protection to uninfected recipients (Arnold et al., 2011a). In addition, *H. pylori*-infected animals were characterized by lung-infiltrating semi-mature DC, which might suggest that Tregs generated during infection have the ability to influence extragastric immune responses by retaining DCs in a semi-mature state (**Figure 1**). An analogous mechanism was recently proposed by Onishi et al. (2008) who found that $FoxP3^+$ Tregs form aggregates

on DCs to actively down-regulate their co-stimulatory molecules CD80 and CD86, thus competing with naïve T-cells for access to DCs and limiting their ability to activate effector T-cell responses. Our finding of the accumulation of Tregs and semi-mature DCs in the lungs of infected, but not asthmatic mice (Arnold et al., 2011a) is in line with this model of asthma prevention (summarized schematically in **Figure 1**).

ASTHMA PROTECTION CONFERRED BY *H. PYLORI* IS LINKED TO THE EXPRESSION OF SPECIFIC VIRULENCE FACTORS AND IS MOST PRONOUNCED IN YOUNG INDIVIDUALS

Although a negative association between CagA-positive *H. pylori* infection status and allergic disease manifestations has been suggested (Chen and Blaser, 2007), protection conferred by *H. pylori* infection in our model was not linked to the expression of a functional type IV secretion system (Arnold et al., 2011a). Interestingly, the mucosal or systemic administration of the *H. pylori* neutrophil-activating protein (HP-NAP) in a therapeutic model of asthma was shown to inhibit bronchial inflammation through agonistic ligation of toll-like receptor 2 (TLR2; Codolo et al., 2008). HP-NAP delivery reduced lung eosinophilia in response to repeated ovalbumin challenge and decreased the production of IL-4, IL-5, and GM–CSF in the bronchioalveolar fluid (Codolo et al., 2008). In *H. pylori*-infected individuals, stimulation of lamina propria lymphocytes with HP-NAP further increased IL-10 production compared to uninfected controls, while reducing the proliferative and IFN-γ responses of stimulated PBMC (Windle et al., 2005). Because IL-10 is a key cytokine in the resolution of asthmatic inflammation (Xystrakis et al., 2006; Ogawa et al., 2008), this raises the possibility that specific *H. pylori* virulence

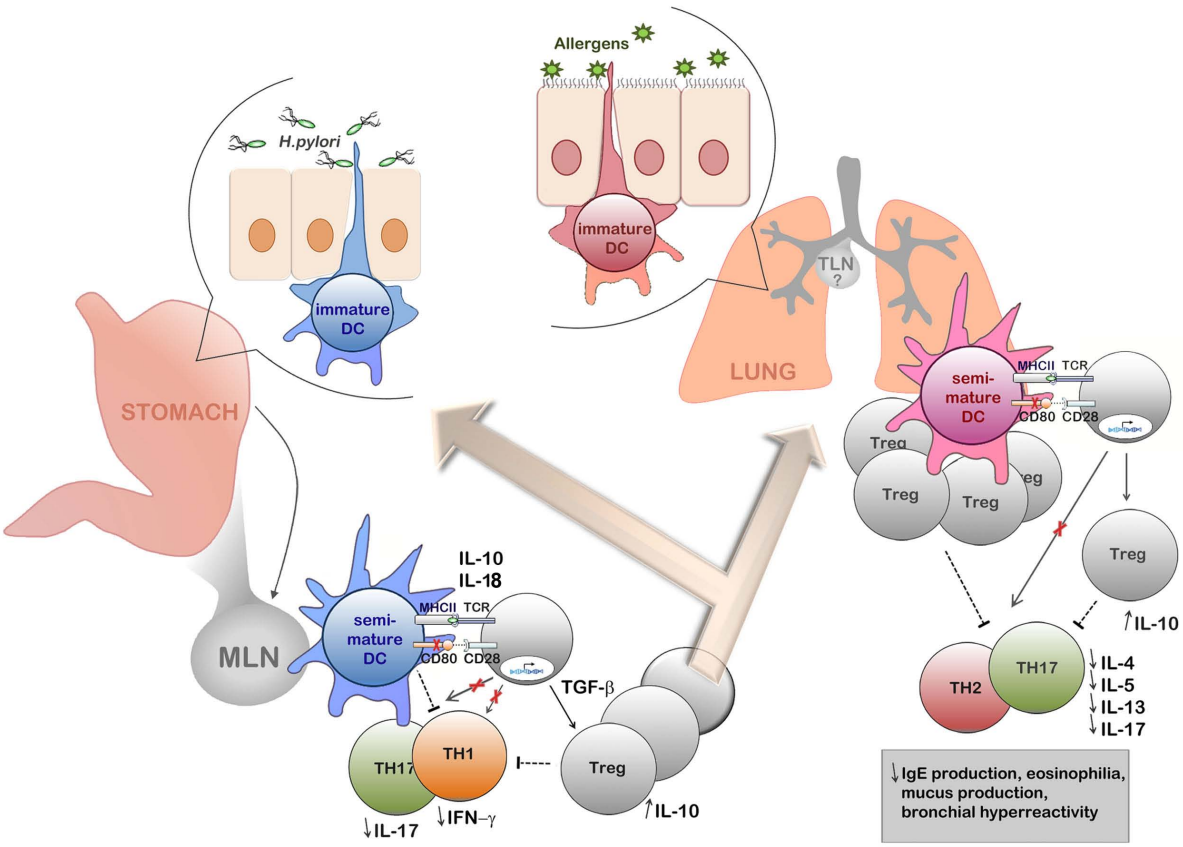

FIGURE 1 | Schematic representation of the current model of *H. pylori*-induced immune tolerance and asthma protection. Tolerogenic dendritic cells and *H. pylori*-induced regulatory T-cells act in concert to prevent adaptive Th1/Th17-driven immunity to the infection and to inhibit allergen-specific Th2 responses. In chronically infected humans, *H. pylori* resides exclusively in the gastric mucosa, where it is presumably encountered and detected by tissue-resident DC populations extending dendrites into the gastric lumen. *H. pylori*-experienced DCs migrate to the gut-draining mesenteric lymph nodes, where they act as potent inducers of TGF-β-dependent FoxP3$^+$ regulatory T-cells, but fail to prime *H. pylori*-specific Th1 and Th17 responses. Induced Tregs may further perpetuate the tolerogenic effects of *H. pylori*-experienced DCs by retaining mesenteric lymph node DCs in a semi-mature state and by directly suppressing *H. pylori*-specific gastric Th1 and Th17 responses, thereby protecting the host from excessive gastric immunopathology. Newly induced Tregs further migrate to the lung, where they suppress allergen-specific Th2 and Th17 responses involved in the pathogenesis of asthma. The generation of allergic T-cell responses may be blocked either through the tolerogenic effects of Tregs on DCs (retaining DCs in a semi-mature state) or directly through suppression of Th2 and Th17 responses via Treg/T-effector cell contact or via soluble cytokines, in particular IL-10. The ultimate outcome of gastric *H. pylori* infection on the allergen-challenged lung is reduced eosinophilia, mucus production and airway hyper-responsiveness. The involvement of the tracheal lymph nodes in *H. pylori*-induced asthma suppression is likely, but currently not well understood.

factors promote protection against allergic diseases by stimulating the Treg-specific production of IL-10.

Early onset asthma in children and adolescents is rare in the *H. pylori*-infected population (Chen and Blaser, 2008). *H. pylori*-infected children are known to preferentially launch Treg responses to the pathogen (Harris et al., 2008), which may account for the particularly beneficial effects of *H. pylori* in this population. Similarly, experimental *H. pylori* infection induces a continuum of protection against airway inflammation that is negatively correlated with age at the time of infection. Protection was most evident in neonatally infected mice, which develop *H. pylori*-specific immunological tolerance mediated by long-lived, inducible Tregs (Arnold et al., 2011b). Because the neonatal period of life is a unique developmental stage in which immune responses are highly plastic and inherently biased toward tolerance, Tregs generated during the first weeks and months of life are thought to differ qualitatively in their suppressive potential compared to their adult counterparts. Indeed, murine neonatal $CD4^+$ T-cells were shown to intrinsically differentiate into $CD4^+FoxP3^+$ Tregs in response to TCR stimulation and TGF-β signals (Wang et al., 2010). They further stably express high levels of FoxP3, which renders them particularly suppressive. It is therefore tempting to speculate that the observed inverse correlation between *H. pylori* colonization and allergic asthma is mechanistically linked to neonatally acquired immune tolerance to the bacterium (see The Beneficial Effects of *H. pylori* on Allergic and Chronic Inflammatory Disorders are Mediated by Tregs and Tolerogenic DCs).

H. PYLORI PROTECTS AGAINST INFLAMMATORY BOWEL DISEASE

THE PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE

Other immune system disorders for which inverse associations with *H. pylori* have been examined are the inflammatory bowel diseases (IBDs), chronic inflammatory conditions of unknown etiology of the gastrointestinal (GI) tract. Two main types of IBDs–Crohn's disease and ulcerative colitis are distinguished based on the affected GI region and transmural involvement. Crohn's disease is characterized by transmural inflammation of the bowel preferentially involving the terminal ileum and right colon. Ulcerative colitis manifests as a chronic inflammatory condition of the colonic mucosa; in its most limited form it may be restricted to the distal rectum, while the entire colon is involved in its most advanced form. The main symptoms of both IBDs are diarrhea, abdominal pain, and weight loss. Standard medications for both IBDs include salicylates, corticosteroids, and other immunomodulators; surgery is required for the treatment of bowel stenosis, abscesses, and internal fistulas in Crohn's disease patients. Curative treatment is currently not possible as the etiology of both IBDs is unclear.

In healthy individuals, intestinal mucosal homeostasis is controlled by $FoxP3^+CD4^+$ Tregs, which efficiently suppress pathogenic T-cell responses through IL-10 and TGF-β (Maloy et al., 2003; Kamanaka et al., 2006; Li et al., 2007a). The failure of regulatory networks to control excessive T-cell responses breaks this equilibrium and leads to chronic inflammation. In patients with ulcerative colitis, the intestinal production of IL-4 and IL-13 cytokines is reminiscent of an atypical Th2 adaptive response (Fuss et al., 1996), whereas Crohn's disease patients present a Th1-polarized cytokine profile, with production of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-12 (Fuss et al., 1996). In addition, both types of diseases are characterized by the accumulation of IL-17-producing $CD4^+$ T-cells, termed Th17 cells (Annunziato et al., 2007). These cells differentiate from naïve T-cells through the synergistic effects of IL-6 and TGF-β, IL-1, and IL-21 (Veldhoen et al., 2006; Korn et al., 2007) and require IL-23 for their maintenance and expansion (Ouyang et al., 2008). Recent work has shown that IL-23 drives chronic intestinal inflammation in a mouse model of colitis by directly targeting T-cells, and induces their proliferation and accumulation in the colon (Hue et al., 2006). IL-23 further favors the emergence of an immunogenic $IL\text{-}17A^+IFN\text{-}\gamma^+$ double-positive $CD4^+$ T-cell subset (Hue et al., 2006; Ahern et al., 2010) and inhibits the differentiation of $FoxP3^+$ Treg cells (Kullberg et al., 2006; Ahern et al., 2010), suggesting that the IL-23/Th17 axis is a major determinant in the pathogenesis of IBD. The key role of IL-23 is also supported in humans, as increased expression of IL-23 is detected in IBD patients and mutations in the *IL23R* gene increases susceptibility to both Crohn's disease and ulcerative colitis (Ahern et al., 2010). IL-23 can also drive inflammation in the absence of T-cells. In lymphocyte-deficient $Rag^{-/-}$ mice, the development of colitis following *Helicobacter hepaticus* infection or treatment with agonistic anti-CD40 antibody depended on IL-23 (Hue et al., 2006; Yen et al., 2006). This inflammation was further attributed to an IL-23-responsive innate lymphoid population that expresses IL23R, Thy1 and RORγt, and secretes IL-17 and IFN-γ (Buonocore et al., 2010). The detection of a similar innate lymphoid cell population in the inflamed intestine of patients provided evidence for a functional role of IL-23-responsive innate cells in the pathogenesis of IBD (Buonocore et al., 2010).

HELICOBACTER INFECTION IS INVERSELY CORRELATED WITH IBD

Several epidemiological studies have examined a possible inverse correlation between IBDs and *Helicobacter* infection. A Hungarian study investigating 133 IBD patients (both Crohn's and colitis patients) and similar numbers of controls found a significant inverse association with *H. pylori* infection (Pronai et al., 2004); whereas only 13% of IBD patients carried *H. pylori*, the rates ranged from 39 to 67% in various control groups (Pronai et al., 2004). This result was confirmed in a Polish study examining 94 pediatric IBD patients (both types of IBD), which revealed a lower *H. pylori* colonization rate in patients compared to healthy controls (9.6 vs. 38.4%, $p < 0.0001$; Sladek et al., 2007). A recent meta-analysis conducted by Luther et al. (2010) of 30 articles examining such a possible link confirmed that *H. pylori* infection may indeed confer some level of protection against IBD, with only 27% of IBD patients showing evidence of *H. pylori* infection compared to 41% of patients in the control group. The authors caution, however, that the heterogeneity among examined studies and the possibility of publication bias may limit the certainty of their findings. It is therefore all the more interesting that the same group has provided experimental evidence of protective effects of *H. pylori* infection on *Salmonella typhimurium*-induced colitis. Upon co-infection of both bacteria, *H. pylori* suppressed *Salmonella*-specific Th17 responses in the cecum, and reduced cecal inflammation caused by

Salmonella infection (Higgins et al., 2011). The protective effects were linked to increased levels of IL-10 in the mesenteric lymph nodes of co-infected over *Salmonella*-only infected mice, suggesting that this regulatory cytokine modulates the differentiation and/or activity of Th17 cells (Higgins et al., 2011). The same group has attributed the protective effects of *H. pylori* on colitis to the bacteria's chromosomal DNA, which appears to exhibit a high ratio of immunoregulatory to immunostimulatory sequences (Luther et al., 2011) and is by itself sufficient to prevent sodium dextran sulfate-induced colitis. In their experimental protocol, Luther et al. (2011) treated mice with one orally administered dose of 20–50 μg *H. pylori* DNA prior to subjecting them to an acute and a chronic protocol of colitis induction; in both models, administration of the DNA reduced the pathology and also attenuated other parameters of DSS-induced colitis such as bleeding and weight. The protective properties of *H. pylori* DNA were attributed to inhibition of cytokine production by DC, which upon addition of the DNA failed to produce type I interferon and IL-12 in response to *E. coli* DNA (Luther et al., 2011). Whether *H. pylori* DNA is indeed the relevant factor conferring protection against IBD in humans or mice remains to be elucidated in more detail; in fact, data showing protection by live infection in IBD models other than acute *Salmonella*-induced colitis are currently not available.

H. PYLORI MAY PROTECT AGAINST OTHER T-CELL-DRIVEN AUTO-IMMUNE DISEASES

Given the documented protective effects of *H. pylori* infection on asthma and other allergic disease manifestations on the one hand, and IBD on the other, the possibility has been raised that the presence or absence of this infection may also influence the risk of developing additional T-cell-driven immunological or metabolic disorders (Blaser and Falkow, 2009). The incidence of auto-immune diseases caused by the aberrant activation of aggressive autoreactive T-cells, such as multiple sclerosis (MS) and type I diabetes mellitus, has increased sharply in the second half of the twentieth century (Bach, 2002), i.e., in the time frame in which *H. pylori* has begun to disappear – at least in recent birth cohorts – from human populations in most developed countries (Blaser and Falkow, 2009). Socioeconomic conditions favoring lower *H. pylori* transmission and infection rates, such as frequent use of antibiotics in childhood, small family size, and non-crowded housing (Blaser and Falkow, 2009) have all also been found to be associated with a higher prevalence of allergic and auto-immune diseases (Bach, 2002). While a possible inverse correlation between *H. pylori* infection and auto-immune diseases remains largely speculative at this point in time, it is tempting to postulate that the same immunomodulatory and immunoregulatory mechanisms protecting infected individuals from allergic asthma may also be operative against excessive T-cell-driven auto-immune activation. The pathogenesis of auto-immune diseases has been studied most thoroughly in MS, an auto-immune disorder directed against the myelin sheath of neuronal axons, causing demyelination and a broad spectrum of CNS symptoms. In MS, as in IBD (see *H. pylori* Protects Against Inflammatory Bowel Disease), the Th17 subset of helper T-cells is thought to be the driving force behind the chronic (neuro-) inflammation causing disease symptoms. It is now widely accepted that Th17 cells, not Th1 cells as believed previously (Gutcher and Becher, 2007), are the main encephalitogenic population in auto-immune neuro-inflammation in experimental auto-immune encephalomyelitis (EAE), the standard mouse model of MS. Pathogenic auto-immune Th17 cells are characterized by the secretion of the cytokines IL-22, IL-21, IL-17A, IL-17F, and GM–CSF (Littman and Rudensky, 2010). A recent study has reported a dominant role for Th17-derived GM–CSF in auto-immune CNS inflammation based on the evidence that autoreactive helper T-cells specifically lacking GM–CSF failed to initiate neuro-inflammation despite expression of IL-17A and IFN-γ, whereas GM–CSF secretion by *Ifng*$^{-/-}$Il17a$^{-/-}$ helper T-cells was sufficient to induce EAE (Codarri et al., 2011).

Very little solid epidemiological data is available to date to support a protective effect of *H. pylori* on the development of MS. One study has found an inverse correlation of *H. pylori* infection with MS in the Japanese population (Li et al., 2007b), and some studies point to a higher prevalence of MS in adults with a record of having been afflicted with asthma in childhood (Ponsonby et al., 2006). In the study by Li et al. (2007b) examining 105 MS patients and 85 healthy controls, *H. pylori* seropositivity was significantly lower in patients with conventional MS (22.6%) relative to the healthy controls (42.4%). This result obviously remains to be confirmed in larger patient cohorts, and should also be experimentally examined in the EAE model of MS. In EAE, CNS inflammation and progressive paralysis of the tail and hind limbs is entirely driven by myelin-specific auto-aggressive T-cells (Codarri et al., 2011), and should therefore be susceptible to *H. pylori*-induced, Treg-mediated immunoregulation. Other experimental models of auto-immune disease, such as the non-obese diabetic model of type I diabetes (Chaparro et al., 2006) may provide informative results as well.

THE BENEFICIAL EFFECTS OF *H. PYLORI* ON ALLERGIC AND CHRONIC INFLAMMATORY DISORDERS ARE MEDIATED BY TREGS AND TOLEROGENIC DCs

TREGS PROMOTE *H. PYLORI* PERSISTENCE, LIMIT GASTRIC INFECTION-ASSOCIATED IMMUNOPATHOLOGY AND PREVENT AIRWAY HYPER-RESPONSIVENESS IN MICE

Numerous recent reports have implicated Tregs and DCs with tolerogenic activity in mediating the systemic immunomodulatory effects of *H. pylori* infection, both in human carriers, and in experimentally infected animals. In a seminal study examining human gastric T-cell responses to *H. pylori* infection, Robinson et al. (2008) showed that patients with peptic ulcer disease exhibited stronger Th1 and Th2 responses to *H. pylori* than asymptomatic carriers; conversely, the latter group predominantly mounted Treg responses to the infection. IL-10-expressing Tregs were particularly abundant in the gastric mucosa of the normal carriers compared to the peptic ulcer disease patients; interestingly, mucosal IL-10 levels were directly correlated with bacterial densities, with asymptomatic carriers showing high IL-10 expression receiving the highest colonization scores and peptic ulcer disease patients with low IL-10 expression receiving comparatively low colonization scores (Robinson et al., 2008). In a similar study conducted by Harris et al. (2008) the relatively mild gastritis typical of *H. pylori*-infected children could also be linked to Treg-predominant T-cell responses. Children with a mild form of gastritis exhibited higher

gastric mucosal Treg numbers and higher levels of the regulatory cytokines IL-10 and TGF-β than adults with more severe gastritis (Harris et al., 2008). Evidence for a functional role for Tregs and Treg-derived cytokines in promoting *H. pylori* persistence on the one hand, and in mediating *H. pylori*-induced immunomodulation on the other has been provided in experimental infection models. The earliest such evidence came from IL-10$^{-/-}$ mice, which are able to spontaneously clear *Helicobacter* infections, but suffer from – at least temporarily – strongly enhanced Th1 mediated gastritis (Ismail et al., 2003). The depletion of Tregs in mice infected with *Helicobacter* at 6 weeks of age using a CD25-specific antibody resulted in a strong reduction in colonization, and in accelerated and enhanced gastritis (Sayi et al., 2011). The depletion of Tregs in a genetic mouse model in which the diphtheria toxin receptor is expressed under the Treg-specific *foxp3* promoter (*foxp3*-DTR-transgenic mouse) also resulted in clearance of the infection and in severe gastritis, even accompanied by the development of preneoplastic gastric lesions (Arnold et al., 2011b). Mice whose CD4$^+$ T-cells cannot respond to TGF-β due to transgenic expression of a dominant-negative form of TGF-β receptor II also develop strongly enhanced pathology and spontaneously reduce bacterial burdens (Arnold et al., 2011b), indicating that TGF-β-dependent inducible Tregs, but not TGF-β-independent natural Tregs, are predominantly involved in maintaining persistence and in mediating *H. pylori*-specific immunomodulation. Interestingly, the systemic depletion of Tregs in the *foxp3*-DTR-transgenic model improved the clearance of *H. pylori* by vaccinated mice, suggesting that the efficacy of an *H. pylori* vaccine is significantly hampered by the Treg-mediated suppression of protective effector T-cell responses (Hitzler et al., 2011). The latest evidence for an important role of Tregs in the immunomodulation conferring protection against asthma was provided by the finding that the depletion of Tregs abrogates asthma protection (Arnold et al., 2011a). Conversely, as mentioned above, purified Tregs alone were sufficient to transfer protection from *H. pylori*-infected donors to uninfected recipients (Arnold et al., 2011a). Whereas as few as 100'000 Tregs isolated from neonatally infected donors were suppressive in the asthma model, Tregs from uninfected or adult-infected mice failed to confer protection (Arnold et al., 2011a). The selective suppressivity of Tregs from neonatally infected mice can be attributed to the fact that neonatal exposure to *H. pylori* induces immune tolerance to the bacteria (Arnold et al., 2011b).

H. PYLORI RE-PROGRAM DCs TOWARD TOLEROGENICITY

In contrast to natural Tregs, which originate from the thymus, inducible Tregs are generated in the periphery. Certain populations of poorly immunogenic DCs are believed to initiate and maintain peripheral immune tolerance through the induction of anergy, deletion of autoreactive T-cells and the instruction and differentiation of inducible Tregs (Maldonado and von Andrian, 2010). Such tolerogenic DCs function by converting naive T-cells into FoxP3$^+$ Tregs through antigen presentation in the absence of co-stimulatory signals or cytokines, either alone or in combination with the production of soluble and membrane-bound tolerogenic factors such as IL-10, TGF-β, retinoic acid, and programmed death ligands (PD-Ls; Kretschmer et al., 2005; Maldonado and von Andrian, 2010). DCs appear to indeed play a central role in the induction and maintenance of *H. pylori*-specific immune tolerance (**Figure 2**). The depletion of DCs impairs vaccine-induced protective immunity to a similar degree as the depletion of Tregs in the same model (Hitzler et al., 2011). In experimental infection models using adult-infected mice, the depletion of DCs improves the control of the infection and strongly enhances gastric T-cell infiltration and chronic gastritis (Hitzler et al., 2011). DCs isolated from the mesenteric lymph nodes of neonatally infected, tolerant mice exhibit tolerogenic properties *ex vivo*, i.e., they act as poor inducers of Th1 or Th17 cells, and excellent inducers of Tregs (Oertli et al., 2012). The depletion of DCs from neonatally infected, tolerant mice is consequently sufficient to break tolerance (Oertli et al., 2012). Whereas a functional role for DCs in balancing tolerance and immunity in *Helicobacter* infection is thus well established, the underlying mechanism is much less thoroughly understood. Evidence from *in vitro* infection of bone-marrow-derived DCs suggests that *H. pylori* possesses the ability to profoundly re-program DCs toward tolerogenicity (**Figure 2**; Kao et al., 2010; Oertli et al., 2012). DCs that have been exposed to *H. pylori* fail to undergo maturation upon stimulation with *E. coli* LPS; the IL-12 secretion and up-regulation of the co-stimulatory molecules CD80, CD86, and CD40 that are hallmarks of LPS-matured DCs are prevented by the infection (**Figure 2**; Oertli et al., 2012). *H. pylori*-exposed DCs further efficiently induce FoxP3 expression in co-cultured naive T-cells in a TGF-β-dependent manner, but fail to prime Th1 or Th17 cells (Kao et al., 2010; Oertli et al., 2012). The ability of *H. pylori* to re-program DCs in such a manner requires direct contact (Oertli et al., 2012), but is independent of the virulence factor CagA (Kao et al., 2010).

THE TOLEROGENICITY OF DCs REQUIRES THE SYNTHESIS, PROCESSING, AND SECRETION OF INTERLEUKIN-18

The tolerogenic activity of DCs requires the DC-intrinsic expression and processing of IL-18 (**Figure 2**), as demonstrated by the inability of IL-18$^{-/-}$ DCs to induce FoxP3 expression in co-cultured T-cells and the failure of IL-18 receptor-deficient (IL-18R$^{-/-}$) T-cells to convert to FoxP3$^+$ Tregs upon co-culturing with *H. pylori*-infected wild type DCs (Oertli et al., 2012). Indeed, IL-18$^{-/-}$ as well as IL-18R$^{-/-}$ mice exhibit significantly lower Treg numbers in their mesenteric lymph nodes than wild type mice under conditions of *Helicobacter* infection, and generate stronger Th17 responses and develop more severe infection-associated immunopathology (Oertli et al., 2012). CD4$^+$CD25$^+$ cells isolated from infected IL-18$^{-/-}$ or IL-18R$^{-/-}$ donors fail to prevent allergen-induced asthma, indicating that not only the differentiation, but also the suppressive activity of Tregs depends on IL-18 signaling (Oertli et al., 2012). The current model thus assumes that the availability of IL-18 dictates whether naive T-cells co-cultured with DCs differentiate into Th1, Th17, or Treg cells; whereas Th1 and Treg differentiation depend crucially on IL-18, Th17 cells develop under conditions where IL-18 is lacking (**Figure 2**). The results obtained in the *H. pylori* model are reminiscent of the hypersusceptibility of IL-18$^{-/-}$ animals toward experimentally induced colitis, which has been attributed to the lack of Nlrp6 inflammasome activation (Elinav et al., 2011). Which nod-like receptors are involved in the innate immune recognition of *H. pylori* leading to caspase-1 activation and IL-18 processing remains

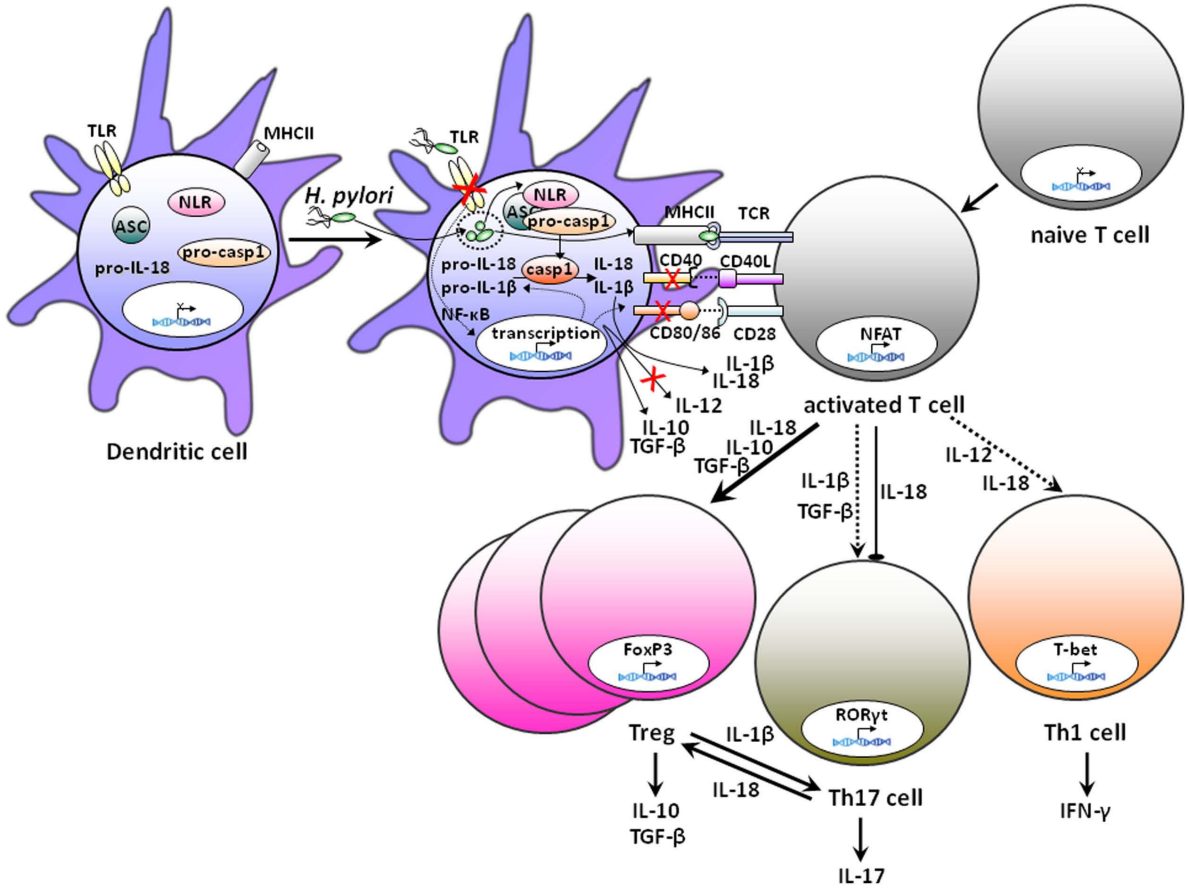

FIGURE 2 | Schematic representation of the effects of *H. pylori* exposure on DCs and the DC/T-cell interaction. Exposure to *H. pylori* induces semi-mature DCs with high expression of MHC class II, but only low to moderate expression of the co-stimulatory molecules CD40, CD80, and CD86, and of the cytokine IL-12. In contrast, IL-10 is made in large quantities by *H. pylori*-experienced DCs. Inflammasome activation by *H. pylori* through as yet uncharacterized cytoplasmic nod-like receptors (NLRs) leads to caspase-1 activation and the processing and secretion of IL-1β and IL-18. IL-1β promotes Th17 differentiation, whereas IL-18 is required for Th1 and Treg differentiation. *H. pylori*-experienced DCs actively induce the conversion of naive T-cells to FoxP3$^+$ Tregs in a process that requires IL-18, TGF-β, and possibly IL-10. In contrast, *H. pylori*-experienced DCs are poor inducers of Th17 and Th1 differentiation. The documented lack of *H. pylori* TLR ligands in conjunction with efficient inflammasome activation by the bacteria suggests that the relative availability of pro-IL-1β (low level expression due to lack of transcriptional activation) and pro-IL-18 (high levels due to constitutive expression) for caspase-1 processing may dictate the outcome of the DC/T-cell interaction.

to be determined. It is interesting to note in this context that *H. pylori* lacks many TLR ligands shared by other gram-negative, pathogenic bacteria. *H. pylori* flagellin is a poor ligand of TLR5 (Gewirtz et al., 2004) due to mutations in the TLR5 recognition site of the N-terminal D1 domain of flagellin (Andersen-Nissen et al., 2005). The bacterium's LPS consists predominantly of the tetra-acylated lipid A variety, which is known to exhibit 1000-fold reduced bioactivity as compared to *E. coli* LPS (Moran et al., 1997). While *H. pylori* harbors TLR2 ligands (Rad et al., 2009; Sayi et al., 2011), these exhibit predominantly anti-inflammatory properties *in vivo* (Sayi et al., 2011). The combined results imply that the lack of (pro-inflammatory) TLR signaling in conjunction with high level inflammasome activation and IL-18 secretion may favor Treg over Th17 differentiation during *H. pylori* infection. Our recent finding that addition of *E. coli* LPS can reverse the tolerogenic effects of *H. pylori* on DCs (Oertli et al., 2012) lends further support to this model. LPS is a strong inducer of IL-1β, which in turn is required for Th17 polarization (**Figure 2**). The relative availability of IL-1β and IL-18, which is influenced by TLR-/Myd88- and NF-κB-dependent transcriptional activation of IL-1β expression (IL-18, in contrast, is preformed and stored in granules, and not subject to extensive transcriptional regulation), thus dictates whether Tregs or Th17 cells are preferentially induced. In the context of *H. pylori* exposure of DCs, IL-18 is produced in copious amounts due to efficient inflammasome activation; in contrast, due to the concomitant lack of TLR-mediated transcriptional activation, IL-1β is not available for caspase-1-mediated processing, leading to the preferential differentiation of naive T-cells into Tregs as opposed to Th17 cells (**Figure 2**).

As Th17 cells have been implicated in adaptive immunity to *H. pylori* infection (DeLyria et al., 2009; Velin et al., 2009; Hitzler et al., 2011), the preferential induction of Tregs over Th17 cells may have conferred a selective advantage to the bacteria in the 60000 years

of co-evolution of *H. pylori* with its human host (Moodley et al., 2009) and explains why infected individuals with strong gastric Treg, but weak T-effector responses show the highest levels of colonization and the least severe gastric pathology (Robinson et al., 2008). An only recently recognized bystander effect of the strong *H. pylori*-specific Treg induction is cross-suppression of allergen- or autoantigen-specific T-cell responses (Arnold et al., 2011a), which results in the above-discussed protective effects against asthma and other allergies, as well as against IBDs.

CONCLUSION AND PERSPECTIVES

Two avenues of active research in the *Helicobacter* field will likely benefit most from integrating the concepts outlined here into current models and strategies; on the one hand, it is obvious that *H. pylori* – or at least its tolerance-promoting properties – should be harnessed for the development of new preventive or therapeutic strategies in the treatment of asthma and other allergies, and of IBDs. Whether an infection-independent strategy sometimes referred to as "tolerizing vaccination" will work in this context remains to be seen. On the other hand, vaccine development efforts directed at eradicating *H. pylori* (therapeutically or prophylactically) must take into account the need to overcome the immunomodulatory properties of this infection if sterilizing immunity is to be achieved. Finally, it will be interesting to compare the strategies that *H. pylori* has evolved to establish and maintain persistent infection to those exploited by other chronic bacteria such as mycobacteria and *Salmonella*. Overriding the immune evasion and – modulation strategies of persistent bacterial infections is a crucial first step in breaking the asymptomatic carrier state and in successfully interrupting the transmission cycle that perpetuates the worldwide public health problems associated with these persistent infections.

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Campylobacter polysaccharide capsules: virulence and vaccines

Patricia Guerry[1]*, Frédéric Poly[1], Mark Riddle[1], Alexander C. Maue[1], Yu-Han Chen[2] and Mario A. Monteiro[2]

[1] Enteric Diseases Department, Naval Medical Research Center, Silver Spring, MD, USA
[2] Department of Chemistry, University of Guelph, Guelph, ON, Canada

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Mark Estes, University of Georgia, USA
Christine M. Szymanski, University of Alberta, Canada

***Correspondence:**
Patricia Guerry, Enteric Diseases Department, Naval Medical Research Center, 503 Robert Grant Avenue, Silver Spring, MD 20910, USA.
e-mail: patricia.guerry@med.navy.mil

Campylobacter jejuni remains a major cause of bacterial diarrhea worldwide and is associated with numerous sequelae, including Guillain Barré Syndrome, inflammatory bowel disease, reactive arthritis, and irritable bowel syndrome. *C. jejuni* is unusual for an intestinal pathogen in its ability to coat its surface with a polysaccharide capsule (CPS). These capsular polysaccharides vary in sugar composition and linkage, especially those involving heptoses of unusual configuration and *O*-methyl phosphoramidate linkages. This structural diversity is consistent with CPS being the major serodeterminant of the Penner scheme, of which there are 47 *C. jejuni* serotypes. Both CPS expression and expression of modifications are subject to phase variation by slip strand mismatch repair. Although capsules are virulence factors for other pathogens, the role of CPS in *C. jejuni* disease has not been well defined beyond descriptive studies demonstrating a role in serum resistance and for diarrhea in a ferret model of disease. However, perhaps the most compelling evidence for a role in pathogenesis are data that CPS conjugate vaccines protect against diarrheal disease in non-human primates. A CPS conjugate vaccine approach against this pathogen is intriguing, but several questions need to be addressed, including the valency of CPS types required for an effective vaccine. There have been numerous studies of prevalence of CPS serotypes in the developed world, but few studies from developing countries where the disease incidence is higher. The complexity and cost of Penner serotyping has limited its usefulness, and a recently developed multiplex PCR method for determination of capsule type offers the potential of a more rapid and affordable method. Comparative studies have shown a strong correlation of the two methods and studies are beginning to ascertain CPS-type distribution worldwide, as well as examination of correlation of severity of illness with specific CPS types.

Keywords: *Campylobacter*, capsules, capsule conjugate vaccines, virulence

INTRODUCTION

Campylobacter jejuni, one of the most common causes of bacterial diarrhea worldwide, is biologically distinct from other enteric pathogens, such as *Salmonella*, *Shigella*, and *Vibrio*. A member of the epsilon proteobacteria, *C. jejuni* is more similar to Gram negative mucosal pathogens such as *Haemophilus influenzae* and *Neisseria meningitidis* in that it is microaerophilic, naturally transformable, and encapsulated. The polysaccharide capsule (CPS), which is the topic of this review, is unique for an enteric pathogen, and the *C. jejuni* capsular polysaccharides are unique compared to most others.

CAMPYLOBACTER CAPSULES: STRUCTURES AND GENETICS

During the 1990s, Aspinall and co-workers discovered that *Campylobacter* species (*C. jejuni*, *C. coli*, and *C. lari*) exposed polysaccharides (PSs) that were considered to be O-chain PS regions of cell-wall lipopolysaccharides (LPSs; Aspinall et al., 1992, 1993, 1995a,b; McDonald, 1993; Aspinall, 1998). However, structural data obtained from some *Campylobacter* species showed that these moieties were not associated with a LPS component, but were of the same type as teichoic acid PSs, as in *C. jejuni* serotype HS1 (for Heat Stable serotype; McDonald, 1993) and capsule PSs (CPSs), as in *C. lari* (Aspinall, 1998). Subsequently, genomic analysis provided confirmation that the observed PSs of *C. jejuni* were capsule (CPSs; Parkhill et al., 2000). Corcoran et al. (2006) demonstrated the phospholipid anchor in three CPS types (HS3, HS6, and HS23/36) was dipalmitoyl-glycerophosphate, with ester-linked hexadecanoic acids. *Campylobacter* species, like other Gram negative mucosal pathogens and unlike other enteric pathogens, express a CPS and lipooligosaccharide (LOS; core → lipid A) in lieu of a full length LPS (O-chain → core → lipid A). The Penner serotyping scheme is a passive slide hemagglutination that is based primarily on CPSs, although other structures, including LOS can contribute to serotype specificity (Penner and Hennessy, 1980; Preston and Penner, 1989; Karlyshev et al., 2000). A total of 23 serotypes were initially described in the original publication (Penner and Hennessy, 1980), and this was quickly extended to 47 serotypes for *C. jejuni*, although many are found in related, cross-reacting complexes.

The structure of eight CPS types of *C. jejuni* have been published (Aspinall et al., 1992, 1995c; Hannify et al., 1999; Muldoon et al., 2002; Karlyshev et al., 2005; McNally et al., 2005, 2007; Gilbert

et al., 2007; Chen et al., 2008), and these vary in sugar composition and linkage. The expression of (i) heptoses of unusual configuration (i.e., *altro*, *ido*, *gulo*, *talo*) and (ii) *O*-methyl phosphoramidate (MeOPN) are key structural markers of the capsules of *Campylobacter* species, especially *C. jejuni* (**Figure 1**). The structural complexity of the heptoses is further enhanced by the introduction of a deoxy function at the C-6 position, in that, within a single CPS polysaccharide chain, it is common to observe the presence of the heptose and its complementary 6-deoxy-heptose, for example, D-*glycero*-D-*altro*-heptose and 6-deoxy-*altro*-heptose in *C. jejuni* strains that belong to serotype complex HS23/36 (Aspinall et al., 1993; Kanipes et al., 2006). MeOPN has been identified on most *C. jejuni* CPSs, although it is found attached in different linkages to different sugars in each (Karlyshev et al., 2005; McNally et al., 2007). MeOPN is also found in non-stoichiometric amounts, likely because of phase variation (see below).

Campylobacter jejuni capsules are assembled via an ABC transporter mechanism, similar to class 2 and class 3 capsules of *E. coli* K1 and K5, *N. meningitidis* and *H. influenzae*, and the genetic organization of capsule genes in *C. jejuni* is similar to those found in these bacteria, as shown in **Figure 2**. Capsule genes in these groups are organized in three regions, where the conserved regions 1 and 3 are involved in capsule assembly and transport, and the variable region 2 encodes genes responsible for synthesis of the polysaccharides. The major difference between CPS groups 2 and 3 is the organization of the *kps* genes, and the presence of an additional gene involved in thermoregulation of capsule synthesis, *kpsU*, in group 2 capsule strains. *C. jejuni* is more similar to the group 3 capsules by its absence of the *kpsU* gene and apparent lack of thermoregulation (Stintzi, 2003), but appears to be a hybrid between groups 2 and 3 due to differences in gene organization (**Figure 2**). Although the *kpsM* gene of 81–176 has been shown to functionally complement the corresponding mutation in *E. coli* K1 (Bacon et al., 2001), no functional studies of *C. jejuni* *kps* gene products have been reported. The function of the products of Class 2 and Class 3 *kps* genes have been reviewed recently (Vimr and Steenbergen, 2009).

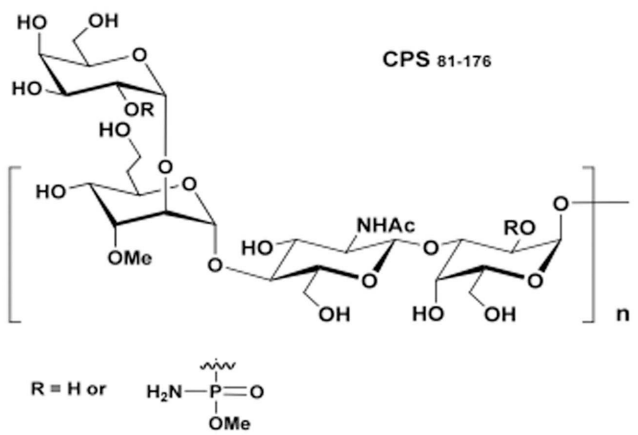

FIGURE 1 | Covalent structure of the CPS from *C. jejuni* strain 81–176 (serotype HS23/36). The trisaccharide repeating block is composed of → 3)-D-Gal*p*-(1 → 2)-3-Me-6d-α-D-altro-Hep*p*-(1 → 3)-β-D-Glc*p*NAc-(1 → . The methylated heptose is sometimes substituted by 6-deoxy-*altro*-heptose. The *O*-methyl phosphoramidate side chain is connected to the C-2 position of Gal in non-stoichiometric amounts (Kanipes et al., 2006).

Region 2, located between *kpsC* and *kpsF* in *C. jejuni*, contains genes that are responsible for biosynthesis of specific polysaccharides (see **Figure 3**). Variability of this region reflects the array of CPS structures/Penner serotypes of *C. jejuni*. A total of 18 CPS loci from distinct Penner types have been sequenced to date (Parkhill et al., 2000; Pearson et al., 2004; Fouts et al., 2005; Karlyshev et al., 2005; Poly et al., 2011). Region 2 in these strains contains from 11 to 30 genes and ranges in size to from 11 to 34 kb. Variation in this region reflects horizontal gene transfer, gene duplication, deletion, and fusion, all of which participate in the observed variability of CPS structures (Karlyshev et al., 2005). Despite the high level of overall variability in region 2, some genes are highly conserved. These include genes for synthesis of heptose (*hddC*, *gmhA*, *hddA*) and deoxyheptose (*dmhA*) as well of genes involves in MeOPN biosynthesis. McNally et al. (2007) showed that four genes (CJ1415c–1418c) are required for MeOPN synthesis in NCTC 11168, and these genes are highly conserved in most strains of *C. jejuni*. Two other genes, Cj1419c and Cj1420c, annotated as methyl transferases, are always found adjacent to the MeOPN synthesis genes, although a function has not been ascribed to them. **Figure 2** indicates that 61.5% (8/13) of published CPS loci contain homologs of Cj1415c–Cj1420c. BLAST analysis reveals the presence of these genes not just in *C. jejuni* strains, but also in other

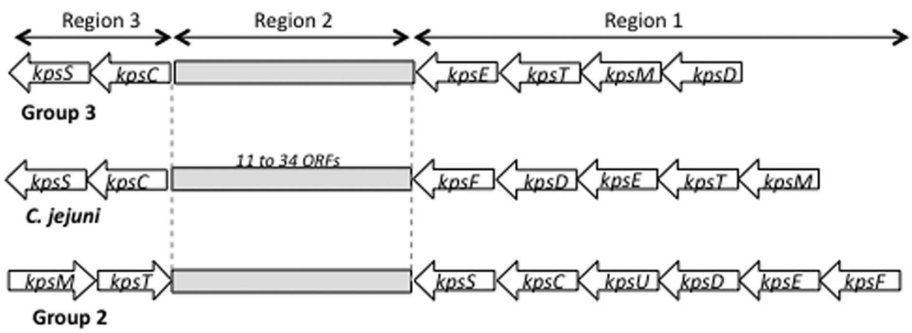

FIGURE 2 | Schematic of the capsule regions of *E. coli* group 2 and group 3 capsules compared to *C. jejuni*.

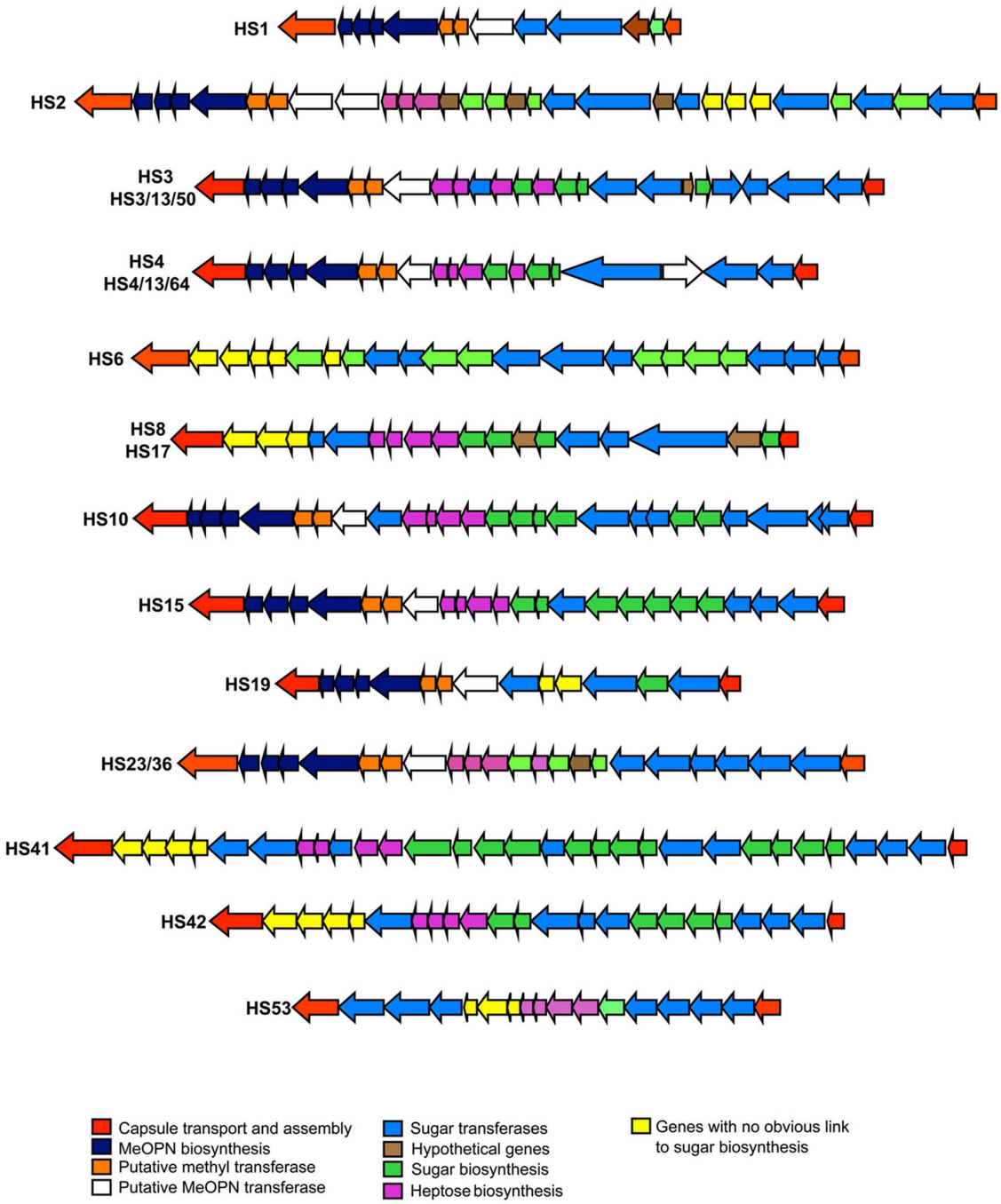

FIGURE 3 | Schematic of variable region 2 CPS loci from sequenced representative Penner serotype loci. The function or putative function of genes is color-coded, as indicated.

epsilon proteobacteria, including other species of *Campylobacter*, *Wolinella succinogenes*, and *Helicobacter* spp. other than *H. pylori*. Production of CPS in those strains in uncertain, but the high degree of protein identity and gene order of the homologs suggests a common source of origin (lateral transfer) of those genes as well as a putative biosynthesis of MeOPN by a wider number of species than originally thought (McNally et al., 2007).

McNally et al. (2007) also identified two genes adjacent to the MeOPN gene cluster, Cj1421c and Cj1422c, as transferases responsible for addition of MeOPN to two distinct sites in the CPS of an HS2 CPS (the C-3 of β-D-Gal*f*NAc and to the C-4 of D-*glycero*-α-L-*gluco*-heptopyranose residues). Based on similarity to these MeOPN transferases, other putative MeOPN transferases can be identified in additional *C. jejuni* CPS loci, all of which appear

to be adjacent to the MeOPN biosynthesis genes. These putative MeOPN transferases contain a conserved N-terminal region sequence and a variable C-terminal region. The conserved region likely encodes the region of the enzyme that recognizes MeOPN, while the variable region likely reflects the different sugar residues to which MeOPN is attached.

The genes encoding enzymes for biosynthesis of heptose and deoxyheptose are also highly conserved in various CPS loci. CPS heptoses are synthesized by the products of *hddC* (putative D-glycero-D-manno-heptose 1-phosphate guanosyltransferase), *gmhA2* (phosphoheptose isomerase), *hddA* (putative D-glycero-D-manno-heptose 7-phosphate kinase). Biosynthesis of deoxyheptose has been demonstrated in *C. jejuni* (Karlyshev et al., 2005) and linked by homology of sequence to *dmhA* a putative GDP-mannose 4,6-dehydratase involves in the conversion of heptose to deoxyheptose. The enzymatic properties of DmhA from strain 81 to 176 were recently characterized (McCallum et al., 2011).

REGULATION OF CPS EXPRESSION

The ability of *C. jejuni* to undergo phase variations by slip strand mispairing during replication at homopolymeric tracts of bases is well established (Linton et al., 2000; Guerry et al., 2001; Hendrixson, 2006). *C. jejuni* CPS expression undergoes phase variable changes at two levels. Expression of CPS undergoes an on/off phase variation, likely due to slip strand mismatching in one or more genes in region 1 and 3 (Bacon et al., 2001; Guerry and Szymanski, 2008). Other phase variations can modulate the CPS structure. Thus, GC tracts are commonly found in *dmhA*, Cj1420c (one of the genes adjacent to the MeOPN genes, see above), as well as putative MeOPN transferases. Thus, phase variable changes in *dmhA* may regulate the amount of deoxyheptoses in the capsules of HS23/36 complex, and changes in the MeOPN transferases are thought to be responsible for the non-stoichiometric amounts of this modification on the CPS.

There is also some evidence that CPS expression is regulated transcriptionally. Experimental determination of the transcriptional patterns of the capsule genes has not been reported, but *in silico* studies by Petersen et al. (2003) suggested a *rpoD* (sigma^{70})-regulated operon that started with *kps*M and extended into region 2. Transcriptional profiling studies have suggested that some of the genes involved in CPS biosynthesis are regulated differentially. Thus, four genes in region 2 were reported to be up-regulated under growth conditions with high iron (Palyada et al., 2004), and some CPS genes in both region 2 and region 3 were down regulated *in vivo* in the stomach of pigs (Reid et al., 2008). *In vitro* studies showed that four genes involved in CPS transport and three genes involved in polysaccharide biosynthesis were down regulated when *C. jejuni* was grown in the presence of HCT-8 human intestinal epithelial cells (Corcionivoschi et al., 2009). The significance of these changes to capsule structure and virulence remain to be determined.

ROLE OF CAPSULES IN VIRULENCE

The ability to turn CPS expression on and off suggests that CPS expression may be advantageous at some points during the *C. jejuni* lifestyle and disadvantageous at others, suggesting a role in virulence. Similarly, the ability to phase vary the structure of the CPS, such as modifying the levels of MeOPN, likely serves a biological purpose. Polysaccharide capsules are important in virulence for virtually all bacteria that express these structures, but surprisingly little is understood about the role that CPS plays in *C. jejuni*-mediated disease. Bacon et al. (2001) showed that a *kpsM* mutant of strain 81–176 showed a modest (about 10-fold) reduction in invasion of intestinal epithelial cells *in vitro*. Similar results were obtained using non-encapsulated mutants of other strains by Bachtiar et al. (2007) and Corcionivoschi et al. (2009). The latter study also demonstrated that capsule expression by both 81–176 and NCTC 11168 was reduced by passage with HCT-8 intestinal cells in culture, as mentioned above. This result was somewhat unexpected, but the passaged cells showed reduced invasion that correlated with reduced capsule expression. However, the passaged cells represented those not attached to the monolayer; the authors did not examine capsule expression on adhered or invaded cells.

There have been a few studies that have examined the interaction of the *C. jejuni* CPS with various components of the host innate immune response. *C. jejuni* shows levels of resistance to complement killing that are comparable to those of other mucosal pathogens, and, like other mucosal pathogens, the CPS contributes to resistance to complement killing (Bacon et al., 2001; Keo et al., 2011). However, it is not clear if complement resistance is required for intestinal colonization or after invasion of the intestinal epithelium. Zilbauer et al. (2005) showed that *C. jejuni* 81–176 and NCTC 11168 induced human β-defensins 2 and 3 (hBD2 and hBD3) from intestinal epithelial cells in culture, but that both wildtype and *kpsM* mutants of both strains were equally sensitive to the action of these β-defensins. This is consistent with work of Keo et al. (2011) who showed that the LOS core played a role in resistance to β-defensins and polymyxin, but that the CPS played a limited role. In contrast, capsules on other mucosal pathogens have been shown to protect against β-defensins (Campos et al., 2004). Rose et al. (2011) showed that non-encapsulated mutants showed increased production of IL-6, IL-10, and TNF-alpha from murine dendritic cells. A similar effect was seen with mutant that lacked MeOPN on the capsule, suggesting an important biological role for this unusual modification.

There are limited small animal models for *C. jejuni* disease. Champion et al. (2010) have developed a model using the larvae of the wax moth, *Galleria mellonella*. Injection of three different strains of *C. jejuni* into the larvae resulted in rapid killing. Using specific mutants the authors showed that the polysaccharide CPS of strain NCTC 11168, and specifically, the MeOPN modification, was responsible for this lethality. This may be related to the fact the MeOPN is structurally related to organophosphorus pesticides, but the significance of this finding to human disease remains to be determined. Chickens, which can become colonized with *C. jejuni* without disease, have been used to study commensalism. Two groups have shown that encapsulated strains colonize chickens better than unencapsulated mutants (Grant et al., 2005; Bachtiar et al., 2007). Unfortunately, there are few diarrhea models of disease for *C. jejuni*, but Bacon et al. (2001) showed that a *kpsM* mutant of 81–176 was attenuated in diarrheal disease in an infant ferret model. More convincing evidence of a role in diarrheal disease has come from vaccination studies in non-human primates (see below).

THE BURDEN OF *C. JEJUNI* DISEASE

Campylobacter jejuni infection, which occurs through exposure to contaminated food and water, is a major global health problem. In the developing world it is estimated that 40–60% of children under the age 5 will develop at least one symptomatic infection, usually occurring during the first year of life (reviewed in Coker et al., 2002). Epidemiologic studies are lacking in geographically diverse settings, but those that do exist suggest that repeated pediatric infections are not uncommon. Recent data suggest that *Campylobacter* may be even more important than previously thought. The Global Enterics Multi-Center Study (GEMS), a prospective, multi-center, case–control study of acute diarrhea in children 0–59 months of age funded by the Bill and Melinda Gates Foundation, has found in 2-year follow-up data that *Campylobacter* infections in Asia (Bangladesh, India, Pakistan) represent important enteric infections leading to severe diarrhea in the first years of life (Levine, 2011). A recent passive clinical surveillance study in a defined catchment population from Karachi, Pakistan reports an annual incidence of *C. jejuni* infection of 29 per 1,000 person-years, with peak incidence at around 2 years of age (Soofi et al., 2011). It is notable that *C. jejuni* infections were more frequently diagnosed than *Shigella*, though *Shigella* was more often associated with dysentery. Additionally, for the developing world, one must consider the association between *Campylobacter* and HIV infection where incidence may be increased, and morbidity and mortality are found to be higher among HIV positive individuals (Sorvillo et al., 1991; Molina et al., 1995; Tee and Mijch, 1998). In a case series of 38 patients with HIV and *Campylobacter* infection, acute diarrhea, fever, and abdominal pain was a predominant symptom complex, however 11% had bacteremia, and 8% experienced chronic diarrhea (Molina et al., 1995). Furthermore, long-term carriage of *Campylobacter* can occur, sometimes associated with recurrent attacks of enteritis and bacteremia among HIV infected individuals. Given the growing HIV pandemic, it is estimated that the burden of campylobacteriosis in developing countries may be among the top 10 in 2020.

In the industrialized world, general population incidence estimates based on passive surveillance vary depending on geography and over time with rates in the US averaging around 15/100,000 with a slow decline over the past decade, and rates in Europe around 50–90/100,000 with rising trends (reviewed in Janssen et al., 2008). Some countries in Eastern Europe and New Zealand have higher incidence rates of 300–400/100,000 per year. Due to underreporting of these types of illness, true rates of campylobacteriosis are considered to be 10–100 times higher than those reported historically. To this end, recent population-based studies in a number of developed countries have employed varied study designs to estimate overall incidence of *Campylobacter* infections through use of cohort studies, state of the art and standardized identification methods, surveys, and active surveillance networks (**Table 1**). These studies confirm the underreporting and case ascertainment bias associated with passive surveillance studies and find that incidence of *Campylobacter* infection ranges from 3 to 15 cases per 1,000 person-years.

Finally, travelers represent unique populations that are at high risk for *Campylobacter*, where globally it occurs in 5–15% of diarrheal cases (Riddle et al., 2006; Shah et al., 2009). *Campylobacter* infections are considerably more frequent in some areas of SE Asia. Ravel et al. (2011) recently reported data from an enhanced, passive surveillance system of travel-related diseases caused by enteropathogens within a Canadian community from June 2005 to May 2009. Of the 446 cases of travel-related disease

Table 1 | Global estimates of *Campylobacter* incidence in developed countries.

Reference	Kubota et al. (2008)	Tam et al. (2012)	De Wit et al. (2001)	Hall et al. (2008)	Scallan et al. (2011)
Country	Japan	UK	Netherlands	Australia	US
Year of study	2006–2007	2008–2009	1998–1999	2000–2004	2006
Study design	Two 2-week cross-sectional, population-based telephone surveys combined with catchment area surveillance	Prospective, community cohort study and prospective study of general practice presentation in national surveillance system	Prospective population-based study with nested case–control study in general population	Empirical model based on published and unpublished data from multiple active/passive surveillance sources	Empirical model based on published and unpublished data from multiple active/passive surveillance sources
Numbers	4,247 Household interviews, 8,462 laboratory confirmed cases ascertained in active surveillance	6,836 Cohort participants, 800,000 catchment area for national surveillance	4,860 Patients enrolled in cohort	Not applicable	Not applicable
Incidence estimate (95% CI), per 1,000 person-years	15.1 (7.4–28.6)	10.9 (7.4–15.9)	4.8 (1.7–10.4)	11.8 (7.6–26.7)	2.8 (not given)
Foodborne illness rank	1 of 3 overall	4 of 12 overall; 1 of 5 bacterial	1 of 5 bacterial	1 of 3 overall	4 of 31 overall; 3 of 21 bacterial

due to enteropathogens reported, *Campylobacter* was the most frequently identified cause ($n = 123$, 28%). Similarly, in a report from the GeoSentinel Travel Network, a clinician-based sentinel surveillance data for 17,353 ill returned travelers, *Campylobacter* was identified in 8.5 per 100 diarrheal cases and was the leading bacterial etiology (Freedman et al., 2006).

Taken together, these data highlight the clear importance of *Campylobacter* in global populations in terms of disease incidence, but do not completely portray the enormity of *Campylobacter* as a public health problem. An increasing number of studies are highlighting *Campylobacter*-associated chronic health sequelae (**Table 2**). Beyond the well described association with Guillain–Barré Syndrome (GBS), the leading cause of acute flaccid paralysis in the developing world for which up to a third can be attributed to *Campylobacter*, reactive arthritis, inflammatory bowel disease (particularly Crohn's Disease), and irritable bowel syndrome are also recognized as post-campylobacteriosis sequelae in industrialized populations, although there is lack of information on these sequelae in the developing world. Furthermore, it will be of interest to explore possible associations with CPS type as well as other virulent mechanisms and these health sequelae.

From a public health perspective, some country-level approaches have been attempted to quantify and characterize the burden and cost associated with acute diarrheal infections and their chronic consequences. For example, in New Zealand it is estimated that major foodborne illness and its chronic health consequences costs society approximately $86 million per year, and 90% of this cost is attributed to lost productivity due to absence from work (Lake et al., 2010), and campylobacteriosis is shown to account for approximately 90% of the estimated cost of foodborne illness. Cost and burden of disease studies in Australia and the Netherlands have also been conducted outlining substantial individual and societal costs in which acute disease is either matched or exceeded by the chronic consequences (Mangen et al., 2004). Beyond the country-level perspective focusing on domestically acquired foodborne infections, populations at high risk for acute infectious diarrhea (e.g., travelers, military) should also be considered in estimating the full burden of disease and the potential value of current and novel interventions. It should be noted, while not as well studied, the chronic consequences of *Campylobacter* infections in developing world populations should not be underestimated. Given the known mucosal barrier disruption effects of *Campylobacter* infection (Beltinger et al., 2008), the contribution of this particular infection may emerge as an important factor to malnutrition and associated consequences in the developing world. An international group of investigators are collaborating on a project entitled The Interactions of Malnutrition and Enteric Infections (MAL–ED): Consequences for Child Health and Development (http://mal-ed.fnih.org/), involving a network of eight sites that are exploring the factors associated with a child's risk of enteric infection, chronic diarrhea, malnutrition, as well with impaired gut function, vaccine response, and cognitive and physical development (Lorntz et al., 2006; Tarleton et al., 2006; Santos et al., 2008; Oria et al., 2010). Given the known mucosal barrier disruption effects of *Campylobacter* infection, it will be important to follow.

While more study utilizing improved diagnostics and characterizing the pathogenesis of acute and chronic health effects of *Campylobacter* infections is needed, the data that is available suggest that control of *Campylobacter* should be considered a global public health priority. Promotion and strengthening of food safety systems, good manufacturing practices and educating retailers and consumers about appropriate food handling and avoiding contamination are needed now and can be employed. Education of consumers and training of food handlers in safe food handling is one of the most critical interventions in the prevention of foodborne illnesses. Due to the challenges of implementing these aforementioned strategies, however, vaccines for use in travelers, military personnel, and pediatric populations in the developing world are much needed. To this end, we have explored the feasibility of CPS conjugate vaccines against *C. jejuni*.

CPS CONJUGATE VACCINES AS A STRATEGY AGAINST *C. JEJUNI*

CPS-based vaccine strategies have been very successful at reducing the overall disease incidence of several encapsulated bacteria including *Streptococcus pneumoniae*, *N. meningitidis*, and *H. influenzae* (reviewed in Lesinski and Westernick, 2001a; Knuf et al.,

Table 2 | Summary evidence of post-*Campylobacter* infection risk of select chronic health consequences.

Sequelae	Post-infective attributable risk*	Comment	Reference
Guillain Barré syndrome	1 per 1,000	14–32% of GBS cases can be attributed to *C. jejuni*	Nachamkin et al. (2000)
Reactive arthritis	1–5%	5% of *C. jejuni* ReA may be chronic or relapsing	Pope et al. (2007)
Inflammatory bowel disease	3–4 per 10,000	Recent evidence suggests that *C. jejuni* can breach the intestinal barrier and may prime the intestine for chronic inflammatory responses in susceptible individuals (Kalischuk and Buret, 2010)	Gradel et al. (2009), Jess et al. (2011), Rodriguez et al. (2006)
Irritable bowel syndrome	1–10%	IBS developed in 36% of patients associated with a large waterborne outbreak of mixed *Campylobacter* and STEC in Walkerton, Canada. Symptoms persist in approximately 40–50% at 5–7 years	Rodriguez and Ruigomez (1999), Thornley et al. (2001), Dunlop et al. (2003), Marshall et al. (2006)

**Post-infective attributable risk considers the absolute difference of the rate of sequelae after C. jejuni compared to the rate of the sequelae in an unexposed population.*

2011). Given the success of these capsular-based vaccines, we have begun studies to determine the feasibility of a capsule conjugate vaccine approach to prevent disease caused by *C. jejuni*.

Most capsular polysaccharides are thymus-independent (TI) antigens meaning that helper T cells are needed to generate robust, long-lived antibody responses. Although adults can generate some antibody responses to purified capsules administered as a vaccine (Lesinski and Westernick, 2001b), children <2 years of age do not develop strong responses to TI antigens (Rijkers et al., 1998). As most carbohydrate antigens cannot directly activate naïve T cells, a carrier protein is often included to optimize immune responses (Knuf et al., 2011). This vaccine strategy effectively converts a TI antigen into a thymus dependent (TD) antigen that allows boosting of the immune response, IgG antibody class switching, and the generation of memory cells possessing antibody with higher avidity for CPS (Lesinski and Westernick, 2001b).

In the initial studies, CPS was purified from *C. jejuni* strains 81–176 (HS23/36) and CG8486 (HS4 complex) and conjugated to the carrier protein, CRM_{197}, which is a mutant diphtheria toxin subunit, using reductive amination (Monteiro et al., 2009). BALB/c mice were immunized subcutaneously with escalating doses of *C. jejuni* CPS–CRM_{197}. Maximal titers were generated following three doses of vaccine. One month after the last dose of vaccine, immunized mice were challenged with each respective strain using an intranasal infection model to determine if vaccination conferred protection. Immunized mice exhibited significantly lower levels of sickness following challenge suggesting that antibodies generated against the capsule afforded some protection against infection with *C. jejuni* in this model. However, testing in a more relevant model was still required.

The New World monkey, *Aotus nancymaae*, has been shown to develop diarrheal disease that mimics aspects of human illness following orogastric challenge with *C. jejuni* (Jones et al., 2006). To determine if immunization with CPS–CRM_{197} could prevent diarrheal disease, monkeys were immunized with 2.5 μg of polysaccharide adjuvanted with alum three times with an interval of 6 weeks between subcutaneous injections. Nine weeks following the third immunization, monkeys were challenge orogastrically with approximately 10^{11} CFU of *C. jejuni* 81–176. The diarrheal attack rate for non-immunized control monkeys was 70%. However, 100% of vaccinated animals (14/14) were protected against diarrheal disease (Monteiro et al., 2009). This work was the first demonstration that a capsule conjugate vaccine could protect against enteric disease and also suggests that the capsule plays a critical role in virulence.

These proof-of-concept studies proved the feasibility of using a capsule conjugate vaccine to prevent illness caused by *C. jejuni*. However, before developing a vaccine for practical use, the number of CPS types needed to achieve broad coverage against the most prevalent strains of *C. jejuni* needs to be determined

CPS TYPING BY MULTIPLEX PCR

The likely target population for a vaccine against *C. jejuni* would be civilian and military travelers to endemic regions and pediatric populations living in endemic areas. However, while there are extensive data on Penner types of *C. jejuni* strains from the developed world, particularly the Europe and Canada, there are limited data from less developed countries (LDC) where the incidence of *C. jejuni* diarrhea is high. **Figure 4** summarizes published data on Penner types from sporadic clinical cases based on >16,000 clinical isolates from the developed world and about 700 from LDC. A non-systemic review of Penner typing for sporadic clinical cases identified HS4, HS2, and HS1 as the most common globally. The HS4 complex was the most prevalent, accounting for 21.2 and 13.2% of all cases for the developed and LDC, respectively, followed by HS2 (16.4 and 7.96% for developed and LDC, respectively) and the HS1 complex (10.7 and 6.81% for developed and LDC, respectively). Penner types HS3, HS5, and HS8 were also prevalent worldwide, while other serotypes tend to show geographic and population-based variability. More than 10% of isolates from the developed world and >21% from the developing world were non-typeable in the Penner scheme. This could reflect the existence of CPS types not covered in the Penner typing scheme, but it is also in partly due to the fact that Penner serotyping requires CPS expression and a certain percentage of these isolates have turned capsule expression off by phase variation (Poly, unpublished).

To overcome the limited data on CPS distribution in LDC's, a *C. jejuni* CPS multiplex-based PCR was recently developed and validated. The current CPS multiplex is composed of a total of 14 primer pairs, separated into two mixes, alpha and beta (Poly et al., 2011). Primers were designed on specific sequences of available *C. jejuni* class 2 loci (**Figure 2**). Most of the Penner serotypes that fall into related complexes are recognized with the same pair of primers. Thus, the multiplex system does not distinguish between HS23 and HS36 and other similar complexes of Penner types, which have very similar CPS structures that crossreact immunologically. Attribution of CPS type is deciphered based on the length of the PCR product after electrophoresis on a 2% agarose gel along with a molecular weight ladder. This technology was validated on a total of 244 strains of known serotype and shown to have sensitivities and specificities ranging from 90 to 100%. A perfect correlation between CPS type and Penner type was demonstrated. This technology is still expanding and is currently being evaluated in multiple laboratories worldwide. One advantage of the multiplex system over classical serotyping is that the multiplex is independent of phase variation in capsule expression. Collection of additional data by the multiplex PCR will provide a more accurate picture of *C. jejuni* CPS distribution, particularly in LDCs, that will dictate the final formulation of an effective CPS conjugate vaccine. The ability to rapidly determine capsule type will also facilitate studies to determine if specific CPS types are associated with more severe acute diarrheal disease and with the chronic sequelae, particularly functional bowel disorders.

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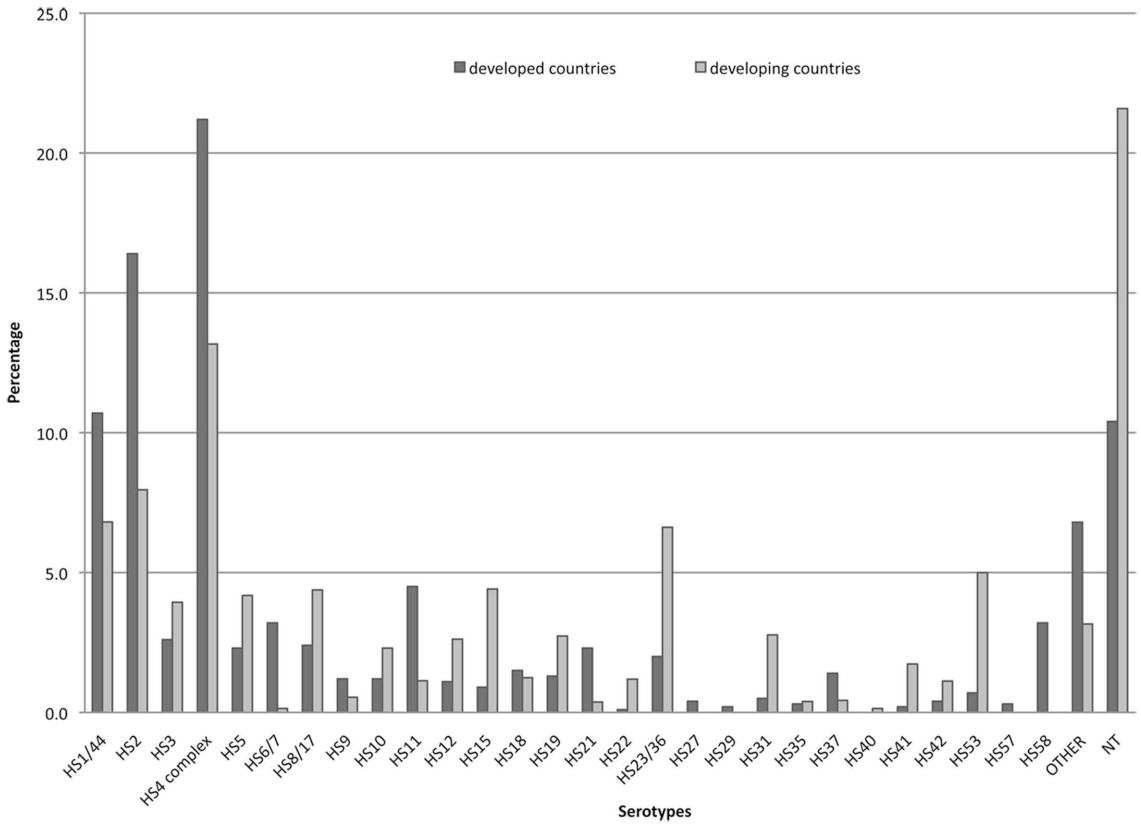

FIGURE 4 | Penner serotype distribution worldwide. The data represent a total of >16,000 strains from developed countries (Penner and Hennessy, 1980; McMyne et al., 1982; Penner et al., 1983; Jones et al., 1985; Patton et al., 1985; Mills et al., 1991; Albert et al., 1992; Skirrow et al., 1993; Marshall et al., 1994; Owen et al., 1994; Nielsen et al., 1997, 2006; Frost et al., 1998; McKay et al., 2001; Oza et al., 2002; Wareing et al., 2002; Woodward and Rodgers, 2002; Karenlampi et al., 2003; Siemer et al., 2004; Cornelius et al., 2005; Fussing et al., 2007; McTavish et al., 2008); and far fewer strains (711) from less developed countries, including Central Africa (Georges-Courbot et al., 1989), South Africa (Lastovica et al., 1986), South Africa (Lastovica et al., 1986), Egypt (Poly et al., 2011), Bangladesh (Neogi and Shahid, 1987), Kuwait (Sjogren et al., 1989), and Thailand (Poly et al., 2011).

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Phase variable expression of capsular polysaccharide modifications allows *Campylobacter jejuni* to avoid bacteriophage infection in chickens

Martine C. Holst Sørensen[1†], Lieke B. van Alphen[2†], Christopher Fodor[2], Shauna M. Crowley[2], Bjarke Bak Christensen[3], Christine M. Szymanski[2]* and Lone Brøndsted[1]*

[1] *Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg, Denmark*
[2] *Department of Biological Sciences, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada*
[3] *National Food Institute, Technical University of Denmark, Søborg, Denmark*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Jun Lin, The University of Tennessee, USA
Duncan Maskell, University of Cambridge, UK

****Correspondence:***
Christine M. Szymanski, Department of Biological Sciences, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada T6G 2E9.
e-mail: christine.szymanski@ualberta.ca;
Lone Brøndsted, Department of Veterinary Disease Biology, University of Copenhagen, Stigbøjlen 4, DK-1879 Frederiksberg C, Denmark.
e-mail: lobr@life.ku.dk

†Martine C. Holst Sørensen and Lieke B. van Alphen have contributed equally to this work.

Bacteriophages are estimated to be the most abundant entities on earth and can be found in every niche where their bacterial hosts reside. The initial interaction between phages and *Campylobacter jejuni*, a common colonizer of poultry intestines and a major source of foodborne bacterial gastroenteritis in humans, is not well understood. Recently, we isolated and characterized a phage F336 resistant variant of *C. jejuni* NCTC11168 called 11168R. Comparisons of 11168R with the wildtype lead to the identification of a novel phage receptor, the phase variable *O*-methyl phosphoramidate (MeOPN) moiety of the *C. jejuni* capsular polysaccharide (CPS). In this study we demonstrate that the 11168R strain has gained cross-resistance to four other phages in our collection (F198, F287, F303, and F326). The reduced plaquing efficiencies suggested that MeOPN is recognized as a receptor by several phages infecting *C. jejuni*. To further explore the role of CPS modifications in *C. jejuni* phage recognition and infectivity, we tested the ability of F198, F287, F303, F326, and F336 to infect different CPS variants of NCTC11168, including defined CPS mutants. These strains were characterized by high-resolution magic angle spinning NMR spectroscopy. We found that in addition to MeOPN, the phase variable 3-*O*-Me and 6-*O*-Me groups of the NCTC11168 CPS structure may influence the plaquing efficiencies of the phages. Furthermore, co-infection of chickens with both *C. jejuni* NCTC11168 and phage F336 resulted in selection of resistant *C. jejuni* bacteria, which either lack MeOPN or gain 6-*O*-Me groups on their surface, demonstrating that resistance can be acquired *in vivo*. In summary, we have shown that phase variable CPS structures modulate phage infectivity in *C. jejuni* and suggest that the constant phage predation in the avian gut selects for changes in these structures leading to a continuing phage–host co-evolution.

Keywords: bacteriophage, *Campylobacter jejuni*, capsular polysaccharide, phase variation, phosphoramidate, methylation

INTRODUCTION

Bacteriophages (phages) are the most abundant group of organisms on the planet and are estimated to outnumber their bacterial hosts by 10-fold (Brüssow and Hendrix, 2002). The first step of phage infection is through the specific recognition and attachment of the phage particle to the host cell (Weinbauer, 2004). In general, phages bind to unique host-specific structures, thus allowing them to recognize a suitable host in a mixed bacterial population (Rakhuba et al., 2010). Knowledge of phage receptors is required for implementation of efficient phage therapies against human pathogens – an intervention strategy that has been promoted by the increasing occurrence of antibiotic resistance (Levy and Marshall, 2004; Kutateladze and Adamia, 2010), yet only a few bacterial phage receptors have been identified in Gram-negative bacteria (for example, Gemski and Stocker, 1967; Stirm and Freund-Molbert, 1971; Gemski et al., 1975; Yu and Mizushima, 1982; Morona et al., 1984).

Capsular polysaccharides (CPS) are highly hydrated polymeric gels that provide a thick (400 nm or more) layer protecting bacteria from hostile environments and host immune defenses, but the CPS can also be a target for phage binding and infection (Stirm and Freund-Molbert, 1971; Roberts, 1996; Comstock and Kasper, 2006). The capsule of the zoonotic foodborne pathogen, *Campylobacter jejuni*, is required for serum resistance, invasion of human epithelial cells, colonization of chickens, and diarrheal disease in ferrets (Bacon et al., 2001; Jones et al., 2004). Furthermore, mutation of several *C. jejuni* CPS genes resulted in phage resistance against a group of phages, but the actual capsular moieties involved

Abbreviations: β-D-Rib, ribose; β-D-Gal*f*NAc, *N*-acetylgalactosamine in the furanose configuration; α-D-Glc*p*A6(NGro), glucuronic acid with 2-amino-2-deoxyglycerol at C-6; MeOPN, *O*-methyl phosphoramidate; Hep*p*, heptose. The 2-amino-2-deoxyglycerol can also be substituted with an *N*-ethanolamine modification at C-6.

were not identified (Coward et al., 2006). Only recently a specific component of the *C. jejuni* capsule was identified as the phage receptor for this species. We showed that phage F336 relies on the *O*-methyl phosphoramidate (MeOPN) modification of the *C. jejuni* NCTC11168 CPS for adsorption to the host cell surface and infection (Sørensen et al., 2011).

The CPS of *C. jejuni* is the major component recognized by the Penner serotyping scheme for which 47 different serotypes are known (Guerry et al., 2012). Thus, the CPS are highly diverse in structure and this is reflected by the gene content of the capsular loci in various *C. jejuni* strains (Karlyshev et al., 2005). In addition, a number of CPS genes possess homopolymeric tracts making them prone to phase variation which allows a rapid on/off switching of the genes and promotes variations in CPS even when the strains have identical gene contents (Linton et al., 2001; St Michael et al., 2002; Karlyshev et al., 2005; McNally et al., 2005; Chen et al., 2008). The CPS of *C. jejuni* 11168 consists of -2)-β-D-Rib*f*-(1-5)-3-MeOPN-β-D-Gal*f*NAc-(1-4)-α-D-Glc*p*A6(*N*Gro)-(1-backbone with a 3,6-di-*O*-methyl-4-MeOPN-D-*glycero*-α-L-*gluco*-Hep*p* side chain (St Michael et al., 2002; Szymanski et al., 2003; McNally et al., 2005). The CPS biosynthetic locus in strain NCTC11168 includes 28 genes and 6 of these genes (*cj1420*, *cj1421*, *cj1422*, *cj1426*, *cj1429*, and *cj1437*) contain homopolymeric G (polyG) tracts (Parkhill et al., 2000). Even though the function of only three out of the six gene products has been identified, a highly variable presence/absence of methyl, ethanolamine, aminoglycerol, and phosphoramidate modifications of the CPS have been observed (St Michael et al., 2002; Szymanski et al., 2003; McNally et al., 2005), suggesting that the remaining genes could encode enzymes involved in synthesis or transfer of these modifications to the CPS glycans. Recently, the *cj1421* and *cj1422* genes were shown to encode MeOPN transferases attaching MeOPN to the Gal*f*NAc and the Hep residues, respectively (McNally et al., 2007). Similarly, *cj1426* was demonstrated to encode the 6-*O*-Me transferase enzyme (Sternberg et al., submitted).

We previously discovered that resistance toward phage F336 evolved with a high frequency in *C. jejuni* NCTC11168 due to loss of the MeOPN receptor on the bacterial surface as a result of phase variation in the *cj1421* gene encoding the MeOPN–Gal*f*NAc transferase. In addition, deletion of the *cj1421* gene in NCTC11168 resulted in phage resistance, proving that MeOPN attached to Gal*f*NAc was a receptor for phage F336 (Sørensen et al., 2011). The aim of the present study was to determine if other phages rely on the MeOPN moiety for infection of *C. jejuni* and to explore the role of other CPS phase variable modifications for phage infection. Knowledge of phage receptors and development of phage resistance *in vivo* is essential for implementation of efficient phage therapy against *C. jejuni* in the chicken gut. We found that not only the MeOPN moiety, but also the phase variable 3-*O*-Me and 6-*O*-Me groups in the CPS of *C. jejuni* NCTC11168 may influence phage sensitivity. Co-infection of chickens with *C. jejuni* NCTC11168 and phage F336 resulted in bacterial acquisition of phage resistance through a loss of MeOPN attached to Gal*f*NAc of the capsule or the acquisition of the 6-*O*-Me group. We propose that the constant exposure of *C. jejuni* to naturally occurring phages in the avian gut selects for different phase variable structures of the CPS resulting in a continuous phage-host co-evolution.

MATERIALS AND METHODS

BACTERIAL STRAINS, MEDIA, AND GROWTH CONDITIONS

The *C. jejuni* strains used in this study are listed in **Table 1** and were routinely grown on blood agar Base II (Oxoid) supplemented

Table 1 | *Campylobacter jejuni* strains.

	Serotype	Description	Reference
1447	HS:4c	Chicken isolate	Hansen et al. (2007)
NCTC12662	HS:5j	Origin not known	National Collection of Type Cultures, UK
NCTC11168 (MP21)	HS:2	Wild type	Sørensen et al. (2011)
NCTC11168 MP24	HS:2	NCTC11168 variant passaged	This study
NCTC11168 MP25	HS:2	NCTC11168 variant passaged	This study
NCTC11168 MP26	HS:2	NCTC11168 variant passaged	This study
NCTC11168 V26	HS:2	NCTC11168 variant	Carrillo et al. (2004)
kpsM	Untypeable	NCTC11168 *kpsM::kan* does not have a capsule	Karlyshev et al. (2000)
11168R		Phage F336 resistant NCTC11168	Sørensen et al. (2011)
S11168R		Phage-sensitive 11168R	Sørensen et al. (2011)
11168H	HS:2	Hypermotile NCTC11168	Jones et al. (2004)
11168HΔ*1421*		Loss of MeOPN on Gal*f*NAc	McNally et al. (2007)
11168HΔ*1421*/*1422*		Loss of MeOPN on Gal*f*NAc Loss of MeOPN on Hep	McNally et al. (2007)
11168HΔ*1422*		Loss of MeOPN on Hep	McNally et al. (2007)
11168HΔ*1421*/*1422*+ *1422*		Loss of MeOPN on Gal*f*NAc Gain of MeOPN on Hep	McNally et al., 2007
11168H1		11168H variant Loss of 6-*O*-Me and MeOPN on Hep	This study

Table 2 | Bacteriophages.

	Category[a]	*Hha*I restriction pattern[b]	Origin	Reference
F198	III	c	Broiler intestine	Hansen et al. (2007)
F287	III	d	Duck intestine	Hansen et al. (2007)
F303	III	d	Duck abattoir	Hansen et al. (2007)
F326	III	d	Duck intestine	Hansen et al. (2007)
F336	III	b	Duck intestine	Hansen et al. (2007)

[a] *Category is based on genome size (all are 140 kb) as defined in Sails et al. (1998).*
[b] *HhaI restriction pattern as determined in Hansen et al. (2007) b, 5+2 weak bands; c, 4 bands; d, 3 bands.*

with 5% calf blood (BA) or in brain heart infusion (BHI) broth under microaerobic conditions (6% CO_2, 6% O_2, 84% N_2, and 4% H_2) at 37°C. For chicken experiments, bacteria were grown on Karmali agar (Oxoid) supplemented with Campylobacter selective supplement under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2) at 37°C.

BACTERIOPHAGES

Bacteriophages used in this study are listed in **Table 2**. They all belong to the family of Myoviridae and have genome sizes of approximately 140 kb, but show different *Hha*I restriction patterns. Phages were propagated on *C. jejuni* NCTC12662, except for phage F336 where *C. jejuni* 1447 was used (Hansen et al., 2007). Stock bacteriophages were kept in SM buffer (0.05 M Tris–Cl, pH 7.5 supplemented with 5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, and 5 ml gelatin, 2% w/v solution) at 4°C.

BACTERIOPHAGE PROPAGATION

Bacteriophage propagation was performed as described by Sørensen et al. (2011) using a plate lysis method developed from Frost et al. (1999), Hansen et al. (2007). The propagating strains were grown overnight on blood agar Base II plates and harvested in BHI supplemented with 1 mM $CaCl_2$ and 10 mM $MgSO_4$ (CBHI). Bacterial suspensions were adjusted to an optical density of $OD_{600} = 0.35$, incubated for 4 h at 37°C under microaerobic conditions, mixed with phages at a multiplicity of infection (MOI) of 0.01 and incubated for 15 min at 37°C to allow bacteriophage adsorption. Subsequently, 0.6 ml of the adsorbed suspensions were mixed with 5 ml of NZCYM overlay agar [NZCYM (Sigma) broth with 0.6% agar] at 45°C and poured onto NZCYM 1.2% agar plates. After incubation, plates with lysis were flooded with sterile SM buffer and the phages eluted overnight with gentle shaking at 4°C. Phage suspensions were harvested and filtered through a sterile 0.2 μm membrane filter.

BACTERIOPHAGE TITRATION

Bacteriophage titration was performed as previously described (Sørensen et al., 2011). Briefly, bacterial lawns were made by mixing 5 ml NZCYM overlay agar tempered at 45°C with 0.5 ml bacteria harvested in CBHI from Base II agar plates and incubated for 4 h as described above and poured onto NZCYM plates. Phage solutions were spotted manually with three times 10 μl of 10^0–10^{-7} phage dilutions in SM buffer. Plates were incubated at 37°C for 20–24 h under microaerobic conditions and the number of plaque-forming units per milliliter (PFU/ml) of undiluted phage suspensions was calculated. Usually, phage titers of 10^8–10^9 PFU/ml could be obtained on both the propagating strains and *C. jejuni* MP21.

PLAQUE ASSAY

Susceptibility of a *C. jejuni* strain to the bacteriophages was performed using the plaque assay protocol described under Bacteriophage titration and determined as the efficiency of plaquing (EOP) in percent by dividing the PFU/ml on the test strain by the PFU/ml on the control strain (NCTC11168) multiplied by 100. The values represented are the mean count standard deviations derived from two to four independent experiments.

HIGH-RESOLUTION MAGIC ANGLE SPINNING NMR SPECTROSCOPY

High-resolution magic angle spinning (HR-MAS) NMR analysis of intact bacterial cells was performed as previously described (McNally et al., 2005).

IN VIVO EXPERIMENTS

One-day-old chickens were obtained from the Poultry Research Facility, Department of Agriculture, Food and Nutritional Science, University of Alberta. On the day of arrival, chickens were checked for *C. jejuni* colonization by cloacal swabs and divided in groups of four chickens. On day 2, chickens were first orally gavaged with either 10^7 F336 phages in SM buffer or with SM buffer alone. After approximately 30 min, the chickens were orally gavaged with a suspension containing 10^6 *C. jejuni* NCTC11168 in PBS or with PBS alone. Six days post infection, chickens were euthanized and the contents from one cecum were collected aseptically. The cecal contents were weighed and resuspended in phosphate-buffered saline at a final concentration of 0.1 g/ml. This suspension was subjected to 10-fold serial dilutions and plated onto Karmali selective agar plates to determine bacterial colonization levels. One hundred microliters of the 10^{-1} dilution was plated separately onto selective plates and all resulting bacterial colonies were pooled together. Half of this pool was used for sequencing and phage sensitivity and the other half was used for NMR analysis. The experiment was repeated twice with consistent results. PCR of gene *cj1421* was performed as previously described (Sørensen et al., 2011) while sequencing was done using the following primer: 1421-seq2-F (5′-CTCGAGTTATAAGGATATTTTAGATGAG-3′). Gene *cj1426* was PCR-amplified using the primers: 1426-PCR-F (5′-TTGAGAATTATGATAAGATGAAGG-3′) and 1426-PCR-R (5′-TTTCCTAAGAATTCTTTACTTTCG-3′) and then sequenced using the primers: 1426-seq-F (5′-AAGATCCAGATAAAAGAGAT TATTTGG-3′) and 1426-seq-R (5′-ATCAGGAGAATCAAAAA TGATTTTTCC-3′).

RESULTS

THE MeOPN MOIETY OF THE CPS OF *C. jejuni* NCTC11168 IS A RECEPTOR FOR SEVERAL PHAGES

We recently demonstrated that phage F336 requires the *O*-methyl phosphoramidate (MeOPN) moiety attached to the Gal*f*Nac of the *C. jejuni* NCTC11168 CPS for infection (Sørensen et al., 2011).

Table 3 | Efficiency of plaquing (EOP) of phages related to F336 as compared to NCTC11168.

Phage	NCTC11168	11168R	S11168R	*kpsM*
F198	100[a]	–[b]	82.5 ± 24.7	–
F287	100	0.04 ± 0.03	2300 ± 282.8	–
F303	100	–	350 ± 113.1	–
F326	100	0.02 ± 0.02	11.6 ± 6.7	–
F336	100	–	331.8 ± 64.3	–

[a] *EOP is calculated in percent as the PFU/ml of the phages on the test strain divided by the PFU/ml obtained on NCTC11168 multiplied by 100.*

[b] *–No plaques formed.*

In our phage collection (Hansen et al., 2007) we found that five phages, including F336, rely on capsular moieties for infection of *C. jejuni* NCTC11168, since they do not form plaques on a *kpsM* capsule mutant (**Table 3**). In our previous study, we isolated a phage F336 resistant NCTC11168 variant *in vitro* (11168R), which lost the MeOPN attached to the Gal*f*NAc residue due to the presence of an additional G in gene *cj1421* resulting in an off version of the gene (Sørensen et al., 2011). Here we found that 11168R had gained cross-resistance to all of the four phages: F198, F287, F303, and F326 (**Table 3**). While phages F198 and F303 did not form plaques on *C. jejuni* 11168R at all, plaques were observed with F287 and F326, but with a much lower EOP compared to wild type NCTC11168. Hence, the MeOPN structure is important for infection by the four other phages, although two of these phages (F287 and F326) were able to infect a strain not expressing the MeOPN modification. Similar results were obtained when screening a *cj1421* MeOPN transferase deletion mutant in the NCTC11168 wild type background (data not shown). Supporting this, the EOP of four of these phages, including F336, were fully restored to wild type NCTC11168 levels, while the EOP of phage F326 was partially restored, when we tested the phages against a 11168R revertant containing an intact *cj1421* gene and returned expression of the CPS MeOPN (*C. jejuni* S11168R; Sørensen et al., 2011; **Table 3**). Thus, several phages rely on the MeOPN moiety attached to the Gal*f*NAc residue of CPS for infection of *C. jejuni* NCTC11168.

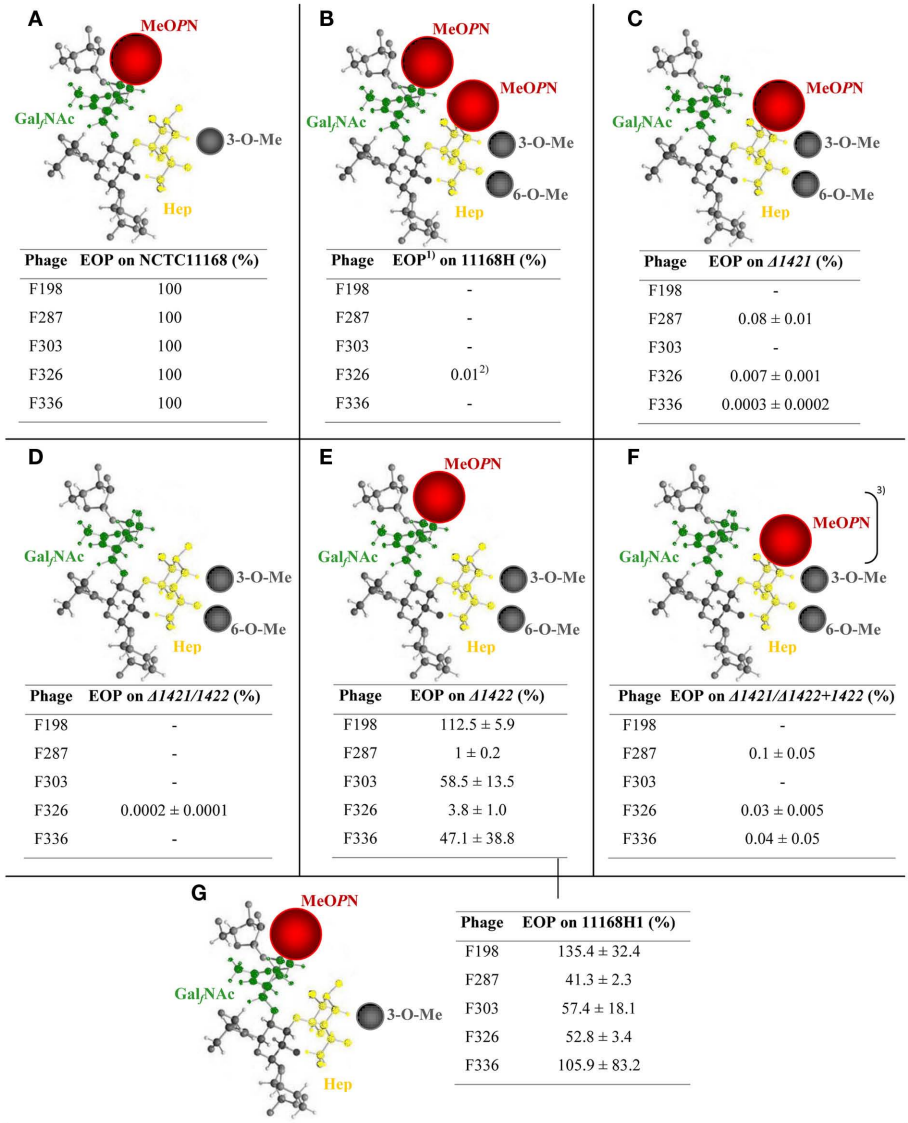
THE LOCATION OF THE MeOPN ON THE CPS IS IMPORTANT FOR PHAGE INFECTION

The MeOPN moiety of *C. jejuni* NCTC11168 can be attached to the Gal*f*NAc and the Hep residues in the CPS (McNally et al., 2007) and we speculated that the attachment site of MeOPN may be important for phage infection. We therefore conducted plaque assays and determined EOPs of the five phages F198, F287, F303, F326, and F336 on the hypermotile NCTC11168 variant 11168H as well as on defined mutants in the *cj1421* and *cj1422* MeOPN transferase genes in this background and compared to the NCTC11168 wild type strain. All of these strains have previously been characterized by HR-MAS NMR and ^{1}H–^{31}P heteronuclear single-quantum correlation (HSQC) HR-MAS NMR for the presence/absence of MeOPN in their CPS structure (McNally et al., 2007). Our results showed that the attachment site of MeOPN in the CPS clearly influenced the efficiency of the phages to infect a particular strain (**Figure 1**). In general, all phages showed a higher EOP when MeOPN was situated on the Gal*f*NAc residue (**Figures 1A,E**) as compared to MeOPN attached to the Hep residue in the CPS (**Figure 1C**). A lower EOP obtained with MeOPN attached to the Hep residue was further confirmed by screening a *cj1421* + *cj1422* deletion strain complemented with *cj1422* (McNally et al., 2007) with our five phages (**Figure 1F**). Interestingly, the four phages F198, F287, F303, and F336 were not able to infect *C. jejuni* 11168H, which is the only strain expressing MeOPN on both Gal*f*NAc and Hep (**Figure 1B**), suggesting that two MeOPN moieties may inhibit phage infection. Finally, we observed that the presence of two other phase variable CPS modifications, the 3-*O*-Me and 6-*O*-Me groups influenced the plaquing efficiency of the phages. We also isolated a 11168H single colony variant (11168H1) that by HR-MAS NMR and ^{1}H–^{31}P HSQC HR-MAS NMR analysis showed the same CPS profile as the NCTC11168 wild type strain (data not shown). The phage sensitivity profile observed with this strain (**Figure 1G**) confirmed the results obtained with NCTC11168 (**Figure 1A**). Thus, the presence of the 6-*O*-Me group on the CPS reduced the plaquing efficiencies of phages F287 and F326 (**Figures 1A,E**), suggesting that other phase variable CPS modifications besides MeOPN affect phage infection.

C. jejuni PHAGES REQUIRE DIFFERENT COMBINATIONS OF PHASE VARIABLE CPS MODIFICATIONS FOR SUCCESSFUL INFECTION

Phase variable expression of the capsular modifications in *C. jejuni* NCTC11168 is well documented (Parkhill et al., 2000; St Michael et al., 2002; Szymanski et al., 2003). Three frozen stocks of NCTC11168 (MP24, MP25, and MP26) that had been passaged a number of times in our laboratory were analyzed by HR-MAS NMR and ^{1}H–^{31}P HSQC HR-MAS NMR (**Figure 2**). This analysis revealed differences in the four phase variable modifications of the CPS structure, i.e., the 3-*O*-Me, 6-*O*-Me, MeOPN-Gal*f*NAc, and MeOPN-Hep as compared to our NCTC11168 wild type (**Table 4**). We also included another NCTC11168 variant (V26) previously shown to have yet another different set of modifications (St Michael et al., 2002; Carrillo et al., 2004).

Plaque assays using our five phages showed that these *C. jejuni* strains had distinct phage sensitivity profiles (**Figures 3B–E**). Interestingly, *C. jejuni* MP26 that expresses both MeOPN moieties, similar to the phage-resistant *C. jejuni* 11168H (**Figure 1B**), could be infected by four of the five phages although with different efficiencies (**Figure 3D**). However, CPS of *C. jejuni* MP26 only contains the 3-*O*-Me group, whereas 11168H CPS contains both the 3-*O*-Me and 6-*O*-Me groups (**Figures 1B** and **3D**). These results indicate that the presence of 6-*O*-Me may inhibit phage infection. This was further supported by the observation that none of the phages formed plaques when the 3-*O*-Me was exchanged with the 6-*O*-Me while retaining the MeOPN modification attached to Gal*f*NAc (**Figures 3A,E**). Furthermore, the presence of the 6-*O*-Me group in combination with a MeOPN situated on the Gal*f*NAc and a 3-*O*-Me group clearly inhibited infection by phage F287 and F326 (**Figures 1E,G**). Thus, our data suggest that the 6-*O*-Me group inhibits plaque formation *in vitro* and that all five phages require MeOPN attached to Gal*f*NAc together with the 3-*O*-Me to obtain the most efficient infection. However, different combinations of the phase variable CPS modifications appear to influence



Phage	EOP on NCTC11168 (%)
F198	100
F287	100
F303	100
F326	100
F336	100

Phage	EOP[1)] on 11168H (%)
F198	-
F287	-
F303	-
F326	0.01[2)]
F336	-

Phage	EOP on *Δ1421* (%)
F198	-
F287	0.08 ± 0.01
F303	-
F326	0.007 ± 0.001
F336	0.0003 ± 0.0002

Phage	EOP on *Δ1421/1422* (%)
F198	-
F287	-
F303	-
F326	0.0002 ± 0.0001
F336	-

Phage	EOP on *Δ1422* (%)
F198	112.5 ± 5.9
F287	1 ± 0.2
F303	58.5 ± 13.5
F326	3.8 ± 1.0
F336	47.1 ± 38.8

Phage	EOP on *Δ1421/Δ1422+1422* (%)
F198	-
F287	0.1 ± 0.05
F303	-
F326	0.03 ± 0.005
F336	0.04 ± 0.05

Phage	EOP on 11168H1 (%)
F198	135.4 ± 32.4
F287	41.3 ± 2.3
F303	57.4 ± 18.1
F326	52.8 ± 3.4
F336	105.9 ± 83.2

FIGURE 1 | Capsular polysaccharide structures expressed by defined MeOPN transferase mutants and their affect on phage sensitivity. The CPS structure is schematically illustrated (modified from Guerry and Szymanski, 2008). Phage sensitivity patterns are calculated as the [1]efficiency of plaquing (EOP) as compared to plaque formation on the NCTC11168 wild type strain. **(A)** NCTC11168; **(B)** 11168H; **(C)** 11168HΔ*1421*; **(D)** 11168HΔ*1421*/Δ*1422*; **(E)** 11168HΔ*1422*; **(F)** 11168HΔ*1421*/Δ*1422*+*1422*; and **(G)** 11168H1. [2]Plaques were only detected in one out of three experiments. [3]MeOPN detected in lower amounts due to partial complementation. Abbreviations are: Gal*f*NAc, *N*-acetylgalactosamine in the furanose configuration; MeOPN, *O*-methyl phosphoramidate; Hep, heptose; and *O*-Me, *O*-methyl.

binding of the phages differently. In particular, phage F326 showed the lowest EOP values (**Table 3**; **Figures 1** and **3**) suggesting that other combinations of receptors, not investigated in this study, might be optimal for the binding of this phage.

SELECTION FOR PHAGE-RESISTANT VARIANTS DURING *C. jejuni* COLONIZATION OF CHICKENS

To assess whether *C. jejuni* is capable of becoming resistant to bacteriophage F336 *in vivo* during colonization of chickens, we orally gavaged 2-day-old chickens with F336 and subsequently with *C. jejuni* NCTC11168. After 6 days, the chickens were euthanized and the bacteria from the ceca were analyzed. We observed similar levels of colonization after 6 days of infection between the groups receiving bacteria only, as compared to those receiving bacteria and bacteriophages (i.e., 10^9 CFU/g cecal content, **Table 5**). The bacteria grown from the cecal contents were directly pooled from plates and analyzed by HR-MAS NMR. All bacteria obtained from chickens infected with *C. jejuni* alone showed an NMR profile that was the same as the NMR spectrum of the inoculum: the bacteria expressed one MeOPN attached to Gal*f*NAc and the 3-*O*-Me group bound to Hep. However, bacteria from the group of chickens that received both bacteria and bacteriophages had lost the

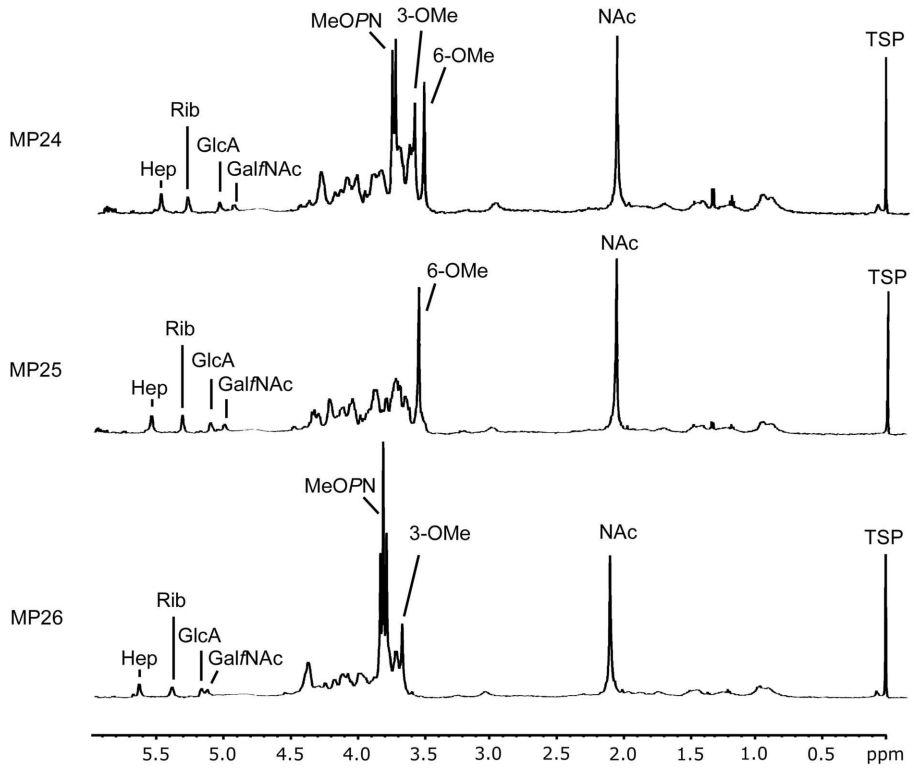


FIGURE 2 | High-resolution magic angle spinning NMR spectra showing the differences in CPS modifications between NCTC11168 variants MP24, MP25 and MP26. ^{1}H CPMG spectra of intact *C. jejuni* cells. Abbreviations are: Hep, D-*glycero*-α-L-*gluco*-Hep; Rib, β-D-Rib*f*; GlcA, α-D-Glc*p*A6(*N*Gro); Gal*f*NAc, β-D-Gal*f*NAc; MeOPN, *O*-methyl phosphoramidate; OMe, *O*-methyl; NAc, *N*-acetyl; TSP, trimethylsilyl propionic acid standard.

Table 4 | ppm Values for ^{1}H HR-MAS NMR spectra for NCTC11168 variants and MeOPN transferase status.

C. jejuni strain	Hep[a]	Rib	GlcA	Gal*f*NAc	3-*O*-Me	6-*O*-Me	NAc	MeOPN	*cj1421*/*cj1422* (Gal*f*NAc/Hep)[b]
NCTC11168 MP24	5.582	5.376	5.103	5.015	3.629	3.557	2.071	3.772	−/+
NCTC11168 MP25	5.599	5.363	5.148	5.039	–	3.569	2.066	- (3.772)[c]	−/−
NCTC11168 MP26	5.610	5.361	5.143	5.101	3.630	–	2.068	3.768/3.743	+/+

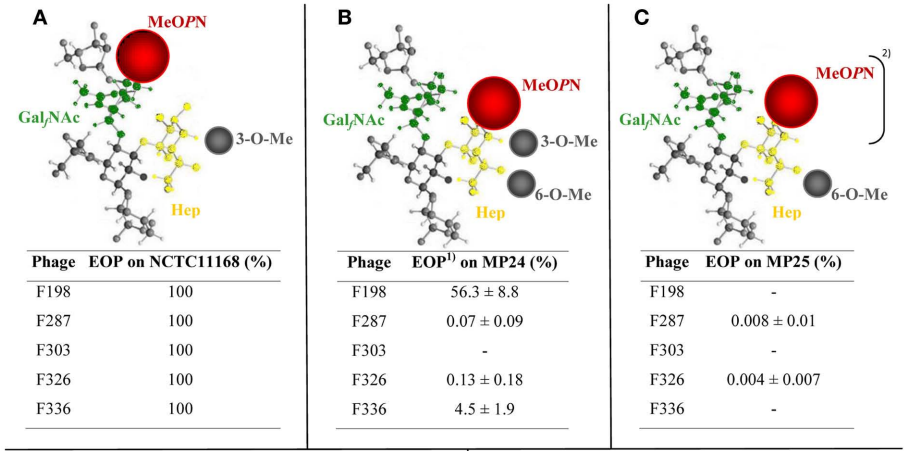
[a] *Only anomeric proton resonances for carbohydrate residues are listed.*

[b] *Summary of sequencing results indicating the "on" (+) and "off" (−) status of the genes encoding the MeOPN transferases onto GalfNAc (cj1421) and Hep (cj1422).*

[c] *Only trace amounts of MeOPN detected.*

MeOPN in all instances, except for one (**Figure 4**; **Table 5**). In chicken #238, infected with phage F336 and *C. jejuni*, the bacteria showed reduced levels of MeOPN present on both Gal*f*NAc and Hep, but the bacteria also expressed high levels of 6-*O*-Me on the Hep (**Figure 4**).

To test whether the observed changes in CPS modifications resulted in phage resistance, we performed bacterial titration assays with all of the isolates recovered from the chickens. All isolates from the F336 infected birds had gained resistance to phage F336 and still showed similar levels of colonization (**Table 5**). Previously, we have shown that a loss of MeOPN on Gal*f*NAc can be the result of a switch in the polyG region of the MeOPN transferase gene *cj1421* (Sørensen et al., 2011). To determine whether the loss of MeOPN and subsequent phage resistance were the result of a switch in the polyG region, we sequenced gene *cj1421* from the pooled colonies from both experiments. All phage-sensitive isolates showed 9 G residues in the polyG region, while all phage-resistant isolates (except from chick 238) showed either 8 or 10 G residues in this region, which in both cases results in a non-functional transferase, due to a premature stop codon 1 or 5 amino acids after the polyG region, respectively (data not shown). Examination of the pooled colonies from chick 238 showed 9 G residues in the polyG region of *cj1421*, consistent with the observed MeOPN expression. However, sequencing of the polyG region of *cj1426* from the pooled colonies from chick 238 showed 10 G residues indicating that the 6-*O*-Me transferase enzyme was functional (again consistent with the acquired expression of the Me modification in **Figure 4**) compared to 11 G residues in



Phage	EOP on NCTC11168 (%)
F198	100
F287	100
F303	100
F326	100
F336	100

Phage	EOP[1)] on MP24 (%)
F198	56.3 ± 8.8
F287	0.07 ± 0.09
F303	-
F326	0.13 ± 0.18
F336	4.5 ± 1.9

Phage	EOP on MP25 (%)
F198	-
F287	0.008 ± 0.01
F303	-
F326	0.004 ± 0.007
F336	-

Phage	EOP on MP26 (%)
F198	0.06 ± 0.09
F287	29.8 ± 18.4
F303	14.2 ± 12.1
F326	3.8 ± 6.6
F336	-

Phage	EOP on V26 (%)
F198	-
F287	-
F303	-
F326	-
F336	-

FIGURE 3 | Capsular polysaccharide structures expressed by NCTC11168 variants and the corresponding phage sensitivity profiles. The CPS structure is schematically illustrated (modified from Guerry and Szymanski, 2008). Phage sensitivity patterns are calculated as the [1]efficiency of plaquing (EOP) as compared to plaque formation on the NCTC11168 wild type strain. **(A)** NCTC11168; **(B)** NCTC11168 MP24; **(C)** NCTC11168 MP25; **(D)** NCTC11168 MP26; and **(E)** NCTC11168 V26. [2]MeOPN only detected in minor amounts. Abbreviations as above are: Gal*f*NAc, *N*-acetylgalactosamine in the furanose configuration; MeOPN, *O*-methyl phosphoramidate; Hep, heptose; and *O*-Me, *O*-methyl.

the parental strain lacking this modification. Thus, *C. jejuni* can become resistant to phage F336 during *in vivo* colonization of chickens by changing the expression of its CPS phase variable modifications.

DISCUSSION

Implementation of efficient phage therapies to target pathogenic bacteria requires detailed knowledge of the bacterial receptors recognized by the phages, as this bacteria–phage interaction is the first event in a process leading to lysis of the host organism. We previously identified the *C. jejuni* NCTC11168 CPS phase variable MeOPN modification as the receptor for phage F336 (Sørensen et al., 2011). The goal of the present study was to further explore the role of MeOPN and other *C. jejuni* phase variable CPS modifications, such as *O*-methylation in phage infection. We compared five different phages, including F336 that were unable to proliferate in an acapsular mutant of *C. jejuni* NCTC11168, and determined their ability to infect NCTC11168 variants containing the same CPS backbone sugars as NCTC11168, but with different modifications.

We found that the phages F198, F287, F303, F326, and F336 all infected *C. jejuni* most efficiently when the 3-*O*-Me modification on Hep and a MeOPN moiety was present, with higher EOPs observed when the MeOPN modification was attached to Gal*f*NAc rather than Hep. Interestingly, when the 3-*O*-Me was exchanged with the 6-*O*-Me while retaining the MeOPN on Gal*f*NAc, none of the phages formed plaques at all, demonstrating a clear role for the *O*-methyl Hep modifications for phage infection, in addition to the previously identified phage receptor, MeOPN (Sørensen et al., 2011). By looking at the predicted three dimensional (3D) structure of the *C. jejuni* NCTC11168 CPS (Guerry and Szymanski, 2008), it is possible that the presence or absence of one or more CPS modifications may lead to conformational changes that interfere with the phage binding process. Indeed it has been found that changes in the 3D conformation of the outer membrane protein OmpA, which serves as the receptor for many of the T-even phages infecting *E. coli*, leads to phage resistance (Riede and Eschbach, 1986). Furthermore, the spatial arrangement of the modifications may be important for phage recognition and some modifications may potentially block the phage binding site when

Table 5 | Presence of MeOPN and phage sensitivity of *C. jejuni* isolated after colonization of chickens.

	Infected with	Bacterial counts (CFU/g)	MeOPN on Gal*f*NAc	Phage sensitivity[a]
MP21 Inoculum	NR	NR	+	Yes
MP21 Karmali	NR	NR	+	Yes
Chick 405	MP21	5.7×10^9	+	Yes
Chick 406	MP21	4.1×10^9	+	Yes
Chick 407	MP21	9.9×10^8	+	Yes
Chick 408	MP21	1.1×10^9	+	Yes
Chick 505	MP21 + F336	7.9×10^8	–	No
Chick 506	MP21 + F336	5.0×10^9	–	No
Chick 507	MP21 + F336	5.8×10^9	–	No
Chick 508	MP21 + F336	8.5×10^8	–	No
Chick 343	MP21	2.0×10^9	+	Yes
Chick 344	MP21	2.1×10^9	+	Yes
Chick 345	MP21	1.6×10^9	+	Yes
Chick 346	MP21	3.6×10^9	+	Yes
Chick 237	MP21 + F336	1.2×10^9	–	No
Chick 238	MP21 + F336	5.5×10^9	+[b]	No
Chick 239	MP21 + F336	4.7×10^9	–[c]	No
Chick 240	MP21 + F336	4.1×10^9	–	No

Chicks 405–408 and 505–508 are from the first experiment, while chicks 343–346 and 237–240 are from the second experiment.

[a]*Yes: plaques formed on isolated strain, No: no plaques formed on isolated strain.*

[b]*Bacteria isolated from this chick showed reduced MeOPN levels (of both MeOPN groups) and enhanced levels of 6-O-Me.*

[c]*MeOPN levels barely above the baseline could be detected from bacteria isolated from this chick.*

NR, not relevant.

they are expressed. Certainly masking the phage binding site is a well known resistance mechanism in other bacteria. One example is *Staphylococcus aureus* that masks its phage receptor with protein A, a cell-wall-anchored virulence factor for this bacterium (Nordstrom and Forsgren, 1974). In agreement with this hypothesis, we observed that when MeOPN was present on both the Gal*f*NAc and Hep residues together with the 3-*O*-Me and 6-*O*-Me Hep modifications, then four of the five phages could no longer infect that particular *C. jejuni* 11168 strain. Thus, both the presence and absence of the capsular modifications appears to modulate phage infectivity of the NCTC1168 strain.

Our results demonstrate that the phages infecting *C. jejuni* are highly specific in the recognition of the CPS and that each phage has evolved to recognize a particular combination of capsular modifications. One might speculate that the phages included in this study originated from the same ancestral phage that has co-evolved with *C. jejuni* to recognize different capsular surface structures by minor modifications in the phage receptor binding proteins (RBP). In support of this, sequencing of viral proteins from the *C. jejuni* phage CP220 revealed at least two distinct tail structures in the mature virions although propagated from a single plaque (Timms et al., 2010), suggesting a possibility for the phage to recognize multiple receptors. A similar phenomena has been observed for capsular phages infecting *E. coli* (Scholl et al., 2001). Indeed RBPs of other phages often have hypervariable regions in the receptor recognizing domains, reflecting the ability of the phage to easily accumulate mutations thereby changing the structure of the RBP to recognize modified receptors or new types of receptors, such as switching from protein to carbohydrate recognition (Montag et al., 1990; Drexler et al., 1991; Hashemolhosseini et al., 1994). The *C. jejuni* phages investigated in this study have similar genome sizes and morphologies and were previously isolated from duck and broiler intestines and duck abattoirs (Hansen et al., 2007). Although these phages all are able to infect the NCTC1168 strain, they do show different host ranges for *C. jejuni* strains of different Penner serotypes (Hansen et al., 2007). In addition, enzymatic restriction cleavage of the phage genomes revealed differences among the phages (**Table 2**, Hansen et al., 2007). Thus, although a conserved head and tail architecture is maintained, it is apparent that genomic dissimilarities exist between the five phages compared in this study and this may account for the differences in host specificity.

In *C. jejuni,* the CPS are highly diverse, due to differences in the genetic compositions of the CPS loci, but also because many of the genes in these loci contain homopolymeric tracts making them prone to phase variation (Linton et al., 2001; St Michael et al., 2002; Szymanski et al., 2003; Karlyshev et al., 2005; McNally et al., 2005; Chen et al., 2008). Based on the number of phase variable modifications in the capsule of *C. jejuni* NCTC11168 alone, this may result in the expression of >700 CPS structural variants. Our results demonstrate that phase variable CPS surface structures clearly influence the phage attachments sites in *C. jejuni* NCTC11168. Phase variable expression also affects phage adsorption in *Bordetella* spp. These bacteria use phase variable expression of the BvgAS two-component regulatory system to alter their surfaces through the regulation of colonization and virulence factors

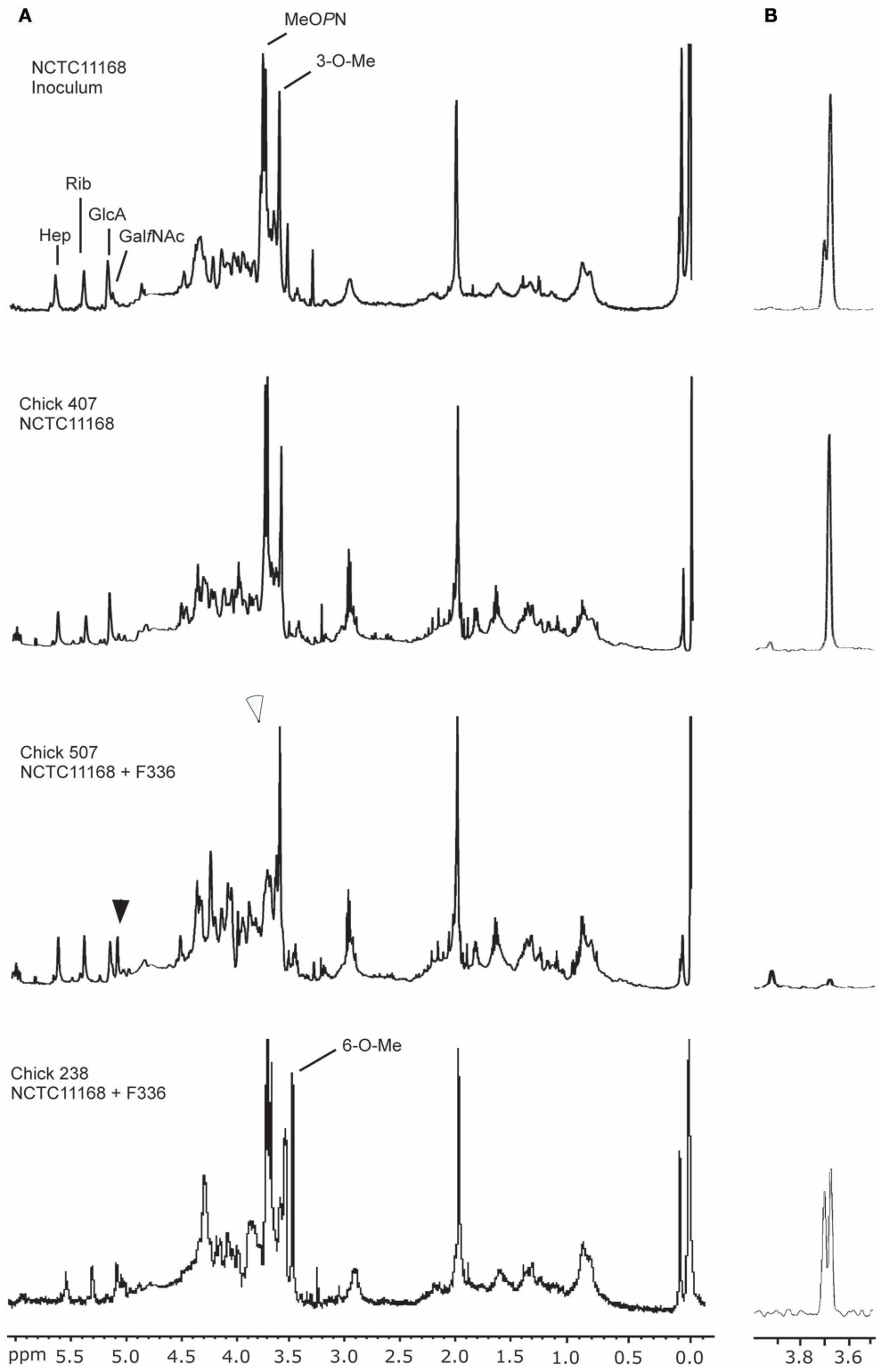


FIGURE 4 | NMR analysis of intact *C. jejuni* cells recovered from chicken cecal contents. (A) ^{1}H HR-MAS NMR CPMG spectra, displaying the capsular profile and **(B)** corresponding 1D ^{1}H–^{31}P HSQC spectra specifically demonstrating the phosphoramidate resonances. Black arrowhead: note the change in the Gal*f*NAc resonance due to the absence of MeOPN. White arrowhead: note the absence of the MeOPN specific resonance.

and also expression of the phage BPP-1 receptor Prn (pertactin autotransporter; Liu et al., 2002). To combat these changes, the BPP-1 phage has a sophisticated mechanism of varying its RBP ($\sim 10^{+13}$ possible sequences) that approaches the levels of diversity observed for immunoglobulins (Liu et al., 2002; Doulatov et al., 2004), and the phage is thus able to overcome the resistance by an equally efficient mechanism.

It was previously shown that CPS expression is important for *C. jejuni* colonization of the chicken gut as well as being important for interactions with human models of disease (Bacon et al., 2001; Jones et al., 2004). Here we show for the first time that the presence of phages selects for specific CPS variants *in vivo* in the chicken gut without altering the colonization levels, even though MeOPN expression influences *C. jejuni* invasion of human cells (van Alphen, unpublished results). Thus, the roles of phase variable CPS modifications, such as the MeOPN and the *O*-methyl groups, may not only be linked to adaptation to the human or chicken hosts, but may serve an important function for *C. jejuni* survival in the avian gut; an environment that contains a high number of bacteria as well as phages (Connerton et al., 2004; Hansen et al., 2007). Scott et al. (2007) also described that certain isolates of *C. jejuni* containing Mu-like prophage DNA sequences were capable of developing bacteriophage resistance in the chicken gut through a mechanism of reversible genomic rearrangement. Remarkably, these isolates inverted genomic segments up to 590 kb which also led to a decrease in chicken colonization and production of a functional Mu-like bacteriophage (Scott et al., 2007). The authors speculated that the development of phage resistance could be due to alterations in the bacterial surface receptor or through saturation of the receptor sites if the Mu phage is recognizing the same structures. Here, we have shown that phase variable modifications of the CPS influence phage infectivity, suggesting that the constant exposure of *C. jejuni* to phages in the avian gut selects for changes in the phase variable structures of the CPS leading to a continuing co-evolution of *C. jejuni* polysaccharides and phage RBPs. This should be considered when developing future strategies for phage therapy against *C. jejuni* in the chicken host and when determining the kinetics of resistance development and the timing for therapy administration. Since the alteration of bacterial surface receptors that reduces or eliminates phage adsorption to the host cell appears to be the most common mechanism of phage resistance (Bohannan and Lenski, 2000), continued investigations into the diversity of phage receptors in *C. jejuni* is required to select the most efficient combinations of phages for therapy.

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Glycoconjugates play a key role in *Campylobacter jejuni* infection: interactions between host and pathogen

***Christopher James Day*†, *Evgeny Alexander Semchenko*† *and Victoria Korolik* ***

Institute for Glycomics, Griffith University, Gold Coast, QLD, Australia

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Marina Šanti, University of Rijeka, Croatia
Christine M. Szymanski, University of Alberta, Canada

***Correspondence:**
Victoria Korolik, Institute for Glycomics, Griffith University, Gold Coast, QLD 4222, Australia.
e-mail: v.korolik@griffith.edu.au

†*Christopher James Day and Evgeny Alexander Semchenko have contributed equally to this work.*

Glycan based interactions between host and pathogen are critical in many bacterial and viral diseases. Glycan interactions range from initial receptor based adherence to protecting the infective agent from the host's immune response through molecular mimicry. *Campylobacter jejuni* is an ideal model for studying the role of glycans in host–pathogen interactions, as well as the role of bacterial surface glycoconjugates in infection. Using glycan array analysis, *C. jejuni* has been shown to interact with a wide range of host glycoconjugates. Mannose and sialic acid residues appear to play a role in initial interactions between host and pathogen following environmental exposure, whereas fucose and galactose based interactions are likely to be required for prolonged colonization. Other studies have highlighted potential decoy receptor type interactions between host's intestinal mucins and *C. jejuni*, demonstrating the importance of host glycoproteins as defense against *C. jejuni* infection as well as the role for glycoconjugates found in human breast milk in protection of breast feeding infants from infection with *C. jejuni*. *C. jejuni* can produce N- and O-linked glycoproteins, capsular polysaccharide (CPS) and/or lipooligosaccharide (LOS) which results in *C. jejuni* presenting its own diverse sugar coated displays on the cell surface. Bacterial glycans play an important and versatile role in infection and disease. Of these, the best understood is the molecular mimicry of human gangliosides presented by *C. jejuni*'s LOS and its link to the onset of autoimmune neuropathies such as the Guillain Barrè syndrome (GBS). However, the role of glycoconjugates presented by *C. jejuni* extends beyond expression of sialylated ganglioside structures involved in initiation of GBS. Expression of surface glycans by *C. jejuni* may also relate to the ability of this organism to interact with the glycoproteins for initial host–pathogen interactions and continued infectivity.

Keywords: glycans, LOS, glycoconjugates

INTRODUCTION

Glycosylation is a ubiquitous decoration of proteins, lipids, and other molecules (such as organic acids) with carbohydrate structures by all living cells (Lehmann et al., 2006). For pathogenic bacteria both the presentation of surface glycans and the recognition of host cell glycans are crucial for the survival in, colonization, and continued infection of host tissues (Lehmann et al., 2006). *Campylobacter jejuni* has been shown to present a wide array of glycans to aid in the evasion of the host immune responses and to protect itself from host defenses, including proteolytic degradation (Karlyshev et al., 2005b). Recently *C. jejuni* has also been shown to bind to a diverse range of host glycans that are potentially crucial for the initial attachment to and continued colonization of the host (Morrow et al., 2005; Day et al., 2009). Here we discuss recent data on both, the factors involved in the heterogeneous expression of lipooligosaccharide (LOS) by *C. jejuni* and the importance of the interactions of *C. jejuni* with host glycans for colonization and infection.

IMPORTANCE OF GLYCANS FOR INFECTION

To infect a host, bacterial pathogens must be equipped with multiple factors to assist in colonization and depending on the site of infection, these factors vary widely. Some bacterial species must be highly motile and sensitive to small chemical gradients to successfully locate their niche, while other species require no self motility to successfully infect the host tissues they prefer. Initial contact between host and pathogen occurs most frequently through bacterial surface components that mediate adherence which are collectively called adhesins. As the host cell surfaces display multitude of glycoconjugates, it is not surprising that a large number of bacteria express carbohydrate specific adhesins and that expression of specific lectin style adhesins is thought to be responsible for the tissue tropism of pathogens (Sokurenko et al., 1998; Lehmann et al., 2006; Lloyd et al., 2007).

A diverse range of glycoconjugates has now been identified as targets of pathogenic microbes including fucosylated glycans (e.g., *Pseudomonas aeruginosa*), gangliosides, and lactoceramides (e.g., *Helicobacter pylori*), sialylated glycans (e.g., *Streptococcus gordonii*), and mannosylated structures (e.g., *Escherichia coli*; Gilboa-Garber et al., 1994; Thomas et al., 2002; Lehmann et al., 2006; Yajima et al., 2008). One of the best defined glycan dependent interactions with host tissues is the recognition of mannosylated glycoconjugates by the FimH protein of *E. coli* (Sokurenko et al., 1994, 1998; Klemm et al., 1996; Aprikian et al., 2007; Rosen et al.,

2008; Sepehri et al., 2009). Not only has this been defined as a key interaction required for colonization, commensalism, and infections with *E. coli*; but also depending on the chain length of the mannose recognized by FimH, it identifies the favored niche of the *E. coli* strain (Sokurenko et al., 1998; Pouttu et al., 1999; Rosen et al., 2008). *E. coli* strains that interact through FimH with short repeat mannosylated glycoconjugates tend to be uropathogenic, while those that recognize longer mannose repeats tend to be found as either commensals or pathogens in intestinal and colonic tissues (Sokurenko et al., 1998; Rosen et al., 2008). Many other bacteria, including *Streptococcus* spp. and *Neisseria* spp., have been shown to require interactions with host glycans for successful infection (Lehmann et al., 2006; Lloyd et al., 2007; Sharon, 2008), however, the factors involved in these interactions have not always been elucidated (Scharfman et al., 1999; Day et al., 2009).

DEFINED GLYCAN BINDING IN THE EPSILONPROTEOBACTERIA: *HELICOBACTER PYLORI*

Carbohydrate based interactions have not been fully solved for *C. jejuni*, but the related species *Helicobacter pylori* has been extensively studied and the lectins involved in carbohydrate dependent interactions have been well characterized (Utt and Wadstrom, 1997; Ilver et al., 1998; Mahdavi et al., 2002; Cooksley et al., 2003; Hynes et al., 2003; Bennett and Roberts, 2005; Walz et al., 2005). *H. pylori* exhibits broad complexity in carbohydrate-binding specificity, owing to the expression of six different lectins/adhesins with specificities for sialylated oligosaccharides, Lewis B antigen, lactosylceramide and ganglioseries glycolipids, and heparan sulfate (Utt and Wadstrom, 1997; Ilver et al., 1998; Mahdavi et al., 2002; Cooksley et al., 2003; Hynes et al., 2003; Bennett and Roberts, 2005; Walz et al., 2005). Of these six lectins, only four (SabA, BabA, HapA, and Hbp) are actually involved in adherence of the bacteria to host tissues, while the other two (HP-NAP and HP0721) appear to be involved in the "mopping up" of free sialic acid in chronically infected tissue. It has been proposed for *H. pylori* that initial interactions with host tissues may be achieved through binding to non-sialylated glycoconjugates present in the normal gastric epithelium (e.g., Lewis B antigen and glycosphingolipids). While, the sialic acid binding capacity of *H. pylori* is likely to enable persistence of *H. pylori* infection by mediating adhesion through the sialic acid binding lectin, SabA, to the already diseased epithelium of the stomach of chronically infected hosts (**Figure 1**; Mahdavi et al., 2002).

CAMPYLOBACTER JEJUNI GLYCAN DEPENDENT INTERACTIONS WITH HOST TISSUES

Like *H. pylori*, *C. jejuni* has been reported to have a broad binding specificity for glycans and is known to interact with mucins and other glycoproteins (Hugdahl et al., 1988; McAuley et al., 2007; Tu et al., 2008; Stahl et al., 2011). Through glycan array based analysis, we have identified the binding of *C. jejuni* strain NCTC11168 to galactose, fucose, sialic acid, mannose, glucosamine, and glycosaminoglycans, however, the interactions with glycans were dependent on the conditions that *C. jejuni* were grown or maintained (Day et al., 2009). *C. jejuni* NCTC11168 was found to bind glycans terminating in galactose regardless of linkage (both α-

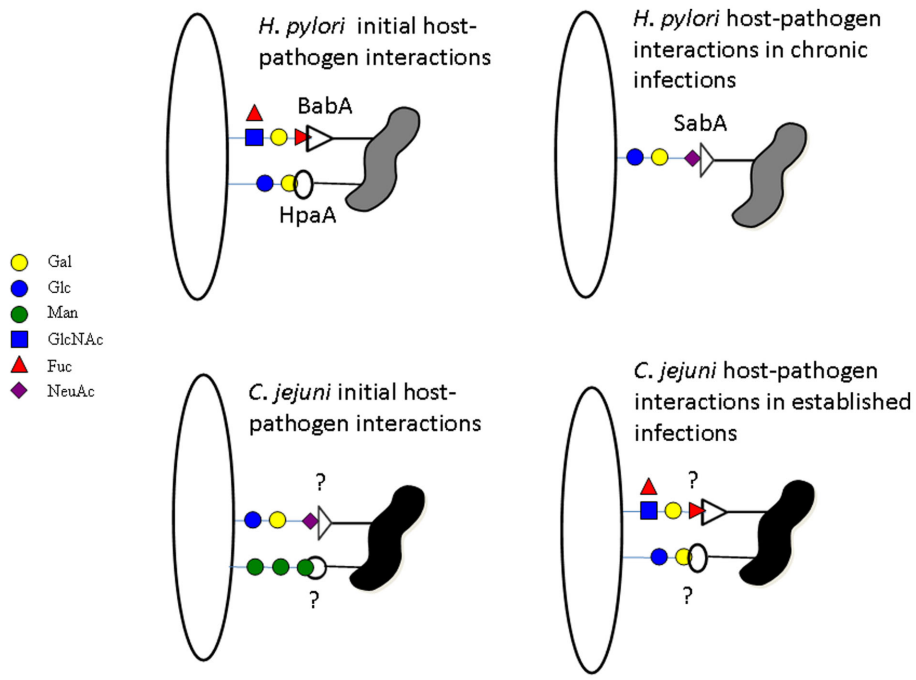

FIGURE 1 | *Campylobacter jejuni* and *H. pylori* host glycoconjugate recognition. *H. pylori* recognizes lactoceramides and fucosylated structures for the initial contact swapping to sialylated structures during chronic infection. *C. jejuni* does the opposite recognizing sialic acid and mannose for initial contact and swapping to fucose and galactose after establishing the infection. The proteins involved in *H. pylori* glycoconjugate recognition are known but in *C. jejuni* they have yet to be elucidated.

and β-linked) and fucosylated glycans when grown under conditions mimicking mammalian and avian hosts. Less binding was observed to fucosylated and terminal galactose structures when maintained in conditions mimicking environmental stress (Day et al., 2009). Conversely, direct interactions between *C. jejuni* NCTC11168 and sialic acid or mannose containing glycoconjugates was observed in glycan array experiments but only after the bacteria were put under environmental stress (Day et al., 2009). The glycan based interactions observed for *C. jejuni* NCTC11168 under the different conditions has been replicated in numerous other strains (unpublished data). Of the 11 other *C. jejuni* strains [human isolates: 11168; 351; 375; 520; 81116 and 81-176 and chicken isolates: 008; 019; 108; 331; 434 and 506] tested so far at all three conditions (environmental, 37, and 42°C)], nine have been found to bind to almost identical structures under the same conditions as *C. jejuni* NCTC11168. The two exceptions are atypical *C. jejuni* strains, still under investigation. These glycan dependent interactions and the way in which *C. jejuni* is able to modulate its binding of glycoconjugates under different conditions, allows us to hypothesize that mannose and sialic acid based adherence is required for initial contact with host tissues after exposure to the external environment, with long term infection resulting from interactions with fucose and terminal galactose containing glycoconjugates (**Figure 1**; Day et al., 2009).

It is interesting to note the differences in the way that *C. jejuni* and *H. pylori* interact with glycoconjugates may explain why bacteria that are closely related infect different niches within the same host (**Figure 1**). For *H. pylori*, normal stomach mucosal tissue is mostly glycosylated with fucosylated structures such as Lewis B and is rich in lactoceramides, while being poor in sialylated glycoconjugates. The inflamed stomach, however, produces a highly sialylated mucosal layer therefore requiring *H. pylori* to recognize sialic acid in order to maintain chronic infections (Mahdavi et al., 2002). In contrast, for *C. jejuni*, the initial interactions in the intestinal tissues are with proteins such as MUC1, a highly sialylated and mannosylated structure. MUC1, a cell surface bound mucin, is a decoy receptor and is released from by the intestinal epithelium into the intestinal lumen when recognized by pathogens such as *C. jejuni* (McAuley et al., 2007; Linden et al., 2008). Due to this the continued binding of sialic acid by *C. jejuni*, once it has begun to adjust to the host environment, would be detrimental to long term infection. The crypts of the intestinal epithelium, *C. jejuni*'s favored niche, are rich in a gel forming mucin MUC2, a protein with significantly less sialylation than MUC1. It is here in the crypts and at the epithelial surface, away from the lumen of the intestinal tract, that *C. jejuni* would gain advantage from recognizing fucosylated and terminal galactose structures as these become the predominant structures presented by the host (Day et al., 2009).

We have also shown that adherence to cultured Caco-2 colonic epithelial cells could be significantly reduced when *C. jejuni* binding was competed against commercially available lectins, particularly those lectins with specificity for fucose and galactose (Day et al., 2009). Almost complete inhibition of adherence to Caco-2 cells was observed when cells were pre-incubated with the galactose recognizing lectin ECA (90% inhibition of 11168-O grown at 42°C) or the fucose binding lectin UEA-1 (95–100% inhibition of 11168-O; Day et al., 2009). Previous studies had already identified fucosylated glycans as crucial for interactions between *C. jejuni* and host tissues (Morrow et al., 2005). These studies identified that fucosylated glycoproteins, present in human breast milk, could inhibit *C. jejuni* colonization, with Lewis B, and 2-fucosylactose being identified as the key glycans recognized by *C. jejuni* (Morrow et al., 2005). These data together with our findings highlight the crucial nature of glycan based interactions for *C. jejuni* infection and colonization. It is therefore crucial that the factors involved in the recognition of host glycans be elucidated.

Intriguingly, *C. jejuni* does not contain any orthologs or homologs of the *H. pylori* SabA, BabA, Hbp HapA, or HP0721 lectins but does contain a protein with 65% identity to the sialic acid binding lectin HP-NAP (Parkhill et al., 2000; Gundogdu et al., 2007). Whether this HP-NAP homolog can act as an adhesin for *C. jejuni* is yet to be determined. To date, bioinformatic analyses have failed to identify definite glycan recognizing proteins and the mechanisms for the recognition of such a diverse range of glycans by *C. jejuni* remains a mystery. Mechanisms, other than protein based adherence, however, may be involved in this diverse glycan recognition. One such mechanism may rely on the *C. jejuni*'s own surface exposed glycosylation such as LOS and capsular polysaccharides (CPS) which have both been linked to adherence of *C. jejuni* to host cells in culture, indicating a potential role for the bacterially produced glycans in the recognition of host tissues (Fry et al., 2000).

CAMPYLOBACTER JEJUNI SURFACE GLYCOSYLATION

Campylobacter jejuni was the first bacteria to be identified with the mechanism for N-linked protein glycosylation and displays a range of glycosylated structures including N- and O-linked glycoproteins, LOS, and CPS (Karlyshev et al., 2005b). N-linked glycans can either be surface expressed structures or produced as a free oligosaccharide (Nothaft and Szymanski, 2010; Nothaft et al., 2010). Interestingly, though the flippase involved in the attachment of the N-linked glycan to the surface is non-specific for the structure it translocates, *C. jejuni* produces an N-linked glycan structure that is highly conserved between strains (Young et al., 2002; Linton et al., 2005; Kelly et al., 2006). O-linked glycosylation in *C. jejuni* is exclusively found on the flagella and is critical for correct flagella assembly, motility, and flagella dependent adherence (Thibault et al., 2001; Szymanski et al., 2003; Logan et al., 2009). While there is some diversity in the expression of the O-linked glycans produced by different strains, the greatest diversity in glycosylation structures is observed for both CPS and LOS (Moran, 1997; Moran and Penner, 1999; Karlyshev et al., 2005a,b). CPS and LOS are also one of the most immunogenic of the surface glycosylations presented by *C. jejuni* explaining much of the strain specific immunity commonly seen with *C. jejuni* infections (Karlyshev et al., 2005b; Perera et al., 2007; Monteiro et al., 2009). The glycan structures of the CPS are variable between strains with the heat stable antigen typing, which types the combination of CPS and LOS present, being one of the ways in which *C. jejuni* strains can be differentiated from one another (Moran and Penner, 1999). However, while structure of CPS is differential between strains and can be heterogenous in the same strain, the structure of the outer core sugars of the LOS is almost always heterogeneous within a population of *C. jejuni* of the same strain (Linton et al., 2000a; Guerry et al., 2002; Semchenko et al., 2010).

LIPOOLIGOSACCHARIDE BIOSYNTHESIS CLUSTERS: LINK TO GUILLAIN–BARRÉ SYNDROME

Lipooligosaccharide are glycolipids expressed by Gram negative bacteria as part of their outer membrane. LOS is comprised of a lipid A molecule attached to a polysaccharide chain with a conserved inner core and a variable outer core. The outer core may be present as a single copy (LOS) as seen by the pathogen *Neisseria meningitides* or may repeat multiple times to produce lipopolysaccharide (LPS) as seen in a range of bacteria including *E. coli* (Zhu et al., 2002; Wang and Quinn, 2010). The LOS of *C. jejuni* has been widely studied and has been hypothesized to play important roles in cellular survival, host evasion, and adherence to host tissues (Karlyshev et al., 2005b). Strain to strain variation observed for LOS structures presented by *C. jejuni* has been linked to the diversity of the genes present within the LOS biosynthesis clusters (Gilbert et al., 2002). More then 20 different LOS biosynthesis clusters have been identified in *C. jejuni* strains, including the five capable of sialylation and ganglioside mimicry (A, B, C, M, and R; Gilbert et al., 2008). One biosynthesis cluster type, type E (*C. jejuni* 81116), also produces a larger LPS molecule rather than an LOS (Gilbert et al., 2008). The type E biosynthesis cluster is divergent from those seen in other *C. jejuni* precursor clusters contributing to the generation of a differential core oligosaccharide (Gilbert et al., 2008).

To date, the five clusters with mechanisms for ganglioside molecular mimicry (A, B, C, M, and R), have been the major focus of research into the *C. jejuni* LOS (Karlyshev et al., 2005b; Yuki, 2007; Gilbert et al., 2008). The most commonly used model strains for analyzing *C. jejuni* NCTC11168 and 81–176 both have clusters capable of ganglioside molecular mimicry, falling into cluster types C and B, respectively. There appears to be a bias in research toward sialylated LOS structures of *C. jejuni*. This is mostly due to the focus on the strains producing LOS with ganglioside mimicry due to their clinical significance in the induction of Guillain–Barré syndrome (GBS; Yuki, 1997). For example, recent studies have demonstrated that *C. jejuni* with sialylated LOS binds a sialoadhesin, which in turn leads to a cascade of events that result in production of cross-reactive antibodies (Louwen et al., 2008; Mortensen et al., 2009; Heikema et al., 2010; Kuijf et al., 2010).

Campylobacter jejuni infection is usually self-limiting, but in rare cases the infection precludes a debilitating, polyneuropathic disorders GBS, or the oculomotor variant Fisher syndrome (FS; Godschalk et al., 2007). *C. jejuni* is the most common antecedent infection in these neuropathies and expression of LOS mimicking host gangliosides is considered necessary for the neuropathy development since such mimicry can induce the production of self-reactive antibodies (Godschalk et al., 2007). Gangliosides are glycosphingolipids occurring in high concentration in the peripheral nervous system, particularly in the nerve axon but are also present on the epithelial cells of the entire gut, stomach through to the colon (Nachamkin et al., 1998; Ang et al., 2004). The production of self-reactive antibodies against these glycolipids plays a central role in GBS and FS development (Yuki, 1997). *C. jejuni* has been shown to produce ganglioside mimicries, including those implicated in GBS (GM_1, GM_{1b}, GD_{1a}, GalNAc–GD_{1a}) and FS (GQ_{1b}, GT_{1a}; Yuki, 1997; Ang et al., 2004). Supporting a pathogenic role of *C. jejuni* in GBS, *C. jejuni* LOS-induced anti-GM1 ganglioside antibodies react at the nodes of Ranvier, where the axon is exposed in the nerve fiber (Moran et al., 2005), resembling the pathology observed in GBS patients, and inoculation of *C. jejuni* GM_1-mimicking LOS has been reported to induce GBS-like symptoms in a rabbit model (Yuki et al., 2004). Although mimicry of gangliosides by *C. jejuni* LOS has been extensively studied structurally over the last two decades, it is important to note that these characterization studies were performed on strains grown almost exclusively at 37°C. The research focus on the growth temperature of 37°C is completely at odds with the lifestyle *C. jejuni* which colonizes a variety of niches whose temperatures range from 25 to 42°C (Blaser, 1997), and with previous studies that have highlighted the role the growth temperature plays in gene regulation, expression, enzymatic function, protein folding, and general bacterial metabolism (Stintzi, 2003; Semchenko et al., 2010). All this taken together indicates that there is a need to investigate LOS genetic and structural diversity at temperatures which represent different hosts.

LOS STRUCTURE AND HETEROGENEITY: HOW *C. JEJUNI*'S ENVIRONMENT CAN EFFECT STRUCTURE

Campylobacter jejuni exists in wide range of temperatures and atmospheric conditions, from normal atmospheric temperatures, and oxygen levels, when in the environment, to 42°C and virtually anaerobic in parts of the avian gut. While *C. jejuni* is thought not to replicate when in the environment, it is capable of growth at temperatures ranging from 30 to 47°C and therefore is capable of growth at the body temperatures of mammalian and avian hosts, 37 and 42°C, respectively (Blaser, 1997). It is not surprising that gene expression changes have been noted under these varying conditions including the regulation of genes found in LOS biosynthesis clusters (Stintzi, 2003). Recently, however, we have shown that gene expression may not be the only change that affects the overall LOS structure under varying conditions (Semchenko et al., 2010).

Different temperature environments may trigger events to accommodate the colonization, commensalism, pathogenesis, or dormancy of this bacterium. The lack of knowledge of the structure of *C. jejuni* LOS at 42°C compared to 37°C prompted us to examine the effect of incubation temperature on the phenotypic variation of LOS, including the mimicry of gangliosides, in *C. jejuni* strain NCTC11168. Variation in LOS structure was assessed by electrophoretic analysis and immunoblotting and confirmed by nuclear magnetic resonance (NMR) spectroscopy. Carbohydrate epitopes produced were assessed for ganglioside mimicry using various anti-ganglioside ligands (i.e., antibodies, lectins, and cholera toxin) as probes. In addition, LOS structural variation at these two incubation temperatures was examined in minimally subcultured *C. jejuni* isolates from humans and chickens. Importantly, notable differences were observed in the relative production by *C. jejuni* 11168 of varying size and ganglioside mimicries at 37 and 42°C. At 37°C, the LOS of *C. jejuni* strain 11168 was observed to be relatively homogenous, with 90% of the total LOS produced with structure mimicking GM1 (Semchenko et al., 2010). This changed dramatically at 42°C, with diversity in the LOS increasing, including an increased expression of asialo-GM1) resulting in

just over 50% of the LOS structure present at 42°C consisting of the GM1-like structure seen to be dominant at 37°C (Semchenko et al., 2010). The diversity in the structures seen at different temperatures may be the result of changes in gene expression, enzymatic functionality, and metabolic changes, all potentially contributing. In addition, the *C. jejuni* LOS biosynthesis clusters are also known to contain several phase variable genes which are subject to change. *C. jejuni* strains have also been observed to functionally alter some of their glycosyltransferases through non-phase variable alterations, such as point mutations, on/off status, and substrate–acceptor specificity (Linton et al., 2000a; Guerry et al., 2002; Semchenko et al., 2010).

The LOS structures of two strains of *C. jejuni*, NCTC11168 and 81-176, have been extensively studied to identify the different forms of gangliosides they can mimic and what genes/conditions alter the host cell mimicry they can present (Linton et al., 2000a; Guerry et al., 2002; Semchenko et al., 2010). *C. jejuni* NCTC11168 has class C biosynthesis cluster that typically produces LOS that mimics monosialylated gangliosides such as GM1–2 as a result of differential expression of galactosyltransferase – *wlaN* (Linton et al., 2000a; St. Michael et al., 2002; Semchenko et al., 2010, 2011). However, recently we have demonstrated that cluster C strains are also capable of regulating LOS sialylation via inactivation of the sialytransferase – *cst* with a transient mutation in the gene reading frame (Semchenko et al., 2010, 2011). Thus cluster C strains are capable of producing LOS structures with GM1, GM2, asialo-GM1, and asialo-GM2 like mimicries, and depending on what type of *cst* gene (*cstII* or *cstIII*) they encode, they may also mono- or di-sialylate the LOS, bringing the total of potential structures to six from a single cluster type.

Campylobacter jejuni 81-176 has a class B biosynthesis cluster that, depending on the sialytransferase expressed, encodes the machinery to produce ganglioside mimic ranging from monosialylated GM2–3 to GD structures with GM2 and GM3 being the most commonly observed in the wild-type organism (Guerry et al., 2002). The genes most responsible for this mimicry are the genes coding for biosynthesis of sialic acid from *N*-acetylmannose to CMP-Neu5Ac (NeuA, NeuB, NeuC) and a sialic acid transferase (CstII or CstIII; Linton et al., 2000b; Gilbert et al., 2002, 2008). Interestingly, the class C LOS biosynthesis cluster of *C. jejuni* has a gene that encodes both NeuA (CMP-Neu5Ac synthase) and CgtA (GalNAc transferase) in a single enzyme (Gilbert et al., 2008). We hypothesize that the presence of this enzyme is the reason for some of the heterogeneity observed between different growth conditions. Only half the LOS of *C. jejuni* 11168 is sialylated when grown under conditions mimicking the commensal avian host but nearly 90% being sialylated at conditions mimicking mammalian hosts (Semchenko et al., 2010, 2011). This change in sialylation is not necessarily related to a change in the on/off expression status of *cst*, but, we believe, can be due to the differences in optimal or suboptimal enzymatic function under different environmental stresses. This mechanism allows production of LOS that mimics various gangliosides in a host dependant manner, without needing to phase vary its sialic acid biosynthesis genes (Semchenko et al., 2010, under revision). This allows the entire population to produce different range of molecules, rather then only a few cells that underwent a phase change, thus avoiding a population bottleneck and increasing the fitness of the infecting population.

In other pathogens variability of surface antigens such as LOS and protein glycosylation enable the pathogen to better evade the immune system (Jarvis and Vedros, 1987; Platt et al., 1994; Khatua et al., 2010). This works by the host selecting or enriching for the bacteria that present surface antigens that have not yet triggered an immune response (**Figure 2A**). However, the way that LOS is synthesized in *C. jejuni*, may result in heterogenous surface expression of LOS structures on a single bacteria. Single colonies of *C. jejuni* NCTC11168, when screened with anti-GM_1 antibodies or cholera toxin, show uniform reactivity with the lectin/antibody, suggesting uniform expression of the surface GM_1 epitope. However, when the LOS preparations from these colonies are separated electrophoretically, and then screened with anti-GM_1 antibodies or cholera toxin, there is heterogeneity in each sample with both GM_1 and asialo-GM_1 present (Semchenko et al., 2010, 2011). We therefore propose that *C. jejuni* are likely to be heterogenous for LOS presentation on a single bacteria limiting host based selection of successful *C. jejuni* clones and potentially this heterogenic LOS

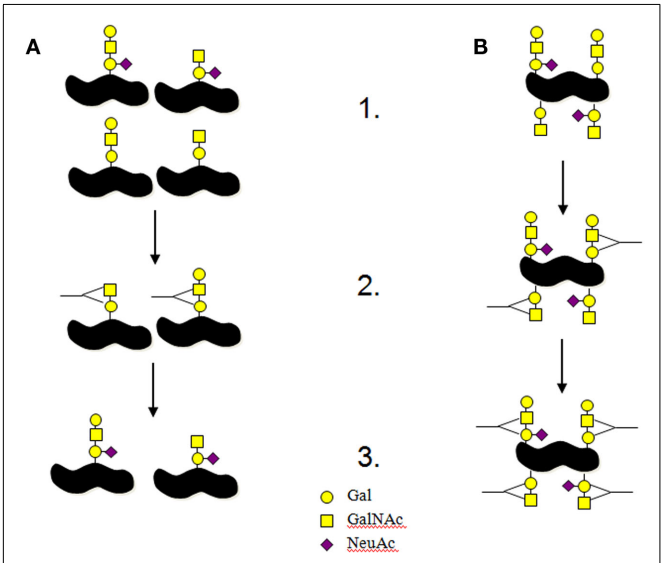

FIGURE 2 | Interactions between bacterial antigens and host immune system. (A) This is the normal process for the interactions between host immune systems and pathogenic bacteria. **(1)** A portion of the bacterial colony produces different antigens on their surfaces. **(2)** The immune system targets the surface structures that are presented in a part of the population resulting in other antigens being selected to remain. In this example antibodies are produced to non-sialylated LOS structures. **(3)** The antigens not targeted by the immune system are allowed to expand causing the infection. **(B)** This is the model we think is happening for *C. jejuni* LOS. **(1)** Each bacterium produces all potential surface antigens in different concentrations. **(2)** The immune system targets the surface structures that are presented in the population resulting antibody production against a portion of the surface antigens. In this example antibodies are produced to non-sialylated LOS structures. **(3)** The antigens not targeted by antibodies are still present on the targeted bacteria resulting in the increased likelihood of producing antibodies to those other LOS antigens. If the host has a defect in the mechanisms of ensuring antibodies are not produced against self then *C. jejuni* producing multiple antigens per bacteria offers a greater risk for the production of anti-self antibodies.

may actually contribute to the production of anti-self antibodies seen in GBS/FS (**Figure 2B**).

CONCLUSION

Glycosylation of both the host surface and the bacterial surface is crucial for the host–pathogen interactions observed for *C. jejuni*. A better understanding of the factors involved in glycan expression and recognition by both the bacteria and the host may offer new insights into the mechanisms involved in *C. jejuni* commensal colonization, pathogenic infection, and the induction of polyneuropathic disorders GBS and FS.

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The *Campylobacter jejuni* CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells

Jason M. Neal-McKinney and Michael E. Konkel*

School of Molecular Biosciences, Washington State University, Pullman, WA, USA

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Stacey Gilk, National Institutes of Health, USA
Susan Logan, National Research Council of Canada, Canada

****Correspondence:***
Michael E. Konkel, School of Molecular Biosciences, Washington State University, 1770 NE Stadium Road, Biotechnology Life Sciences Building, Room 302c, Pullman, WA 99164–7520, USA.
e-mail: konkel@vetmed.wsu.edu

Campylobacter jejuni is a leading cause of bacterial gastroenteritis worldwide. Acute *C. jejuni*-mediated disease (campylobacteriosis) involves *C. jejuni* invasion of host epithelial cells using adhesins (e.g., CadF and FlpA) and secreted proteins [e.g., the *Campylobacter* invasion antigens (Cia)]. The genes encoding the Cia proteins are up-regulated upon co-culture of *C. jejuni* with epithelial cells. One of the Cia proteins, CiaC, is required for maximal invasion of host cells by *C. jejuni*. Previous work has also revealed that CiaC is, in part, responsible for host cell cytoskeletal rearrangements that result in membrane ruffling. This study was performed to test the hypothesis that CiaC is delivered to the cytosol of host cells. To detect the delivery of CiaC into cultured epithelial cells, we used the adenylate cyclase domain (ACD) of *Bordetella pertussis* CyaA as a reporter. In this study, we found that export and delivery of the *C. jejuni* Cia proteins into human INT 407 epithelial cells required a functional flagellar hook complex composed of FlgE, FlgK, and FlgL. Assays performed with bacterial culture supernatants supported the hypothesis that CiaC delivery requires bacteria-host cell contact. We also found that CiaC was delivered to host cells by cell-associated (bound) bacteria, as judged by experiments performed with inhibitors that specifically target the cell signaling pathways utilized by *C. jejuni* for cell invasion. Interestingly, the *C. jejuni flgL* mutant, which is incapable of exporting and delivering the Cia proteins, did not induce INT 407 cell membrane ruffles. Complementation of the *flgL* mutant with plasmid-encoded *flgL* restored the motility and membrane ruffling. These data support the hypothesis that the *C. jejuni* Cia proteins, which are exported from the flagellum, are delivered to the cytosol of host cells.

Keywords: T3SS, flagellum, effector proteins, adenylate cyclase, membrane ruffling

INTRODUCTION

Campylobacter jejuni remains a leading bacterial cause of gastroenteritis, with an estimated annual incidence of 2.4 million cases per year in the US (Mead et al., 1999), and 400–500 million cases worldwide (Konkel et al., 2001; Ruiz-Palacios, 2007). Most infections with *C. jejuni* are linked to the consumption of undercooked poultry or foods that have been contaminated with raw poultry (Konkel et al., 2001). This is due to the fact that *C. jejuni* is a commensal organism in chickens and is able to colonize the ceca of birds at levels greater than 10^8 CFU/gram of cecal contents (Sahin et al., 2002). Infection with *C. jejuni* (campylobacteriosis) is characterized by diarrhea containing blood and/or leukocytes, fever, and abdominal cramps. Although campylobacteriosis is usually self-limiting, some individuals require antibiotic treatment. Infection with *C. jejuni* can also lead to Guillain–Barré syndrome, an acute demyelinating neuropathy characterized by flaccid paralysis (Konkel et al., 2001).

Type III secretion systems (T3SS) are complex macromolecular structures that allow Gram-negative bacteria to secrete proteins across the inner and outer membranes without a periplasmic intermediate (Cornelis, 2006; Desvaux et al., 2006). Classical T3SS possessed by pathogenic bacteria such as *Yersinia*, *Shigella*, and *Salmonella* allow these organisms to deliver effector proteins from the bacterial cytosol directly to the cytosol of a target human cell (Sory et al., 1995; Cornelis, 2006; Galan and Wolf-Watz, 2006). The delivered effector proteins then modulate host cell behavior to facilitate uptake of the organism and subsequent disease. These classical T3SSs can be described as a "molecular syringe" and are composed of a protein complex spanning both bacterial membranes, which is attached to a needle complex that extends from the cell. The needle complex is a hollow conduit with a tip containing translocon proteins, which incorporate into the membrane of the host cell, allowing for delivery of effector proteins into the host cytosol (Cornelis, 2006; Galan and Wolf-Watz, 2006).

C. jejuni-mediated disease is dependent on many factors, including motility, cell adherence, and cell invasion (Konkel et al., 1999, 2001; Guerry, 2007; Flanagan et al., 2009). The flagellum, which is a T3SS with components homologous to the classical T3SS, plays a central role in *C. jejuni* pathogenesis. The flagellum is composed of a basal body, hook, and filament. The basal body spans both the inner and outer membranes. The hook is a short, curved conduit extending from the basal body. It is composed primarily of the protein FlgE, and is capped by the hook-filament

junction proteins FlgK and FlgL. The components comprising the basal body and hook are assembled as they are exported (Ferris and Minamino, 2006). After the hook is completed by the incorporation of FlgK and FlgL, the export specificity of the flagellar T3SS changes to allow for secretion of FlaA and FlaB, which form the flagellar filament (Minamino et al., 1999; Ferris and Minamino, 2006). Deletion of *flgK, flgL*, or *flgE* abolishes the ability of the organism to form a flagellar filament (Konkel et al., 2004; Fernando et al., 2007). The flagellar filament is composed of FlaA and FlaB and extends approximately three microns from the bacterial cell (Grant et al., 1993; Guerry, 2007). The flagellar filament is capped by the FliD protein, which allows flagellin subunits to polymerize into the growing flagellum (Yokoseki et al., 1995).

Multiple research groups have concluded that proteins secreted from the flagellum play a role in both commensal colonization of poultry and *C. jejuni* invasion of human epithelial cells (Konkel et al., 1999; Ziprin et al., 2001; Fernando et al., 2007). Proteins found to be exported via the flagellum of *C. jejuni* include CiaB (Konkel et al., 1999), CiaC (Christensen et al., 2009), CiaI (Buelow et al., 2010), FlaC (Song et al., 2004), and FspA (Poly et al., 2007). The first report of a non-flagellar protein secreted through the *C. jejuni* flagellum was CiaB (Konkel et al., 1999). A mutation in the *ciaB* gene abolishes export of the other Cia proteins and reduces bacterial invasion into host cells. Complementation of the *ciaB* mutant restores host cell invasion *in vitro* and virulence *in vivo* (Rivera-Amill et al., 2001; Raphael et al., 2005a). FlaC binds to epithelial cells and is required for maximal cell invasion (Song et al., 2004). The FspA protein induces apoptosis of INT 407 cells (Poly et al., 2007). Konkel et al. demonstrated that the Cia proteins (i.e., CiaC and CiaI) modulate host cell signaling and alter *C. jejuni* intracellular trafficking (Konkel et al., 2004; Christensen et al., 2009; Buelow et al., 2010; Eucker and Konkel, 2011). As one might predict, the *ciaB, ciaC*, and *ciaI* genes up-regulated in response to conditions encountered in the gastrointestinal tract (i.e., bile salt deoxycholate), and have been shown to contain sequences for export through the flagellar T3SS (Malik-Kale et al., 2008; Christensen et al., 2009). Other research groups have also reported that the expression of *ciaC* and *ciaI* is linked to virulence (Carrillo et al., 2004; Stintzi et al., 2005). These findings are in agreement with the proposal that the Cia proteins contribute to *C. jejuni* virulence. Interestingly, flagellar proteins can be exported from the classical T3SS and *vice versa* (Lee and Galan, 2004; Badea et al., 2009). Moreover, proteins exported from the classical T3SS have been reported to ultimately result in the modulation of host cell signaling pathways (Sun et al., 2007). In bacteria that possess both flagellar and classical T3SS, the export of proteins is controlled via well-orchestrated gene regulatory mechanisms and chaperone specificity (Lee and Galan, 2004). The only T3SS present in *C. jejuni* is the flagellum (Parkhill et al., 2000).

C. jejuni invasion of host cells is a complex event that is initiated by *C. jejuni* binding to cells via several adhesins, including CadF, FlpA, Cj1349c, and CapA (Konkel et al., 1997; Ashgar et al., 2007; Flanagan et al., 2009; Konkel et al., 2010). While *C. jejuni* binding alone to host cells is sufficient to stimulate host cell signaling pathways, this event in itself is not sufficient to facilitate *C. jejuni* invasion of host cells. We have shown that the Cia proteins, which are synthesized and secreted by *C. jejuni* when co-cultured with epithelial cells, are required for maximal cell invasion (Christensen et al., 2009). The observation that chloramphenicol, an inhibitor of bacterial protein synthesis, retards *C. jejuni* invasion is consistent with the hypothesis that the Cia proteins contribute to cell invasion (Konkel and Cieplak, 1992; Oelschlaeger et al., 1993; Rivera-Amill et al., 2001). We propose the Cia proteins participate in cell invasion by triggering cell signaling events necessary for actin cytoskeletal rearrangement. Maximal *C. jejuni* invasion of host cells requires activation of the host cell integrin receptors, components of the focal complex, and Rac1 (Eucker and Konkel, 2011; Krause-Gruszczynska et al., 2011). Together, these events result in membrane ruffling and bacterial uptake.

The purpose of this study was to elucidate the mechanism of Cia protein delivery to host cells. Upon contact with epithelial cells, *C. jejuni* secrete ~18 Cia proteins (Larson et al., 2008). It is not known if the Cia proteins act on the surface of the epithelial cell or if they are delivered to the cytosol of a target epithelial cell. We chose to use CiaC to investigate the mechanism of Cia protein delivery to host cells (Christensen et al., 2009). In this study, we present evidence that supports the hypothesis that CiaC is delivered to the cytosol of host cells by cell-associated bacteria.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE METHODS

C. jejuni wild-type strains F38011 (Raphael et al., 2005b), 81116 (Black et al., 1988), and 81–176 (Black et al., 1988) were used in this study. Bacteria were grown on Mueller–Hinton agar plates supplemented with 5% citrated bovine blood (MHB) and in Mueller–Hinton (MH) broth with constant shaking. Deoxycholate was added to a concentration of 0.01% to broths where indicated. The bacteria were incubated in a microaerobic environment (85% N_2, 10% CO_2, 5% O_2), at 37°C. The *C. jejuni* were passaged onto fresh media every 48 h. The *E. coli* Inv-α (Invitrogen) and S17–1 λ-*pir* (Tascon et al., 1993) strains were cultured on Luria–Bertani agar plates (LB) or in LB broth with shaking. The cultures were incubated at 37°C in an aerobic environment. All cloning steps were performed in *E. coli* TOP10 (Invitrogen), and the ACD fusion vectors were transformed into *E. coli* S-17 for conjugation into *C. jejuni*.

CELL CULTURE

INT 407 cells (ATCC #CCL-6) were grown in Minimal Essential Medium (MEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories), 1% glutamine (Invitrogen), and 1% sodium pyruvate (Cellgro). Cultures were maintained at 37°C in a humidified 5% CO_2 incubator. Cells were grown to confluency and passaged using 2.5% trypsin (Sigma). For ACD delivery assays, a 24-well plate was seeded with 1.5×10^5 cells/well 18 h prior to infection.

GENERATION OF *C. jejuni* MUTANTS

The *C. jejuni* F38011 *fliD, flaAflaB, fliD, ciaC*, and *ciaB* mutants were generated as previously described (Konkel et al., 1999, 2004; Christensen et al., 2009; Buelow et al., 2010). The *C. jejuni*

F38011 *flgL*, F38011 *flgK*, F38011 *flgE*, 81116 *flaAflaB*, and 81–176 *flaAflaB* mutants were generated by double-crossover recombination using the pBSK-Kan2 suicide vector. The pBSK-Kan2 plasmid is identical to the pBluescript vector (Stratagene), except that the original ampicillin resistance cassette was replaced with the *apha-3* kanamycin resistance cassette, which functions in both *E. coli* and *C. jejuni* (Christensen et al., 2009). Approximately 1 kilobase of DNA flanking the target genes was amplified by PCR using the primers indicated in **Table A1** and sequentially cloned into pBSK-Kan2 using SacI and SacII sites for the upstream fragment and SacII and XhoI sites for the downstream fragments. A tetracycline (Tet) resistance cassette was amplified using the primers indicated in **Table A1** and inserted into the SacII site between the two flanking regions. The suicide vectors were introduced into *C. jejuni* via electroporation, and transformants were selected for on MHB plates supplemented with 2 μg/mL Tet. The suicide vectors described in this study were confirmed by nucleotide sequencing. Inactivation of the target genes in *C. jejuni* was confirmed by PCR (not shown).

The *flgL* mutant was complemented by PCR amplifying the native promoter and ORF using the primers listed in **Table A1**, and cloning the PCR product into the pRY111 vector using XhoI and EcoRI. The *flgL* complementation plasmid was electroporated into *E. coli* S-17, and subsequently conjugated into the *C. jejuni* F38011 *flgL* mutant.

MOTILITY ASSAYS

Overnight cultures of *C. jejuni* were harvested from MHB agar plates and re-suspended in MH broth to 1.0 OD_{540}/mL. Ten μL of the bacterial suspension was spotted onto the center of a MH agar plate containing only 0.4% agar, and incubated for 48 h. The zone of motility was measured from the edge of the inoculum spot to the edge of growth. Each sample was assayed in triplicate. A separate motility plate was stab-inoculated with each strain for visual comparison of motility zones.

TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy was performed as previously described (Neal-Mckinney et al., 2010). Briefly, *C. jejuni* taken from MHB plates were re-suspended in water, added to formvar coated copper grids, and stained with 2% phosphotungstic acid. The grids were visualized using a FEI Tecnai G2 20 Twin microscope.

Cia SECRETION ASSAY

Secretion of the Cia proteins into culture supernatants was assayed as previously described, with minor modifications (Konkel et al., 2004). Briefly, *C. jejuni* grown on MHB agar plates overnight were harvested and washed three times in MEM without methionine or FBS (Cellgro). Three mL of a 0.3 OD_{540}/mL suspension was added to a T25 flask, followed by 10 μL of Express Protein Labeling Mix [β-^{35}S]-methionine (Perkin Elmer) and 30 μL of dialyzed FBS, and incubated for 3 h at 37°C in a 5% CO_2 incubator. After incubation, 1.5 mL of culture was centrifuged for 5 min in a benchtop centrifuge, and 1 mL of supernatant was filtered through a 0.2 μm filter and transferred to a new tube. The proteins in the supernatant were precipitated using 1 mM HCl-acetone and re-suspended in 100 μL (10-fold concentration). Concentrated supernatants were diluted 1:2 in 2X sample buffer, boiled, and separated by SDS-PAGE. The proteins were transferred to a PVDF membrane and dried overnight. The PVDF membrane was exposed to X-ray film (Kodak Biomax) for 48 h and developed. The secretion assay was repeated at least three times, and a representative image is shown.

GENERATION OF ADENYLATE CYCLASE DOMAIN FUSION VECTORS

The ACD (amino-terminus) of *Bordetella pertussis cyaA* was PCR amplified from the pJB2581 vector (Bardill et al., 2005), using the primers shown in **Table A1** and cloned into the pRY111 vector using BglII and KpnI. Inverse PCR was then performed using the primers indicated in **Table A1**, to create a SacI-PstI-XhoI-BamHI-EcoRI-BglII multiple cloning site immediately upstream of the ACD to create the pRY111-ACD vector. The nucleotide sequences of *ciaC, ciaI, flaA, flaG, flgB*, and *metK* were amplified without the termination codon, using the primers shown in **Table A1** and cloned into pRY111-ACD using SacI and EcoRI. The vectors were sequenced, and electroporated into *E. coli* S-17 for conjugation into *C. jejuni*.

ADENYLATE CYCLASE DOMAIN IMMUNOBLOTS

Immunoblots were performed to detect ACD fusion proteins in whole-cell lysates (WCL) of *C. jejuni*. Bacteria were harvested after overnight growth in MH broth supplemented with 0.01% deoxycholate and suspended in PBS to a density of 10 OD_{540}/mL, diluted 1:2 in 2X sample buffer, boiled, and separated by SDS-PAGE. The proteins were transferred to PVDF membranes and probed with a 1:1000 dilution of a mouse anti-ACD antibody (sc-13582, Santa Cruz Biotechnology). Bound mouse antibody was detected using 1:2000 diluted rabbit anti-mouse IgG conjugated to HRP (Sigma), and the blot was developed using Western Lightning ECL reagent (Perkin Elmer).

ADENYLATE CYCLASE DOMAIN REPORTER DELIVERY ASSAYS

INT 407 epithelial cells were seeded at a density of 1.5×10^5. *C. jejuni* were grown for 18 h in MH broth supplemented with 0.01% DOC, and harvested by centrifugation. The pellets were re-suspended and washed twice in PBS, then pelleted and re-suspended to a density of 0.3 OD_{540}/mL in MEM with 1% FBS. The cell suspension was diluted 1:20, and one mL of the diluted suspension was used to infect a well of INT 407 cells in a 24-well plate. After incubation at 37°C in a humidified 5% CO_2 incubator for 30 min, the cells were rinsed with PBS, and 300 μL of 0.1 M HCl was added to each well. The INT 407 cells were lysed by incubating the sealed 24-well plate atop boiling water for 15 min, and the lysates were collected. After centrifugation at 16,000 × *g* for 2 min to pellet cell debris, 100 μL of each sample was assayed for cAMP using an enzyme-linked immunosorbant assay cAMP kit (Enzo, New York, NY) according to the manufacturer's instructions. Each sample was tested in triplicate. The assays were performed at least three times to ensure reproducibility.

Supernatants from a *C. jejuni* wild-type strain harboring *ciaC-ACD* were collected by inoculating the strain to 0.6 OD_{540}/mL in MEM with 1% FBS, and incubating for 1 h. The cultures were centrifuged and supernatants filtered through a 0.2 μm filter to remove any *C. jejuni*. The presence of CiaC-ACD in the

supernatants was confirmed using an *in vitro* ACD activity assay and immunoblotting (not shown). For assays using a filter to block contact with host cells, a 6-well tissue culture plate was seeded with 3×10^5 cells/well and incubated overnight. The culture media was replaced with MEM with 1% FBS, and a Thincert (Greiner Bio-One) well insert with a 0.2 μm pore size was added to the wells. Two mL of *C. jejuni* (0.3 OD_{540}/mL) in MEM with 1% FBS were used to inoculate the upper chamber of the inserts. TAE226 (Novartis) was dissolved in methanol. For assays using TAE226 to inhibit *C. jejuni* internalization, 10 μM TAE226 was added to the wells of a 24-well plate 30 min prior to infection with *C. jejuni* (0.3 OD_{540}/mL). To increase the sensitivity of cAMP detection, we used a high inoculum (0.3 versus 0.015 OD_{540}/mL used in the initial assays) in assays using filters to block *C. jejuni* contact with host cells and assays using TAE226 to inhibit invasion. The samples were assayed in triplicate, and the assay was repeated at least three times. A representative assay is shown.

INTERNALIZATION ASSAY (GENTAMICIN PROTECTION ASSAY)

Binding and internalization assays were performed as described elsewhere (Christensen et al., 2009). Briefly, each well of a 24-well plate was inoculated with MEM 1% FBS containing 0.03 OD_{540}/mL of *C. jejuni* wild-type strain harboring pRY107 (kanamycin resistant), *ciaC* mutant (chloramphenicol resistant), or a mixture of the two strains. The concentration of the *ciaC* mutant strain was held constant at 0.03 OD_{540}/mL, while increasing ratios (i.e., 1:2, 1:5, 1:10) of the wild-type strain were added. Gentamicin was added to the wells 3 h post-infection at a concentration 250 μg/mL, a concentration that has been shown previously to efficiently kill extracellular bacteria without affecting intracellular bacteria (Monteville et al., 2003). INT 407 cell lysates were serially diluted and plated on both MHB Kan and MHB Cm plates to select for the wild-type strain and *ciaC* mutant, respectively. Where indicated, TAE226 was added to cells 30 min prior to infection. Statistical significance was evaluated using a two-tailed Student's *t*-test ($P < 0.05$). The samples were assayed in quadruplicate wells, and the assay was performed four times.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) was performed to observe membrane ruffling, as described elsewhere (Eucker and Konkel, 2011). Two independent, blinded observers quantified the number of INT 407 cells with membrane ruffling in four fields of view at 1000× magnification.

RESULTS

SECRETION OF THE Cia PROTEINS REQUIRES THE FLAGELLAR HOOK

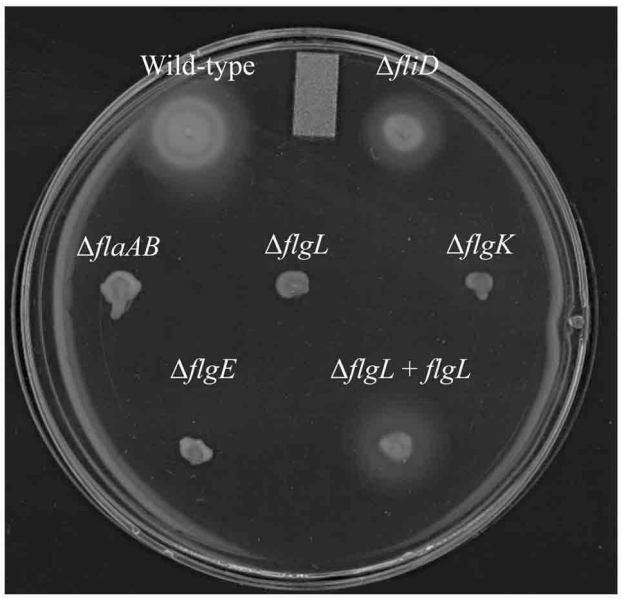
Flagellar gene deletions were generated in *C. jejuni* F38011 by homologous recombination, resulting in the deletion of the *fliD, flaAflaB, flgL, flgK*, and *flgE* loci. Motility assays were performed to ensure that the motility phenotypes of the flagellar mutant were in agreement with previously published data (Konkel et al., 2004; Fernando et al., 2007; Neal-Mckinney et al., 2010). Inactivation of the *flaAflaB, flgL, flgK*, or *flgE* loci completely abolished motility compared to the *C. jejuni* F38011 wild-type strain and isogenic *fliD* mutant (**Figure 1**). The reduced motility of the *C. jejuni fliD* mutant in motility agar compared to the *C. jejuni* wild-type strain is in agreement with the hypothesis that FliD helps incorporate exported FlaA and FlaB monomers into the growing flagellar filament. The flagellin monomers can diffuse away from the flagellar filament when the *C. jejuni fliD* mutant is grown in broth, reducing flagellum length, and bacterial motility (Yokoseki et al., 1995). Based on the results of the Cia secretion assays (see below), experiments were performed to specifically complement the *flgL* mutant by transforming the mutant with a plasmid harboring a wild-type copy of the gene. In contrast to the *flgL* mutant, motility was restored in the transformed isolate, although not to the same amount as the wild-type strain. This is due possibly to differences in transcriptional regulation of the *flgL* gene in the wild-type strain (chromosomal) and *flgL* complemented strain (multi-copy plasmid). Transmission electron microscopy was used to confirm the results of the motility assays, and revealed that the wild-type strain and *flgL* mutant transformed with the *flgL* plasmid produced complete flagella, while the *flgL* mutant did not produce a flagellar filament (**Figure A1**). This finding demonstrates that the phenotype displayed by the mutant was due solely to the absence of the FlgL protein.

Secretion of the Cia proteins from the *C. jejuni fliD, flaAflaB, flgK, flgL*, and *flgE* mutants was assayed by autoradiography of protein supernatants from *C. jejuni* grown in the presence of 1% FBS to stimulate Cia secretion (**Figure 2A**) (Konkel et al., 2001, 2004). The proteins in the supernatant were concentrated, and probed with antibody reactive against the ACD (**Figure 2B**). Supernatants and whole cell lysates (**Figures 2C,D**, respectively) of each of the isolates were probed with an anti-CysM serum to ensure that bacterial cell lysis was not responsible for the proteins observed in the supernatants by autoradiography. The Cia proteins were secreted from the *fliD* and *flaAflaB* mutants but not the *flgL, flgK*, and *flgE* mutants, as judged by autoradiography. The ∼66 kDa band visible in the *flgL, flgK*, and *flgE* mutant secretion profiles is background from the FBS used to stimulate Cia secretion, and was not observed in samples without 1% FBS (not shown). In agreement with the results of the autoradiograph, CiaC-ACD was detected in the supernatant of the wild-type strain, as well as the *fliD* and flaAB mutants (the *flgL* mutant transformed with the *flgL* plasmid did not contain the *ciaC-ACD* gene). Interestingly, the *fliD* and *flaAB* mutants had a more intense secretion profile than wild-type.

The results obtained for the secretion assay performed with the newly generated *C. jejuni* F38011 *flaAflaB* mutant were not consistent with our previous findings using a *C. jejuni* 81116 mutant with a partial *flaAflaB* gene deletion (Grant et al., 1993). We regenerated new *C. jejuni* 81116 and 81–176 *flaAflaB* mutants whereby the entire *flaA* and *flaB* ORFs were deleted. All three of the *C. jejuni flaAflaB* mutants (F38011, 81116, and 81–176) were non-motile, and exported the Cia proteins into the supernatants as judged by the secretion assay (**Figure A2**).

Based on the results of the secretion assay and the fact that FlgL was the most distal component of the flagellar apparatus that appeared necessary for Cia protein secretion, we tested whether the *flgL* complement strain could secrete the Cia proteins. Complementation of the *flgL* mutant with a plasmid encoding *flgL* restored secretion of the Cia proteins. Based on these data, we

A



Strain	Zone of motility (mm)
Wild-type	14.0 ± 1.0
Δ*fliD*	11.3 ± 0.58
Δ*flaAflaB*	Non-motile
Δ*flgL*	Non-motile
Δ*flgK*	Non-motile
Δ*flgE*	Non-motile
Δ*flgL* + *flgL*	11 ± 1.0

B

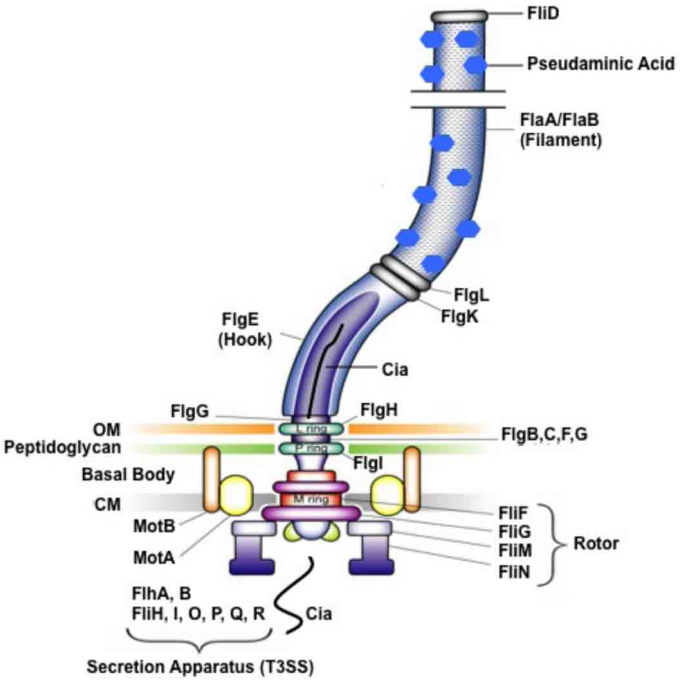


FIGURE 1 | Motility of the *C. jejuni* flagellar mutants. Motility assays were performed using *C. jejuni* F38011 wild-type strain, the isogenic *fliD, flaAflaB, flgL, flgK*, and *flgE* mutants, and the *flgL* mutant complemented with *flgL* **(A)**. The table contains zones of motility ± one standard deviation obtained from a quantitative motility assay performed in triplicate. The zone of motility was determined by measuring from the edge of the inoculum spot to the edge of growth. A diagram of the *C. jejuni* flagellum is also included, and was adopted from [Larson et al. (2008)] **(B)**. OM, outermembrane; CM, cytoplasmic membrane.

concluded that the hook and hook-associated proteins, but not the flagellar filament, are required for Cia protein secretion.

C. jejuni CiaC IS DELIVERED TO HOST CELLS

We hypothesized that CiaC, which is a secreted from the flagellar apparatus and required for maximal invasion of host cells, is delivered to the cytosol of epithelial cells. To test this hypothesis, we used the adenylate cyclase domain (ACD) of the *Bordetella pertussis* CyaA protein as a reporter for host cell delivery (Glaser et al., 1988; Sory and Cornelis, 1994; Sory et al., 1995). The ACD, which is activated only in the presence of calmodulin found in eukaryotic cells, catalyzes the production of cAMP from ATP. We generated a pRY111-based (Yao et al., 1993) shuttle plasmid whereby a number of *C. jejuni* genes, including *ciaC*, were cloned in-frame with the ACD. As a negative control, the *metK* gene driven from its native promoter was also cloned

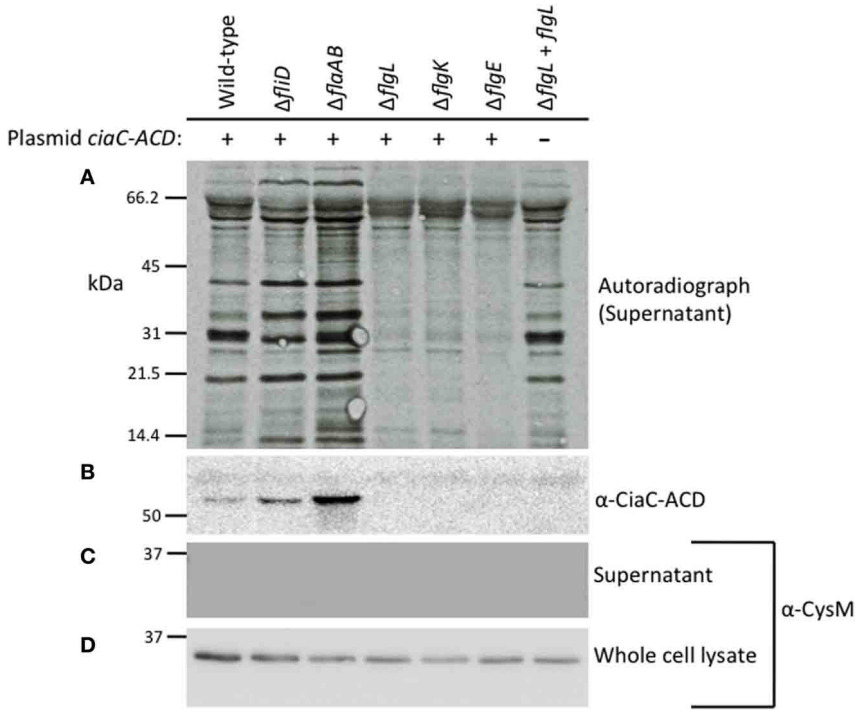


FIGURE 2 | The Cia proteins are secreted from the *C. jejuni* F38011 wild-type strain and *fliD* and *flaAflaB* mutants, but not *flgL, flgK,* and *flgE* mutants. The *C. jejuni* F38011 wild-type strain and isogenic *fliD, flaAB, flgL, flgK,* and *flgE* mutants were assayed for secretion of the Cia proteins as well as for secretion of plasmid-encoded CiaC-ACD. Newly synthesized proteins were labeled with [^{35}S]-methionine and Cia secretion was stimulated by the addition of 1% FBS. Proteins in the supernatants were concentrated 10-fold, separated by SDS-PAGE, transferred to PVDF membranes, and exposed to X-ray film **(A)** or probed with an anti-ACD antibody **(B)**. Faint bands present in all lanes represent bovine serum albumin (66.5 kDa) and degradation products. The molecular mass of the pre-stained protein standards are listed in kDa. Supernatants **(C)** and whole-cell lysates **(D)** were probed with anti-CysM antibody to confirm that cell lysis did not occur.

in-frame with the ACD. The *C. jejuni* MetK protein, which is an S-adenosylmethionine synthetase, is localized in the cytoplasm (Parkhill et al., 2000). The *ciaC-ACD* and *metK-ACD* plasmids were introduced into the *C. jejuni* F38011 wild-type strain and isogenic flagellar mutants, and ACD delivery assays were performed.

INT 407 cells were infected at a MOI of ∼500, similar to that used in our gentamicin protection assays. The amount of intracellular cAMP produced by the INT 407 cells was assayed at 30 min post-infection, as preliminary experiments revealed this time-point resulted in the greatest level of cAMP (data not shown). Infection of INT 407 cells with the *C. jejuni* F38011 wild-type strain, *fliD* mutant, and *flaAflaB* mutant harboring *ciaC-ACD* resulted in a significant increase in intracellular cAMP compared to the amount of cAMP produced by cells inoculated with the *flgL, flgK*, and *flgE* mutants harboring *ciaC-ACD* (**Figure 3**). Only minimal levels of cAMP were detected in INT 407 cells inoculated with the *C. jejuni* F38011 wild-type strain and *flgE* mutant harboring *metK-ACD*. Noteworthy is that the delivery of CiaC to INT 407 cells was also demonstrated using MOI of ∼5000 and ∼100, whereas a significant increase in cAMP levels (as compared to the flgE mutant negative control) was not detected in INT 407 cells for MetK regardless of the MOI (data not shown). These results are consistent with the hypothesis that the CiaC protein is delivered to the cytosol of host cells. Further, these results indicate that a flagellar hook and hook-associated proteins (FlgE, FlgK, and FlgL) are required for Cia delivery.

C. jejuni CiaI IS DELIVERED TO HOST CELLS

Previous studies have suggested that multiple flagellar and Cia proteins are secreted upon cultivation with epithelial cells, some of which function to modulate cell behavior and facilitate pathogenesis (Konkel et al., 1999; Christensen et al., 2009; Buelow et al., 2010). To test whether other flagellar exported proteins are delivered to host cells, the ACD reporter assay was used to examine delivery of a known Cia protein (i.e., CiaI) (Buelow et al., 2010), as well as secreted flagellar components (i.e., FlaA, FlaG, and FlgB) (Hendrixson and Dirita, 2003; Larson et al., 2008; Christensen et al., 2009) (**Figure 4**). Each ACD construct was transformed into the *C. jejuni* wild-type strain and *flgE* mutant and assayed for cAMP production in INT 407 cells after 30 min infection. Infection with *C. jejuni* F38011 wild-type harboring *ciaI-ACD* resulted in a significant increase in cAMP, compared to the *flgE* mutant harboring *ciaI-ACD*. The *flaA-ACD, flaG-ACD*, and *flgB*-ACD plasmids in the *C. jejuni* F38011 wild-type strain did not result in an increase in cAMP levels compared to the *flgE* mutant. The CiaI-ACD, FlgB-ACD, FlaA-ACD, and FlaG-ACD proteins were exported, as judged by immunoblot analysis

of supernatants using anti-ACD and anti-CysM antibodies (**Figure A3**). Collectively, these results demonstrate that the CiaC and CiaI proteins are exported and delivered to host cells in a flagellar T3SS dependent manner. The flagellar proteins FlaA, FlaG, and FlgB are either not delivered to host cells or delivered in low concentrations undetectable by our ACD reporter assay.

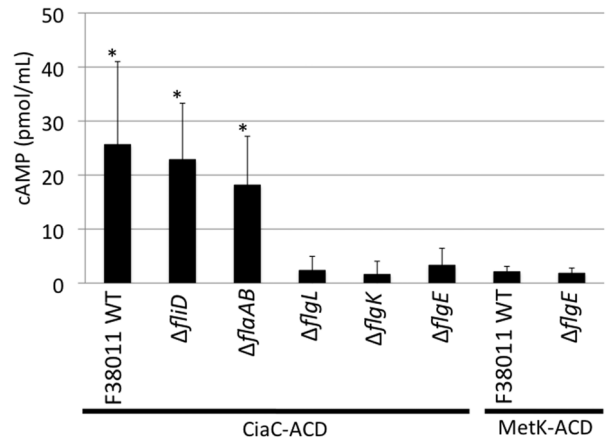


FIGURE 3 | The CiaC virulence proteins is delivered to the cytosol of INT 407 cells from the *C. jejuni* F38011 wild-type strain, *fliD* mutant, and *flaAflaB* mutant, but not *flgL, flgK,* and *flgE* mutants. Delivery of the CiaC-ACD fusion protein from the cytosol of *C. jejuni* to the cytosol of INT 407 cells was assayed in the *C. jejuni* F38011 wild-type strain and isogenic *fliD, flaAflaB, flgL, flgK,* and *flgE* mutants, and delivery of MetK-ACD was assayed in the *C. jejuni* F38011 wild-type strain and *flgE* mutant. The concentration of cAMP within the INT 407 cells was assayed 30 min post-infection. The error bars represent one standard deviation from mean. The asterisk indicates values that are significantly different from the *flgE* mutant harboring *ciaC-ACD* as judged by the Student's *t*-test ($P < 0.05$). Data from two biological replicates were combined.

To ensure that each of the ACD fusion proteins were synthesized at equivalent levels in the *C. jejuni* wild-type strain and *flgE* mutant, whole cell lysates of each strain were probed by immunoblot, using an anti-ACD antibody (**Figure 5**). The intensity of the reactive bands in the wild-type strain and *flgE* mutant were similar, indicating that production of the ACD fusion proteins was not significantly affected by mutation of *flgE*.

PROTEIN DELIVERY REQUIRES BACTERIA-HOST CELL CONTACT

To address whether the Cia proteins are delivered to host cells from outer membrane vesicles released into the culture supernatants from *C. jejuni* or via another mechanism not requiring bacteria-host cell contact, we cultured *C. jejuni* in medium containing 1% FBS for 1 h and then prepared bacterial cell-free supernatants. The purpose of adding FBS to the medium was to stimulate the synthesis and export of the Cia proteins into the supernatants (Rivera-Amill et al., 2001). For these assays, *C. jejuni* were suspended in medium at a level that was 40-fold greater (0.6 OD_{540}/mL) than used for the previous *C. jejuni*-cell infection assays, in order to increase the amount of Cia proteins in the supernatants. Using this procedure, we were readily able to detect CiaC-ACD in the cell-free supernatant by immunoblots and by an *in vitro* activity assay whereby the supernatants were mixed with activity buffer containing ATP and calmodulin (data not shown). The cell-free supernatant was then added to INT 407 epithelial cells and cAMP levels of the cells were measured after 30 min (**Figure 6**). The results were compared to those obtained for cells infected with a wild-type strain (positive control) or *flgE* mutant (negative control) harboring *ciaC-ACD* (Panel A). No cAMP production was observed in cells treated with CiaC-ACD containing supernatants, indicating that the secreted CiaC-ACD protein was not delivered to the host cell cytosol. An additional experiment was performed using a 0.2 μm pore filter to block physical contact of *C. jejuni*

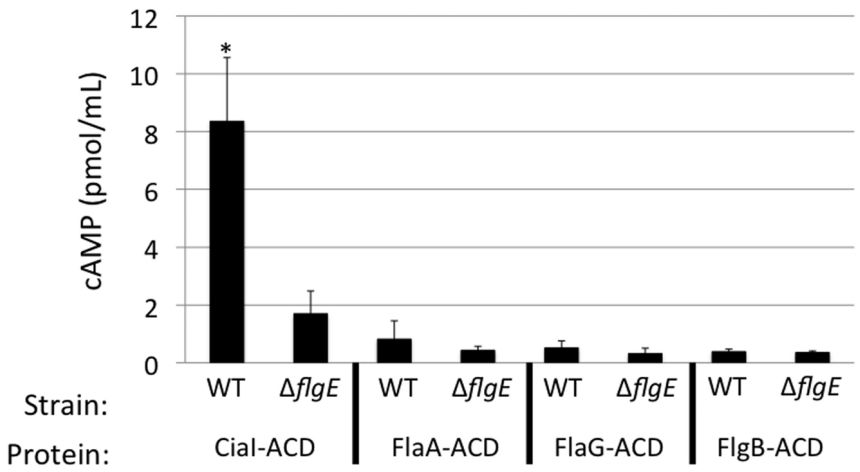


FIGURE 4 | CiaI is delivered from the *C. jejuni* F38011 wild-type strain to INT 407 cells. Delivery of the CiaI-ACD, FlaA-ACD, FlaG-ACD, and FlgB-ACD fusion proteins was assayed in the *C. jejuni* F38011 wild-type strain and isogenic *flgE* mutant. INT 407 cells were inoculated with the *C. jejuni* F38011 wild-type strain and *flgE* mutant harboring the various ACD plasmids. The concentration of cAMP within the INT 407 cells was assayed 30 min post-infection. The error bars represent one standard deviation from mean. The asterisk indicates that the amount of cAMP produced in the wild-type strain was significantly greater than the value obtained from the *flgE* mutant, as judged by the Student's *t*-test ($P < 0.05$).

with the epithelial cells (Panel B). The filter used created two chambers within the tissue culture well, a bottom chamber containing the epithelial cells and an apical chamber containing a *C. jejuni* wild-type strain harboring *ciaC-ACD*. While proteins were secreted from *C. jejuni* and entered the bottom chamber, no cAMP production was observed. These results suggest that *C. jejuni* binding to host cells is necessary for CiaC protein delivery.

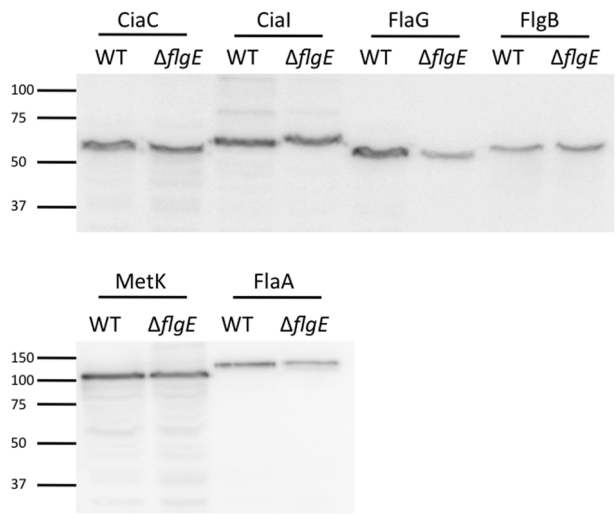


FIGURE 5 | Immunoblot using anti-ACD antibody reveals equivalent protein synthesis of ACD fusion proteins in *C. jejuni* F38011 wild-type strain and *flgE* mutant. The amount of ACD fusion proteins produced in the *C. jejuni* F38011 wild-type strain and isogenic *flgE* mutant was examined by immunoblot. Whole cell lysates were prepared from bacteria grown overnight in MH broth supplemented with 0.01% deoxycholate, transferred to PVDF membranes, and probed with an anti-ACD antibody. The molecular mass of the pre-stained protein standards are listed in kDa.

Cia PROTEIN DELIVERY DOES NOT REQUIRE BACTERIAL INTERNALIZATION

It had previously been shown that effector proteins can be exported by pathogens located both inside and outside of the target cell (Coburn et al., 2007). To determine whether Cia protein delivery was occurring by cell-associated (bound) bacteria, the delivery of CiaC-ACD to host cells was assayed in the presence of TAE226 to inhibit bacterial invasion (**Figure 7**). TAE226 is a specific inhibitor of Focal Adhesion Kinase, which is required for maximal *C. jejuni* invasion of INT 407 cells (Eucker and Konkel, 2011). The viability of INT 407 cells treated with TAE226 or methanol (vehicle control) was assessed using trypan blue (Sigma) staining, and neither treatment affected the viability of the cells (data not shown). Treatment of cells with TAE226 significantly reduced invasion of INT 407 cells by *C. jejuni* to a level below that of the *ciaB* mutant (invasion deficient control) in a dose-dependent manner (**Figure 7A**). The addition of TAE226 did not significantly reduce the level of cAMP produced in response to delivery of CiaC-ACD (**Figure 7B**). These data demonstrate that *C. jejuni* delivers proteins to epithelial cells prior to internalization.

A FUNCTIONAL FLAGELLAR EXPORT APPARATUS IS REQUIRED FOR *C. jejuni* TO INDUCE MEMBRANE RUFFLING OF INT 407 CELLS

Delivery of the Cia virulence proteins modulates signaling pathways in host cells, leading to cytoskeletal rearrangement mediated in part by the Rho GTPase Rac1 (Eucker and Konkel, 2011). Rearrangement of the host cell cytoskeleton causes the formation of membrane ruffles that can be visualized and quantified using SEM. We performed SEM of cells infected with the *C. jejuni* F38011 wild-type strain, *flgL* mutant, and *flgL* mutant complemented with *flgL* to confirm that the Cia virulence proteins were delivered to host cells in a flagellar T3SS dependent manner (**Figure 8**). In the absence of bacteria, membrane ruffling was

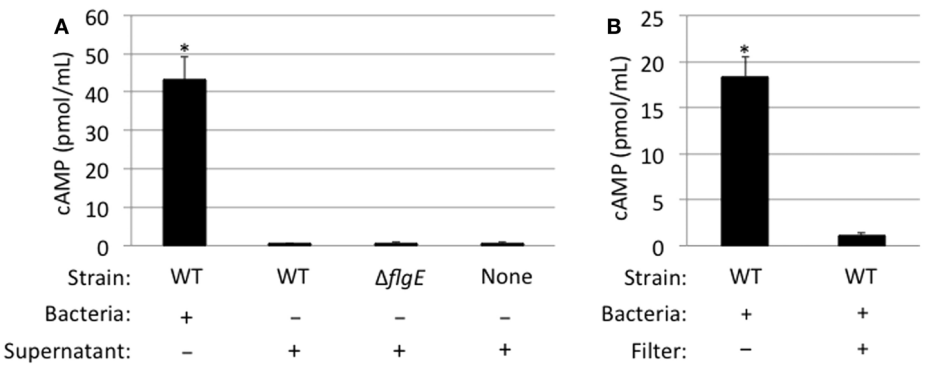


FIGURE 6 | CiaC-ACD delivery into INT 407 cells requires bacteria-host cell contact. The delivery of CiaC-ACD into INT 407 cells assayed using whole bacteria and CiaC-ACD containing supernatants **(A)**, as well as a filter to block physical contact of bacteria with INT 407 cells **(B)**. **(A)** Cell-free supernatants of *C. jejuni* F38011 wild-type strain and isogenic *flgE* mutant were harvested from bacteria incubated in MEM supplemented with 1% FBS for 1 h. The *C. jejuni* F38011 strain harboring the *ciaC-ACD* plasmid or cell-free supernatants collected from this strain were added to INT 407 cells and cAMP was measured 30 min post-inoculation. **(B)** A delivery assay was performed in the presence and absence of a 0.2 μm pore filter. To accommodate the 0.2 μm pore filter, six-well tissue culture plates (rather than 24-well) were used. The value shown for *C. jejuni* F38011 wild-type strain was normalized by subtracting the background cAMP value obtained for the *flgE* mutant, with or without a 0.2 μm pore filter. The error bars represent one standard deviation from mean. The asterisk indicates the amount of cAMP produced in the wild-type strain was significantly greater than the value obtained from the *flgE* mutant as judged by the Student's *t*-test ($P < 0.05$).

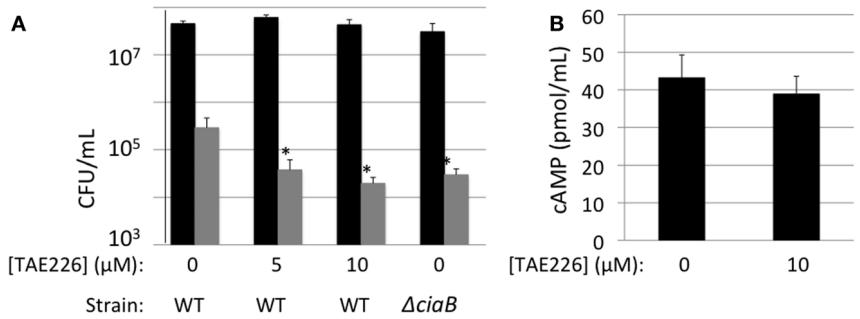


FIGURE 7 | CiaC-ACD is delivered into INT 407 cells by extracellular bacteria. A bacterial invasion assay **(A)** and delivery assay **(B)** were performed in the presence of TAE226, an inhibitor of Focal Adhesion Kinase. Focal adhesion kinase is required for maximal invasion of host cells by *C. jejuni*. **(A)** The gentamicin protection assay was used to determine the number of cells of *C. jejuni* F38011 wild-type strain and its isogenic *ciaB* mutant internalized in the presence of 0, 5, or 10 μM TAE226. Gentamicin was added 3 h post-infection to kill extracellular bacteria. The INT 407 cells were then lysed and the bacteria were enumerated by serial dilution and plating. Shown are the number of cell-associated bacteria (black bars) and internalized bacteria (gray bars). The number of bacteria internalized in the presence of TAE226 was significantly reduced, when compared to *C. jejuni* infected cells in the absence of inhibitor, as judged by the Student's *t*-test ($P < 0.05$). **(B)** Delivery of the CiaC-ACD fusion protein was assayed at 30 min post-infection in the presence of 0 or 10 μM TAE226. The values shown were normalized by subtracting the background cAMP value obtained for the *flgE* mutant (i.e., Cia secretion negative) containing each ACD fusion construct from the values obtained for the wild-type strain. The error bars represent one standard deviation from the mean. The amount of cAMP produced in the presence of TAE226 was not significantly different from the value obtained in the absence of TAE226, as judged by the Student's *t*-test ($P < 0.05$). Cells were treated with methanol (vehicle control), and trypan blue staining was performed to confirm that the solvent used for TAE226 did not affect cell viability (not shown).

observed in 33.5% (# of cells examined = 212) of the INT 407 cells examined (Panel H). In contrast, 63.7% (# of cells examined = 216) of the INT 407 cells infected with the *C. jejuni* wild-type strain exhibited membrane ruffling (Panels A and B). Consistent with our previous findings demonstrating that FlgL is required for CiaC export and delivery, the amount of membrane ruffling observed in cells inoculated with the *flgL* mutant (Panels C and D) was indistinguishable from that of non-infected INT 407 cells (membrane ruffling was observed in 35% of the cells, # of cells examined = 356), whereas the complemented *flgL* mutant (Panels E and F) caused membrane ruffling in 58.5% of cells (# of cells examined = 232). The addition of cell-free supernatants containing the Cia proteins to INT 407 cells (Panel G) did not stimulate membrane ruffling (27.3% of cells, # of cells examined = 157). Importantly, bacteria-host cell contact was promoted by centrifugation of the tissue culture plates to eliminate the effects of motility on adherence. Despite the fact that the *flgL* mutant was in contact with the epithelial cells, contact alone did not appear sufficient to induce membrane ruffling. These results support the hypothesis that the delivery of the Cia proteins induces cytoskeletal rearrangement, and the delivery of the Cia proteins depends on a functional flagellar export apparatus.

A *C. jejuni* WILD-TYPE STRAIN CANNOT RESCUE THE INVASION OF A *ciaC* MUTANT

As we observed intense membrane ruffling in response to delivery of the Cia proteins, we sought to determine whether the Cia proteins delivered by a wild-type bacterium could induce global changes in the cell architecture to facilitate the internalization of an invasion deficient bacterium. We performed internalization assays with various ratios of a *C. jejuni* wild-type strain and the *ciaC* mutant (**Figure 9**). In this experiment, the amount of the *ciaC* mutant added to each well was kept constant while increasing amounts of the *C. jejuni* wild-type strain were added. Consistent with previous work (Christensen et al., 2009), a significant reduction in invasion was noted for the *C. jejuni ciaC* mutant when compared to the wild-type strain. However, the number of internalized *ciaC* mutant bacteria did not change in response to additional wild-type bacteria. These results suggest that the Cia proteins act in a localized manner within the cell, and that the membrane ruffling induced by wild-type *C. jejuni* is not sufficient to facilitate the uptake of a bacterium attached to another location on the host cell.

DISCUSSION

While the precise roles of virulence proteins in facilitating bacterial invasion of host epithelial cells remain to be elucidated, it is clear that virulence proteins are secreted from the cell in a flagellar dependent manner (Konkel et al., 1999, 2004; Song et al., 2004; Poly et al., 2007). While some secreted virulence proteins likely act on the surface of the cell, others are presumably delivered to the cytosol, based on their biological functions. Prior to this study, it was not known whether the Cia proteins: (1) modulate host cell activities from the extracellular environment; (2) are delivered to the cytosol of host cells from the flagellum; or (3) are exported to the extracellular environment and then taken into the cytosol via a mechanism independent of the flagellum. Although we are unable to elucidate the precise mechanism of CiaC delivery, we demonstrate that cell-associated *C. jejuni* are necessary for delivery of CiaC to the cytosol of host cells. The delivery of CiaC is dependent on a functional flagellar T3SS. The flagellar proteins FlaA, FlaG, and FlgB were not delivered to host cells, suggesting that the mechanism of delivery of some Cia proteins to a host cell is specific. The addition of cell-free supernatants containing CiaC-ACD did not result in cAMP production, indicating

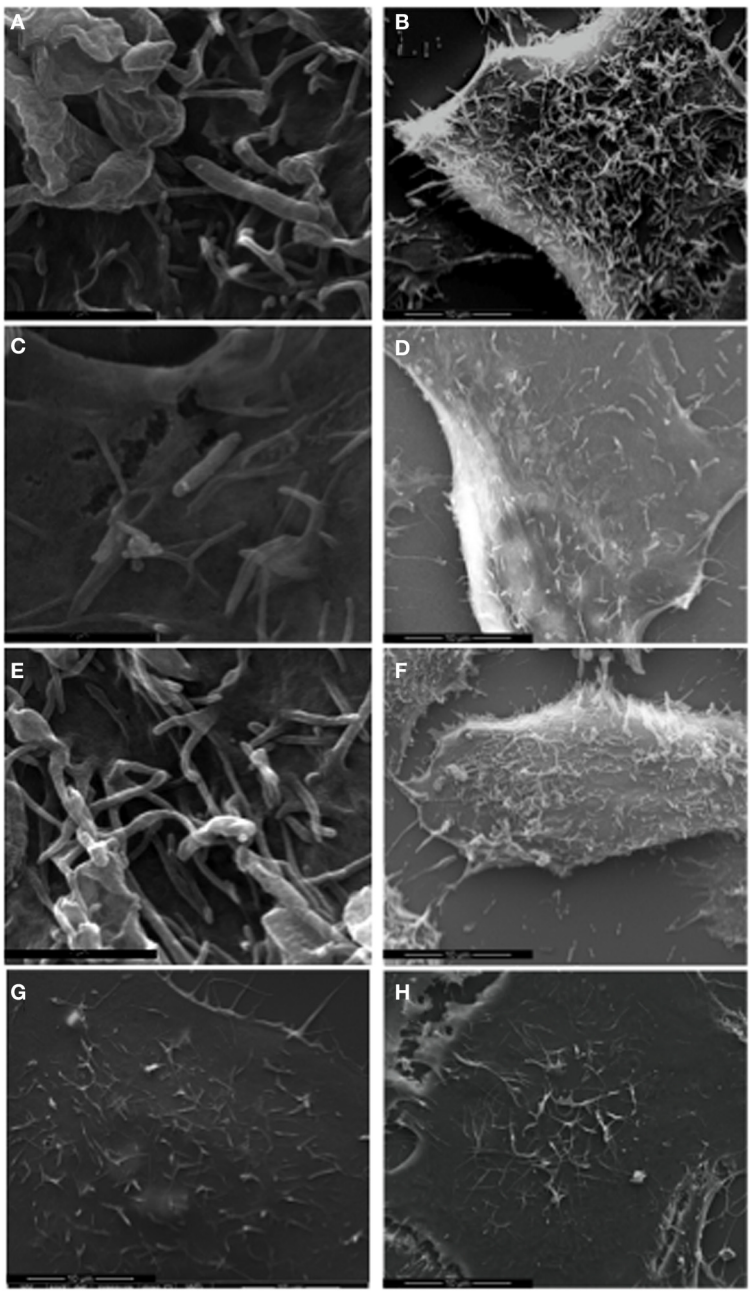


FIGURE 8 | *C. jejuni* F38011 wild-type induces membrane ruffling. INT 407 cells were infected with the *C. jejuni* F38011 wild-type strain **(A** and **B)**, *flgL* mutant **(C** and **D)**, *flgL* mutant harboring a plasmid containing native *flgL* **(E** and **F)**, cell-free supernatants containing the Cia proteins **(G)**, or were uninfected **(H)**. The cells were fixed and removed at 15 min post-infection, and visualized by SEM at 50,000× **(A, C,** and **E)** and 7000× **(B, D, F, G,** and **H)** magnification.

that CiaC is delivered by a mechanism without an extracellular intermediate.

In a previous study, it was found that inactivation of the *flaAflaB* locus in *C. jejuni* 81116 (GRK7 strain) abolished secretion of the Cia proteins (Grant et al., 1993). Noteworthy is that the *C. jejuni* 81116 GRK7 *flaAflaB* mutant was constructed via a different method than the *C. jejuni* F38011 *flaAflaB* mutant, such that the *C. jejuni* 81116 *flaAflaB* mutant chromosome still possessed a 5′ portion of the *flaA* gene. Surprisingly, when we completely deleted the *flaA* and *flaB* locus in the *C. jejuni* F38011 strain, the Cia proteins were still secreted. To determine whether this phenotype was strain-specific, we generated identical mutations in *C. jejuni* strains F38011, 81116, and 81–176. When the *flaAflaB* locus was completely deleted, the three mutant strains were still able to secrete the Cia proteins. Our results demonstrate that FlaA and FlaB are not required for secretion of

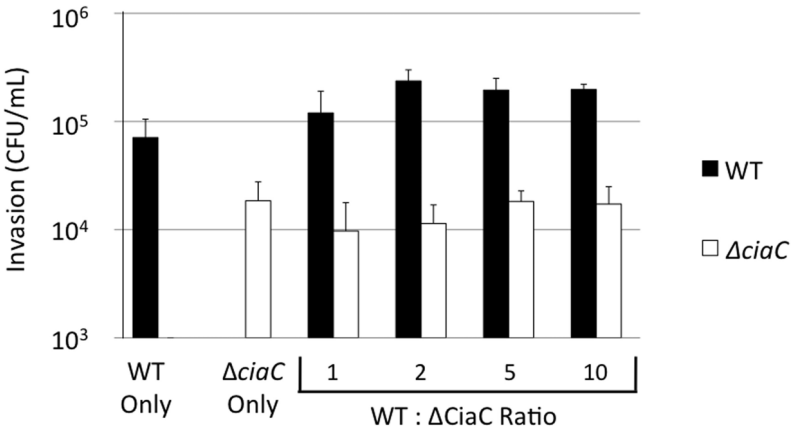


FIGURE 9 | Cia delivery by the *C. jejuni* F38011 wild-type strain cannot rescue the invasion of a *ciaC* mutant. A bacterial invasion assay was performed using the *C. jejuni* F38011 wild-type strain, the isogenic *ciaC* mutant, or a mixture of both strains. The numbers of internalized *C. jejuni* F38011 wild-type (black bars) and *ciaC* mutant (white bars) bacteria were determined at 3 h post-infection using the gentamicin protection assay. The numbers below the graph indicate the quantity of *C. jejuni* F38011 wild-type and *ciaC* mutant bacteria in each well, in units of 0.03 OD_{540}. The error bars represent one standard deviation. The number of *C. jejuni ciaC* mutant bacteria internalized did not change in response to increasing amounts of wild-type bacteria, as judged by the Student's *t*-test ($P < 0.05$).

the Cia proteins. Based on this finding, it may be useful to re-examine previous studies that have examined the role of FlaA and FlaB in motility, human disease, and commensal colonization of chickens, to ensure that the observed phenotypes are due solely to loss of motility and not loss of protein secretion through the T3SS (Grant et al., 1993; Konkel et al., 2004).

One of the first questions we sought to address in this study is whether the Cia proteins were actually delivered to the cytosol of host cells. Using the ACD reporter assay, we found that Cia secretion competent *C. jejuni* strains (i.e., *C. jejuni* F38011 wild-type, *fliD* mutant, and *flaAB* mutant) were able to deliver CiaC-ACD and CiaI-ACD to host cells, resulting in an increase in cAMP levels. *C. jejuni* strains that could not secrete the Cia proteins (i.e., *C. jejuni flgL* mutant, *flgK* mutant, and *flgE* mutant) were unable to deliver CiaC-ACD to host cells. Other proteins secreted through the flagella (i.e., FlaA, FlgB, and FlaG) are either not delivered to the host cell, or delivered in minute quantities compared to CiaC or CiaI, as the FlaA-ACD, FlgB-ACD, and FlaG-ACD proteins did not result in significant increases in cAMP. Noteworthy is that CiaI-ACD, FlaA-ACD, and FlaG-ACD were secreted from *C. jejuni* at similar levels as judged by immunoblot (**Figure A3**), while CiaI-ACD was the only protein delivered to host cells. While the precise mechanism of Cia delivery via the flagellum remains to be elucidated, it is clear that the Cia proteins are delivered to the cytosol of host cells.

To our knowledge, *C. jejuni* is the only organism that uses a flagellar T3SS to deliver effector proteins to the cytosol of target cells. While it had been hypothesized that the Cia proteins could be delivered to host cells via the flagellar filament, a critique of this hypothesis was the inequality of the injectisome needle and flagellar filament. The injectisome needle complex is typically ∼1 μm in length, rigid, and stationary (Sory et al., 1995; Cornelis, 2006). In contrast, the flagellar filament of *C. jejuni* can be longer than 3 μm, is flexible, and is rapidly rotating during bacterial movement (Grant et al., 1993). Interestingly, many components of the flagellar T3SS have orthologs in injectisome T3SS (Desvaux et al., 2006; Erhardt et al., 2010). In fact, the basal body and hook structure of the flagellum is similar in structure to the basal body and needle of the injectisome (Cornelis, 2006; Erhardt et al., 2010). This study revealed that the flagellar cap protein FliD and filament proteins FlaA and FlaB are dispensable for Cia protein secretion, while the hook protein FlgE and hook-filament junction proteins FlgK and FlgL are required. The secretion negative phenotype of the *flgK* mutant is in agreement with a previous study by Fernando et al. (Fernando et al., 2007). Thus, it is possible that the *C. jejuni* flagellar T3SS without a filament could function analogous to an injectisome. We hypothesize that there may be additional proteins that interact with the flagellar hook (i.e., FlgE, FlgK, and/or FlgL) to enable direct delivery of CiaC, similar to a translocon complex that interacts with the tip of the injectisome needle.

Treatment of INT 407 cells with the FAK inhibitor TAE226 inhibits *C. jejuni* internalization in a dose-dependent manner. In this study, we found that delivery of CiaC-ACD was not affected by TAE226 at concentrations that reduced invasion ∼10-fold, demonstrating that CiaC can be delivered without invasion. In the *Yersinia* Ysc T3SS, protein delivery to host cells requires intimate contact with host cells (Sory et al., 1995). In this study, when the bacteria-host cells contact was prevented (i.e., supernatants only or filter to block contact), no protein delivery was detected. These results suggest that the delivery of the Cia proteins from *C. jejuni* to a host cell requires intimate contact. Additionally, the results of the mixed infection internalization assay (**Figure 9**) suggest that the Cia proteins act in a localized fashion within the host cell. While these findings are in agreement with the hypothesis that the flagellar T3SS of *C. jejuni* directly delivers proteins to the host cell cytosol, they do not eliminate the possibility that the Cia proteins are exported by the flagellar T3SS and taken into the host cell indirectly by a mechanism dependent on bacteria host-cell contact. However, the finding that not all flagellar exported proteins are

delivered to the host cell cytosol suggests a specific mechanism for the delivery of CiaC and CiaI by the flagellum. Regardless, identification of a flagellar-associated translocon complex is necessary to conclude that the flagellar T3SS of *C. jejuni* also functions as an injectisome.

In our current model of disease, *C. jejuni* enters the intestine where it encounters environmental stimuli (i.e., deoxycholate) that up-regulate the transcription of virulence genes, including the genes encoding the Cia proteins (Stintzi et al., 2005; Malik-Kale et al., 2008). *C. jejuni* then bind to host cells via adhesins (e.g., CadF, CapA, and FlpA) (Konkel et al., 1997, 2010; Ashgar et al., 2007; Flanagan et al., 2009). The binding of *C. jejuni* to host cells allows the Cia proteins to be delivered to the host cell cytosol, where they modulate host cell signaling events (Eucker and Konkel, 2011). The observation that chloramphenicol reduces *C. jejuni* internalization into host cells by preventing *de novo* synthesis of the Cia proteins supports the proposal that *C. jejuni* binding to host cells via the constitutively synthesized adhesins is not sufficient for host internalization, but rather sets the stage for delivery of the Cia proteins (Konkel and Cieplak, 1992). Our model of Cia protein delivery is comparable to the canonical T3SS-dependent virulence strategy used by other intestinal pathogens (Gauthier and Finlay, 1998). Pathogens that utilize T3SS often secrete and deliver multiple proteins that contribute to the processes of colonization, invasion, modulation of immune function, cytotoxicity, and tight junction disruption (Coburn et al., 2007). *C. jejuni* secretes ∼18 Cia proteins, and the majority have yet to be identified and characterized. It is likely that these proteins contribute to different stages of virulence. The results of this study demonstrate for the first time that CiaC and CiaI are delivered to the cytosol of host cells, and provide a framework for the dissection of *C. jejuni* pathogenesis.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX

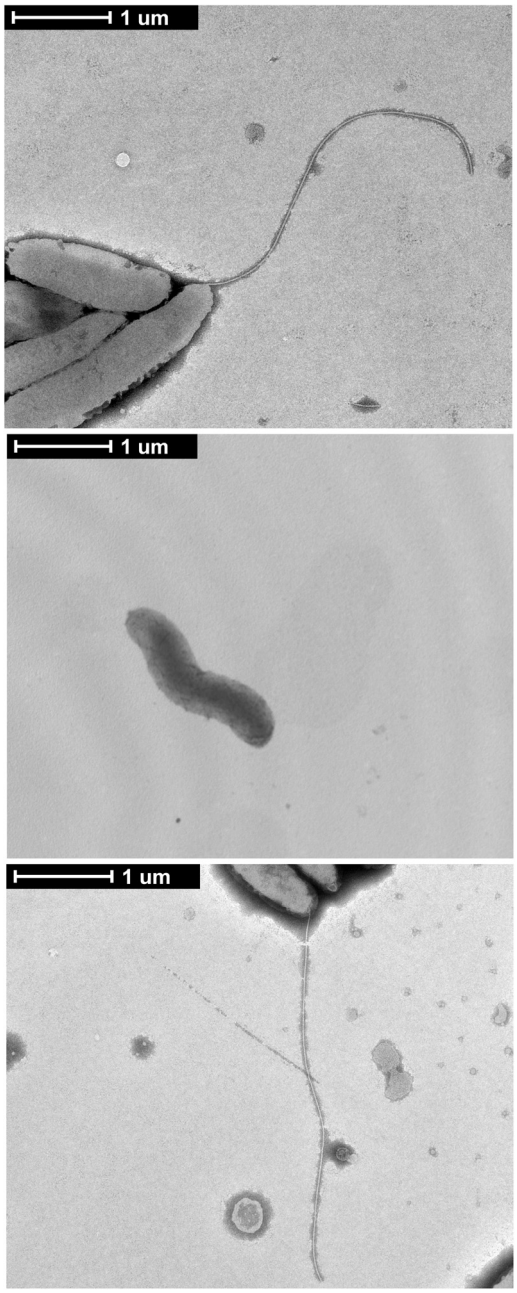

FIGURE A1 | Complementation of the *C. jejuni flgL* mutant with a plasmid harboring *flgL* restores the flagellar filament. *C. jejuni* wild-type strain, the *flgL* mutant, and *flgL* mutant harboring plasmid-encoded *flgL* were examined by TEM. *C. jejuni* were spotted onto formvar coated copper grids, stained with 2% phosphotungstic acid, and visualized at 6500× magnification. The scale bar represents one micron.

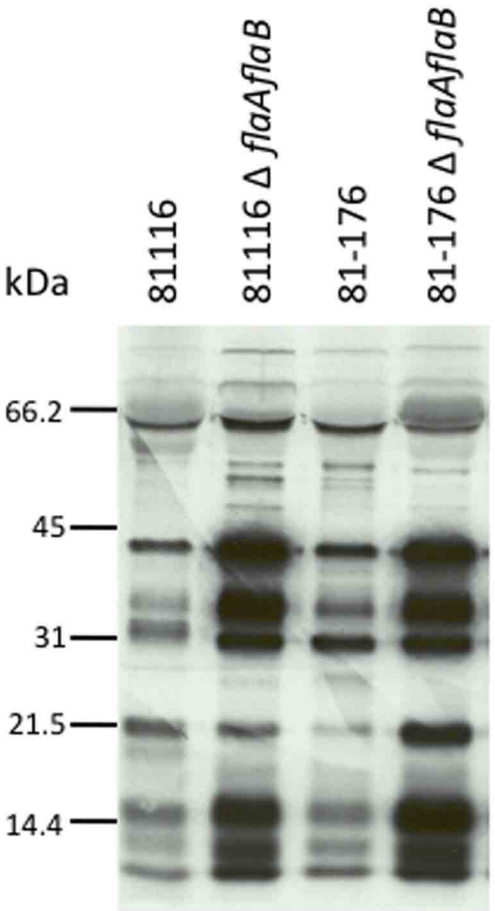

FIGURE A2 | *C. jejuni* 81116 and 81–176 *flaAflaB* mutants secrete the Cia proteins. Secretion of the Cia proteins from 81116 and 81–176 wild-type strains and isogenic *flaAflaB* mutants was stimulated by incubation with 1% FBS, and the secreted proteins were analyzed by autoradiograph.

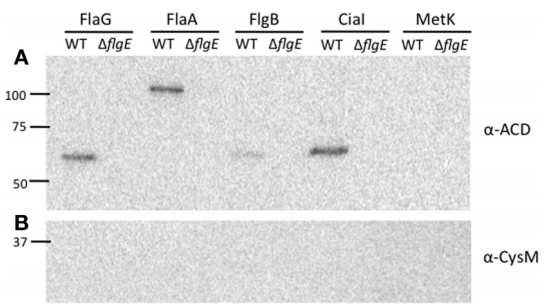

FIGURE A3 | FlaG, FlaA, FlgB, and CiaI are secreted through the flagellar T3SS. Secretion of the FlaG-, FlaA-, FlgB-, CiaI-, and MetK-ACD fusion proteins from the *C. jejuni* wild-type strain and *flgE* mutant was examined. The *C. jejuni* strains were transformed with the various ACD plasmids, and Cia secretion was stimulated by the addition of FBS. After 3 h, the proteins in the supernatant fractions were precipitated and an immunoblot was performed using an anti-ACD **(A)** and anti-CysM **(B)** antibodies. MetK-ACD was included as a negative control, as it is not secreted from *C. jejuni*. The molecular mass of the pre-stained protein standards are listed in kDa.

Table A1 | Primers used in this study.

Name	Sequence 5′–3′	Purpose
MEK2474	ATATACCGCGGTTAAGTTTATTTGTAATTCTCATTTTTTTCC	F38011 *flgL* suicide vector upstream fragment
MEK2475	ATATAGAATTCCAAGACTTAAAAGAAATGAAGGACG	F38011 *flgL* suicide vector upstream fragment
MEK2476	ATATACCGCGGTGCATATCAAGCTTCTCTTAAAGC	F38011 *flgL* suicide vector downstream fragment
MEK2477	ATATAGGTACCACGCAATAAAGCTTGGTTAATAGC	F38011 *flgL* suicide vector downstream fragment
MEK2478	ATATACCGCGGGTTCCAAAAATACCCATTATAAATCC	F38011 *flgK* suicide vector upstream fragment
MEK2479	ATATAGAGCTCCAAGAGTTTTAGAACAAGATATAGC	F38011 *flgK* suicide vector upstream fragment
MEK2480	ATATACCGCGGCTTACTAGGGCTTAAATCTTAAGC	F38011 *flgK* suicide vector downstream fragment
MEK2481	ATATACTCGAGCATTAGTTTTTAATACAAGTTTTGAGC	F38011 *flgK* suicide vector downstream fragment
MEK2466	ATATAGAGCTCCTATGATCCCATGTGCAAAACG	F38011 *flgE* suicide vector upstream fragment
MEK2467	ATATACCGCGGATATTGCTTTAAAAAATTTAACGCTTTGG	F38011 *flgE* suicide vector upstream fragment
MEK2468	ATATACCGCGGTAATCCAGCTTAAACAATAATTTAAGC	F38011 *flgE* suicide vector downstream fragment
MEK2470	ATATACTCGAGGTGGAGGTTGATTTCATTAATGC	F38011 *flgE* suicide vector downstream fragment
MEK1874	ATATAGAGCTCTTGTAGTGGAAAAATTTATCTTGTGG	81116 *flaAB* suicide vector upstream fragment
MEK1875	ATATACCGCGGATAATTTCAAACTCATCCATGAGC	81116 *flaAB* suicide vector upstream fragment
MEK1876	ATATACCGCGGTCAAGCTAACGCTGTACAGC	81116 *flaAB* suicide vector downstream fragment
MEK1877	ATATACTCGAGTAAAAACTTATCAAATTAATTTACATTCACC	81116 *flaAB* suicide vector downstream fragment
MEK1866	ATATAGAGCTCAAGAAAGAGTAAATTTACAACTTAGG	81-176 *flaAB* suicide vector upstream fragment
MEK1867	ATATACCGCGGAAATAATTTCAAACTCATCCATGAGC	81-176 *flaAB* suicide vector upstream fragment
MEK1868	ATATACCGCGGAAACTATTACAATAATCTTTCTAAAGAGC	81-176 *flaAB* suicide vector downstream fragment
MEK1869	ATATACTCGAGAATAATAATATAGCAGAGTTAATTTTTGG	81-176 *flaAB* suicide vector downstream fragment
MEK2066	AAACCGCGGTATTGCGAAAACGATGAAGAAC	Tetracycline cassette amplification
MEK2074	AAACCGCGGGTCTCTAATCCTTTCAAATCTC	Tetracycline cassette amplification
MEK3030	ATATACTCGAGTATATCAAGAAATAGGTATTAACAAGC	*flgL* mutant complementation
MEK3031	ATATAGAATTCGAAAATAAAATCAAAGCCAAATTATACC	*flgL* mutant complementation
MEK2319	ATATAGATCTCAGCAATCGCATCAGGCTG	Adenylate cyclase domain amplification
MEK2382	ATATGGTACCTTATCCATAGCCGGAATCCTG	Adenylate cyclase domain amplification
MEK2599	CCGCTCGAGCTGCAGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGG	ACD vector inverse PCR
MEK2629	ATACTGCAGCTCGAGGGATCCGAATTCAGATCTCAGCAATCGCATCAG	ACD vector inverse PCR
MEK2913	ATATGAGCTCTTCATTTGCTCCTTGCTTGCC	*ciaC* ACD fusion construct
MEK2620	ATATAGAATTCCTTTGTATTGTTTGTGGCATTTCC	*ciaC* ACD fusion construct
MEK2740	ATATGAGCTCTTCATTTGCTCCTTGCTTGCC	*ciaI* ACD fusion construct
MEK2666	ATATAGAATTCGGCGTAAAGATTTAAACTATCATC	*ciaI* ACD fusion construct
MEK2917	ATATAGAGCTCAAAGATTTTTAGACGATGCTTTAGC	*flaA* ACD fusion construct
MEK2918	ATATAGAATTCTAAAACATTTTGTTGAACAGAATTAGC	*flaA* ACD fusion construct
MEK2919	ATATAGAGCTCATATTTTTATCAAAGAAGAAAGACTTGG	*flaG* ACD fusion construct
MEK2920	ATATAGAATTCACTCTCCTTATCAAATATCATTCC	*flaG* ACD fusion construct
MEK2921	ATATAGAGCTCGGTATGAAACTTCAAATGGATGG	*flgB* ACD fusion construct
MEK2922	ATATAGAATTCAGAGCTTGCATCAAGTATAGAAC	*flgB* ACD fusion construct
MEK2931	ATATAGAGCTCAACAAGCATAGGAGCGATAGC	*metK* ACD fusion construct
MEK2932	ATATAGAATTCTAGCTTTTTAAACTGTTCTAGAGC	metK ACD fusion construct

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Major host factors involved in epithelial cell invasion of *Campylobacter jejuni*: role of fibronectin, integrin beta1, FAK, Tiam-1, and DOCK180 in activating Rho GTPase Rac1

Manja Boehm[1,2†], Malgorzata Krause-Gruszczynska[2†], Manfred Rohde[3], Nicole Tegtmeyer[1], Seiichiro Takahashi[4], Omar A. Oyarzabal[5] and Steffen Backert[1,2]*

[1] School for Biomedical and Biomolecular Science, Belfield Campus, University College Dublin, Dublin, Ireland
[2] Department of Microbiology, Otto von Guericke University, Magdeburg, Germany
[3] Department of Medical Microbiology, Helmholtz Center for Infection Research, Braunschweig, Germany
[4] Department of Molecular Medicine, Max-Planck-Institute for Biochemistry, Martinsried, Germany
[5] Department of Biological Sciences, Alabama State University, Montgomery, AL, USA

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Stefan Bereswill, Charité-University Medicine Berlin, Germany
Christof R. Hauck, Universitaet Konstanz, Germany
Nick Dorrell, London School of Hygiene and Tropical Medicine, UK

***Correspondence:**
Steffen Backert, School of Biomolecular and Biomedical Sciences, University College Dublin, Ardmore House, Belfield Campus, Dublin, Ireland.
e-mail: steffen.backert@ucd.ie

†Manja Boehm and Malgorzata Krause-Gruszczynska have contributed equally to this work.

Host cell entry by the food-borne pathogen *Campylobacter jejuni* has been reported as one of the primary reasons of tissue damage in infected humans, however, molecular invasion mechanisms and cellular factors involved in this process are widely unclear. Here we used knockout cell lines derived from fibronectin$^{-/-}$, integrin beta1$^{-/-}$, and focal adhesion kinase (FAK)$^{-/-}$ deficient mice and corresponding wild-type (WT) controls, to study *C. jejuni*-induced signaling cascades involved in the bacterial invasion process. Using high resolution scanning electron microscopy, GTPase pull-downs, G-LISA, and gentamicin protection assays we found that each of these host cell factors is indeed required for activation of the small Rho GTPase member Rac1 and maximal host cell invasion of this pathogen. Interestingly, membrane ruffling, tight engulfment of bacteria and invasion were only seen during infection of WT control cells, but not in fibronectin$^{-/-}$, integrin beta1$^{-/-}$, and FAK$^{-/-}$ knockout cell lines. We also demonstrate that *C. jejuni* activates FAK autophosphorylation activity at Y-397 and phosphorylation of Y-925, which is required for stimulating two downstream guanine exchange factors, DOCK180 and Tiam-1, which are upstream of Rac1. Small interfering (si) RNA studies further show that DOCK180 and Tiam-1 act cooperatively to trigger Rac1 activation and *C. jejuni* invasion. Moreover, mutagenesis data indicate that the bacterial fibronectin-binding protein CadF and the intact flagellum are involved in Rho GTPase activation and host cell invasion. Collectively, our results suggest that *C. jejuni* infection of host epithelial target cells hijacks a major fibronectin → integrin beta1 → FAK → DOCK180/Tiam-1 signaling cascade, which has a crucial role for Rac1 GTPase activity and bacterial entry into host target cells.

Keywords: Rho family GTPases, molecular pathogenesis, cellular invasion, signaling, virulence

INTRODUCTION

Despite the sophisticated human immune system, some microbial food-borne pathogens have co-evolved with their hosts during evolution to overcome protective cellular barriers and to establish short- or long-term infections in the gut. *Salmonella*, *Campylobacter*, *Escherichia*, *Shigella*, *Listeria*, and other bacteria as well as some parasites and enteric viruses are the most common food-borne pathogens (Fang et al., 1991; Salyers and Whitt, 1994; Sougioultzis and Pothoulakis, 2003; Eckmann and Kagnoff, 2005; Lamps, 2007). Importantly, infections with these organisms represent one of the leading causes of morbidity and death in the human population worldwide. Reports by the World Health Organization (WHO) revealed that humans suffer from 4.5 billion incidences of diarrhea causing about 1.8 million deaths annually (World Health Organization, 2004). These infections are especially problematic in infants, young children, or immunocompromised patients, while the majority of enteric infections in healthy adults seem to be self-limiting. Upon ingestion, such pathogens commonly pass through the stomach in sufficient numbers in order to infect the small intestine or colon. To establish and maintain a successful infection in the gastrointestinal tract, microbial pathogens have evolved various strategies to invade tissues, avoid or resist the innate immunity, disturb the normal gut flora, damage the cells, and multiply in specific niches (Thanassi and Hultgren, 2000; Burns et al., 2003; Alouf and Popoff, 2005). Interestingly, most but not all food-borne bacterial pathogens can be classified with invasive phenotypes, which are able to induce their own uptake into epithelial cells that are normally non-phagocytic. According to specific features of the invasion process, we distinguish between the "zipper"- and "trigger"-mechanism, respectively (Cossart and Sansonetti, 2004). Whereas the "zipper"-mechanism is initiated by a bacterial surface protein (commonly by an adhesin) which binds to a specific host cell receptor followed by internalization of the bacterium, the "trigger"-mechanism involves injected bacterial

factors by type III/IV secretion systems which often mimic or highjack specific host cell factors to trigger the bacterial uptake process.

Campylobacter infections of the human gastrointestinal tract have been recognized as the leading causes of enteric bacterial infection (Nachamkin et al., 2008). They may be responsible for as many as 400–500 million bacterial gastroenteritis cases per year worldwide (Friedman et al., 2000). Statistical data indicate that *Campylobacter* infections of humans cause considerable use of medication and health service burden. In the USA, it has been estimated that *Campylobacter*-associated illnesses cost up to 6.2 billion dollar per year (Forsythe, 2000). Remarkably, in many studies in the USA and other industrialized countries, *Campylobacters* were found to cause diarrheal disease more than 2–7 times as frequently as *Salmonella* and *Shigella* species or pathogenic *E. coli* (Allos, 2001; Tam, 2001). In particular, two *Campylobacter* species, *Campylobacter jejuni* and *C. coli*, are most frequently isolated from infected humans. *C. jejuni* is a typical zoonotic pathogen as it can be found as part of the normal intestinal flora in numerous mammals and birds. Thus, *C. jejuni* can contaminate poultry, beef, veal, pork, water, and milk during food processing, and is mainly transmitted by the fecal–oral route (Potturi-Venkata et al., 2007). *Campylobacters* remain highly motile in the intestinal mucus, and their microaerophilic nature ensures its survival in the mucus layer. As a consequence of infection, the bacteria colonize the ileum and colon, where they can interfere with the normal functions of the gastrointestinal tract. This may cause some intestinal diseases typically associated with fever, malaise, abdominal pain, and watery diarrhea, often containing blood cells (Wassenaar and Blaser, 1999; Poly and Guerry, 2008). In addition, individuals exposed to *C. jejuni* may develop postinfection sequelae including Reiter's reactive arthritis or peripheral neuropathies including Miller–Fisher and Guillain–Barrè syndromes (Blaser and Engberg, 2008). Accumulating research activities over the last few years indicated that *C. jejuni* perturbs the normal absorptive capacity of the intestine by damaging epithelial cell functions either directly by cell invasion and/or the production of toxin(s), and indirectly by triggering inflammatory responses (Ketley, 1997; Wooldridge and Ketley, 1997).

Early reports of intestinal biopsies from patients and *in vitro* infection of cultured human intestinal epithelial cell lines have shown that *C. jejuni* is able to enter gut tissue cells (van Spreeuwel et al., 1985; Oelschlaeger et al., 1993; Wooldridge et al., 1996). Numerous studies indicated that *C. jejuni* encode a variety of adhesins including CadF, FlpA, JlpA, and PEB1 (Pei et al., 1998; Konkel et al., 2001; Poly and Guerry, 2008). For example, CadF and FlpA are well-characterized bacterial outer membrane proteins which bind fibronectin, an important extracellular matrix protein and bridging molecule to integrin receptors (Moser et al., 1997; Konkel et al., 2010). It has been postulated that host cell invasion by *C. jejuni* is one of the main reasons for tissue damage, and this process may proceed in a microtubule-dependent and/or actin-dependent fashion (Oelschlaeger et al., 1993; Hu and Kopecko, 1999; Biswas et al., 2004). Infection with *C. jejuni* triggers membrane ruffling in infected INT-407 intestinal epithelial cells followed by its entry exhibiting features of both the trigger and zipper mechanisms (Krause-Gruszczynska et al., 2007). Maximal adherence and invasion of INT-407 cells by *C. jejuni* requires CadF and is accompanied with increased levels of tyrosine phosphorylation of some host cell proteins (Biswas et al., 2004; Hu et al., 2006) such as the integrin-associated protein paxillin (Monteville et al., 2003), but the importance of these observations for the invasion process are unknown. Interestingly, CadF maybe also involved in the activation of the small Rho GTPases Rac1 and Cdc42, but the exact mechanism remained unclear (Krause-Gruszczynska et al., 2007). In addition, it has been shown that mutation of genes in the flagellar export system and *ciaB* (*Campylobacter* invasion antigen B), as well as deletion of *kpsS* and *waaF* genes, playing a role in the biosynthesis of capsular polysaccharide and lipooligosaccharide, respectively, resulted in reduced bacterial adhesion and invasion *in vitro*, indicating that these proteins could also play roles in host cell invasion (Karlyshev et al., 2000; Kanipes et al., 2004; Konkel et al., 2004; Guerry, 2007; Hu and Kopecko, 2008; Larson et al., 2008). However, some of these data are conflicting in the literature. For example, it is still unclear if the role of the flagellum during invasion is restricted to bacterial motility or secretion of bacterial factors into the medium; especially the role of secreted CiaB in invasion was called into question (Novik et al., 2010).

Pharmacological inhibitor studies have indicated that host heterotrimeric G proteins and certain protein kinases [EGF- and PDGF-receptor, phosphatidylinositol 3-kinase (PI3-K), and others] are also involved in epithelial cell entry of *C. jejuni* (Wooldridge et al., 1996; Hu et al., 2006; Watson and Galán, 2008). In addition, expression of dominant-negative mutants of caveolin-1 but not dynamin-II significantly decreased *C. jejuni* internalization suggesting that caveolin-1 or caveolae may also play a role in the uptake process (Watson and Galán, 2008). Once internalized in epithelial cells, *C. jejuni* co-localize with microtubules (Hu and Kopecko, 1999), they can survive for considerable time and consequently induce a cytotoxic response *in vitro* (Konkel et al., 1992; Day et al., 2000). The *C. jejuni*-containing intracellular vacuole deviates from the canonical endocytic pathway, and thus may avoid delivery into lysosomes and subsequent bacterial killing (Watson and Galán, 2008). Intracellular survival of *C. jejuni* may support the ability to evade host immune responses, causes relapse of the acute infection, and may establish long-term persistent infections (Lastovica, 1996; Day et al., 2000; Hofreuter et al., 2008). However, the molecular mechanism of early *C. jejuni* host cell invasion as well as the complex interplay of different bacterial and host factors at the pathogen–host cell interface is still not clear. Here we show that the activation of small Rho GTPase Rac1 is a major pathway in *C. jejuni* invasion. Using mouse knockout cell lines in Rac1 activation screens, invasion assays as well as scanning electron microscopy, we demonstrate a crucial role of fibronectin, integrin β1, focal adhesion kinase (FAK), and two guanine exchange factors, Tiam-1 (T-lymphoma invasion and metastasis gene-1) and DOCK180 (180-kDa dedicator of cytokinesis 1), in activating Rac1 and the induction of membrane ruffling during *C. jejuni* infection. Using *C. jejuni* knockout mutants we further investigated the potential role of the fibronectin-binding protein CadF and an intact flagellum in this process.

MATERIALS AND METHODS

BACTERIAL STRAINS

The *C. jejuni* strains 81–176, 84–25, and F38011 were used in this study. The isogenic F38011Δ*cadF*, 81–176Δ*cadF*, 81–176Δ*flaA/B*, and 81–176Δ*flhA* mutants were kindly provided by Konkel et al. (1997) and Patricia Guerry (Goon et al., 2006), respectively. All *C. jejuni* strains were grown on Campylobacter blood-free selective Agar Base (Oxoid) containing Campylobacter growth supplement (Oxoid) or, when appropriate, on Mueller–Hinton (MH) agar amended with 50 μg/ml kanamycin or 30 μg/ml or chloramphenicol at 37°C under microaerobic conditions (generated by CampyGen, Oxoid) for 48 h.

KNOCKOUT FIBROBLASTS AND OTHER CELL LINES

Human embryonic intestinal epithelial cells (INT-407), obtained from the American Type Culture Collection (ATCC CCL-6), were grown in Eagle's minimum essential medium (MEM) containing L-glutamine and Earle's salts (Gibco). Several mouse fibroblast knockout cell lines were cultured in RPMI1640 or DMEM medium, respectively, which were supplemented with 10% fetal calf serum (Gibco). Generation of the floxed $FN^{+/+}$ mouse fibroblast cells and $FN^{-/-}$ knockout cells has been described (Nyberg et al., 2004; Schroeder et al., 2006). The $FN^{-/-}$ cells were grown in DMEM supplemented with 10% FCS or alternatively, in serum replacement medium for bacterial infection studies (Sigma Aldrich). Monolayers of GD25 mouse fibroblasts (integrin beta1$^{-/-}$) or GD25 cells stably transfected with integrin β1A (GD25β1A) were grown in 10% fetal bovine serum (Wennerberg et al., 1996). Mouse knockout cells deficient in ($FAK^{-/-}$ cells) as well as stable expression of WT FAK in $FAK^{-/-}$ cells were described previously (Sieg et al., 1999). After reaching a confluency of about 70%, the cells were washed two times with phosphate-buffered saline (PBS), and then starved for 12 h.

INFECTION STUDIES

For the infection assays, the different cell lines were seeded to give 4×10^5 cells in 12-well tissue culture plates. The culture medium was replaced with fresh MEM without antibiotics 1 h before infection. Bacteria were suspended in (PBS, pH 7.4), added to the cells at a multiplicity of infection (MOI) of 100 and co-incubated with host cells for the indicated periods of time per experiment.

INHIBITOR STUDIES

The pharmacological inhibitors methyl-beta cyclodextrin (MβCD, Sigma, 1–10 mM) and PF-573228 (Tocris; 10 μM) were added 30 min prior to infection and kept throughout the duration of the incubation period. All control cells were treated with the corresponding solvent for the same length of time. We have carefully checked the viability of cells in every experiment to exclude toxic effects resulting in loss of host cells from the monolayers. The experiments were repeated at least three times with similar results.

PLASMID DNA TRANSFECTION

The pcDNA3.1-FAK WT and corresponding Y397F, K454R, ΔPR1/ΔPR2, and Y925F mutants were kindly provided by the lab of D. Schlaepfer (Sieg et al., 1999). Transfection of plasmid constructs was performed using GeneJammer transfection reagent according to manufacturer's instructions (Stratagene). After 48 h, transfected INT-407 cells were infected with *C. jejuni* strains. All FAK constructs were expressed as C-terminal HA-tag fusion proteins as described (Sieg et al., 1999). The efficiency of transfection was verified both by immunofluorescence microscopy and Western blotting using an α-HA antibody (NEB).

siRNA TRANSFECTION

siRNA(s) directed against human DOCK180, Trio and control siRNA containing scrambled sequence were purchased from Santa Cruz. siRNAs targeted against human Rac1 and RhoA were synthesized as follows. Rac1 target sequence (5′-AAAACTTGCCTACTGATCAGT-3′), RhoA target sequence (5′-TACCTTATAGTTACTGTGTAA-3′) were utilized. For down-regulation of Tiam-1 both the siRNA from Santa Cruz and another one obtained from MWG-Biotech [Tiam-1 target sequence (5′-ACAGCTTCAGAAGCCTGAC-3′)], were used simultaneously. Transfection of siRNAs was performed using siRNA Transfection Reagent as recommended by the manufacturer (Santa Cruz).

GENTAMICIN PROTECTION ASSAY

Infection of eukaryotic cells at a density of 4×10^5 cells per well was carried out as described above. After infection, the cells were washed three times with 1 ml of pre-warmed MEM medium per well to remove non-adherent bacteria. To determine the colony-forming units (CFU) corresponding to intracellular bacteria, the INT-407 cell monolayers were treated with 250 μg/ml gentamicin (Sigma) at 37°C for 2 h, washed three times with medium, and then incubated with 1 ml of 0.1% (w/v) saponin (Sigma) in PBS at 37°C for 15 min. The treated monolayers were resuspended thoroughly, diluted, and plated on MH agar. To determine the total CFU corresponding to host-associated bacteria, the infected monolayers were incubated with 1 ml of 0.1% (w/v) saponin in PBS at 37°C for 15 min without prior treatment with gentamicin. The resulting suspensions were diluted and plated as described above. For each strain, the level of bacterial adhesion and uptake was determined by calculating the number of CFU. All experiments were routinely performed in triplicates. In parallel control experiments, 250 μg/ml gentamicin killed all extracellular bacteria (data not shown).

Rac1-GTP PULL-DOWN ASSAY

Rac1 activation in infected cells was determined with the Rac1 activation assay kit (Cytoskeleton), based on a pull-down assay using the Cdc42–Rac1 interactive binding domain of PAK1 fused to glutathione S-transferase, GST–CRIB (Sander et al., 1998). Briefly, host cells were grown to 70% confluency and serum-starved overnight. Subsequently, cells were incubated in PBS (pH 7.4) as a control or infected with *C. jejuni* (MOI of 100) in a time course. Uninfected and infected host cells were washed with PBS, resuspended in the assay buffer of the kit, and detached from dishes with a cell scraper. For a positive and negative control, a portion of the uninfected cell lysate was mixed with GTPγ-S and GDP for 15 min, respectively. Cell lysates (treated with bacteria, GTPγ-S, GDP or untreated) were mixed with the PAK–RBD slurry (1 h, 4°C). Finally, the beads were collected by centrifugation and washed three times with assay buffer. Activated Rac1 was then visualized by immunoblotting as described below. To confirm equal

amounts of protein for each sample, aliquots of the lysates from different time points were also analyzed by immunoblotting. The GTPase activities were quantitated as band intensities representing the relative amount of active Rac1-GTP using the Lumi-Imager F1 software program (Roche).

G-LISA ASSAY

Rac1 activation in infected cells was also determined by a second approach, the G-LISA™ Rac1-activation assay (Cytoskeleton). Host cells were grown to 70% confluency in tissue culture petri dishes and serum depleted overnight. The cells were infected with *C. jejuni* for the indicated times per experiment. Subsequently, cells were washed with PBS, resuspended in lysis buffer of the kit and harvested from the dishes with cell scraper. Total protein concentration in each lysate was determined by protein assay reagent of the kit. The G-LISA's contains a Rac1-GTP-binding protein immobilized on provided microplates. Bound active Rac1 was detected with a specific antibody and luminescence. The luminescence signal was quantified by using a microplate reader (SpectraFluor Plus, Tecan).

GENERATION OF THE POLYCLONAL α-FLAGELLIN AND α-MOMP ANTIBODIES

Polyclonal antiserum (α-FlaA and α-MOMP) was raised according to standard protocols (Biogenes GmbH) by immunization of two rabbits with a conserved FlaA-derived peptide (corresponding to amino acids 93–106: KTKATQAAQDGQSL) or a MOMP-derived peptide (amino acids 400–413: NLDQGVNTNESADH), which were conjugated to *Limulus polyphemus* hemocyanin carrier protein. The resulting antisera were affinity-purified and the specificity against their antigens was confirmed by Western blotting.

MOTILITY ASSAY

Motility phenotypes of strains were tested in MH media containing 0.4% agar. Bacterial cells were harvested from a 36 h culture on conventional agar plates and resuspended in PBS to obtain an optical density at 600 nm of 0.45 (approximately 1×10^9 cfu/ml). The bacteria were incubated for 30 min in the presence or absence of 20 μg/ml α-FlaA antibody or pre-immune serum as control. To ensure that equal amounts of antibody were present on the entire agar surface in the α-FlaA sample, 50 μl of the antibody solutions were plated onto the corresponding plates. Subsequently, 2 μl of a bacterial suspension of 2×10^8 cfu/ml (±antibody) were stabbed into motility agar. Plates were incubated at 37°C under microaerophilic conditions for 24 h, followed by measuring the diameter of the resulting swarms. The results were the mean of at least five separate measurements from three experiments.

SDS-PAGE AND IMMUNOBLOTTING

Proteins from transfected and/or infected host cells were separated on 10–15% polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Staining with primary antibodies against Rac1 (Upstate), FAK-PY-397 (Biomol), FAK-PY-925 (NEB), FAK, RhoA, fibronectin, β1 integrin, Tiam-1, DOCK180, or GAPDH (all Santa Cruz) was performed according to the manufacturer's instructions. As a secondary antibody, horseradish peroxidase-conjugated α-mouse, α-rabbit, or α-goat IgG was used (DAKO). Immunoreactive bands were visualized by ECL plus Western Blotting Detection System (Amersham Biosciences). Relative FAK kinase activities were quantitated as band intensities of the FAK-PY397 signals related to its FAK control blot using the Lumi-Imager F1 software program (Roche).

FESEM (FIELD EMISSION SCANNING ELECTRON MICROSCOPY)

Host cell monolayers grown on coverslips were infected with *C. jejuni* strains for either 4 or 6 h, then fixed in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose; pH 6.9) containing 5% formaldehyde and 2% glutaraldehyde, and subsequently washed several times with cacodylate buffer. Samples were dehydrated with a graded series of acetone (10, 30, 50, 70, 90, and 100%) on ice for 15 min for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change of 100% acetone. Samples were then subjected to critical-point drying with liquid CO_2 (CPD030, Bal-Tec, Liechtenstein). Dried samples were covered with a 10 nm thick gold film by sputter coating (SCD040, Bal-Tec, Liechtenstein) before examination in a field emission scanning electron microscope (Zeiss DSM-982-Gemini) using the Everhart Thornley SE detector and the inlens detector in a 50:50 ratio at an acceleration voltage of 5 kV.

STATISTICAL ANALYSIS

All data were evaluated using Student's *t*-test with SigmaStat statistical software (version 2.0). Statistical significance was defined by $^*p \leq 0.05$ and $^{**}p \leq 0.005$. All error bars shown in figures and those quoted following the ± signs represent SD.

RESULTS

CAMPYLOBACTER JEJUNI INVASION IS TIME-DEPENDENT AND REQUIRES Rac1, FIBRONECTIN, INTEGRINS AND FAK

In our previous studies we have shown that small Rho GTPases such as Rac1 and Cdc42 are activated by *C. jejuni*, and inhibitors as well the expression of dominant-negative constructs have indicated that active Rac1 maybe one important host factor required for bacterial entry into host cells (Krause-Gruszczynska et al., 2007). Here we aimed to identify the signaling pathway leading to Rac1 activation. First, we confirmed that Rac1 is activated in infected non-phagocytic intestinal epithelial cells (INT-407) using a novel commercial assay, called G-LISA (see Materials and Methods). The results show that wild-type (WT) *C. jejuni* strains 81–176, 84–25, or F38011 induced the generation of active Rac1-GTP during the course of infection (**Figure 1A** and data not shown). To confirm that Rac1 is indeed necessary for *C. jejuni* invasion, we downregulated Rac1 expression by siRNA. Down-regulation of Rac1 expression by about 90% lead to a significant drop in the number of intracellular bacteria as determined by gentamicin protection assays (**Figure 1B**). As controls, a scrambled siRNA or siRNA against RhoA, another Rho GTPase member, did not suppress *C. jejuni* invasion (**Figures 1B,C**). These results indicate that penetration of *C. jejuni* into cultured cells specifically requires Rac1.

Previous studies have shown that addition of methyl-beta cyclodextrin (MβCD), an agent that sequesters cholesterol,

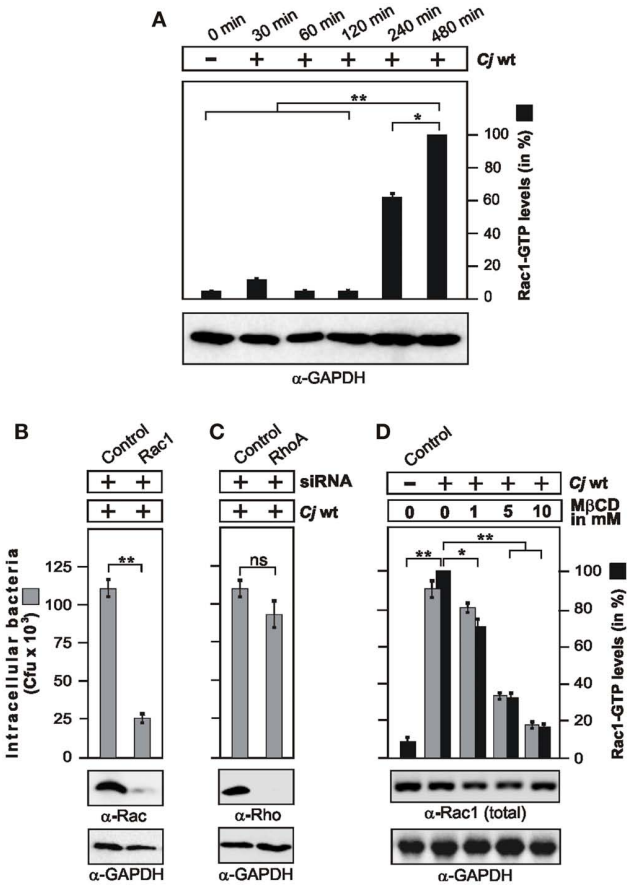

FIGURE 1 | *Campylobacter jejuni* invasion is time-dependent, and requires Rac1 and lipid rafts. (A) Quantification of Rac1 activity during the course of infection. INT-407 cells were infected with WT *C. jejuni* 81–176 for indicated periods of time. The presence of active Rac1-GTP was quantified by G-LISA. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level. Similar amount of cells at every time point was confirmed by α-GAPDH Western blotting. **(B,C)** Effect of Rac1 and RhoA expression knockdown on *C. jejuni* invasion. INT-407 cells were transfected with Rac1-siRNA **(B)** or RhoA-siRNA **(C)**, as well as a scrambled siRNA as control. After 48 h, cells were infected with *C. jejuni* for 6 h. Intracellular bacteria were quantified by gentamicin protection assays. Immunoblotting with α-Rac1 or α-RhoA antibodies confirmed down-regulation of the proteins. GAPDH expression levels were determined as control. **(D)** Effects of MβCD targeting lipid rafts on host cell internalization of *C. jejuni*. INT-407 monolayers were pre-incubated with the indicated concentrations of MβCD for 30 min, followed by 6 h infection with *C. jejuni*. Total intracellular *C. jejuni* were quantified by gentamicin protection assays. The presence of active Rac1-GTP was analyzed by CRIB–GST pull-down and quantified. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level (right lane). Similar quantities of total Rac1 and GAPDH were confirmed by Western blotting. $^{*}p < 0.05$ and $^{**}p \leq 0.005$ were considered as statistically significant as compared to the control.

decreased the ability of *C. jejuni* to enter several cultured epithelial cell lines, suggesting that lipid rafts may be required for efficient host cell invasion (Watson and Galán, 2008). Thus, we investigated if intact lipid rafts may be required for *C. jejuni*-triggered Rac1 activation. Indeed, addition of MβCD blocked *C. jejuni*-induced Rac1 activation and internalization into INT-407 cells in a dose-dependent manner (**Figure 1D**), suggesting that a lipid raft-associated host receptor maybe targeted by *C. jejuni* to trigger downstream signaling leading to Rac1 activation. Since *C. jejuni* encodes CadF, an outer membrane protein binding to the extracellular matrix protein fibronectin (Konkel et al., 2001), we proposed that a classical fibronectin → integrin-β1 → FAK pathway could be involved in activating Rac1. To investigate this question, we utilized fibroblast cell lines derived from fibronectin$^{-/-}$, integrin-β1$^{-/-}$ (so called GD25 cells) and FAK$^{-/-}$ knockout mice (Wennerberg et al., 1996; Sieg et al., 1999; Nyberg et al., 2004), which have the advantage that the cells are completely devoid of expressing the proteins of interest (**Figures 2A–C**). As controls, we infected in parallel experiments floxed fibronectin$^{+/+}$ cells, GD25 cells re-expressing WT integrin-β1A (GD25β1A) and FAK$^{-/-}$ cells re-expressing WT FAK. Infection studies showed that WT *C. jejuni* can effectively enter the control cells, while the knockout cells exhibited significant deficiency for bacterial uptake (**Figures 2A–C**). In addition, transient transfection of FAK$^{-/-}$ knockout cells with HA-tagged WT FAK restored the capability of *C. jejuni* to enter these cells, while expression of FAK mutants that were either not capable of autophosphorylation (FAK Y397F), impaired kinase activity (FAK K454R), lacking several proline residues in two PxxP-motifs (FAKΔPR1/2) necessary for association with SH3-containing proteins such as p130CAS or Graf, or lacking the Grb2-binding site (FAK Y925F; Sieg et al., 1999; Hauck et al., 2002), significantly reduced the internalization of *C. jejuni* ($p \leq 0.001$) by about 35–50% (**Figure 2D**). This indicates an important role of FAK signaling downstream of fibronectin and integrins in facilitating efficient uptake of *C. jejuni*.

CAMPYLOBACTER JEJUNI INDUCES PROFOUND MEMBRANE RUFFLING AND INVASION IN WT CELLS BUT NOT IN FIBRONECTIN, INTEGRIN, AND FAK KNOCKOUT CELLS

Next we infected the WT fibroblasts and their corresponding fibronectin$^{-/-}$, GD25, and FAK$^{-/-}$ knockout cell lines and analyzed the interaction of *C. jejuni* with the surface of host cells by high resolution FESEM. First, we confirmed that the WT *C. jejuni* strains had single bipolar flagella (**Figure 3A**, white arrows). After infection, the micrographs revealed that the bacteria were able to attach to the host cell surface, followed by cellular invasion which was observed predominantly after 4–6 h of infection (**Figure 3B**). Interestingly, similar to our earlier observations with infected INT-407 cells (Krause-Gruszczynska et al., 2007), we found that *C. jejuni* entered the WT fibroblast cells in a very specific fashion, first with its leading flagellar tip followed by the opposite flagellar end (**Figure 3B**, yellow arrows). Tight engulfment of the bacteria and membrane ruffles (red arrows), filopodia-like structures (blue arrows) as well as the appearance of elongated microspikes (green arrowheads) were also regularly observed. In contrast, infection of fibronectin$^{-/-}$, GD25 and FAK$^{-/-}$ knockout cell lines also revealed bound bacteria at the surface of the cells with short microspikes present, but no membrane ruffles or invading *C. jejuni* could be detected (**Figure 3C**). The predominant observation of membrane ruffling in WT cells confirms the typical occurrence of Rac1 GTPase

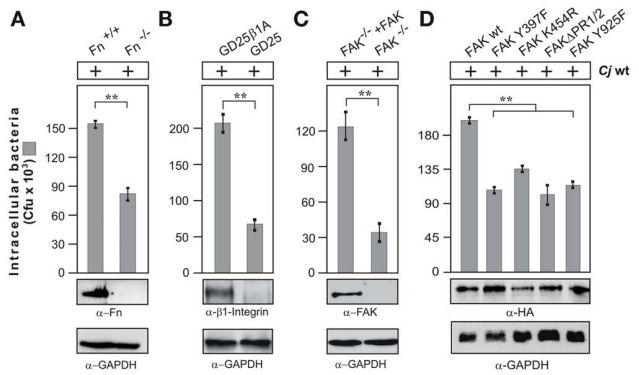

FIGURE 2 | Importance of fibronectin, integrin β1, and FAK expression on *C. jejuni* invasion. The following cells lines were infected with WT *C. jejuni* 81–176 for 6 h. **(A)** Fibronectin-deficient cells ($Fn^{-/-}$) and corresponding floxed WT cells ($Fn^{+/+}$), **(B)** integrin β1-deficient cells (GD25) and GD25 stably re-expressing WT integrin β1A (GD25-β1A) cells, and **(C)** FAK-deficient cells ($FAK^{-/-}$) and $FAK^{-/-}$ cells stably re-expressing WT FAK. **(D)** $FAK^{-/-}$ cells transiently transfected with the indicated HA-tagged FAK constructs. Intracellular *Campylobacter* cells were quantified by gentamicin protection assays. $^{**}p \leq 0.005$ was considered as statistically significant. Fibronectin, integrin β1, and FAK expression was analyzed by immunoblotting. Expression of the individual FAK constructs was verified using α-HA antibody. GAPDH expression levels were determined as loading control.

activation followed by dynamic membrane rearrangements during the *C. jejuni* invasion process, and this depends on the expression of three crucial host cell factors, fibronectin, integrin β1, and FAK.

WT BUT NOT CadF MUTANT *C. JEJUNI* INDUCE PROFOUND FAK ACTIVATION

Focal adhesion kinase is an intracellular non-receptor tyrosine kinase and important modulator of integrin-dependent focal cell contacts, thereby orchestrating well-known integrin-initiated outside-in signaling events (Sieg et al., 1999; Hauck et al., 2002). FAK localizes with β1-integrins and becomes activated by autophosphorylation at position Y-397, and downstream signaling can be transmitted through subsequent phosphorylation of FAK at Y-925. The next experiment was therefore to examine whether *C. jejuni* can stimulate the autophosphorylation of FAK. For this purpose, FAK WT cells were infected with WT *C. jejuni* for different time periods and cell lysates were analyzed with an antibody specific for FAK phosphorylated at position Y-397. Quantification data show that *C. jejuni*-induced FAK autophosphorylation profoundly, and this correlated with increasing amounts of intracellular bacteria over time (**Figure 4A**). In addition, *C. jejuni*-induced the phosphorylation of FAK at Y-925 in a time-dependent manner (**Figure 4B**). To investigate the potential contribution of the CadF protein for FAK phosphorylation events, the effect of an isogenic Δ*cadF* mutant was examined under identical conditions. The results show that FAK autophosphorylation was widely impaired in infections with the Δ*cadF* mutant (**Figures 4A,B**). These findings suggest that expression of CadF maybe involved in *C. jejuni*-induced FAK kinase activity.

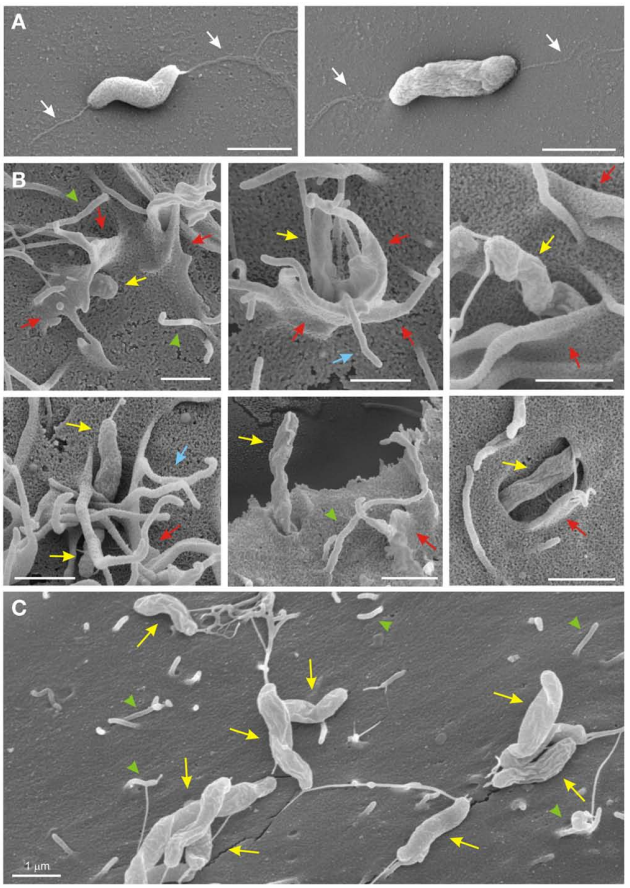

FIGURE 3 | High resolution field emission scanning electron microscopy of *C. jejuni* invasion. (A) *C. jejuni* strains have single bipolar flagella (white arrows). **(B)** *C. jejuni* 81–176 infected for 4–6 h were able to induce their entry into the WT fibroblast target cells and were regularly associated with membrane ruffles (red arrows), filopodia-like structures (blue arrows) as well as elongated microspikes (green arrowheads). Invading bacteria were also marked (yellow arrows). **(C)** Infection of GD25 knockout cells with WT *C. jejuni* 81–176 for 6 h also revealed bacterial attachment, but membrane dynamics or invasion were not induced. Similar results were observed for the infected Fibronectin-deficient and FAK-deficient cells (data not shown).

INDUCTION OF MAXIMAL Rac1-GTP LEVELS REQUIRES THE cad*F* GENE AND IS STRONGLY IMPAIRED IN $FAK^{-/-}$ KNOCKOUT CELLS

Next we aimed to investigate if FAK is required for *C. jejuni*-induced Rac1 activation. For this purpose, $FAK^{-/-}$ knockout cells and $FAK^{-/-}$ cells re-expressing FAK were infected under the same conditions as shown in **Figures 4A,B**, followed by CRIB–GST pull-down assays. While growing levels of activated Rac1 were detected in FAK-positive cells over time, no detectable activation of Rac1 was found in $FAK^{-/-}$ cells during the entire course of infection (**Figure 4C**), indicating the involvement of FAK in signaling upstream of Rac1 activation during *C. jejuni* invasion. Furthermore, significantly reduced Rac1-GTP levels were observed in both FAK-positive and $FAK^{-/-}$ cells infected with the Δ*cadF* mutant (**Figure 4C**). These findings further support the view that *cadF* plays a role in signaling leading to FAK-mediated activation of

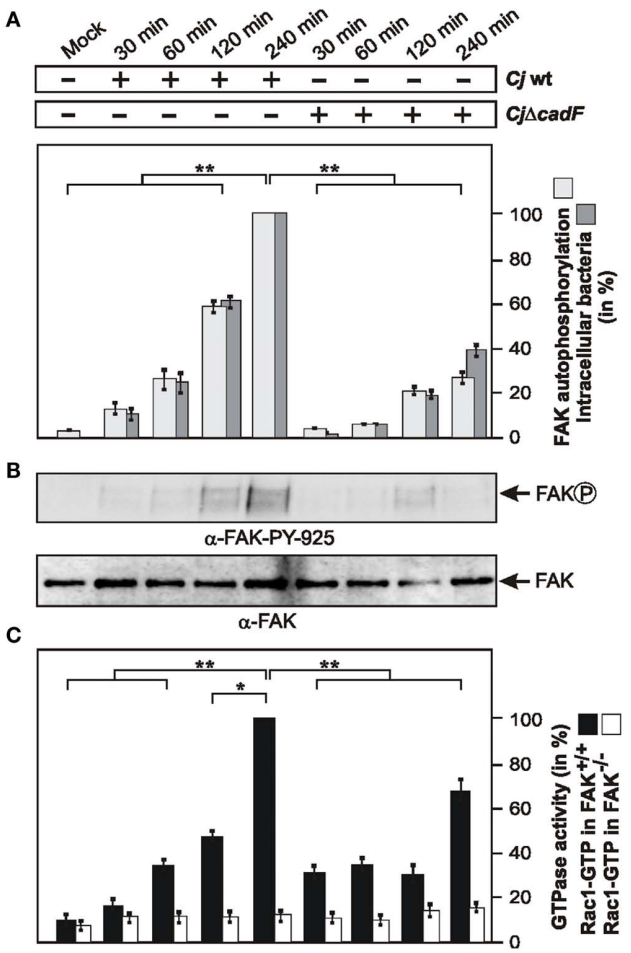

FIGURE 4 | Importance of FAK for *C. jejuni*-induced Rac1 activation and role of the bacterial CadF protein. (A) FAK-positive fibroblasts were infected with WT *C. jejuni* F38011 or isogenic F38011Δ*cadF* for indicated periods of time. Quantification of FAK kinase activity during the course of infection. One hundred percentage of activity corresponds to the highest amount of detected FAK phosphorylated at Y-397 (lane 5). **(B)** Cell lysates were also analyzed by immunoblotting with α-FAK-PY-925. Total FAK expression levels were determined as control. **(C)** Quantification of Rac1 GTPase activity during the course of infection. FAK-positive and FAK-deficient cells were infected with *C. jejuni* WT F38011 or F38011Δ*cadF* mutant for the indicated periods of time. The presence of bound, active Rac1-GTP or was analyzed in CRIB–GST pull-down assays. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level. $^{*}p < 0.05$ and $^{**}p \leq 0.005$ were considered as statistically significant as compared to the control.

Rac1. However, the Δ*cadF* mutant was still able to induce some GTPase activation in FAK-positive cells suggesting that other bacterial factor(s) are also implicated in this signaling (**Figures 4A–C**, right lanes).

THE GUANINE EXCHANGE FACTORS TIAM-1 AND DOCK180 ARE REQUIRED FOR RAC1 ACTIVATION AND *C. JEJUNI* INVASION

Our next aim was to determine additional signaling components downstream of FAK and upstream of Rac1 activation during infection with *C. jejuni.* Cycling of Rho GTPases between the inactive and active forms is positively regulated by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase activating proteins (GAPs). GEFs stimulate the exchange of GDP for GTP to generate the active form of a given GTPase, which is then capable of recognizing downstream targets (Schmidt and Hall, 2002; Hsia et al., 2003; Tomar and Schlaepfer, 2009). To identify which GEFs are involved in *C. jejuni* invasion, the expression of typical GEFs including DOCK180, Tiam-1, or Trio was downregulated using target-specific siRNA. While down-regulation of Tiam-1 and DOCK180 led to the significant reduction in *C. jejuni* internalization (**Figures 5A,B**), both down-regulation of Trio (**Figure 5C**) and transfection with non-targeting scrambled control siRNA sequence had no significant effect on *C. jejuni* uptake as quantified by gentamicin protection assays (**Figures 5A–C**). Importantly, the drop in invasion rates correlated with significantly reduced Rac1 GTP levels in CRIB-pull-down assays of Tiam-1 and DOCK180 siRNA-treated cells, but not in Trio siRNA-treated cells (**Figures 5D–F**). This suggests that two GEFs, Tiam-1 and DOCK180 (but not Trio), play a role in *C. jejuni*-induced Rac1 activation and invasion.

TIAM-1 AND DOCK180 ACT COOPERATIVELY TO TRIGGER RAC1 ACTIVATION AND *C. JEJUNI* INVASION DOWNSTREAM OF FAK

We have noted that down-regulation of the individual GEFs Tiam-1 or DOCK180 did not lead to a complete blockade of Rac1 activity and *C. jejuni* uptake. We therefore proposed that both GEFs may act together in *C. jejuni*-infected cells. To investigate this question, Tiam-1 and DOCK180 expression was downregulated by siRNA, either alone or simultaneously, followed by G-LISA to determine Rac1 activity. The results show that simultaneous down-regulation of Tiam-1 and DOCK180 led to a profound block of *C. jejuni*-induced Rac1 activity (**Figures 6A,B**). A similar strong blockade of Rac1 levels was achieved in infected $FAK^{-/-}$ cells (**Figure 4C**) or by infection in the presence of the FAK kinase inhibitor PF-573228 (**Figure 6C**). As expected, simultaneous down-regulation of Tiam-1 and DOCK180 resulted not only in profound inhibition of Rac1 activity but also profound blockade of *C. jejuni* invasion (**Figure 6D**). These data suggest that one important pathway of *C. jejuni* host cell entry proceeds by activation of a FAK → Tiam-1/DOCK180 → Rac1 signaling cascade.

THE FLAGELLUM IS ALSO INVOLVED IN *C. JEJUNI*-INDUCED RAC1 ACTIVATION AND INVASION

Since *cadF* is not the sole bacterial gene involved in *C. jejuni*-induced GTPase activation, the following aim was to search for other factor(s) playing a role in this signaling. The flagellar apparatus was reported to be one of the most intensively investigated pathogenicity determinant in *C. jejuni* (Konkel et al., 2004; Guerry, 2007). For this purpose, host cells were infected with WT strain 81–176 and its isogenic mutants Δ*fla*A/B, lacking the two major flagella subunits FlaA and FlaB (Goon et al., 2006), and Δ*flhA*, a key element involved in the coordinate regulation of late flagellar genes and other factors in *C. jejuni* (Carrillo et al., 2004). First, we confirmed the absence of flagella in both mutants (**Figure 7A**), followed by infection assays. As expected activated Rac1 was detected in FAK-positive cells between 2 and 4 h after infection with WT

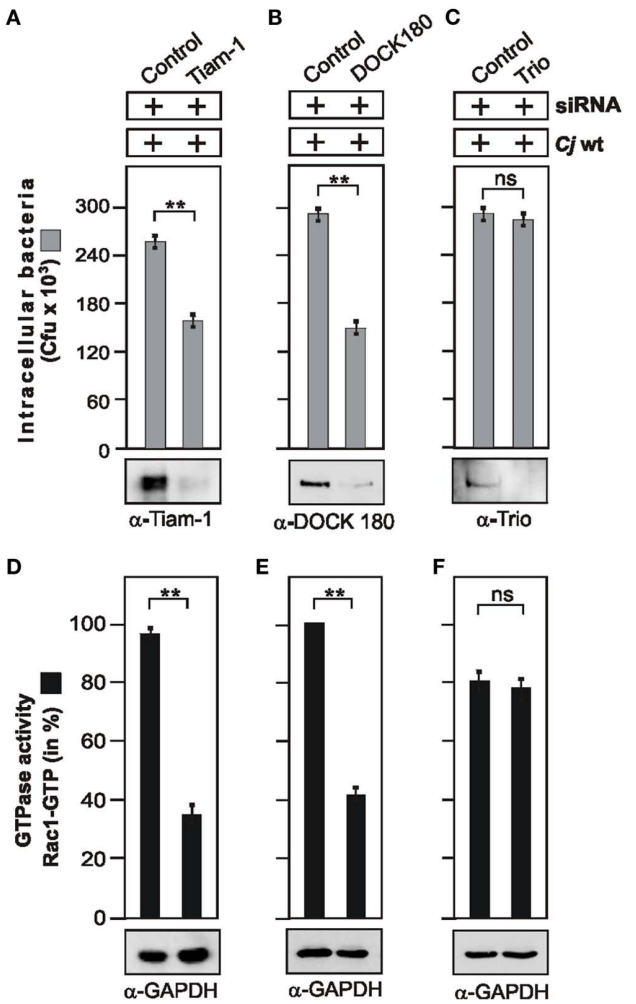

FIGURE 5 | Importance of guanine exchange factors Tiam-1, DOCK180, and Trio for *C. jejuni*-induced Rac1 activation and bacterial invasion. INT-407 cells were transfected with siRNA against Tiam-1 **(A)**, DOCK180 **(B)**, or Trio **(C)**, as well as a scrambled siRNA as control. After 48 h, cells were infected with WT *C. jejuni* 81–176 for 6 h. Intracellular bacteria were quantified by gentamicin protection assays. Immunoblotting with indicated antibodies confirmed successful knockdown of respective proteins. To detect Tiam-1 expression, immunoprecipitation with α-Tiam-1 antibody was performed. GAPDH expression levels were determined as control. **(D–F)** The presence of bound, active Rac1-GTP was analyzed in CRIB–GST pull-down assays for each indicated GEF. Quantification of Rac1 GTPase activity during the course of infection. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level. $^{**}p \leq 0.005$ was considered as statistically significant as compared to the control.

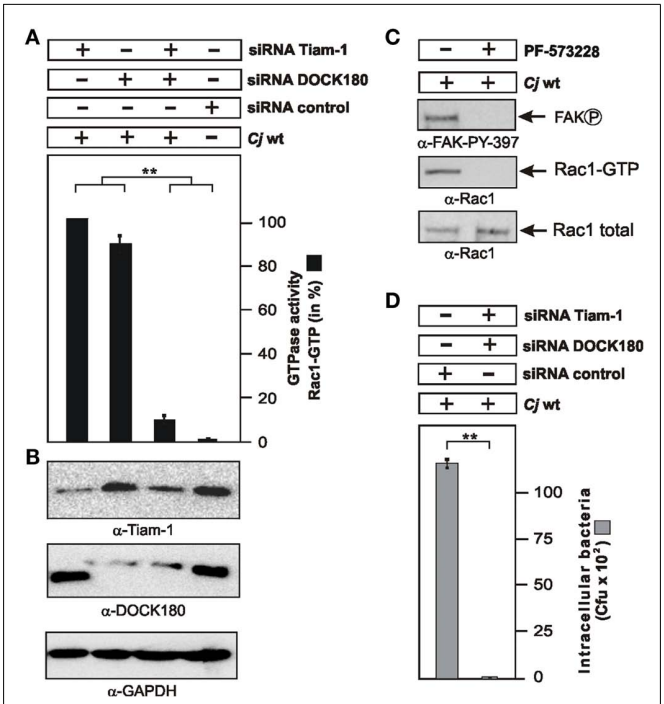

FIGURE 6 | Importance of Tiam-1, DOCK180, and FAK activation on *C. jejuni*-induced Rac1 activation and bacterial invasion. (A) INT-407 cells were transfected for 48 h with siRNA for Tiam-1, DOCK180, or a scrambled siRNA as control. Quantification of Rac1 GTPase activity after infection with WT *C. jejuni* 81–176 for 6 h. The presence of bound, active Rac1-GTP or was analyzed by G-LISA. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level (lane 1). **(B)** Immunoblotting with the indicated antibodies confirmed knockdown of the proteins. GAPDH expression levels were determined as control. **(C)** Addition of FAK kinase inhibitor PF-573228 during a 6 h infection led to the disappearance of Rac1-GTP and FAK phosphorylated at Y-397. **(D)** Intracellular bacteria were quantified by gentamicin protection assays in the indicated siRNA-treated cells. $^{**}p \leq 0.001$ were considered as statistically significant as compared to the control.

C. jejuni (**Figures 7B,C**). In contrast, no detectable Rac1 activation was found in cells infected with Δ*fla*A/B or Δ*flhA* mutants during the entire course of infection (**Figures 7B,C**), indicating an important role of these flagellar genes in activating Rac1 by *C. jejuni*, in addition to the contribution by *cadF* as shown above.

There is some controversy in the literature of whether the *C. jejuni* flagellum-mediated bacterial motility is important for invasion or if the flagellum can secrete invasion-related bacterial factors in the supernatant (Konkel et al., 2004; Novik et al., 2010). To investigate if the flagellar effect on Rac1 is direct or indirect, we were searching for a condition in which the flagellar motility is not affected, but invasion can be impaired. We have generated an α-FlaA antibody against a conserved region at the N-terminus of FlaA, and this antibody recognizes *C. jejuni* FlaA proteins in Westernblots (**Figure 8A**). We then pre-incubated WT *C. jejuni* with the α-FlaA antibody or pre-immune serum as control followed by motility assays in soft agar (see Materials and Methods). Treatment of any of the used *C. jejuni* strains with the pre-immune serum revealed no significant differences in bacterial motility or invasion as compared to non-treated bacteria (data not shown). The results also showed that while presence of the α-FlaA antibody had a slight but no significant effect on motility of WT *C. jejuni* or Δ*cadF* mutant (**Figures 8A,B**), bacterial invasion was significantly impaired as determined by gentamicin protection assay (**Figures 8C,D**). This suggests that the flagellum of *C. jejuni* has a motility-independent activity leading to bacterial entry into host target cells.

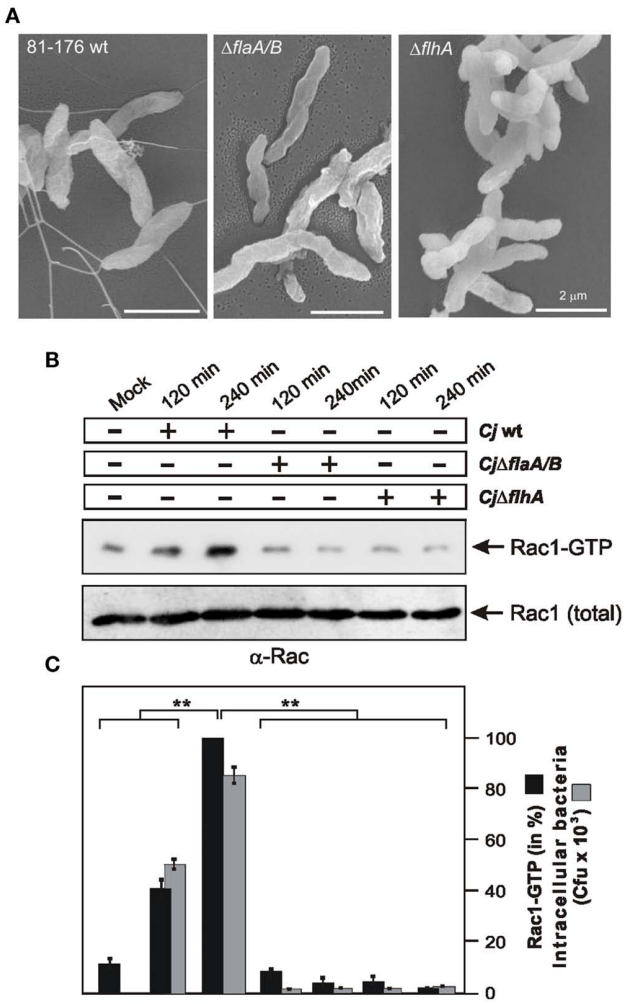

FIGURE 7 | Importance of the flagellar apparatus for *C. jejuni*-induced activation of Rac1 and bacterial invasion. (A) High resolution field emission scanning electron microscopy of *C. jejuni* WT 81–176, 81–176ΔflaA/B, and 81–176Δ*flhA* mutants. **(B)** FAK-positive cells were infected with the indicated strains in a time course. The presence of bound, active Rac1-GTP was analyzed in CRIB–GST pull-down assays followed by Western blotting using α-Rac1 antibody. Similar quantities of individual GTPases at every time point were confirmed by Western blotting using equivalent volumes of cell lysates. **(C)** Quantification of Rac1-GTP levels during the course of infection. One hundred percentage of activity corresponds to the highest amount of detected GTPase-GTP level. The amount of intracellular bacteria was quantified by gentamicin protection assays.

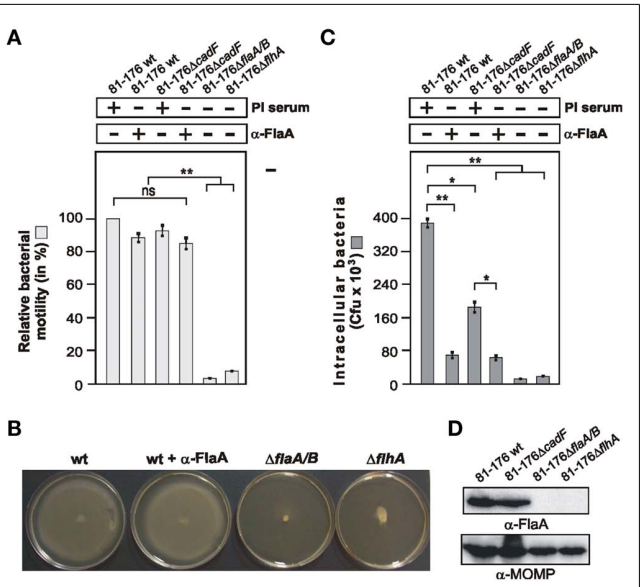

FIGURE 8 | Different effects of an α-FlaA antibody on *C. jejuni* motility and host cell invasion. (A) Quantification of motility using the indicated WT and mutant strains in the presence of 20 μg/ml α-FlaA antibody or pre-immune (PI) serum as control. One hundred percentage of motility corresponds to the highest motility presented by *C. jejuni* WT strain 81–176. **(B)** Examples of motility phenotypes with indicated strains. **(C)** INT-407 cells were infected with the indicated WT and mutant strains for 6 h in presence or absence of 20 μg/ml α-FlaA antibody or PI serum as control. Intracellular *Campylobacter* cells were quantified by gentamicin protection assays. **(D)** The α-FlaA immunoblot shows that flagellin is expressed in WT and Δ*cadF C. jejuni*, but not in flagellar mutant bacteria. The α-MOMP immunoblot served as loading control. $^{*}p < 0.05$ and $^{**}p \leq 0.005$ were considered as statistically significant as compared to the control.

DISCUSSION

Host cell entry is an important process in the pathogenesis of many pathogenic bacteria and involves numerous steps such as bacterial binding at specific receptor sites, signaling to the host cell, re-programming of intracellular host signal transduction pathways, membrane and cytoskeletal rearrangements, and eventual engulfment of the bacterium, which commonly involves the activity of one or more small Rho GTPases. Rho family members, including Rac1, Cdc42, and RhoA, are small GTP-binding proteins that serve as guanine nucleotide-regulated switches which transmit external stimuli to modulate different cellular functions (Tran Van Nhieu et al., 1999; Schmidt and Hall, 2002; Cossart and Sansonetti, 2004; Rottner et al., 2005; Tomar and Schlaepfer, 2009). Host cell entry of *C. jejuni* is considered as one of the primary reasons for bacterial-caused tissue damage, however, the molecular mechanism of *C. jejuni* invasion is widely unclear. We have recently shown that *C. jejuni* infection of INT-407 cells is accompanied by time-dependent activation of small Rho GTPases, predominantly Rac1 (Krause-Gruszczynska et al., 2007). Using specific GTPase-modifying toxins, inhibitors and GTPase expression constructs we have also demonstrated that Rac1 activity is clearly involved in *C. jejuni* invasion. Here we aimed to identify the signaling pathway leading to *C. jejuni*-induced Rac1 activation. Using knockout cell lines of several host receptors (fibronectin$^{-/-}$, GD25 integrin-β1$^{-/-}$) and kinases (FAK$^{-/-}$), siRNA transfection, scanning electron microscopy, Rho GTPase pull-downs, G-LISA, and gentamicin protection assays, we demonstrate that *C. jejuni* hijacks a major fibronectin → integrin beta1 → FAK → DOCK180/Tiam-1 signaling cascade (Hsia et al., 2003), which is crucial for triggering Rac1 GTPase activity followed by bacterial entry of host target cells. The major findings of this study are summarized in a proposed model (**Figure 9**) and are discussed below.

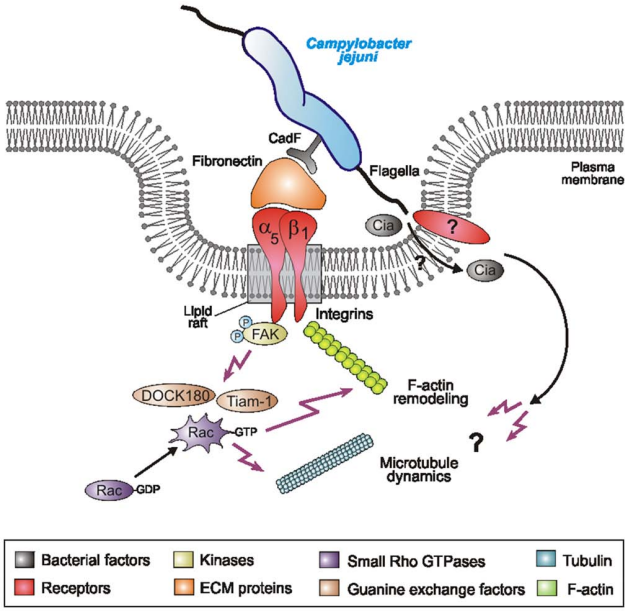

FIGURE 9 | Proposed model for *C. jejuni*-induced signaling leading to Rac1 activation and bacterial invasion. *C. jejuni* adheres to host cells via the fibronectin-binding protein CadF which acts as a bridge engaging the integrin β1 receptor. Integrin occupancy and clustering in lipid rafts leads to recruitment and activation of the non-receptor tyrosine kinase FAK. Phosphorylation of FAK triggers a cascade of signals resulting in the formation of protein complexes leading to activation of GEFs, including DOCK180 and Tiam-1. Activated DOCK180 and Tiam-1 then induce the activation of Rac1. This potentially causes localized actin and/or microtubule rearrangements at the site of *C. jejuni* entry, resulting in bacterial uptake. In addition to *C. jejuni cadF*, some flagellar genes also appear to play a role. If the flagellum participates by sole bacterial motility, by translocating bacterial Cia effector proteins or targeting a host receptor directly is not yet clear.

Most of the recent studies investigating *C. jejuni* invasion utilized pharmacological inhibitors or dominant-negative constructs which may have side-effects, and thus only provide very limited clarity on host factors playing crucial roles in the bacterial entry process. Here we used for the first time a series of three knockout cell lines for infection assays. These cell lines have the great advantage that the respective genes of interest were completely deleted in the chromosomes. Thus, not even small traces of protein are expressed, allowing clear answers if certain genes are involved in a given response or not. In fact, *C. jejuni* has two reported fibronectin-binding proteins, CadF and FlpA (Moser et al., 1997; Konkel et al., 2010), and host cell entry of *C. jejuni* shown here was widely dependent on the expression of fibronectin, integrin β1 and FAK. Since integrin β1 is the natural receptor of fibronectin, our data suggest a cascade of signaling events in a fibronectin → integrin β1 → FAK dependent fashion. In addition, we found that Rac1-GTP levels induced by *C. jejuni* were significantly elevated in infected FAK-expressing but not in FAK-deficient cells, and Rac1 activation was confirmed conclusively by two independent approaches, CRIB-pull-down and G-LISA. In line with these observations, we observed membrane dynamics, ruffling, and engulfment of *C. jejuni* upon infection with WT control cells, but not in any of the infected knockout cell lines. Together, these new findings provide clear evidence that fibronectin, integrin β1, and FAK are major host factors playing not only a role in Rac1 activation but also host cell entry of the bacteria. By comparison, this scenario is very similar to that reported for *Staphylococcus aureus*, a Gram-positive pathogen expressing various fibronectin-binding proteins, because infected fibronectin$^{-/-}$ or FAK$^{-/-}$ cells were also severely impaired in their ability to internalize the latter bacteria (Agerer et al., 2005; Schroeder et al., 2006). Furthermore, integrin-mediated uptake of *S. aureus* depends on the integrity of membrane microdomains (Hoffmann et al., 2010), and this is in line with the finding that MβCD-treatment blocks internalization of *C. jejuni*. Our studies would suggest that *S. aureus* may also activate Rac1 by a similar sequence of events to trigger their uptake process.

By a strategy engaging fibronectin and integrin β1, *C. jejuni* seems to exploit the ability of this receptor complex to dynamically associate with the intracellular cytoskeleton and to generate the necessary pulling forces to promote bacterial uptake by the host cell. In non-infected healthy tissues, integrin β1-containing fibrillar cell adhesions are important for the organization of the extracellular matrix, as they co-align with fibronectin fibrils, and genetic elimination of integrin β1 in GD25 cells results in defects in the assembly of a fibrillar meshwork of extracellular fibronectin (Wennerberg et al., 1996; Danen and Yamada, 2001; Leiss et al., 2008). Cellular pulling forces generated via an integrin β1-mediated linkage to the actin-myosin network seem to be critical for fibronectin fibril formation, as force-triggered conformational changes are essential to expose cryptic oligomerization motifs within the fibronectin molecules (Sechler et al., 2001). Importantly, FAK has been shown to play a key role in the formation of a fibrillar fibronectin extracellular matrix. Cultured FAK$^{-/-}$ cells *in vitro*, as well as FAK$^{-/-}$ mouse embryos *in vivo*, fail to properly assemble fibronectin fibrils (Ilic et al., 2004; Leiss et al., 2008). In line with the fact that FAK$^{-/-}$ cells are unable to properly organize the extracellular fibronectin matrix, we found that these cells are deficient in the ability to internalize *C. jejuni*, suggesting that fibronectin/integrin-linkages to the actin-myosin network are disrupted and pulling forces are not provided. As one would expect from these results, *C. jejuni* profoundly stimulated FAK kinase activity during infection. The activation of FAK has also been described for other pathogens targeting integrins for bacterial invasion or other purposes, including *S. aureus* (Agerer et al., 2005), *Yersinia pseudotuberculosis* (Alrutz and Isberg, 1998; Eitel et al., 2005), and *Helicobacter pylori* (Kwok et al., 2007; Tegtmeyer et al., 2010), thus FAK seems a favorite target in bacterial pathogenesis.

The observation that FAK expression and activation is required for *C. jejuni*-triggered Rac1 activity and invasion, led us to investigate the involved signaling in more detail. In fact, *C. jejuni*-induced the phosphorylation of FAK at Y-397 and Y-925, and expression of FAK Y397F, K454R, Y925F, or ΔPR1/2 mutants in FAK$^{-/-}$ cells did not restore bacterial uptake as compared to WT FAK. A well-described GEF downstream of FAK is DOCK180 (Hauck et al., 2002; Hsia et al., 2003). The signaling cascade involves p130Cas, an adapter molecule binding to proline-rich residues in the carboxyl-terminal domain of FAK. p130Cas then associates

with the adapter protein Crk and this complex activates DOCK180. In addition, it has been shown that expression of phospho-mimetic FAK-Y925E enhanced cell protrusions together with activation of the same DOCK180-dependent Rac1 signaling pathway, thus, phosphorylation of FAK at Y-925 is also involved this scenario (Deramaudt et al., 2011). These observations are in well agreement with our findings, suggesting that *C. jejuni* activates a classical FAK → p130CAS → Crk → DOCK180 → Rac1 signaling pathway. However, siRNA knockdown of DOCK180 expression was not sufficient to completely eliminate Rac1 activity and bacterial invasion. The other GEF required for *C. jejuni*-induced Rac1 activation was Tiam-1, but the molecular mechanism how FAK can target Tiam-1 is not yet clear. Interestingly, syndecan-2-mediated cell migration was diminished when cells were transfected with non-phosphorylatable FAK Y397F mutant or siRNA against Tiam-1, suggesting that a FAK → Tiam-1 → Rac1 signaling pathway is activated (Park et al., 2005). If FAK can stimulate Tiam-1 directly or via another factor, however, needs to be investigated in future studies.

The profound activation of Rac1 is a hallmark of numerous highly invasive pathogens such as *Shigella flexneri* and *Salmonella enterica* (Hardt et al., 1998; Tran Van Nhieu et al., 1999). These bacteria induce extensive membrane ruffling by the "trigger mechanism" requiring different Rho GTPases including Rac1, which are targeted directly through bacterial GEFs injected into the host cell by a type III secretion system (T3SS). Interestingly, *C. jejuni* does not encode a classical T3SS as reported above (Konkel et al., 1999; Hofreuter et al., 2006). This is in line with our observations that eukaryotic GEFs are required for *C. jejuni*-induced Rac1 activation, while homologs of well-known *S. flexneri* and *S. enterica* GEFs are absent in *C. jejuni* genomes. Our recent studies showed that *C. jejuni* pathogenicity factors such as plasmid pVir, cytolethal distending toxin (CDT), certain capsular genes and the adhesin PEB1 are not involved in *C. jejuni*-induced Rac1 activation nor invasion, and even some mutants including Δ*kpsS* or Δ*peb1* even produced higher GTPase-GTP levels as compared to WT *C. jejuni* (Krause-Gruszczynska et al., 2007). Importantly, we found here that an isogenic Δ*cadF* mutant less efficiently induced activation of Rac1 as compared to WT bacteria. Although this is an indirect approach, this data suggests that the fibronectin-binding protein CadF, at least in part, could be involved in Rac1 activation. Thus, we propose that CadF does not only act as a canonical adhesin for bacterial binding to fibronectin, but can also stimulate integrins and FAK kinase activity, which subsequently activates downstream GEFs and Rac1, important in *C. jejuni* invasion. If the other fibronectin-binding protein FlpA may also play a role needs to be investigated in future studies. However, another proposed *C. jejuni* factor involved in Rac1 activation reported here is the flagellum, because Δ*flaA/B* or Δ*flhA* knockout mutants lacking FlaA expression and flagella induced only very little Rac1-GTP levels. The flagellar apparatus represents one of the most intensively investigated pathogenicity determinants in *Campylobacter*. FlaA/B proteins have been shown to be required for bacterial colonization in a number of animal models (Morooka et al., 1985; Wassenaar et al., 1993; Hendrixson and DiRita, 2004), and they play an active but yet unknown role in the invasion of epithelial cells (Wassenaar et al., 1991; Grant et al., 1993; Yao et al., 1994). The possible impact of the flagellum in invasion is controverse in the literature. One hypothesis is that the flagella, like their evolutionary related T3SS counterparts, can secrete invasion-triggering factors such as CiaB and others into the culture supernatant (Konkel et al., 1999, 2001, 2004). The other hypothesis is that flagella-mediated motility is the driving force to trigger *C. jejuni* invasion, but CiaB is not involved (Novik et al., 2010). We show here that flagella-driven motility is not significantly hampered by addition of an α-Flagellin antibody, but the invasive properties of *C. jejuni* were significantly inhibited. This suggests that motility and invasion are important, but not necessarily coupled processes. We propose that the flagellum, unlike its role in motility, may transport bacterial effectors into the medium or into the host cell, and/or the flagellum itself may bind a host cell receptor directly to trigger signaling involved in invasion (**Figure 9**). Future studies should investigate these different possibilities.

Taken together, the work presented here demonstrates several lines of evidence for a clear role of fibronectin, integrin β1, FAK, DOCK180, Tiam-1, and Rac1 during host cell entry of three different isolates including the *C. jejuni* model strain 81–176. By comparison to other bacterial infection models, our electron microscopic studies of infected INT-407 and other cell lines reported here support the view that *C. jejuni* do not enter host cells by a robust trigger mechanism as known for *Shigella* and *Salmonella*, nor a classical "zipper mechanism" as described for *Yersinia*, but rather by a very specialized pathway sharing features of both mechanisms. Our electron microscopic pictures, showing that *C. jejuni* enter cells first with the leading flagellum (**Figure 3B**), may underline the important role of the flagellum in this context. Our findings suggest that the flagellum may act cooperatively with the CadF adhesin in order to establish initial host cell contact and trigger host cell signaling leading to Rac1 activation and invasion. Furthermore, there appears to be a separate, flagellum-mediated uptake mechanism, which is independent of CadF and which allows invasion in the absence of this adhesin. Together, these results add another important aspect to our understanding of the mechanism of *C. jejuni* pathogenesis. Since the exact pathway of *C. jejuni* invasion into host target cells is still not fully understood, it will be highly interesting to study in future the precise mechanism of how active Rac1 regulates actin rearrangements and/or microtubule dynamics involved in the bacterial entry process. It will be also interesting to investigate the role of another small Rho GTPase member, Cdc42, which is also activated by *C. jejuni* (Krause-Gruszczynska et al., 2007). Experiments are underway in our laboratory to investigate these signaling pathways in more detail.

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Identification of genetic loci that contribute to *Campylobacter* resistance to fowlicidin-1, a chicken host defense peptide

Ky Van Hoang[1], Ying Wang[2] and Jun Lin[1]*

[1] *Department of Animal Science, The University of Tennessee, Knoxville, TN, USA*
[2] *Department of Biosystems Engineering and Soil Science, The University of Tennessee, Knoxville, TN, USA*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Eric Déziel, Institut National de la Recherche Scientifique, Canada
Lone Brøndsted, University of Copenhagen, Denmark

***Correspondence:**
Jun Lin, Department of Animal Science, The University of Tennessee, 2640 Morgan Circle Drive, Knoxville, TN 37996-4574, USA.
e-mail: jlin6@utk.edu

Antimicrobial peptides (AMPs) are critical components of host defense limiting bacterial infections at the gastrointestinal mucosal surface. Bacterial pathogens have co-evolved with host innate immunity and developed means to counteract the effect of endogenous AMPs. However, molecular mechanisms of AMP resistance in *Campylobacter*, an important human food-borne pathogen with poultry as a major reservoir, are still largely unknown. In this study, random transposon mutagenesis and targeted site-directed mutagenesis approaches were used to identify genetic loci contributing *Campylobacter* resistance to fowlicidin-1, a chicken AMP belonging to cathelicidin family. An efficient transposon mutagenesis approach (EZ::TN™ <KAN-2> Transposome) in conjunction with a microtiter plate screening identified three mutants whose susceptibilities to fowlicidin-1 were significantly increased. Backcrossing of the transposon mutations into parent strain confirmed that the AMP-sensitive phenotype in each mutant was linked to the specific transposon insertion. Direct sequencing showed that these mutants have transposon inserted in the genes encoding two-component regulator CbrR, transporter CjaB, and putative trigger factor Tig. Genomic analysis also revealed an operon (*Cj1580c-1584c*) that is homologous to *sapABCDF*, an operon conferring resistance to AMP in other pathogens. Insertional inactivation of *Cj1583c* (*sapB*) significantly increased susceptibility of *Campylobacter* to fowlicidin-1. The *sapB* as well as *tig* and *cjaB* mutants were significantly impaired in their ability to compete with their wild-type strain 81–176 to colonize the chicken cecum. Together, this study identified four genetic loci in *Campylobacter* that will be useful for characterizing molecular basis of *Campylobacter* resistance to AMPs, a significant knowledge gap in *Campylobacter* pathogenesis.

Keywords: *Campylobacter*, fowlicidin, antimicrobial peptide, resistance

INTRODUCTION

Campylobacter species including *C. jejuni* and *C. coli* are the most common bacterial causes of human gastroenteritis in the United States and other developed countries (Allos, 2001). Human *Campylobacter* illnesses are caused primarily by *C. jejuni* (∼90%) and secondarily by *C. coli* (∼10%). Poultry are the major reservoir of *Campylobacter* and thus the main source for human campylobacteriosis (Friedman et al., 2000). As a comensal, *Campylobacter* could establish persistent, benign infections in chickens (up to 10^{10} CFU per gram of feces). To successfully colonize and persist in intestinal tracts of animals and humans, *Campylobacter* must have evolved a variety of mechanisms to counteract hash *in vivo* conditions as well as host innate immunity. However, we know less about *Campylobacter* pathogenicity than we do about other prevalent pathogens, which impeded the development of efficient intervention strategies to prevent and control *Campylobacter* infections in humans and animal reservoirs.

Endogenous antimicrobial peptides (AMPs) belong to the most ancient and efficient components of host defense. Defensins and cathelicidins are two major families of AMPs in humans and animals. Both defensins and cathelicidines are important defense AMPs expressed by epithelium in small intestine, consequently limiting bacterial infections at the gastrointestinal mucosal surface (Wehkamp et al., 2007). Despite the existence of a broad diversity in AMP sequences and structures, the vast majority of AMPs share a common theme in the mechanism of killing action by disruption of membrane integrity (Yeaman and Yount, 2003; Peschel and Sahl, 2006). Generally, AMPs directly interact with target cells via initial electrostatic and hydrogen bond attraction, then disrupt the structure or function of bacterial membrane by permeating lipid bilayers, forming transmembrane pore, and ultimately lead to cell death. However, transmembrane pore forming is not the only mechanism of bacterial killing by AMP. AMPs may also have intracellular targets (e.g., DNA gyrase, ribosome) and

Abbreviations: AMP, antimicrobial peptide; F1, fowlicidin-1.

AMP-mediated bacterial death may occur as a result of several independent or cooperative mechanisms of action (Yeaman and Yount, 2003). Expression of defensins and cathelicidins is either constitutive or inducible in gastrointestinal tract (Wehkamp et al., 2007). Epithelial AMPs have been observed to be induced in human intestinal epithelia upon infection by *C. jejuni* (Zilbauer et al., 2005). Recently, a full panel of chicken defensins and cathelicidins (designated as "fowlicidins") has been identified and characterized (Lynn et al., 2004; Xiao et al., 2004, 2006; Bommineni et al., 2007; van Dijk et al., 2007). These chicken AMPs displayed potent and broad spectrum of antibacterial activity, highlighting their role in host innate defense and in the development of novel antimicrobial (peptide antibiotic) (Hancock and Sahl, 2006).

Bacterial pathogens have co-evolved with host innate defense and developed means to curtail the effect of endogenous AMPs (Yeaman and Yount, 2003; Peschel and Sahl, 2006). Different AMP resistance mechanisms have been well characterized in various enteric pathogens such as *Salmonella enterica serovar* Typhimurium and *Escherichia coli* (Yeaman and Yount, 2003; Peschel and Sahl, 2006). The best studied AMP resistance mechanisms in Gram-negative bacteria involve (1) electrostatic repulsion of AMPs by modification of cell surface, (2) proteolytic cleavage of AMPs, and (3) active extrusion of AMPs by drug efflux pumps. Other mechanisms, such as capsule production, changes in the composition of lipid fatty acids in membrane and maintenance of appropriate membrane potential, have been also implicated in AMP resistance. Covalent modification of LPS in outer membrane via two-component regulatory systems (e.g., PhoP/PhoQ), which consequently reduces the negative charge and/or membrane fluidity of LPS, has been identified as a major mechanism in these Gram-negative pathogens (Ernst et al., 2001; Yeaman and Yount, 2003; Peschel and Sahl, 2006). However, as an important strategy to evade killing by innate immunity and by potential peptide antibiotics, AMP resistance mechanisms are still largely unknown in *Campylobacter*. Recently, it has been observed that lipid A modification (Cullen and Trent, 2010; van Mourik et al., 2010) and lipooligosaccharide (LOS) production (Lin et al., 2009; Naito et al., 2010) could confer *Campylobacter* resistance to polymyxin B, an AMP produced by bacterial species *Paenibacillus polymyxa,* that has been successfully used as a model peptide to study AMP resistance in many Gram-negative pathogens. However, the polymyxin B resistance gene identified from our recent study (Lin et al., 2009) did not contribute *C. jejuni* resistance to endogenous AMPs (e.g., fowlicidins and bacteriocins), strongly suggesting that polymyxin B may not be a good surrogate for studying *Campylobacter* resistance to structurally unrelated but physiologically relevant AMPs.

In this study, we chose fowlicidin-1 (F1), a cathelicidin expressed in chicken intestine, as a model peptide to examine mechanisms of AMP resistance and identified three genetic loci (*cbrR*, *cjaB*, and *tig*) involved in F1 resistance in *C. jejuni* using an efficient *in vivo* transposon mutagenesis system. In addition, genomic analysis revealed an operon *cj1580c-1584c* that shared homology to the *sap* (sensitivity to antimicrobial peptide) operon that conferred resistance to AMP-mediated killing of different pathogens such as *Salmonella* (Parra-Lopez et al., 1993). Site-directed mutagenesis of the *sap* operon showed that *sapB* (*cj1583c*) is involved in F1 resistance in *C. jejuni*. Chicken experiments further demonstrated that these genes play an important role in the colonization of *Campylobacter* in the intestine.

MATERIALS AND METHODS

BACTERIAL STRAINS, PLASMIDS, AND GROWTH CONDITIONS

The major bacterial strains and plasmids used in this study are listed in **Table 1**. Among the 174 *Campylobacter* isolates used for susceptibility test to chicken AMP F1, 154 isolates were *C. jejuni* and 20 isolates were *C. coli*. These *Campylobacter* strains were isolated from different hosts and geographically diverse locations described our previous study (Hoang et al., 2011a). *Campylobacter* strains were routinely grown in Mueller–Hinton (MH) broth (Difco) or MH agar at 42°C under microaerophilic conditions generated by using CampyGen Plus gas pack (Oxoid) in an enclosed jar. *E. coli* strains were grown in Luria–Bertani (LB) broth with shaking (250 rpm) or on agar at 37°C overnight. Antibiotics kanamycin (Kan) or chloramphenicol (Cm) was added in medium at a desired concentration when needed.

AMP SUSCEPTIBILITY TESTING

The susceptibilities of *C. jejuni* and *C. coli* isolates to F1 were determined by a standard microtiter broth dilution method with an inocula of 10^6 bacterial cells/ml as described previously (Lin et al., 2002). Minimum inhibitory concentrations (MICs) were determined by the lowest concentration of specific antimicrobial showing complete inhibition of bacterial growth after 24 h of incubation at 42°C. The chicken cathelicidin F1 (>95% purity) was synthesized by Bio-Synthesis (Lewisville, TX).

In vitro SELECTION OF F1 RESISTANT *C. jejuni*

F1 was used as the selective agent to obtain spontaneous F1 resistant mutants *in vitro*. Briefly, *C. jejuni* 81–176 was grown on F1-free MH agar plates overnight. The cells were harvested and suspended in MH to final $OD_{600\,nm}$ about 1.2. The cell suspensions were plated on MH agar plates containing 16, 32, or 64 μg/ml of F1. Following four days of incubation under microaerophilic conditions at 42°C, the plates were checked for the emergence of F1 resistant mutants. The F1 pre-adapted cells were also used for *in vitro* selection of F1 resistant mutants. Briefly, *C. jejuni* 81–176 F1 were grown in MH broth containing sub-lethal concentration of F1 (4 μg/ml) for five consecutive passages (2 days per passage). Following the fifth passages, cultures were centrifuged, and the pellets were suspended in MH broth to final $OD_{600\,nm}$ about 1.2. The bacterial cells suspensions were also plated MH agar plates with increasing concentrations of F1 for selecting F1 resistant mutant as described above. Mutants were randomly selected, grown in MH broth, and subjected to MIC test together with the parent strain *C. jejuni* 81–176.

DNA ISOLATION AND NATURAL TRANSFORMATION

Chromosomal DNA was isolated from *Campylobacter* using the Wizard Genomic Purification Kit (Promega) according to the manufacturer's instructions. Natural transformation (biphasic method) was performed following standard procedure (Wang and Taylor, 1990).

Table 1 | Major bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source and reference
C. jejuni		
JL241	NCTC 11168, human isolate	Parkhill et al., 2000
JL28	81–176, human isolate with poor colonization ability in chicken	Black et al., 1988
JL242	81–176, human isolate with high colonization ability in chicken	Black et al., 1988
S3B	*C. jejuni* strain isolated from a chicken	Hoang et al., 2011a
JL599	JL28 derivative, *cbrR*::Kan	This study
JL601	JL28 derivative, *cjaB*::Kan	This study
JL602	JL28 derivative, *tig*::Kan	This study
JL656	JL242 derivative, *cbrR*::Kan	This study
JL657	JL242 derivative, *cjaB*::Kan	This study
JL658	JL242 derivative, *tig*::Kan	This study
JL623	JL241 derivative, *cbrR*::Kan	This study
JL665	JL241derivative, *cjaB*::Kan	This study
JL629	JL241derivative, *tig*::Kan	This study
JL668	JL656/pCbrR	This study
JL694	JL657/pCjaAB	This study
JL695	JL658/pTig	This study
JL624	*C. jejuni* S3B derivative, *cbrR*::Kan	This study
JL631	*C. jejuni* S3B derivative, *tig*::Kan	This study
JL805	*C. jejuni* S3B derivative, *cjaB*::Kan	This study
JL697	JL242 derivative, *cj1583c*::Cm (*sapB*$^-$)	This study
JL706	JL241 derivative, *cj1583c*::Cm (*sapB*$^-$)	This study
JL719	JL242 derivative, *cj1584c*::Cm (*sapA*$^-$)	This study
JL793	JL242 derivative, *cj1581c*::Cm (*saAD*$^-$)	This study
JL792	JL242 derivative, *cj1580c*::Cm (*sapF*$^-$)	This study
E. coli		
DH5α	F$^-$ *ϕ80lacZΔM15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_k^-, m_k^+) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
JL690	DH5α containing pCjaAB	This study
JL691	DH5α containing pTig	This study
JL652	DH5α containing pCbrR	This study
JL692	DH5α containing pcmSapB	This study
JL48	Conjugation helper strain, DH5α containing plasmid RK2013	Lin et al., 2005
PLASMIDS		
pRY111	*E.coli-C.jejuni* shuttle vector, Cm^r	Yao et al., 1993
pCbrR	pRY111 derivative containing 1.5 kb *cbrR* gene plus its promoter region	This study
pTig	pRY111 derivative containing 1.6 kb *tig* gene plus its promoter region	This study
pCjaAB	pRY111 derivative containing 2.7 kb *cjaAB* operon plus its promoter region	This study
pGEM-T Easy	PCR cloning vector, Amp^r	Promega
pSapB	pGEM-T Easy containing 2.0 kb *Cj1583c* (*sapB*) gene of JL241	This study
pcmSapB	pSapB with Cm resistant gene inserted in *Cj1583c* (*sapB*) gene	This study

RANDOM TRANSPOSON MUTAGENESIS AND SCREENING OF MUTANTS WITH INCREASED SUSCEPTIBILITIES TO F1

C. jejuni 81–176 (JL28 in **Table 1**, F1 MIC = 8 μg/ml) was subjected to *in vivo* transposon mutagenesis using EZ::Tn5™ <KAN-2> Transposome (Epicentre) as detailed in our previous publication (Lin et al., 2009). Briefly, one microliter of EZ-Tn5 <KAN-2> TnP transposome complex containing 25 ng transposon was used to electroporate *C. jejuni* JL28 competent cells. The Kan^r transformants were individually picked and inoculated in 96-well microplates. Following 24 h of incubation, cultures of mutants were replicated into microtiter plates containing 4 μg/ml of F1. Those mutants that could not grow in F1-containing media were selected from the initial plates and subjected to a second screening to confirm increased sensitivities to F1. To confirm specific genetic linkage between the transposon insertion and the increased F1 susceptibility of each mutant, backcrossing of the transposon mutations into wild-type *C. jejuni* 81–176 was performed using natural transformation. The MICs of the backcrossed mutants for F1 were determined together with parent strain. The specific transposon insertion site of each mutant was

determined by directly sequencing the genomic DNA (Lin et al., 2009). Sequence analysis was performed using DNAStar software package.

PCR AND RT-PCR

PCR was performed in a volume of 50 μl containing 1 mM each deoxynucleoside triphosphate, 200 nM primers, 2.0 mM $MgCl_2$, 100 ng of *Campylobacter* genomic DNA, and 2.5 U of *Taq* DNA polymerase (Promega) or *Pfu Turbo* DNA polymerase (Stratagene). Cycling conditions varied according to the estimated annealing temperature of the primers and the expected sizes of the products (available upon request). RNA was extracted from *C. jejuni* strains using RNeasy Mini Kit (Qiagen) followed by treatment of RNA samples with RNase-free DNase (Qiagen). RT-PCR was conducted using the MasterAmp kit (Epicentre). Cycling conditions for RT-PCR included an initial incubation at 55°C for 30 min and 94°C for 2 min followed by 40 cycles of 94°C for 15s, 52°C for 30s, and 68°C for 1 min, and a final extension at 68°C for 5 min. A RT-PCR mixture lacking the RT was included as a negative control.

COMPLEMENTATION *IN trans*

To complement *cjaB* mutation in isogenic CjaB mutant JL657, a 2.7-kb complete *cjaAB* operon including its 174-bp upstream and 193-bp downstream regions, was PCR amplified from NCTC 11168 using primer pairs of CjaAB-F and CjaAB-R (**Table 2** and **Figure 1**). The PCR was performed using *pfu* DNA polymerase (Stratagene) and the blunt-ended PCR product was purified and ligated to shuttle vector pRY111 (Yao et al., 1993), which was digested with *Sma*I prior to ligation. The ligation

Table 2 | Key oligonucleotide primers used in this study.

Primer	DNA Sequence (5′–3′)	Product size (kb)[a]	Gene/Operon amplified[a]
Tf-F	TCATGAATTTCACCACTTAGCA	1.6	*Cj0193c* (*tig*)
Tf-R	TGCTATCATTGAAGGCAAATTTTA		
CjaAB-F	TCGCCTAATGCCAAAGTTTC	2.7	*cjaAB*
CjaAB-R	TCACCATCTGCATTGCATTTA		
Cj0643-F	GCAATGCGTATCAACAATCC	1.5	*cbrR*
Cj0643-R	AAAAATTTCCTTTCTTTTGAAAAC		
Cj1583c-F	AAAAAGCCGAGGATTTGCTT	2.0	*Cj1583c* (*sapB*)
Cj1583c-R	CTGTGGCTATAGCATGAACGA		
Cj1584c-F	CGGGTATATCTTGGCAGCAT	1.9	*Cj1584c* (*sapA*)
Cj1584c-R	GAAACCCCTAAGTCCCCTTTT		
Cj1582c-F	CCTGTTTTGGTGCTCGTTTTA	1.5	*Cj1582c* (*sapC*)
Cj1582c-R	GCTCTGCATCTTGCAAAACA		
Cj1581c-F	CGCTTTAATTCATTGGTGTTTC	1.5	*Cj1581c* (*sapD*)
Cj1581c-R	TTAAAATTTTCCAAACCATCTTG		
Cj1580c-F	TTGATCGTTTGTGGCATTCT	1.5	*Cj1580c* (*sapF*)
Cj1580cR	AAAAATCAAAGCCCAAGGAAA		
CmF	CGATTTAAATGCTCGGCGGTGTTCCTTT	0.8	*cat*
CmR	CGATTTAAATGCGCCCTTTAGTTCCTAAAG		
Sap4-F	GTG CTA AAA CGCTTA GTTTTTAGTATT	0.6	*sapB*
Sap4-R	AATCAAATGCTCTAAACGATTTAA AAA		
Sap5-F	GATGCAGTG ATTAATCTTGTATTT TCAGG	0.5	*sapA*
Sap5-R	TCCATTTTACAAATTTATAAGGACCTG		
Cj1583c-RT-F	GGGCTTGATAAGCCTTTGCT	0.41	*Cj1583c*
Cj1583c-RT-R	AAAACGAGCACCAAAACAGG		
Cj1582c-RT-F	TTTTTAGCCTTGCCTGCTTT	0.40	*Cj1582c*
Cj1582c-RT-R	CCACCAAGCGCCTATAAAAA		
Cj1581c-RT-F	ACAAAGTGGAGTGGGCAAAA	0.38	*Cj1581c*
Cj1581c-RT-R	AATCCAAACTAGGCTCAC		
Cj1580c-RT-F	GTGGTGCAGGTAAAAGCACA	0.32	*Cj1580c*
Cj1580c-RT-R	GGCTTCTCCTCCGCTTAACT		
ClpP-RT-F	ATGATGAACTTGCCGCTTCT	0.40	*Cj0192c*
ClpP-RT-R	GCTTCTTGTGCTGACATGAAAA		
Cj0644-RT-F	CAGGGGTGCATCCTTATGAA	0.32	*Cj0644*
Cj0644-RT-R	GCAAATGTTCGCTTGCATTA		
Cj0645-RT-F	ATAATTGCGGAAATGGCAAG	0.41	*Cj0645*
Cj0645-RT-R	GCTTCTTGTGCTGACATGAAAA		

[a] *Product sizes and amplified genes refer to those of the relevant primer pair.*

mix was introduced into DH5α by transformation. One transformant (JL690) with a plasmid bearing intact *cjaAB* operon (pCjaAB) was created. The pCjaAB from JL690 was then transferred to JL657 by tri-parental conjugation using JL48 as a helper strain (Lin et al., 2005). Similar approach was used to complement *tig* and *cbrR* mutations using primer pairs of Td-F/Td-R and Cj0643-F/Cj0643-R, respectively (**Table 2** and **Figure 1**). The complemented strains, together with other related strains, were subjected to AMP killing assay as described below.

CONSTRUCTION OF ISOGENIC *sap* MUTANTS

The putative *sap* genes (*Cj1580c* to *cj1584c*, **Figure 1**) were inactivated by insertional mutagenesis according to our previous publication (Lin et al., 2005). Taking *sapB* (*Cj1583c*) as an example, an approximately 2 kb fragment was PCR amplified from genomic DNA of 11168 (JL241) by using primer pair Cj1583c-F and Cj1583c-R (**Table 2**). The PCR product was cloned into pGEM-T Easy vector (Promega) to generate pSapB (**Table 1**). The chloramphenicol resistance gene cassette (*cat*) was PCR amplified from plasmid pRY111 (Yao et al., 1993) by using *PfuUltra*® High-Fidelity DNA polymerase (Stratagene) and primers of CmF and CmR (**Table 2**). The resulting blunt-ended PCR product was purified and ligated into pSapB vector, which was digested with *Swa*I prior to ligation, to generate mutant construct pcmSapB (**Table 1**). The construct pcmSapB, which serves as suicide vector, was then introduced into 81–176 (JL242) by natural transformation. One transformant, designated JL697, was selected on MH agar containing 5 μg/ml of Cm. The inactivation of putative *sapB* (*Cj1583c*) in JL697 was confirmed by PCR (data not shown). A similar site-directed mutagenesis approach was used to create isogenic *Cj1584c* (*sapA*), *Cj1582c* (sapC), *Cj1581c* (*sapD*), and *Cj1580c* (*sapF*) mutants of 81–176 by using primers pairs Cj1584c-F/R Cj1582c-F/R, Cj1581c-F/R, and Cj1580c-F/R, respectively (**Table 2**). Notably, due to the absence of a transcriptional terminator downstream of the *cat* gene and the same orientation of the *cat* gene with respect to the inactivated gene, inactivation of specific gene likely has no polar effect on the downstream genes; thus, construction of the individual isogenic *sap* mutants may help us to evaluate their relative contribution to fowlicidin resistance.

AMP KILLING ASSAY

The *C. jejuni* mutants together with their parent strains were subjected to AMP killing assay using 96-well plate as described previously (Mount et al., 2010) with minor modifications. The AMPs used in killing assay include chicken cathelicidin F1, chicken defensin AvBD9 (kindly provided by Dr. Guolong Zhang, Oklahoma State University), bacteriocin OR-7 and E-760 (Lin et al., 2009), and those purchased from Sigma (polymyxin B, colistin, cecropin A, gramicidin, and maganannin). Briefly, *Campylobacter* strains were grown in MH broth to mid-log phase, and the cells were washed with MH broth and diluted to approximately 10^6 CFU/ml in MH broth. A volume of 180 μl of the diluted cells was mixed with 20 μl MH broth (control) or AMP stock solution (concentration of 10-fold MIC of specific AMP) (treatment). The plates were incubated under microaerophilic conditions at 42°C for 2 h. After 2 h incubation, 20 μl of bacterial culture were taken and serially diluted in MH broth and plated onto MH agar plates. The number of CFU was enumerated after two days of incubation under microaerophilic conditions at 42°C. Percentage survival was calculated by dividing the CFU number of bacteria incubated with AMP relative to those incubated in the presence of MH broth and then multiplied by 100. All assays were carried out in triplicate and two independent experiments were

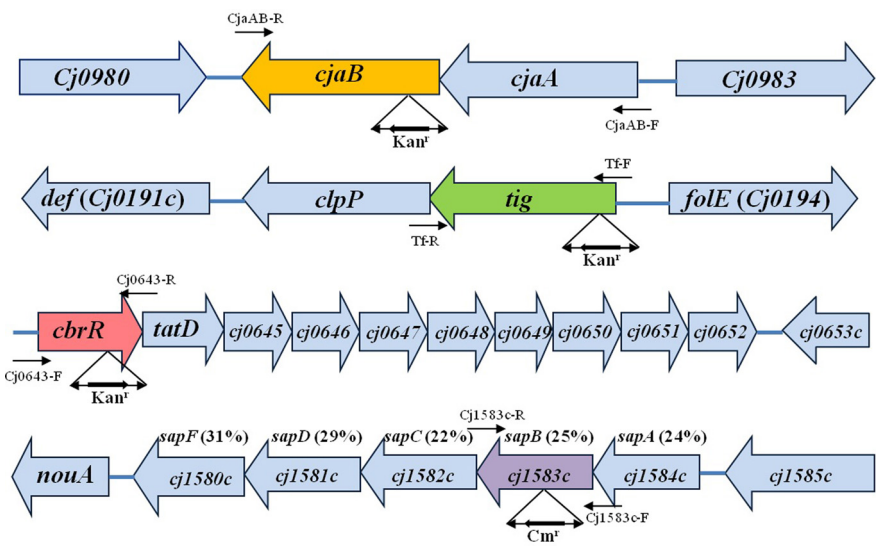

FIGURE 1 | Genomic organization and features of the *cjaAB, tig, cbrR,* and *sap* operons in *C. jejuni* 81–176. The identified ORFs are indicated by boxed arrows. The corresponding gene loci in *C. jejuni* NCTC 11168 are described in boxed arrows. The locations of major primers used in this study are indicated by arrows. The location and orientation of antibiotic resistance cassette are indicated below each inactivated gene. For *sap* operon, the aa identity of the putative sap gene to its homolog in *S. enreica serovar* Typhimurium LT2 is listed in parentheses.

performed. The significance of differences in susceptibility was determined using the Student's *t*-test.

CHICKEN COLONIZATION EXPERIMENT

Because *cbrR* mutant has been evaluated for its colonization ability in a previous study (Raphael et al., 2005), only *sapB* as well as *tig* and *cjaB* mutants were evaluated together with their parent strain 81–187 in this study. The chicken study was approved by the Institutional Animal Care and Use Committee at The University of Tennessee. Briefly, one-day-old broiler chickens were obtained from a commercial hatchery (Hubbard Hatchery, Pikesville, TN). The chickens were negative for *Campylobacter* as determined by culturing cloacal swabs prior to use in this study. These chickens were randomly assigned into three treatment group (10 or 11 chicks/group). At four days of age, each chicken was orally inoculated with a 1:1 mixture of wild type 81–176 (JL242) and its isogenic *cjaB* mutant (group I), *tig* mutant (group II), or *sapB* mutant (group III), with a dose of approximately 10^7 CFU of bacteria per chick. For each group, five birds were euthanized and cecal contents were collected at 3, and 10 days post-inoculation (DPI). The cecal contents from each bird were weighed and diluted in MH broth. The cecal suspensions were duplicate plated onto MH agar plates with *Campylobacter*-specific selective supplements (Oxoid, UK) for total *Campylobacter* enumeration and onto selective plates supplemented with appropriate antibiotics (30 μg/ml of Kan or 6 μg/ml of Cm) for the specific mutant numbers in each sample. The plating media were tested prior to use to ensure that they supported the growth of the mutant strains. Notably, before inoculation, the motility of the wild-type and its isogenic mutants were confirmed to be at a comparable level.

The number of CFU per gram of cecal contents was calculated for each chicken and was used as an indicator of the colonization level. The detection limit of the plating methods was 100 CFU/g of cecal contents. The bird from which no *Campylobacter* colonies were detected was assigned a conservative value of 99 CFU/g of cecal contents for the purpose of calculating means and for statistical analysis. Student's *t*-test was used to examine the significance of differences in *Campylobacter* colonization levels (log transformed CFU). A *P*-value of <0.01 was considered significant.

RESULTS

EMERGENCE OF ACQUIRED F1 RESISTANCE IN *Campylobacter*

Emergence of acquired AMP resistance is an important issue related to bacterial pathogenesis and development of sustainable peptide antibiotics (Yeaman and Yount, 2003; Hancock and Sahl, 2006; Peschel and Sahl, 2006). Previous studies suggested that high-level resistance to host endogenous AMP is difficult to develop (Peschel and Sahl, 2006), which is also supported by our recent *Campylobacter* work on the development of acquired resistance to polymyxin B (a widely used model peptide) and bacteriocins (the AMPs produced by bacteria) (Lin et al., 2009; Hoang et al., 2011a,b). Thus, in this study we first performed a screening to determine if high-level F1 resistance is observed in various *Campylobacter* isolates. Standard MIC test using MH broth showed that majority of *C. jejuni* and *C. coli* isolates exhibited low MIC to F1, ranging from 4 μg/ml to 8 μg/ml. Among the 154 tested *C. jejuni* isolates, 58 and 85 showed MICs of 4 μg/ml and 8 μg/ml, respectively. Only one *C. jejuni* strain showed slightly higher MIC of 16 μg/ml and only 6.5% of *C. jejuni* isolates (10 out of 154) showed MICs $\leq$ 2 μg/ml (eight isolates with MIC of 2 μg/ml and 2 with MIC of 1 μg/ml). *C. coli* showed similar pattern as *C. jejuni* and displayed MICs of 0.5 μg/ml (1 isolate), 1 μg/ml (three isolates), 2 μg/ml (three isolates), 4 μg/ml (eight isolates), and 8 μg/ml (five isolates). Together, the MIC survey here showed that none of the strains displayed high-level acquired resistance to F1.

Our recent *Campylobacter* studies (Lin et al., 2009; Hoang et al., 2011a,b) and work in other bacteria have demonstrated that availability of AMP resistant mutant was very helpful for examination of acquired AMP resistance using functional genomics approaches, such as random transposon mutagenesis and microarray. Thus, in this study we also intended to obtain an *in vitro* selected F1 resistant mutant. Different *in vitro* selection methods consistently led to the emergence of F1 resistant colonies on the selective plates containing 32, or 64 μg/ml of F1. Total 24 colonies were randomly selected for MIC test together with their parent strain 81–176. However, after being cultured in F1 free MH broth, none of these isolates displayed higher MIC than 81–176 for F1, indicating all these mutants were false-positive. Despite extensive efforts, no mutant with stable acquired F1 resistance was selected *in vitro*. In summary, these findings strongly suggest that endogenous AMPs are not favorable for the development of high-level acquired AMP resistance in *Campylobacter*.

IDENTIFICATION OF GENETIC LOCI CONTRIBUTING TO F1 RESISTANCE BY RANDOM TRANSPOSONE MUTAGENESIS

Although we failed to obtain *C. jejuni* mutants with acquired F1 resistance, examination of intrinsic AMP resistance in wild-type *C. jejuni* strains would fill a significant knowledge gap and also likely shed light on the mechanisms of acquired AMP resistance in *Campylobacter*. Thus, in this study a library containing 4800 Kan^r mutants were generated for screening the mutants with an increased susceptibility to F1. Three mutants displaying increased sensitivity to F1 were identified. Backcrossing of the transposon mutations into *C. jejuni* 81–176 further confirmed that the F1 sensitive phenotype in each mutant was linked to the gene with a specific transposon insertion. Direct sequencing of the mutant genomic DNA using transposon-specific primers showed the EZ::TN <KAN-2> transposon inserted into *Cj0981c*, *Cj0193c*, and *Cj0643* (**Figure 1**). All the transposon insertions occurred in the coding regions of corresponding genes. The orientations of Kan^r cassette within transposon in each mutant were the same as the corresponding disrupted gene (**Figure 1**). The *Cj0981c*, *Cj0193c*, and *Cj0643* encode transporter CjaB, putative trigger factor Tig, and the response regulator CbrR, respectively. F1 killing assay further confirmed that the insertional inactivation of *cbrR*, *tig*, or *cjaB* led to significantly increased susceptibility of *C. jejuni* 81–176 to F1 (**Figure 2A**). This phenotype was also observed in other strain background (*C. jejuni* NCTC 11168 and S3B) (**Figure 2B**). However, mutation of these genes did not confer significantly enhanced susceptibility of these mutants to other tested AMPs (data not shown).

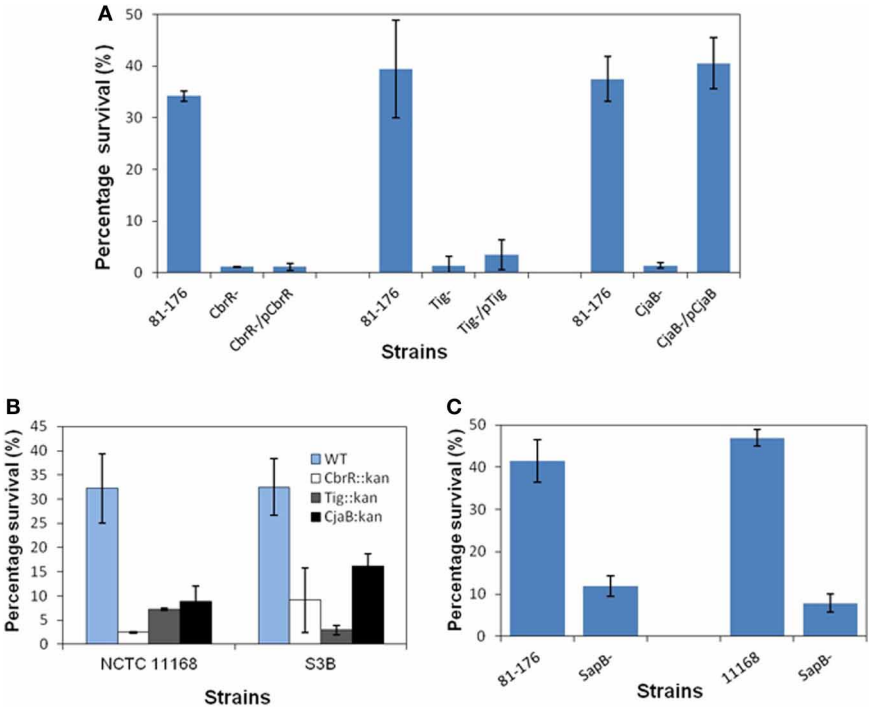

FIGURE 2 | Role of *cbrR, tig, cjaB* and *sapB* in F-1 resistance in different *C. jejuni* strains. (A) Susceptibilities of wild-type 81–176 and its *cbrR, tig,* and *cjaB* mutant constructs to F1. **(B)** Effects of mutations in *cbrR, tig,* and *cjaB* on the susceptibilities of *C. jejuni* NCTC 11168 and S3B to F1. **(C)** Inactivation of *sapB* increased susceptibilities of 81–176 and NCTC 11168 to F1. Log-phase cells in MH broth were supplemented with F1 at final concentration of 8 μg/ml and incubated for 2 h at 42°C under microaerophic condition. Samples were diluted and plated on MH agar plates to determine bacterial viability. Percentage survival was calculated by dividing the CFU number of bacteria incubated with AMP relative to those incubated in the presence of MH broth and then multiplied by 100. Each data point represents the mean value obtained from two independent experiments with triplicate measurements in each independent experiment.

Complementation of *cjaB* mutation in JL657 completely restored F1 resistance level back to the level of parent strain 81–176 (**Figure 2A**), which is consistent with the report that the *cjaB* and its upstream gene *cjaA* formed an operon (Wyszynska et al., 2006; see **Figure 1**). Complementation of CbrR and Tig mutant had little effect on sensitivity of the mutants to F1 (**Figure 2A**), likely due to the polar effect of the transposon mutation on the downstream genes in the same operon. The *cbrR*, a gene encoding a two-component response regulator required for bile salt resistance of *Campylobacter* (Raphael et al., 2005), appears to form an operon together with its nine downstream genes (**Figure 1**). For example, the stop codon of *cbrR* and start codon of *tatD* overlap by 10 nucleotides (**Figure 1**). There is no intergenic space between the stop codon of *tig* and the start codon of downstream clpP, suggesting these two genes are organized into an operon. The *clpP* is separated from its downstream gene *def* by a 28 bp intergenic region which is predicted to contain promoter sequence. Thus, *tig* operon likely contains two genes (*tig* and *clpP*) (**Figure 1**). RT-PCR using specific primers (**Table 2**) showed that *clp* and *tatD*/*Cj0645* were barely transcribed in *tig* and *cbrR* mutants, respectively, while all the genes were expressed normally in wild-type 81–176 (data not shown), indicating that the insertional mutation in *tig* and *cbrR* caused a polar effect on the downstream genes.

SapB CONTRIBUTES TO F1 RESISTANCE IN *C. jejuni*

The *sap* (sensitivity to antimicrobial peptide) operon confers resistance to AMP-mediated killing of different pathogens such as *Salmonella* (Parra-Lopez et al., 1993) and *Haemophilus influenza* (Mason et al., 2005) although the exact mechanisms of how Sap transporters protect the cells from AMP attack remain unclear. Analysis of NCTC 11168 genome revealed that the operon *Cj1580c-Cj1584c* shared homology to the identified *sap* operon in *S. enreica serovar* Typhimurium LT2 with identical genetic components and organization, which include (**Figure 1**), in which *sapA* (*Cj1584c*) encodes a putative periplasmic peptide binding protein and *sapB* (*Cj1583c*) encodes a membrane permease. Comparative genomic analysis of published *C. jejuni* genomes showed that the putative *sapABCDF* operon is highly conserved in *C. jejuni* with nucleotide sequence identity ranging from 98 to 100%. This observation was further confirmed by our PCR survey using *sapB* specific primer pair (Sap4-F/R, **Table 2**) and *sapA* specific primer pair (Sap5-F/R, **Table 2**) for 27 diverse *Campylobacter* strains (21 *C. jejuni* strains and 6 *C. coli* isolates), in which majority of strains contain *sap* operon as reflected by the positive PCR results (23 out of 27).

Isogenic SapA, SapB, SapD, and SapF mutants of 81–176 were successfully created in *C. jejuni* 81–176 (**Table 1**). Mutation in SapC could not be generated after repeated attempts. RT-PCR

analysis using specific primers (**Table 2**) indicated that insertional inactivation using Cm resistance gene marker did not cause polar effect on the transcription of the genes (*Cj1582c*, *Cj1581c*, and *Cj1580c*) downstream of specific mutated gene (data not shown). As shown in **Figure 2C**, inactivation of SapB caused significantly in increased sensitivity to F1 in both 81–176 and 11168 strain background. However, mutation of other individual *sap* genes including *sapA* (*Cj1584c*), *sapD* (*Cj1581c*), and *sapF* (*Cj1580c*) (**Figure 1**) did not alter sensitivity of the mutants to F1 (data not shown). In addition, isogenic *sapB* mutant did not display significantly increased susceptibilities to other tested AMPs with diverse sequence and structure (data not shown).

INACTIVATION OF SapB, CjaB, AND Tig REDUCED *Campylobacter* COLONIZATION IN CHICKENS

It has been demonstrated that *cbrR* is required for optimal colonization of *Campylobacter* in chickens (Raphael et al., 2005). In this study, we compared colonization ability of the isogenic *sapB*, *cjaB*, and *tig* mutants to their parent strain 81–176 using mixed infection in chicken. As shown in **Figure 3**, inactivation of the *sapB*, *cjaB*, or *tig* gene greatly impaired the colonization ability of *C. jejuni* 81–176 in chickens. Specifically, when 81–176 and its specific isogenic mutant were co-inoculated into a group of chickens, the colonization level of the specific mutant was significantly lower than that of 81–176 at 3 and 10 days post-inoculation (DPI). In particular, by 10 DPI, the specific mutant of *C. jejuni* 81–176 (*tig*$^-$, *cjaB*$^-$, or *sapB*$^-$ mutant) was no longer detected in any of the cecal samples collected from the chickens inoculated with mixture of 81–176 and its isogenic mutant. It is important to mention that both 81–176 and the isogenic mutants showed similar growth patterns in MH broth (data not shown). We also performed an identical *in vitro* competition assay in MH broth; all the mutants were not outcompeted by 81–176 and the isogenic mutant displayed an *in vitro* competitive index (ratio of the mutant to wild-type strain at early stationary phase) of about 1, indicating that growth rate of these mutants is similar to its wild-type 81–176.

DISCUSSION

Development of AMP resistance is an important strategy utilized by many bacterial pathogens including various enteric bacteria to evade host innate immunity and to colonize in various host niches successfully (Ernst et al., 2001; Yeaman and Yount, 2003; Peschel and Sahl, 2006). However, it is still largely unknown how important AMP resistance is for *Campylobacter* infection and what is the molecular basis of AMP resistance in *Campylobacter*.

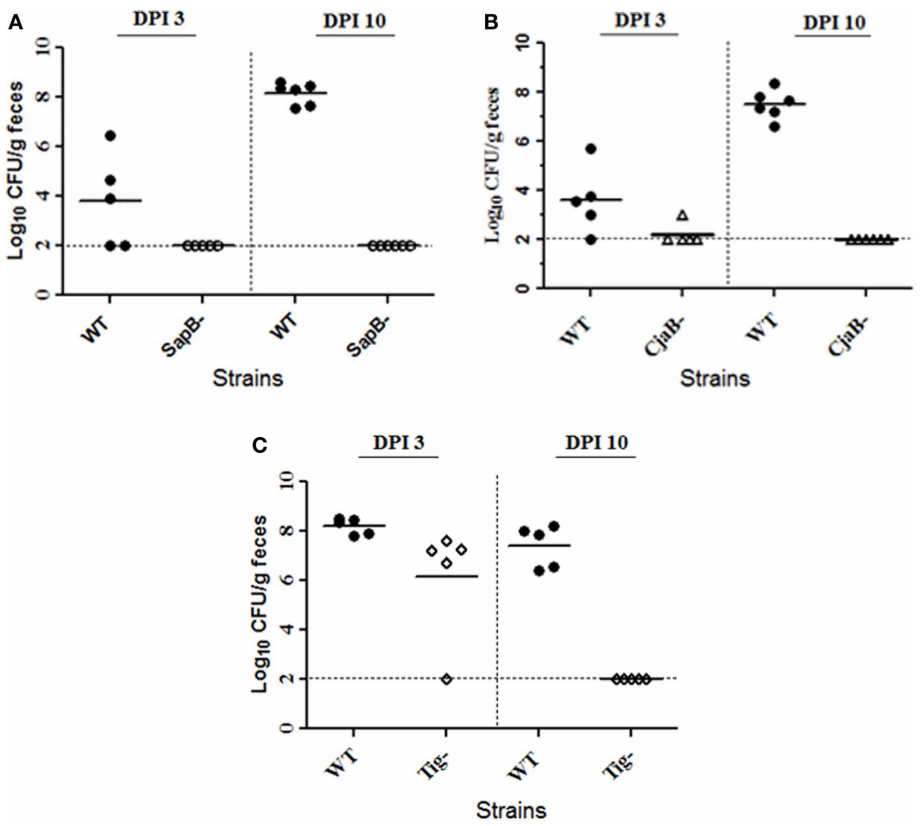

FIGURE 3 | Inactivation of SapB, CjaB, or Tig impaired *C. jejuni* 81–176 colonization of chickens. Three chicken groups were inoculated with a 1:1 mixture of wild type 81–176 and its isogenic SapB mutant **(A)**, CjaB mutant **(B)**, or Tig mutant **(C)**. For each group, five to six birds were euthanized at the indicated days post-inoculation (DPI) and the cecal contents were collected and used for CFU enumeration. Each symbol indicates the $\log_{10}$ number of CFU/g of cecal contents for a single chicken. The horizontal bars indicate the means of groups at the indicated times. The horizontal dash line indicates the limit of detection.

To date, there are no any animal knockout experiments demonstrating the critical role of specific host AMP in defense against *Campylobacter*. However, it has been well established that AMPs (primarily defensins and cathelicidins) are a major component of host innate immunity systems against enteric pathogens. For example, mice deficient in cathelicidin or defensins production are more susceptible to intestinal infections by enteric pathogens (Wilson et al., 1999; Iimura et al., 2005). Thus, both defensins and cathelicidins are important AMPs expressed in the small intestine to protect host against pathogens (Wehkamp et al., 2007). Zilbauer et al. (2005) also observed that epithelial AMPs were induced in human intestinal epithelia upon infection by *C. jejuni*. In chickens, although different AMPs including the F1 used in this study are expressed in the intestine, the concentrations of specific AMP in any of intestinal niches are still unknown. Specific knock out model should be developed to determine if chicken deficient in F1 production is more susceptible for *Campylobacter* infection. Notably, colonization of *Campylobacter* in chicken and other hosts is determined by delicate interactions between *Campylobacter* and host in which many determinants in addition to AMP are involved. However, given the intimate interaction of *Campylobacter* with intestinal mucosa and the intracellular stage during systemic *Campylobacter* infection in humans (invasion of epithelial cells and engulfment by phagocytes), *Campylobacter* should have co-evolved with host innate defense and developed means to curtail the effect of endogenous AMPs for successful colonization as observed in other enteric pathogens (Ernst et al., 2001; Yeaman and Yount, 2003; Peschel and Sahl, 2006). However, mechanisms of AMP resistance in *Campylobacter* are still largely unknown.

We have initiated determination of AMP resistance mechanisms in *Campylobacter* using polymyxin B as a model peptide and have defined seven genetic loci (e.g., *galU* required for LOS production) contributing to *C. jejuni* resistance to polymyxin B (Lin et al., 2009). Recent work also showed that production of specific LOS (Naito et al., 2010) and lipidA modification (Cullen and Trent, 2010; van Mourik et al., 2010) were required for polymyxin B resistance in *C. jejuni*, likely due to their role in electrostatic shielding against polymyxin B. Polymyxin B has been successfully used as a model peptide to study AMP resistance in many Gram-negative pathogens although polymyxin B bears little structural resemblance to the host defense AMPs (Ernst et al., 2001; Yeaman and Yount, 2003; Peschel and Sahl, 2006). However, our previous study (Lin et al., 2009) strongly suggested that polymyxin B may not be a good surrogate for studying AMP resistance in *Campylobater*. In addition, examination of *Campylobacter* resistance to bacteriocins revealed a different set of genes which are not involved in resistance to polymyxin B and other endogenous AMPs (Hoang et al., 2011a). Therefore, direct usage of natural host AMP as a model peptide may generate physiologically relevant information for *Campylobacter* pathogenesis. We chose to use F1 as a surrogate of host defense peptides because of following reasons. First, unlike defensins, the short α-helical fowlicidin-1 (F1) can be easily synthesized at reasonable cost for large scale screening. Second, cathelicidins including F1 are important AMPs expressed in intestine and displayed potent bactericidal effect (Lynn et al., 2004; Xiao et al., 2004, 2006; Peschel and Sahl, 2006; Bommineni et al., 2007). In addition, some genes (e.g., *phoPQ*) required for cathelicidin resistance could also involve resistance to structurally unrelated AMPs such as defensins (Ernst et al., 2001; Winfield and Groisman, 2004). Thus, genes contributing to F1 resistance may be important for *in vivo* colonization of *C. jejuni* in the host. Finally, F1 is produced by chicken, an animal model used in this study, which would make us well correlate the findings obtained from *in vitro* and *in vivo* studies.

In this study, inactivation of SapB, CjaB, Tig, and CbrR led to increased susceptibilities to F1 in different *C. jejuni* strain background. In addition, chicken experiment also showed impaired colonization ability of these mutants in the intestine. The Sap operon encode ABC-type transporter system and have been demonstrated to involve AMP resistance in various Gram-negative bacteria; mutation of the *sap* operon also resulted in impaired colonization in animal models (Parra-Lopez et al., 1993; Lopez-Solanilla et al., 1998; Chen et al., 2000; McCoy et al., 2001; Lupp et al., 2002; Mason et al., 2005, 2006; Mount et al., 2010). The exact mechanisms of how Sap transporters protect the cells from AMP attack remain unclear. In this study, we demonstrated the role of *sapB* in *Campylobacter* resistance to F1; however, mutation of *sapB* in *C. jejuni* did not lead to significantly increased susceptibilities to other natural AMPs. We should interpret this finding cautiously. The *C. jejuni sap* may still interact to other structurally unrelated AMPs with lower efficiency than F1; however, limited expression level of Sap in our *in vitro* killing assay system may diminish the susceptibility difference between Sap mutant and wild-type strain. Mason et al. (2003, 2005) reported that *sap* expression of *H. influenza* is greatly up-regulated *in vivo*. Therefore, the *sap* operon of *Campylobacter* is possible up-regulated during *in vivo* infection and such enhanced expression of *sap* may ultimately confer *C. jejuni* resistance to various endogenous AMPs, leading to greatly impaired intestinal colonization observed in this study (**Figure 3A**). This hypothesis needs to be examined in the future. In terms of CjaAB operon, our chicken study has clearly showed its role in *C. jejuni* colonization in the intestine, likely due to its contribution to AMP resistance. The CjaB was predicted as an inner membrane transporter with unknown function in *Campylobacter* (Wyszynska et al., 2006). The underlying mechanism for CjaAB conferring F1 resistance is not clear. It has been observed that efflux pumps are involved in AMP resistance in various bacteria (Yeaman and Yount, 2003; Peschel and Sahl, 2006). Recently, we also demonstrated that CmeABC efflux pump is required for *Campylobacter* resistance to bacteriocins (Hoang et al., 2011a). Therefore, it is likely that CjaAB function as an efflux pump to extrude F1 out of cells for resistance. However, it is equally possible that CjaAB may function as an influx transporter and facilitate transportation of F1 to *Campylobacter* cytoplasm where F1 is targeted for degradation.

Given the failure of complementation and lack of expression of the genes downstream of the inactivated *tig* and *cbrR*, the phenotypes of isogenic *tig* and *cbrR* mutants observed in this study are likely caused by other gene(s) in the same operon or the coordination of all genes within the operon (**Figure 1**). Trigger factor Tig was found highly conserved in eubacteria,

functioning as a chaperon to interact with newly synthesized polypeptides, assist protein folding, and plays a vital role in bacterial virulence (Rassow and Pfanner, 1996; Martinez-Hackert and Hendrickson, 2009). The gene immediately downstream of *tig* in *Campylobacter* was annotated as *clpP* that function as a protease to degrade misfolding peptides in other bacteria (Frees et al., 2007). Notably, the ClpP homolog of *Bacillus anthracis* has been demonstrated to be involved in resistance to AMPs human cathelicidin LL-3 (McGillivray et al., 2009). Therefore, since degradation of AMPs by protease is one of mechanisms used by bacteria to resist endogenous AMPs (Yeaman and Yount, 2003), the *C. jejuni* ClpP may involve proteolytic cleavage and play a role in virulence by mediating the role in AMP resistance. This hypothesis needs to be examined in the future. Recently, Cohn et al. (2007) has characterized the *C. jejuni* ClpP and observed that ClpP influenced heat tolerance of *C. jejuni*. Therefore, the impaired colonization of *tig* mutant observed in this study also could be attributed to the temperature sensitivity due to inactivation of ClpP. Similarly, *cbrR* and nine of its downstream genes appear to organize into an operon (**Figure 1**) and some downstream genes may be involved in AMP resistance in *C. jejuni*. For example, *Cj0649* encodes a beta-barrel LptD-like protein and the LptD in *Pseudomonas aeruginosa* appeared to act as a dominant resistance marker against cathelicidins likely due to its function in the assembly of LPS in the outer leaflet of the outer membrane (Srinivas et al., 2010). However, the two component regulators identified in this study (CbrR) and in our recent work (RacR) (Lin et al., 2009) may still involve *Campylobacter* resistance to AMPs. In response to environmental cues via two-component regulatory systems (e.g., PhoP/PhoQ), Gram-negative bacteria can add covalent modification to LPS and consequently reduce the negative charge and/or membrane fluidity of LPS and protect themselves from attack by AMPs, which has been a major AMP resistant mechanism in enteric bacteria (Ernst et al., 2001; Yeaman and Yount, 2003; Peschel and Sahl, 2006). Mutations occurred in two-component regulators could result in sustained, acquired AMP-resistance. To hunt potential two-component regulators required for AMP resistance, we have performed sequencing of nine regulators, five histidine kinases, and six signal transduction systems in polymyxin B resistant mutant; but no sequence difference was observed in all selected regulators between wild-type 81–176 and its polymyxin B resistant derivatives (Hoang, 2010). Despite accumulating studies on the functional characterization of two-component regulators in *C. jejuni*, it is still unknown which environmental signals to which these regulators respond. We speculate that some two-component regulatory systems in *C. jejuni* can sense and integrate multiple environmental cues in the host into a coordinated cellular response and promote *Campylobacter* resistance to AMPs in different host niches. Testing this hypothesis relies on the identification of specific cues activating two-component regulatory systems, a challenge issue to elucidate physiology and pathogenesis role of two-component regulatory systems in bacterial pathogens.

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Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism?

Tadhg Ó Cróinín* and Steffen Backert*

UCD School of Biomolecular and Biomedical Sciences, University College Dublin, Dublin, Ireland

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Hazel Marjory Mitchell, The University of New South Wales, Australia
Michael Konkel, Washington State University, USA

****Correspondence:***
Tadhg Ó Cróinín and Steffen Backert, UCD School of Biomolecular and Biomedical Sciences, University College Dublin, Belfield Campus, Dublin-4, Ireland.
e-mail: tadhg.ocroinin@ucd.ie; steffen.backert@ucd.ie

Campylobacter jejuni, a spiral-shaped Gram-negative pathogen, is a highly frequent cause of gastrointestinal foodborne illness in humans worldwide. Clinical outcome of *C. jejuni* infections ranges from mild to severe diarrheal disease, and some other complications including reactive arthritis and Guillain–Barré syndrome. This review article highlights various *C. jejuni* pathogenicity factors, host cell determinants, and proposed signaling mechanisms involved in human host cell invasion and their potential role in the development of *C. jejuni*-mediated disease. A model is presented which outlines the various important interactions of *C. jejuni* with the intestinal epithelium, and we discuss the pro's and con's for the "zipper" over the "trigger" mechanism of invasion. Future work should clarify the contradictory role of some previously identified factors, and should identify and characterize novel virulence determinants, which are crucial to provide fresh insights into the diversity of strategies employed by this pathogen to cause disease.

Keywords: molecular pathogenesis, cellular invasion, signaling, virulence

INTRODUCTION

Campylobacter jejuni and related species are commensals in many birds and domestic mammals, and have been recognized as being the most important cause of bacterial food poisoning worldwide. Infections with *C. jejuni* are the major cause of human bacterial gastroenteritis, and may be responsible for as many as 400–500 million cases worldwide each year (Friedman et al., 2000). Disease outcomes vary from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea lasting for several weeks, but *C. jejuni* is also associated with the development of the reactive arthritis and peripheral neuropathies, the Miller–Fisher and Guillain–Barrè syndromes in a minority of individuals (Young et al., 2007). The publication of numerous complete genome sequences of different *C. jejuni* strains has revealed an organism that displays a large degree of strain to strain variation. This natural heterogeneity has made studying the pathogenicity of this pathogen particularly challenging. However, significant progress has been made in recent years which has contributed to our understanding of the role of several key factors including the cytolethal distending toxin (Lara-Tejero and Galán, 2000; Ge et al., 2008) as well as glycosylation and molecular mimicry processes (Guerry and Szymanski, 2008; Nothaft and Szymanski, 2010). One of the key differences between infection of humans and chickens by *C. jejuni* is the apparently increased number of bacteria invading epithelial cells in the human host (Young et al., 2007). This suggests that both bacterial adherence to and entrance into epithelial cells may be critical steps that are essential for disease development. Thus, the identification of factors involved in these processes is the key for developing therapeutics to treat infections as well as enhancing our understanding of the pathogenesis.

There are two general strategies which the multitude of enteric bacterial pathogens use to enter host target cells. According to specific characteristics of the invasion process, we can distinguish between the classical "zipper"- and "trigger"-mechanisms, respectively (Cossart and Sansonetti, 2004). The "zipper"-mechanism is initiated by one or more bacterial surface proteins (commonly comprising adhesins and invasins) which bind to one or more specific host cell receptors followed by internalization, as reported for *Yersinia* or *Listeria* species (**Figure 1A**). On the other hand, the "trigger"-mechanism involves type-III and type-IV secretion systems (T3SSs and T4SSs) injecting bacterial proteins which often mimic or hijack specific host cell factors to trigger the bacterial uptake process, as described for *Salmonella* and *Shigella* (**Figure 1B**). Genome analyses revealed a notable absence of these classical pathogenicity factors in *C. jejuni*, making predictions very difficult. Since a suitable animal model system mimicking human infection is not available, a wide variety of *in vitro* cell culture models have been applied to identify the *C. jejuni* factors that play a role in adherence and invasion (**Table 1**). Unfortunately, the use of different *C. jejuni* strains and various cell models of infection led to substantial confusion and controversies in the literature. This review aims to summarize recent developments and to outline the experimental evidence for factors with proposed roles in adhesion and invasion. We discuss the pro's and con's of these findings in order to see whether *C. jejuni* may utilize a "zipper" or "trigger" mechanism of host cell invasion.

BACTERIAL MOTILITY AND ROLE OF THE FLAGELLA

Campylobacter jejuni is a highly motile organism with bipolar flagella which has been reported to be essential for colonization both in humans and animal models (Guerry, 2007). The organisms regulate motility through a complex chemotaxis system that allows to swim toward attractants and away from repellants (Lertsethtakarn et al., 2011). Early studies revealed that *C. jejuni* motility is required for invasion of epithelial cells *in vitro* as well as showing that increases in mucosal viscosity led to enhanced motility,

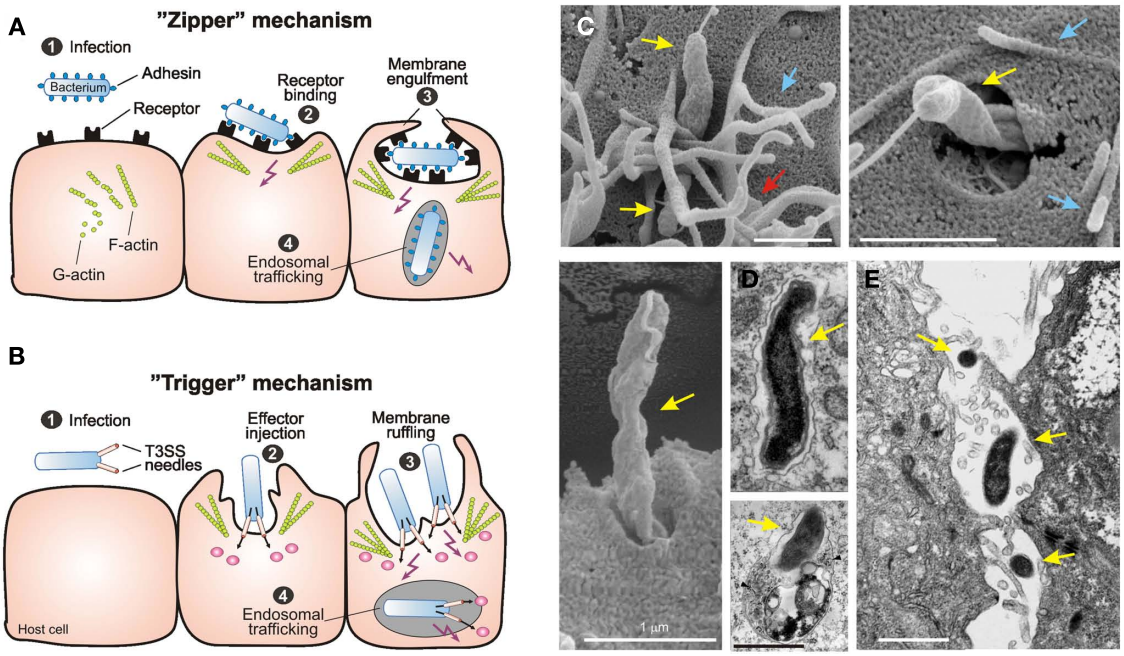

FIGURE 1 | Primary mechanisms of bacterial invasion into non-phagocytic host cells. Schematic representation of the two different routes of entry by intracellular bacterial pathogens. The pathogens induce their own uptake into target cells by subversion of host cell signaling pathways using the "zipper" and "trigger" invasion mechanism, respectively. **(A)** Bacterial gastrointestinal pathogens commonly colonize the gastric epithelium [step 1]. The "zipper" mechanism of invasion involves the high-affinity binding of bacterial surface adhesins to their cognate receptors on mammalian cells [step 2], which is required to initiate cytoskeleton-mediated zippering of the host cell plasma membrane around the bacterium [step 3]. Subsequently the bacterium is internalized into a vacuole. Some bacteria have developed strategies to survive within, or to escape from this compartment [step 4]. **(B)** The "trigger" mechanism is used by *Shigella* or *Salmonella* spp. which also colonize the intestinal epithelium [step 1]. These pathogens use sophisticated type-III or type-IV secretion system to inject various effector proteins into the host cell cytoplasm [step 2]. These factors manipulate a variety of signaling events including the activation of small Rho GTPases and cytoskeletal reorganization to induce membrane ruffling and subsequently bacterial uptake [step 3]. As a consequence of this signaling, the bacteria are internalized into a vacuole [step 4], followed by the induction of different signaling pathways for intracellular survival and trafficking. This figure was adapted from Tegtmeyer et al. (2011) with kind permission from Springer Publishing. **(C)** Scanning electron microscopy of *C. jejuni* 81-176 invasion. Invading bacteria (yellow arrows) were regularly associated with membrane ruffles (red arrows) and filopodia-like structures (blue arrows). This figure was adapted from Boehm et al. (2012). **(D)** Electron micrographs of *C. jejuni*–containing vacuoles (CCVs) that do not co-localize with BSA-gold (left) and CCVs that co-localize with BSA-Gold and resemble lysosomes (right, arrows) are shown. The two pictures were kindly provided by Dr. Galan (Watson and Galán, 2008). **(E)** The electron micrograph of translocating *C. jejuni* across polarized Caco-2 cells by the paracellular pathway was kindly provided by Dr. Konkel (Konkel et al., 1992b).

adherence, and invasion (Szymanski et al., 1995). This apparent direct association between motility and invasion combined with the absence of an identifiable classical T3SS or T4SS led some groups to speculate that the flagellum could be also be used as a secretory device for invasion-associated effector molecules. In fact, the flagellum represents a T3SS by definition (Desvaux et al., 2006), and a series of papers have been published reporting the extracellular release of *C. jejuni* proteins in the presence of fetal calf serum (FCS) or upon host cell contact, which was abolished when crucial flagellar apparatus genes were inactivated. Additional support came from secretion experiments of *Yersinia* or *C. jejuni* virulence factors through their respective flagellar T3SS model system, suggesting that type-III protein secretion by the flagellum may be a general mechanism for the transport of proteins that influence bacterial–host interactions (Young et al., 1999; Christensen et al., 2009; Neal-McKinney et al., 2010).

The first identified secreted factor was the *Campylobacter* invasion antigen B (CiaB), a 73-kDa protein with weak homology to T3SS effectors of other pathogens (Konkel et al., 1999). This paper showed that although *ciaB* mutants had no reduction in adherence to non-polarized INT-407 cells, a significant reduction of intracellular bacteria was observed. CiaB expression was also shown to be essential for the secretion of a whole family of other secreted Cia proteins that were induced in the presence of FCS (Rivera-Amill and Konkel, 1999). The CiaB protein was reported to be translocated into the cytoplasm of host cells, suggesting that it is a true effector molecule facilitating invasion (Konkel et al., 1999). A later study using a variety of mutants in flagellar subunits reported that the CiaB protein appeared to require at least one of the flagellar subunits for its secretion (Konkel et al., 2004). CiaB synthesis is reported to be induced by bile salts (Malik-Kale et al., 2008) and both synthesis and secretion can be induced by host cell components suggesting that the protein is under strict environmental regulation (Rivera-Amill et al., 2001). However, a very recent study has reported no significant reduction in invasion by a *ciaB* mutant in the model strain 81-176 suggesting that further work is required to confirm the role played by this protein during infection (Novik et al., 2010). Since the discovery of CiaB we have

Table 1 | Bacterial factors and proposed roles in *C. jejuni* infection.

Bacterial factor	Proposed function	Applied experimental methods	Strains used	Cell system used	Reference
AspA, AspB	Aspartate ammonia lyase and amino transferase	Infection *in vitro*, GPA, ISA	81-176	T84	Novik et al. (2010)
CadF	Adhesin to fibronectin	Infection *in vitro* and in chickens, GPA, FBA, CBA, ELISA	F38011, 11168, 81-176	INT-407, T84, LMH	Konkel et al. (1997), Ziprin et al. (1999), Monteville et al. (2003), Scott et al. (2010)
CapA	Adhesin/Invasin	Infection *in vitro* and chickens, GPA	11168, F38011	Caco-2, LMH	Ashgar et al. (2007), Flanagan et al. (2009)
CDT	Cytolethal distending toxin	Treatment of cells *in vitro*, MI, FACS, IFM, DNAse assays	81-176	COS-1, REF52, Henle-407	Lara-Tejero and Galán (2000)
CiaB, CiaC	Invasin	Infection *in vitro*, GPA, MLA, T3SS assays	F38011, 11168	INT-407	Konkel et al. (1999), Christensen et al. (2009)
CiaI	Intracellular survival	Infection *in vitro*, GPA, MLA, IFM	F38011	INT-407, HeLa	Buelow et al. (2011)
CJ0977[a]	Invasion	Infection *in vitro* and ferrets, EM, MA, GPA	81-176	INT-407	Goon et al. (2006)
CstII	LOS sialylation	Infection *in vitro*, GPA	GB2, GB11, GB19	Caco-2, T84	Louwen et al. (2008)
FlaC	Invasin	Infection *in vitro*, MA, EM, cell fractionation, GPA, IFM	TGH9011	HEp-2	Song et al. (2004)
FlpA	Adhesin to fibronectin	Infection *in vitro* and in chickens, FBA, ABB, GPA, IFM, ELISA	F38011	INT-407, Hela, LMH	Flanagan et al. (2009), Konkel et al. (2010), Eucker and Konkel (2012)
FspA	Apoptosis	BRP, AA	81-176, CG8486	INT-407	Poly et al. (2007)
GGT	Gamma-glutamyl transpeptidase	Infection *in vitro*, mice and chickens, cell fractionation, MA, GPA, HPS, AA	RM1221, 81-176, 81116, 11168	INT-407, CCD841 CoN	Hofreuter et al. (2006, 2008), Barnes et al. (2007)
HtrA	Periplasmic protease and chaperone	Infection *in vitro*, GPA	11168	INT-407	Baek et al. (2011)
JlpA[b]	Adhesin to HSP90-α, proinflammatory responses	Infection *in vitro*, GPA, BRP, ABB, ligand overlays, geldanamycin inhibitor, p38/NFκB activation using AABs	TGH9011	HEp-2	Jin et al. (2001), Jin et al. (2003)
KpsE, KpsM, KpsT	Capsule proteins, invasion	Infection *in vitro*, infection of chicken and ferrets, GPA	81-176, 81116	INT-407	Bacon et al. (2001), Bachtiar et al. (2007)
Peb1, Peb3, and Peb4[c]	Transport proteins and chaperones	Infection *in vitro* and in mice, BRP, GPA	81-176, 11168	Hela, INT-407	Leon-Kempis Mdel et al. (2006), Min et al. (2009), Asakura et al. (2007), Kale et al. (2011)
PflA	Motility	Infection *in vitro*, GPA	81-176	INT-407	Yao et al. (1994)
PorA (MOMP)	Major outer membrane protein	Infection *in vitro*, BRP	K22, 1767	INT-407	Schröder and Moser (1997), Moser et al. (1997)
pVIR	Invasion	Infection *in vitro* and in ferrets, GPA	81-176, VC83	INT-407	Bacon et al. (2000)
SodB	Superoxide dismutase	Infection *in vitro* and in mice, GPA, ISA	81-176	T84	Novik et al. (2010)
VirK	Intracellular survival	Infection *in vitro* and in mice, GPA, IFM	81-176	T84, COS-7	Novik et al. (2009)

AA, apoptosis assay; AB, antibody; AAB, activation-specific antibodies; ABB, antibody blocking; BRP, binding assays using recombinant or purified protein; CBA, competitive binding assay; EM, electron microscopy; FACS, fluorescence-activated cell sorting; FBA, fibronectin binding assay; GPA, gentamicin protection assay; HPS, hydrogen peroxide susceptibility test; IFM, immunofluorescence microscopy; ISA, intracellular survival assay; MI, microinjection of proteins; MA, motility assay on agar; MLA, ^{35S}-methionine labeling assay; pVIR, mobilizable plasmid in some strains; T3SS assays, translocation assay using the Yersinia flagellar type-III secretion apparatus.

[a]Another report indicated that the Cj0097 mutant has a deficiency in motility in liquid broth (Novik et al., 2010).

[b]Identified as an adhesin in strain TGH9011, but no effect observed with jlpA mutants in either 11168 or 81-176 strains (van Alphen et al., 2008; Novik et al., 2010).

[c]These structural studies along with other assays suggest primary roles in protein transport. Originally, the Peb's were identified as putative adhesins (Pei et al., 1991).

seen the emergence of other potential Cia proteins which have been speculated to be important virulence factors. CiaC is such an example and reported to be essential for maximal invasion of INT-407 cells (Christensen et al., 2009; Eucker and Konkel, 2012). CiaI has also been recently identified as a protein secreted through the flagellar transport system, and appears to play a role in intracellular survival (Buelow et al., 2011). Another protein which has been postulated by one group to be secreted through the flagellum is FlaC. This protein has strong homology with the flagellin subunit proteins FlaA and FlaB, and has been shown to play a role in invasion using the HEp-2 *in vitro* cell model (Song et al., 2004). The FspA protein which has been shown to induce apoptosis in INT-407 cells has also been reported to be secreted through the flagellum (Poly et al., 2007).

CadF AND FlpA FIBRONECTIN BINDING PROTEINS

Perhaps the most well characterized *C. jejuni* factor interacting with host cells is the CadF protein. CadF was first identified as a 37-kDa protein that bound the extracellular matrix (ECM) protein fibronectin, in either immobilized or soluble form (Konkel et al., 1997). The sequence of the *cadF* gene product revealed a protein with homology to an adhesin from *Pseudomonas fluorescens*. In the same year another group confirmed the observation that recombinant CadF bound to fibronectin as well as reporting that the protein could bind to membrane fractions of INT-407 cells *in vitro* (Moser et al., 1997). A later study revealed the importance of CadF for colonization, as *cadF* mutants were unable to infect chickens (Ziprin et al., 1999). Using the polarized cell line T84 it was reported that *C. jejuni* crossed the epithelial layer between neighboring cells (paracellular route) and invasion might occur through the basolateral but not apical surface (Monteville and Konkel, 2002). This study also revealed that CadF expression was required for maximal T84 cell binding. A later study identified the fibronectin binding domain in CadF as four surface-exposed amino acids at position 134–137 (Konkel et al., 2005). Mutations in these residues significantly reduced binding to fibronectin and INT-407 cells while peptides containing this putative fibronectin binding site exhibited blocking capacity for binding. Interestingly, Krause-Gruszczynska et al. (2007a) have reported that a 39-bp insertion in the *cadF* gene of *C. coli* strains leads to a larger protein and that these strains bind and invade INT-407 cells less efficiently than *C. jejuni*. In *C. jejuni*, CadF expression has been reported to be downregulated in response to human mucin Muc2, suggesting that levels of expression of the protein may vary during the infection cycle (Tu et al., 2008). Interestingly, most strains of *C. jejuni* exhibit two bands in Western blots using anti-CadF antibodies, suggesting that distinct forms of the protein may exist. A recent study using mass spectroscopy proposed that CadF may undergo post-translational processing steps, which result in certain cases in the removal of the immunogenic epitope but retention of the fibronectin binding site (Scott et al., 2010). The exact role that these different CadF forms play during infection is not yet fully understood.

Recently, a second potential fibronectin binding protein has been identified in *C. jejuni* called FlpA. FlpA was first described as being a protein which played a role in adherence to chicken epithelial cells as well as playing a significant role in colonization of chickens (Flanagan et al., 2009). The protein was later described as being capable of binding fibronectin and that *flpA* mutants showed a significant decrease in binding to INT-407 cells as compared to the wild-type strain (Konkel et al., 2010). However, neither CadF nor FlpA or any Cia protein were found in a global screen for invasion-related genes in strain 81-176 (Novik et al., 2010). A recent study has proposed that CadF and FlpA may act together to target fibronectin for bacterial binding and subsequent invasion by *C. jejuni* (Eucker and Konkel, 2012). This indicates that certain additive effects of genes involved in cell adhesion and invasion may play a role. However, inhibitor studies using chloramphenicol showed that bacterial protein synthesis is required for maximal *C. jejuni* invasion (Konkel and Cieplak, 1992; Oelschlaeger et al., 1993). Chloramphenicol retards the expression of Cia proteins but not CadF or FlpA (Christensen et al., 2009; Eucker and Konkel, 2012), suggesting that constitutive expression of these adhesins alone is not sufficient for the bacterial uptake process.

ROLE OF JlpA

JlpA is a 42-kDa lipoprotein which can be *N*-linked glycosylated at two residues (Scott et al., 2009). This surface-exposed lipoprotein has also been implicated as an important adhesin for *C. jejuni*. An initial study in 2001 revealed that *jlpA* mutants exhibit a decreased ability to bind to HEp-2 cells (Jin et al., 2001). It was also reported in this study that binding of the wild-type strain could be inhibited by either pre-incubation with recombinant JlpA protein or antibodies raised against JlpA. A later study revealed that recombinant JlpA bound to HEp-2 cells and that the corresponding receptor appeared to be a 90-kDa protein, which was identified as a heat shock protein, HSP90-α (Jin et al., 2003). Furthermore, the *jlpA* gene has been shown to be upregulated in response to human mucin along with other *C. jejuni* genes suggesting the protein may play a key role in pathogenicity (Tu et al., 2008). However, two other studies have been unable to show a role for JlpA in adherence of *C. jejuni*. One study, using T84 cells, revealed a minimal reduction in invasion with a *jlpA* mutant when compared to the wild-type strain (Novik et al., 2010), while the other study which used a chicken epithelial cell line revealed no reduction in invasion of a *jlpA* mutant (Flanagan et al., 2009). This study also reported no defect in colonization of broiler chickens by a *jlpA* mutant. Whether these conflicting results are due to strain variation or differences in experimental approaches is not yet clear (**Table 1**).

ROLE OF Peb PROTEINS

One group of *C. jejuni* surface molecules that were originally implicated as being involved in adhesion to epithelial cells are the Peb proteins. These proteins were first described as "CBF proteins," which were observed to range from between 26 to 30-kDa and were shown to be present in bacterial outer membrane fractions which bound to Hela cells (Fauchere et al., 1989). These proteins were originally described as being major immunogenic antigens of *C. jejuni* (Pei et al., 1991). Peb1 (originally called CBF1) was first described as playing a key role in the adherence to epithelial cells (Fauchere et al., 1989). Later studies revealed that Peb1 was surface-exposed and showed that purified Peb1 bound to HeLa cell membranes *in vitro*, and that co-incubation with anti-Peb1 antibodies significantly reduced adherence of *C. jejuni* to intact HeLa

cells (Kervella et al., 1993). This role in adherence was confirmed when a *peb1* mutant was shown to display a clear reduction in adherence to HeLa cells, invasion of INT-407 cells as well as a significant reduction in its ability to colonize the mouse intestine as compared to the wild-type strain (Pei et al., 1998). However, later studies reported that a *peb1* mutant exhibited a minimal reduction in invasion of T84 cells or adherence to chicken epithelial cells, suggesting that the importance of Peb1 in adherence or invasion may vary depending on the assay and cell system used (Flanagan et al., 2009; Novik et al., 2010).

When the gene encoding Peb1 was originally identified and sequenced, it revealed some homology to proteins involved in amino acid transport systems in other bacteria (Pei and Blaser, 1993). A more recent study has confirmed that Peb1 binds aspartate and glutamic acid, and suggested that the protein was predominantly found in the periplasm and not in the inner or outer membranes (Leon-Kempis Mdel et al., 2006). These studies may implicate that the primary role of the Peb1 protein may be the utilization of certain amino acids. The recently published crystal structure of Peb1 has identified key domains which may indeed play a role in binding amino acids and may provide a basis for furthering our understanding of how this protein carries out this function and how this affects the ability of the organism to adhere to or invade epithelial cells (Muller et al., 2007).

A similar dual role has also been suggested for other reported Peb proteins (**Table 1**). Like Peb1, Peb4 had been implicated as a potential adhesin of *C. jejuni* (Kervella et al., 1993; Asakura et al., 2007). When the nucleotide sequence of Peb4 was first deduced it revealed a gene with homology to factors involved in protein export (Burucoa et al., 1995). This role in protein export has since been confirmed by a study showing profound effects on the outer membrane profile in a *peb4* mutant including a reduction in CadF protein levels (Rathbun and Thompson, 2009). The recently published crystal structure of Peb4 has confirmed that the protein appears to be a chaperone and may thus play a key role in exporting proteins to the outer membrane and that this may explain its role in adherence (Kale et al., 2011). A later study revealed that Peb3 may be a transport protein involved in the utilization of 3-phosphoglycerate (Min et al., 2009). These reports highlight the difficulty in identifying the specific mechanism of action of these proposed "adhesins" and further studies are required to discover what exact role the Peb proteins play in *C. jejuni* adherence or invasion.

OTHER PROPOSED FACTORS INVOLVED IN ADHESION

Several other genes have also been reported as playing a role in either adherence or invasion by *C. jejuni*. The CapA (*Campylobacter* protein A) protein was initially identified as an autotransporter protein and a *capA* mutant displayed reduced adherence to and invasion of Caco-2 cells as well as an inability to colonize chickens (Ashgar et al., 2007). A later study confirmed the role of this protein as a putative adhesin or invasin using chicken epithelial cells but revealed no colonization defect in chickens (Flanagan et al., 2009). About 40% of human isolates and 39% of chicken isolates in this study lack the *capA* gene, which can explain the reduced bacterial binding to LMH cells as compared to *capA* expressing control strains. Two other proteins, KpsE and KpsM, which are involved in capsule production have also been implicated as playing a role in invasion. *In vivo* experiments using *kpsE* and *kpsM* mutants in the ferret model have indicated the role played by the capsule in colonization (Bacon et al., 2001; Bachtiar et al., 2007). Another proposed adhesin is Cj0977 (Goon et al., 2006), but later studies have shown that reduced binding maybe due to a deficiency in motility in liquid broth (Novik et al., 2010), although the mutants were motile on soft agar plates (Goon et al., 2006). Furthermore, another study has revealed the role played by lipooligosaccharide (LOS) sialylation as mutants in the *cstII* gene showed reduced ability to enter epithelial cells (Louwen et al., 2008). Other proteins recently identified as affecting adherence and invasion levels also include the chaperone and serine protease HtrA (Baek et al., 2011) and VirK (Novik et al., 2009). Baek et al. showed that the chaperone activity of HtrA in the periplasm may affect proper folding of adhesins involved in host cell binding, whereas Novik et al. pointed out that the reduction of intracellular bacteria observed in the gentamycin protection assay (GPA) with a *virK* mutant may be primarily due to a ∼100-fold increase in susceptibility to certain antimicrobial peptides. Finally, although some genes have been identified on the pVIR plasmid in strain 81-176 to play a role in invasion, these genes seem to be strain-specific as they are only present in a very small subset (∼10%) of *C. jejuni* isolates (Bacon et al., 2000, 2002).

THE CELLULAR INVASION PROCESS

Early reports which investigated intestinal biopsies from human patients indicated that *C. jejuni* is able to enter gut tissue cells *in vivo* (van Spreeuwel et al., 1985). Numerous studies have then shown that *C. jejuni* can invade and survive within a number of cultured cell lines *in vitro* (**Table 2**). Various *C. jejuni* factors permitting motility, glycosylation, capsular synthesis, and adherence have been implicated in the invasion process (Grant et al., 1993; Yao et al., 1994; Pei et al., 1998; Bacon et al., 2001; Konkel et al., 2001; Szymanski et al., 2002). Very commonly, *C. jejuni* with introduced mutations in these genes exhibited various degrees of deficiency in binding to and entering human target cells, or in colonizing certain animal models including chicken and mice (Hendrixson and DiRita, 2004; Kakuda and DiRita, 2006; Watson and Galán, 2008; Novik et al., 2010). A common laboratory method to monitor adherence and invasion is GPA, but also immunofluorescence and electron microscopy (EM) approaches have been utilized in some but not all studies (**Table 2**). However, it is not yet clear which mechanism of invasion, "zipper" or "trigger," is used by *C. jejuni*. High resolution EM investigation of infected non-polarized INT-407 and other cell lines indicated that *C. jejuni* induces membrane ruffling in a contact-dependent manner followed by host cell entry, first with its flagellar tip followed by the opposite flagellar end (Krause-Gruszczynska et al., 2007b, 2011; Boehm et al., 2012), surprisingly sharing some features of both "zipper"- and "trigger"-mechanisms (**Figure 1C**).

PUTATIVE HOST CELL RECEPTORS AND KINASES INVOLVED IN THE INVASION PROCESS

Lipid rafts are specific microdomains of plasma membrane of eukaryotic host cells which are enriched in cholesterol and sphingolipids, and favor the interactions of receptor molecules and

Table 2 | Host factors and proposed roles in *C. jejuni* infections.

Host factor	Proposed function	Applied experimental methods	Strains used	Cell system used	Reference
Actin filaments	Invasion	Infection *in vitro*, cytochalasin D and mycalolide B inhibitors, GPA, IFM	81116, HP5100, CCUG7800, F38011, 81-176	INT-407	Biswas et al. (2003), Monteville et al. (2003)
Calcium	Invasion	Infection *in vitro*, BAPTA inhibitors, GPA	81-176	INT-407	Hu et al. (2005)
Cdc42	Invasion	Infection *in vitro*, CA and DN constructs, GMT, CRIB-PD, GPA, IF, FESEM	81-176, 84-25, F38011	INT-407; $^{-/-}$ cell lines	Krause-Gruszczynska et al. (2007b, 2011)
Caveolae	Invasion	Infection *in vitro*, filipin-III and MβCD inhibitors, DN constructs, GPA, IFM	N82, 81-176	Caco-2, INT-407, Cos-1	Wooldridge et al. (1996), Hu et al. (2006), Watson and Galán (2008)
DOCK180	Rac-1 activation, invasion	Infection *in vitro*, siRNA, GPA	F38011, 81-176, 84-25	HeLa, INT-407	Eucker and Konkel (2012), Boehm et al. (2012)
Dynein	Invasion, intracellular trafficking	Infection *in vitro*, nocodazole and o-Van inhibitor, GPA, IFM	81-176	INT-407	Hu and Kopecko (1999)
EGF receptor	Invasion	Infection *in vitro*, ABB, PD168393 and erlotinib inhibitors, DN constructs, GPA	F38011, 81-176, 84-25	INT-407	Eucker and Konkel (2012), Krause-Gruszczynska et al. (2011)
FAK	Invasion signaling	Infection *in vitro*, TAE226 and PF573228 inhibitors, DN and other constructs, GPA, FESEM	F38011, 81-176, 84-25	INT-407, FAK$^{-/-}$cells	Krause-Gruszczynska et al. (2011), Eucker and Konkel (2012), Boehm et al. (2012)
Fibronectin	Adhesion, invasion	Binding and infection *in vitro*, CBA, TWA, ABB, use of *cadF* mutant, GPA, FESEM	F38011, 81-176, 84-25	INT-407, T84, Fn$^{-/-}$cells	Monteville and Konkel (2002); Monteville et al. (2003), Krause-Gruszczynska et al. (2011), Boehm et al. (2012)
G proteins	Invasion	Infection *in vitro*, pertussis and cholera toxin treatments, GPA	N82, 81-176	Caco-2, INT-407	Wooldridge et al. (1996), Hu et al. (2006)
Integrin β1	Adhesion, invasion signaling	Infection *in vitro*, CRIB-PD, G-lisa, GPA, FESEM	81-176, 84-25, F38011	Integrin β1$^{-/-}$ cells	Boehm et al. (2012)
Lysosomes	Intracellular trafficking	Infection *in vitro*, IFM with EEA-1, Lamp-1, Rab4, and Rab5	81-176	Cos-1	Watson and Galán (2008)
MAPK	Inflammatory signaling, invasion	Infection *in vitro*, binding of GST-JlpA *in vitro*; AABs for Erk, JNK, and p38; MAPK inhibitors	THG9011, 81-176, 11168	HEp-2, T84, Caco-2, human colonic explants, INT-407	Jin et al. (2003), MacCallum et al. (2005), Chen et al. (2006), Hu et al. (2006)
Microtubule filaments	Invasion	Infection *in vitro*; nocodazole inhibitor	81-176, VC84	INT-407	Oelschlaeger et al. (1993)
Mucin (chicken)	Inhibition of bacterial virulence	Binding studies, GPA	81-176	HCT-8	Alemka et al. (2010)
Myd88	Colonization controlled by TLRs	Colonization of Myd88$^{-/-}$ but not wt control mice	81-176	Myd88$^{-/-}$mice	Watson et al. (2007)
NF-κB	Inflammatory signaling	Binding of GST-JlpA *in vitro*, AABs, cytokine release	THG9011	HEp-2	Jin et al. (2003)
Nramp1	Colonization of mice	Colonization enhanced in Nramp1$^{-/-}$ mice	81-176	Nramp1$^{-/-}$mice	Watson et al. (2007)

(Continued)

Table 2 | Continued

Host factor	Proposed function	Applied experimental methods	Strains used	Cell system used	Reference
Occludin	Impaired epithelial barrier functions	Infection *in vitro*, TER, hyperphosphorylation of occludin, NF-κB activation, AABs, GPA	81-176, 11168	T84	Chen et al. (2006)
Paxillin	Invasion	Phosphorylation of paxillin, infection *in vitro*, IP, AAB, GPA	F38011	INT-407	Monteville et al. (2003)
PDGF receptor	Invasion	Infection *in vitro*, AG370 inhibitor, DN constructs, GPA	F38011, 81-176, 84-25	INT-407	Krause-Gruszczynska et al. (2011)
PI3-kinase	Invasion	Infection *in vitro*, LY294002 and wortmannin inhibitors, GPA, Vav2 constructs	N82, 81-176, 27 clinical strains	INT-407	Wooldridge et al. (1996), Hu et al. (2006), Biswas et al. (2000), Krause-Gruszczynska et al. (2011)
PKC	Invasion	Infection *in vitro*, calphostin C inhibitor, GPA	81-176, 27 clinical strains	INT-407	Hu et al. (2005), Biswas et al. (2000)
Rac-1	Invasion	Infection *in vitro*, CA and DN constructs, GMT, CRIB-PD, G-lisa, GPA, IFM, FESEM	81-176, F38011, 84-25	INT-407, $^{-/-}$ cell lines	Krause-Gruszczynska et al. (2007b), Eucker and Konkel (2012), Boehm et al. (2012)
Src kinases	Invasion	Infection *in vitro*, PP2 inhibitor, GPA	F38011	INT-407, SYF cells	Eucker and Konkel (2012), Krause-Gruszczynska et al. (2011)
Tiam-1	Rac-1 activation, invasion	Infection *in vitro*, DN and other constructs, siRNA, GPA	81-176, F38011, 84-25	INT-407, $^{-/-}$ cells	Boehm et al. (2012)
Vav2	Cdc42 activation, invasion	Infection *in vitro*, DN and other constructs, siRNA, GPA	81-176, F38011, 84-25	INT-407, Vav$^{-/-}$ cells	Krause-Gruszczynska et al. (2011)

AB, antibody; AAB, activation-specific antibody; ABB, antibody blocking; CBA, competitive binding assay; MβCD, methyl-beta cyclodextrin; CA constructs, constitutive-active constructs; DN constructs, dominant-negative constructs; IP, immunoprecipitation; CRIB-PD, pull-down experiments to quantify GTPase-GTP levels; EEA-1, early endosomal marker 1; FAK, focal adhesion kinase; FESEM, field emission scanning electron microscopy; Fn, Fibronectin, G-lisa, ELISA-based GTPase-GTP quantification system; GPA, gentamicin protection assay, GST-JlpA, glutathione-S- transferase-tagged JlpA; GMT, GTPase-modifying toxins such as toxin B or CNF, which either inhibit or activate GTPases; IFM, immunofluorescence microscopy; IP, immunoprecipitation; MAPK, mitogen-activated protein kinases; MyD88, myeloid differentiation factor 88; o-Van, ortho-vanadate inhibitor; PKC, protein kinase C; SYF, Src$^{-/-}$, Yes$^{-/-}$, Fyn$^{-/-}$ triple knockout cells; TER, transepithelial resistance; TWA, transwell assays with polarized cells; Vav2, guanine exchange factor of the Vav family; wt, wild-type; $^{-/-}$, knockout cells.

the regulation of downstream signaling pathways. Pharmacological inhibitor studies using the lipid raft-disrupting compounds filipin-III or MβCD as well as certain toxins have indicated that host heterotrimeric G proteins and caveolae may be involved in epithelial cell entry of *C. jejuni* (Wooldridge et al., 1996; Hu et al., 2006; Watson and Galán, 2008). Expression of dominant-negative mutants of caveolin-1 but not dynamin-II significantly decreased *C. jejuni* internalization (Watson and Galán, 2008), suggesting that caveolin-1 in caveolae structures may play a role in the uptake process (**Figure 2**). Interestingly, *C. jejuni* is severely hampered in its ability to invade cell lines derived from fibronectin$^{-/-}$, integrin-β1$^{-/-}$, focal adhesion kinase (FAK)$^{-/-}$, and Src$^{-/-}$/Yes$^{-/-}$/Fyn$^{-/-}$ (SYF) triple knockout mice using EM and/or GPA, but can efficiently enter wild-type control cells (Krause-Gruszczynska et al., 2011; Boehm et al., 2012). Infection of integrin-β1$^{-/-}$ knockout cells stably expressing integrin-β1 point mutants with well-known defects in extracellular fibronectin fibril formation or intracellular FAK signaling also exhibited severe deficiencies in *C. jejuni* invasion (Krause-Gruszczynska et al., 2011). Novel data also showed that FAK and certain receptor protein tyrosine kinases such as EGF receptor (EGFR) and platelet derived growth factor receptor (PDGFR) are activated by *C. jejuni* and involved in the uptake process (Krause-Gruszczynska et al., 2011; Boehm et al., 2012; Eucker and Konkel, 2012). Another focal adhesion signaling molecule, paxillin, also exhibited increased levels of tyrosine phosphorylation upon *C. jejuni* infection, which was not seen during infection with mutants of the fibronectin binding protein CadF (Monteville et al., 2003). These data collectively suggest that the CadF → fibronectin → integrin-β1 → FAK → paxillin pathway is another major signaling cascade involved in *C. jejuni*-mediated host cell entry (**Figure 2**). In addition, inhibitor studies combined with GPA have suggested that phosphatidylinositol 3-kinase (PI3-kinase), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) are involved in events controlling *C. jejuni* internalization (Wooldridge et al., 1996; Biswas et al., 2000; Hu

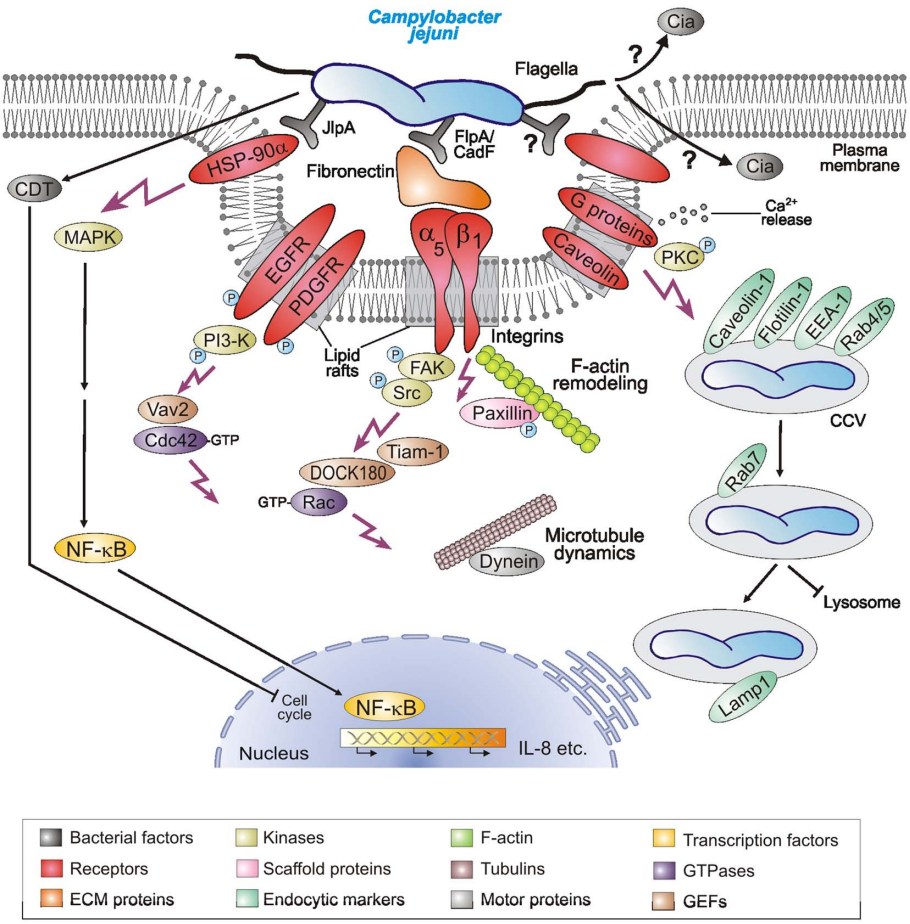

FIGURE 2 | Hypothetical model for *C. jejuni*-induced signaling events leading to bacterial invasion and establishing infections. *C. jejuni* adheres to host cells via numerous reported and unknown factors. Several indicated host cell receptors have been proposed to play a role in the uptake of the bacteria. This potentially causes localized F-actin and/or microtubule rearrangements at the site of *C. jejuni* entry, resulting in engulfment and bacterial uptake. Several indicated host cell signaling molecules and pathways including the intracellular survival in *Campylobacter*-containing vacuoles (CCVs) have been reported in *in vitro* infection models and may play a role during pathogenesis *in vivo*. For more details, see tables and text.

et al., 2006). Other inhibitor-based work has also shown that Ca^{2+} release from host intracellular stores is essential for efficient *C. jejuni* recovery by GPA (Hu et al., 2005). However, whether most of the latter factors are directly involved in the bacterial entry step or trafficking linked to intracellular bacterial survival is not yet clear.

FUNCTION OF GEFs AND SMALL Rho GTPases IN *C. jejuni* INVASION

The Rho family of small GTPases, including Rac-1, Cdc42, and RhoA, are small GTP-binding proteins that serve as guanine nucleotide-regulated switches which transmit external stimuli to modulate different normal cellular functions as well as invasion of multiple bacterial pathogens (Cossart and Sansonetti, 2004). The internalization of *C. jejuni* into INT-407 cells is accompanied by a time-dependent activation of both Rac-1 and Cdc42 (Krause-Gruszczynska et al., 2007b, 2011; Boehm et al., 2012; Eucker and Konkel, 2012). Using specific GTPase-modifying toxins, inhibitors, and GTPase expression constructs it was shown by immunofluorescence, GPA, and other studies that Rac-1 and Cdc42, but not RhoA, are involved in *C. jejuni* invasion (Krause-Gruszczynska et al., 2007b). Interestingly, the activation of these GTPases and induction of membrane ruffling by *C. jejuni* was severely inhibited in infected fibronectin$^{-/-}$, integrin-β1$^{-/-}$, FAK$^{-/-}$, and SYF$^{-/-}$ knockout cell lines, although the bacteria had intact flagella and bound to their cell surface (Krause-Gruszczynska et al., 2011; Boehm et al., 2012). Maximal GTPase–GTP levels induced by *C. jejuni* also involve EGFR, PDGFR, FAK, Src, and PI3-kinase activities as supported by inhibitor studies, and the bacterial fibronectin binding protein CadF as well as the flagellum and one secreted *Campylobacter* invasion antigen, CiaC (Krause-Gruszczynska et al., 2007b, 2011; Eucker and Konkel, 2012). Immunofluorescence data also indicated that CiaC is essential for recruitment of Rac-1 to the site of *C. jejuni* adherence (Eucker and Konkel, 2012). The exact role of CiaC, however, is not yet clear, but it would appear that it does not encode a guanine exchange factor (GEF) for activating Rac-1. CiaC shares no sequence homology with the well-known GEFs from *Shigella*

flexneri or *Salmonella enterica*, which are directly injected into the host cell by a T3SS and known to induce extensive membrane ruffling by the "trigger" mechanism (Cossart and Sansonetti, 2004). In agreement with these observations, two host cell GEFs (DOCK180 and Tiam-1) were identified to trigger Rac-1 activity downstream of FAK based on small inhibitory RNA (siRNA) knockdown, inhibitors, and the use of $FAK^{-/-}$ knockout cell experiments (Boehm et al., 2012; Eucker and Konkel, 2012), while another GEF (Vav2) is downstream of FAK, EGFR, PDGFR, and PI3-kinase to trigger Cdc42 GTPase activity (Krause-Gruszczynska et al., 2011). Collectively, these results suggest that *C. jejuni* actively triggers signaling to stimulate their own uptake by host target cells using a unique mechanism and the activation of the Rho GTPase members Rac-1 and Cdc42 plays a crucial role in this entry process (**Figures 1C** and **2**).

ROLE OF MICROFILAMENTS VERSUS MICROTUBULES DURING INVASION

Microfilaments (MFs) and microtubules (MTs), which are composed of actin or tubulin subunits, respectively, play an important role in cell architecture and other basic cellular processes. Using inhibitor and GPA studies, *C. jejuni* internalization has been variously reported to require MTs (Oelschlaeger et al., 1993; Hu and Kopecko, 2008), MFs (De Melo et al., 1989; Konkel and Joens, 1989), both MTs and MFs (Oelschlaeger et al., 1993; Biswas et al., 2003; Monteville et al., 2003), or neither MTs or MFs (Russell and Blake, 1994). Other studies investigated the co-localization of infecting *C. jejuni* with MTs and the molecular motor protein dynein (Hu and Kopecko, 1999), but also with MFs (Konkel et al., 1992a; Rivera-Amill et al., 2001; Krause-Gruszczynska et al., 2007b). This available data, although not sufficient to allow definitive conclusions, suggest that different *C. jejuni* strains may prefer MT-dependent or MF-dependent pathways for host invasion, while others may depend on both MTs and MFs during invasion. Remarkably, after about 20 years of research, the *C. jejuni*-mediated triggers which result in MT and/or MF rearrangements to facilitate bacterial uptake are still not clear. It will therefore be important to investigate how activated caveolae, FAK, EGFR, PDGFR, PI3-kinase, Rac-1, Cdc42, or other host factors could potentially stimulate MFs and/or MTs dynamics to mediate the *C. jejuni* entry process.

INTRACELLULAR TRAFFICKING AND SURVIVAL OF *C. jejuni*

Various intracellular pathogens utilize a variety of strategies to live and replicate within host cells. GPA and EM studies have shown that *C. jejuni* can survive for extended periods of time in several cell lines and tissues (**Table 2**). Following entry into intestinal epithelial cells, *C. jejuni* appears to localize in a specific compartment in the cytoplasm, which seems to be distinct from the lysosomes (Watson and Galán, 2008). It was found that the *C. jejuni*-containing vacuole (CCV) deviates from the canonical endocytic pathway immediately after host cell entry, thus avoiding delivery into lysosomes (**Figure 1D**). The CCV appears to interact with early endosomal compartments because it associates with early endosomal marker protein EEA-1 and two trafficking GTPases, Rab4, and Rab5 (**Figure 2**, right). However, this interaction seems only transient and does not progress inside the canonical endocytic pathway (Watson and Galán, 2008). The CCV can be also stained with Lamp-1, a late endosomal marker, although this compartment appears to be unique and clearly distinct from lysosomes (**Figure 2**). CCVs were not stainable with the lysosomal marker protein cathepsin B and it is also not accessible to certain endocytic tracers (Watson and Galán, 2008). Taken together, the acquisition of Lamp-1 occurring very early during maturation of CCVs, appears to proceed by an unusual pathway not requiring the GTPases Rab5 or Rab7, although recruited to the CCV. More studies are required to elucidate in more detail the mechanism by which *C. jejuni* modulates intracellular trafficking and survival.

The subset of *C. jejuni* genes which are important for intracellular trafficking and survival are widely unknown, but a couple of potential factors are emerging. One of these factors is CiaI, a reported secreted protein (Buelow et al., 2011). A *ciaI* mutant was impaired in intracellular survival by GPA. Immunofluorescence examination indicated that the *ciaI* mutant more frequently co-localized with Cathepsin D in the CCVs as compared to wild-type bacteria, and is probably killed in these compartments. In another recent study, insertional mutation of three novel genes named *aspA*, *aspB*, and *sodB* also exhibited clear defects in intracellular recovery by GPA (Novik et al., 2010). Further characterization of these mutants revealed that the reduction in recovered bacteria was due to a combined defect in adhesion and impaired intracellular viability (SodB) or undetermined effects on bacterial physiology (AspA and AspB). These studies highlight the importance of the use of multiple assays to identify and characterize genes which play potential roles in *C. jejuni* adherence and invasion.

URGENT PROBLEMS TO BE SOLVED

Campylobacter jejuni is a remarkable foodborne microbe, but by comparison to other well-known enteric pathogens, we know very little about the bacterial and host factors involved in establishing infection and triggering disease. This dilemma is in part due to the clear absence of classical bacterial adhesins, toxins, or typical T3SSs or T4SSs in the sequenced *C. jejuni* genomes. The other enormous handicap is the large amount of highly conflicting data in the literature. For almost every reported factor proposed to be involved in a given host response, there is at least one other study showing the opposite. It is possible that the reported results depend on the specific strains used, how the bacteria were grown, but also on differences in the experimental conditions and applied methodology (**Tables 1** and **2**). In addition, many studies using single mutants lack genetic complementation of the corresponding wild-type gene, which although technically very difficult in *C. jejuni*, would be very useful to restore the phenotypes reported for many of the aforementioned pathogenicity factors. Furthermore, one must be very careful when using methods such as GPA as an accurate measurement of bacterial invasion. GPA determines the number of viable CFU protected from gentamicin, and thus represents a direct measurement of intracellular surviving bacteria, rather than a direct measure of the invasion process itself? Some studies have identified factors which appear to play a direct role in invasion when studied using GPA, but when studied using other methods are actually revealed to have an indirect role. These include AspA and AspB whose primary role was shown to be in

the production of fumarate but when mutated displayed a reduction in invasion by the GPA assay (Novik et al., 2010). However, this effect could be reversed by the addition of fumarate showing that this reduction was due to physiological effects rather than these proteins functioning as adhesins or invasins directly (Novik et al., 2010). Studies such as these highlight how important it is to use a variety of experimental approaches to identify and study any factors found to play a role in host cell interactions. The same arguments apply for certain studies using pharmacological inhibitors. Besides such problems as the lack of controls, it should be asked if these inhibitors or their solvents alone have specific activities on the host or bacteria over time and, if properly performed, does a given compound really inhibit the entry process itself or does it interfere with bacterial survival in the CCVs? GPA certainly does not discriminate between these different possibilities either. Thus, in future, more direct methods such as novel microscopic technologies are necessary to investigate in detail the involvement of certain host factors in bacterial engulfment and uptake, but also in intracellular survival processes.

Although several of the discussed GPA studies have identified certain *C. jejuni* gene mutants exhibiting severe defects in entering or surviving in cells, there is no direct evidence yet that any of the identified gene products can directly trigger invasion (**Tables 1** and **2**). For example, it is still controversial if the role of the flagellum during invasion is restricted to bacterial motility or secretion of bacterial Cia proteins into the medium or even injection into the host cell. This model of Cia protein secretion through the flagellum is very tempting and would support the idea that *Campylobacter* uses a "trigger mechanism" of invasion involving the secretion of effector proteins directly into the cell to induce their uptake, similar to *Salmonella* and *Shigella*. Some electron microscopic evidence exists that would support this model (**Figure 1C**), but it should be noted that a recent study has suggested that CiaB plays a minimal or no role in invasion (Novik et al., 2010). Thus, much more work is required to confirm the role of the flagellum as a secretion system for effector proteins involved in invasion.

Confusion also exists as to the exact role played by some of the previously proposed adhesins. Proteins such as JlpA have been described by some authors as being an important adhesin (Jin et al., 2001), whereas other groups have been unable to show any significant decrease in invasion in a *jlpA* mutant (Novik et al., 2010). Furthermore, factors such as the PEB proteins which were originally described as adhesins (Pei et al., 1991) now appear to primarily have roles as transporters (Leon-Kempis Mdel et al., 2006) or as chaperones (Kale et al., 2011), suggesting that they may not directly interact with the host cell but play a more indirect role. One protein that is very well characterized and appears to clearly play a role in adherence to host epithelial cells is CadF (Monteville et al., 2003). This protein along with the recently described FlpA protein (Flanagan et al., 2009) appears to bind to fibronectin and specific fibronectin binding sites have been identified in CadF (Konkel et al., 2005). The importance of CadF has been observed in a large number of strains and using a variety of different experimental approaches (**Table 1**). Thus, targeting the fibronectin/integrin receptor could explain why *C. jejuni* may try to reach basolateral surfaces during infection (van Alphen et al., 2008). How the bacteria breach this epithelial barrier, by a transcellular route (**Figure 1D**) or a paracellular route (**Figure 1E**), is also under much debate and not yet clear (Konkel et al., 1992b; Grant et al., 1993; Brás and Ketley, 1999; Monteville and Konkel, 2002; Hu et al., 2008; Kalischuk et al., 2009). However, the underlined importance of CadF and the fibronectin/integrin might give support to a "zipper"-like mechanism of invasion as used by *Listeria* or *Yersinia* species.

CONCLUDING REMARKS

In conclusion it is very difficult at present to conclusively state how *C. jejuni* facilitates its uptake into host epithelial cells. There is evidence in the literature and specifically EM images which give some support for both the "zipper" and "trigger" mechanisms of invasion, underlining the concept that *C. jejuni* enters epithelial cells by a unique novel mechanism. It may be that *C. jejuni* has developed during evolution a strategy which shares features of both of these mechanisms, but more work is clearly required to pinpoint the exact pathways used by this important pathogen to enter and survive in intestinal epithelial cells. At the moment, we favor a model where at least two major receptor-involved pathways give rise to *C. jejuni* invasion, the fibronectin/integrin, and caveolae structures (**Figure 2**). We also consider that caveolae and integrin may act cooperatively in GTPase signaling as shown for other cell systems (del Pozo et al., 2005; del Pozo and Schwartz, 2007). Future studies should investigate how this occurs and what are the forces triggered by the host cell that mediate engulfment, uptake, and also membrane closure behind the entering bacteria. It will be also important to investigate, in more detail, the mechanisms by which *C. jejuni* survives and spreads intracellularly as well as how it causes extra-intestinal infection in organs such as the liver in various animals (Hofreuter et al., 2008; Cox et al., 2009). It therefore appears that *C. jejuni* will continue to be a fascinating and rewarding research subject in the future.

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A fluoroquinolone resistance associated mutation in *gyrA* affects DNA supercoiling in *Campylobacter jejuni*

Jing Han[1], Yang Wang[1,2], Orhan Sahin[1], Zhangqi Shen[1], Baoqing Guo[1], Jianzhong Shen[2] and Qijing Zhang[1]*

[1] *Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, IA, USA*
[2] *China Agricultural University, Beijing, China*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Marcelo Tolmasky, California State University Fullerton, USA
Odile Tresse, French National Institute for Agricultural Research/Nantes-Atlantic National College of Veterinary Medicine, Food Science and Engineering, France

***Correspondence:**
Qijing Zhang, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, 1116 Veterinary Medicine Complex, Ames, IA 50011, USA.
e-mail: zhang123@iastate.edu

The prevalence of fluoroquinolone (FQ)-resistant *Campylobacter* has become a concern for public health. To facilitate the control of FQ-resistant (FQ^R) *Campylobacter*, it is necessary to understand the impact of FQ^R on the fitness of *Campylobacter* in its natural hosts as understanding fitness will help to determine and predict the persistence of FQ^R *Campylobacter*. Previously it was shown that acquisition of resistance to FQ antimicrobials enhanced the *in vivo* fitness of FQ^R *Campylobacter*. In this study, we confirmed the role of the Thr-86-Ile mutation in GyrA in modulating *Campylobacter* fitness by reverting the mutation to the wild-type (WT) allele, which resulted in the loss of the fitness advantage. Additionally, we determined if the resistance-conferring GyrA mutations alter the enzymatic function of the DNA gyrase. Recombinant WT gyrase and mutant gyrases with three different types of mutations (Thr-86-Ile, Thr-86-Lys, and Asp-90-Asn), which are associated with FQ^R in *Campylobacter*, were generated in *E. coli* and compared for their supercoiling activities using an *in vitro* assay. The mutant gyrase with the Thr-86-Ile change showed a greatly reduced supercoiling activity compared with the WT gyrase, while other mutant gyrases did not show an altered supercoiling. Furthermore, we measured DNA supercoiling within *Campylobacter* cells using a reporter plasmid. Consistent with the results from the *in vitro* supercoiling assay, the FQ^R mutant carrying the Thr-86-Ile change in GyrA showed much less DNA supercoiling than the WT strain and the mutant strains carrying other mutations. Together, these results indicate that the Thr-86-Ile mutation, which is predominant in clinical FQ^R *Campylobacter*, modulates DNA supercoiling homeostasis in FQ^R *Campylobacter*.

Keywords: *Campylobacter*, fluoroquinolone resistance, GyrA mutation, DNA supercoiling, fitness

INTRODUCTION

Campylobacter jejuni, a Gram-negative microaerophilic bacterium, has emerged as a leading bacterial cause of foodborne gastroenteritis in the United States and other developed countries (Slutsker et al., 1998). Antibiotic treatment using fluoroquinolone (FQ) or erythromycin is recommended when the infection by *Campylobacter* is severe or occurs in immunocompromised patients (Engberg et al., 2001; Oldfield Iii and Wallace, 2001). However, *Campylobacter* is increasingly resistant to FQ antimicrobials, which has become a major concern for public health (Engberg et al., 2001; White et al., 2002; Gupta et al., 2004). As a zoonotic pathogen, *C. jejuni* is highly prevalent in food producing animals and poultry, and is exposed to antibiotics used in agricultural settings. There has been a concern on the use of FQ antimicrobials in poultry production since the use selectively enriches FQ-resistant (FQ^R) *Campylobacter* that can be transmitted to humans via the food chain (Luangtongkum et al., 2009). This concern led to the withdrawal of FQ antimicrobials from poultry production in the U.S. in 2005. Despite the ban, FQ^R *Campylobacter* continues to persist on poultry farms (Price et al., 2005, 2007; Luangtongkum et al., 2009), suggesting that FQ^R *Campylobacter* does not show a fitness cost in the absence of antibiotic selection pressure.

In Gram-negative bacteria, DNA gyrase, a type II topoisomerase, is the primary target of FQ antibiotics (Hooper, 2001). Once inside bacterial cells, FQ antimicrobials form stable complex with the target enzymes and trap the enzymes on DNA, resulting in double-stranded breaks in DNA, and bacterial death (Willmott et al., 1994; Shea and Hiasa, 1999; Drlica and Malik, 2003). Bacterial DNA gyrase is essential for bacterial viability. It catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (Champoux, 2001). The enzyme consists of two subunits (subunits A and B) that combine into an A_2B_2 complex to form a functional enzyme. The two subunits are encoded by *gyrA* and *gyrB*, respectively. In *Campylobacter*, the resistance to FQ antimicrobials is mediated by point mutations in the quinolone resistance-determining region (QRDR) of *gyrA* in conjunction with the function of the multidrug efflux pump CmeABC (Bachoual et al., 2001; Engberg et al., 2001; Luo et al., 2003; Ge et al., 2005). No mutations in *gyrB* have been implicated in FQ^R in *Campylobacter* (Bachoual et al., 2001; Payot et al., 2002; Piddock et al., 2003). Specific mutations at positions Thr-86, Asp-90, and Ala-70 in GyrA have been linked to FQ^R in *C. jejuni* (Wang et al., 1993; Engberg et al., 2001; Luo et al., 2003). The Thr-86-Ile change (mediated by the C257T mutation

in the *gyrA* gene) is the most commonly observed mutation in FQ^R *Campylobacter* isolates and confers high-level (ciprofloxacin minimum inhibitory concentration (MIC) $\geq$ 16 μg/ml) resistance to FQ, whereas the Thr-86-Lys and Asp-90-Asn mutations are less common and are associated with intermediate-level FQ^R (Gootz and Martin, 1991; Wang et al., 1993; Ruiz et al., 1998; Luo et al., 2003).

Resistance-conferring mutations in target genes are often associated with changes in physiological processes, which may result in reduced growth rate and fitness in the absence of antibiotic selection (Andersson and Levin, 1999; Levin et al., 2000; Andersson, 2003; Kugelberg et al., 2005). However, bacteria can develop compensatory mutations to ameliorate the fitness cost associated with antimicrobial resistance (Bjorkman et al., 1998; Bjorkholm et al., 2001; Nagaev et al., 2001; Normark and Normark, 2002; Andersson, 2003). In some cases, antibiotic-resistant mutants show little or no fitness cost even without compensatory mutations (Sander et al., 2002). Previously Luo et al. (2005) showed that FQ^R *Campylobacter* carrying the Thr-86-Ile substitution in GyrA subunit was able to outcompete the FQ-susceptible (FQ^S) strains in the absence of antibiotic usage, suggesting that acquisition of FQ^R enhances the *in vivo* fitness of FQ^R *Campylobacter*.

DNA supercoiling modulates gene expression and affects bacterial adaptive responses to environmental challenges (Tse-Dinh et al., 1997; Lopez-Garcia, 1999; Prakash et al., 2009). Alteration in DNA supercoiling status may conceivably affect the physiology and fitness of bacterial organisms. Previous work conducted in *Escherichia coli* and *Pseudomonas* revealed that antibiotic resistance-conferring mutations in GyrA reduced the supercoiling activity of the enzyme in these organisms (Barnard and Maxwell, 2001; Kugelberg et al., 2005). However, it is unknown if the GyrA mutations conferring FQ^R in *Campylobacter* influences the function of GyrA and modulate the DNA supercoiling status within the bacterial cells. In this study, we confirmed the specific role of the Thr-86-Ile mutation in GyrA in influencing *Campylobacter* fitness in chickens by reverting the mutation to a wild-type (WT) allele. We then evaluated the effects of three different types of GyrA mutations [C257T (Thr-86-Ile), C257A (Thr-86-Lys), and G268A (Asp-90-Asn)] on DNA supercoiling using *in vitro* and *in vivo* (within *Campylobacter* cells) assays. Our results showed that the Thr-86-Ile change in GyrA was directly linked to the fitness change in *Campylobacter* and greatly reduced the supercoiling activity of GyrA, while other mutations, although reduced the susceptibility of *Campylobacter* to ciprofloxacin, did not affect the supercoiling activity of DNA gyrase. Together, these results provide new insights into the molecular mechanisms underlying the fitness of the FQ^R *Campylobacter*.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

The FQ^S strains and FQ^R mutant strains (carrying different point mutations in *gyrA*) used in this study are listed in **Table 1**. The strains were routinely grown in Mueller–Hinton (MH) broth (Difco) or agar at 42°C under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2). *Campylobacter*-specific growth supplements and selective agents (Oxoid) were added to media when needed to recover *Campylobacter* from chicken feces. MH media were supplemented with kananmycin (50 μg/ml) or chloramphenicol (4 μg/ml) as needed. *E. coli* cells were grown at 37°C with shaking at 200 r.p.m. in LB medium which was supplemented with ampicillin (100 μg/ml) or kanamycin (30 μg/ml).

REVERSION OF THE *gyrA* MUTATION (Thr-86-Ile)

To formally define the role of the C257T mutation in influencing *Campylobacter* fitness, the specific mutation (C257T) in *gyrA* of isolates 62301R33 was reverted to WT sequence by using a method reported by Ge et al. (2005). *Cj1028c* and *gyrA* are tandemly positioned on the chromosome of *C. jejuni*. An 850-bp fragment containing the 3′ region of *Cj1028c*, the intergenic region, and the *gyrA* sequence up to the mutation site was amplified by PCR using *C. jejuni* 62301R33 as a template and gyrA1028F and gyrA257WR (corresponding to the WT *gyrA* sequence) as primers (**Table 2**) and then cloned into a pGEMT-Easy (Promega). The construct was linearized using *Eco*RV, which cuts once within the cloned *cj1028c* sequence, ligated with a blunt-ended Cm^r cassette, and electroporated into *E. coli* JM109. Recombinant plasmids were purified from *E. coli* JM109 and used to transform the parent strains *C. jejuni* 62301R33 by electroporation. Transformants were selected on MH agar containing chloramphenicol 10 mg/l and analyzed by PCR and DNA sequencing, confirming the insertion of the *cat* cassette

Table 1 | Bacterial strains used in this study.

	Strains	aa mutation in GyrA	nt mutation in *gyrA*	Description	Source or reference
FQ^S strains	62301S2	None	None	Wild-type; isolated from chicken	Luo et al. (2005)
	62301R33S	None	None	62301R33 derivative; 257T in *gyrA* was reverted to wild-type 257C; *Cj1028c: Cm*^{r}	This study
	NCTC 11168	None	None	Wild-type; isolated from human	Parkhill et al. (2000a)
	11168 (S)	None	None	NCTC 11168 derivative; *Cj1028c: Cm*^{r}	This study
FQ^R strains	62301R33	Thr-86-Ile	C257T	Isolated from chicken	Luo et al. (2005)
	52901-II 1	Thr-86-Lys	C257A	Isolated from chicken	Luo et al. (2005)
	62301R37	Asp-90-Asn	G268A	Isolated from chicken	Luo et al. (2005)
	62301R33R	Thr-86-Ile	C257T	62301R33 derivative; *Cj1028c: Cm*^{r}	This study
	11168CT	Thr-86-Ile	C257T	NCTC 11168 derivative; C257T mutation in *gyrA*	Yan et al. (2006)
	11168 (R)	Thr-86-Ile	C257T	NCTC 11168 derivative; *Cj1028c: Cm*^{r} and C257T mutation in *gyrA*	This study

Table 2 | Key PCR primers used in this study.

Primer name	Sequence (5′–3′)	Target gene /cluster
gyrA1028F	TGCTCTGCTTTTGTGAATTA	*Cj1028c* and *gyrA* (ACA, Thr-86)
gyrA257WR	AAACTGCTGTATCTCCATGT	
gyrA1028F	TGCTCTGCTTTTGTGAATTA	*Cj1028c* and *gyrA* (ATA, Ile-86)
gyrA257m (T)	AAACTGCTATATCTCCATGT	
gyrA-F-2	ACATGCATGCGAGAATATTTTTAGCAA	Whole ORF of *gyrA*
gyrA-R	ACGCGTCGACTTATTGCAAATCTAAACC	
gyrB-F-J2	ACATGCATGCCAAGAAAATTACGGTGCG	Whole ORF of *gyrB*
gyrB-R-J	ACGCGTCGACTTACACATCCAAATGCTT	
topA-F	GCTGCTTTAAATCCGGACTT	Whole ORF of *topA*
topA-R	CAGCTTCGCAAACATACTCA	
cj1686c-F1	AAGCCAAAGATGCCAAAGAA	*topA*
cj1686c-R1	TGCGTGGTAAGGTGTTTTCA	

into *Cj1028c* and the simultaneous replacement of the mutant *gyrA* with the WT allele. This revertant was named 62301R33S. To make an isogenic pair for the revertant for *in vivo* competition, the *cat* gene was also inserted into *Cj1028c* of isolate 62301R33 without changing the C257T mutation in *gyrA*. Primers gyrA1028F and gyrA257m(T) were used for this purpose (**Table 2**). The obtained construct was named 62301R33R. The ciprofloxacin MIC in the revertant 62301R33S was restored to the WT level (0.125 μg/ml), while 62301R33R with the *cat* gene inserted into *Cj1028c* retained the ciprofloxacin MIC at 32 μg/ml. Using the same strategy, a point mutation (C257T) in *gyrA* was introduced into FQ^S NCTC 11168. The generated FQ^R mutant strain and its isogenic FQ^S strain, both of which contained a Cm^r insertion in *cj1028c*, were named 11168 (R) and 11168 (S), respectively (**Table 1**).

To measure the motility of these constructs, they were grown overnight on fresh MH plates and then were collected from MH plates using MH broth. The optical density at 600 nm (OD_{600}) was adjusted to 0.3. Approximately 1 μl of this suspension was then stabbed into a MH motility plate (MH broth + 0.4% Bacto Agar). Following microaerobic growth at 42°C for approximately 30 h, the radius of growth halo was measured for each strain. The results showed that these FQ^S and FQ^R strains were equally motile as determined by the motility assay (data not shown).

IN VIVO COLONIZATION AND PAIRWISE COMPETITION

The chicken experiments were performed using pairwise competition as described previously (Luo et al., 2005). Three groups (10–11 birds/group) of *Campylobacter*-free chickens were inoculated with approximately 10^7 CFU (per bird) of 62301R33S, 62301R33R, or a mixture (approximately 1:1) of the two strains via oral gavage. During the entire experiment, antibiotic-free feed and water were given to the chickens. Thus, antibiotic selection pressure was not involved in the competition. After inoculation, cloacal swabs were collected from the chickens at days 3, 6, and 9 for culturing *Campylobacter*. Each fecal suspension was serially diluted in MH broth and plated simultaneously onto two different types of culture media: conventional *Campylobacter* selective plates for recovering the total *Campylobacter* colonies and the selective MH plates supplemented with 4 μg/ml ciprofloxacin for recovering FQ^R *Campylobacter* colonies. To confirm the results from the differential plating, 10–15 *Campylobacter* colonies were selected randomly for each group from the conventional selective plates (no ciprofloxacin) at each sampling time and tested for ciprofloxacin MICs with *E*-test strips (AB Biodisk, Solna, Sweden). In the second chicken experiment, the same pairwise experiment was performed using the isogenic pair of 11168(S) and 11168(R). In both experiments, the detection limit of the plating methods was 100 CFU/g of feces. The statistic analyses that were used to determined the significance of differences in the level of colonization between the two groups were performed as described in a previous publication (Han et al., 2008).

All animals were handled in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Iowa State University (A3236-01). All efforts were made to minimize suffering of animals.

PRODUCTION AND PURIFICATION OF GyrA AND GyrB

Full-length histidine (His)-tagged recombinant gyrases including WT GyrA, three mutant GyrAs, and GyrB were produced in *E. coli* by using the pQE-30 vector of the QIAexpress expression system (Qiagen), which allows the tagging of a recombinant protein with 6× His at the N-terminus. The complete coding sequences of *gyrA* in strains 62301S2 (no mutation in GyrA), 62301R33 (with the Thr-86-Ile change in GyrA), 52901-II2 (with the Thr-86-Lys change in GyrA), and 62301R37 (with the Asp-90-Asn change in GyrA) were amplified using primers gyrA-F-2 and gyrA-R. The complete coding sequence of *gyrB* in strain 62301S2 was amplified using primers gyrB-F-J2 and gyrB-R-J. A restriction site (underlined in the primer sequences) was attached to the 5′ end of each primer to facilitate the directional cloning of the amplified PCR product into the pQE-30 vector. The amplified PCR product was digested with *Sph*I and *Sal*I, and then ligated into the pQE-30 vector, which was previously digested with the same enzymes. Each plasmid in the *E. coli* clone expressing a recombinant peptide was sequenced, revealing no undesired mutations in the coding sequence. These N-terminal His-tagged recombinant GyrA and GyrB proteins were expressed and purified to near-homogeneity under native conditions by following the procedure supplied with the pQE-30 vector. Then these proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). To remove imidazole, the purified proteins were washed extensively with 10 mM Tris–HCl using Centricon YM-50 (Millipore).

IN VITRO SUPERCOILING ASSAY

DNA supercoiling activity was assayed by monitoring the conversion of relaxed pBR322 to its supercoiled form. WT or mutant

GyrA was mixed with WT GyrB in a ratio of 1:1. Each supercoiling reaction contained 500 ng of relaxed pBR322 DNA (TopoGEN, Inc.) and 300 μM gyrases in 1× assay buffer (TopoGEN, Inc.). The reaction mixtures were incubated at 37°C for 1 h. Assays were terminated by the addition of 0.2 volume of the stop buffer (TopoGEN) and 1 volume of chloroform–isoamyl alcohol (24:1). The reactions were analyzed on 1.0% agarose gels. The gels were stained with ethidium bromide for 30 min and then destained in 1× TAE buffer for 1 h.

MEC DETERMINATIONS

To determine the susceptibility of various gyrases to the inhibitory effect of ciprofloxacin, the *in vitro* supercoiling assay using recombinant gyrases was also performed with added ciprofloxacin as descried previously (Martin, 1991). Ciprofloxacin was added into each reaction prior to the addition of DNA gyrases. The minimum effective concentration (MEC) was defined as the lowest concentration of ciprofloxacin that shows an observable inhibition on supercoiling of the plasmid DNA.

EXAMINATION OF *IN VIVO* SUPERCOILING

To determine if the mutations in GyrA changed the DNA supercoiling in *Campylobacter* cells, the shuttle plasmid pRY107 was used as a reporter to monitor the relative difference in the levels of DNA supercoiling between the FQ^S and FQ^R strains. pRY107, which carries a kanamycin resistance marker (Yao et al., 1993), was transferred into 62301S2, 62301R33, 52901-II2, 62301R37, 11168, and 1168CT by conjugation. Plasmid DNA was then isolated using QIAGEN Plasmid Midi kit (Qiagen Inc.) and analyzed by agarose gel electrophoresis in the absence or presence of chloroquine diphosphate salt, as described previously (Mizushima et al., 1997; Kugelberg et al., 2005). Agarose gels (1%) were run for 20 h at 2 V/cm in 1× TAE buffer containing 20 μg/ml of chloroquine and were washed for 4 h in distilled water before staining with ethidium bromide. The topoisomers of the plasmid DNA from each strain were visualized using a digital imaging system (Alpha Innotech). The relative amounts of supercoiled vs. relaxed DNA in each sample reflecting the level of DNA supercoiling in each strain were determined by densitometry scanning and were used to indicate the difference in DNA supercoiling between the FQ^S and FQ^R strains.

SEQUENCE DETERMINATION OF *topA*

The entire *topA* gene from *C. jejuni* 62301S2 and 62301R33 were amplified by PCR with the forward primer topA-F and reverse primer topA-R. The forward primer is 286 bp upstream of the AUG start codon, and the reverse primer extends 239 bp beyond the UAG stop codon of *topA*. The PCR was performed in a volume of 50 μl containing 100 μM each deoxynucleoside triphosphate (dNTP), 200 nM primers, 2.5 mM MgSO4, 100 ng of *Campylobacter* genomic DNA, and 5 U of Taq DNA polymerase (Promega). The cycling conditions consisted of an initial polymerase activation step at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min, with a final extension at 72°C for 10 min. The amplified PCR products were purified with the QIAquick PCR purification kit (Qiagen) and subsequently sequenced.

REAL-TIME QUANTITATIVE RT-PCR

The transcription of *topA* in 62301S2 and 62301R33 was compared by qRT-PCR. The primers used for qRT-PCR are listed in **Table 2** and the method was performed as described in a previous work (Lin et al., 2005).

ANTIBIOTIC SUSCEPTIBILITY TEST

The MIC of ciprofloxacin was determined by using *E*-test strips (AB Biodisk, Solna, Sweden) following the manufacturer's instructions. The detection limit of the *E*-test for ciprofloxacin was 32 μg/ml.

RESULTS

DIRECT ROLE OF THE Thr-86-Ile CHANGE IN THE ENHANCED FITNESS

A previous study by Luo et al. (2005) showed that FQ^R *Campylobacter* carrying the Thr-86-Ile substitution GyrA outcompeted its isogenic FQ^S strains in chickens, suggesting that acquisition of FQ^R enhances the *in vivo* fitness of FQ^R *Campylobacter*. The previous work was done using FQ^R mutants generated from antibiotic selection or natural transformation. To further confirm the link of this specific mutation with the enhanced fitness, we reverted the mutation back to the WT allele and conducted pairwise competition in chickens using 62301R33S and 62301R33R (**Table 1**). After inoculation with either of the two isolates or mixed populations, all of the groups of birds were colonized by *Campylobacter* at similar levels (**Figure 1A**). When separately inoculated into chickens, the two strains showed no significant differences ($P > 0.05$) in the level of colonization (the number of *Campylobacter* shed in feces; **Figure 1A**). Differential plating by using ciprofloxacin-containing plates showed that the group inoculated with the FQ^S revertant alone shed homogeneous FQ^S *Campylobacter*, whereas the group inoculated with FQ^R strain shed homogeneous FQ^R *Campylobacter* during the entire course of the experiment. However, in the group inoculated with mixtures of the two strains, the FQ^S revertant strain was outcompeted by its parent FQ^R *Campylobacter* ($P < 0.05$; **Figure 1B**). *E*-test of *Campylobacter* colonies (10∼15 colonies per group per time point) randomly selected from non-antibiotic plates confirmed the results of differential plating. This result clearly indicates that once the C257T mutation in *gyrA* is reverted, *Campylobacter* loses its fitness advantage in chickens, confirming the previous results using clonally related and isogenic strains (Luo et al., 2005). To further show the specific effect of the C257T mutation on fitness, we introduced this mutation into a different strain background (NCTC 11168) and conducted chicken competition using the isogenic pair of constructs. As shown in **Figure 1C**, 11168(S) was outcompeted by 11168(R) ($P < 0.05$), indicating the mutation had the same impact on fitness in a *C. jejuni* strain different from the previously tested ones. These new findings plus our previously published studies (Luo et al., 2005) convincingly showed that the C257T mutation in *gyrA* is directly responsible for the enhanced fitness of *Campylobacter* in chickens.

NO COMPENSATORY MUTATIONS IN *topA*

Campylobacter jejuni has only two types of topoisomerases, type I (TopA) and type II (gyrase). The gyrase introduces negative supercoiling to DNA, while TopA relaxes DNA to prevent excessive supercoiling. Thus, the two enzymes are the key proteins modulating the level of DNA supercoiling in *Campylobacter*. To determine

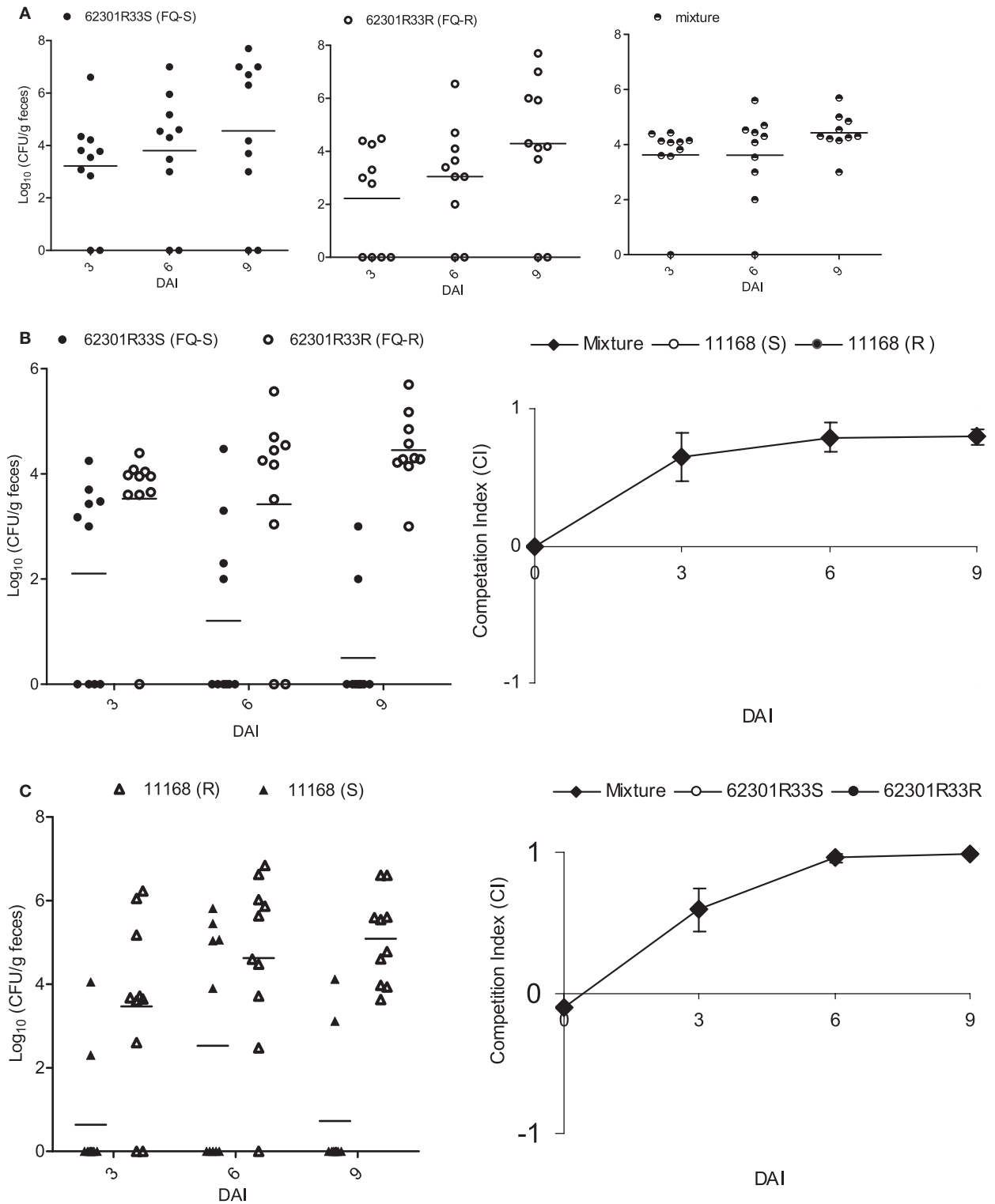

FIGURE 1 | Pairwise competitions between FQ^S and FQ^R strains in chickens using isogenic pairs of 62301R33S/62301R33R (A,B) and 11168(S)/11168(R) (C). (A) The colonization level of total *Campylobacter* in three groups of chickens that were inoculated with 62301R33S (solid circle), 62301R33R (open circle), or 1:1 mixture of the two stains. **(B)** Differential enumeration of FQ^S (solid circle) and FQ^R (open circle) *Campylobacter* in the group inoculated with a mixture of 62301R33S and 62301R33R. **(C)** A second chicken experiment shows the competition between FQ^S (solid triangle) and FQ^R (open triangle) *Campylobacter* in a different strain background. The chickens were inoculated with a 1:1 mixture of 11168(S) and 11168(R). In all panels, each symbol represents the colonization level of a single chicken and the median level of each group is indicated by a horizontal bar. The detection limit of the plating method was about 100 CFU/g of feces and a negative sample was arbitrarily assigned a value of zero. DAI, days after inoculation.

if there were any mutations in TopA that might potentially offset the impact of the Thr-86-Ile mutation in GyrA, we sequenced *topA* and compared its transcription between isogenic strains. The whole ORF of *topA* was PCR amplified from 62301R33 and sequenced, which showed that the *topA* sequence was identical to that in 62301S2. In addition, the expression level of *topA* was not different in 62301R33 and 62301S2 as determined by qRT-PCR (data not shown). These results suggest that the Thr-86-Ile mutation in GyrA was not accompanied by changes in *topA* sequence or expression.

EFFECT OF GyrA MUTATIONS ON *IN VITRO* SUPERCOILING

To determine the impact of the resistance-conferring mutations on the enzymatic activities of GyrA, we produced the recombinant forms of various gyrases. As estimated by SDS-PAGE, the recombinant GyrA and GyrB were 97 and 87 kDa, respectively, comparable with the calculated molecular masses of the two proteins. The recombinant GyrA carrying a Thr-86-Ile, Thr-86-Lys, or Asp-90-Asn mutation migrated at a position similar to the recombinant GyrA of the WT strain on SDS-PAGE (data not shown), indicating that the point mutations did not affect the migration of GyrA on SDS-PAGE. The supercoiling activity of the recombinant DNA gyrases from the WT and mutant strains was analyzed by an *in vitro* assay. Both GyrA and GyrB are required for producing negative supercoiling and no detectable supercoiling activity was observed when only one subunit (either GyrA or GyrB) was used in the assay (data not shown). The recombinant GyrA from mutant strains carrying the Thr-86-Lys or Asp-90-Asn mutation exhibited a supercoiling activity similar to that of the recombinant GyrA from the WT strain, whereas the negative supercoiling activity of the GyrA from the mutant strain carrying the Thr-86-Ile change was substantially reduced compared with the GyrA from the WT strain (**Figure 2**). In fact, the most supercoiled band was absent with this mutant GyrA (**Figure 2**). The results were consistently shown in multiple experiments with different concentrations of gyrases (up to 1200 nM; data not shown) and indicate that the Thr-86-Ile mutation, but not other mutations, alters the negative supercoiling activity of GyrA.

EFFECT OF THE GyrA MUTATIONS ON *IN VIVO* SUPERCOILING

The recombinant GyrA carrying the Thr-86-Ile mutation showed reduced supercoiling activity *in vitro*, but it is important to determine if the same mutation also affects DNA supercoiling *in vivo* (in *Campylobacter* cells). For this purpose, we used pRY107, a shuttle plasmid, as a reporter plasmid to monitor the relative levels of DNA supercoiling in the FQ^S and FQ^R strains. Plasmid pRY107 was transferred into 62301S2 (WT strain), 62301R33 (carrying the Thr-86-Ile mutation in GyrA), 52901-II2 (carrying the Thr-86-Lys mutation in GyrA), and 62301R37 (carrying the Asp-90-Asn mutation), which were clonally related and all derived from strain S3B (Luo et al., 2005). Plasmid DNA was re-isolated from these constructs and analyzed by agarose gel electrophoresis in the presence of chloroquine. Under the condition utilized in this study (20 μg/ml chloroquine), negatively supercoiled forms of pRY107 migrated slower than relaxed topoisomers. This pattern of migration was confirmed by using commercially available plasmid pBR322 (supercoiled and relaxed; data not shown). Compared to the pRY107 from 62301S2, the plasmid topoisomers from 62301R33 harboring the Thr-86-Ile mutation in GyrA shifted to lower positions, indicating less supercoiling (**Figure 3A**). As determined by densitometrical analysis, the percentage of the most supercoiled DNA in the total population of the plasmid topoisomers extracted from 62301S2 and 62301R33 was 87.4 and 20.2%, respectively. The plasmids from 52901-II2 and 62301R37 showed topoisomer distribution patterns similar to that of 62301S2 (**Figure 3A**). These results indicate that the GyrA mutant with the Thr-86-Ile mutation reduced DNA supercoiling in *Campylobacter* cells, while the mutants with the Thr-86-Lys or Asp-90-Asn changes in GyrA did not alter DNA supercoiling. To further confirm the impact of the Thr-86-Ile change on DNA supercoiling, we introduced pRY107 to a different strain background, NCTC 11168 and 11168CT. The only known difference between this pair of isolates is the C257T mutation in *gyrA* (**Table 1**). Similar to the result from 62301R33, the plasmid topoisomers from 11168CT shifted to lower positions compared with NCTC 11168 (**Figure 3B**), indicating reduced negative supercoiling. The *in vivo* supercoiling results were consistent with the findings from the *in vitro* supercoiling assay and demonstrated that the Thr-86-Ile mutation in GyrA reduced DNA supercoiling in *Campylobacter*.

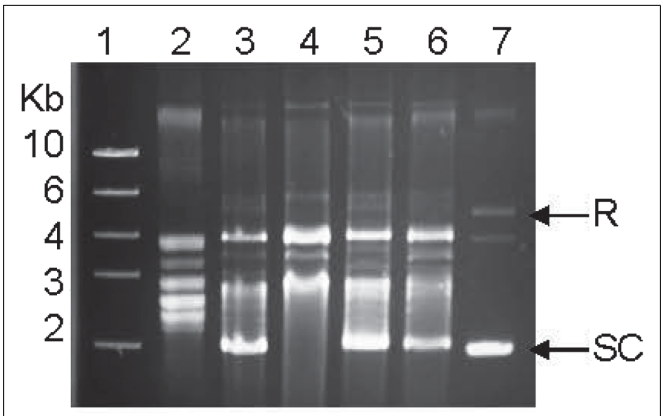

FIGURE 2 | Supercoiling activities of the recombinant WT gyrase and the three recombinant mutant gyrases measured by an *in vitro* assay. Lane 1, DNA ladder; Lane 2, relaxed pBR322 (control); Lane 3, WT GyrA + GyrB; Lane 4, mutant GyrA (Thr-86-Ile) + GryB; Lane 5, mutant GyrA (Thr-86-Lys) + GryB; Lane 6, mutant GyrA (Asp-90-Asn) + GryB; and Lane 7, supercoiled pBR322 (control). SC indicates supercoiled DNA and R represents relaxed DNA.

GyrA MUTATIONS REDUCE THE SUSCEPTIBILITY TO CIPROFLOXACIN

The GyrA mutations confer resistance to FQ antimicrobials and it is likely that the resistance is due to the reduced susceptibility of the mutant gyrases to the antibiotics. This possibility was determined using the *in vitro* supercoiling assay. As shown in **Figure 4**, the MEC of ciprofloxacin to the recombinant WT GyrA is 32, while the MEC of the two recombinant mutant gyrases carrying the Thr-86-Lys or Asp-90-Asn mutations were 1024, indicating that the two mutations significantly reduced the susceptibility of GyrA to the inhibition by ciprofloxacin. With the mutant GyrA carrying

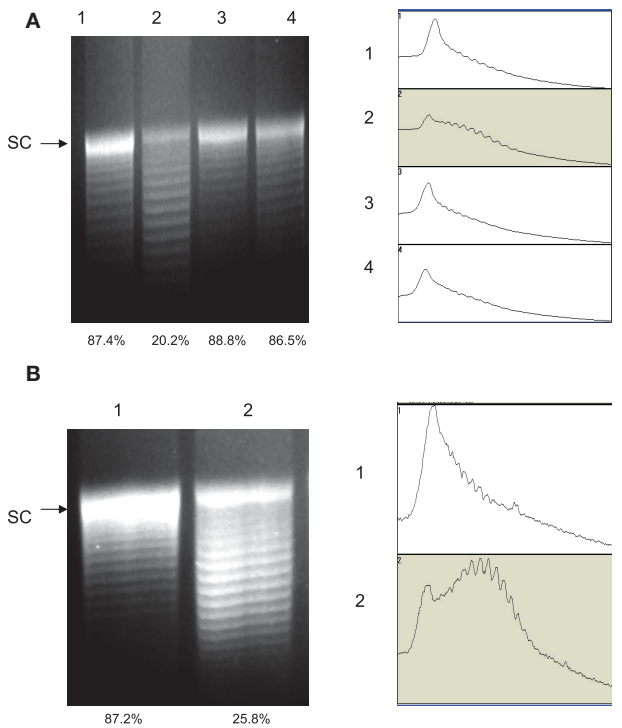

FIGURE 3 | Agarose gel electrophoresis analysis of plasmid topoisomers extracted from different strain background. The plasmid DNA was run on a 1.0% agarose gel containing 20 μg/ml chloroquine. **(A)** Plasmid pRY107 isolated from strain S3B derivatives. Lane 1, pRY107 from 62301S; Lane 2, pRY107 from 62301R (carrying the Thr-86-Ile mutation in GyrA); Lane 3, pRY107 from 52901-II2 (carrying the Thr-86-Lys mutation in GyrA); and Lane 4, pRY107 from 62301R37 (carrying the Asp-90-Asn mutation in GyrA). **(B)** Plasmid pRY107 isolated from strain 11168 and its derivatives. Lane 1, pRY107 from NCTC 11168; and Lane 2, pRY107 from 11168CT (carrying the Thr-86-Ile mutation in GyrA). The results of densitometric scanning are shown to the right of each gel image. The numbers on the left of the densitometric scanning correspond to the lane numbers of the gel image. The number below each lane indicates the percentage of the most supercoiled DNA in the total population of plasmid topoisomers as measured by densitometry.

the Thr-86-Ile change, the inhibitory effect of ciprofloxacin was not measurable because the mutation itself abolished the ability of the enzyme to form the supercoiled band in the *in vitro* assay (**Figure 4**). These findings suggest that the resistance-conferring mutations in GyrA desensitize the inhibitory effect of ciprofloxacin and provide a molecular basis for the reduced susceptibility of FQ^R mutants to ciprofloxacin.

DISCUSSION

Several reports have documented the variable fitness changes associated with topoisomerase mutations in bacteria (Bagel et al., 1999; Gillespie et al., 2002; Giraud et al., 2003; Ince and Hooper, 2003; Kugelberg et al., 2005). In *S. typhimurium*, the FQ^R mutants selected by *in vitro* plating were highly resistant to FQs, but grew significantly slow in culture media, and failed to colonize chickens (Giraud et al., 2003). On the contrary, the *in vivo* selected FQ^R *Salmonella* isolates exhibited intermediate susceptibility to FQs, had normal growth in liquid medium, and were able to colonize chickens as efficiently as or lower than that of the WT strains (Giraud et al., 2003; Zhang et al., 2006). In the case with FQ^R *E. coli*, single mutations in DNA gyrase or topoisomerase IV conferred a low-level resistance to FQs and did not incur a significant fitness cost, while accumulation of multiple mutations in the enzymes resulted in a high-level resistance and a significant fitness disadvantage (Bagel et al., 1999; Komp Lindgren et al., 2005; Morgan-Linnell and Zechiedrich, 2007). A recent study using isogenic constructs further demonstrated the variable effects of single and multiple GyrA mutation on the fitness of *E. coli* (Marcusson et al., 2009). Thus, depending on the types of mutations, bacterial organisms, and the environment in which fitness is measured, FQ^R-conferring GyrA mutations either have no effects on bacterial fitness or incur a fitness cost.

In contrast to the findings in other bacteria, Luo et al. (2005) using clonally related isolates and isogenic transformants showed that FQ^R *Campylobacter* outcompeted FQ^S strains in chickens in the absence of FQ antimicrobials and the enhanced fitness was linked to the single point mutation (Thr-86-Ile) in *gyrA*, which confers on *Campylobacter* a high-level resistance to FQ antimicrobials. To exclude the possible involvement of compensatory mutation and further confirm the specific role of this resistance-conferring mutation in the enhanced fitness, we constructed a revertant of FQ^R *Campylobacter*, in which the C257T change was reverted. The *in vivo* results showed that once the Thr-86-Ile mutation in GyrA of 62301R33 was reverted, *Campylobacter* lost its fitness advantage in chickens (**Figure 1**), confirming the specific effect of the point mutation on *Campylobacter* fitness. In addition, introducing the Thr-86-Ile mutation into the GyrA of *C. jejuni* NCTC11168, which is a different strain and divergent from the S3B derivatives used in the previous work (Luo et al., 2005), also enhanced its fitness in chickens. These new results along with the previous findings conclusively establish that the Thr-86-Ile mutation in GyrA modulates fitness of *C. jejuni* in chickens.

Previously it was shown in other bacteria that antibiotic resistance-conferring mutations in GyrA affected the supercoiling activity of GyrA (Barnard and Maxwell, 2001; Kugelberg et al., 2005). For example, Barnard and Maxwell (2001) showed that FQ^R *E. coli* mutant carrying a single (Ala^{87}) or double mutations (Ala^{83} Ala^{87}) in GyrA exhibited reduced supercoiling compared to that of the WT strain. In *Pseudomonas aeruginosa*, it was also shown that FQ^R GyrA Ile^{83} mutant and Tyr^{87} mutant had decreased supercoiling, which was associated with reduced growth rate (Kugelberg et al., 2005). In this study, we demonstrated that in *Campylobacter* the recombinant GyrA with the Thr-86-Ile change showed a greatly reduced supercoiling activity compared with that of the WT enzyme (**Figure 2**). The mutation also reduced DNA supercoiling in *Campylobacter* cells (**Figure 3**). The results from the *in vitro* and *in vivo* supercoiling assays are consistent and indicate that acquisition of the Thr-86-Ile mutation in GyrA impacts DNA supercoiling homeostasis within *Campylobacter*. In contrast, the supercoiling activity of the mutant gyrases harboring the Thr-86-Lys or the Asp-90-Asn mutation was comparable to that of the WT gyrase (**Figure 2**), suggesting that these two types of mutations do not affect the physiological function of the enzyme.

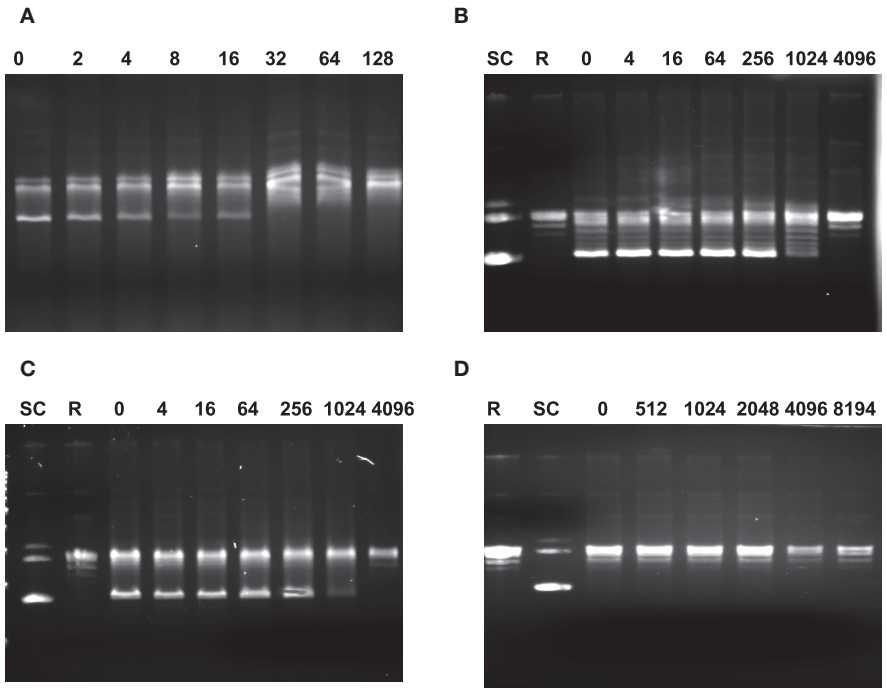

FIGURE 4 | Agarose gel electrophoresis showing the inhibition of DNA gyrase supercoiling activities by ciprofloxacin. In each reaction, the relaxed pBR322 was incubated with different enzymes in the presence of various concentration of ciprofloxacin. **(A)** Reactions with recombinant GyrA from the WT strain and GyrB; **(B)** reactions with mutant GyrA (Thr-86-Lys) and GyrB; **(C)** reactions with mutant GyrA (Asp-90-Asn) and GyrB; and **(D)** reactions with mutant GyrA (Thr-86-Ile) + GyrB. The numbers on top of each panel are the concentrations of ciprofloxacin (μg/ml) used in the reaction. SC indicates supercoiled DNA and R represents relaxed DNA (TopoGen). In each lane, the most supercoiled band migrates faster than the other topoisomers.

Interestingly, the Thr-86-Ile and Thr-86-Lys mutations occurred at the same place, but had a different impact on the enzyme function. This is probably due to the fact that Thr and Lys are both hydrophilic amino acids, while Ile is a hydrophobic residue. Thus the Thr-86-Ile change would conceivably more disruptive than the Thr-86-Lys mutation to the function of GyrA. In *Campylobacter* GyrA, Thr-86 is equivalent to Ser-83 in *E. coli* GyrA (Wang et al., 1993). It has been known that Ser-83 is located in the QRDR and Ser-83 is involved in the interaction of gyrase–quinolone–DNA complex (Barnard and Maxwell, 2001). Thus changes in the QRDR of GyrA in *Campylobacter* likely prevent FQ binding, producing resistance to FQ antimicrobials. Indeed, we demonstrated that the mutant gyrases carrying the Thr-86-Lys or the Asp-90-Asn mutation had significantly higher MECs of ciprofloxacin than the WT GyrA (**Figure 4**.), indicating that the mutant gyrases are more resistant to the inhibition by ciprofloxacin. Interestingly, the MECs of ciprofloxacin with the enzymes were significantly (at least 250 times) higher than the MICs of ciprofloxacin in these strains. This finding was consistent with the results from other studies (Gootz and Martin, 1991; Martinez et al., 2006) in which MECs and MICs were found to differ by one or two orders of magnitude. Due to the lack of supercoiling activity in the absence of ciprofloxacin, the MEC of the mutant gyrase with the Thr-86-Ile mutation was not measurable using the *in vitro* supercoiling assay (**Figure 4**). However, the MEC of this mutant gyrase is expected to be much higher than those of the other two mutant gyrase as the mutation Thr-86-Ile produces a higher level of FQ^R than the other types of mutations. These findings provide a molecular explanation for the resistance FQ antimicrobials in *Campylobacter*.

In *Campylobacter*, the genes encoding topoisomerase IV (*parC/parE*) are absent (Parkhill et al., 2000b), and the supercoiling homeostasis is controlled by topoisomerase I (TopA) and topoisomerase II (GyrA and GyrB). Our previous study has shown that the FQ^R isolates had no compensatory mutations in GyrA and GyrB (Luo et al., 2005). Since topoisomerase I, encoded by *topA*, is involved in the relaxation of DNA supercoiling and also plays an important role in maintaining the topological state of DNA, it is necessary to determine if there are any compensatory mutations in *topA* and whether the expression of the *topA* is changed in FQ^R *Campylobacter*. Our results showed that the coding sequence of *topA* was identical between strains 62301S2 and 62301R33 and there was no difference in the expression of *topA* between these two strains, which excluded the possibility that mutations or altered expression of *topA* contributed to the fitness change in FQ^R *Campylobacter*.

In conclusion, the present study shows that the Thr-86-Ile mutation in GyrA is directly linked to the enhanced fitness of FQ^R *Campylobacter* and no compensatory mutations in *topA* are associated with this fitness change. The Thr-86-Ile mutation, not other types of mutations, reduces the supercoiling activity of GyrA and modulates DNA supercoiling homeostasis in *Campylobacter*. Given that DNA supercoiling is important for gene expression in bacteria (Dorman and Corcoran, 2009; Booker et al., 2010), it is tempting to speculate that the altered DNA supercoiling might be linked to the fitness change in FQ^R *Campylobacter*. This possibility

awaits further investigation in future studies. Findings from this study provide new insights into the molecular mechanisms associated with the enhanced fitness in FQ^R *Campylobacter*, which continue to persist in the absence of antibiotic selection pressure.

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Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates

Virginie Dufour[1†], Bachar Alazzam[1], Gwennola Ermel[1†], Marion Thepaut[1], Albert Rossero[2,3], Odile Tresse[2,3] and Christine Baysse[1†]*

[1] Duals Team, UMR6026-CNRS, University of Rennes 1, Rennes, France
[2] INRA UMR1014 SECALIM 1014, Nantes, France
[3] LUNAM Université, Oniris, University of Nantes, Nantes, France

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Qijing Zhang, Iowa State University, USA
Kelli L. Hiett, Agricultural Research Service, USA

***Correspondence:**
Christine Baysse, DUALS Team, UMR6026-CNRS, University of Rennes 1, 262, Avenue du Général Leclerc, F-35042 Rennes, France.
e-mail: christine.baysse@univ-rennes1.fr

†Present address:
Virginie Dufour, Gwennola Ermel and Christine Baysse, Microbiology Team, EA1254, University of Rennes 1, 35042 Rennes, France.

Food-borne human infection with *Campylobacter jejuni* is a medical concern in both industrialized and developing countries. Efficient eradication of *C. jejuni* reservoirs within live animals and processed foods is limited by the development of antimicrobial resistances and by practical problems related to the use of conventional antibiotics in food processes. We have investigated the bacteriostatic and bactericidal activities of two phytochemicals, allyl-isothiocyanate (AITC), and benzyl isothiocyanate (BITC), against 24 *C. jejuni* isolates from chicken feces, human infections, and contaminated foods, as well as two reference strains NCTC11168 and 81-176. AITC and BITC displayed a potent antibacterial activity against *C. jejuni*. BITC showed a higher overall antibacterial effect (MIC of 1.25–5 $\mu g\,mL^{-1}$) compared to AITC (MIC of 50–200 $\mu g\,mL^{-1}$). Both compounds are bactericidal rather than bacteriostatic. The sensitivity levels of *C. jejuni* isolates against isothiocyanates were neither correlated with the presence of a GGT (γ-Glutamyl Transpeptidase) encoding gene in the genome, with antibiotic resistance nor with the origin of the biological sample. However the *ggt* mutant of *C. jejuni* 81-176 displayed a decreased survival rate compared to wild-type when exposed to ITC. This work determined the MIC of two ITC against a panel of *C. jejuni* isolates, showed that both compounds are bactericidal rather than bacteriostatic, and highlighted the role of GGT enzyme in the survival rate of *C. jejuni* exposed to ITC.

Keywords: *Campylobacter jejuni*, isothiocyanates, gamma glutamyl transpeptidase, antimicrobials, plant extract, glucosinolate

INTRODUCTION

Campylobacter jejuni is a food-borne pathogen responsible of severe gastrointestinal diseases worldwide. In the US, the incidence of *C. jejuni* infections is the second largest after *Salmonella* cases (Gillis et al., 2011), whereas in European Union, *Campylobacter* infections are the most commonly reported bacterial gastrointestinal diseases (European-Food-Safety-Authority, 2011). *C. jejuni* can colonize poultry, cattle, pigs, and sheep asymptomatically, and poultry is a particular common source of humans contamination (Friedman et al., 2004): humans are exposed to *C. jejuni* infection through handling and consuming contaminated meat, water, or raw milk. Infections result in severe diarrhea; moreover, serious sequels such as reactive arthritis and Guillain–Barré syndrome, a neurodegenerative complication, can result from *C. jejuni* infections (Nachamkin, 2002).

The research on natural preservatives to reduce meat contamination is therefore a major interest, and volatile substances like isothiocyanates (ITC), that may not influence processed food, are promising candidates for pathogen reduction. ITC are degradation products from glucosinolates, secondary metabolites which constitute a group of more than 140 different compounds, found in all plants belonging to the *Cruciferae* family (Fahey et al., 2001). Glucosinolates are stored in the cell vacuole and come into contact with the enzyme myrosinase (a thioglucosidase) located in cell wall or cytoplasm during tissue damage (Fenwick et al., 1983; Poulton and Moller, 1993; Magrath et al., 1994). Glucosinolates are then hydrolyzed to a number of products, ITC being the quantitatively dominant compound. It is known that glucosinolates degradation products possess biological activities including beneficial effect on human health, fungicidal, herbicidal, and nematocidal properties (Fahey et al., 1997; Bonnesen et al., 1999; Lazzeri et al., 2004; Keum et al., 2005). Amongst them, ITC exhibit biocidal activities against various bacterial pathogens. There is now ample evidence for the antimicrobial properties of ITC (Aires et al., 2009a,b), but reports of suppression of bacteria by ITC are still limited to some bacteria, and nothing is known about their activity against *C. jejuni*.

Allyl ITC (AITC) is already used as preservative in food industry (Delaquis and Mazza, 1995; Masuda et al., 2001). AITC is generated from its precursor, allyl glucosinolate, namely, sinigrin (1-thio-L-D-glucopyranose 1-*N*-(sulfoxy)-3-buteneimidate; Kawakishi and Namiki, 1969; Masuda et al., 1996) which is particularly abundant in horseradish (*Armoracia lapathifolia*) and wasabi (*Wasabia japonica*). AITC reportedly has antimicrobial activity against a wide range of microorganisms (Kyung and Fleming, 1997; Lin et al., 2000a,b; Masuda et al., 2001).

Jang et al. (2010) recently reported a greater antimicrobial activity of aromatic isothiocyanates, such as Benzyl isothiocyanate (BITC), compared to aliphatic ones, using four Gram-positive

bacteria (*Bacillus cereus* KCCM 11204, *Bacillus subtilis* KCCM 11316, *Listeria monocytogenes* KCCM 40307, and *Staphylococcus aureus* KCCM 12214) and seven Gram-negative bacteria (*Aeromonas hydrophila* KCTC 2358, *Pseudomonas aeruginosa* KCTC 1636, *Salmonella choleraesuis* KCCM 11806, *Salmonella enterica* KCTC12400, *Serratia marcescens* KCTC 2216, *Shigella sonnei* KCTC 2009, and *Vibrio parahaemolyticus* KCCM 11965). Recent data also had shown a bactericidal effect of BITC against Gram-negative periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Sofrata et al., 2011). The vegetable source of BITC is the glucotropaeolin glucosinolate, found in Cabbage (Tian et al., 2005), Wasabi (Sultana et al., 2003), Papaya (Kermanshai et al., 2001), and Mustard (Dorsch et al., 1984).

MPITC was first isolated from the seeds of *Iberis sempervirens* (Kjaer et al., 1955) and more recently from the Egyptian plant *Capparis cartilaginea* (Hamed et al., 2007). MPITC and AITC are both constituents in horseradish and are on the GRAS list permitted for such flavors (Waddell et al., 2005). In nature, AITC constitute 37% of horseradish volatiles while MPITC is a minor constituent (about 1%). The antibacterial effect of this ITC is not yet determined.

Sulforaphane is generated from glucoraphanin, an abundant glucosinolate in some varieties of broccoli. It was found to be active against *Helicobacter pylori*, a microaerophilic epsilon proteobacteria such as *C. jejuni*, which dramatically enhances the risk of gastric cancer in infected patients (Fahey et al., 2002).

The antimicrobial activity of ITC is suggested to involve a reaction with thiol groups of glutathione or redox-active proteins, with subsequent inhibition of sulfhydryl enzyme activities and inhibition of redox-based defenses (Tang and Tang, 1976; Kolm et al., 1995; Jacob and Anwar, 2008). The addition of exogenous thiol groups can suppress the antimicrobial effect of ITC (Tajima et al., 1998). The activity of ITC varies with the structure of the molecule, but variations are also noticed amongst identical bacterial species for one ITC. Therefore, we can postulate that the efficacy of the ITC may depend on both the rate of spontaneous degradation of ITC-thiol conjugates and of the detoxification mechanisms of the bacterial isolate. The specific processes for ITC resistance of bacteria are still unknown. In rats and possibly other mammals, benzylITC (BITC) is degraded via conjugation with glutathione by the glutathione-*S*-transferase (GST), and transformed to cysteinyl glycine and cysteine conjugate by the Gamma Glutamyl Transpeptidase (GGT). The latter is N-acetylated to form mercapturic acid excreted in the urine (Brusewitz et al., 1977). In bacteria, the only report on ITC detoxification concerned cyanobacteria, and pointed out the role of GST and glutathione (Wiktelius and Stenberg, 2007).

Some strains of *C. jejuni*, including the highly virulent strain 81-176, possess the GGT, while all *C. jejuni* strains are unable to synthesize glutathione (Hofreuter et al., 2006). GGT was found to be important for the chick colonization rate and is suspected to contribute to virulence of some *C. jejuni* isolates (Barnes et al., 2007; Hofreuter et al., 2008; Feodoroff et al., 2010). However, its role in the detoxification of electrophilic compound, such as ITC, has not been investigated.

This study aims to analyze the antibacterial activity of two ITC against 24 *C. jejuni* isolates from various origins: chicken feces, human infections (blood or feces) and contaminated processed meats. Additionally, we investigated whether or not the presence of GGT in these isolates affects their sensitivity to ITC.

MATERIALS AND METHODS

BACTERIAL ISOLATES AND GROWTH CONDITIONS

The *C. jejuni* isolates used in this study are listed in **Table 1**. Strains NCTC11168 and 81-176 are widely used reference strains which genome sequences have been published (Parkhill et al., 2000; Hofreuter et al., 2006). Other isolates were selected for having different origins and various antibiotic resistance profiles and were isolated from independent pork or poultry slaughterhouses and processings, or independent human cases. All isolates were streaked on Müeller Hinton agar (MHA) and grown microaerobically at 37°C for 24 h, then the cells were harvested in 2 mL Müeller Hinton broth (MHB) and diluted in the same medium to the appropriate concentration ($OD_{600\,nm} = 0.05$). All cultures were grown under microaerobic atmosphere (CampyGen, Oxoid) at 37°C with 150 rpm shaking.

PULSE FIELD GEL ELECTROPHORESIS

Pulse field gel electrophoresis (PFGE) was performed according to Ribot et al. (2001) and CAMPYNET protocol (http://Campynet.vetinst.dk/PFGE.html). Briefly, isolates were subcultured on Karmali at 42°C for 2–3 days under microaerobic atmosphere. Bacterial colonies were harvested and re-suspended in 1 mL of Tris buffer (100 mol L^{-1} Tris, pH 8, and 100 mmol L^{-1} EDTA). About 200 µL of suspension was subsequently mixed with an equal volume of 2% agarose (BioRad, Marnes-la-Coquette France) at 56°C. The mixture was molded into plugs and allowed to set at 4°C until totally gelified. The agarose plugs were placed in ETSP buffer (50 mmol L^{-1} EDTA, 50 mmol L^{-1} Tris pH 8, 1% Sarcosyl, and 1 mg mL^{-1} Proteinase K) and incubated at 54°C overnight. Then, plugs were washed in TE buffer (10 mmol L^{-1} Tris pH 8, 1 mmol L^{-1} EDTA) four times for 0.5 h. Half of each plug was digested overnight with *Sma*I (New England BioLabs, Saint Quentin en Yvelines, France) at 24°C, and the resulting macrorestriction digests were electrophoresed using CHEF-DRIII system (BioRad) in 1.3% agarose gel in 10 times diluted 5× TBE buffer (0.45 mol L^{-1} Tris–Borate, 0.01 mol L^{-1} EDTA, pH 8.3) at 6 V cm^{-1}. Pulsing was ramped from 6 to 30 s over 21 h then 2–5 s over 3 h at 14°C. Gels were stained with ethidium bromide for 2 h, destained in water for 20 min and photographed under UV light with ChemiDoc ™ XRS (BioRad). A lambda ladder PFG marker (New England BioLabs) was used for fragment size determination. Bands were analyzed using BioNumerics v 3.5 (Applied Maths Kortrijk, Belgique). Pulsotype grouping was performed with the band position tolerance of the Dice coefficient at 2.0%. When identical profiles were observed between strains with *Sma*I, *Kpn*I macrodigestion was performed in the same conditions as described above with the following modifications: electrophoresis was performed in 1.2% agarose gel and a pulsing ramping from 3 to 30 s over 22 h at 14°C.

Table 1 | *Campylobacter jejuni* isolates used in this study and some of their features.

Name	Provided by	Origin	Isolated from	Antibiotic resistance
NCTC11168	A. Stintzi[1]	Clinical isolate	Diarrheic patient, 1977	ND
81-176	O. Tresse[2]	Clinical isolate	Diarrheic patient, 1985	ND
2-77	CNR Bordeaux[3]	Clinical isolate	Human stools sample, 2010	Ampicillin, tetracycline, fluoroquinones
2-78	CNR Bordeaux[3]	Clinical isolate	Human stools sample, 2010	None
2-79	CNR Bordeaux[3]	Clinical isolate	Human blood sample, 2010	Fluoroquinones
2-80	CNR Bordeaux[3]	Clinical isolate	Human blood sample, 2010	Tetracycline
2-81	CNR Bordeaux[3]	Clinical isolate	Human intestinal biopsy, 2010	Fluoroquinones
3-1	ANSES Ploufragan[4]	Environmental	Pork slaughter house, 2009	Streptomycin, tetracycline
3-2	ANSES Ploufragan[4]	Environmental	Pork slaughter house, 2009	Streptomycin, tetracycline
3-3	ANSES Ploufragan[4]	Environmental	Pork slaughter house, 2009	Tetracycline
3-4	ANSES Ploufragan[4]	Environmental	Pork slaughter house, 2009	Ciprofloxacin, nalidixic acid
3-5	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	Tetracycline, nalidixic acid
3-6	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	Tetracycline, ciprofloxacin, Nalidixic acid
3-7	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	Tetracycline, ciprofloxacin, Nalidixic acid
3-8	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	Ciprofloxacin, nalidixic acid
3-9	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	None
3-10	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	None
3-11	ANSES Ploufragan[4]	Environmental	Poultry slaughter house, 2009	Tetracycline
3-12	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	Tetracycline, ciprofloxacin, nalidixic acid
3-13	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	None
3-14	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	Tetracycline
3-15	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	None
3-16	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	None
3-17	ANSES Ploufragan[4]	Food industry	Processed poultry, 2009	None

ND, not determined. These isolates were kindly provided by:

[1]Alain Stintzi, Ottawa Institute of System Biology, Ottawa, Canada.

[2]Odile Tresse, SECALIM, Oniris, Nantes, France.

[3]Francis Mégraud, Centre National de Référence des Campylobacters et Helicobacters, Bordeaux, France.

[4]Katell Rivoal, Hygiene, and Quality of Poultry and Pork Products, ANSES, Ploufragan, France.

GROWTH ANALYSES

A quantity of 500 μL of each isolates was inoculated at $OD_{600\,nm} = 0.05$ in triplicate on 48-wells plates, and incubated microaerobically at 37°C under shaking. The $OD_{600\,nm}$ was measured every 3 h to monitor the growth with an automatic plate reader (BioTek). The growth of each isolate was monitored at least in triplicate.

ISOTHIOCYANATES SOLUTIONS

Isothiocyanate commercial pure solutions [Allyl-isothiocyanate (AITC), benzyl isothiocyanate (BITC), ethyl isothiocyanate (ETIC), and 3-(methylthio)propyl isothiocyanate (MTPITC)] were purchased from Sigma-Aldrich. Pure solutions were diluted in absolute ethanol to 100 mg mL^{-1} (AITC and EITC) or 10 mg mL^{-1} (BITC and MPITC) stock solutions.

MINIMUM INHIBITORY CONCENTRATION DETERMINATION

Minimum inhibitory concentrations (MICs) were determined by two different versions of the agar dilution method.

Briefly, twofold serial dilutions of isothiocyanate stock solutions were added to 50°C molten MHA to get the final desired concentrations (from 6.25 to 200 μg mL^{-1} AITC and EITC or from 0.625 to 20 μg mL^{-1} for BITC and MPITC) and then the media were poured on 45 or 100 mm Petri dishes.

Cells were inoculated at $OD_{600\,nm} = 0.05$ in triplicate in 48-well plates, and incubated microaerobically for 6 h with shaking.

Each 6 h-culture was diluted to $OD_{600\,nm} = 0.01$ and 40 μL, corresponding to approximately 5×10^5 CFU mL^{-1}, were spread on 45 mm plates. MIC was defined as the lowest isothiocyanate concentration in solid MHA where no growth was observed after 48 h of 37°C microaerobic incubation.

Alternatively, 10-fold serial dilutions of 6 h-cultures were made in MHB and 5 μL of each dilution were spotted on 100 mm plates. Hundred microliters of some dilutions were also spread on Columbia agar plates to determine colony-forming units per milliliter concentration of each culture. MIC was defined as the lowest isothiocyanate concentration that inhibited any visible growth of a 10^5- to 5×10^5-CFU spot after 48 h of 37°C microaerobic incubation.

In both cases, ethanol was added to MHA as a negative control and inoculated plates without any addition were used as positive growth controls. Each plate was done in triplicates and each experiment was repeated twice.

Minimal inhibitory concentrations and MBCs were also assayed in liquid medium. Strains were inoculated in 5 mL MHB at $OD_{600\,nm} = 0.05$, then 10 μL of either isothiocyanate dilution

(in absolute ethanol) or absolute ethanol or sterile water (controls) were added. Final ITC concentrations were 10, 5, 2.5, and 1.25 $\mu g\,mL^{-1}$ for AITC, and 1.25, 0.625, 0.312, and 0.156 $\mu g\,mL^{-1}$ for BITC. $OD_{600\,nm}$ were measured before and after 18 h of microaerobic incubation at 37°C with shaking. Minimal Inhibitory Concentration was defined as the lowest concentration of ITC that inhibits any visible growth after 18 h of incubation. Cells were also spread before and after incubation on MHA for colony counting. MBC was defined as the lowest concentration of ITC that kills 99.9% of the bacteria (i.e., 3 log reduction) after 18 h of incubation.

SURVIVAL ASSAYS

Campylobacter jejuni strains were grown in MHB in 50-mL sterile culture flasks. After ITC addition, (final concentrations AITC: 50, 100, or 200 $\mu g\,mL^{-1}$; BITC: 0, 2.5, 5, or 10 $\mu g\,mL^{-1}$), samples were collected at 0, 6, 12, and 24 h of growth, and the viable cells were numbered by plating serial dilutions onto MH agar plates and colony counting after incubation for 24 h at 37°C in microaerobic conditions. The experiment was performed twice with triplicate assays.

CONSTRUCTION OF *C. JEJUNI* 81-176 *ggt* MUTANT

To investigate the roles of GGT, the chromosomal region in *C. jejuni* containing the *ggt* gene was deleted and replaced with the 3′-amino-glycoside phosphotransferase type III gene (*aphA*-3, Km cassette) through a homologous recombination event. The plasmid containing the Δ*ggt::aphA-3cat* gene was constructed as follows. The plasmid pE1509 containing the upstream sequence of the *ggt* gene was obtained by cloning the PCR product amplified with primers ggtD21Lxba (5′-GAAGATAGTATAAAATGCACTCTAG AAAAG-3′) and ggtD22Rnde (5′-ACTTAGCGTGATTGAAATCG CATATGTAG-3′) in the pGEMTeasy (Promega). After digestion by *Nde*I, the insert was introduced into the plasmid pE1520 containing the downstream sequence of the *ggt* gene which was obtained by cloning the PCR product obtained with primers ggtD21Rxba (5′-GCGATGATAATAGGA CTTGCTCTAGACACTAT-3′) and ggtD22Lsac (5′-ATCCAAAAAC TGGAAAAATCCGCGGCTCT-3′) in the pGEMTeasy (Promega) to give the plasmid pE2057. The Km cassette obtained by PCR amplification using primers LKmSma (5′-TGCCCGGGAC AGTGAATTGGAG-3′) and RKmSma (5′-CCCCCGGGCATTGCA ATCCTAA-3′) and restricted by *Sma*I was inserted at the *Pfi*MI site of the plasmid pE2057 between the upstream and downstream regions of the *ggt* gene. The resulting suicide vector pE2088 was electroporated into the *C. jejuni* 81-176 strain. K_m resistant clones were selected on Colombia agar (Oxoid) containing kanamycin (50 $\mu g\,mL^{-1}$). The double crossing over was checked using PCR amplification with either primers ggtV1for (5′-GCTTCCCACCGCAGGATCGC-3′) and KmV1rev (5′-ACCTGGGAGACAGCAACATC-3′); or primers KmV1 for (5′-TTCCTTCCGTATCTTTTACGC-3′) and ggtV1rev (5′-GCTTTTGCTTGTGCTTTTGCGGGA-3′). The construction in *C. jejuni* 81-176 was finally checked by DNA sequencing.

PCR SCREENING OF *ggt* GENE IN *C. JEJUNI* ISOLATES

Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega) for use as a PCR matrix. Presence of a GGT encoding gene on the *C. jejuni* isolates genomes was checked by PCR using two primer pairs specific for both 5′ and 3′ conserved regions of the *ggt* gene in *C. jejuni* and related strains. The *ggt* genes of *C. jejuni* 81-176, *C. jejuni* subsp. *doylei* 269.97, *C. jejuni* subsp. *jejuni* 260.94, *C. jejuni* subsp. *jejuni* HB93-13 and *H. pylori* 26695 were aligned using the MultAlin software (Corpet, 1988; **Figure A1** in Appendix). PCR amplification with ggt1 (5′-CACGCTAAGTTTTGGTGCAG-3′) and ggt4 (5′-GTCCTTCCTTTGCAATA-3′) primers produces a PCR product of 622-bp starting at base 33 from ATG of *C. jejuni ggt* genes, while utilization of ggt3 (5′-TACATGGGCGATCCTGATTT-3′) and ggt6 (5′-GCATTAGCTTCTCCGCCTA-3′) amplifies a 330-bp-long DNA fragment of *ggt* starting at base 960 from ATG of *C. jejuni ggt* genes. Genomic DNA from *C. jejuni* NCTC1168 and 81-176 were used as negative and positive controls respectively.

PCR amplifications with *C. jejuni* 16S RNA gene specific primers, 16SCJA (3′-AGAGTTTGATCCTGGCTCAG-5′) and 16SCJB (5′-TGTCTCAGTTCCAGTGTGACT-3′) were performed on each DNA sample as supplementary control.

STATISTICAL ANALYSES

Data from all three technical replicates of the two independent survival assays were analyzed by Student *t*-test. Possible correlations between origin, antibiotic resistance, presence, or absence of the *ggt* gene, and sensitivity to isothiocyanates of all 24 isolates were assessed by Fisher's exact test. For all tests, p-values < 0.05 were considered significant.

RESULTS

GROWTH RATE OF *C. JEJUNI* ISOLATES AND SELECTION

Twenty four isolates (including the two reference strains *C. jejuni* NCTC1168 and 81-176, plus the *ggt* mutant of 81-176) were selected for their similar growth rate in 48-well plates on MHB medium at 37°C in microaerobic conditions. As shown in **Table 2**, most *C. jejuni* isolates display a mean growth rate constant between 0.29 and 0.15 h^{-1} in such conditions. Two isolates (3-14 and 3-6) with a reduced growth rate as well as an isolate (3-16) with a higher growth rate were selected to evaluate the impact of the generation time on the sensitivity to ITC. However, all cultures reached a similar biomass, i.e., a final $\log_2(100 \times OD_{600\,nm})$ from 4.3 to 6.2, after 24 h growth, indicating that the cell viability was not affected in slower growth isolates (**Table 2**). Moreover, the correlation between $OD_{600\,nm}$ (0.05 and 0.15) and the number of colony-forming units per milliliter was the same for every isolate (data not shown).

PULSE FIELD GEL ELECTROPHORESIS OF *C. JEJUNI* ISOLATES

To assess the genomic diversity of the 24 isolates used in this study, PFGE was performed using *Sma*I and *Kpn*I. Each of the 24 isolates has a different *Sma*I or *Kpn*I pulsotype (**Figure 1**). Two pairs of isolates (3-8 and 3-17, 3-13 and 2-77) displayed very similar *Sma*I pulsotypes (S9 and S9′, S18 and S18′) but had different pulsotypes after *Kpn*I digestion (**Figure A2** in Appendix).

Table 2 | Growth of *C. jejuni* isolates.

Isolates	Growth rate, mean (h^{-1}) ± SD	Final log_2(100*OD), mean ± SD
NCTC1168	0.262 ± 0.046	5.637 ± 0.028
81-176	0.195 ± 0.034	5.786 ± 0.071
2-77	0.292 ± 0.003	5.368 ± 0.048
2-78	0.285 ± 0.043	5.749 ± 0.030
2-79	0.275 ± 0.056	5.809 ± 0.017
2-80	0.322 ± 0.054	5.869 ± 0.072
2-81	0.146 ± 0.026	5.339 ± 0.405
3-1	0.046 ± 0.019	4.345 ± 0.172
3-2	0.158 ± 0.008	6.038 ± 0.043
3.3	0.068 ± 0.010	4.348 ± 0.024
3-4	0.261 ± 0.085	5.357 ± 0.030
3-5	0.259 ± 0.054	5.213 ± 0.090
3-6	0.068 ± 0.006	4.945 ± 0.117
3-7	0.171 ± 0.006	5.492 ± 0.071
3-8	0.236 ± 0.023	6.199 ± 0.037
3-9	0.132 ± 0.044	5.208 ± 0.314
3-10	0.194 ± 0.009	5.368 ± 0.008
3-11	0.285 ± 0.020	5.327 ± 0.035
3-12	0.370 ± 0.056	5.823 ± 0.237
3-13	0.291 ± 0.028	5.056 ± 0.126
3-14	0.239 ± 0.012	5.953 ± 0.150
3-15	0.256 ± 0.041	5.323 ± 0.123
3-16	0.450 ± 0.061	5.190 ± 0.189
3-17	0.353 ± 0.045	5.725 ± 0.039

C. jejuni isolates were grown on 48-well plates in MH medium, in microaerobic conditions (CampyGen, Oxoid) at 37°C. Growth rates were calculated as the slope of the growth curves during logarithmic phase and expressed as h^{-1}. Final OD were measured at 600 nm after 24 h growth. SD (minimum of three replicates).

MINIMAL INHIBITORY CONCENTRATIONS OF ISOTHIOCYANATES AGAINST *C. JEJUNI* ISOLATES

AITC, BITC, and MPITC were preliminary chosen for their chemical properties (**Figure 2**), their occurrence in natural plant compounds and for their reported antibacterial activity.

EITC was selected as a negative control since it has a high volatile property (**Figure 2**) and there is no report of an antibacterial effect such as when tested against intestinal bacteria including *E. coli*, C*lostridia*, *Lactobacilli,* and bifidobacteria (Kim and Lee, 2009).

Although sulforaphane was previously reported to display an inhibitory effect against the *C. jejuni* closely related genus *Helicobacter* (Fahey et al., 2002), and despite preliminary experiments carried-out in our laboratory that demonstrated a MIC of 15 $\mu g\,mL^{-1}$ against *C. jejuni* NCTC1168 (Ermel, G., unpublished), the high cost of chemically purified sulforaphane dissuaded us from using it in a large scale study.

A first assay was performed with the spread-based method to determine the MIC of the four ITC against the reference strain *C. jejuni* NCTC1168. As expected, the MIC of EITC was higher than 200 $\mu g\,mL^{-1}$ (upper limit of the assay) while the MIC of AITC was of 200 $\mu g\,mL^{-1}$. MPITC and BITC displayed an identical MIC of 5 $\mu g\,mL^{-1}$. Therefore, BITC was selected instead of MPTIC for further study according to its widespread natural occurrence.

When the MIC assay was extended to the 24 *C. jejuni* isolates by the spot-based method, two groups of sensitivity appeared (**Figure 1**). The nine more resistant isolates are NCTC1168, 2-77, 2-78, 2-80, 3-2, 3-4, 3-6, 3-16, and 3-17, on which the MIC of AITC was of 200 $\mu g\,mL^{-1}$ and the MIC of BITC was of 5 $\mu g\,mL^{-1}$. For the majority of tested *C. jejuni* isolates ($n = 11$) the MIC were of 100 $\mu g\,mL^{-1}$ for AITC and 2.5 $\mu g\,mL^{-1}$ for BITC. The growth of the most sensitive isolate (3-13) was inhibited at minimal concentrations of 50 $\mu g\,mL^{-1}$ of AITC and 1.25 $\mu g\,mL^{-1}$ of BITC. Additionally, four isolates (3-5, 3-9, 3-12, and 3-15) displayed miscellaneous sensitivity to AITC (from 50 to 200 $\mu g\,mL^{-1}$) and BITC (from 2.5 to 5 $\mu g\,mL^{-1}$). Isothiocyanate sensitivity profiles did not correlate with similarities between isolates as determined by PFGE. Even closely related isolates (3-8 and 3-17, 3-13 and 2-77) have different sensitivities to AITC and BITC (**Figure 1**).

It is interesting to note that the *ggt* mutation in *C. jejuni* 81-176 did not impact on the MIC of AITC and BITC (**Figure 1**).

COMPARISON BETWEEN ITC RESISTANCE AND *ggt* DETECTION ON GENOMES

Using two distinct pairs of primers specific for conserved regions of *ggt* in four *C. jejuni* genomes, DNA fragments were amplified by PCR with both primer pairs in *C. jejuni* isolates 81-176 (positive control), 2.77, and 3.16.

This low prevalence of the *ggt* gene in our *C. jejuni* isolate panel is in accordance with a previous study that described the presence of GGT in only 15 out of 166 *C. jejuni* human isolates (Feodoroff et al., 2010) whereas Gonzalez et al. (2009) identified 36.6% of their chicken isolates (out of 205) as *ggt*-positive.

There is no correlation neither between the MIC of AITC and BITC and the presence of GGT nor between the MIC and the origin or pulsotype of the *C. jejuni* isolates (**Figure 1**; Fisher's exact test, p-value > 0.05).

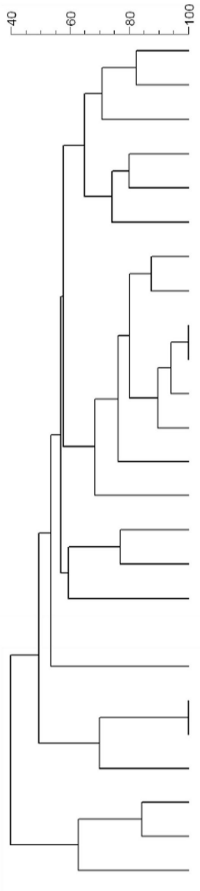
DETERMINATION OF THE MINIMAL BACTERICIDAL CONCENTRATION

For both ITC, MIC, and MBC values were identical (**Table 3**.). Therefore, we can affirm that AITC and BITC have a bactericidal effect on *C. jejuni*. Moreover the MIC values are higher against *C. jejuni* NCTC1168 than against 81-176, and not affected by the *ggt* mutation, as found with the agar dilution method (**Figure 1**).

AITC and BITC MIC and MBC values are the same on wild-type 81-176 and *ggt* mutant. However, MBC are discrete values, so the wild-type and mutant strain can have a different killing rate while displaying the same MBC value. While counting colonies for this MBC assay, we noticed that there were 100–1000 times less *ggt* mutant cells than 81-176 wild-type cells after treatment with 5 $\mu g\,mL^{-1}$ AITC or 0.625 $\mu g\,mL^{-1}$ BITC (corresponding to MBC values). As a consequence, we decided to carry on a more dynamic sensitivity experiment on these two strains.

SURVIVAL ASSAYS OF *C. JEUNI* 81-176 AND *ggt* MUTANT

The standard assay for testing the antibiotic susceptibility of bacteria is MIC, but this method is of limited value in determining the susceptibility kinetic of bacteria and the ratio of cells surviving to MIC. An alternative approach to measurement of the bactericidal activity of antimicrobial agents is the time-kill method, which



Pulsotypes			Minimal inhibitory concentration			
	SmaI	KpnI	Isolate name	*ggt*	AITC	BITC
	S1	ND	3-9		50	2.5
	S2	ND	3-14		100	2.5
	S3	ND	2-79		100	2.5
	S4	ND	3-15		100	5
	S5	ND	3-16	+	200	5
	S6	ND	3-5		200	2.5
	S7	ND	NCTC11168		200	5
	S8	ND	3-7		100	2.5
	S9	K1	3-8		100	2.5
	S9'	K2	3-17		200	5
	S10	ND	3-11		100	2.5
	S11	ND	3-4		200	5
	S12	ND	2-80		200	5
	S13	ND	3-12		100	5
	S14	ND	3-10		100	2.5
	S15	ND	2-81		100	2.5
	S16	ND	81-176	+	100	2.5
			↳ 81-176 Δ*ggt*		100	2.5
	S17	ND	3-6		200	5
	S18	K3	3-13		50	1.25
	S18'	K4	2-77	+	200	5
	S19	ND	2-78		200	5
	S20	ND	3-2		200	5
	S21	ND	3-3		100	2.5
	S22	ND	3-1		100	2.5

FIGURE 1 | AITC and BITC minimal inhibitory concentrations (determined by agar dilution method) on 24 *C. jejuni* isolates. From left to right: dendrogram generated using Dice's coefficient and showing isolates grouping based on *Sma*I PFGE; *Sma*I PFGE profiles; *Sma*I and *Kpn*I pulsotypes of the 24 isolates; *ggt:* +: positive PCR amplification of *ggt* gene with both primer pairs; MIC of AITC and BITC, expressed as the lowest concentration (μg mL^{-1}) inhibiting all visible growth of a 5×10^5-CFU mL^{-1} inoculate. ND: not determined.

examines the impact of antimicrobial exposure on the bacterial population at multiple time-points rather than the single time point used in the MIC method, and evaluates the rate of survival rather than the inhibition of growth.

To determine whether AITC and BITC were as effective in killing the *C. jejuni* 81-176 and *ggt* mutant, cell viability was measured by bacterial plating at concentrations closer to the MIC and at completely lethal concentrations (**Figure 3**).

For the strain *C. jejuni* 81-176 on which AITC displayed a MIC of 100 μg mL^{-1} in solid medium, an exposure of 24 h to 100 μg mL^{-1} AITC is sufficient to kill the whole population. Interestingly, the isogenic *ggt* mutant of 81-176 had a lower survival rate than the wild-type strain when exposed to AITC (**Figure 3A**, $p = 0.0001$); however both populations are erased by 24 h-exposure to 50 μg mL^{-1} AITC. A similar pattern was found when *C. jejuni* 81-176 strain and *ggt* mutant were exposed to BITC in liquid MH although the difference in survival rates between the two strains and mutant were less marked than with AITC but still significant (**Figure 3B**, $p = 0.001$).

DISCUSSION

The development of antibiotic resistance by *C. jejuni* strains, mainly to fluoroquinolone and macrolides, is a major concern for human health and poultry industry (Luangtongkum et al., 2009; Smith and Fratamico, 2010). Although antibiotics have been banned by the poultry industry, there is a persistence of antibiotic resistant strains of *C. jejuni* in animal reservoirs. Zhang et al. (2003a) demonstrated that these resistant strains survived more successfully in their hosts than the non-resistant strains even without selection pressure.

Cross-contamination from various sources during slaughter occurred, but the majority of *Campylobacter* contamination on carcasses appeared to originate from the slaughtered flock itself (Wirz et al., 2010). Therefore, by reducing the colonization of chick intestine by *C. jejuni*, the human infection through contaminated food consumption may be reduced.

It has been suggested that *C. jejuni* was more sensitive to natural antimicrobials such as olive leaf extract compared to other pathogenic microorganisms (Sudjana et al., 2009). Other examples of

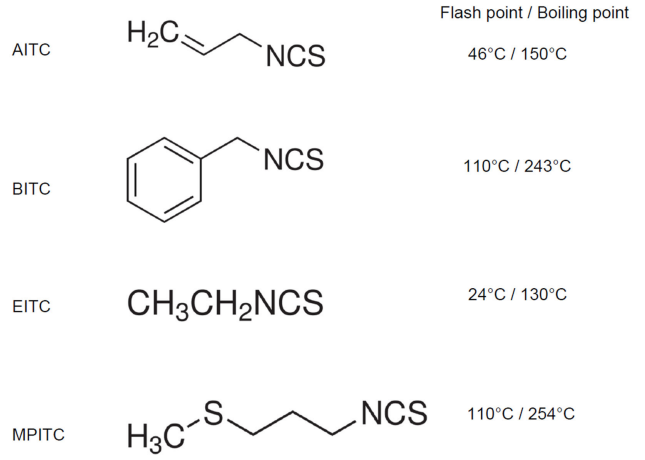


FIGURE 2 | Chemical structure and properties of the four ITC tested in this study. The flash point is the lowest temperature for which the chemical is evaporating to give a combustible concentration of gas and is indicative of the evaporation rate at a given temperature.

Table 3 | Minimal inhibitory (MIC) or bactericidal (MBC) concentrations in broth cultures.

Strain	MIC		MBC	
	AITC	BITC	AITC	BITC
NCTC11168	10	1.25	10	1.25
81-176	5	0.625	5	0.625
81-176 Δ*ggt*	5	0.625	5	0.625

Values are given in $\mu g\ ml^{-1}$.

efficient natural antimicrobials against *C. jejuni* include linalool vapor of bergamot and linalool oils (Fisher and Phillips, 2006) and essential oil from *Origanum minutiflorum* (Aslim and Yucel, 2008).

It is only recently that plant-derived food ingredients have been explored for their antimicrobial properties. So far, essential oils were the most frequently used form of plant extract tested for antimicrobial activity in foods. Isothiocyanates are other plant extracts with flavoring properties that are arising as promising antimicrobial agents, with activities often rivaling synthetic chemicals. Researches are focusing on the use of active packaging with ITC (Shin et al., 2010), or their combination with existing antibiotic treatments (Palaniappan and Holley, 2010). However there is only one report about efficiency of ITC (sulforaphane) against *C. jejuni* (Woelffel, 2003).

Our work assessed *C. jejuni* sensitivity to isothiocyanates using several methods: minimal inhibitory concentration (MIC) determination in solid and liquid media, MBC determination, and survival assays.

Not surprisingly, the behavior of *C. jejuni* strains in liquid culture plus ITC appears different than onto solid medium. MIC values of AITC and BITC are lower when measured in MH broth than in agar dilution method (**Figure 1**; **Table 3**). MIC values are not a biological constant. The MIC is influenced by many factors such as the interaction between the antimicrobial agent, the bacterial cell and the medium, and the physiological status of the cells. It is known that for most antimicrobial, the concentration required to kill sessile bacteria may be greater than those required to kill planktonic bacteria. MIC values can only be compared if they are measured under well standardized conditions, which are not yet been defined for such volatile compounds as ITC. We have adapted the agar dilution methods for plant extracts previously described by Klancnik et al. (2010). These authors already pointed out a discrepancy between the antibacterial activity levels obtained by the agar dilution method and the broth dilution method for Gram-negative bacteria: a lower concentration of antimicrobial was required for growth inhibition in liquid culture. Moreover, the evaporation rate of ITC may be higher in liquid culture than when ITC are embedded into agar medium. Previous data have shown that AITC vapor was more effective as antimicrobial agent than liquid AITC (Shin et al., 2010); thus it may partially explained the differences we have observed between the two methods.

To investigate the bacteriostatic or bactericidal effect of isothiocyanate on *C. jejuni*, we carried on a MIC determination experiment in liquid media on the two reference strains NCTC11168 and 81-176, and the *ggt* mutant. By counting the viable cells at different ITC concentrations, the MBC can be determined. A given compound can be called bactericidal when it kills bacteria rather than it inhibits the metabolism, i.e., when the MIC and the MBC value are identical. The MBC of AITC and BITC was measured as the minimum concentration needed to kill most (99.9%) of the *C. jejuni* cells (NCTC1168 and 81-176 strains) after incubation for 18 h under a given set of conditions: in MH broth at 37°C in microaerobic conditions. While sulforaphane was reported as a bacteriostatic antimicrobial compound (Woelffel, 2003), our work shows that AITC and BITC clearly display bactericidal activities. MIC and MBC of AITC were higher than those of BITC on each of the 24 *C. jejuni* tested isolates.

In addition, the growing number of reports on antibacterial and anticancer activities of ITC has increased their interest as food supplements. Dietary ITC exert a cancer chemopreventive effect in animals. ITC are able to reduce carcinogen-induced tumorigenesis by inducing carcinogen detoxification (Hecht, 1995), to interfere with angiogenesis (Thejass and Kuttan, 2007a,b), and to induce cell cycle arrest and cell death in cancer cells, while decreasing cancer cell invasion and metastasis (Zhang et al., 2003b). The most intensively studied ITC as chemopreventive agents are AITC, BITC, PEITC, and sulforaphane. For a recent review see Zhang (2012). The effect of ITC as chemopreventive agents is predominantly mediated by the formation of inactive labile thiocarbamate adducts by reaction with the thiol groups of target proteins, while the reaction with amine groups to give thiourea derivatives is less common due to a lesser affinity and a significantly lower reaction rate. The reactivity of specific amines and thiols in target proteins also depends on their own pK_a values (Podhradsky et al., 1979). ITC accumulate as GSH conjugates in the cells and binds to target protein via thiols exchange reactions (Zhang, 2000; Mi et al., 2010).

Concerning thiols exchanges reactions, examples of well studied direct ITC targets are the cytochrome P450 monooxygenase

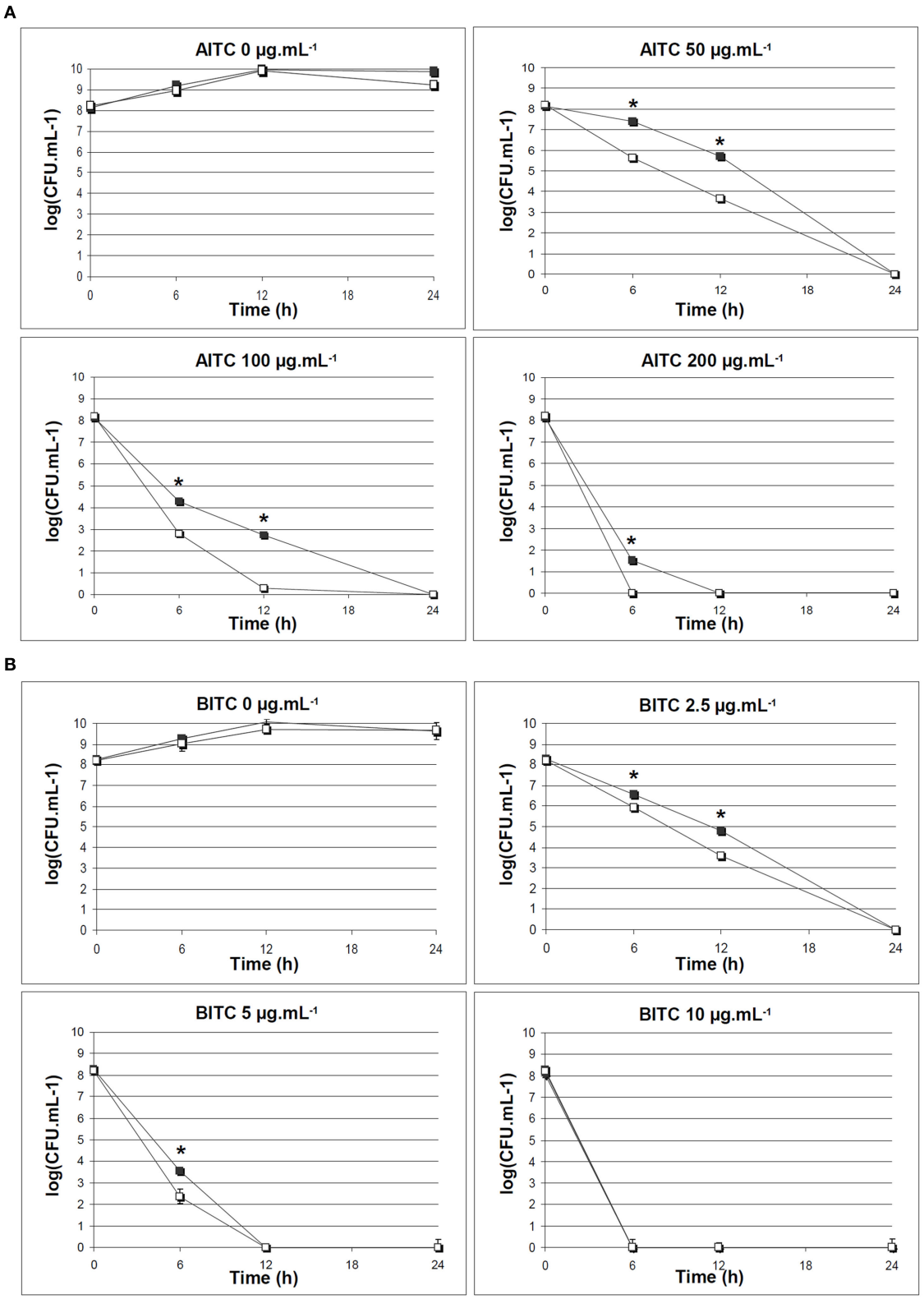


FIGURE 3 | Survival rate of *C. jejuni* 81-176 WT and *ggt* mutant exposed to AITC or BITC. *C. jejuni* strains were grown in MH Broth at 37°C in microaerobic conditions and viable cells were numbered after 6, 12, or 24 h-exposure to ITC. Black squares: *C. jejuni* 81-176 WT; white squares: *C. jejuni* 81-176 *ggt* mutant. **(A)** AITC at 0, 50, 100, or 200 µg mL^{-1}; **(B)** BITC at 0, 2.5, 5, or 10 µg mL^{-1}. The experiment was performed twice with triplicate assays, each point represents mean of 6 data points with SDs. *$p < 0.05$, *t*-test.

(a detoxifying enzyme responsible for the activation of numerous carcinogens; Moreno et al., 1999), the Keap1 protein which inactivation results in the accumulation and activation of the major regulator of the antioxidant response Nrf2 (Zhang and Hannink, 2003), and the α-tubulin thus leading to cell cycle arrest (Smith et al., 2004). ITCs disrupt the redox homeostasis of the cells, both via a long lasting adaptive response depending on the activation of Nrf2 (transcription of antioxidant and repairs enzymes; Jeong et al., 2005) but also by depleting the levels of available intracellular GSH (Zhang, 2000) and by inhibiting glutathione/thioredoxine reductases (Hu et al., 2007). However the biological effect of ITC is dose dependant: at high concentration, the modification of mitochondrial functions leads to proapoptotic cytochrome *c* release, reactive oxygen species (ROS) generation, respiratory alteration, and necrotic cell death. The resulting inflammation may lead to carcinogenic effects (Nakamura et al., 2002).

As we reported in this paper about the MIC of various ITC against *C. jejuni*, variation in the R groups of ITC also accounts for different chemopreventive biological efficiencies of ITC in animals, since the side chain may modify the electrophilicity of – NCS groups, the accessibility to nucleophilic centers, and the lipophilicity of ITC and therefore their location in cell compartments (Zhang, 2012). Our study also showed that the resistance levels of several *C. jejuni* strains for a given ITC are varying, and this variation is probably related to the specific genetic potential of each strain for ITC-conjugate and/or ROS detoxification.

In most living organisms, GGT catalyzes the first step in the degradation of GSH by cleavage and transfer of the γ-glutamyl moiety from GSH to an amino acid acceptor (or hydrolysis to release glutamate). This reaction leads to a release of cysteinyl glycine. GGT enzyme is involved in ITC detoxification in eukaryotes after their conjugation to GSH by GST (glutathione-*S*-transferase) enzymes (Brusewitz et al., 1977); ITC detoxification in cyanobacteria, also involve GST and glutathione (Wiktelius and Stenberg, 2007).

Our result also suggest that the GGT enzyme may be involved in ITC detoxification in *C. jejuni* since a *ggt* mutant displayed a decreased survival rate to ITC compared to its isogenic wild-type. Despite the absence of glutathione biosynthesis and GST in *C. jejuni*, our results suggest that GGT may be able to detoxify conjugates with other low molecular weight thiols present in *C. jejuni* cells, such as cysteine or *N*-acetylcysteine. The exact mechanism of GGT-mediated ITC resistance remains to be unveiled.

However, only few *C. jejuni* isolates encode a *ggt* gene. Additionally, the presence or absence of the *ggt* does not correlate with isothiocyanate sensitivity, indicating that ITC resistance probably depends on several factors, the presence of the *ggt* being only one of many genetic differences between *C. jejuni* isolates. For example, efflux systems could be involved, as it has been described for *Pseudomonas syringae* pathovars *maculicola:* the *sax* genes encoding resistance–nodulation–division efflux systems were found to be required to overwhelm aliphatic isothiocyanate-based defenses of Arabidopsis plants (Fan et al., 2011). Even if homologs of these genes have not been found in *C. jejuni* genomes, Campylobacters possess efflux systems (Lin et al., 2002; Mamelli et al., 2005). Moreover, the resistance levels of *C. jejuni* isolates to ITC do not correlate with their resistance to antibiotics (this study, **Tables 1** and **3**; Woelffel, 2003) suggesting that these resistance mechanisms are distinct.

Although the antibacterial activity of ITC seems to be directed by their non-specific binding to sulfhydryl groups on the active sites of enzymes and of glutathione (Tang and Tang, 1976; Kolm et al., 1995), the exact mechanisms for ITC antibacterial activity are not completely known. It has been shown that AITC inhibited the catalysis by both thioredoxin reductase and acetate kinase in *E. coli* O157:H7 (Luciano and Holley, 2009). ITC also cause membrane damage and leakage of cellular metabolites (Lin et al., 2000b).

From a transcriptomic analysis, it was recently reported that the isothiocyanate iberin from Brassicaceae, specifically blocks expression of quorum sensing regulated genes in *Pseudomonas. aeruginosa* and induced the MEF–OprN efflux pump (Jakobsen et al., 2012).

We are currently investigating the role of several disulfide oxidoreductases of *C. jejuni* in the resistance to AITC and BITC. Moreover, by analyzing the data from a carried-out transcriptomic identification of the *C. jejuni* transcripts induced or repressed by subinhibitory concentrations of BITC, we expect to unravel the whole *C. jejuni* response to ITC.

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APPENDIX

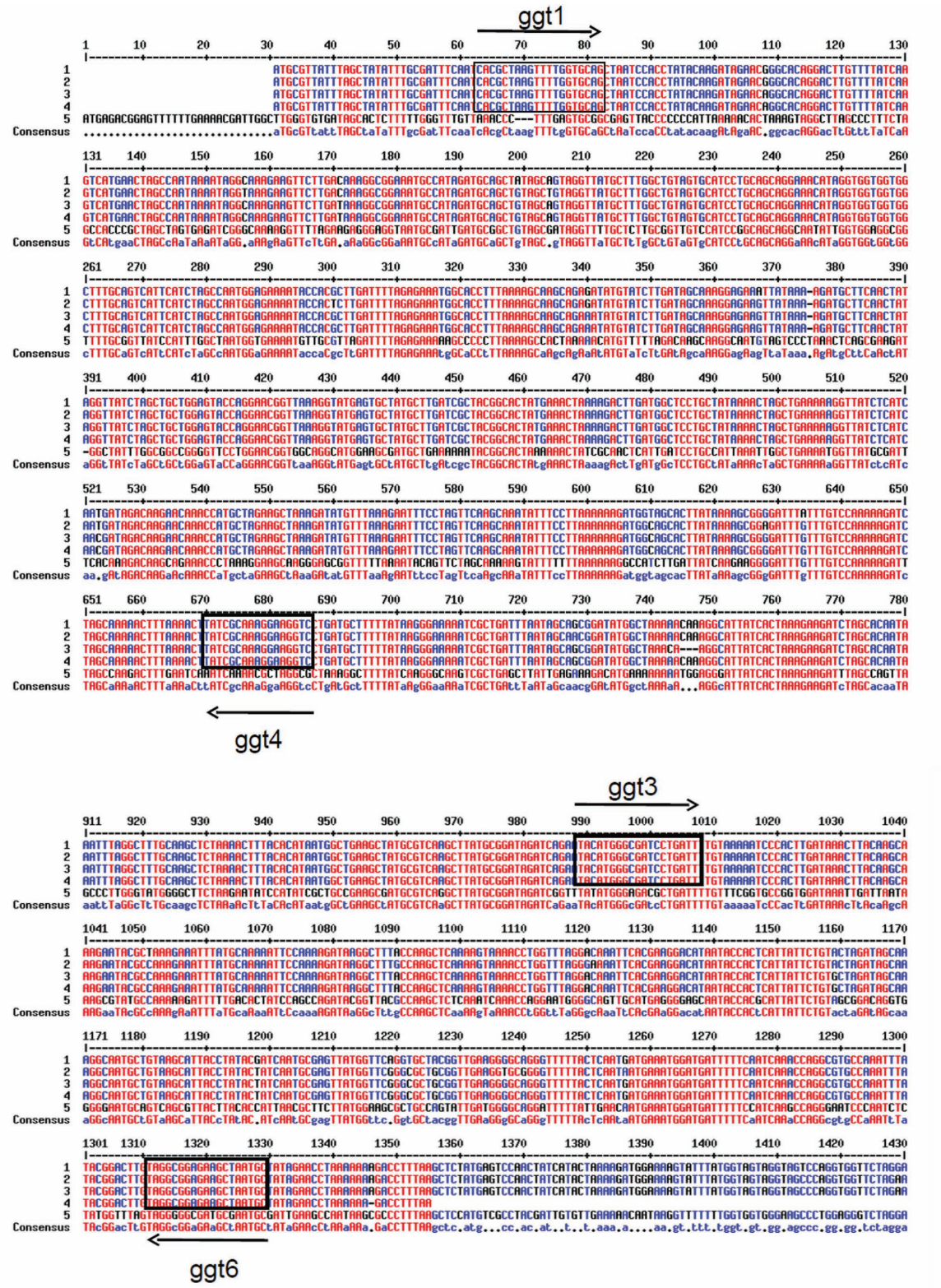

FIGURE A1 | Alignment by MultAlin software (Corpet, 1988) of ggt genes from: 1: *C. jejuni* 81-176; 2: *C. jejuni* subsp. doylei 269.97; 3: *C. jejuni* subsp. jejuni 260.94, 4: *C. jejuni* subsp. jejuni HB93-13; 5: *Helicobacter pylori* 26695 Arrow: position of primers used for PCR amplification of ggt gene in *C. jejuni* isolates.

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A framework for assessing the concordance of molecular typing methods and the true strain phylogeny of *Campylobacter jejuni* and *C. coli* using draft genome sequence data

Catherine D. Carrillo[1], Peter Kruczkiewicz[2], Steven Mutschall[2], Andrei Tudor[1], Clifford Clark[3] and Eduardo N. Taboada[2]*

[1] *Bureau of Microbial Hazards, Food Directorate, Health Canada, Ottawa, ON, Canada*
[2] *Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Lethbridge, AB, Canada*
[3] *National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Mark Estes, University of Georgia, USA
William Miller, USDA–ARS, USA

****Correspondence:***
Eduardo N. Taboada, Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, c/o Animal Diseases Research Institute, Canadian Food Inspection Agency, Twp Rd. 9-1, Lethbridge, Alberta, T1J 3Z4.
e-mail: eduardo.taboada@phac-aspc.gc.ca

Tracking of sources of sporadic cases of campylobacteriosis remains challenging, as commonly used molecular typing methods have limited ability to unambiguously link genetically related strains. Genomics has become increasingly prominent in the public health response to enteric pathogens as methods enable characterization of pathogens at an unprecedented level of resolution. However, the cost of sequencing and expertise required for bioinformatic analyses remains prohibitive, and these comprehensive analyses are limited to a few priority strains. Although several molecular typing methods are currently widely used for epidemiological analysis of campylobacters, it is not clear how accurately these methods reflect true strain relationships. To address this, we have developed a framework and associated computational tools to rapidly analyze draft genome sequence data for the assessment of molecular typing methods against a "gold standard" based on the phylogenetic analysis of highly conserved core (HCC) genes with high sequence quality. We analyzed 104 publicly available whole genome sequences (WGS) of *C. jejuni* and *C. coli*. In addition to *in silico* determination of multi-locus sequence typing (MLST), *flaA*, and *porA* type, as well as comparative genomic fingerprinting (CGF) type, we inferred a "reference" phylogeny based on 389 HCC genes. Molecular typing data were compared to the reference phylogeny for concordance using the adjusted Wallace coefficient (AWC) with confidence intervals. Although MLST targets the sequence variability in core genes and CGF targets insertions/deletions of accessory genes, both methods are based on multi-locus analysis and provided better estimates of true phylogeny than methods based on single loci (*porA*, *flaA*). A more comprehensive WGS dataset including additional genetically related strains, both epidemiologically linked and unlinked, will be necessary to more comprehensively assess the performance of subtyping methods for outbreak investigations and surveillance activities. Analyses of the strengths and weaknesses of widely used typing methodologies in inferring true strain relationships will provide guidance in the interpretation of this data for epidemiological purposes.

Keywords: *Campylobacter* spp., genome, MLST, CGF, *flaA*, *porA*, molecular epidemiology

INTRODUCTION

Campylobacter spp. are the most common cause of bacterial gastroenteritis in Canada (Public Health Agency of Canada, 2009), as around the world, with most cases (>95%) attributed to infection with *C. jejuni* and *C. coli* – at a ratio of ∼6:1, respectively. Yet, despite important public health and socioeconomic impacts of this organism (Thomas et al., 2008), limited progress has been made in defining routes of infection and reducing associated illness. This is in part due to the sporadic distribution of the majority of cases of campylobacteriosis (Government of Canada, 2007, 2010), and the associated difficulties in identifying sources of infection. Furthermore, due to the widespread occurrence of this organism in the intestinal tracts of animals and in the environment, there are many possible sources of exposure.

Molecular epidemiology of *C. jejuni* and *C. coli* remains challenging due to the nature of the genome evolution in these organisms and the extensive genomic and phenotypic diversity within these species. Genome evolution in *C. jejuni* and *C. coli* is largely driven by frequent genomic rearrangements and interstrain genetic exchange (de Boer et al., 2002; Ridley et al., 2008; Wilson et al., 2009a). For these species, recombination appears to affect population structure more rapidly than *de novo* mutation (Dingle et al., 2001; Biggs et al., 2011). To further complicate matters, there is evidence of stability for some clones (Nielsen et al., 2001),

whereas in other cases differences in genetic profiles are observed within a single passage of the organism through an animal host (de Boer et al., 2002). Competition for resources within their gastrointestinal niche likely drives this high rate of evolution through selection of any change that may offer a competitive advantage in this microbe-rich environment (Lefébure and Stanhope, 2009). The rapid evolution of the *C. jejuni* and *C. coli* genomes has important consequences for interpretation of molecular typing information. Outbreak isolates may be missed in cases where small genomic changes result in changes of molecular profiles (Hanninen et al., 1999; Nuijten et al., 2000; Sails et al., 2003; Barton et al., 2007). Conversely, some strains will appear to be clonal, and will be linked by typing methods despite true differences in gene content between isolates and absence of epidemiological linkage (Taboada et al., 2008; Biggs et al., 2011).

Several molecular subtyping schemes have been developed for use in characterization of *C. jejuni* and *C. coli* isolates for epidemiological investigation (reviewed in Klena and Konkel, 2005). Of these, multi-locus sequence typing (MLST), based on DNA sequence analysis of seven housekeeping genes, is currently the leading method, in part due to the ease of comparison of nucleotide sequence-based typing among labs worldwide (Dingle et al., 2001). This typing scheme has greatly contributed to an improved understanding of *Campylobacter* epidemiology. Similarly, DNA sequencing of the flagellin gene short variable region (*flaA*-SVR; Meinersmann et al., 1997, 2005) and the *porA* gene (Clark et al., 2007) is also routinely used. Alternative methods which incorporate analysis of the accessory genome include comparative genomic fingerprinting (CGF), a low cost, high throughput, and high resolution method that is based on the detection of 40 genes using multiplex PCR (Taboada et al., 2012). Current analyses suggest that CGF is highly concordant with MLST, but with a better discriminatory power (Clark et al., 2012; Taboada et al., 2012). Much like sequence-based methodologies, CGF types can be easily compared among laboratories. With the advent of high throughput next generation sequencing (NGS) technologies, whole genome sequence (WGS) analysis has begun to play an increasing role in microbial epidemiology, particularly in high profile outbreak situations (Gilmour et al., 2010; Chin et al., 2011; Rohde et al., 2011). Unfortunately, current costs limit the use of full genome analysis to a few priority strains (e.g., Parkhill et al., 2000; Pearson et al., 2007). While all of these methods have played an important role in improving our understanding of transmission of campylobacters to human hosts, the range of available typing methods leads to difficulties in meta-analysis of study data.

In order to move toward a common, standard molecular typing methodology suitable for most epidemiological studies, robust evaluation of existing typing schemes is needed. High quality, whole genome sequence (WGS) is the true gold standard for molecular characterization of microbes as all of the information necessary to determine molecular types is encoded within the genome. Analysis of this data can be highly discriminatory among closely related strains (Biggs et al., 2011), but can also be used to infer evolutionary relationship for distantly related organisms (Lefébure and Stanhope, 2009; Lefébure et al., 2010). In the near future, WGS will likely become the method of choice for characterization of microbes; however, use of WGS for surveillance activities is currently not feasible for most laboratories. Nonetheless, there is a growing number of full genomes available for analysis. This data can be used to rigorously assess existing typing schemes to help identify those that would work most effectively for public health activities, and to select improved targets for next generation typing schemes. Furthermore, an improved understanding of the performance of each method will assist in the interpretation of existing studies.

We have used publicly available *C. jejuni* and *C. coli* WGS data in the development of a framework to assess performance of MLST, *flaA*, *porA*, and CGF typing schemes compared to the inferred "reference" phylogeny based on conserved core genome elements. Such a framework will provide a basis for future, more expansive molecular typing method evaluation based on WGS data.

MATERIALS AND METHODS

STRAINS USED IN ANALYSIS

A total of 104 strains were included in this study (Table S1 in Supplementary Material). Of these, 24 complete or draft *C. jejuni* and *C. coli* sequences were retrieved from GenBank: *C. jejuni* subsp. *jejuni* [NCTC 11168 (NC_002163), 81–116 (NC_009839), 81–176 (NC_008787), 84–25 (NZ_AANT00000000), CF93-6 (NZ_AANJ00000000), HB93-13 (NZ_AANQ00000000), CG8421 (NZ_ABGQ00000000), CG8486 (NZ_AASY00000000), 260.94 (NZ_AANK00000000), IA3902 (CP001876.1), ICDCCJ07001 (NC_014802), M1 (CP001900.1), S3 (CP001960.1), 1336 (NZ_ADGL00000000), 305 (ADHL00000000.1), 327 (ADHM00000000.1), 414 (NZ_ADGM00000000), DFVF1099 (ADHK00000000.1), D2600 (AGTF00000000.1), NW(AGTE00000000.1)], C. *jejuni* subsp. *doylei* 269.97(NC_009707), C. *jejuni* RM1221 (NC_003912), and C. *coli* [RM2228 (AAFL00000000), JV20 (NZ_AEER00000000)]. Sequence data from 39 strains of C. *jejuni* and 41 strains of *C. coli* were retrieved from the Short Read Archive under accession numbers SRP001790 and SRA010929, respectively (Lefébure et al., 2010).

SEQUENCE ASSEMBLY AND ANNOTATION

Illumina traces from 80 of the *C. jejuni* and *C. coli* genomes sequenced by Lefébure et al. (2010) were assembled using Velvet (version 1.1.06; Zerbino and Birney, 2008) using a hash length of 25 as this was found to give optimal assemblies. The order of the contigs was inferred by comparison with the *C. jejuni* NCTC 11168 reference genome using ABACAS (Assefa et al., 2009). Prediction of coding sequences and annotation was completed using the rapid annotation using subsystem technology (RAST; Aziz et al., 2008).

ASSESSMENT OF WGS DATA QUALITY

In order to generate a measure of quality of WGS data, we examined the *C. jejuni* genomes [closed reference sequence (RefSeq) genomes ($n = 9$), draft RefSeq genomes ($n = 8$), draft 454 genomes ($n = 3$), and draft Illumina genomes ($n = 41$)] using a two-step process to examine truncations in core genes predicted in each genome. In the first step, a set of "core genes" for *C. jejuni* was identified based on a preliminary comparative genomic survey using a subset of RefSeq annotated genomes. Whole genome pair-wise homology searching using BLAST+ (version 2.2.25; Camacho et al., 2009) was performed at the ORF level using the program

BLASTP using the strain NCTC 11168 as a reference. Genes were considered "core" if conserved across all of the genomes analyzed, yielding a set of 1,314 genes. In the second step, the 1,314 genes from strain NCTC 11168 were queried against the predicted ORFs for the set of 61 *C. jejuni* genomes using BLASTP. Alignment lengths were used to identify truncations if shorter than the length of the RefSeq. A one-tailed unpaired *t*-test was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego) to determine statistical significance of increase in number of truncations observed in draft quality genome sequences compared to closed RefSeq.

CORE/ACCESSORY GENOME PHYLOGENETIC ANALYSIS

A semi-automated approach was developed to rapidly infer a core genome phylogeny for the dataset. In the first step, a robust set of "highly conserved core" (HCC) genes for *C. jejuni* and *C. coli* was identified based on a preliminary comparative genomic survey using a subset of RefSeq annotated genomes. Whole genome pair-wise homology searching using BLAST+ (version 2.2.25; Camacho et al., 2009) was performed at the ORF level using the program BLASTP. Genes were considered "core" if conserved across all of the genomes analyzed. A 90% sequence identity cut-off was used to identify HCC genes, yielding a set of 389 genes (Table S3 in Supplementary Material). In the second step, the program CONCATENATOR (Kruczkiewicz et al., 2011), a program written in C# using the.NET Framework 4.0, was used to: (1) identify the homologous sequences for the set of 389 HCC genes in each genome in the dataset using BLASTN; (2) perform individual alignments for each gene using MUSCLE (Edgar, 2004a,b); and (3) concatenate the alignments to produce a single alignment (i.e., a "concatenome"). The reference core genome phylogeny for the dataset was then estimated based on the concatenome using Sea View (Gouy et al., 2010) using uncorrected distances.

IN SILICO TYPING ANALYSIS

The program "microbial *in silico* typer" (MIST) was used to generate *in silico* molecular typing results from whole genome sequence data (Kruczkiewicz et al., 2011). MIST derives several kinds of *in silico* typing data from "raw" genome sequences (i.e., contig assemblies), including MLST (Dingle et al., 2001), *porA* typing (Clark et al., 2007), *flaA* typing (Meinersmann et al., 1997, 2005), and CGF (Taboada et al., 2012). The full implementation of MIST, which was written in the C# programming language using the.NET Framework 4.0, will be described in detail elsewhere; functionalities used in this study will be briefly described here. *Sequence Typing*: the sequence for each of the target genes (i.e., MLST genes: *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, *uncA*; the *porA* gene; and the *flaA* gene) was identified in each of the contig assemblies through homology searching using BLAST+ (version 2.2.25; Camacho et al., 2009). Alleles were inferred for each gene by comparing these sequences against allelic sequences obtained from the *C. jejuni* PubMLST database[1]. MLST allelic profiles were used to determine the sequence type (ST) and clonal complex (CC) for each strain. *Comparative Genomic Fingerprinting*. Presence of targets in the CGF40 scheme (Taboada et al., 2012) was determined by performing a homology search for each target using BLASTN against each WGS and using a sequence identity cut-off of 95% to score the presence/absence of each target. To generate CGF40 clusters, pair-wise profile similarities we computed using the simple matching coefficient and clustered using the unweighted-pair group method using average linkages (UPGMA) in Bionumerics (v.5.1; Applied Maths, Austin, TX, USA), using 100, 95, and 90% fingerprint similarities for cluster definition.

ASSESSMENT OF SNP AND ACCESSORY GENE CONTENT DIFFERENCES

Calculation of pair-wise SNP rates: to estimate pair-wise SNP rates between strains in the dataset, the sequences from the HCC set were concatenated into a single 395,563 bp multiple sequence alignment. All gapped positions resulting from indels or missing data were removed from the alignment, yielding an alignment of 319,428 bp. *Calculation of pair-wise accessory gene content differences:* for each pair of strains, the total number of SNPs was computed and the SNP rate expressed as the average number of SNPs per 1,000 bp. To estimate accessory genome content differences, pair-wise differences in conservation profiles between strains in the dataset were calculated for a set of 3,903 accessory genes that were selected based on the following criteria: absence in at least one or more genomes; presence in at least two genomes (i.e., no "strain-specific" genes); and non-redundancy (i.e., a single gene was chosen from each set of orthologs).

COMPARISON OF MOLECULAR TYPING METHODS

In silico typing results were compared to the reference phylogeny by taking the latter and subdividing it into "phylogenetic clusters" at several levels of resolution targeting a specific average intra-cluster SNP rate (5, 10, and 15 SNPs per 1,000 bp). The adjusted Wallace coefficient (AWC; Severiano et al., 2011) was used to compare the phylogenetic clusters to the genotypic clusters obtained from the various methods using MIST. This and other measures of subtyping method performance (Carriço et al., 2006) were analyzed at the Comparing Partitions server[2].

RESULTS AND DISCUSSION

EFFECT OF WHOLE GENOME SEQUENCE (WGS) DATA QUALITY ON DOWNSTREAM DATA ANALYSIS

The dataset assembled for this study represents a collection of public *C. jejuni* and *C. coli* WGS data that includes both closed and unfinished genomes designated as RefSeq by NCBI (Pruitt et al., 2002, updated May 23, 2011), as well as draft quality genome assemblies with various levels of sequence coverage and in various states of "fragmentation" (i.e., multiple contigs). It includes "earlier generation" sequence data generated through Sanger sequencing and NGS data generated using the 454 and Illumina platforms. The heterogeneous nature of these data enabled the examination of the impact of sequence data quality on downstream analyses, including pan-genome (i.e., core/accessory genome) analysis, core genome based phylogenetic analysis and the derivation of *in silico* typing results. In particular, the high quality closed genome sequence data present a benchmark against which to assess the quality of draft assemblies generated using NGS platforms. The

[1] http://pubmlst.org/campylobacter/

[2] http://www.comparingpartitions.info

latter not only comprise the bulk of WGS data currently available in public databases but, owing to higher-throughput and lower associated costs of data generation, effectively represent the only kind of WGS data currently being generated in laboratories around the world.

Quality assurance remains a significant challenge for NGS data, and is likely to be impacted by the variability in depth of coverage observed for the *C. jejuni* and *C. coli* sequences (Table S1 in Supplementary Material), among other things. Lower coverage may result in erroneous base-calling due to inherent systematic error rates of sequencing platforms (i.e., small insertion/deletions in 454 sequences, miscalls in Illumina sequences; Metzker, 2010). In addition, low coverage may impact assembly of sequence reads into long contigs, resulting in assemblies comprised of larger numbers of short contigs and a concomitant increase in incomplete (i.e., "truncated") gene sequence data during automated gene prediction and sequence annotation. These not only present the potential for allelic miscalls but may also pose significant problems for downstream phylogenetic analysis.

To assess quality of NGS data included in this study, the number of predicted genes that were truncated relative to core genes in *C. jejuni* NCTC11168 was determined. Draft genome assemblies included in the dataset differed in the number of partial or truncated genes identified (Table S1 in Supplementary Material; **Figure 1**). Closed genomes included in NCBI's RefSeq collection had the lowest number of truncated genes and RefSeq genomes that were not closed had significantly more ($p < 0.05$) truncated genes. The closed RefSeq *C. jejuni* genome ICDCCj07001 was not included in the statistical analysis as the quality of the sequence appeared to be much lower than in the other closed genomes (83 truncations), and inclusion of this outlier skewed the results. The 454 draft genomes had very high levels of gene truncation, but only three genomes with low coverage (10× to 20×) were available for analysis. Short read (36 bp) Illumina data from the Lefébure et al. (2010) study were minimally processed beyond assembly and scaffolding prior to analysis. The number of truncations in this dataset was similar to what was observed in non-closed RefSeq genomes, but significantly higher ($p = 0.0006$) than the "gold standard" closed RefSeq genomes. Note that two new *C. jejuni* genomes (D2600 and NW) with 70× sequencing coverage on Illumina had gene truncation numbers similar to those observed in the closed genome sequence data.

Our analysis suggests that assessment of truncation in predicted genes relative to a high quality reference genome may be a rapid and informative assessment of overall genome quality for all sequencing platforms included in this analysis (**Figure 1**). This method was particularly informative in the assessment of quality of reads generated by 454 sequencing technology. Errors in 454 sequencing reads tend to occur in homopolymer repeats (Metzker, 2010), and since *Campylobacter* genomes have high numbers of homopolymeric adenine and thymine tracts, they would be particularly susceptible to this type of error. Nonetheless, this measure was also found to be effective for both Sanger sequence and Illumina sequence. Draft genomes of similar quality tended to have similar overall numbers of randomly distributed gene truncations. A subset of these, likely in the range of 10–15 truncations observed in the high quality genomes, may represent *bona fide* allelic variation due to hypervariable homopolymeric tracts that cause premature stop codons in "contingency genes" containing them (Parkhill et al., 2000; Jerome et al., 2011). In contrast, truncations in which sequence breakpoints appear randomly distributed in individual strains across the dataset are more likely to be due to incomplete assembly or poor sequence data. High levels of apparent erroneous truncation were more prevalent in lower quality genome sequence data. Moreover, closed genomes did not necessarily represent the highest quality sequence. For example, the closed RefSeq *C. jejuni* genome ICDCCJ07001 had an unusually high number of apparently truncated genes compared to other genomes in this category. More extensive analyses of additional species of bacteria in the public database may provide a more complete understanding of how gene truncation may be more generally used as a quality metric.

Despite variability in sequence quality as assessed by gene truncation, most of the sequence typing alleles (i.e., MLST genes, *flaA*, *porA*) matched experimentally determined alleles (Table S2 in Supplementary Material, described in detail below). While Illumina sequencing data is known to have a higher error rate

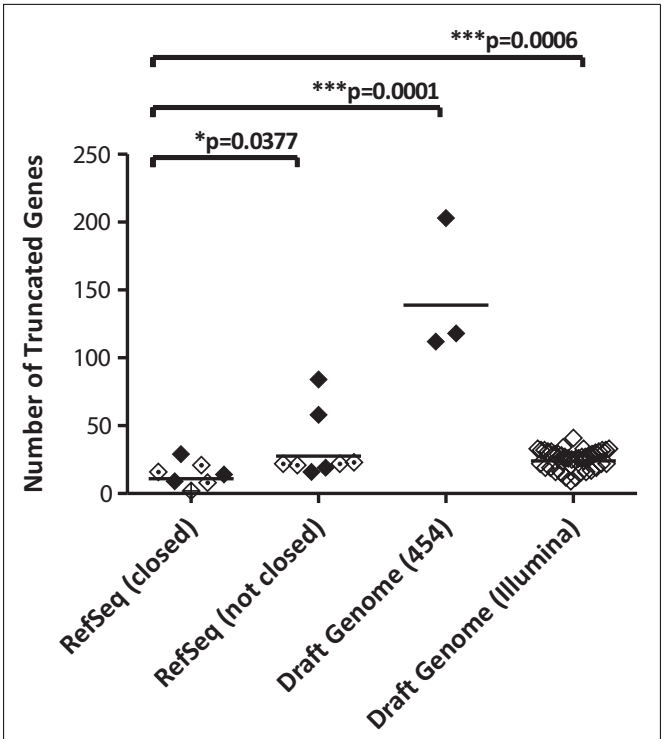

FIGURE 1 | Finishing quality is reflected in the number of truncated predicted genes in *C. jejuni* whole genome sequences. The *C. jejuni* genomes used in this analysis were classified according to finishing levels (reference sequence or draft), and/or sequencing platform used [Sanger open diamonds with a central period), 454 (closed diamonds), or Illumina (open diamonds)]. The number of predicted genes with truncations relative to *C. jejuni* NCTC11168 was calculated for each genome. The mean number of truncations for each group is represented by a line. Closed genomes had significantly fewer truncations than RefSeq genomes that were not closed and draft genomes generated with either 454 or Illumina sequencing technologies.

than the other technologies (Metzker, 2010; Suzuki et al., 2011), increased coverage greatly reduces false SNP identification. Comparisons of error rates in Illumina and 454 sequencing platforms in sequencing an *E. coli* strain (Suzuki et al., 2011) found ∼46 false SNPs in this 4.6 Mb genome. If we assume a similar error rate in *Campylobacter* genomes, we would expect up to 17 false SNPs in each of these smaller genomes. The coverage of the genomes in the Lefébure et al. (2010) strain set was generally much higher than in the Suzuki et al. (2011) study (Table S1 in Supplementary Material); therefore, we would predict a concomitant decrease in error rates in these genomes, as increased coverage is known to decrease these errors (Suzuki et al., 2011). Furthermore, given that this would ultimately represent a small fraction of the inter-isolate SNPs, it is unlikely that this error would substantially impact results of downstream analyses.

FROM WGS TO CORE GENOME PHYLOGENETIC ANALYSIS

In order to facilitate core genome phylogenetic analysis, we designed a pipeline aimed at identifying a subset of HCC genes with high quality across the dataset and to subsequently infer an estimate of the phylogenetic relationship for the strains used in this analysis. The use of large sets of core genes in phylogenetic analysis represents the best estimate of the "true" phylogenetic relationship of bacterial isolates since it can minimize effects of conflicting signal due to recombination. Moreover, by assessing the quality of core genomes prior to phylogenetic analysis, our approach allowed tolerance of minimally processed draft genome sequence data.

A preliminary comparative genomic survey of annotated genomes for *C. jejuni* and *C. coli* was performed to identify a set 389 HCC genes that could be used to derive a reference phylogeny for the dataset. Because differences in the depth of sequence coverage and platform-specific sequencing error bias have the potential to affect the sequence quality of the various draft assemblies, several steps were taken in the automated analysis strategy to maximize the amount of core genome data used while minimizing the potentially adverse effects of erroneous gene sequence data on downstream phylogenetic analysis. For example, although only 215 of the HCC genes had full length predicted gene calls in all 104 genomes analyzed, we identified many cases ($n = 279$; 166 genes) in which a small (i.e., 1–2 bp) indel led to a frameshift and premature stop codon (i.e., an "indel truncation"). In such cases, the sequence downstream of the indel was retrieved up to the full length of the gene if it could be aligned to the original RefSeq from NCTC 11168. At the same time, because of difficulties in differentiating indels due to sequencing errors from those due to biological causes (*bona fide* indels, contingency frameshifts) indel positions were ignored in the phylogenetic analysis. In a smaller number of cases ($n = 127$; 105 genes) we observed the premature truncation of a gene sequence due to proximity to the end of a contig (i.e., a "contig fragmentation"). In these cases, and in cases in which the gene was absent from at least one genome assembly ($n = 39$; 38 genes), gapped positions due to missing sequence data were also ignored from the analysis. These combined approaches allowed us to make use of a large proportion of the sequence data from the 389 HCC gene set (319,428 out of 395,563 bp).

Overall, the dataset was largely comprised of a genetically diverse set of strains. This presented challenges in terms of assessing the overall inferred phylogeny by standard methods such as bootstrapping and maximum likelihood (Felsenstein, 1989; Schmidt and von Haeseler, 2007). Nonetheless, the resulting phylogeny shows a deep split with significant support between the *C. jejuni* and *C. coli* strains (**Figure 2**). The SNP rates observed for the dataset are consistent with the split since the average inter-species SNP rate among the strains in the dataset was ∼108.6 per 1,000 bp whereas average intraspecies SNP rates were an order of magnitude lower (∼13.9 and 17.3 per 1,000 bp in *C. coli* and *C. jejuni* respectively). Although this is in contrast to previous findings based on intraspecies recombinational exchange of MLST alleles (Sheppard et al., 2008) and a 16s rRNA gene phylogeny in which mixing of the two species is observed (data not shown), it is consistent with findings of Lefébure et al. (2010) which suggest that although recombinational exchange between the species occurs, it is of a limited scale and does not remove the dominant phylogenetic signal supporting the species' split.

Within the *C. jejuni* and *C. coli* clades, significant support could be found for a small number of highly conserved sub-branches that were well-supported by the underlying SNP distributions (**Figure 2A**), with strains that form branches with robust bootstrap support sharing significantly lower SNP levels with respect to one another in contrast to unrelated strains in the dataset. At the same time, phylogenetic trees derived for individual core genes do not support the overall consensus phylogeny and this conflicting phylogenetic signal is consistent with significant levels of intraspecific recombinational exchange (results not shown). The HCC gene phylogeny is also compatible with the underlying accessory genome content, with strains within robustly supported branches sharing significantly fewer accessory gene content differences with respect to other strains in the dataset (**Figure 2B**). It thus appears that the accumulation of differences in accessory genome content is consistent with the accumulation of SNP differences in the core genome ($r^2 = 0.9703$).

ASSESSMENT OF AUTOMATED *IN SILICO* TYPING FROM WGS DATA

For draft genome assemblies it is generally assumed that higher levels of coverage and fewer contigs are indicative of better quality data. As can be seen in **Figure 3**, a large proportion of sequence typing alleles (MLST genes, *flaA*, *porA*) was inferred whether from high quality finished genomes or from minimally processed draft assemblies. Thus, the relationship between quality estimates and their effect on downstream analysis is not always straightforward. For example, although Illumina data generally resulted in assemblies with larger numbers of contigs, the resulting sequence data were of sufficient quality to allow high levels of allele identification from *in silico* typing analysis. In most cases, allele identification matched the published or publicly available ST types. For example, among 80 strains with known MLST ST (i.e., from Lefébure et al., 2010 or available from the PubMLST database) concordance was found to be 98.4%, or 551/560 alleles. Overall, nine allelic discrepancies in the *in silico* derived ST were found, affecting data in eight of the genomes. In two of these cases, one of the seven MLST alleles could not be identified, with one of these two missing alleles in the RefSeq for *C. coli*. In the other seven cases there

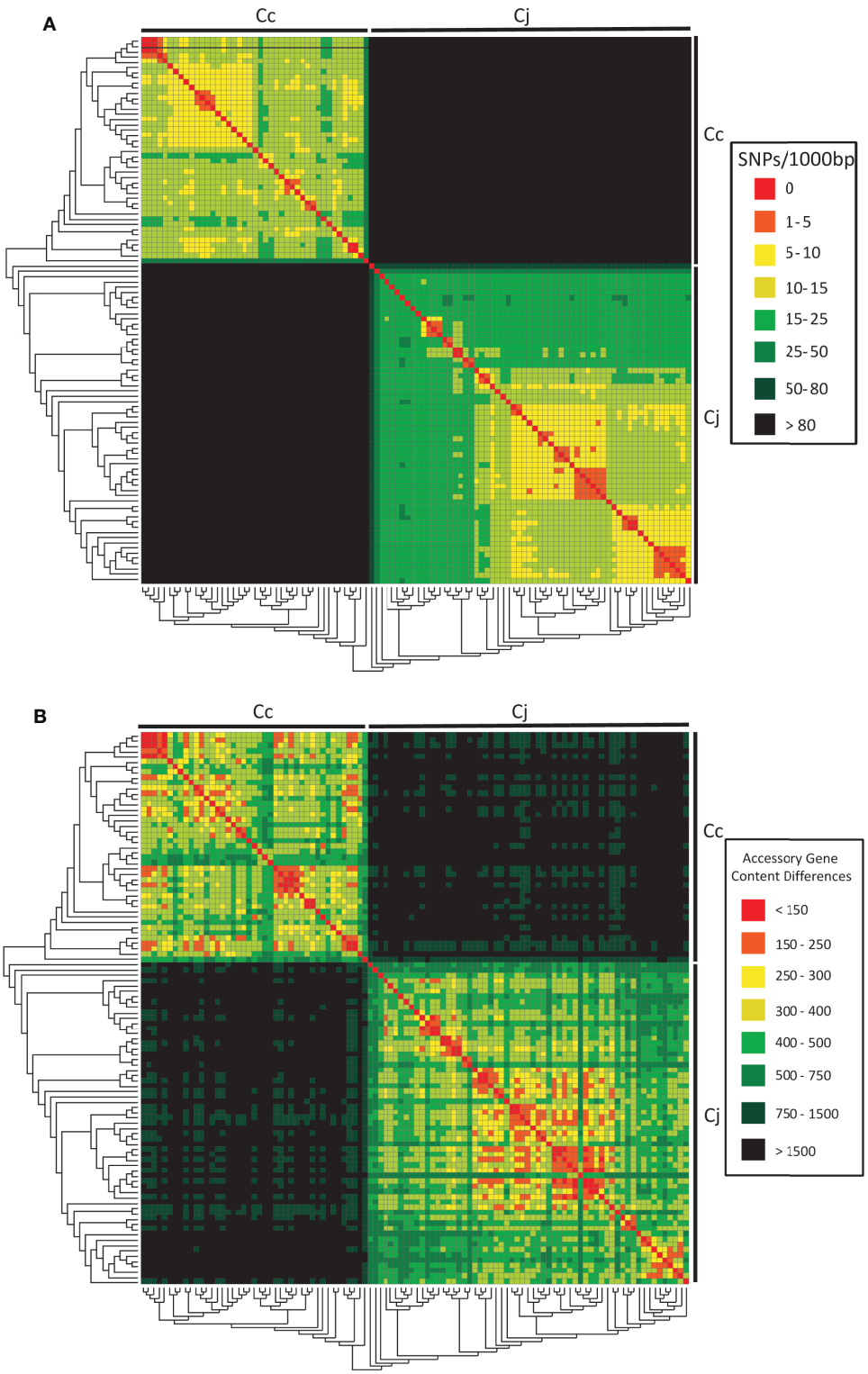

FIGURE 2 | The core genome phylogeny for *C. jejuni* and *C. coli* is consistent with underlying genome-wide SNP rates and accessory genome content. The 104 *C. jejuni* and *C. coli* WGS were analyzed using an automated pipeline for core genome analysis; a core gene phylogeny derived from 389 core genes is shown here. This phylogeny was compared to the underlying **(A)** SNP rates and **(B)** accessory gene content differences. High SNP rates and accessory genome content differences between *C. coli* and *C. jejuni* genomes support a deep split between the species. Conversely, small phylogenetic clusters comprised of highly similar strains are supported by lower differences in SNPs and accessory gene content. A high resolution image of this figure is available in the supplementary material.

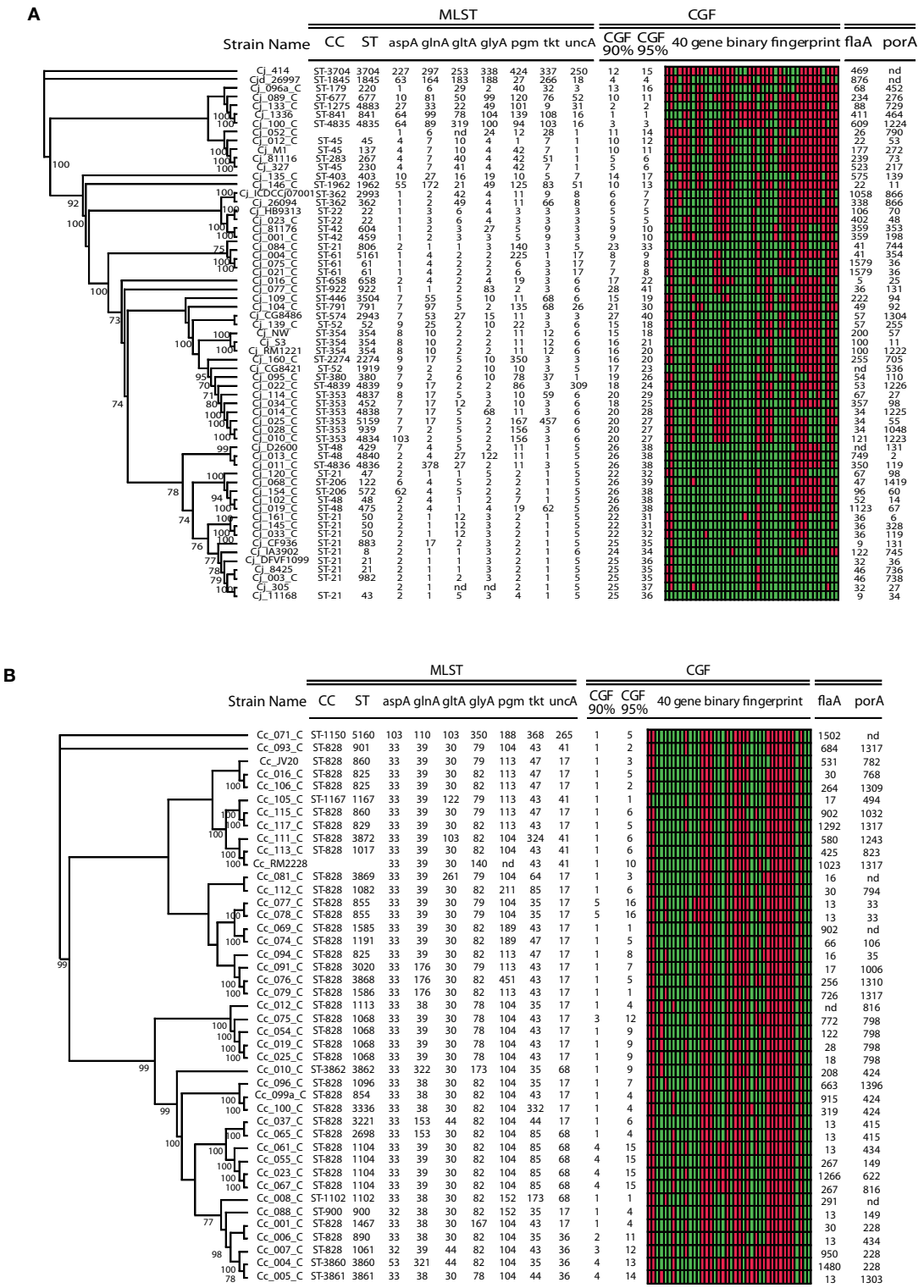

FIGURE 3 | *In silico* typing data derived from *C. jejuni* and *C. coli* and WGS is concordant with core genome phylogeny. Publicly available WGS data for 104 *C. jejuni* **(A)** and *C. coli* **(B)** strains were used to derive typing profiles using an *in silico* typing pipeline. Although the dataset is comprised of highly genetically diverse strains, there is concordance between molecular typing and phylogenetic data: strains sharing similar/identical molecular fingerprints were found clustered in the dendrogram and increasing similarity led to shorter branch lengths. CGF cluster numbers are based on 90 and 95% fingerprint identity; green is positive, red is negative. MLST alleles that could not be determined are noted as "nd."

was a discrepancy at one of the seven loci and it is not possible to ascertain which of the methods gave the incorrect result (Table S2 in Supplementary Material), although in four of these cases the discordant alleles differed by a single SNP; in 3 of the cases alleles were so different (53–70 SNPs) that it is unlikely that sequencing errors were responsible for the observed lack of concordance.

MULTI-LOCUS *CAMPYLOBACTER* SUBTYPING METHODOLOGIES REFLECT CORE GENE PHYLOGENY

Molecular fingerprints obtained by subtyping methods represent a low resolution proxy for the full genome complement of a strain. Thus, one possible approach for comparing subtyping data to the underlying core genome phylogenetic data would be to compare the topologies of dendrograms obtained using each method to the reference phylogeny. Nevertheless, because of the relative paucity of data used, most of the topological information encoded in dendrograms from subtyping data lacks robustness and deeper relationships *between* clusters cannot be reliably inferred. In order to perform the comparison, we deconstructed the reference phylogenetic tree into sets of robust "phylogenetic clusters" reflecting a particular level of genetic similarity (i.e., SNP rate). These could be compared to the clusters obtained by subtyping using measures of concordance that do not rely on overall tree topology. The AWC has recently been proposed as a quantitative measure of congruence of genotypic clusters obtained using different typing methods (Severiano et al., 2011). In order to assess the level of concordance between *in silico* derived subtyping results and the WGS data, we used the AWC to compare the genotypic clusters obtained from *in silico* analysis of the WGS data to phylogenetic clusters in the HCC dendrogram reflecting various SNP rates (5, 10, and 15 bp per 1,000 bp).

The number of partitions, or clusters, obtained using the various methods was very high, with the multi-locus typing methods [i.e., CGF40 (100%), ST, and ST-*porA*] generating unique subtypes for a significant proportion of the strains in the dataset and Simpson's Index of Diversity values approaching 1 (**Table 1**). This genetic variability is in large part due to the nature of the strains for which WGS data were publicly available since there is great interest in the scientific community in sequencing strains that may be unique, or that represent lineages that were previously uncharacterized.

In contrast to methods based on single loci (*flaA*, *porA*), both multi-locus typing methods (MLST, CGF) were highly congruent with core genome phylogeny (**Table 1**). These single locus methods are generally used on their own in the context of short term epidemiological analyses, and have been found to be useful for improving discriminatory power of MLST (Dingle et al., 2008; Clark et al., 2012), but perhaps less suitable for examining long term epidemiology; our results are consistent with this view. It is perhaps not entirely unexpected that MLST results provided a very close approximation of core genome phylogeny (**Table 1**) since the latter is essentially equivalent to a highly extended MLST typing scheme. Indeed, extended typing schemes are being more widely adopted to increase discriminatory power of MLST and to achieve more informative results from such schemes (Dingle et al., 2008; Lang et al., 2010; Zautner et al., 2011). It has been suggested that typing methods that target dispensable genes are better suited to short term epidemiology whereas methods based on core gene sequence such as MLST would more adequately reflect true genetic relationship among strains, and would be more useful for long term epidemiological studies (Wilson et al., 2009b). Given the divergent genomes assessed in this study, which reflect "long term" relationships, the degree to which an accessory genome based binary typing scheme such as CGF40 reflected core genome phylogeny was surprising.

ASSESSMENT OF GENOMIC SIMILARITY FOR GROUPS OF STRAINS WITHIN GENOTYPING CLUSTERS

The underlying structure of the dataset, which did not produce many multi-strain genotypic clusters, presented challenges to the analysis of congruence of molecular typing methods in a phylogenetic context, particularly at the higher levels of resolution. In order to further assess whether genotypic clusters obtained using the various subtyping methods represent groups of highly

Table 1 | Comparison of metrics of subtyping method performance.

Method	Partitions	Simpson's ID (CI)	Phylogenetic clusters		
			15 SNPs per 1000 bp (CI)[1]	10 SNPs per 1000 bp (CI)[1]	5 SNPs per 1000 bp (CI)[1]
CGF40 (100%)[2]	82	0.997 (0.995–0.999)	0.813 (0.682–0.945)	0.833 (0.715–0.951)	0.610 (0.411–0.810)
CGF40 (95%)[3]	53	0.984 (0.978–0.990)	0.654 (0.520–0.787)	0.644 (0.527–0.761)	0.320 (0.203–0.437)
ST	77	0.994 (0.990–0.998)	1.000 (1.000–1.000)	1.000 (1.000–1.000)	0.727 (0.583–0.872)
CC	35	0.860 (0.797–0.922)	0.299 (0.146–0.452)	0.227 (0.123–0.330)	0.071 (0.038–0.104)
porA	73	0.993 (0.989–0.997)	0.515 (0.318–0.712)	0.494 (0.311–0.678)	0.325 (0.169–0.480)
flaA	68	0.988 (0.980–0.997)	0.347 (0.195–0.499)	0.270 (0.138–0.402)	0.221 (0.100–0.342)
ST-porA[4]	89	0.998 (0.995–1.000)	1.000 (1.000–1.000)	1.000 (1.000–1.000)	1.000 (1.000–1.000)

Because of missing data, only 94 isolates could be included in the analysis.

[1]95% Confidence intervals.

[2]Comparative genomic fingerprinting clustered at 100% identity.

[3]Comparative genomic fingerprinting clustered at 95% identity.

[4]Hybrid method using combined MLST+ porA.

genetically related strains, we calculated the average SNP rate per 1,000 bp in the 389 core genes for all sets of strains sharing the same genotypic cluster. The average SNP rate observed for any two strains in the dataset was 61.4 per 1,000 bp and each of the subtyping methods assessed generated genotypic groups with significantly lower SNP rates (i.e., reflecting higher genetic similarity rates), ranging from 2.0 to 15.3 SNPs per 1,000 bp, than the average. Nevertheless, the multi-locus methods generated clusters with consistently lower SNP rates than those observed for the single locus methods (**Figure 4A**). Moreover, whereas the former had relatively uniform distributions the latter showed significant rate variability. This is consistent with the possibility that due to recombination, single locus methods can in some cases lead to genotypic clusters comprised of strains that are quite genetically different.

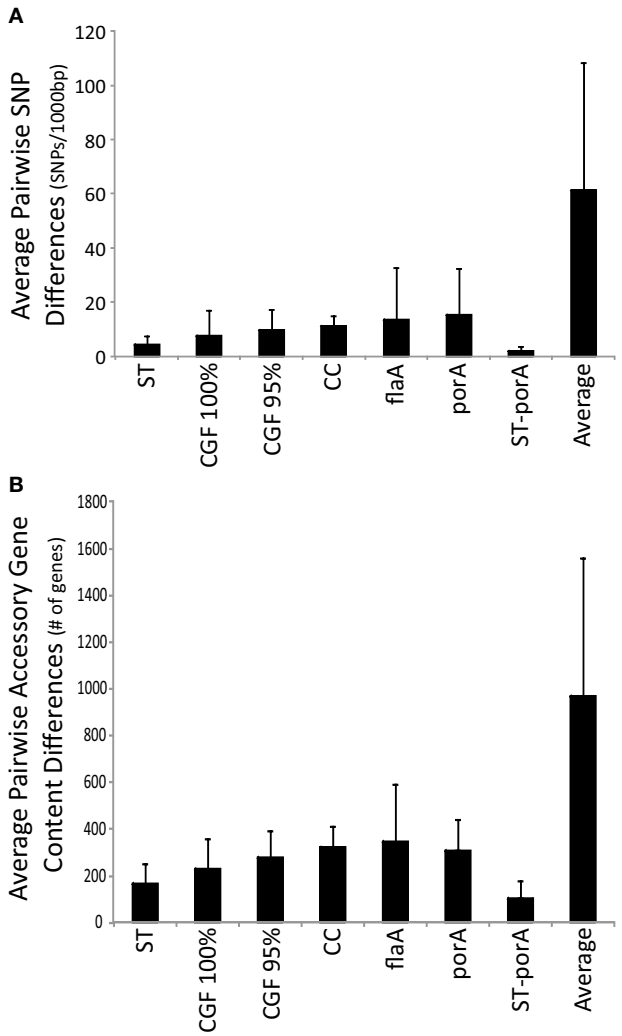

FIGURE 4 | Genotypic clusters are comprised of strains with high genomic similarity. Genotypic clusters obtained using five different molecular typing methods were analyzed for **(A)** average SNP rates or **(B)** accessory gene content differences in order to assess intra- and inter-cluster core and accessory genetic similarities, respectively. Although all methods generated genotypic clusters sharing higher core and accessory genetic similarities than expected by chance alone, multi-locus methods led to genotypic clusters with higher genetic similarity.

We next examined the extent to which strains that are indistinguishable based on current subtyping methods differ at the genomic level by assessing the number of conserved genes between pairs of strains in a set of 3,903 genes from the pooled accessory genome among the 104 genomes in the dataset (**Figure 2B**). As with SNP rates, the average number of accessory gene content differences was consistently lower within genotypic clusters obtained with the various methods (**Figure 4B**), ranging from $n = 103$–343, compared to the average rate observed for any two strains ($n = 964$). Thus, multi-locus methods, whether based on the analysis of core genes (i.e., MLST) or the analysis of accessory genes (i.e., CGF), appear to outperform single locus methods in grouping genetically similar strains.

Although the various subtyping methods generate groups of strains with generally high levels of genetic similarity (i.e., low core gene SNP rates and fewer accessory genome content differences), the WGS data ultimately has the resolution to differentiate the strains within these clusters on the basis of genomic differences in the accessory genome, the core genome or both. Significant genomic differences have been previously observed between strains of the same MLST ST by microarray analysis (Taboada et al., 2008) and more recently by WGS analysis (Biggs et al., 2011). Because genomic differences between clonal strains are likely a reflection of the underlying epidemiology (i.e., separation in time and space), which would allow for the accumulation of such genomic differences, approaches to target such features through laboratory-based assays or to rapidly extract and analyze them from WGS data will become increasingly important in the deployment of genomic-based approaches in an epidemiological context.

DEVELOPMENT AND ASSESSMENT OF NEXT GENERATION TYPING SCHEMES

The need for a new generation of subtyping methods is underscored by a recent study by Biggs et al. (2011), in which the authors used WGS analysis to show significant genomic variation in two isolates that were indistinguishable by MLST and *flaA*-SVR typing. This example illustrates how strains that are linked by low resolution subtyping methods may harbor genomic differences consistent with spatial and/or temporal separation and points to the need for higher resolution methods for strain characterization.

Even subtle genomic changes can significantly impact strain characteristics (Carrillo et al., 2004; Jerome et al., 2011) given recent evidence that genomic change in *Campylobacter* is greatly influenced by positive selection (Lefébure and Stanhope, 2009). Moreover, genomic changes leading to phenotypic traits of public health significance (e.g., antimicrobial resistance, virulence, survival) may significantly impact risk profiles associated with specific genotypes.

Ultimately, whole genome sequence is the gold standard for microbial strain characterization Nonetheless, although rapid high throughput whole genome sequencing is rapidly becoming a feasible option for the investigation of public health events (Gilmour et al., 2010; Chin et al., 2011; Rohde et al., 2011), high throughput

lower-resolution methods are still necessary in the context of epidemiological surveillance.

An increasing body of WGS data could be used to inform the development of enhanced subtyping methods and the *in silico* approach that was used in this study could form the basis for a framework aimed at assessing novel subtyping methods prior to development and experimental implementation. The advantage of such a framework is that it allows for the testing of non-traditional typing targets such that most informative marker combinations could be used to develop enhanced subtyping schemes.

As the cost of sequencing continues to decline, bioinformatics pipelines that enable rapid analysis of draft genome data will enable public health laboratories to not only link WGS data to historical data but to provide optimal strain characterization using "extended MLST" analysis of hundreds of genes comprising core conserved, core variable, and accessory genes. This study demonstrates the feasibility of rapid analysis of minimally processed draft genome sequence data using an automated analytical pipeline.

CONCLUSION

Full genome sequence data provide the means for the evaluation of novel and existing molecular typing tools. In a post-genomics era, there is the opportunity to devise typing schemes that are based on the selection of informative regions that unambiguously provide evolutionary relationship among strains, but with sufficient resolution to capture subtle genomic changes between related strains that might arise through separation in time/space. A higher level of resolution is necessary to get an adequate representation of the true evolutionary relationship between strains that may otherwise appear to be clonal (Biggs et al., 2011).

This study was limited by the publicly available full genomes. While there is a great deal of genetic diversity captured among the strains for which WGS data are currently available, the dataset did not produce many multi-strain genotypic clusters, which made it difficult to analyze the congruence of molecular typing methods to the core genome phylogeny. The inclusion of additional strains with various degrees of genetic and epidemiologic linkage will be required to address whether current methods are sufficiently discriminatory for distinguishing closely related strains that are temporally or spatially unrelated and the analytical approaches that have been developed in this study will facilitate the assessment of molecular typing methods using a phylogenetic framework. To address this gap, we are currently in the process of sequencing a number of strains collected as part of a large-scale epidemiologic survey and that have been linked by epidemiological data and/or various typing data (Clark et al., 2012). The development of epidemiologically relevant reference panels of strains to be characterized by WGS analysis to be used for the assessment and validation of existing and emerging methods for pathogen characterization would be of great benefit to public health agencies and should be a priority for international collaboration.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2012.00057/abstract

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Multilocus sequence typing methods for the emerging *Campylobacter* species *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus*, and *C. curvus*

William G. Miller[1]*, Mary H. Chapman[1], Emma Yee[1], Stephen L. W. On[2], Desmond K. McNulty[3†], Albert J. Lastovica[4], Anne M. Carroll[3], Eleanor B. McNamara[3], Geraldine Duffy[5] and Robert E. Mandrell[1]

[1] *USDA, ARS, WRRC, Produce Safety and Microbiology, Albany, CA, USA*
[2] *Christchurch Science Centre, Institute of Environmental Science and Research Limited, Christchurch, New Zealand*
[3] *Public Health Laboratory, Health Service Executive, Cherry Orchard Hospital, Ballyfermot, Dublin, Ireland*
[4] *Department of Biotechnology, University of the Western Cape, Bellville, South Africa*
[5] *Food Safety Department, Teagasc Food Research Centre, Ashtown, Dublin, Ireland*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Qijing Zhang, Iowa State University, USA
Eduardo Taboada, Public Health Agency of Canada, Canada

***Correspondence:**
William G. Miller, USDA, ARS, WRRC, Produce Safety and Microbiology Research Unit, 800 Buchanan Street, Albany, CA 94710, USA.
e-mail: william.miller@ars.usda.gov

†Present address:
Desmond K. McNulty, ICON Central Laboratories, South County Business Park, Leopardstown, Dublin, Ireland.

Multilocus sequence typing (MLST) systems have been reported previously for multiple food- and food animal-associated *Campylobacter* species (e.g., *C. jejuni*, *C. coli*, *C. lari*, and *C. fetus*) to both differentiate strains and identify clonal lineages. These MLST methods focused primarily on campylobacters of human clinical (e.g., *C. jejuni*) or veterinary (e.g., *C. fetus*) relevance. However, other, emerging, *Campylobacter* species have been isolated increasingly from environmental, food animal, or human clinical samples. We describe herein four MLST methods for five emerging *Campylobacter* species: *C. hyointestinalis*, *C. lanienae*, *C. sputorum*, *C. concisus*, and *C. curvus*. The *concisus*/*curvus* method uses the loci *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *ilvD*, and *pgm*, whereas the other methods use the seven loci defined for *C. jejuni* (i.e., *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*). Multiple food animal and human clinical *C. hyointestinalis* ($n = 48$), *C. lanienae* ($n = 34$), and *C. sputorum* ($n = 24$) isolates were typed, along with 86 human clinical *C. concisus* and *C. curvus* isolates. A large number of sequence types were identified using all four MLST methods. Additionally, these methods speciated unequivocally isolates that had been typed ambiguously using other molecular-based speciation methods, such as 16S rDNA sequencing. Finally, the design of degenerate primer pairs for some methods permitted the typing of related species; for example, the *C. hyointestinalis* primer pairs could be used to type *C. fetus* strains. Therefore, these novel *Campylobacter* MLST methods will prove useful in differentiating strains of multiple, emerging *Campylobacter* species.

Keywords: MLST, emerging, *Campylobacter hyointestinalis*, *Campylobacter lanienae*, *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter sputorum*

INTRODUCTION

Campylobacters are a major cause of human bacterial gastrointestinal illness in the industrialized world (Mølbak and Havelaar, 2008; Olson et al., 2008); campylobacterioses (12.68 cases per 100,000) were second only to *Salmonella* infections (16.2/100,000) in the United States in 2008 (Anonymous, 2009). The majority of *Campylobacter* strains isolated from human clinical samples have been identified as *C. jejuni* subsp. *jejuni* or, to a lesser extent, *C. coli* (Lastovica and Allos, 2008). Recently, pathogenic campylobacters outside of the *C. jejuni*/*C. coli* group, termed here as emerging *Campylobacter* species, have been isolated more frequently from food and/or food animals. Recovery of these more fastidious, emerging *Campylobacter* species from food has not been reported often; isolation of such strains is likely limited by the culture conditions employed, conditions that favor *Campylobacter* species such as *C. jejuni* and *C. coli*. However, Lynch et al. (2011) using novel culture conditions, reported the isolation of multiple emerging *Campylobacter* spp., e.g., *C. concisus*, *C. curvus*, and *C. sputorum*, from chicken, beef, and pork samples. Emerging campylobacters isolated from food animals are often strains of species associated typically with livestock, such as *C. hyointestinalis* in sheep, cattle, and swine (Hakkinen et al., 2007; Salihu et al., 2009; Oporto and Hurtado, 2011), *C. lanienae* in cattle and swine (Sasaki et al., 2003; Inglis et al., 2004; Oporto and Hurtado, 2011), and *C. sputorum* in cattle and sheep (Terzolo, 1988; On et al., 1998).

The clinical relevance of the emerging *Campylobacter* spp. is as yet undetermined. Many of the emerging campylobacters are isolated infrequently from human clinical samples, although, as with isolation from food, recovery of these strains from clinical samples is probably limited by the isolation methods and media used. Nevertheless, emerging *Campylobacter* species are isolated from human clinical samples (Edmonds et al., 1987; Gorkiewicz et al., 2002; Lastovica and Allos, 2008; Bullman et al., 2011). Although the frequency of human illness associated with emerging *Campylobacter* spp. might be quite low, especially when compared to *C. jejuni*-associated gastroenteritis, it is possible that some

emerging species could be associated with more severe illness. One such example is *C. concisus*, for which a strong association with Crohn's disease and ulcerative colitis, has been reported recently (Man et al., 2010; Mahendran et al., 2011; Mukhopadhya et al., 2011).

Although molecular detection methods exist for many of the emerging campylobacters, population analyses, epidemiology, and source tracking of these organisms are limited by the strain typing methods available for these taxa. Molecular typing methods such as amplified fragment length polymorphism (AFLP) analysis and pulsed field gel electrophoresis (PFGE) methods have been employed on emerging *Campylobacter* strains (reviewed in On et al., 2008); however, sequence-based typing methods are not available for many species. One such sequence-based typing method is multilocus sequence typing (MLST). MLST methods amplify and sequence defined regions of moderately conserved housekeeping loci. At each locus, regions with distinct sequences receive arbitrary but unique allele numbers; similarly, each different allelic profile is assigned a unique sequence type (ST). The first *Campylobacter* MLST method was developed for *C. jejuni* (Dingle et al., 2001). This method sequences portions of seven genes: *aspA*, *atpA* (*uncA*), *glnA*, *gltA*, *glyA*, *pgm* (*glmM*), and *tkt*. The *C. jejuni* MLST method has been used in multiple typing studies and has been used successfully for strain typing and characterization, identification of clonal complexes and lineages, epidemiology, and investigation of host/source-associations (reviewed in Maiden and Dingle, 2008). Since the description of the *C. jejuni* MLST method, other *Campylobacter* MLST methods have been constructed that type *C. coli* (Dingle et al., 2005; Miller et al., 2005), *C. lari* (Miller et al., 2005), *C. upsaliensis* (Miller et al., 2005), *C. helveticus* (Miller et al., 2005), *C. fetus* (van Bergen et al., 2005), and *C. insulaenigrae* (Stoddard et al., 2007). Besides the primary use of *Campylobacter* MLST data for strain typing, MLST data for multiple taxa within *Campylobacter* are a valuable resource for studies on lateral gene transfer and evolution. MLST data can be used also to identify putative and perhaps clinically relevant taxonomic subdivisions within a species (Miller et al., 2005); additionally, MLST can provide genotypic information for novel species that are diverse phenotypically (Stoddard et al., 2007), especially those for which the only molecular speciation method is 16S rDNA sequencing.

Development of several *Campylobacter* MLST methods was assisted by the availability of draft genome sequences (Miller et al., 2005). Development of the novel *Campylobacter* MLST methods described in this study utilized recent draft genomes of *C. hyointestinalis*, *C. lanienae*, and *C. sputorum* (Miller et al., unpublished data), in addition to the closed *C. concisus* and *C. curvus* genomes available in the NCBI Microbial Genomes database. We anticipated that the draft genomes would contain some sequencing errors; however, enough reliable sequencing data was available to design MLST primers that could be used to type these five *Campylobacter* species. Therefore, in this study we describe four novel MLST methods that can be used to type: (1) *C. concisus* and *C. curvus*; (2) both subspecies of *C. hyointestinalis* (subspp. *hyointestinalis* and *lawsonii*) and *C. fetus* (subspp. *fetus* and *venerealis*); (3) *C. lanienae*; and (4) all three biovars of *C. sputorum* (bvs. *fecalis*, *paraureolyticus*, and *sputorum*). All four MLST gene sets are identical to the *C. jejuni* gene set [i.e., *aspA*, *atpA*(*uncA*), *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*], with the exception of the *C. concisus*/*C. curvus* MLST method in which *ilvD* replaces *tkt*. A sample set of 213 isolates of diverse geographic origin and source was typed in this study. For all four methods, a total of 163 STs and 729 alleles were identified, indicating that these new MLST methods provide resolution similar to the previous MLST methods described.

MATERIALS AND METHODS

GROWTH CONDITIONS AND CHEMICALS

All *Campylobacter* strains were cultured routinely under microaerobic conditions (1.5% O_2, 10% H_2, 10% CO_2, and 78.5% N_2) at 37°C on Brain Heart Infusion agar (Becton Dickinson, Sparks, MD, USA) or Anaerobe Basal Agar (ABA; Oxoid, Lenexa, KS, USA) supplemented with 5% (v/v) laked horse blood (Hema Resource and Supply, Aurora, OR, USA). PCR enzymes and reagents were purchased from New England Biolabs (Beverly, MA, USA) or Epicentre (Madison, WI, USA). All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA). DNA sequencing chemicals and capillaries were purchased from Applied Biosystems (Foster City, CA, USA). PCR and sequencing oligonucleotides were purchased from Eurofins MWG Operon (Huntsville, AL, USA).

ISOLATION OF *CAMPYLOBACTER* FROM FERAL SWINE AND CATTLE

Cattle feces were inoculated into wells of a six-well microtiter plate containing 6 ml 1× Anaerobe Basal Broth (Oxoid) amended with Preston supplement (Oxoid), using a sterile cotton swab. Plates were placed inside plastic ZipLoc bags and incubated under microaerobic conditions (as above) for 24 h at 37°C, while shaking at 40 rpm. After incubation, a 10-μl loop of each enrichment culture was plated onto ABA amended with 5% laked horse blood and CAT supplement (Oxoid). Feral swine feces were plated directly, using a sterile cotton swab, onto ABA amended with 5% laked horse blood and CAT supplement. All plates were then incubated under microaerobic conditions at 37°C for 24 h. Bacterial cultures were than filtered through 0.2 μm mixed cellulose ester filters onto ABA plates and incubated at 37°C under microaerobic conditions. After 24 h, single colonies were streaked onto new ABA plates and incubated 24–48 h at 37°C for purification.

CAMPYLOBACTER SPECIATION

Campylobacter strains isolated from the feces of California feral swine or cattle were speciated initially by 16S rDNA sequencing, using the primer pairs 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1392R (5′ GAC GGG CGG TGT GTA C 3′; Lane, 1991). However, the 27F/1392R primers were not able to type *C. hyointestinalis* strains past the species level and several strains could not be typed unequivocally. To improve speciation, the atpAF/atpAR primer pairs from Miller et al. (2005) were used. These primers can amplify all *Campylobacter* taxa described currently, with the exception of *C. avium* (data not shown). Using these *atpA* primers, campylobacters of uncertain type were amplified and sequenced; the sequences were then compared by alignments and phylogenetic analyses to strains of known species/subspecies identification, especially those whose genomes had been sequenced. Unlike the 16S primers, the *atpA* primers could speciate unambiguously all

of the strains isolated in this study and could identify clearly both subspecies of *C. hyointestinalis*. The *atpA* primer pairs, however, could not differentiate the three known biovars of *C. sputorum* (i.e., *sputorum*, *fecalis*, and *paraureolyticus*; On et al., 1998). The MLST results provided further confirmatory speciation data; *atpA* speciation agreed completely with subsequent MLST speciation.

DETECTION OF UREASE AND CATALASE ACTIVITY

The biovar *paraureolyticus* can be distinguished from the other two biovars of *C. sputorum* by the production of urease (On et al., 1998). Therefore, to identify putative bv. *paraureolyticus* strains, *C. sputorum* isolates were assayed for urease activity, as follows: a 10-μl loop of an overnight *C. sputorum* culture was resuspended in 2 ml urease reagent (3 mM NaH_2PO_4, 110 mM urea, 7 μg/ml phenol red, pH 6.8) and incubated for 1 h at RT. *C. sputorum* cultures were typed as bv. *paraureolyticus* based on a positive reaction (solution turning from yellow/orange to magenta). Genome-sequenced strains of biovars *sputorum* (strain RM3237) and *paraureolyticus* [strain RM4120 (LMG 11764)] were used as negative and positive controls, respectively. All tests were performed independently at least twice.

The *C. sputorum* biovars are distinguished also by the production of catalase: bv. *fecalis* is catalase-positive while the other two biovars are catalase negative (On et al., 1998). To test for catalase activity, a 10-μl loop of an overnight *C. sputorum* culture was resuspended in 200 μl 3% H_2O_2 on a glass slide. Presence of bubbles indicated a positive reaction. Genome-sequenced strains of biovars *sputorum* (strain RM3237) and *fecalis* [strain RM4121 (CCUG 20703)] were used as negative and positive controls, respectively. All tests were performed independently at least twice.

MULTILOCUS SEQUENCE TYPING

Each MLST amplification mixture contained: 1× MasterAmp PCR buffer (Epicentre, Madison, WI, USA), 1× MasterAmp PCR enhancer (Epicentre), 2.5 mM $MgCl_2$, 250 μM (each) dNTPs, 50 pmol each primer, and 1 U *Taq* polymerase (New England Biolabs). For strains where genomic DNA was extracted using kits or standard isolation protocols, 50 ng purified genomic DNA was added to each reaction tube. Otherwise, 2 μl of a boilate was added. Boilates were prepared by resuspending a 1-μl loop of a pure culture or a single Microbank bacterial storage bead (Pro-Lab, Austin, TX, USA) in 100 μl TE and heating at 80°C for 5 min, then 100°C for 20 min, and cooling to 4°C. MLST amplifications were performed on a Tetrad thermocycler (Bio-Rad, Hercules, CA, USA) with the following settings: 94°C for 30 s, 53°C for 30 s, and 72°C for 2 min (30 cycles). Amplicons were purified on a BioRobot 8000 workstation (Qiagen, Valencia, CA, USA). Cycle sequencing reactions were performed on a Tetrad thermocycler, using the ABI PRISM BigDye terminator cycle sequencing kit (version 3.1; Applied Biosystems) and standard protocols. Cycle sequencing extension products were purified using BigDye XTerminator (Applied Biosystems). DNA sequencing was performed on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems), using POP-7 polymer and ABI PRISM Genetic Analyzer Data Collection and ABI PRISM Genetic Analyzer Sequencing Analysis software. Sequences were trimmed, assembled, and analyzed in SeqMan (v 9.1; DNASTAR, Madison, WI, USA).

ALLELE NUMBER/SEQUENCE TYPE ASSIGNMENT

The Perl program MLSTparser3 (Miller et al., 2009) was modified to include the novel MLST methods for *C. concisus*, *C. curvus*, *C. hyointestinalis*, *C. lanienae*, and *C. sputorum*. The expanded MLSTparser3 was used to identify the MLST alleles and ST of each *Campylobacter* strain typed in this study. New *Campylobacter* MLST databases were created[1]; allele and ST data generated in this study were deposited in this database and are available online. The allelic profiles for all 213 strains are listed in Table S1 in Supplementary Material.

PHYLOGENETIC ANALYSES

A dendrogram of unique *Campylobacter* STs was constructed by concatenating the allele sequences comprising each ST. Allele sequences for each strain were concatenated in the order *aspA-atpA-glnA-gltA-glyA-pgm-tkt* with the exception of *C. concisus* and *C. curvus* allele sequences, that were concatenated in the order *aspA-atpA-glnA-gltA-glyA-ilvD-pgm*. Composite concatenate lengths were 3345 bp (*C. concisus*/*C. curvus*), 3312 bp (*C. fetus*, *C. hyointestinalis*, and *C. lanienae*), or 3321 bp (*C. sputorum*). Sequence alignments were performed using CLUSTALX (ver. 2.1)[2], and dendrograms were constructed using the neighbor-joining method with the Kimura two-parameter distance estimation method (Kimura, 1980). Phylogenetic analyses were performed using MEGA version 5.1 (Tamura et al., 2011). Polymorphic sites and d_n/d_s ratios were calculated using START2 (Jolley et al., 2001)

RESULTS AND DISCUSSION

DESIGN OF THE NOVEL *CAMPYLOBACTER* MLST METHODS

Construction of the novel *Campylobacter* MLST methods was facilitated by the availability of genome sequences for all of the taxa typed in this study. The genome-sequenced strains were: the completed genomes of *C. concisus* strain 13826 (NC_009802.1), *C. curvus* strain 525.92 (NC_009715.1), and *C. fetus* subsp. *fetus* strain 82–40 (NC_008599.1), and the draft genomes of *C. hyointestinalis* subsp. *hyointestinalis* (*Chh*) strain RM4092 (LMG 9260), *C. hyointestinalis* subsp. *lawsonii* (*Chl*) strain RM4096 (CCUG 27631), *C. lanienae* strain RM3663 (NCTC 13004), *C. sputorum* bv. *sputorum* strain RM3237, *C. sputorum* bv. *fecalis* strain RM4121 (CCUG 20703), *C. sputorum* bv. *paraureolyticus* strain RM4120 (LMG 11764), and strain RM6914, exemplar of a novel *C. concisus*-like clade (Mandrell et al., manuscript in preparation).

Primer design based on a sequence from a single strain might not lead to a successful MLST method if the sequence variation within that taxon prevents the design of primer pairs that efficiently amplify all strains. Therefore, MLST gene sequences from related species would be aligned. Based on this alignment, primers would be designed to bind to regions, 100–200 bp upstream and downstream of the allelic endpoints, that demonstrate a high degree of conservation among the aligned taxa. One to four degenerate bases would be incorporated into the MLST primers, if necessary, to optimize primer binding. This approach was used previously to construct successfully other *Campylobacter* MLST

[1] http://pubmlst.org/campylobacter/
[2] http://www.clustal.org/

methods (Miller et al., 2005). Therefore, the full *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt* gene sequences were extracted from the completed and draft genomes and various alignments were performed. Based on sequence similarity between the various *Campylobacter* taxa, we developed four novel MLST methods to type the strains in this study: Method 1 for typing both *C. concisus* and *C. curvus*; Method 2 for typing *C. fetus* and both subspecies of *C. hyointestinalis*; Method 3 for typing *C. lanienae* strains; and Method 4 for typing all three biovars of *C. sputorum*.

The final MLST primer sets are listed in **Table 1**. Methods 2, 3, and 4 use the same seven loci and allelic endpoints of the *C. jejuni* MLST method, i.e., *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm*, and *tkt*. However, for the *C. concisus*/*C. curvus* MLST method, the sequence diversity at the *tkt* locus was too great for the construction of suitable primers. Therefore, *tkt* was replaced by *ilvD* in Method 1; *ilvD* was used in a *C. jejuni* MLST method described previously (Manning et al., 2003). The Method 1 *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, and *pgm* alleles also use the same endpoints of their *C. jejuni* counterparts; the *ilvD* allelic endpoints are unique to this study.

CHARACTERIZATION OF THE FOUR NOVEL *CAMPYLOBACTER* MLST METHODS

A total of 213 strains were typed in this study. Complete descriptions of each strain, including isolation source, date, and location (if known), allelic profiles and STs are listed in Table S1 in Supplementary Material. Strains typed in this study were isolated over a 30-year period (1981–2010) and were also geographically diverse: strains from each species were isolated on two to three continents (Table S1 in Supplementary Material). All of the *C. concisus* and *C. curvus* strains typed were isolated from human clinical samples, whereas nearly all of the *Chl* and *C. lanienae* strains typed were isolated from pigs and feral swine; strains from the other three taxa were a mixture of isolates from humans, cattle, and swine (**Table 2**).

With a few exceptions, the primary MLST primers listed in **Table 1** were able to amplify successfully all seven loci and provide high quality sequence data for all 213 strains to generate unambiguous ST. However, despite our best efforts to overcome sequence variation in the initial primer design, in a few instances (∼1–2%), the main primer pairs did not provide sequence quality high enough for an unambiguous ST. Thus, alternate primer pairs (annotated as "A" in **Table 1**) were used to amplify and sequence these alleles. No strain was excluded from the final strain list because a ST could not be obtained.

GENETIC DIVERSITY

Phylogenetic analysis of the MLST STs validated the taxonomic relationships observed previously (Debruyne et al., 2008) for the six *Campylobacter* species typed in this study. Although the use of different gene sets comprising Method 1 and Methods 2, 3, and 4 prevented the simultaneous analysis of all six species, a clear delineation between *C. concisus* and *C. curvus* strains was observed (**Figure 1**), as well as unambiguous segregation of the other four species (**Figure 2**), that included the related *C. hyointestinalis*, *C. fetus*, and *C. lanienae* taxa. Moreover, the two *C. hyointestinalis* subspecies, *Chh* and *Chl*, formed distinct clusters (**Figure 2**). However, the two *C. fetus* subspecies could not be discriminated by phylogenetic analysis, consistent with previous observations (van Bergen et al., 2005). Divergent STs (*C. lanienae* STs 1 and 4; *C. sputorum* STs 8, 13, 14, and 15) were identified within some strains (see below and **Figure 2**). In *C. sputorum*, these divergent STs formed a cluster (termed Csp_2) distinct from a cluster (termed Csp_1) containing the other *C. sputorum* strains.

Many MLST STs and alleles were identified in this study (**Table 3**). With the exception of *C. fetus*, for which a previous study identified also a relatively small number of highly clonal STs (van Bergen et al., 2005), the majority of strains within each of the remaining five species possess unique STs. Phylogenetic analysis of the STs of these five species indicated that the least amount of variation exists within *Chh* and each of the two *C. sputorum* clades, whereas the greatest amount of variation detected here resides clearly within the *C. concisus* and *C. curvus* strain sets (**Figures 1–3**). Even with the inclusion of the divergent *C. lanienae* and *C. sputorum* STs, the average number of base substitutions per site was less in each case (0.0330, *C. lanienae*; 0.0265, *C. sputorum*; **Figure 3**) than that calculated for the *C. concisus* STs (0.0641; **Figure 3**).

The high degree of variation across the *C. concisus* STs is reflected by the large number of alleles and polymorphic sites identified within this strain set: for the 70 *C. concisus* strains, the number of alleles detected at any locus ranged from 55 (*atpA*) to 64 (*ilvD*; **Table 3**). This high density of alleles translated into the large number of polymorphic sites identified at each of the seven *C. concisus* loci (**Table 4A**). Over 100 polymorphic sites were present within the alleles of each of the *C. concisus* MLST loci (**Table 4A**), a large number when compared to the relatively few polymorphic sites detected within the *Chh* alleles, even when the relative sizes of the strain sets were factored into the comparison. For some species, the numbers of polymorphic sites were inflated by the presence of divergent alleles or strains within the strain set. When these alleles and strains were removed from the appropriate strain sets, the number of polymorphic sites decreased substantially. For example, removal of the divergent *C. sputorum* strains present within *C. sputorum* clade Csp_2 (**Figure 3**) eliminated 23 of 24 polymorphic sites at the *glnA* locus (**Table 4A**).

While MLST is often used as a strain typing tool, it can be used also to investigate the population structure of an organism, to identify lineages, for example, that demonstrate a higher association with disease or a particular host. Genes used for MLST methods, typically core housekeeping genes, are usually under purifying or neutral selection. Positively selected genes would be influenced by external/environmental pressures and would evolve more through recombination rather than through the accumulation of point mutations; thus, such genes are not generally used in MLST methods, since they may not provide an accurate representation of the clonal structure of a bacterial population (Maiden, 2006; Perez-Losada et al., 2011). One method of determining the level of selective pressure on a gene is by calculating the ratio between non-synonymous (d_n) and synonymous (d_s) base substitutions. The rate of synonymous base substitution in genes should equal the neutral substitution rate, in the absence of codon usage bias. Non-synonymous base substitutions (that result in an amino acid change) would be the result of positive

Table 1 | *Campylobacter* MLST primers.

Locus	Allele size (bp)	Oligonucleotide primers				Method					
						1		2		3	4
		Forward (5′–3′)		Reverse (5′–3′)		Co	Cv	Cf	Ch	Cl	Cs
asp	477	aspCCCFN1	GGHCAAAGCACAAATGAYRCTTATCC	aspCCCRN1	GCCWAGDACTGATTTTARGCAAGC	P	P				
		aspCCF1	CAAAGCACAAATGACACTTATCCAA	aspCCR1	GRACTGATTTTAGGCAAGCCTCAGG	A	A				
		HFaspF	CTTTGAWAAAAAAGCAGARGAGTTTAA	HFaspR	GCTGTAACGATACCGATTGAGTTATAA			P	P		
		HYOaspF	AAGAGTKGCTATTTATGAAAGACTTTGTG	HYOaspR	AATGCTGTAACGATACCGATTGAGT				A		
		LANaspF	TTTAGCCACAGCTATGGAGTATCTCAA	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC					P	
		HFLaspXF	AAYATGAAYGCAAACGAAGTTATAGC	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC					A	
		SPUTaspF	GAAACATTTGCAAGACGAACTAGAT	SPUTaspR	AAATGCTGTTACTATACCAACTGATCC						P
atpA	489	atpCCCFN1	GATACYATCATYAAYCAAAAAGGTCAAGA	atpCCCRN2	GGTATTTHGCCTCKATATAWGGATAWAGC	P	P				
		atpCCF1	ACTATCATCAAYCAAAAAGGTCAAG	atpCCR2	GGATAWAGCTCMGCTTCAAATTTTGT	A	A				
		HFatpF	GTATYAAAGCTATWGACGGTTTGGTTC	HFatpR	GAGYGGGCTATAAGGAGGTTG			P	P		
		HYOatpF	ATGTVGCTATMGGTCAAAARCAATC	HYOatpR	TTTCTACWGGRAGYGGGCTATAAGG				A		
		LANatpF	AACCAAAAAGGTCAAGATGTTATATGT	LANatpR	ATTTTCTACTGGAAGTGGGCTATAAGG					P	
		HFLatpXF	CMAAAGGHATYATGGCTAGAAAAT	HFLatpXR	TTCRATATCTTTATCHAGWGCTTTTTT					A	
		SPUTatpF	ACTATCATAAATCAAAAAGGCGGAG	SPUTatpR	TTCTCAATACCAAGRGGTGAATAAG						P
glnA	477	glnCCCFN1	GSTTGGCAYAGCATAAGYTACAAC	glnCCCRN2	GTTYGTGCTWGGGTTTGTRAAGGC	P	P				
		glnCCF1	GSYTGGCACAGCATAAGCTACAAC	glnCCR2	ACCRTCTTTCCARACTGATTGATGC	A	A				
		HFglnF	GGCATCACGTATCKTATAATATAAAAGC	HFglnR	ATGTACGTGCATACCGCTTCC			P	P		
		HYOglnF	TCTTATAATATAAAAGCCGTGAGCGAA	HYOglnR	CCRTCTTTCCATATACTTTGATGTACG				A		
		LANglnF	TGGCAYCAYGTATCWTATAATATAAAAGC	LANglnR	ATGGACRTGCATACCRCTWCCATTATC					P	
		HFLglnXF	TTTYGAATWTTGTRAWGAAAATGAAGT	HFLglnXR	AGAGTAWGTWAGAATGCTTGGKGCTTC					A	
		SPUTglnF	AGGAACTTGGCATCATGTTTCTTAT	SPUTglnR	CCATCTTTCCAAATAGATTGATGAA						P
gltA	402	gltCCCFN1	GGGMTACACCTCRACKGCGATGTG	gltCCCRN2	CBCCRTGWGCCCAGCCCC	P	P				
		gltCCF1	TACATCGACGGKCTAAARGGCGAG	gltCCR2	GATCTCYTCWAGCTGGCGGATGAC	A	A				
		HFgltF	CTATAACRTTTATMGATGGWGAAAAAGG	HFgltR	ATCAACYCTATCTGGAGTTCCTATCAT			P	P		
		HYOgltF	TATCACGTTTATAGATGGTGAAAAAGG	HYOgltR	YCTATCTGGAGTTCCTATCATYTCAAG				A		
		LANgltF	ATGCATAGMGGMTATGATATAGCGTGG	LANgltR	CATCAACTCTATCTGGAGTWCCKATCA					P	
		HFLgltXF	TACTGGTATGTTTACRTTTGATAGGGG	HFLgltXR	GAAATCMACATTTGGATATARATTTCT					A	
		SPUTgltF	AAAAAAGCATATTAAAACATCGTGG	SPUTgltR	TTATCCACACTTCCTATCATTTCTAGTT						P

(Continued)

Table 1 | Continued

Locus	Allele size (bp)	Oligonucleotide primers				Method					
						1		2		3	4
		Forward (5′–3′)		Reverse (5′–3′)		Co	Cv	Cf	Ch	Cl	Cs
glyA	507	glyCCCFN1	ACAAACAAATACGCMGAAGGCTA	glyCCCRN1	GATATCWGCRTCTTTWCCGCTAAA	P	P				
		glyCCF1	AARSAGCTTTTTGGMTGCGAA	glyCCR1	GATRTCWGCGTCTTTTCCGCTAA	A	A				
		HFglyF	GCAAAYGTTCAGCCAAATAGC	HFglyR	TTTATTTACTGTTATTCCWGCRTTACC			P	P		
		HYOglyF	TTGCWAATGTTCAGCCAAATAGC	HYOglyR	ATTCCRGCATTRCCAAGAGCGATAT				A		
		LANglyF	TGCWAATGTTCAGCCAAATAGCG	LANglyR	CAAGAGCGATATCRGCRTCTTTACC					P	
		HFLglyXF	GATWGCTAGTGARAAYTTCACATATCC	HFLglyXR	GYCCTCTTTCATACCYCTTGC					A	
		SPUTglyF	TGCAAATGTTCAACCAAACTCAG	SPUTglyR	GTAATTCCAGCATTTCCTAAAGCAT						P
ilvD	492	ilvCCCFN1	CGACTGGGCTAAAAGACGAGGA	ilvCCCRN1	TATGTGAGCGATRTTTYGGCTGAT	P	P				
		ilvCCF1	CGACYGGGCTAAAAGACGAGGAC	ilvCCR1	CKATGTGAGCGATGTTTTGGCTGAT	A	A				
pgm	501	pgmCCCFN1	CARATMAAAAATTCHTTCCCAAAAGAG	pgmCCCRN1	CTTTTABGCATTTARGGCTTTTAYRAA	P	P				
		pgmCCF1	ATCAAAAATTCMTTCCCAAAAGAGC	pgmCCR1	ATCAMRTTTTCRGTRCCAGAGTATCTAA	A	A				
		HFpgmF	AAAAGGTTTRMGAGTTGTTTTGGACGT	HFpgmR	TAACGTTTTCWGTVCCWGAATATCTAAA			P	P		
		HYOpgmF	GCTTACCTTAAAAGGTTTRCGAGTTGT	HYOpgmR	TCATCACGTCCTTCAAGCAAAAG				A		
		LANpgmF	GCTTACYTTAAAAGGCCTRMGAGTTGT	LANpgmR	AAGAAGCAGYCTAATCAAATTYTCTGT					P	
		HFLpgmXF	CAATMGCRTTTTTAACCGAAGATATG	HFLpgmXR	AATTTTTCYACYTCTTCCATYTTTTTA					A	
		SPUTpgmF	TTCCAAAAGAGCTTACAATGTATGG	SPUTpgmR	TGTTCCTGAATATCTAAATAGTGAGCG						P
tkt	459, 468 (Cs)	HFtktF	TTTTTRTGTGCVGATATGGTTCAAAA	HFtktR	TATGATWTCTTCRCCAAGMGGAGC			P	P		
		HYOtktF	CAATGGGACTTGCTGATTTAATGAG	HYOtktR	TCTTTGCMTCTTTTATGATATCTTCGC				A		
		HFLtktXF	AATAAGATTTTTRTGTGCVGATATGGT	HFLtktXR	AAGAGTGAATTTARMAGCTCTTTTTTA					P	
		LANtktF	CATCTAAAKCAYAATCCMAAAAATCC	LANtktR	ATCTCWKCGCCAAGMGGAGC					A	
		SPUTtktF	TTGCGGATATTATGGTTGTTTTAGC	SPUTtktR	GATTAAATCCTGCGAGTTTTTTTGC						P

All tkt alleles are 459 bp except for C. sputorum (468 bp). Forward and reverse primers were used in both PCR amplification and subsequent amplicon sequencing.

Co, C. concisus; Cv, C. curvus; Cf, C. fetus (both subspecies); Ch, C. hyointestinalis (both subspecies); Cl, C. lanienae; Cs, C. sputorum; P, primary MLST primer; A, alternate MLST primer.

Table 2 | Source of the *Campylobacter* strains typed in this study.

Species	Subspecies	Strains	Human	Cow/cattle	Pig/feral swine	Other/unknown
concisus		70	70	0	0	0
curvus		16	16	0	0	0
fetus		21	6	4	8	3
hyointestinalis	*hyointestinalis*	39	14	16	9	0
hyointestinalis	*lawsonii*	9	0	1	8	0
lanienae		34	1	0	32	1
sputorum		24	2	9	8	5

selection. Thus, the ratio of non-synonymous to synonymous base substitutions (d_n/d_s) would be an indicator of potential positive selection: ratios > 1 would be evidence of possible positive selection, whereas ratios < 1 would be more indicative of purifying or stabilizing selection. The d_n/d_s values for previous *Campylobacter* methods were quite low: the highest d_n/d_s values for *C. jejuni* (0.093), *C. coli* (0.173), *C. lari* (0.047), *C. upsaliensis* (0.097), and *C. insulaenigrae* (0.110) were substantially <1 (Colles et al., 2003; Miller et al., 2005; Stoddard et al., 2007). Similar ratios (highest d_n/d_s values) calculated in this study for *C. concisus* (0.0295), *C. curvus* (0.0468), *Chh* (0.0516), *Chl* (0.0655), *C. lanienae* (0.0562), and *C. sputorum* (0.0426; **Table 4B**) are consistent with the previous methods, indicating that these MLST loci are also not subject to positive selection.

IDENTIFICATION OF PUTATIVE LATERAL GENE TRANSFER EVENTS AND NOVEL TAXA

Characterization of the *Campylobacter* MLST methods also identified putative lateral gene transfer events. An allele that was nearly identical to, and clustered phylogenetically with, alleles from another taxon was determined to represent a putative lateral gene transfer event. For example, strains RM14410 and RM14403 in the *Chh* strain set contain alleles *pgm*-14 and *pgm*-16, respectively (Table S1 in Supplementary Material), that are 97.8–99.6% similar at the nt level to *pgm* alleles from *Chl* but only 95.2–96.2% similar at the nt level to *pgm* alleles from *Chh* (data not shown); these two alleles are also clearly related to other *Chl pgm* alleles, based on phylogenetic analysis (**Figure 4A**). Strains RM14410 and RM14403 also contain the *atpA* alleles *atpA*-2 and *atpA*-13, respectively, that cluster with other *Chh atpA* alleles (**Figure 4B**). Alleles at the other five loci for these two strains cluster also with other *Chh* alleles (data not shown), indicating that RM14403 and RM14410 are *Chh* strains in which a putative lateral transfer event has occurred at the *pgm* locus. The extent of gene transfer in these two strains could not be determined by MLST and will require further genome sequence analyses. It is not surprising that *Chl* alleles were discovered in *Chh* strains: these two taxa are highly related, being subspecies of the same species, and are isolated often from the same food animals (i.e., cattle and swine). No putative lateral transfer events were observed within *C. concisus* or *C. curvus* and no alleles were identified that originated tentatively in another species.

In some instances, phylogenetically divergent alleles within a strain set were indicative of either highly divergent strains or perhaps novel taxa. Here, as in other MLST studies (Miller et al., 2005, 2009), putative lateral gene transfer events were identified at only one of the seven MLST loci. For example, *Chh* STs containing *pgm*-14 or *pgm*-16 (ST-26_{hh} and ST-28_{hh}, respectively; **Figure 2**) were divergent at only the *pgm* locus. The alleles for each of the remaining six loci within each ST were of likely *Chh* origin, and phylogenetic analysis of the concatenated allele sequences clearly placed these two STs within the *Chh* clade. However, some STs (ST-1_{lan}, ST-4_{lan}, ST-8_{sp}, ST-13_{sp}, ST-14_{sp}, and ST-15_{sp}) contain three to seven variant alleles (Table S1 in Supplementary Material). The *C. lanienae* STs ST-1_{lan} and ST-4_{lan} differ substantially from the other typed *C. lanienae* strains: concatenated nucleotide sequences across all seven loci for ST-1_{lan} and ST-4_{lan} are on average only approx. 92 and 88% similar, respectively, to the concatenated sequences of the other *C. lanienae* STs (**Figure 2**), which display an average 98% cross-similarity (data not shown). Therefore, these two *C. lanienae* STs may be exemplars of novel *C. lanienae*-related taxa (for comparison, the concatenated *Chh* nucleotide sequences are approx. 94% similar to those of the other *C. hyointestinalis* subspecies and 87% similar to those of the related species *C. fetus*; **Figure 2**). Additionally, six phylogenetically related, urease-negative strains of *C. sputorum*, all isolated from cattle over a 19-month time period, may be members of a novel taxon. Within these six strains, four divergent ST were identified (ST-8_{sp}, ST-13_{sp}, ST-14_{sp}, and ST-15_{sp}). The concatenated allele sequences of these four STs are 95% similar to STs from the three established *C. sputorum* biovars, that display only 1% sequence divergence across the 3321-bp (**Figure 2**). Thus, it is possible that these six strains are members of a *C. sputorum*-like taxon, perhaps a novel *C. sputorum* subspecies or biovar. Nevertheless, for both the divergent *C. lanienae* and *C. sputorum* strains, additional biochemical and molecular tests will need to be performed to definitively establish their taxonomic position within *Campylobacter*.

SUBTYPING OF *C. CONCISUS* AND *C. SPUTORUM* STRAINS

Previous studies investigating the diversity of *C. concisus* organized strains from this species into two major genetically diverse clusters or genomospecies (GS), based on strain typing using 23S rRNA PCR (Engberg et al., 2005; Kalischuk and Inglis, 2011) or AFLP (Aabenhus et al., 2005; Kalischuk and Inglis, 2011). Included in the *C. concisus* strain set here were several strains characterized previously by AFLP (Aabenhus et al., 2005). In agreement with these previous studies, phylogenetic analysis of the *C. concisus* STs identified two clusters: each cluster contained almost exclusively GS1 or GS2 strains (**Figure 1**). Within *C. concisus*, two to eight

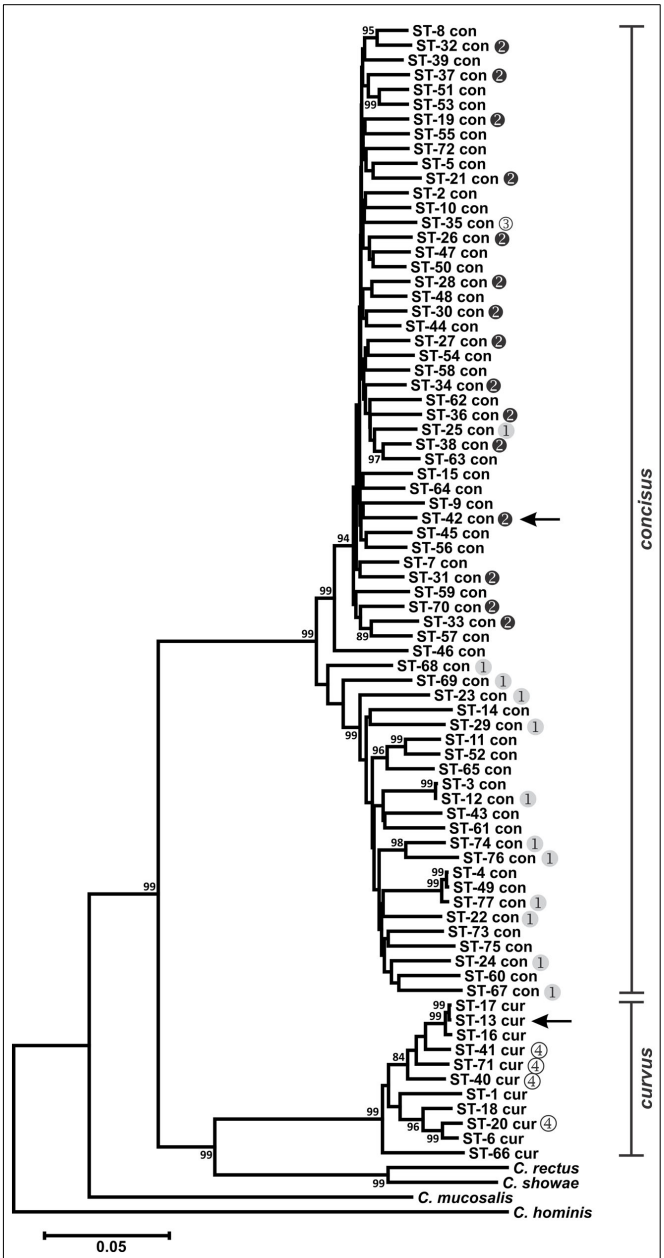

FIGURE 1 | Dendrogram of *C. concisus* and *C. curvus* STs. Allele sequences for each strain were concatenated in the order *aspA-atpA-glnA-gltA-glyA-ilvD-pgm* and aligned using CLUSTALX. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura two-parameter distance estimation method (Kimura, 1980). Bootstrap values >75%, generated from 1000 replicates, are shown at the nodes. Scale bar represents substitutions per site. Genomospecies 1–4 designations, as assigned by Aabenhus et al. (2005), were placed to the right of STs representing strains from that study. Arrows indicate the STs of the *C. concisus* and *C. curvus* genome-sequenced strains. The dendrogram contains also the concatenated *aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *ilvD*, and *pgm* allele sequences of *Campylobacter rectus*, *Campylobacter showae*, *Campylobacter mucosalis*, and *Campylobacter hominis*. These allele sequences were extracted from draft (*C. rectus*, *C. showae*, and *C. mucosalis*) and completed (*C. hominis*) genome sequences.

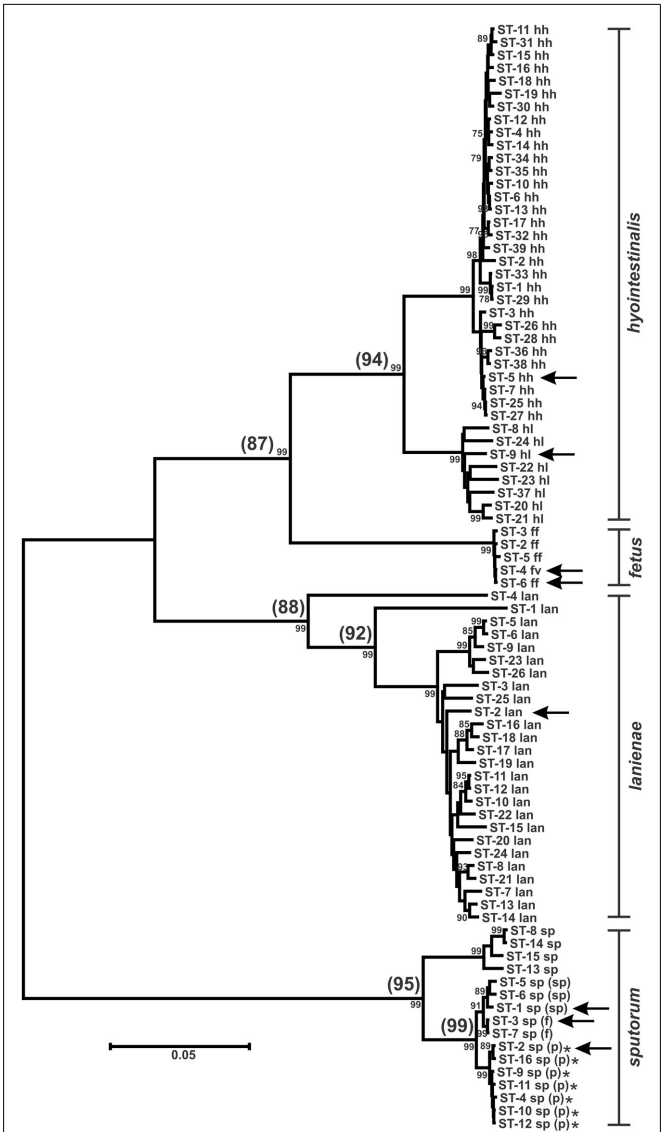

FIGURE 2 | Dendrogram of *C. hyointestinalis, C. fetus, C. lanienae,* and *C. sputorum* STs. Allele sequences for each strain were concatenated in the order *aspA-atpA-glnA-gltA-glyA-pgm-tkt* and aligned using CLUSTALX. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura two-parameter distance estimation method (Kimura, 1980). Bootstrap values >75%, generated from 1000 replicates, are shown at the nodes. Scale bar represents substitutions per site. ST labels indicate taxon: hh, *C. hyointestinalis* subsp. *hyointestinalis*; hl, C. *hyointestinalis* subsp. *lawsonii*; ff, C. *fetus* subsp. *fetus*; fv, *C. fetus* subsp. *venerealis*; lan, *C. lanienae*; sp, *C. sputorum*; sp (sp), *C. sputorum* bv. *sputorum*; sp (f), *C. sputorum* bv. *fecalis*; sp (p), *C. sputorum* bv. *paraureolyticus*. Arrows indicate the STs of the genome-sequenced strains for each taxon. ∗Urease-positive strains. Values in parentheses at the nodes represent the average %nt similarity of the STs split at each node, following pairwise comparisons of the concatenated allele sequences.

alleles at each of the seven MLST loci were identified in more than one ST (Table S1 in Supplementary Material). It is noteworthy perhaps that of these 33 "common" MLST alleles, only one

Table 3 | Sequence types and alleles identified by the novel MLST methods.

Species	Subspecies	Strains	STs	Alleles							
				aspA	***atpA***	***glnA***	***gltA***	***glyA***	***ilvD***	***pgm***	***tkt***
concisus		70	66	60	55	62	59	61	64	59	N/A
curvus		16	11	8	7	6	6	7	9	7	N/A
fetus		21	5	1	4	2	2	2	N/A	1	2
hyointestinalis	*hyointestinalis*	39	31	8	6	7	5	12	N/A	12	13
hyointestinalis	*lawsonii*	9	8	7	8	7	6	7	N/A	7	7
lanienae		34	26	16	9	13	11	13	N/A	16	12
sputorum		24	16	6	6	3	6	9	N/A	6	7

Cf	Chh	Chl	$Clan_{all}$	$Clan_1$	Csp_{all}	Csp_1	Csp_2	
0.0015	0.1446	0.1547	0.2488	0.2481	0.3291	0.3278	0.3329	Cf
	0.0086	0.0629	0.2394	0.2389	0.3405	0.3397	0.3428	Chh
		0.0190	0.2286	0.2279	0.3310	0.3291	0.3366	Chl
			0.0330	NT	0.3383	0.3376	0.3406	$Clan_{all}$
				0.0192	0.3383	0.3376	0.3405	$Clan_1$
					0.0265	NT	NT	Csp_{all}
						0.0073	0.0549	Csp_1
							0.0093	Csp_2

Ccur	Ccon	
0.0408	0.2189	Ccur
	0.0641	Ccon

FIGURE 3 | Genetic distance between and within the *Campylobacter* taxa. Each value represents the average number of base substitutions per site between concatenated allele sequences. Analyses were conducted using the Kimura two-parameter model (Kimura, 1980). The analysis involved 86 nucleotide sequences. Codon positions included were first + second + third+ non-coding. All positions containing gaps and missing data were eliminated. There were a total of 3312 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Branch lengths used to calculate the averages here were used to construct the dendrograms of **Figures 1** and **2**. NT, not tested. The *C. lanienae* and *C. sputorum* strain sets contained divergent STs. The STs $ST\text{-}1_{lan}$ and $ST\text{-}4_{lan}$ were removed from the *C. lanienae* strain set $Clan_{all}$ to create $Clan_1$. Similarly, $ST\text{-}8_{sp}$, $ST\text{-}13_{sp}$, $ST\text{-}14_{sp}$, and $ST\text{-}15_{sp}$ were removed from the *C. sputorum* strain set Csp_{all} to create Csp_2; the remaining STs formed Csp_1.

allele (*glyA*-31) was identified in both GS1 and GS2 strains, suggesting that minimal genetic exchange occurs between the two genomospecies.

Division of *C. concisus* strains into two primary genogroups is not merely an academic exercise. In a study analyzing *C. concisus* strains isolated from diarrheic and non-diarrheic individuals, Kalischuk and Inglis reported that GS1 strains were isolated predominantly from healthy individuals while the GS2 cluster contained isolates primarily from diarrheic individuals (Kalischuk and Inglis, 2011); this correlation between diarrheal disease and GS2 *C. concisus* species was also observed by Aabenhus et al. (2005). Moreover, GS2 strains were reported to exhibit higher levels of epithelial invasion (Kalischuk and Inglis, 2011). Therefore, GS1 or GS2 strains would be predicted to lead possibly to different clinical outcomes, and the proper placement of *C. concisus* isolates into these two genomospecies would be critical not only clinically but also for epidemiological studies. The MLST method described here provides another accurate tool for *C. concisus* genomotyping.

Campylobacter concisus GS4 strains were isolated from severely immunodeficient patients and identified initially by limited phenotyping; comparative AFLP analysis and other DNA-based testing indicated their taxonomic position required clarification (Aabenhus et al., 2005). Given these data, and our MLST results, it is likely that these strains are *C. curvus* and not *C. concisus*. These species share many phenotypic traits (On et al., 1996) and are difficult to distinguish with limited phenotypic testing.

At present, *C. sputorum* biovars are identified by their catalase or urease phenotypes (On et al., 1998). No PCR/sequencing methods exist that differentiate the three described biovars, although whole-genome macro-restriction profiling has been used to suggest biovar clonality (On et al., 1999). However, putative biovar-associated alleles were observed at three of the *C. sputorum* MLST loci (Table S1 in Supplementary Material). For example, at the *atpA* locus, bv. *sputorum* strains are *atpA*-1, bv. *fecalis* strains are *atpA*-3, and bv. *paraureolyticus* strains are either *atpA*-2 or *atpA*-4; strains of the cow-associated *C. sputorum* clade described above are either *atpA*-5 or *atpA*-6. Similar associations exist at the *glyA* and *pgm* loci. Indeed, the three biovars segregate also when all seven loci are examined phylogenetically (**Figure 2**), although the differences are quite small. Obviously, the size of the *C. sputorum* strain set typed here is too small to reach any definitive conclusions, although the associations are intriguing.

CONCLUSION

The four MLST methods described in this study typed successfully all 213 *Campylobacter* strains, representing at least ten *Campylobacter* taxa that included both subspecies of *C. fetus* and *C. hyointestinalis* and all three biovars of *C. sputorum*. Each method identified multiple novel STs; the small number of STs present in some taxa were more likely due to the limited size of the sample sets for those taxa than a limitation of the method itself. These methods were successful despite the high degree of variation in some species, e.g., *C. concisus*. Also, the concatenated *C. concisus* and *C. curvus* ST sequences were only 81% similar at the nt level (**Figure 1**); likewise, the *C. hyointestinalis* and *C. fetus* ST sequences were 87% similar (**Figure 2**), yet MLST Methods 1 and 2 could type either set of strains unequivocally. Indeed, the ability to sequence such variable strains provided an unexpected bonus to these MLST methods. The methods described here identified putative novel *C.*

Table 4 | Diversity within the *Campylobacter* MLST loci.

Species	Subspecies	Strains	*aspA*	*atpA*	*glnA*	*gltA*	*glyA*	*ilvD*	*pgm*	*tkt*
A. POLYMORPHIC SITES										
concisus		70	131	105	120	102	129	140	128	N/A
curvus		16	90	47	47	51	40	63	41	N/A
hyointestinalis	*hyointestinalis*	39	9	9	10	27	15	N/A	30 (17)	24
hyointestinalis	*lawsonii*	9	23	36	10	11	64	N/A	19	21
lanienae		34	67 (29)	86 (51)	58 (19)	57 (26)	71 (26)	N/A	142 (31)	96 (38)
sputorum		24	58 (5)	33 (17)	24 (1)	28 (9)	39 (14)	N/A	18 (10)	20 (8)
B. RATIOS OF NON-SYNONYMOUS (d_n) TO SYNONYMOUS (d_s) BASE SUBSTITUTIONS										
concisus		70	0.0257	0.0028	0.0052	0.0036	0.017	0.0295	0.0095	N/A
curvus		16	0.0417	0.0057	0.008	0.0468	0.0168	0.0149	0.0091	N/A
hyointestinalis	*hyointestinalis*	39	0	0	0	0.0262	0.0516	N/A	0.0249 (0.0110)	0.0381
hyointestinalis	*lawsonii*	9	0.0169	0.0276	0.0216	0	0.0113	N/A	0.0655	0.0251
lanienae		34	0.015 (0.0149)	0.0204 (0.0235)	0.0026 (0)	0.0502 (0.0298)	0.0112 (0.0257)	N/A	0.0562 (0.0748)	0.0421 (0.0419)
sputorum		24	0.041 (0.0715)	0 (0)	0 (0)	0 (0)	0.0102 (0.0511)	N/A	0.0293 (0.0264)	0.0426 (0.1672)

Numbers in parentheses represent polymorphic sites and d_n/d_s ratios recalculated following removal of the divergent pgm-14 and pgm-16 alleles within the Chh profiles and removal of the divergent ST-1_{lan} and ST-4_{lan} (lanienae) and ST-8_{sp}, ST-13_{sp}, ST-14_{sp}, and ST-15_{sp} (sputorum) sequence types.

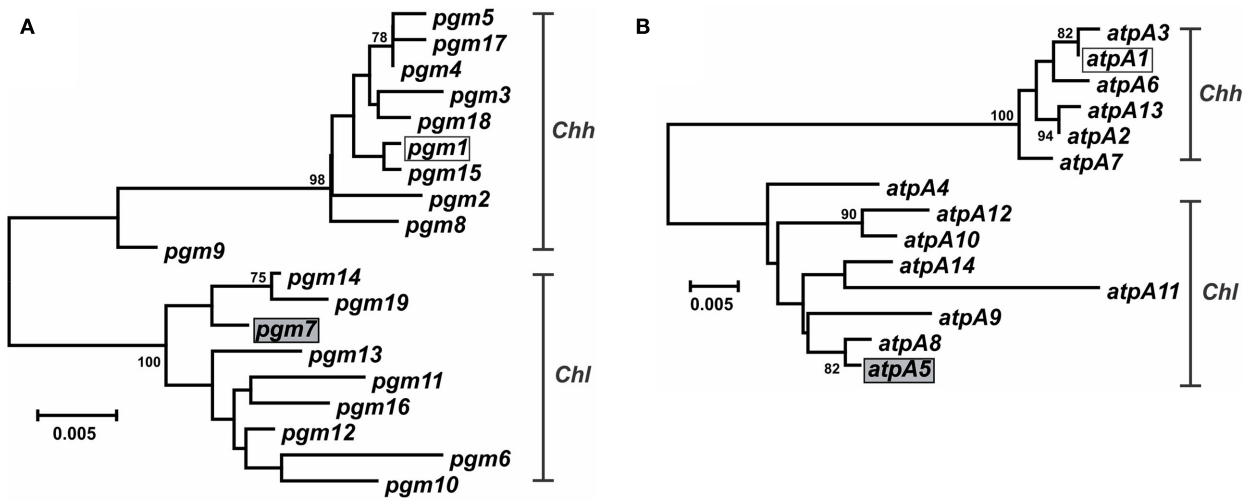

FIGURE 4 | Dendrogram of *C. hyointestinalis pgm* and *atpA* alleles. *C. hyointestinalis pgm* **(A)** and *atpA* **(B)** allele sequences were aligned using CLUSTALX. The dendrogram was constructed using the neighbor-joining algorithm and the Kimura two-parameter distance estimation method (Kimura, 1980). Bootstrap values >75%, generated from 1000 replicates, are shown at the nodes. Scale bar represents substitutions per site. The alleles of the *Chh* (open boxes) and *Chl* (gray boxes) genome sequence strains are indicated.

lanienae- and *C. sputorum*-related taxa and it is likely that these methods could further characterize and type as-yet-undescribed *Campylobacter* species or subspecies. For example, MLST Method 2 has been used to type reptile-associated *C. fetus*-like organisms (data not shown).

For many campylobacters, sequence data is restricted currently to ribosomal rRNA loci. While these rDNA sequences can provide crucial speciation data for many taxa, some groups of campylobacters cannot be differentiated readily by 16S rDNA sequencing. One such example includes *C. hyointestinalis* and *C. lanienae* strains. Some of the strains in this study from these species could not be typed unequivocally by 16S rDNA sequencing; however, MLST could readily place all strains in their proper taxonomic positions. MLST has been shown also to be of value in identifying strains of species with multiple phenogroups, such as *C. insulaenigrae* (Stoddard et al., 2007). In this study, a *C. sputorum* clade was typed that, based on established phenotypic characterization, would likely have been classified as bv. *fecalis*. While additional tests need to be performed, MLST cast some doubt that these strains were *C. sputorum* bv. *fecalis*.

Eighteen of the thirty validly described *Campylobacter* taxa can now be typed by MLST. This number is likely an underestimate, as some of the *C. lari*-like species (e.g., *C. peloridis*) described recently can be typed also using the *C. lari* MLST method (data not shown). The ability of MLST to type and speciate campylobacters, as well as identify putative horizontal gene transfer, indicates that the multiple *Campylobacter* MLST methods now available provide a valuable tool in the epidemiology, typing, and evolution of emerging campylobacters.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2012.00045/abstract

Table S1 | *Campylobacter* strains typed in this study.

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Polynucleotide phosphorylase has an impact on cell biology of *Campylobacter jejuni*

Nabila Haddad[1,2]*, Odile Tresse[1,2], Katell Rivoal[3], Didier Chevret[4], Quentin Nonglaton[1], Christopher M. Burns[5], Hervé Prévost[1,2] and Jean M. Cappelier[1,2]

[1] *LUNAM Université, Oniris, University of Nantes, Nantes, France*

[2] *UMR1014 Sécurité des Aliments et Microbiologie, INRA, Nantes, France*

[3] *Unité Hygiène et Qualité des Produits Aviaires et Porcins, Administración Nacional de la Seguridad Social, Ploufragan, France*

[4] *UMR1319 MICALIS, Plateforme d'Analyse Protéomique de Paris Sud-Ouest, INRA, Jouy-en-Josas, France*

[5] *Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, VA, USA*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Arnoud H. M. Van Vliet, Institute of Food Research, UK
Cecilia M. Arraiano, Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Portugal

****Correspondence:***
Nabila Haddad, ONIRIS, UMR1014 Sécurité des Aliments et Microbiologie, INRA, rue de la Géraudière, 44322 Nantes, France.
e-mail: nabila.haddad@oniris-nantes.fr

Polynucleotide phosphorylase (PNPase), encoded by the *pnp* gene, is known to degrade mRNA, mediating post-transcriptional regulation and may affect cellular functions. The role of PNPase is pleiotropic. As orthologs of the two major ribonucleases (RNase E and RNase II) of *Escherichia coli* are missing in the *Campylobacter jejuni* genome, in the current study the focus has been on the *C. jejuni* ortholog of PNPase. The effect of PNPase mutation on *C. jejuni* phenotypes and proteome was investigated. The inactivation of the *pnp* gene reduced significantly the ability of *C. jejuni* to adhere and to invade Ht-29 cells. Moreover, the *pnp* mutant strain exhibited a decrease in *C. jejuni* swimming ability and chick colonization. To explain effects of PNPase on *C. jejuni* 81-176 phenotype, the proteome of the *pnp* mutant and parental strains were compared. Overall, little variation in protein production was observed. Despite the predicted role of PNPase in mRNA regulation, the *pnp* mutation did not induce profound proteomic changes suggesting that other ribonucleases in *C. jejuni* might ensure this biological function in the absence of PNPase. Nevertheless, synthesis of proteins which are involved in virulence (LuxS, PEB3), motility (*N*-acetylneuraminic acid synthetase), stress-response (KatA, DnaK, Hsp90), and translation system (EF-Tu, EF-G) were modified in the *pnp* mutant strain suggesting a more specific role of PNPase in *C. jejuni*. In conclusion, PNPase deficiency induces limited but important consequences on *C. jejuni* biology that could explain swimming limitation, chick colonization delay, and the decrease of cell adhesion/invasion ability.

Keywords: *Campylobacter jejuni*, polynucleotide phosphorylase, *in vitro* virulence tests, chick colonization, 2D-electrophoresis

INTRODUCTION

Campylobacter jejuni is considered as the leading cause of human bacterial gastroenteritis in the world (Skirrow, 1994; Altekruse et al., 1999). The main reservoir of *C. jejuni* is the guts of avian species with up to 10^9 CFU/g of feces (Newell and Fearnley, 2003). Although gut colonization is asymptomatic in mammals and birds, this bacterium causes diarrhea, fever, and abdominal pain in humans. Moreover, in rare but significant cases, *C. jejuni* triggers autoimmune disease such as Guillain–Barré syndrome (Nachamkin et al., 1998). Consumption and handling poultry meat products are the major sources of campylobacteriosis in developed countries (Cohn et al., 2007).

To analyze gene regulation in *C. jejuni* and its impact on cellular functions, several regulators have been identified and characterized. They are involved in oxidative stress (PerR; Palyada et al., 2009), in iron homeostasis (Fur; Holmes et al., 2005), in host cells interaction (Andersen et al., 2005; Fields and Thompson, 2008), in colonization (Guo et al., 2008; Palyada et al., 2009), and other have pleiotropic functions such as HspR (Andersen et al., 2005). Besides these regulators, RNA degradation mechanisms have shown to be determinant for the post-transcriptional control of gene expression and are also known to be involved in cellular functions. RNases mediate the processing, decay and quality control of RNA, and could be endo- or exoribonucleases (Arraiano et al., 2010). In bacteria, many times mRNAs are first degraded into smaller fragments by endonucleases including RNase E, RNase G, RNase III, and RNase P (Mackie, 1998; Linton et al., 2000; Deutscher, 2006) and subsequently they are degraded in the 3′–5′ direction by exonucleases such as RNase II, polynucleotide phosphorylase (PNPase), and RNase R, (Donovan and Kushner, 1986; Deutscher, 2006; Andrade et al., 2009). Complexes of RNases have been also described as RNA-degrading machines namely degradosomes, involved in RNA degradation control of bacteria including *Escherichia coli* (Carpousis, 2007; Arraiano et al., 2010). Such a protein complex has not been yet described in *C. jejuni* and little is known about post-transcriptional regulation mediated by ribonucleases. In *C. jejuni*, RNase E and RNase II responsible for the major RNA degradation in Gram-negative bacteria are absent, however a *pnp* gene has been identified in all sequenced genomes of the species. PNPase encoded by *pnp* gene is a 3′–5′ exoribonuclease

identified in a large number of both eukaryotic and prokaryotic organisms. It has both 3′–5′ exoribonuclease activity and 3′-terminal oligonucleotide polymerase activity (Mohanty and Kushner, 2000) and plays important roles in mRNA degradation, tRNA processing, and small RNA (sRNA) turnover. In most of bacterial sequenced genomes, orthologous genes to *pnp* have been identified (Leszczyniecka et al., 2004).

Polynucleotide phosphorylase does not seem to be essential at optimal temperature in *E. coli*, unless other exoribonucleases (RNase II or RNase R) are also missing. PNPase is however essential for *E. coli* growth at low temperatures (Luttinger et al., 1996; Zangrossi et al., 2000): It is required to grow after a cold shock in *E. coli* (García-Mena et al., 1999; Beran and Simons, 2001; Briani et al., 2007; Matus-Ortega et al., 2007) and it is involved in cold adaptation of *Bacillus subtilis* (Wang and Bechhofer, 1996), *Salmonella enterica* (Clements et al., 2002), and the psychrotrophic bacterium *Yersinia enterocolitica* (Goverde et al., 1998). *C. jejuni* can not grow below 30°C, but can survive several weeks at 4°C. In this context, we have previously shown that PNPase was also involved in the survival at refrigerated temperatures (under 10°C; Haddad et al., 2009). The role of PNPase in the other cellular functions in diverse bacterial species has been also investigated. It was reported to be implicated in antibiotic resistance and competence of *B. subtilis* (Luttinger et al., 1996; Bechhofer and Stasinopoulos, 1998; Bechhofer and Wang, 1998), and in resistance to oxidative stress in *E. coli* (Wu et al., 2009). This exoribonuclease enzyme also regulates virulence determinants such as host cell invasion, type III secretion systems, and infection in mouse in *S. enterica*, *Y. pestis*, and *Y. pseudotuberculosis* pathogens (Clements et al., 2002; Rosenzweig et al., 2005, 2007; Ygberg et al., 2006).

Considering the wide potential cellular functions of PNPase in the bacterial world and the absence of gene encoding the major exoribonuclease RNase II in *C. jejuni*, the aim of this study was to characterize the phenotypic effects of a *pnp* mutation in *C. jejuni* strain on the proteome, cell motility, *in vitro* cell adhesion/invasion and chick *in vivo* colonization.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Campylobacter jejuni 81-176 and F38011 strains and its derivative mutants defective in PNPase (*C. jejuni* 81Δ*pnp* and F38 Δ*pnp*) were described in our previous paper (Haddad et al., 2009). The *pnp* mutant was obtained by double crossover homologous recombination. *C. jejuni* was routinely cultured on Karmali agar plates (Oxoid, Basingstoke, Hampshire, England), or in brain and heart infusion (BHI) broth (Merck, Darmstadt, Germany) under 110 rpm shaking. *C. jejuni* was grown under microaerobic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) at 37° or 42°C. *C. jejuni* enumeration was performed on Karmali agar plates incubated 48 h at 42°C under microaerobic conditions. Kanamycin (Km) 50 μg/ml was used for *C. jejuni* 81Δ*pnp* strain growth. Strains and plasmids used in this study are listed in **Table 1**.

To determine the doubling time of the parental and the mutant strains, we first determined the growth rate in exponential growth phase. *C. jejuni* cultures were grown in BHI and incubated at different temperatures (32, 34, 37, and 42°C). Experiments were repeated three times independently.

COMPLEMENTATION

As described in Haddad et al. (2009), *trans*-complementation of the derivative strains was done using the entire *C. jejuni pnp* gene and its natural promoter cloned into pRY111 vector. The recombinant vector was introduced in the *C. jejuni* derivative strain (Δ*pnp*) by electroporation, and transformants resistant to both kanamycin at 50 μg/ml and chloramphenicol at 15 μg/ml were isolated.

MOTILITY ASSAY

The parental strains (*C. jejuni* 81-176 and F38011), the mutant strains (*C. jejuni* 81Δ*pnp* and F38Δ*pnp*) and the F38Δ*pnp* complemented strain were grown overnight on Karmali plates at 42°C under microaerobic conditions. They were harvested using BHI broth to obtain a cell suspension (OD_{600} = 0.1). One microliter of the cell suspension was deposited in the center of BHI soft agar plates (0.25 or 0.4% agar). After 48 h incubation in microaerobic conditions at 37 or 42°C, the ability of the strain to move in the soft agar was evaluated by measuring the diameter of the spread colonies. The assays were done four times independently.

Ht-29 CELL INFECTION

Bacterial adhesion and invasion into Ht-29 cells was studied using the gentamicin protection assay as described previously (Haddad et al., 2010). Briefly, microplate wells were seeded with 2×10^5 Ht-29 cells then incubated for 5 days at 37°C in a

Table 1 | Strains and plasmids used in this study.

Strain or plasmid	Genotype and plasmid property	Resistance	Source or reference
C. jejuni 81-176	Wild-type strain		Korlath et al. (1985)
C. jejuni 81Δ*pnp*	Isogenic *pnp* mutant of 81-176	Km	Provided by C. M. Burns
pBluescript	*E. coli* cloning vector	Amp	Stratagene
pUC4K	*E. coli* cloning vector	Amp, Km	
pGA28	pBluescript containing *C. jejuni* 81-176 *pnp* gene	Amp	Provided by C. M. Burns
pGA29	pBluescript containing *C. jejuni* 81-176 *pnp* gene disrupted with *aph*A3 gene	Amp, Km	Provided by C. M. Burns

Km, kanamycin; Amp, ampicillin.

humidified, 5% CO_2 incubator. After washing, the Ht-29 monolayers were infected with a suspension of approximately 2 to 5×10^7 CFU *Campylobacter*. To measure cell adhesion, the infected monolayers incubated for 1 h at 37°C in a humidified 5% CO_2 incubator were washed, then Ht-29 adherent *Campylobacter* were enumerated on Karmali agar. To evaluate bacterial invasion, the infected monolayers were incubated during 3 h and consecutively 2 h with gentamicin (250 μg/ml). After washes and Ht-29 cell lyses, intracellular *Campylobacter* was enumerated on Karmali agar. Each experiment was done in duplicate and performed at least three times independently. The significance of differences in adhesion and invasion efficiency was determined using Student's *t*-test. A *P*-value <0.05 was defined as significant.

CHICK COLONIZATION

The chick model was used to determine colonization potential of *C. jejuni*. Specific pathogen free (SPF) 2 days old chickens were obtained from the high-security livestock at the French Agency of Food, Environmental and Occupational Health Safety (ANSES). Chicks were housed and treated in accordance with the regulation of the local veterinary office (Direction des Services Vétérinaires des Côtes d'Armor, France). All the animals were reared in isolators with controlled airflow. Sixty SPF chickens were distributed into three groups of 20 animals and maintained in separate isolators with unlimited food and water. One group of 20 animals was kept as negative controls and placed in a separate isolator. At 2 days old, chicks were each dosed orally, by gavage, with 10^7 CFU of *C. jejuni* in 0.1 ml of 0.1 M PBS, pH 7.2. During 4 weeks, every 7 days, chicks were sacrificed by cervical dislocation. Ceca were aseptically collected then *C. jejuni* enumerated on Karmali agar plate. The non-parametric Mann–Whitney test was used to assess the statistical significance of differences in colonization levels ($P < 0.01$).

A second independent experiment was performed using the same protocol but with 5 days old chicks.

BACTERIAL RNA ISOLATION AND PURIFICATION

To study expression of *pnp* gene versus growth phase and temperature incubation, *C. jejuni* 81-176 was grown in BHI broth at 37 or 42°C until the mid-log phase (5 h) or the stationary phase (16 h). *C. jejuni* cells from 10 ml of mid-log phase and from 1 ml of stationary phase cultures were harvested (3,300 *g*, 6 min, 4°C) then resuspended in 1 ml of Extract All (Eurobio, Courtaboeuf, France) and 0.2 volumes of chloroform. After centrifugation at 12,000 *g* for 15 min at 4°C, the aqueous phase was removed. Total RNA was precipitated in isopropanol, rinsed in cold ethanol 75% then solubilized in 50 μl of sterile water. Isolation of total RNA was performed in triplicate from three independent cultures. DNA was degraded by treatment with DNase I Amplification Grade (Sigma-Aldrich, Saint Quentin Fallavier, France) then DNA removal was checked by PCR using MO001 primers (**Table 2**). Quality and quantity of RNA were checked using a NanoDrop 2000 (2000c Spectrophotometer, Thermo Scientific). The integrity of RNA samples was checked on a 2% agarose gel; electrophoresis was carried out in Tris–acetate–EDTA buffer for 45 min at 100 V. RNA concentrations were then standardized to 20 ng/μl for each sample prior to reverse transcription.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR

cDNA synthesis was performed using M-MLV (Moloney murine leukemia virus) Reverse Transcriptase (Promega, Charbonnieres, France) with random hexamer primers according to the manufacturer's instructions. Quantitative real-time PCR assay was performed using an Applied Biosystems 7300 Real-time PCR System and MO002 to MO007 primers (**Table 2**). The composition of the PCR mix was as follows: 5 μl of sample, reverse primer (1 μM), forward primer (1 μM), and qsp 25 μl of SYBR Green I Master Mix (Applied Biosystems, Foster City, CA, USA). Amplification program included an initial denaturing step at 95°C (10 min), followed by 40 cycles of 95°C (15 s) and 60°C (1 min). A negative control (without cDNA) was included to each run. A melting curve was obtained from a first step starting from 60 to 95°C, to control specificities of quantitative PCR reaction for each primer pair. Efficiency of amplifications was determined by running a standard curve with serial dilutions of cDNA. The efficiency (E) was calculated with the formula $E = [10\,(1/-s)] \times 100$, where "$s$" is the slope of the standard curve. The C_T (critical threshold) stood for the first PCR cycle during which the fluorescence value was significantly distinct from the background and corresponded to a signal. Results were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of target RNA was adjusted to a reference [internal target RNA,

Table 2 | Primers used in this study.

Primer	Sequences		Gene amplified	Amplicon size (bp)
	Forward (5′–3′)	Reverse (5′–3′)		
MO001	GGATTTCGTATTAACACAAATCGTGC	CTGTAGTAATCTTAAAACATTTTG	*fla*B	1703
MO002	CCAGGTTTTTCAGTAGGCGA	CCACGCTTGGATAAAGTGCT	*pnp*	100
MO003	CGAGCTTGCTTTGATGATGAGTG	AGTTCCCACAGGAAAACCTA	*rpo*A	109
MO004	TAATTGGAACACCTGATGAG	TCCGCATTGGTAGATATTAAG	*lux*S	116
MO005	CCAAATAGTGGAAGTGCAAGA	GTTCCTATGTCAGGATTGCTTT	*peb*3	98
MO006	CAAACAGCTATGATAATAGCC	GGAGCATATCTTTGTGCTACG	*kat*A	87
MO007	CCATAGCAAAAAGCGGTACTAA	CTAGCCACCATAAAAGCAGAA	*hsp*90	118

*rpo*A (51)]. Relative expression level was obtained by the following formula: $2\Delta\Delta C_T$ (37). RT-qPCR was performed in triplicate with, at least, three templates of RNA extracted from independent cultures.

PROTEIN EXTRACTION

To avoid possible misinterpretation of results, proteins were extracted from *C. jejuni* 81-176 or 81Δ*pnp* cultures at the late exponential phase showing similar optical density (OD) at 600 nm and cell counts on agar. Thus, the two strains were assumed to be in the same physiological state. Prior to each experiment, one colony grown on Karmali agar for 48 h at 42°C was resuspended in 50 ml of BHI broth then incubated at 42°C for 24 h in microaerobic conditions with shaking at 110 rpm. Two milliliters of the resulting culture were used to inoculate 200 ml of BHI broth incubated for 7 h in the same conditions (until OD_{600} reached 0.2). Cells were harvested by centrifugation at 7,000 *g* for 20 min, washed consecutively with 200 mM glycine solution (Sigma-Aldrich) and 100 mM Tris–HCl pH 7.0 solution (Sigma-Aldrich) then resuspended in 10 ml of a 10 mM Tris–HCl pH 7.0 buffer. Cells were disrupted by series of 6 × 30 s sonications with 6 min intervals on ice (Vibracell 72434, Bioblock Scientific, Illkirch, France). To eliminate cell debris, samples were centrifuged twice at 10,000 *g* for 20 min at 4°C. Then, cytoplasmic proteins were separated from membrane fractions by ultracentrifugation at 188,000 *g* for 1 h at 4°C. The cytoplasmic protein fraction in the supernatant was treated with protease inhibitor cocktail tablets Complete™ (Roche Diagnostics, Mannheim, Germany) and nuclease solution containing RNase and DNase (Sigma-Aldrich). Protein samples were dialyzed using cellulose membrane tubing with a cut-off at 12,000 Da (Sigma-Aldrich) against Milli-Q water at 4°C in shaking conditions during 3 days, refreshing the dialyze bath each day. Total protein concentration was determined using the Micro BCA™ Protein Assay Kit (Perbio-Science, Brebieres, France).

TWO-DIMENSIONAL GEL ELECTROPHORESIS OF BACTERIAL PROTEINS

A quantity of 100 μg of protein was concentrated using Concentrator 5301 (Eppendorf, Le Pecq, France), at room temperature. Samples were diluted with 275 μl of a solution of 6 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, bromophenol blue (BB; Sigma-Aldrich) and 2% Biolyte 3/10 (Bio-Rad, Marnes-la-Coquette, France). Proteins in the rehydratation solution were absorbed overnight by 17 cm pH 4–7 or 6–11 IPG strips (Bio-Rad, GE Healthcare, Orsay, France). Then, the iso-electro-focalization (IEF) was performed using the Bio-Rad IEF program as follows: from 50 to 250 V for 3 h, from 250 to 6,000 V for 3 h, and at 6,000 V until reaching 54,000 Vh. Finally, each strip was soaked for 20 min in equilibration buffer [1.5 ml of a 6-M urea, 2% SDS, 50 mM Tris–HCl (pH 8.8), 30% glycerol and BB] supplemented with 2% DTT and subsequently for 20 min in equilibration buffer with 4% iodoacetamide (Bio-Rad). The second dimension was performed in 12% acrylamide gels covered with 1% low-melting point agarose (Bio-Rad) and run at 40 mA/gel at 14°C using a Protean II xi cell (Bio-Rad) until the migration reached the base of the gels. Proteins in gels were finally silver stained and scanned with a GS-800 densitometer (Bio-Rad) operated with the Quantity One® software (Bio-Rad) at the resolution of 42.3 μm.

IMAGE, STATISTICAL ANALYSIS, AND PROTEIN IDENTIFICATIONS

The image analysis was performed using the Progenesis Samespots® software (NonLinear Dynamics, Newcastle upon Tyne, UK). The statistical analysis of the results was performed with two technical replicates of three independent experiments resulting in six gels for each strain. Differences between the parental and mutant strain proteome (2 × 6 gels) were validated by principal component analysis (PCA) and differences among matched spot intensities were statistically validated by ANOVA (at a 5% significance level) and by using a Power superior to 0.8. The PCA analysis ensured the reproducibility of the experiments and has shown a significant effect of the *pnp* disruption on *C. jejuni* proteome. Over- and down-expressed proteins were taken into account only if the mean difference in spot intensities passed the threshold of 1.8. When spots of interest were located, 2D-electrophoresis gels were performed again using 700 μg of proteins and were stained with BioSafe colloidal Coomassie blue (Bio-Rad). Spots of interest were excised from preparative gels and protein identification was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using PAPPSO platform facilities[1]. Gel plugs were first washed twice with 50% (v/v) acetonitrile, 25 mM ammonium carbonate in water, and then dried in a vacuum speed concentrator. Aliquot (10 μl) of a trypsin solution (Promega, 12.5 ng/μl in 50 mM NH_4HCO_3) was added to each sample and digestion was performed for 6 h at 37°C. A 1-μl aliquot of each supernatant was spotted directly onto the MALDI plate then dried at room temperature before adding 1 μl of the matrix solution [α-cyano-4-hydroxycinnamic acid, 3 mg/ml in 50% (v/v) acetonitrile 0.1% (v/v) trifluoroacetic acid]. Mass spectra were acquired on a Voyager-DE-STR (Applied Biosystems, Framingham, MA, USA), equipped with a nitrogen laser (Laser Science, Franklin, MA, USA). Tryptic autodigestion ion peaks, $(M + H)^+ = 2211.104$ and 842.509 Da, were used for internal calibration of spectra. The peptide mass lists were used for protein identification in a *C. jejuni*, strain 81-176, amino acid sequence database (UniProtKB, 09.09.2010), using the Protein Prospector v 3.2.1 software set with the following parameters: mass tolerance <20 ppm, four peptides required to match, one missed cleavage, oxidized methionine and carbamidomethylated cysteine respectively as possible and fixed modifications.

POLY(A) DEGRADING ACTIVITY IN *C. jejuni* CELL LYSATES

Campylobacter jejuni was grown in Mueller-Hinton medium to late log phase, lysates prepared using EasyLyse (Epicenter), and assayed for ribonuclease activity as described (Deutscher and Reuven, 1991), except that the reactions were done in a buffer optimized for measurement of RNase R (Cheng et al., 1998). Degradation of [^{3}H]poly(A) (Amersham Product TRK.480) was quantified by scintillation counting [^{3}H]nucleotide released into acid soluble form. Dependence of the reaction on lysates of *C. jejuni* parental and *pnp* mutant strains was measured in the presence and absence of 10 mM KPO_4 to distinguish hydrolytic from phosphorolytic activities. Less than 10% of substrate was consumed in the reactions.

[1]http://pappso.inra.fr

IN SILICO ANALYSIS

DNA and protein homology searches were performed by BLAST analysis[2]. Oligonucleotide primers were designed using Primer 3 program[3].

RESULTS

BACTERIAL GROWTH CURVES AND *pnp* GENE EXPRESSION IN *C. jejuni* 81-176

To determine the expression profile of *pnp* gene, quantitative RT-PCR experiments were assayed in four different growth conditions. *C. jejuni* 81-176 was grown in BHI broth at 37 or 42°C until the mid-log phase (5 h) or the stationary phase (16 h). C_T values were determined in four replicates from cDNA synthesized from RNA extracted from three independent cultures for each growth condition. The specificity of each primer pair (MO002 to MO007) was controlled by analyzing melting curves. PCR efficiencies of the primer pair used were homogenous and all over 90%. The *rpo*A gene was used as an internal control (Ritz et al., 2009) and the expressions at 37°C in mid-log phase were used as calibrators to calculate relative expression levels. No significant difference ($P < 0.05$) in expression of *pnp* gene was observed for the four growth conditions tested (**Figure 1**).

There is a difference between the growth of the parental and the mutant strain, however this difference is only significant below 35°C as shown in **Figure 2**.

To check that phenotypic changes in the *pnp* mutant strain were specifically due to PNPase absence, we attempted genetic complementation of *pnp* in *C. jejuni* 81-176. Despite several attempts, we never obtained such a complemented strain in *C. jejuni* 81-176, but were able to complement the *pnp* mutation in *C. jejuni* strain F38011 (Haddad et al., 2009).

EFFECT OF *pnp* MUTATION ON *C. jejuni* 81-176 MOTILITY

Motility is a common bacterial virulence factor involved in colonization and invasion of host cells by pathogens. Swimming abilities of *C. jejuni* 81-176 parental and *C. jejuni* 81Δ*pnp* mutant strains are presented **Figure 3** on soft agar plate 0.4%. Results indicated that inactivation of *pnp* gene caused a 5.3-fold reduction in motility on agar plates compared to the parental strain, at both 37 and 42°C. Because motility is phase variable in *C. jejuni*, measurements were achieved using independent cultures on different days. The results of four experiments were essentially identical, suggesting that reduced motility of the mutant strain is unlikely due to phase variation. To test if the motility defect observed is specifically linked to the inactivation of the *pnp* gene, we also tested the effect of genetic complementation of the *pnp* mutant with the wild-type *pnp* gene. As we were unable to complement the *pnp* mutation in *C. jejuni* 81-176, we used the complemented mutant of strain F38011 (Haddad et al., 2009). As shown in **Figure 3C**, complementation of the *pnp* mutation in trans restored partially wild-type motility, confirming that absence of PNPase results in reduced motility and associated virulence phenotypes.

Moreover, impaired mutant strain motility was also observed on soft agar plate 0.25% (data not shown).

[2]http://www.ncbi.nlm.nih.gov

[3]http://frodo.wi.mit.edu/primer3/

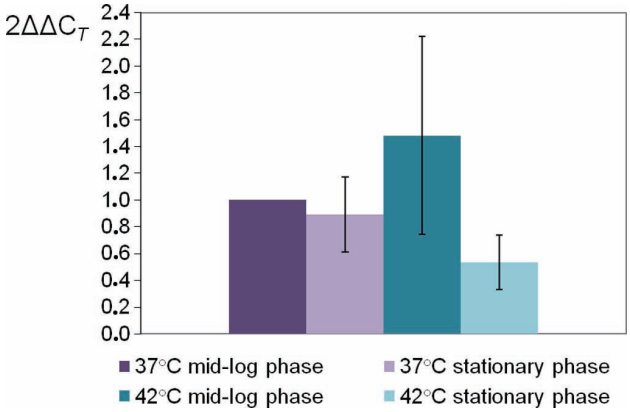

FIGURE 1 | Relative expression levels of *C. jejuni* 81-176 *pnp* gene at different conditions. Gene expression has been estimated using RT-qPCR and the comparative critical threshold ($\Delta\Delta C_T$) method. The *rpo*A gene was used as the internal control, and the expression at 37°C in mid-log phase as the calibrator. Error bars represent the standard deviations from the mean of three independent experiments.

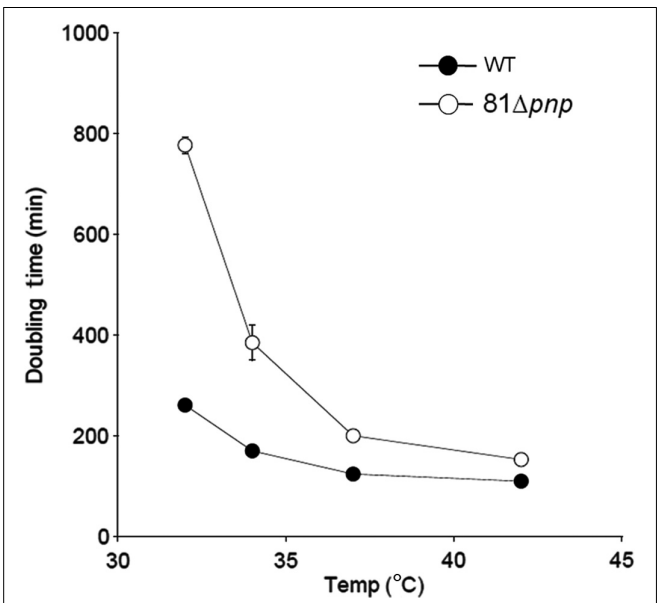

FIGURE 2 | Mean generation time of the parental strain (solid circles) versus the mutant strain (open circles). *C. jejuni* was cultivated in BHI broth and incubated in microaerobic condition at 32, 34, 37, and 42°C. Experiments were performed three times independently.

EFFECT OF *pnp* MUTATION ON ADHESION TO AND INVASION IN Ht-29 CELLS

In *C. jejuni*, motility is critical both for invasion of gastrointestinal epithelial cells and for colonization of the mucus lining of the gastrointestinal tract (Yao et al., 1994). To examine the role of the *pnp* gene in *C. jejuni* infection, the *in vitro* adhesion and invasion abilities of the *pnp* mutant and parental *C. jejuni* 81-176 strains were compared. The *C. jejuni* 81Δ*pnp* mutant exhibited a significant ($P \leq 0.02$) decrease in adhesion ability compared to the parental strain (**Figure 4A**), and a 3 log-fold decrease in invasion

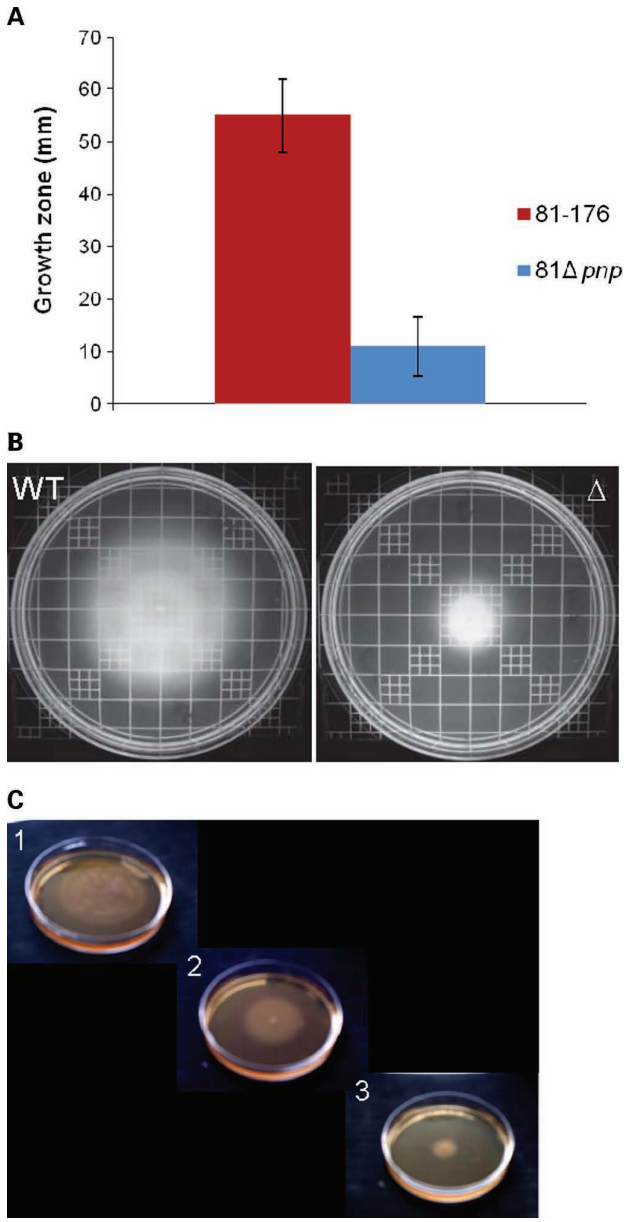

FIGURE 3 | Motility phenotypes of *C. jejuni* strains. Swimming ability was assessed on BHI agar containing 0.4% agar. Strains were inoculated into BHI motility agar and incubated for 48 h at 37°C under microaerobic conditions. **(A)** Mean diameters of four independent experiments were represented. **(B)** Motility agar obtained after an incubation at 37°C during 48 h. **(C)** Motility assay of the parental *C. jejuni* F38011 strain (1), the F38Δ*pnp* complemented strain (2), and the mutant strain F38Δ*pnp* (3), on BHI plates containing 0.4% agar.

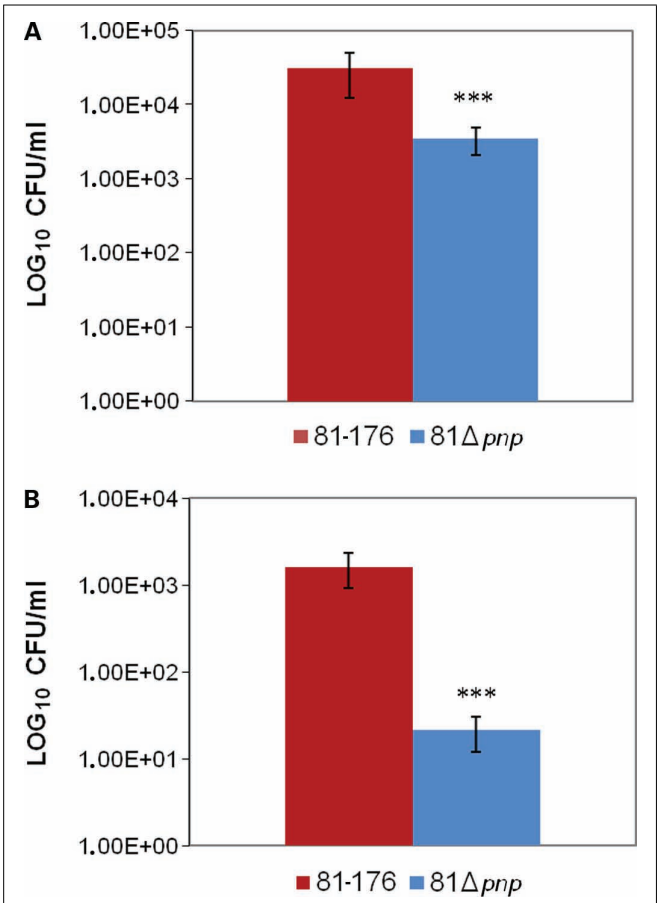

FIGURE 4 | Adhesion and invasion of *C. jejuni* in Ht-29 cells. (A) Effect of mutation of the *pnp* locus on *C. jejuni* adhesion to Ht-29 human intestinal epithelial cells. **(B)** Ht-29 cell invasion assay. Cells were infected with the parental and mutant strains for 3 h, and invasion values were calculated from the number of bacteria that survived 2 h of incubation in the presence of gentamicin. The experiment was performed in duplicate from at least three independent cultures. The values plotted represent means ± standard deviations (error bars). Statistical significance was assessed with an unpaired Student's *t*-test. ***$P < 0.02$.

of Ht-29 cells ($P < 0.02$; **Figure 4B**). No difference in susceptibility to gentamicin for both strains was observed, indicating that the invasion phenotypes of the strains were not due to differences in gentamicin susceptibilities (data not shown). Results of invasion assays are consistent with the lowered adherence of the mutant strain and indicate that inactivation of the *pnp* locus indirectly affects the interaction of *C. jejuni* with epithelial cells, most likely via the reduced motility resulting from the absence of PNPase.

EFFECT OF PNPase DEFICIENCY ON COLONIZATION OF CHICK GUT BY *C. jejuni*

According to the motility and adhesion results, colonization experiments were also conducted. To determine the relative importance of PNPase in chicken colonization, we inoculated 2 days old chicks with the parental and PNPase mutant strains. Three groups of five birds were infected with 10^7 CFU of parental or mutant strains. On day 7, viable bacteria were found in all five chickens fed with the parental strain, whereas viable mutant bacteria were recovered from only two. In addition, the mean colonization concentration of the chicken inoculated with the parental strain was 1.5×10^7 CFU/g on day 7 and was significantly lower (2.4×10^5 CFU/g; $P < 0.01$) in the chickens inoculated with the mutant strain. On days 14, 21, and 28,

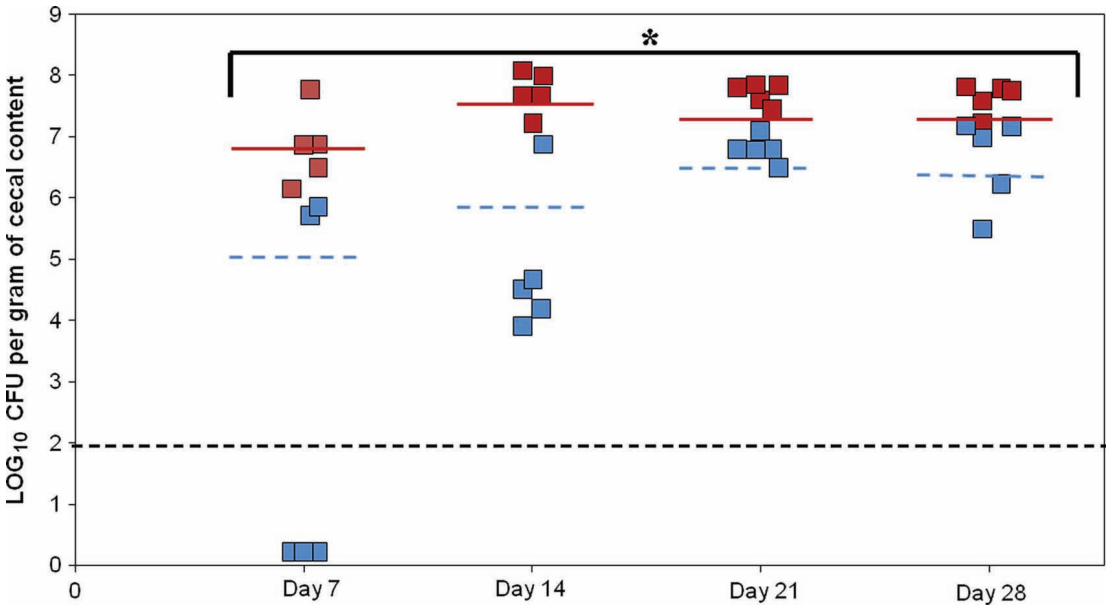

FIGURE 5 | Effect of the PNPase mutation on chick gut colonization by *C. jejuni*. The experiment included three groups of birds. Two days old chicks were orally gavaged with 1.4 to 1.5 $\times$ 10^7 CFU of *C. jejuni* 81-176 (red squares) or 81-176 derivative strain lacking production of PNPase (blue squares). Colonization levels were measured by enumeration of bacteria present in ceca on days 7, 14, 21, and 28 post-inoculation (LOG_{10} CFU/g of ceca). Each dot represents the load of *C. jejuni* in the cecum of an individual chick. The geometric mean of the bacterial loads from each set of chicks is denoted by the horizontal bar. The line designates the detection limit of 2 $\times$ 10^2 CFU/g of ceca. Statistical analysis was performed using the Mann–Whitney test (mean and *P*-value <0.01). *Significant difference between the parental and *pnp* mutant strains.

viable mutant bacteria were detected in all the chickens. However, throughout the experiment, the mean colonization level of the chicken inoculated with the mutant strain was significantly reduced compared to those inoculated with the parental *C. jejuni* (**Figure 5**; $P < 0.01$). The defect in colonization of the *pnp* mutant strain was confirmed by a second experiment using SPF 5 days old chicken in which, the mutant strain was unable to colonize chicken gut, while the parental strain reached a colonization level of 9.3 $\times$ 10^7 CFU/g at 29 days (data not shown).

COMPARATIVE PROTEOMIC ANALYSIS

In order to pinpoint the effect of *pnp* mutation at a protein level, proteomic analyses were performed. Proteomes of the *pnp* mutant and its parental strains were compared using Progenesis SameSpots® software. Examples of digital images from 2-DE gels achieved in the 4–7 and 6–11 p*I* ranges are given in **Figures 6A,B**. This comparative analysis resulted in nine preponderant proteins showing a significant difference in expression, amongst which three were over-expressed and six were repressed in the *pnp* mutant strain compared to the parental strain (**Table 3**). As expected, spot occurrences corresponding to PNPase were absent in the mutant strain (**Table 3**). Among the down-expressed proteins, the heat-shock protein Hsp90, catalase KatA, the elongation factor EF-Tu, the *N*-acetylneuraminic acid (NANA) synthetase and, an antigenic peptide, PEB3 were identified. Among the over-expressed proteins, the elongation factor EF-G, the chaperone DnaK and S-ribosylhomocysteinase, LuxS were found (**Table 3**).

EFFECT OF *pnp* MUTATION ON *lux*S, *peb*3, *kat*A, AND *hsp*90 GENES EXPRESSION

To analyze if our proteomic data are correlated to gene expression, a quantitative RT-PCR analysis was performed on four genes known to be involved in adhesion, invasion, or colonization ability of *C. jejuni*. Therefore, gene expression of *lux*S, *peb*3, *kat*A, and *hsp*90 was measured (**Figure 7**). Levels of *peb*3 and *kat*A mRNA was significantly decreased in the *pnp* mutant strain as compared to the parental strain, showing the same trend of protein expression, while gene expression of *lux*S and *hsp*90 remained unaffected by *pnp* mutant, but not protein expression.

RNA DECAY IN *C. jejuni*

An established method to assess the relative contributions of phosphorolytic and hydrolytic degradation in bacterial lysates was employed to characterize behavior of *C. jejuni*. Reactions were done to confirm measurements were made in the linear range of the assay. **Figure 8** shows that poly(A) can be degraded in the parental *C. jejuni* cell lysates by a hydrolytic mechanism, revealing the existence of hydrolytic ribonucleases. However, phosphorolytic RNA degradation predominates as phosphate addition greatly increased activity. Moreover, poly(A) degradation by lysates of the *pnp* mutant strain was almost totally non-responsive to phosphate addition (**Figure 8**), indicating that the majority of phosphorolytic activity, and therefore the majority of poly(A) degrading activity in the parental cells, depends on the *pnp* gene. Comparison of hydrolytic poly(A) degrading activity in parental and mutant strains lysates revealed that *pnp* deletion increases this activity by almost fivefold.

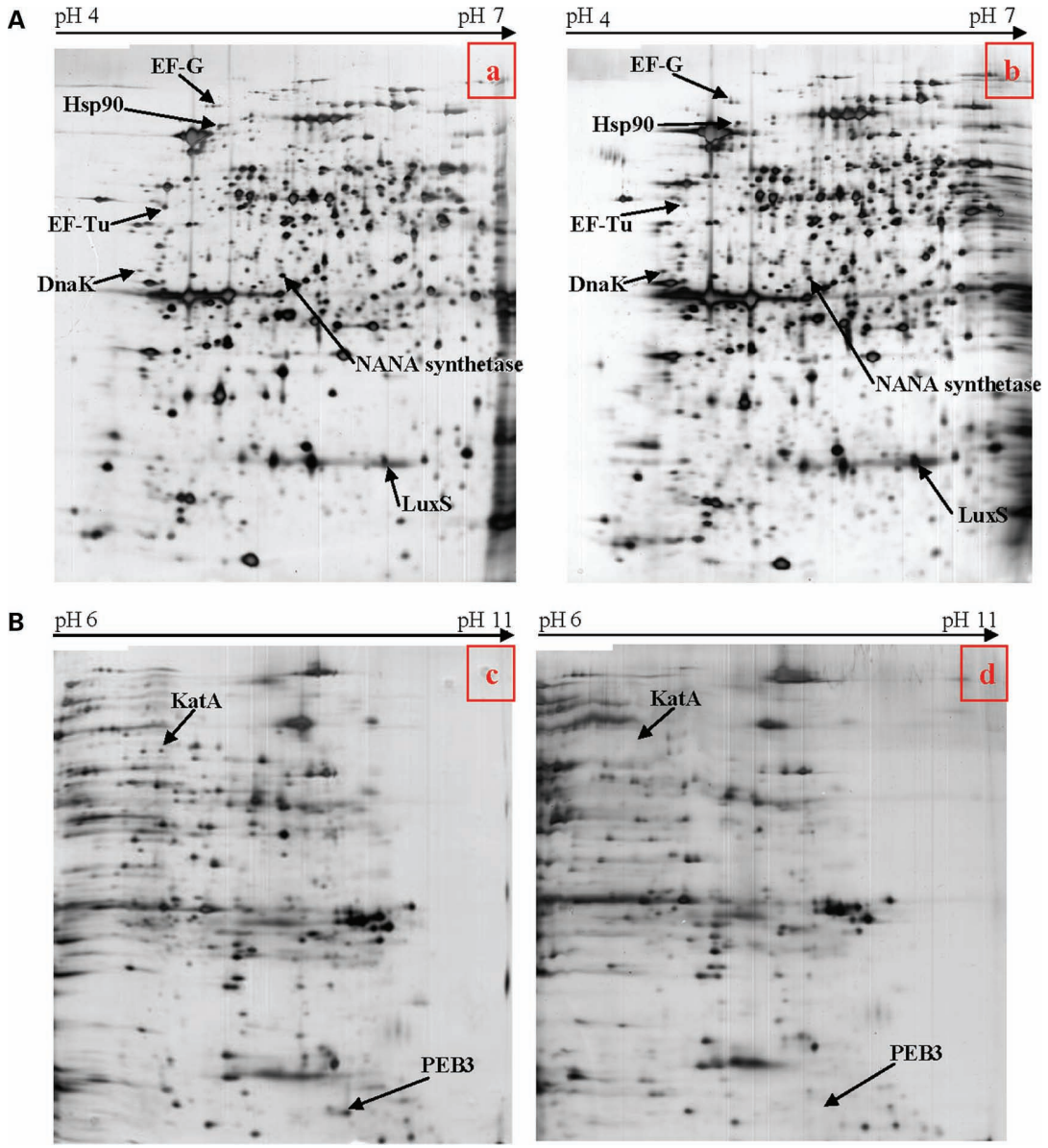

FIGURE 6 | (A) Two-dimensional electrophoresis gels of acidic proteins extracted from *C. jejuni* strain 81-176 (a) and the *pnp* mutant (b) derived from this strain. Proteins were extracted from bacteria cultivated in BHI at 42°C collected when OD_{600} reached 0.2. **(B)** Two-dimensional protein gel analysis of basic proteins extracted from *C. jejuni* strain 81-176 (c) and the *pnp* mutant (d) derived from this strain. Proteins were extracted from bacteria cultivated in BHI at 42°C collected when OD_{600} reached 0.2.

DISCUSSION

Ribonucleases are enzymes involved in post-transcriptional regulation of genes by mediating RNA degradation. In some Gram-negative bacteria, the endoribonuclease RNase E is essential for cellular growth and inactivation of *rne* gene (encoding RNase E) impedes processing and prolongs the lifetime of bulk mRNA (Cam et al., 1996; Ow and Kushner, 2002). For the exoribonuclease activity, RNase II is responsible for the majority of RNA phosphodiester bond cleavage in *E. coli* as most RNA (90%) is degraded via a hydrolytic mechanism (Deutscher and Reuven, 1991). Genes encoding RNase E and RNase II enzymes are absent in the *C. jejuni* genome, suggesting a major role is played by other ribonucleases like PNPase. Because of the pleiotropic functions of PNPase described in various bacteria (Luttinger et al., 1996; Wang and Bechhofer, 1996; Bechhofer and Stasinopoulos, 1998; Goverde et al., 1998; Yamanaka et al., 1999; Clements et al., 2002; Rosenzweig et al., 2005, 2007; Ygberg et al., 2006; Wu et al., 2009), the present study broadly analyzed the role of PNPase in *C. jejuni*, beyond its role in long-term survival to cold (Haddad et al., 2009).

In the current study, we have examined the role of the putative post-transcriptional regulator PNPase and its consequences on *C. jejuni* proteome, which may then affect some biological

Table 3 | *Campylobacter jejuni* over- and down-expressed proteins in the *pnp* mutant as compared to the parental strain.

Protein identification	*C. jejuni* no.	Accession number	MW (kDa)/p*I*	MOWSE score	No. peptides (% masses matched)	Fold in *pnp* mutant
Heat-shock protein 90 (Hsp90)	CJJ81176_0546	YP_001000221	69.6/5.1	144	6/43 (13%)	−2.4
N-acetylneuraminic acid synthetase	CJJ81176_1334	YP_001000992.1	38.7/5.4	27.6	4/23 (17%)	−1.8
Polynucleotide phosphorylase (PNPase)	CJJ81176_1269	YP_001000929.1	79.1/5.3	893	9/64 (14%)	−3.5
Truncated PNPase	CJJ81176_1269	YP_001000929.1	79.1/5.3	153	6/56 (10%)	−2.2
Truncated PNPase	CJJ81176_1269	YP_001000929.1	79.1/5.3	47.9	4/47 (8%)	−2.2
Elongation factor EF-Tu	CJJ81176_0499	YP_001000177.1	46.6/5.1	4.8E + 07	15/65 (23%)	−2
Major antigenic peptide PEB 3	CJJ81176_0315	YP_001000003.1	27.5/9.4	33	6/29 (20%)	−3.1
Catalase (KatA)	CJJ81176_1387	YP_001001043.1	54.3/7.3	108	4/27 (14%)	−2.5
S-ribosylhomocysteinase (LuxS)	CJJ81176_1213	YP_001000873.1	18.3/5.9	557	6/30 (20%)	2.4
Elongation factor EF-G	CJJ81176_0513	YP_001000189.1	76.7/5.1	307	5/28 (17%)	2.3
Molecular chaperone DnaK	CJJ81176_0775	YP_001000443.1	67.4/5	2.3E + 04	7/64 (10%)	1.8

MW, molecular weight; pI, isoelectric point; No. peptides, number of peptides identified.

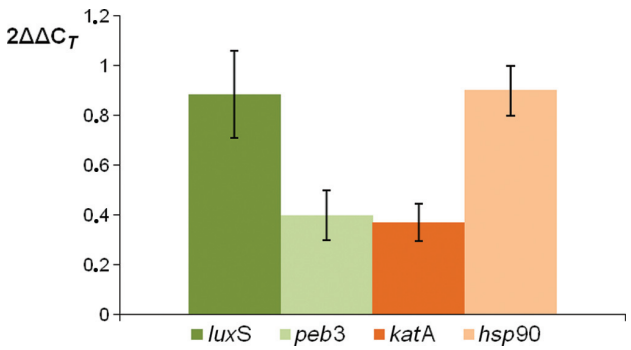

FIGURE 7 | Comparison of relative transcript levels of *lux*S, *peb*3, *kat*A, and *hsp*90 genes between *C. jejuni pnp* mutant and the parental strain, assessed by quantitative RT-PCR. Each bar represents relative expression level of a gene in the mutant strain according to the $2\Delta\Delta C_T$ method described by Livak and Schmittgen (2001). The internal reference gene used was *rpo*A and the calibrator condition was the expression in the wild-type strain (equivalent to 1). Each value is the mean of at least three independent RNA extractions and error bars indicate the standard deviations.

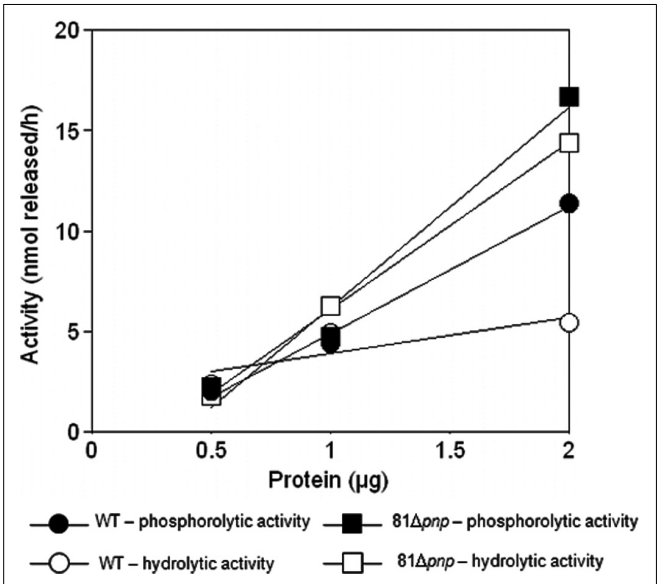

FIGURE 8 | Poly(A) degrading activity in *C. jejuni* cell lysates. Degradation of [^{3}H]poly(A) was quantified by scintillation counting [^{3}H]nucleotide released into acid soluble form. Dependence of the reaction on lysates of the parental strain (circle) and the *pnp* mutant (square) was measured in the presence (closed) and absence (open) of 10 mM KPO_4 to distinguish hydrolytic from phosphorolytic activities.

functions of the pathogen. Growth curve data show that, inactivation of the *pnp* gene has no significant effect on *C. jejuni* 81-176 growth rate at optimal temperature. Generation time increases with the decrease of the temperature of incubation, a finding that confirms the hypothesis that PNPase is involved in *C. jejuni* survival at temperatures below the optimal temperature of growth.

Transcript analyses revealed that expression of *C. jejuni* 81-176 *pnp* gene is not influenced by growth phase or by optimal temperatures of growth. In order to define the role of PNPase in the cellular biology of *C. jejuni*, proteomic profiles of parental and PNPase mutant strains were compared. The *pnp* gene mutation affects synthesis of proteins in *C. jejuni* 81-176; however, only few proteins vary significantly between the parental and the *pnp*-deleted strains Although comparison of proteome of these two strains verified the non-essential role of *pnp* gene in *C. jejuni* 81-176 strain, the majority of phosphorolytic ribonuclease activity seems to depend on PNPase. Moreover, deletion of *pnp* gene increases hydrolytic ribonuclease activity. These results suggest that PNPase either down regulates, or antagonizes function, of a hydrolytic ribonuclease in *C. jejuni*. The enzyme responsible for hydrolytic poly(A) degrading activity is unknown, but comparative sequence analysis predicts a single hydrolytic mRNA degrading exoribonuclease encoded by the *C. jejuni* genome, RNase R.

The swarming ability of the *C. jejuni pnp* mutant strain is reduced. As motility is directly linked to *C. jejuni* pathogenic features, we confirmed that the motility defect observed with the *pnp* mutant strain is due to the *pnp* mutation using the F38011 complemented strain. The motility of *C. jejuni* is conferred by polar flagella, and approximately 40 genes are predicted to be involved in flagella biosynthesis or function (Yao et al., 1994; Parkhill et al., 2000). Flagella-mediated motility is responsible for colonization of the mucous lining of the mammalian and avian gastrointestinal tracts as well as invasion of intestinal epithelial cells (Wassenaar et al., 1991; Grant et al., 1993; Rosenzweig et al., 2007). In proteomic analysis, the abundance of protein related to flagella was not affected by the mutation however a decrease in NANA synthetase production was observed in the *pnp* mutant strain, a factor supposed to be involved in *C. jejuni* motility. NANA synthetase, encoded by the *neu*B gene, catalyzes formation of NANA, the main component of *C. jejuni* lipo-oligosaccharide (LOS). Linton et al. (2000) hypothesized that *neu*B3 gene is involved in the post translational modification of the flagellin subunit and in motility, as inactivation of *neu*B3 leads to non-motile and aflagellate cells (Linton et al., 2000). This information is particularly interesting in that for the *C. jejuni* 81-176 genome, *neu*B is located in a gene locus encoding proteins involved in the formation of flagella or mobility. Because motility is a prerequisite to eukaryotic cells invasion, we expected that the *pnp* mutant would be less invasive than its parental strain. Studies using Ht-29 cells confirmed that mutation of *pnp* decreased the invasiveness of *C. jejuni* and also a decrease in adhesion ability. The putative adhesion PEB3 was 3.1-fold decreased in the *pnp* mutant and quantitative RT-PCR also revealed a diminution in *peb*3 mRNA synthesis. PEB3 protein shares 51.8% sequence identity to Paa, an *E. coli* adhesion (Batisson et al., 2003) and 49% identity to AcfC, an accessory colonization factor from *Vibrio cholerae* (Peterson and Mekalanos, 1988). PEB3 of *C. jejuni* is considered as an adhesion based on the work performed with Hep-2 cells by Wren et al. (Drs. B. W. Wren and R. Langdon, personal communication; Rangarajan et al., 2007).

Interestingly, PNPase was described as a factor affecting *S. enterica* invasion and establishment of chronic infection in a mouse model (Clements et al., 2002). In contrast with results obtained in *C. jejuni*, *S. enterica* pathogenicity islands are over-expressed in PNPase mutants, resulting in increased invasion into epithelial MDCK cells (Clements et al., 2002). However, the cell line used in Clements et al. (2002) study has been isolated from canine kidney and is largely different from those utilized in the current study. Moreover, studies on *S. enterica* PNPase use the typhoid strain of the bacteria while the current study showed interaction of *C. jejuni* with intestinal cells. Other ribonucleases have been shown to be involved in bacterial virulence. RNase R of *Shigella flexneri* has been reported to be required for the expression of the invasion factors IpaB, IpaC, and IpaD (Cheng et al., 1998).

As chickens are the principal origin of campylobacteriosis in humans, the potential colonization of the parental and mutant strains were also investigated in the avian intestine. Initial colonization was markedly different between the variants, indicating that PNPase is required to establish colonization. Hanel et al. (2004) have used a chick model to study the colonization ability of different strains of *C. jejuni* (Hanel et al., 2004). Three groups of strains have been determined. The first group corresponds to strains which could not be re-isolated after chicken inoculation; the second one contains weak or delayed colonizers; the last group includes strong colonizers that could be re-isolated from all animals 7 days after inoculation. In the current study, the wild-type strain *C. jejuni* 81-176 could be considered as a "strong colonizer" because this strain was present in all animals up to 7 days after inoculation. However, *pnp* disruption results in a delayed colonization of the *C. jejuni* mutant strain, which can be linked to the group 2 of Hanel et al. (2004). This defect in colonization could be due to the defect in motility and adhesion abilities of the mutant strain. Moreover, the observed lower expression of *kat*A gene and KatA protein in the *pnp* mutant strain could be linked to the deficiency in colonization. *C. jejuni* expresses a single heme-containing catalase, KatA, which is part of a defense system against oxidative stress (Haas and Goebel, 1992). In addition, KatA contributes to intramacrophage survival of *C. jejuni* and chick gut colonization (Day et al., 2000; Palyada et al., 2009). A Δ*kat*A mutant is unable to colonize the chick ceca after 4 days of incubation (Palyada et al., 2009).

Proteomic experiments showed that LuxS, another protein involved in colonization, is 2.4-fold overproduced in the PNPase mutant strain, but no variation was observed in *lux*S mRNA level. Being the last enzyme in the AutoInducer-2 (AI-2) biosynthesis pathway, LuxS controls production of AI-2. While Holmes et al. (2009) have shown that AI-2 does not function as a quorum sensing molecule in *C. jejuni* during exponential growth *in vitro* (Holmes et al., 2009), LuxS seems to be involved in *C. jejuni* motility by modifying transcription rate of flagellin *fla*A mRNA without change the level of total flagellin protein in the cells (Jeon et al., 2003). Quiñones et al. (2009) have also demonstrated the implication of LuxS in chick gastrointestinal tract colonization and adherence to avian cells LMH, which could be correlated with phenotypic results obtained in this work (Jeon et al., 2005; Quiñones et al., 2009).

Proteomic results indicated that synthesis of two heat-shock proteins were modified by inactivation of the *pnp* gene suggesting a probable stress-like response in the mutant strain. Hsp90 was down-expressed, whereas DnaK chaperone was up-regulated in the *pnp* mutant. In the *C. jejuni* mutant strain, Hsp90 was 2.4-fold less abundant than in parental strain; however, transcript analysis did not reveal such variation. Transcript *hsp*90 could be more stable in the absence of PNPase enzyme and accumulation of this mRNA would lead to a greater production of the protein. In *E. coli*, Hsp90 participates in *de novo* protein folding during a heat-shock condition by facilitating the ability of the DnaK–DnaJ–GrpE complex to interact with newly synthesized polypeptides (Thomas and Baneyx, 2000); however, Homuth et al. (2000) suggested that the probable role of this protein is in response to acid or oxidative stress, because Hsp90 is not over-expressed in *Helicobacter pylori* following a heat-shock (Homuth et al., 2000). Moreover, Hsp90 has been implicated in *Salmonella* pathogenesis by contributing to bacterial survival within host cells (Osman et al., 2009).

In the case of DnaK, this protein is known to be involved in DNA replication, protein folding, and in stress-response. DnaK

was up-regulated in the *C. jejuni pnp* mutant compared to the parental strain, suggesting accumulation of denatured proteins in the mutant cells. Interestingly in *E. coli*, DnaK is preferentially associated to PNPase in the degradosome, a multiprotein complex involved in RNA degradation (Regonesi et al., 2006); however, no degradosome has been yet described in *C. jejuni* particularly because the major component of this complex, RNase E, is not detected in the genome of *C. jejuni*. Production of two elongation factors (EF-Tu and EF-G) was also modified in the mutant strain, suggesting a role of PNPase in the translation mechanism or a rearrangement of protein production in absence of this exoribonuclease.

In conclusion, deficiency in PNPase production in *C. jejuni* results in a defect in its ability to swim, to adhere and invade intestinal cells, and to colonize chick gut. All these phenotypic features could be linked to the motility or export function of the flagella. Further analyses should focus on a possible link existing between PNPase and *C. jejuni* flagella. Moreover, mutation of the *pnp* gene induces a stress-like state of the bacterial cell, observed with overproduction of proteins such as Hsp90, DnaK, KatA, suggesting that PNPase could be involved in *C. jejuni* resistance to stress. Proteomic experiments showed that PNPase inactivation causes little variation in *C. jejuni* protein production, but, specific proteins such as PEB3 and KatA are nonetheless affected. These data suggest that in *C. jejuni*, PNPase is involved in gene expression in multiple ways, in addition to directly degrading the primary transcript; however, PNPase is responsible for adding nucleotides to an existing poly(A) tail of mRNA through a poly(A) polymerase activity (Li and Deutscher, 1994; Mohanty and Kushner, 2000; Oussenko et al., 2005; Viegas et al., 2007). The heteropolymeric tails synthesized by PNPase may further enhance the degradation of mRNA intermediates targets. Further molecular studies using mRNA stability assays will be conducted to verify if the regulation of the genes identified in this study are potential targets of PNPase, either directly or through an alternative mechanism. Indeed, RNase R can be substituting PNPase since the *pnp* mutant strain has the hydrolytic activity increased. This could explain the little variation observed in proteomic results. In *E. coli*, RNase II antagonizes PNPase action on polyadenylated transcripts. In *C. jejuni*, PNPase may antagonize RNase R action. However, direct measurement of hydrolytic ribonuclease gene expression or combinatorial *in vitro* RNA degradation assays may also be required.

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LuxS and quorum-sensing in *Campylobacter*

Paul J. Plummer[1,2]*

[1] Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA
[2] Department of Veterinary Microbiology and Preventative Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Margaret E. Bauer, Indiana University School of Medicine, USA
Martin R. Roop II, East Carolina University, USA

***Correspondence:**
Paul J. Plummer, 2426 Lloyd Veterinary Medical Center, College of Veterinary Medicine, Iowa State University, Ames, IA 50011, USA.
e-mail: pplummer@iastate.edu

Several intercellular bacterial communication mechanisms have been identified in a broad range of bacterial species. These systems, collectively termed quorum-sensing systems, have been demonstrated to play significant roles in a variety of bacterial processes including motility, biofilm formation, expression of virulence genes, and animal colonization. *Campylobacter jejuni* is known to possess a LuxS/ autoinducer-2 (AI-2) mediated system that have been partially characterized over the last decade. AI-2 is formed as a byproduct of the activated methyl recycling pathway, specifically by the LuxS enzyme. Previous work in our laboratory and that of others has demonstrated that this gene is involved in a variety of physiologic pathways of *C. jejuni* including motility, autoagglutination, cytolethal distending toxin (CDT) expression, flagellar expression, oxidative stress, and animal colonization. This review article will summarize the current research associated with LuxS in *C. jejuni* and will provide insights into the role of this system in the metabolism and intercellular communication of this organism. Additionally, the evidence for other quorum-sensing pathways in *Campylobacter* will be discussed.

Keywords: *Campylobacter*, quorum-sensing, AI-2, HSL, virulence

GENERAL MECHANISMS OF QUORUM-SENSING

The first evidence of bacterial communication came from the study of density-dependent bioluminescence of *Vibrio fischeri* (Engebrecht et al., 1983; Engebrecht and Silverman, 1984). The induction of bioluminescence was found to be mediated by the concentration-dependent action of small signaling compounds produced by the bacteria and termed autoinducers (AIs). This work provided the basis for the quorum-sensing model in Gram-negative bacteria where an acyl-homoserine lactone (HSL) autoinducer (AI-1) accumulates in the extracellular environment and freely diffuses into the bacterial cytoplasm. When intracellular AI levels reach a critical threshold concentration it binds to a cellular transcriptional activator (*luxR* homolog) that is responsible for luciferase gene induction (Bassler, 1999). Subsequent work has demonstrated a similar system in over 30 species of Gram-negative bacteria where it exerts control over a variety of cell density-dependent processes. In contrast, Gram-positive species have been demonstrated to produce small peptide signaling molecules that are secreted through the action of an ABC transporter protein system (Bassler, 1999). The extracellular AIs are then recognized by a cognate two-component sensor kinase that initiates a phosphorelay signal transduction cascade. Perhaps most intriguing is the identification of a third quorum-sensing mechanism that appears to be highly conserved over a variety of gram-negative and gram-positive organisms. In these organisms the *luxS* gene is responsible for the synthesis of a novel AI termed autoinducer-2 (AI-2), which has mechanisms reminiscent of both the Gram-positive and Gram-negative systems. The model system, *Vibrio harveyi*, uses a well described two component sensor kinase system (LuxP/Q) to initiate a phosphorelay signal transduction event that ultimately leads to dephosphorylation of the response regulator (LuxO) and changes in gene expression (Bassler et al., 1993). In contrast, *Salmonella* and, more recently, *E. coli* have both been demonstrated to rely on a periplasmic binding protein (LsrB) that recognizes the AI-2 and leads to internalization via an ABC transporter (encoded by the lsrACDBFGE operon) where the AI-2 is phosphorylated and regulates gene expression via LsrR (Taga et al., 2003; Xavier and Bassler, 2005b). According to genomic sequences, *C. jejuni* does not possess acyl-HSL synthetases and the AI-1 sensing system, but possesses a *luxS* homolog (Parkhill et al., 2000; Fouts et al., 2005). For the purposes of this review, we will first review what is known about LuxS and AI-2 production in *C. jejuni* since this has been the primary focus of quorum-sensing research in this species. Following the discussion of LuxS we will summarize the current understanding of alternative quorum-sensing mechanisms in *Campylobacter*, including the HSL/AI-1 system.

LuxS AND AUTOINDUCER-2 PRODUCTION

AI-2 production by the *S*-ribosylhomocysteinase, LuxS, was first described in *Vibrio harveyi* as a result of work on bioluminescence by this organism (Bassler et al., 1993). It was discovered that as the bacterial population grew, the environmental concentrations of AI-2 increased and that the presence of a critical concentration of this signal was necessary for bioluminescence of the organism. LuxS cleaves *S*-ribosylhomocysteine to form homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) which is then spontaneously cyclized to form AI-2 (Winzer et al., 2002; De Keersmaecker et al., 2005; Rajan et al., 2005). As this system has been evaluated in a number of species it has become clear that the spontaneous cyclization of DPD does not produce a single molecular compound but instead can produce multiple molecules with varying abilities to bind AI-2 receptors. This has led to the use of the term "AI-2" to collectively represent a variety

of molecular variants that result from spontaneous changes to DPD and act as ligands of AI-2 receptors (Vendeville et al., 2005). Recent work even suggests that these molecules are able to interconvert allowing for the inclusion of mixed bacterial populations in the quorum recognized by a single species (Xavier and Bassler, 2005a). The molecular form of AI-2 produced by *C. jejuni* is unknown at this time, however, work by our group and others groups has demonstrated that it is active as a *luxP* ligand during *in vitro* bioluminescence assays with a *Vibrio harveyi* reporter strain (Elvers and Park, 2002; Jeon et al., 2003; Quinones et al., 2009; Plummer et al., 2011b).

THE STRUCTURE AND ACTIVITY OF LuxS

Crystal structures of the LuxS homologs suggest that the protein forms a homodimer with a highly conserved active site (Vendeville et al., 2005). Structural studies have demonstrated that the LuxS protein forms a secondary structure containing a four-stranded antiparallel β sheet interacting with four α helices (Hilgers and Ludwig, 2001; Lewis et al., 2001; Ruzheinikov et al., 2001). The LuxS structural fold observed in all of these crystals is highly conserved and appears to be a new type of fold in the alpha-beta family (Lewis et al., 2001). At the interface of the homodimer two identical active sites are present with a tetrahedrally coordinated Fe^{2+}. Homodimerization also leads to the formation of two deep pockets adjacent to the active sites. The function of the pockets is still unclear, but they were demonstrated to hold a covalently bound homocysteine molecule in at least one study (Ruzheinikov et al., 2001). The proposed enzymatic activity involves a series of proton transfer reactions, catalyzed by two Lewis acid/bases and the divalent metal ion that will be discussed further in the next paragraph (Zhu et al., 2004). Although the LuxS protein of *Campylobacter jejuni* has not been crystallized, the gene sequence shows a high degree of homology to other sequenced strains (74% aa identity to LuxS from *Vibrio harveyi*, 71% aa identity to LuxS from *E. coli*, and 73% aa identity to LuxS from *Pasteurella multocida*) and would be expected to have a similar structure (Elvers and Park, 2002).

LuxS is a Fe^{2+} metalloenzyme whose catalytic mechanism has been extensively studied and is well defined (Zhu et al., 2003; Pei and Zhu, 2004; Zhu et al., 2004; Rajan et al., 2005). Two universally conserved residues, Cys-84 and Glu-57 (amino acid numbering in *E. coli* LuxS), have been shown to act as critical general acids/bases during catalysis. In addition, several other active-site residues including Ser-6, His-11, and Arg-39 are also important for full activity of the LuxS enzyme (Pei and Zhu, 2004; Zhu et al., 2004). Recently we described the identification of a naturally occurring G92D mutation in some clones of *C. jejuni* 81116 (Plummer et al., 2011b). The glycine at the 92 position is invariant in enzymatically active enzymes described to date. Strains with this mutation fail to produce significant levels of AI-2 in the biological systems utilized. Importantly, not all clones of 81116 harbor this mutation and several prior publications have demonstrated the presence of a functional LuxS enzyme and AI-2 production in this strain (Elvers and Park, 2002; Jeon et al., 2003). This finding underlines that significance of fully characterizing isolates used for quorum-sensing research even if they are strains previously reported in the literature to have functional LuxS systems.

METABOLIC ROLE OF LuxS

The primary function of LuxS in bacterial cells is as a critical component of the *s*-adenosyl-homocysteine recycling pathway. Functionally, LuxS is an *s*-adenosyl-homocysteinase responsible for hydrolysis of s-adenosylhomocysteine to homocysteine which is then further metabolized to reclaim s-adenosylmethionine (SAM). As a consequence, mutagenesis of LuxS results in both a loss of normal SAM recycling and a loss of AI-2 synthesis. This fact has greatly hindered the ability to specifically attribute any phenotypic differences associated with mutagenesis of LuxS with a specific loss of quorum-sensing ability. It is clear that mutagenesis of *luxS* results in alteration of the extracellular concentration of other SAM pathway metabolites. Using a recombinant LuxS protein bioassay we have demonstrated that the extracellular concentration of *s*-ribosylhomocysteine, the SAM intermediate on which LuxS functions, increases significantly in the culture supernatant of strains deficient in normal LuxS activity (Plummer et al., 2011b). Based on these findings, it is likely that many of the SAM pathway intermediates are present in altered concentrations both intracellularly and extracellularly in *luxS* mutant strains.

The SAM pathway is the primary means of methyl recycling in bacteria (Parveen and Cornell, 2011). Methyl groups donated by SAM are critical for the activity of methyl transferases that are intimately associated with bacterial DNA methylation, chemotaxis, motility, and a variety of other metabolic and biosynthetic reactions. SAM is also critical in bacterial polyamine formation and vitamin synthesis (Parveen and Cornell, 2011). As a consequence, alteration of SAM recycling associated with *luxS* mutagenesis can have significant impacts on bacterial metabolism.

REGULATION OF LuxS EXPRESSION

Hwang et al. (Hwang et al., 2011) recently reported that antisense-mediated gene silencing of CosR, an essential response regulator of *Campylobacter jejuni*, upregulated LuxS expression by 1.7 fold. Similarly, over expression of the CosR protein resulted in a 2.2-fold down regulation of LuxS expression. Collectively there results suggest that the CosR response regulator negatively regulates the expression of LuxS. Building on these findings the group was also able to demonstrate that CosR bindings to the promoter region of LuxS by showing that the incubation of labeled LuxS gene probes with recombinant CosR protein resulted in a concentration-dependent electrophoretic mobility shift of the probes. Finally, using DNase I fingerprinting methods the group was able to develop a consensus binding site for CosR, and they identified a putative binding site located 10 bp upstream of the LuxS start codon. Collectively, these studies provide strong evidence for CosR playing a role in the regulation of LuxS expression. As discussed later in this review, LuxS has been associated with the cellular response of *Campylobacter* to oxidative stress, thus additional studies focused on the role of CosR in this response are warranted.

ENVIRONMENTAL SENSING OF AI-2

Variation has been demonstrated in the mechanism of cellular recognition of environmental AIs. Recent evidence suggest that

chemical interconversion of AI-2 may also allow the signal of one species to effect the response of another species (Xavier and Bassler, 2005b). At present, no mechanisms of cellular recognition of AI-2 are known for *Campylobacter*. In the initial report of AI-2 production by *C. jejuni* Elvers and Park (2002) showed that peak AI-2 concentrations were reached at 18 h and maintained at that level for at least 42 h. This finding would suggest that *Campylobacter* does not have an active "LSR type" system that pumps the compound into the intracellular matrix. More recently, two studies have demonstrated that AI-2 supernatent concentrations peak in mid- to late-exponential growth and decline during stationary phase, more similar to the pattern observed in *Salmonella* and *E. coli* (Cloak et al., 2002; Quinones et al., 2009). Using a slightly different approach, Holmes et al. demonstrated that AI-2 was either inactivated or taken up by *Campylobacter* after the addition of exogenous AI-2 to cultures, although the levels of bioluminescence observed in the *Vibrio* bio assay remained similar to the levels produced endogenously by wildtype strains (Holmes et al., 2009). Collectively, these findings leave uncertainty regarding the fate of AI-2 in the culture supernatant of *Campylobacter*. There are no LSR homologs identified using comparative genomics, however, *Campylobacter* does encode a number of ABC type transporters that could be involved in uptake of AI-2. There is a need for additional work in this area to further define the mechanisms, if any, for sensing or uptake of AI-2 in this species.

PHENOTYPES ASSOCIATED WITH *LuxS* MUTAGENESIS

Alterations in biofilm formation, growth rates, antimicrobial production, motility, toxin secretion, and colonization have been observed with *luxS* mutants of different bacterial species (Vendeville et al., 2005). For an excellent review of the currently described phenotypic changes in more than 15 species see the paper by Vendeville et al. (Vendeville et al., 2005). The experimental design of many of these studies involves mutation of *luxS* and subsequent evaluation for phenotypic changes. As LuxS is required for both homocysteine production and AI-2 synthesis, the phenotypic changes associated with the *luxS* mutants could be due to AI-2-mediated quorum-sensing or the loss of metabolic function of LuxS. Most studies demonstrate the phenotypic changes in the presence of reduced AI-2 levels but fail to demonstrate the direct link of the AI presence with the phenotype. This issue of dissecting the significance of metabolic changes versus AI-2 synthase activity (and the subsequent presence of AI-2 in the environment) has been addressed by several authors and represents a significant challenge in this field of research (Vendeville et al., 2005; De Keersmaecker et al., 2006). A recent study in *Listeria monocytogenes* demonstrated that the increased biofilm thickness observed in *luxS* mutant strains may be associated with increased levels of s-ribosylhomocysteine in the media of the mutant strain presumably associated with the metabolic deficiency of the enzyme and not the lack of AI-2 (Challan Belval et al., 2006). Despite the shortcomings of some experimental designs at differentiating these roles, many of the observed phenotypic changes associated with AI-2 in other species involve essential molecular processes or virulence factors. As such, the findings remain scientifically significant and relevant regardless of the exact mechanisms for the phenotypic changes. The following sections will focus on a review of the current understanding of *Campylobacter* phenotypes associated with *luxS* mutagenesis. **Table 1** includes a summary of phenotypes that have been associated with *luxS* mutagenesis in this species.

CONFIRMATION OF LuxS AND AUTOINDUCER-2 IN *Campylobacter*

The publication of the first full genome sequence of *C. jejuni* in 2000 (Parkhill et al., 2000) allowed for the *in silico* identification of a *luxS* ortholog (*Cj1198*) in 2002 (Elvers and Park, 2002). Elvers and Park were able to demonstrate the presence of AI-2 activity in growth media of *C. jejuni* using the AI-2 bioluminesence assay reported by Bassler et al. (1993) and were able to eliminate the AI-2 activity by insertional mutation of the *luxS* gene. The authors were unable to demonstrate any differences in growth rate, resistance to oxidative stress, or *in vitro* invasion assays, but did show a decrease in motility of the mutant compared to the parent strain. Cloak et al. (2002) subsequently confirmed the presence of a functional *luxS* gene and production of active AI-2 using the same bioluminescence assay. Additionally, they were able to demonstrate measurable levels of AI-2 in milk and chicken broth inoculated with *Campylobacter*, suggesting that the nutrient requirements necessary for AI-2 are present in common food sources. Collectively these results provide strong evidence for the presence of a functional *luxS* gene capable of producing AI-2 in *C. jejuni*. Recently, Tazumi et al. (2011) screened 20 isolates of *C. jejuni*, *C. coli*, *C. upsaliensis, C. fetus*, and *C. lari* to determine how widely conserved the gene was in the genus. Interestingly, hybridization assays along with PCR demonstrated the presence of LuxS homologs in all isolates of *C. jejuni*, *C. coli*, *C. upsaliensis*, and *C. fetus* but failed to identify LuxS homologs in any of the four *C. lari* isolates. Furthermore, AI-2 bioluminescence assays showed the production of functional AI-2 products in all strains except the *C. lari* isolates. Based on these results it is clear that with the exception of *C. lari*, the majority of *Campylobacter* spp. have functional LuxS enzymes and produce AI-2.

LuxS AND GROWTH

Elvers and Park were the first group to evaluate the growth phenotypes of *luxS* mutants in *C. jejuni*. Using the NCTC 11168 strain they demonstrated that the mutant had a similar doubling time compared to the wildtype strain (103 min for the mutant compared to 110 min for the wildtype) (Elvers and Park, 2002). Furthermore, both strains required approximately 20 h to enter stationary phase. Subsequently, other studies have demonstrated essentially equal growth rates between the wildtype and LuxS mutant strains of *C. jejuni* 81116 (Jeon et al., 2003), M129 (Reeser et al., 2007), and NCTC 11168 grown in both Mueller Hinton broth and MEM-α broth(Holmes et al., 2009). Interestingly, He et al. (2008) showed that in *C. jejuni* 81–176 the LuxS mutant had a statistically longer doubling time compared to the wildtype strain when the isolates were incubated at 37°C, but not at 42°C when the growth rates were not different. Recently another publication demonstrated that the LuxS mutant of the 81–176 strain had a decreased growth rate during the exponential phase but reached stationary phase at the same time as its wildtype counterpart when grown at 42°C (Quinones et al., 2009). Based on

Table 1 | *Campylobacter jejuni* phenotypes associated with mutagenesis of LuxS.

Growth	No significant difference	Elvers and Park, 2002 Jeon et al., 2003 Reeser et al., 2007 He et al., 2008
	No difference at 42°C but decreased at 37°C	Holmes et al., 2009
	Decreased in exponential phase but reach stationary at the same time	Quinones et al., 2009
Motility	Decreased in *luxS* mutant	Elvers and Park, 2002 Jeon et al., 2003 Holmes et al., 2009 Quinones et al., 2009 Plummer et al., 2011b
	Motility able to be complemented	Quinones et al., 2009 Plummer et al., 2011b
	Motility decreased in mutant at 37°C but not at 42°C	He et al., 2008
	Motility not changed	Guerry et al., 2006
	Decrease in *flaA* transcription in mutant	Jeon et al., 2003 Holmes et al., 2009
Host colonization	Decreased colonization of chickens beyond day 4 post inoculation using 81–176	Quinones et al., 2009
	Unchanged when monoinoculated NCTC 11168	Plummer et al., 2011a
	Decreased competitive fitness of mutant	Plummer et al., 2011a
	Complete absence of colonization in chicks using IA3902 mutant	Plummer et al., 2011a
	Complete absence of colonization in guinea pigs using IA3902 mutant	Plummer et al., 2011a
In vivo virulence	Decreased survival of mutant in meat juice stored at 5°C	Ligowska et al., 2011
	Complete loss of abortion phenotype in guinea pig abortion model using IA3902, fully restored with complementation	Plummer et al., 2011a
Biofilm formation	Decreased biofilm formation in mutant	Reeser et al., 2007
Cytolethal distending toxin	Decrease in *cdt* gene transcription	Jeon et al., 2005
Autoagglutination	Altered kinetics in mutant with decreased autoagglutination	Jeon et al., 2003
	Decreased autoagglutination	Guerry et al., 2006
Cellular adherence and invasion	No difference in wildtype and mutant on Caco-2 cells	Elvers and Park, 2002
	Decreased adherence and invasion of mutant on LMH cells	Quinones et al., 2009
Oxidative stress	No difference with hydrogen peroxide and paraquat	Elvers and Park, 2002
	Increased sensitivity of mutant to cumene hydroperoxide and hydrogen peroxide using disk diffusion	He et al., 2008
	Increased sensitivity of mutant to hydrogen peroxide using MIC method	He et al., 2008
Chemotaxis	Increased chemotaxis of mutant to amino acids	Quinones et al., 2009
	Decreased chemotaxis of mutant to organic acids	Quinones et al., 2009

these collective results it appears that most strains of *C. jejuni* do not show significant growth defects associated with mutagenesis of LuxS enzyme, however, strain 81–176 seems to have additional complexity associated with the growth kinetics of mutant strains. Given that there are significant differences in how the bacteria were grown in each of these studies (Mueller–Hinton agar versus anaerobe basal agar, microaerophilic conditions established by Oxoid CampyGen versus anaerobic chamber) the reader should be cautious in trying to compare the differences observed between studies. In the case of 81–176, based on He et al. findings there was a temperature dependent difference in growth. Since the majority of other studies did not evaluate the growth kinetics under two temperatures in the same growth controlled growth conditions, it is not clear if similar differences might exist in other strains. Furthermore, not all of the studies (Elvers and Park, 2002; He et al., 2008) performed complementation of the mutant strains, thus secondary mutations associated with the changes in growth kinetics cannot be excluded.

LuxS AND MOTILITY

As mentioned previously, Elvers and Park (2002) provided the first experimental evidence for a role of LuxS in the motility of *C. jejuni*. Using semisolid media they demonstrated that the LuxS mutant formed a statistically smaller halo after incubation when compared to the wildtype strain. Subsequently, many studies have demonstrated that motility on semisolid media is decreased in *luxS* mutant strains (Jeon et al., 2003; Holmes et al., 2009; Quinones et al., 2009; Plummer et al., 2011b). In several cases this motility defect could be at least partially restored by genetic complementation of the *luxS* gene (Quinones et al., 2009; Plummer et al., 2011b). Similar to growth studies, *C. jejuni* strain 81–176 seems to show a temperature dependent difference in motility with a statistically significant decrease in the motility halo at 37°C but a non-statistically significant reduction in motility at 42°C (He et al., 2008). In contrast, a report of a *luxS* mutant of 81–176 generated to study autoagglutination demonstrated no loss of motility (Guerry et al., 2006). In that study, however, there is

no genetic confirmation presented to demonstrate the construction of a loss of function mutant and no complementation studies were performed, thus the results should be interpreted with due caution.

In an attempt to explain the decreased motility of *luxS* null mutants Jeon et al. (2003) performed phase-contrast microscopy on the wildtype and *luxS* mutant strains and failed to observe any differences in motility speed or motility pattern. To study the molecular basis for the motility phenotype they evaluated transcription of the two major flagellin genes (*flaA* and *flaB*) using a primer extension assay. Using this assay they observed a 57% reduction in the transcription of *flaA* in the *luxS* mutant background comparing to the wildtype strain. They were unable to demonstrate a difference in FlaA protein levels using western blotting and did not see a difference in flagellar structure based on transmission electron microscopy. Additionally, they were able to demonstrate decreased agglutination ability of the mutant strain. This change is presumably mediated by altered expression of surface proteins or flagella since a previous study has demonstrated a complete loss of agglutination in flagellar ablated strains (Golden and Acheson, 2002a). No significant changes were observed in the expression of *flaB* using the primer extension assay. Subsequently, Holmes et al. (2009) have confirmed a down regulation of *flaA* (4.2-fold down-regulated in *luxS* mutant compared to wildtype) using cDNA microarray.

LuxS AND HOST COLONIZATION

Two published reports have evaluated the role of LuxS in the colonization of the gastrointestinal system of animals. Quinones et al. (2009) utilized *C. jejuni* strain 81–176 to evaluate the colonization of the chicken gastrointestinal tract. They demonstrated a trend toward decreased colonization of the LuxS deficient strain starting at day 4 post inoculation and a statistically significant decrease of colonization of the mutant strain by 7 days post inoculation in two separate experiments. Although differences in mean colonization levels were observed there were several birds inoculated with mutant strain that maintained colonization levels similar to that observed in the wildtype throughout the study. The authors suggested that in these birds the exogenous levels of AI-2 produced by other enteric flora might be providing the support necessary to maintain colonization (Quinones et al., 2009). Our group has performed similar studies using the *C. jejuni* NCTC 11168 strain and the IA3902 strain of *C. jejuni*, a sheep abortion isolate extensively characterized by our group (Burrough et al., 2009; Sahin and Zhang, 2010). In our study, inoculation of chickens with mono-cultures of wildtype and *luxS* mutant strains of 11168 and the wildtype strain of IA3902 resulted in similar levels of colonization while the *luxS* mutant of IA3902 completely failed to colonize the gastrointestinal tract of chickens (Plummer et al., 2011a). A genetically complemented strain of the IA3902 carrying a chromosomally encoded *luxS* gene restored colonization to wildtype levels. Given that the LuxS deficient strain of 11168 colonized to normal levels, we proceeded to test this strain along with its wildtype counterpart in a competitive fitness experiment where we demonstrated a decreased fitness of the LuxS mutant associated with statistically significant decreases in recovery of the mutant compared to the wildtype populations (Plummer et al., 2011a).

We have also evaluated the role of LuxS in the colonization of the guinea pig gastrointestinal tract using strain IA3902. Following oral inoculation of the wildtype strain IA3902, its *luxS* mutant and a chromosomally encoded complement strain we saw a significant defect in the ability of the mutant strain to colonize the GI tract while the wildtype and complement strains showed high quantity and prolonged colonization based on fecal culture (Plummer et al., 2011a).

LuxS AND VIRULENCE

Very few studies have evaluated the role of LuxS in the virulence of *C. jejuni*. Using the IA3902 strain and the guinea pig model we have recently evaluated the role of LuxS in the virulence of this strain as a cause of abortion. When the wildtype IA3902 strain or its *luxS* mutant were inoculated by the intraperitoneal route into pregnant guinea pigs there was no difference observed in the abortion rates of the groups (Plummer et al., 2011a). In contrast, when the strains were inoculated by the oral route the *luxS* mutant strain was completely attenuated in its ability to produce abortions in the guinea pigs (5/9 aborted in wildtype versus 0/7 in the mutant) (Plummer et al., 2011a). Furthermore, genetic complementation of the mutant strain resulted in full restoration of the abortion phenotype. Collectively these results suggest that LuxS plays an important role in the translocation of *C. jejuni* out of the intestine and into the systemic vasculature where it can move to the placenta and induce abortions.

LuxS AND ENVIRONMENTAL ADAPTATION

Ligowska et al. (2011) evaluated the survival of the NCTC 11168 strain in chicken meat juice incubated at 5°C. They demonstrated that the *luxS* mutant of 11168 started decreasing significantly in number after six days of incubation while the wildtype numbers remained unchanged for the 27-day duration of the study. Based on these results they suggest that LuxS is important for the environmental adaptation to meat juice and cold temperatures.

LuxS AND BIOFILM FORMATION

In 2007, Reeser et al. demonstrated that *luxS* and flagellar structure may be important for *C. jejuni* biofilm growth since isolates that were mutagenized in either the flagellar proteins or *luxS* had significantly decreased biofilm formation using a simple static biofilm assay. Using *C. jejuni* strain M129, a human clinical isolate, they demonstrated that the *luxS* mutant had a statistically significant reduction in crystal violet staining following a 48 h and 72 h incubation at 37°C in a 10% CO_2 environment. Given that similar reductions in crystal violet staining were observed in a *flaAB* mutant it is unclear if the changes in biofilm formation were a direct response to the loss of a functional LuxS enzyme or secondary to altered transcription of *flaA* as demonstrated by Jeon et al. (2003). The overall significance of biofilms in the physiology and survival of *C. jejuni* is still not well understood, so the importance of these findings to bacterial survival or persistence in the environment is unclear presently.

LuxS AND CYTOLETHAL DISTENDING TOXIN

Jeon et al. (2005) evaluated the role of AI-2 in expression of the cytolethal distending toxin (CDT), a putative virulence factor for *C. jejuni*. Using a primer extension assay they were able to demonstrate a 39% decrease in *cdt* gene transcription when *luxS* was mutated. One biological function of CDT involves the arresting of the cell cycle in the G_2/M phase (Whitehouse et al., 1998), and they were able to demonstrate a statistically significant decrease in number of cells arrested in this phase in the *luxS* mutant (Jeon et al., 2005). Recently, another study was published demonstrating that the expression of LuxS was up-regulated following incubation in chicken meat juices along with an up-regulation in the expression of CDT (Ligowska et al., 2011). It is unclear what mechanism underlies the up-regulation of CDT, however, the authors suggest that one possible mechanism would be through an up-regulation of LuxS expression. Additional work is necessary to further define the role of LuxS in the expression of CDT.

LuxS AND AUTOAGGLUTINATION

Jeon et al. (2003) also studied the role of *luxS* in autoagglutination of *Campylobacter* in an effort to determine if the mutation was associated with changes in surface properties exhibited by the bacteria. They were able to demonstrate a significant change in autoagglutination kinetics of the mutant strain compared to the wildtype, with the *luxS* mutant having a decreased autoagglutination. Similarly, Guerry et al. demonstrated that a *luxS* mutant of *C. jejuni* strain 81–176 had a decreased autoagglutination compared to the wildtype parent strains (Guerry et al., 2006). Autoagglutination has previously been associated with the presence of flagellar assemblies (Misawa and Blaser, 2000; Golden and Acheson, 2002b) and it is unclear if the observed change in autoagglutination is mediated by changes in flagellar assembly. Flagella are required for autoagglutionation, but are not sufficient by themselves to induce agglutination (Golden and Acheson, 2002b), therefore, other mechanisms of altered surface structure cannot be ruled out at this time.

LuxS AND CELLULAR ADHERENCE AND INVASION

In their initial description of the phenotypes associated with *luxS* mutagenesis, Elvers and Park (2002) failed to see a difference in adherence and invasion of Caco-2 cells between the wildtype and mutant strains. In their study using *C. jejuni* NCTC 11168 they saw an internalization of 0.08% for the wildtype and 0.05% for the *luxS* mutant, which did not prove to be statistically different. More recently Quinones et al. evaluated the adherence of *C. jejuni* to LMH cells (Quinones et al., 2009). In their study they demonstrated a significant reduction ($P < 0.01$) in adherence of the *luxS* mutant of 81–176 when compared to the wildtype strain and that defect could be fully complemented with the addition of another *luxS* ORF. Given the variability of invasion phenotypes observed in *C. jejuni*, it is likely that the impact of *luxS* mutagenesis is determined by both the strain being tested and the eukaryotic cell type used for the assay. At present, no studies have evaluated the role of LuxS in the subvasion and invasion of eukaryotic monolayers, however, such experiments would likely provide useful information regarding the role of this gene in pathogenicity.

LuxS AND OXIDATIVE STRESS

Two independent studies have evaluated the role of LuxS in the cellular response to oxidative stress of *C. jejuni*. Elvers and Park (2002) used a well diffusion assay to test the oxidative stress of paraquat and hydrogen peroxide on *C. jejuni* NCTC 11168 and its *luxS* mutant. Using parquet concentrations between 1–40 mM and hydrogen peroxide concentrations between 0.015 and 0.3% they could not demonstrate any differences in sensitivity (Elvers and Park, 2002). More recently He et al. compared the sensitivity of *C. jejuni* 81–176 and its *luxS* mutant to cumene hydroperoxide (CHP) and hydrogen peroxide using both a disk inhibition assay and a minimum inhibitory concentration (MIC) assay. Using the disk diffusion method they demonstrated a significant (P ranging from <0.001 to <0.0029) increase in sensitivity to both compounds in a dose dependent fashion (He et al., 2008). Using the MIC methodology they found that MIC of the *luxS* mutant was two to four-fold lower than the MIC of the wildtype strain for hydrogen peroxide, consistent with the disk diffusion method. In contrast, the MIC of the *luxS* mutant and wildtype were the same for CHP. The group also demonstrated that the transcriptional expression of the *aphC* and *tpx* genes, two stress response genes, appeared to be down regulated in the *luxS* mutant strain (He et al., 2008). The reason for the differences in response to hydrogen peroxide between the two studies is not clear. Neither study performed genetic complementation of the mutants to rule out secondary mutations and the methodologies used for testing the response were different (well diffusion in Elvers and Park versus disk diffusion/MIC in He et al.) making direct comparisons difficult.

LuxS AND CHEMOTAXIS

Quinones et al. used the hard-agar plug method to test the chemotactic response of *C. jejuni* 81–176 to both amino acids and organic acids. They demonstrated that zones of accumulation for the amino acids aspartate, asparagine, glutamate, and glutamine were all significantly increased in the *luxS* mutant strain compared to the wildtype (Quinones et al., 2009). They hypothesized that these changes might be a response to altered metabolic activity of the *luxS* mutant that resulted in disrupted amino acid metabolism and carbon compound catabolism. In contrast, the wildtype strain showed an increased chemotactic behavior for all tested organic acids when compared to the *luxS* mutant. Given that organic acids are important energy sources of *C. jejuni* in the chicken gastrointestinal tract they hypothesized that this decreased chemotactic activity may contribute to the decreased chicken colonization that they observed with this isolate.

LuxS AND TRANSCRIPTIONAL REGULATION

Two studies have been published that evaluated the role of LuxS mutagenesis in transcriptional regulation of *C. jejuni* (He et al., 2008; Holmes et al., 2009). Both studies performed a cDNA microarray analysis comparing the mRNA levels of wildtype versus *luxS* mutant. Furthermore, He et al. explored the role of LuxS in the presence of hydrogen peroxide used to induce oxidative stress while Holmes et al. evaluated transcriptional changes associated with the addition of exogenous AI-2. The results of both studies demonstrate altered expression of several flagellar,

SAM metabolism, and ABC transporter genes in the *luxS* mutant, which would be consistent with the previously published phenotypes. Interestingly, He et al. observed that the mutant strain was more sensitive to the toxic effects of hydrogen peroxide and CHP. This phenotype was confirmed by altered expression of both the *ahpC* (encoding alkyl hydroperoxide reductase) and *tpx* (encoding thiol peroxidase) genes (He et al., 2008). Collectively these findings would suggest that the *luxS* gene or AI-2 plays a role in the response of *C. jejuni* to oxidative stress. During this study they did not compare the differential transcriptional regulation of *C. jejuni luxS* mutants in the presence or absence of exogenous AI-2, so it is difficult to dissect how much of the observed change was associated with the metabolic role of *luxS* as opposed to its role in AI-2 synthesis. More recently, Holmes et al. reported a microarray experiment designed to further evaluate the issue of AI-2 sensing by *C. jejuni*. In their experiment they added exogenous AI-2 to the culture media of *luxS* mutant strains and evaluated transcriptional changes in gene expression. They were unable to find a significant number of genes that were differentially expressed in the presence of exogenous AI-2 and concluded that most of the transcriptional changes observed in mid-exponential growth *luxS* mutants of *C. jejuni* were the result of metabolic disturbances induced by altering the SAM recycling pathway. They concluded that AI-2 mediated quorum-sensing is not an important mechanism in mid-exponential growth of *C. jejuni* cultured in MH media (Holmes et al., 2009). Interestingly, they also performed the microarray analysis on samples recovered following a minimal media (MEM-α as opposed to MH broth) incubation. They demonstrate that there was a media dependent association of gene expression with almost twice as many genes being differentially expressed in the complex media versus the minimal media (131 vs. 60 genes for the MHB media and MEM media, respectively) (Holmes et al., 2009). Of those genes there was a subset of 20 genes that were differentially expressed in both media types.

In general, transcriptional changes in *C. jejuni luxS* mutants are less extensive then those demonstrated in other bacterial species. For instance, LuxS appears to act as a global regulator of *E. coli* where 5–10% of the genes in the genome are expressed differentially in the mutant compared to wildtype (Delisa et al., 2001; Sperandio et al., 2001). In *C. jejuni* less than 2% of the genes are differentially regulated suggesting that LuxS is not a global regulator in this species (He et al., 2008; Holmes et al., 2009).

OTHER QUORUM-SENSING COMPOUNDS IN *C. jejuni*

AUTOINDUCER-1 AND *Campylobacter*

As mentioned previously *C. jejuni* does not encode obvious homologs of AI-1 synthase genes. Moorhead et al. performed a BLASTN search against *C. jejuni* strains 11168 and RM1221 using the *luxI* and *ainS* synthase genes as queries and did not identify any putative homologs (Moorhead and Griffiths, 2011). However, the sequence similarity of these genes has been reported to be very low, often with values lower than 35% for pair-wise identity, making identification difficult (Fuqua et al., 1996). Using a screen of eight AI-2 biosensors they were able to demonstrate that two *C. jejuni* strains (81–176 and cj11) both produced positive reactions in the *V. harveyi* BB886 sensor system. This result suggests that these strains are able to produce an HSL (AI-1) product similar to *N*-(3-hydroxy-butanoyl)-L-HSL (3-OH-C4-HSL) (Moorhead and Griffiths, 2011). The investigators were able to fractionate the samples and obtain a purified fraction that demonstrated the biological activity in the HSL sensor bioassay. They named this fraction cjA. Subsequently, they tested a panel of six different HSLs and cjA for their ability to induce changes in the formation of a viable but non-culturable state (VBNC), biofilm formation, virulence gene transcription, and IL-8 stimulation of INT-407 cells. In summary, the short chained HSLs, along with cjA prolonged the onset of the VBNC state although the difference was fairly small. Likewise the addition of 3-OH-C4-HSL or cjA disrupted the ability of strain 81–176 to form microscopic biofilms evaluated using confocal laser scanning microscopy (CLSM) while the addition of the five other HSL compounds had minimal impact. Using RT-PCR the authors then evaluated the expression of five previously described virulence genes associated with *C. jejuni* pathogenesis. Included in the study were *cadF, cdtB, ciaB, flaA,* and *iamA*. The additional of several of the HSL compounds resulted in significant changes in transcriptional levels of these virulence genes with the cjA fraction purified from *C. jejuni* cell-free media inducing a 7-fold up-regulation of *cadF*, a 6.60-fold increase in *cdtB*, a 1.98-fold increase in *ciaB*, a 2.27-fold increase in *flaA* and a 10.0-fold decrease in *iamA*. Collectively these results suggest that *C. jejuni* is able to either produce a HSL compound or a mimic that is capable of inducing a response in bioassays for C4-HSL. Furthermore, the differences in biofilm formation and gene expression following exogenous addition of cjA and other HSL compounds suggest that the organism is capable of sensing and responding to these compounds in the environment. Additional work is necessary to define the role of HSL signaling in *C. jejuni* and to determine the molecular mechanisms responsible for the synthesis and sensing of these compounds.

AUTOINDUCER-3 AND *Campylobacter*

Work conducted by Sperandio et al. (2003) demonstrated that *in vitro* synthesized AI-2 was unable to induce the expression of the locus of enterocyte effacement (*lee*) in *E. coli* and did not complement the type III secretion system phenotype observed in EHEC *luxS* mutants. Furthermore, they demonstrated that using column purification they could induce *lee* expression and restore the type III secretion system with a purified fraction different from the fraction exhibiting AI-2 activity by the *Vibrio* bioluminescence assay. They associated this activity with a new AI named AI-3 and developed a *LEE1::lacZ* fusion for assaying cell free supernatents for the presence of this compound. Importantly, mutation of the *luxS* gene in *E. coli* leads to the loss of both AI-2 and AI-3 activity (52). Testing of *Campylobacter* cell free supernatants for the presence of AI-3 using the *LEE1::lacZ* fusion kindly provided by Dr. V. Sperandio have failed to demonstrate such activity in *Campylobacter* (unpublished data from our laboratory).

CONCLUSIONS

It is clear from multiple studies that the mutagenesis of the LuxS enzyme in *C. jejuni* results in repeatable phenotypes, some of which can significantly alter its virulence. Based on the dual function of LuxS as both a key metabolic enzyme in the SAM recycling

pathway and the synthase protein for the precursor of AI-2 it is more difficult to determine which of these mechanisms underlies the phenotypic changes. Holmes et al. have argued that the lack of a transcriptional response to exogenous AI-2 would suggest that *Campylobacter* is not readily able to recognize and respond to AI-2 as a quorum-sensing molecule in the conditions tested (Holmes et al., 2009). We must consider the fact that the conditions tested were significantly different from the microenvironments present in natural host and as such do not exclude the role of AI-2 as a sensing compound in other environments.

The recent identification of a putative HSL type compound produced by *C. jejuni* demonstrates that one cannot rely exclusively on comparative genomics as a means of studying quorum-sensing in this species. There remain a lot of significant questions regarding this potential HSL compound including: What gene is responsible for production of cjA? How the product is sensed? Are the observed changes in phenotype specific to the compound? Future work in these areas will provide critical information regarding the role of this compound in the pathogenesis of *C. jejuni*.

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Defense and adaptation: the complex inter-relationship between *Campylobacter jejuni* and mucus

Abofu Alemka[1], Nicolae Corcionivoschi[2] and Billy Bourke[2,3]*

[1] *Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada*
[2] *The National Children's Research Centre, Our Lady's Children's Hospital, Crumlin, Dublin, Ireland*
[3] *Alimentary Glycosciences Research Group, School of Medicine and Medical Science, Conway Institute, University College Dublin, Dublin, Ireland*

Edited by:
Alain Stintzi, Ottawa Institute of Systems Biology, Canada

Reviewed by:
Charlene Kahler, University of Western Australia, Australia
Stuart A. Thompson, Medical College of Georgia, USA

***Correspondence:**
Billy Bourke, The National Children's Research Centre, Our Ladys Children's Hospital, Crumlin, Dublin 12, Ireland.
e-mail: billy.bourke@ucd.ie

Mucus colonization is an essential early step toward establishing successful infection and disease by mucosal pathogens. There is an emerging literature implicating specific mucin sub-types and mucin modifications in protecting the host from *Campylobacter jejuni* infection. However, mucosal pathogens have evolved sophisticated mechanisms to breach the mucus layer and *C. jejuni* in particular appears to harbor specific adaptations to better colonize intestinal mucus. For example, components of mucus are chemotactic for *C. jejuni* and the rheological properties of mucus promote motility of the organism. Furthermore, recent studies demonstrate that mucins modulate the pathogenicity of *C. jejuni* in a species-specific manner and likely help determine whether these bacteria become pathogenic (as in humans), or adopt a commensal mode of existence (as in chickens and other animals). This review focuses on recent advances in understanding the complex interplay between *C. jejuni* and components of the mucus layer.

Keywords: *Campylobacter*, mucus, mucins, pathogenicity, motility, chickens

INTRODUCTION

MUCUS COMPOSITION

The visco-elastic mucus gel layer consists of an outer loose layer containing gut microbiota and an inner more compact layer devoid of any bacteria (Johansson et al., 2008). The thickness of the mucus layer ranges from 10 μm in the eye to 700 μm in the intestine (Linden et al., 2008b). Mucin glycoproteins are the main components of mucus while others include; cathelicidins, defensins, lysozyme, protegrins, collectins, antibodies, nitric oxide, and histatins. Collectively these constituents of mucus form a physical barrier, participate in bacterial clearance, and display antimicrobial activity (Linden et al., 2008b; McGuckin et al., 2011). Other protective bioactive molecules in mucus include trefoil factors involved in the repair and restitution of the epithelial mucosa (Taupin and Podolsky, 2003), and resistin-like molecule β (Relmβ) which contribute to the barrier properties of mucus by enhancing mucin gene expression and preventing bacteria from breaching the mucus layer (Hogan et al., 2006; Krimi et al., 2008). In general, mucus protects underlying epithelial cells from chemical, microbial, enzymatic, and mechanical damage.

The main component of mucus, mucin glycoproteins, are heavily *o*-glycosylated in large peptide domains harboring repeats of the amino acids serine and threonine. These areas are known as variable number tandem repeat (VNTR) regions and vary in length both between individuals and different mucin types, leading to VNTR polymorphisms. Carbohydrates constitute approximately 70% of the mass of mucin glycoproteins, conferring mucins with water holding capacity, rigidity, protease resistance, and a high charge density (Klein et al., 1993; Moncada et al., 2003). Up to 16 human mucins have been described, falling into three subfamilies: secreted mucins (MUC7), cell surface mucins (MUC1, MUC4, MUC3A/B, MUC12, MUC13, MUC15, MUC16, MUC 17, MUC20), and gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19). A cluster of four genes on chromosome 11 encode gel-forming mucins, which are the main mucin subfamily in mucus and confer its visco-elastic properties. Cysteine residues at the N- and C-termini of gel-forming mucins form intermolecular and intramolecular disulfide bonds, leading to homo-oligomerization of mucins in regions known as von Willebrand factor-like or D domains (Moncada et al., 2003; Linden et al., 2008b). In the gastrointestinal tract, mucin types are differentially expressed. Mucin sub-types in the colon include MUC2, MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC17, and MUC20. The stomach contains MUC5AC, MUC6, MUC1, MUC4, MUC12, MUC13, and MUC17, while small intestinal mucins include MUC2, MUC3A/B, MUC12, MUC13, MUC15, and MUC17 (Moran et al., 2011).

MUCUS AND ENTERIC BACTERIAL PATHOGENS

Enteric pathogens have to colonize and circumvent the mucus layer prior to causing disease. To establish infection, these bacteria have to bypass both physical and immunological barriers in the gastrointestinal tract. The visco-elastic mucus gel layer protects underlying epithelial cells by trapping pathogens in the loose mucus and clearing them both during gut movements and mucus turnover. In addition, mucin glycoproteins bind and retard bacteria in mucus, preventing them from accessing epithelial cells. Furthermore, antimicrobial peptides present in mucus and gut microflora are directly antagonistic to pathogens. Intestinal microbiota also competitively exclude gut pathogens by occupying mucin receptors, hence preventing pathogen access to the mucosa (reviewed in Linden et al., 2008b).

Enteric pathogens typically breach the mucus layer by employing mucus degradative enzymes. Mucinases, sialidases, *N*-acetyl neuraminate lyases, glycosulfatases, and sialate *o*-acetylesterases are some of the mucinolytic enzymes employed by gut pathogens (Corfield et al., 1992, 1993; Haider et al., 1993; Homer et al., 1994). For example *Vibrio cholerae* expresses HapA which has both mucinolytic and cytotoxic activity (Silva et al., 2003). In addition, chemotaxis and flagellar motility facilitate colonization and penetration of the mucus barrier by enteric pathogens (discussed below for *Campylobacter jejuni*).

In response to the presence of gut microbes, mucin synthesis and glycosylation are regulated. Adherence by probiotic bacteria and products from some bacteria increase the synthesis of mucins from epithelial cells (Smirnova et al., 2003). For example, colonic Muc1 expression is upregulated in both mouse and human intestinal bacterial infections (Linden et al., 2008a). Furthermore, mucin glycosylation changes have been documented following *Helicobacter pylori* infection (reviewed in Moran et al., 2011) and *H. pylori* lipopolysaccharide (LPS) inhibits mucin synthesis in gastric epithelial cells (Slomiany and Slomiany, 2006). *H. pylori* and the stomach gel-forming mucin MUC5AC have been shown to colocalize, and these bacteria bind the dimeric form of the human recombinant trefoil factor 1 (TFF1; Van den Brink et al., 2000; Clyne et al., 2004). Recently, it has been shown that *H. pylori* binds TFF1 by means of its rough form LPS in a pH dependent manner (Reeves et al., 2008). The mechanism of bacterial LPS binding to TFF1 is not known. However, TFF1 harbors a hydrophobic patch of solvent-exposed residues on its surface which may act as a possible carbohydrate binding domain (Reeves et al., 2008). In addition, a pH of between 5 and 6 was found to be optimal for the interaction between TFF1 and *H. pylori*. This pH range is typical of that found in the mucosal niche occupied by *H. pylori in vivo* supporting the possibility that *H. pylori* binding to TFF1 may promote bacterial colonization in the stomach (Schreiber et al., 2004; Reeves et al., 2008).

From the above it is clear that while on the one hand components of mucus influence bacterial colonization and virulence properties, on the other, the presence of bacteria has a marked effect on mucin expression. Therefore, while the mucus layer and its constituents form part of the innate defense against pathogens, mucosal organisms have evolved remarkable strategies to circumvent (or even subvert for their own advantage) this barrier.

THE INTERACTION OF *C. JEJUNI* WITH MUCUS AND MUCINS

MUCUS AND MUCINS AS A BARRIER IN *C. JEJUNI* INFECTION

Much of our understanding of how intestinal mucins protect the mucosa from infection with *C. jejuni* has evolved only recently, led by groundbreaking work from McGuckin and colleagues at the University of Queensland (McAuley et al., 2007; Linden et al., 2008a,b; Dawson et al., 2009). The cell surface mucin, Muc1, was found to be upregulated following infection with both the natural mouse pathogen *Citrobacter rodentium* and with *C. jejuni* (McAuley et al., 2007; Linden et al., 2008a). Muc1 negative mice were shown to have a marked increase in translocation of *C. jejuni* to systemic organs. In addition Muc1 is upregulated in human colonic biopsies following naturally occurring *C. jejuni* infection (Linden et al., 2008a). A not dissimilar role for mucin sulfation in the protection of mice from *C. jejuni* systemic infection (a marker of intestinal barrier function) subsequently was demonstrated using a mouse Nas1 knockout model (Dawson et al., 2009). Interestingly, while Muc1 also has a role in protecting mice from *H. pylori* infection, it does not help protect mice from the pathologic effects of *H. felis* infection (Every et al., 2008). From these experiments, it is obvious that the mucus barrier has evolved a complex array of protective mechanisms against infection and these appear not to be simply generic antibacterial barriers. Nonetheless, it will be important to attempt to document the role of these mucus characteristics in *Campylobacter* models that approximate more closely those of human infection, a problem that continues to bedevil the wider field of *Campylobacter* pathogenesis.

ADAPTATIONS OF *C. JEJUNI* TO LIFE IN THE MUCUS LAYER

Ultimately, *C. jejuni* is very successful in overcoming the intricate barrier structures posed by mucus and mucins in order to establish enteric infection, at least in the human intestinal tract. While the exact details of how *C. jejuni* establishes infection remain elusive, the complex interplay between bacterium and host mucosal surface has been the subject of intensive research in recent years and an understanding is beginning to emerge of the role of mucus in that relationship.

Motility/chemotaxis

Flagellar motility and chemotaxis toward components of mucus are thought to be crucial in the life cycle of *C. jejuni*, both as a commensal and as a pathogen. Virulence in the ferret model, host colonization, adhesion, and invasion all require flagellar motility (Young et al., 2007). Chemotaxis receptors together with other elements of the *C. jejuni* chemotaxis and motility machinery are known to be required for colonization in a chick model of infection (Hendrixson and DiRita, 2004). Likewise, flagellar genes and regulators of flagellar biosynthesis such as FlgR, σ^{28}, and σ^{54} are all required for chick colonization (Hendrixson, 2006). Chemoattractants for *C. jejuni* include amino acids such as L-glutamate, L-serine, L-cysteine, L-aspartate, and the sugar fucose (Hugdahl et al., 1988; Szymanski et al., 1999).

In solutions of increased viscosity comparable to mucus, *C. jejuni* is highly motile, demonstrating peaks of motility with longer path lengths (Ferrero and Lee, 1988; Szymanski et al., 1995). The corkscrew-like shape of these bacteria and the relatively short O-side chains of its lipooligosaccharide (LOS) are purported to prevent non-specific binding of *C. jejuni* to mucin glycoproteins (McSweegan and Walker, 1986). This suggests an adaptation of these bacteria to the environment of mucus, thus conferring *C. jejuni* with an ecological advantage while in mucus (Ferrero and Lee, 1988). Increased motility of *C. jejuni* in highly viscous solutions correlates with increased virulence *in vitro*. Pre-incubation of *C. jejuni* in media of increased viscosity increases bacterial binding and internalization into Caco-2 cells (Szymanski et al., 1995).

Reproduction in mucus

Campylobacter jejuni has been shown to reproduce in chicken mucus (Van Deun et al., 2008). Van Deun et al. showed that *C. jejuni* multiplies in PBS supplemented with mucus but not in

PBS alone or chicken cecal contents. In agreement with these findings, we have recently demonstrated *C. jejuni* replication in growth medium supplemented with mucus derived from a mucus-adherent subclone of methotrexate treated HT29 cells, HT29MTXE12 (E12; Alemka et al., 2010a). We also showed that *C. jejuni* reproduced following colonization of E12 cells (with mucus), compared with HT29 cells that lacked a mucus layer. Taken together, these studies indicate that *C. jejuni* grows and thrives in the mucus milieu, an adaptation that likely helps to ensure its persistence in the host.

Adhesion/invasion and pathogenicity

Conventional tissue culture has contributed greatly to our understanding of how enteric pathogens such as *C. jejuni* elaborate disease. For instance, infection studies with intestinal epithelial cells led to the identification of *C. jejuni* adhesins such as CadF. CadF, also required for chick colonization (Ziprin et al., 1999), binds fibronectin located at the basolateral aspects of cells, and facilitates bacterial internalization (Monteville et al., 2003; Krause-Gruszczynska et al., 2007). Likewise, JlpA, a lipoprotein on the surface of *C. jejuni*, interacts with heat shock protein 90α leading to the activation of p38 mitogen activated protein kinases (MAPK) and NF-κB, thereby eliciting pro-inflammatory responses in Hep-2 cells (Jin et al., 2003). In addition, both microtubules (Hu and Kopecko, 1999) and the actin cytoskeleton (Monteville et al., 2003; Krause-Gruszczynska et al., 2007) are involved in *C. jejuni* invasion. Furthermore, these bacteria translocate cultured intestinal epithelial cells by migrating through both the paracellular (Monteville and Konkel, 2002) and transcellular routes (Bras and Ketley, 1999; Hu et al., 2008).

Despite the utility of conventional cell lines, the absence of an adherent mucus layer on these models is a significant weakness. In order to overcome this, we have attempted to better understand the role of mucus in infection by characterizing the interaction of *C. jejuni* with E12 cells (Behrens et al., 2001; Alemka et al., 2010a). E12 cells were first validated as an appropriate *in vitro* model of *Campylobacter*–host interactions by demonstrating mucus colonization (**Figure 1**), adhesion, invasion, and translocation to the basolateral aspects of cells. Compared with parental HT29 cells that lack a mucus layer, there was enhanced bacterial binding and internalization into E12 cells (Alemka et al., 2010a). Combined with previous studies (de Melo and Pechere, 1988; Szymanski et al., 1995; Byrne et al., 2007) these findings indicate that *C. jejuni* not only has evolved mechanisms to breach the protective mucus barrier layer, but also appears to have subverted the mucus to enhance pathogenicity. In support of this concept, the presence of MUC2 (the main intestinal gel-forming mucin) has been shown to upregulate a number of *C. jejuni* virulence genes, including those involved in motility (*flaA*), adhesion (*jlpA*), invasion (*ciaB*), and toxin formation (*cdt*; Tu et al., 2008).

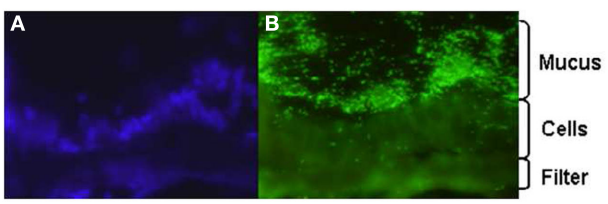

FIGURE 1 | Fluorescence microscopy image of *C. jejuni* colonization of E12 mucus. E12 cells were grown for 14 days in transwell inserts and infected with *C. jejuni*. The filter was excised and wrapped between two pieces of frozen liver and sections probed with DAPI **(A)** to reveal cell nuclei and **(B)** anti-*Campylobacter* antibodies. Mag: ×400.

Which component or characteristic of mucus and mucin that modulates *C. jejuni* pathogenicity is not known. However, recent studies have suggested some intriguing possibilities. It has long been known that *C. jejuni* does not metabolize glucose due to the absence of the enzyme phosphofructokinase in its genome (Parkhill et al., 2000; Guccione et al., 2008) and, because of the absence of genes involved in sugar metabolism (Parkhill et al., 2000; Muraoka and Zhang, 2011), it has generally been assumed that this organism is unable to use any form of carbohydrate as a substrate. Rather these bacteria use amino acids such as glutamate, asparagine, proline, serine, and aspartate as a source of carbon (Guccione et al., 2008; Hofreuter et al., 2008).

However, *C. jejuni* was recently shown to metabolize fucose (Muraoka and Zhang, 2011; Stahl et al., 2011). In these studies, growth of *C. jejuni* was enhanced in media supplemented with fucose. Several strains of *C. jejuni* now have been shown to possess a genomic island that is linked with the ability to take up and utilize fucose as a source of carbon (Stahl et al., 2011). In light of the fact that *Campylobacter* displays chemotaxis to mucin (which harbors fucosylated glycans), binds to mucin (Sylvester et al., 1996), and the finding that these bacteria reproduce in both human and chicken mucus (Van Deun et al., 2008; Alemka et al., 2010a), it is tempting to speculate that *C. jejuni* acquires and utilizes constituents of mucus to sustain its commensal and pathogenic modes of existence, and that differences in the composition of mucus across species may contribute to *C. jejuni* pathogenicity in humans as opposed to commensalism in other animals (discussed below). Furthermore, the importance of the interaction of *C. jejuni* with fucosylated structures possibly could be exploited as a novel antimicrobial therapy in *C. jejuni* infections (Ruiz-Palacios et al., 2003).

Influence of gut microbiota in the mucus layer

In the gut, microbiota are known to colonize the outer loose mucus layer (Johansson et al., 2008). It is thought that microbiota protect the gut from invading enteric pathogens by competing for nutrients and mucosal binding sites with pathogens, by modulating the mucosal immune response and also through direct antagonism (Neish, 2009). Many commensal derived bacteria have been proposed as potential anti infective probiotics. On this basis, we investigated the effect of probiotic colonization of the mucus of E12 cells on *C. jejuni* pathogenicity (Alemka et al., 2010a). Individual probiotic strains (*Lactobacillus rhamnosus*, *L. salivarius*, *Bifidobacterium longum*), or cocktails of these probiotics, reduced *C. jejuni* colonization, invasion, and translocation of mucus-adherent E12 cells in a strain dependent manner. Pre-colonization of E12 mucus with probiotics was required to attenuate *C. jejuni* virulence, suggesting that probiotics have a potential as prophylactic agents against *C. jejuni* infections. The potential use of probiotics to reduce

Campylobacter numbers in chickens and thereby prevent zoonotic transmission may be a particularly attractive strategy to reduce the burden of illness in humans.

CHICKEN MUCUS AND *CAMPYLOBACTER* COMMENSALISM

The consumption of contaminated poultry is the principal mode of transmission of *C. jejuni* to humans. Up to 100% of point-of-sale chicken has been shown to be contaminated with *C. jejuni* (Madden et al., 1998; Pearson et al., 2000). Despite heavy colonization (up to 10^{10} colony forming units per gram of infected intestine) in chickens, *C. jejuni* does not cause disease. These bacteria avidly colonize the mucus layer of deep cecal crypts, but do not adhere to or invade chicken intestinal epithelial cells (reviewed in Young et al., 2007).

Why *C. jejuni* is pathogenic in the human population but establishes a commensal mode of existence in chickens is uncertain. An obvious difference between humans and chickens is body temperature. The human body temperature is 37˚C while it ranges from 41 to 45˚C in chickens. Campylobacters are therefore adapted to higher temperatures in chickens but the effect of temperature on the pathogenicity of *C. jejuni* is not yet fully understood. The two-component system RacRS has been implicated as possibly influencing the adaptation of *C. jejuni* to temperature. Mutants in this regulatory pathway show defects in viability at 42˚C, suggesting that it may contribute to the survival and growth of *C. jejuni* at higher temperatures (MacKichan et al., 2004). In addition, differences in *Campylobacter* gene expression have been reported following growth at 37˚C compared with 42˚C. In one such study (Stintzi, 2003), at 42˚C, there was an increase in the expression of chemotaxis and motility genes and an overall 20% general difference in gene expression compared with 37˚C. Therefore, body temperature may influence the commensal versus pathogenic lifestyles of *C. jejuni*. However, temperature alone is unlikely to account for the difference in clinical manifestation across species.

We originally hypothesized that species-specific tissue tropism might contribute to the differences in clinical outcome of *Campylobacter* infection in humans and chickens. However, we (Byrne et al., 2007) and others (Van Deun et al., 2008) subsequently showed that *C. jejuni* both adheres to and invades chicken primary intestinal cells. These data indicate that *Campylobacter* commensalism in chickens as opposed to pathogenicity in humans is not due to lack of the relevant adhesin–receptor interactions that mediate invasiveness in the chicken intestinal epithelium. In light of these findings we investigated the possibility that intestinal mucus contributes to the inhibitory effect of the chicken intestinal milieu on *C. jejuni* virulence. We demonstrated that crude chicken intestinal mucus, markedly attenuates *C. jejuni* binding and internalization (Byrne et al., 2007). Consistent with these findings, mucus colonization of the human mucus-adherent E12 cell line increased *C. jejuni* adhesion and invasion of E12 cells (Alemka et al., 2010a). Subsequently we demonstrated that purified chicken intestinal mucins, when pre-incubated with *C. jejuni*, preferentially attenuate bacterial internalization into HCT-8 cells (**Figure 2**). The effect depends on the region of the chicken gut from where mucins are isolated (Alemka et al., 2010b). In the same study, attenuation of bacterial invasion by chicken mucins appeared to have been mediated by mucin glycans, since sodium metaperiodate treatment of chicken mucins abolished the attenuating effect of purified mucins on bacterial internalization. Intriguingly, removal of terminal fucose or sialic acid residues failed to inhibit the effect of chicken mucin on *C. jejuni* invasion. However, as chicken mucin glycoproteins are heavily sulfated compared with human mucins (unpublished observations), sulfation may have limited the activity of the fucosidase and sialidase enzymes used in our study (Alemka et al., 2010b). Conversely, it is possible that sulfation of mucin oligosaccharides may in itself influence *C. jejuni* mucus colonization and pathogenicity across species. Taken together, these data indicate that mucus and mucins modulate *C. jejuni* virulence properties and may contribute to the differential clinical manifestations exhibited by *C. jejuni* in different hosts (**Figure 3**).

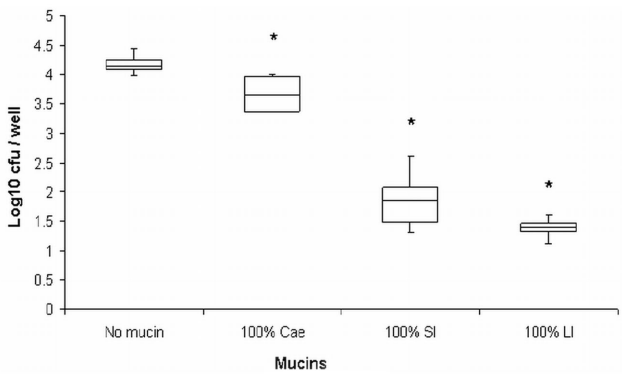

FIGURE 2 | Purified chicken intestinal mucins attenuate *C. jejuni* invasion depending on the region of the chicken gut from which mucins were isolated (Alemka et al., 2010b, reproduced with permission). *C. jejuni* was pre-incubated with mucins prior to infecting HCT-8 cells. Cae, mucin from the cecum; SI, small intestinal mucins; LI, large intestinal mucins. *Denotes significant difference compared to control. Cae $p < 0.05$, $p < 0.005$ for SI and LI. Hundred percentage mucin represents 1 mg/ml.

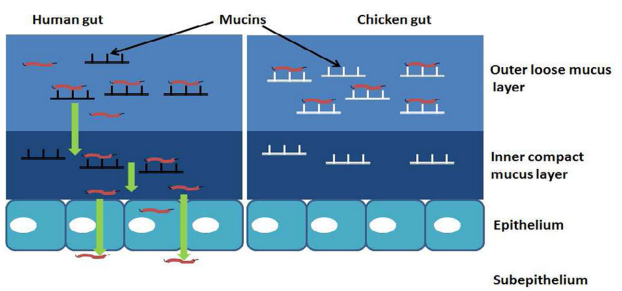

FIGURE 3 | Hypothetical model of how mucus and mucins modulate the pathogenicity of *C. jejuni* in a species-specific manner: in mucus *C. jejuni* displays chemotaxis and flagellar motility toward components of mucin. The characteristic spiral shape and the ability of *C. jejuni* to replicate in mucus suggests an adaptation of these bacteria to the mucus milieu. Interaction with human mucin enhances bacterial binding and internalization into underlying epithelial cells. In chickens, mucin glycoproteins prevent *C. jejuni* association with intestinal epithelial cells, possibly by interfering with their transit through the inner mucus layer and/or onto the appropriate host cell receptors.

CONCLUSION AND FUTURE PERSPECTIVES

The visco-elastic mucus gel layer is part of the innate defense mechanism against infecting bacteria. Mucus colonization by enteric pathogens influences the outcome of infection with these bacteria employing a variety of mechanisms to circumvent the mucus layer and gain access to underlying epithelial cells. *C. jejuni* is attracted to and metabolizes components of mucus. These bacteria are highly motile with a characteristic spiral shape and corkscrew-like motility that suggests an adaptation to the environment of mucus. Recent studies indicate that mucus and mucins influence *C. jejuni* in a species-specific manner with chicken mucins attenuating *C. jejuni* pathogenicity. Therefore, differences in the composition of mucus and mucins across species appear to contribute to *C. jejuni* disease causing lifestyle in the human population, as opposed to commensalism in chickens. Increased knowledge and understanding of the interaction of *Campylobacter* with mucus and mucins has the potential to lead to the development of intervention strategies that take advantage of the propensity of these bacteria to inhabit the intriguing supramucosal niche that is the mucus layer.

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Nutrient acquisition and metabolism by *Campylobacter jejuni*

Martin Stahl, James Butcher and Alain Stintzi*

Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada

Edited by:
D. Scott Merrell, Uniformed Services University of the Health Sciences, USA

Reviewed by:
Erin Gaynor, University of British Columbia, Canada
Arnoud H. M. Van Vliet, Institute of Food Research, UK

***Correspondence:**
Alain Stintzi, Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5.
e-mail: astintzi@uottawa.ca

The gastrointestinal pathogen *Campylobacter jejuni* is able to colonize numerous different hosts and compete against the gut microbiota. To do this, it must be able to efficiently acquire sufficient nutrients from its environment to support its survival and rapid growth in the intestine. However, despite almost 50 years of research, many aspects as to how *C. jejuni* accomplishes this feat remain poorly understood. *C. jejuni* lacks many of the common metabolic pathways necessary for the use of glucose, galactose, or other carbohydrates upon which most other microbes thrive. It does however make efficient use of citric acid cycle intermediates and various amino acids. *C. jejuni* readily uses the amino acids aspartate, glutamate, serine, and proline, with certain strains also possessing additional pathways allowing for the use of glutamine and asparagine. More recent work has revealed that some *C. jejuni* strains can metabolize the sugar l-fucose. This finding has upset years of dogma that *C. jejuni* is an asaccharolytic organism. *C. jejuni* also possesses diverse mechanisms for the acquisition of various transition metals that are required for metabolic activities. In particular, iron acquisition is critical for the formation of iron–sulfur complexes. *C. jejuni* is also unique in possessing both molybdate and tungsten cofactored proteins and thus has an unusual regulatory scheme for these metals. Together these various metabolic and acquisition pathways help *C. jejuni* to compete and thrive in wide variety of hosts and environments.

Keywords: *Campylobacter jejuni*, metabolism, metal transport, nutrient acquisition

INTRODUCTION

Campylobacter jejuni is the most common cause of gastroenteritis in the developed world. The most frequent sources of infection are through contaminated food, particularly poultry products, or through contaminated raw milk or drinking water (Skirrow, 1991). When consumed by its potential host, *C. jejuni* will pass through the stomach and establish itself in the host intestinal tract. *C. jejuni* causes gastroenteritis in humans, with infections typically characterized by diarrhea (sometimes bloody), abdominal pain, and fever (Galanis, 2007). However, *C. jejuni* will colonize to high levels in poultry or other birds without eliciting an immune response. This commensal colonization allows *C. jejuni* to become established in poultry flocks, which often serve as a reservoir for human infection (Lee and Newell, 2006). Poultry meat is easily contaminated during processing, and if the resulting meat is not properly prepared, *C. jejuni* can cause infection in humans with a dose as small as a few hundred bacterial cells (Robinson, 1981).

While we have acquired significant knowledge about *Campylobacter*, especially since its genome was first sequenced in 2000 (Parkhill et al., 2000), many aspects of its biology have remained a mystery. Recent work on *C. jejuni* has allowed us to better understand how *C. jejuni* acquires and metabolizes key nutrients (Velayudhan et al., 2004; del Rocio Leon-Kempis et al., 2006; Hofreuter et al., 2008; Stahl et al., 2011), but we are still only beginning to gain a full appreciation for the role these nutrients play in host colonization. To be successful, *C. jejuni* must possess the ability to survive in a wide variety of hosts, from an avian host, to its pathogenic colonization of a human host. *C. jejuni* must also be able to survive in the environment, in water or food, before being ingested by potential hosts. This requires a certain degree of metabolic flexibility to make sufficient use of the different metabolites that are available in each niche. The extent of *C. jejuni's* metabolic flexibility is particularly impressive given the relatively small number of different metabolic pathways initially identified in its genome (Parkhill et al., 2000).

CARBON AND NITROGEN ACQUISITION AND METABOLISM

GLYCOLYTIC PATHWAYS

Unlike most other bacteria, *C. jejuni* lacks the ability to use many common carbohydrates as carbon sources (Parkhill et al., 2000). Aside from lacking the proper transporters to take up sugars like glucose or galactose, it also lacks several key enzymes within the glycolytic pathway. Although *C. jejuni* possesses the complete set of enzymes necessary for the synthesis of glucose-6-phosphate from pyruvate via gluconeogenesis (Velayudhan and Kelly, 2002), it lacks any form of glucokinase to phosphorylate extracellular glucose. *C. jejuni* is also missing the 6-phosphofructokinase enzyme that is required for the irreversible phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate during glycolysis. Interestingly, it still possesses pyruvate kinase, an enzyme that is required for another irreversible step of glycolysis, but not gluconeogenesis (Velayudhan and Kelly, 2002). It has been hypothesized that *C. jejuni* may catabolize some molecules such as glycerol-3-phosphate, which would require the presence of only

the final portions of the glycolytic pathway. The use of glycerol-3-phosphate is supported by the presence of a glycerol-3-phosphate transporter (GlpT) in the strain 81–176 (Hofreuter et al., 2006). In addition to this, although *C. jejuni* possesses the enzymes transaldolase, transketolase, ribulose-3-phosphate epimerase, and ribose-5-phosphate isomerase, which make up the non-oxidative portion of the pentose phosphate pathway, it completely lacks the oxidative portion of the pathway (Velayudhan and Kelly, 2002; Line et al., 2010). This precludes the possibility that it may be able to metabolize pentose sugars through this pathway (Line et al., 2010), but the function of the remaining genes in *C. jejuni* remains unknown.

The annotation of the *C. jejuni* genome also failed to identify any additional carbohydrate metabolic pathways, however, both Muraoka and Zhang (2010) and Stahl et al. (2011), independently identified a novel L-fucose pathway present within certain strains of *C. jejuni.* These reports identified a L-fucose permease (Cj0486/FucP), present within a genomic island encompassing the genes *cj0480c–cj0490.* This permease has 37.2% amino acid identity to the L-fucose permease identified in *E. coli* and contains three highly conserved amino acids (Asp46, Glu135, and Asn162), which have been found previously to be critical for L-fucose active transport (Dang et al., 2010). Also contained within this genomic island is a second transporter, Cj0484, annotated as a MFS superfamily efflux pump. However, it does not bear any significant similarities to L-fucose permeases and the function of this transporter remains unknown. Despite strong similarities between the *E. coli* and *C. jejuni* permeases themselves, the rest of the pathway in *C. jejuni* does not resemble the known L-fucose metabolic pathways characterized in *E. coli* (Baldoma and Aguilar, 1988) or *Bacteroides* spp. (Coyne et al., 2005). The *C. jejuni* pathway does however show some similarities to a L-fucose dehydrogenase dependent pathway found in *Xanthomonas campestris* (Yew et al., 2006; Stahl et al., 2011). Despite these similarities, the precise mechanisms of this pathway remain unknown, and will require further study.

Although this pathway was completely necessary for the uptake and metabolism of L-fucose *in vitro*, when colonizing the intestine, its utility appears to be host dependent. Chick colonization assays using mutants in the FucP permease did not show any impairment for colonization relative to the wild-type strains. When the same colonization assay was performed in a colostrum-deprived piglet model of infection, a roughly 15- to 100-fold reduction in the mutant relative to wild-type was observed. This reduction relative to the wild-type demonstrates that this pathway is advantageous for colonization of certain hosts, but not completely necessary for colonization (Stahl et al., 2011).

AMINO ACIDS

Among the primary nutrient sources for *C. jejuni,* are a handful of amino acids that it can acquire while growing within the gut. *C. jejuni* also will draw on available amino acids in a sequential manner, with serine, aspartate, asparagine, and glutamate being preferred in that order, although this will vary between strains. Proline will also be metabolized, but only after other nutrients have begun to become exhausted (Wright et al., 2009). It should also be noted that serine, aspartate, glutamate, and proline make up some of the most common amino acids found in chick excreta, perhaps explaining why they play such a central role in *C. jejuni* metabolism (Parsons, 1984).

Serine

Serine metabolism is achieved due to the action of the SdaC (Cj1624c) and SdaA (Cj1625c) proteins (Mendz et al., 1997; Velayudhan et al., 2004). SdaC acts as a transporter for serine into the cell, while SdaA is a L-serine dehydratase. Work by Velayudhan et al. (2004) found that the SdaC transport protein acts as a high capacity, low affinity L-serine transporter. Deletion mutants into this gene completely impaired serine uptake into the cell, indicating that SdaC is the sole transporter capable of transporting significant amounts of L-serine into the cell. Additionally, due to SdaC's low specific affinity for L-serine, SdaC also exhibits some ability to transport other amino acids into the cell as well, particularly L-threonine, raising the possibility this transporter plays a broader role in amino acid uptake (Velayudhan et al., 2004).

In the same study, the SdaA protein was confirmed to be an L-serine dehydratase and was necessary for the metabolism of L-serine (Velayudhan et al., 2004). Interestingly, unlike most other L-serine dehydratases, the protein found in *C. jejuni* was not pyridoxal-5′-phosphate dependent, but rather utilized a [4Fe–4S] cluster in a manner more reminiscent of many anaerobic bacteria (Velayudhan et al., 2004). This enzyme is one among many enzymes in key metabolic pathways that contain oxygen sensitive iron–sulfur complexes (**Figure 1**). It has been proposed that the presence of these iron–sulfur containing enzymes is one of the key reasons for *C. jejuni*'s sensitivity to oxygen, as molecular oxygen or reactive oxygen species, in sufficient quantity will oxidize and damage the iron–sulfur complexes (Flint et al., 1993).

L-Serine utilization has also been found to be absolutely critical for host colonization, with mutants in SdaC or SdaA being completely impaired for colonization in chicks (Velayudhan et al., 2004). However, these same mutants were able to successfully grow *in vitro.* This result suggests that while L-serine is not essential for *C. jejuni* growth, it is necessary in the environment of the gut, where L-serine usage must provide *C. jejuni* with a key carbon source while establishing itself within the intestinal microbiota (Velayudhan et al., 2004).

Aspartate/glutamate

Aspartate and glutamate metabolism are very similar to each other. Both amino acids are taken up through the Peb1 system, composed of the proteins Cj0919c, Cj0920c, Cj0921c (Peb1A), and Cj0922c (PebC). Work by del Rocio Leon-Kempis et al. (2006) has confirmed the function of Peb1A as the transporter responsible for most, if not all, of the uptake of aspartate and glutamate into the cell. The Peb1a protein itself had been previously identified by Fauchere et al. (1989) as linked to cell adhesion and further work using mutational analysis also found that mutants in Peb1A were significantly impaired for adhesion to epithelial cells (Pei et al., 1998). del Rocio Leon-Kempis et al. (2006) confirmed that this protein was found in the periplasm, and mutants in the gene for Peb1a were mostly impaired for L-aspartate uptake and were completely impaired for L-glutamate uptake. The remaining uptake of L-aspartate in the mutant strain was attributed to the function of the C4-dicarboxylate transporters (DctA, DcuA, and DcuB), but

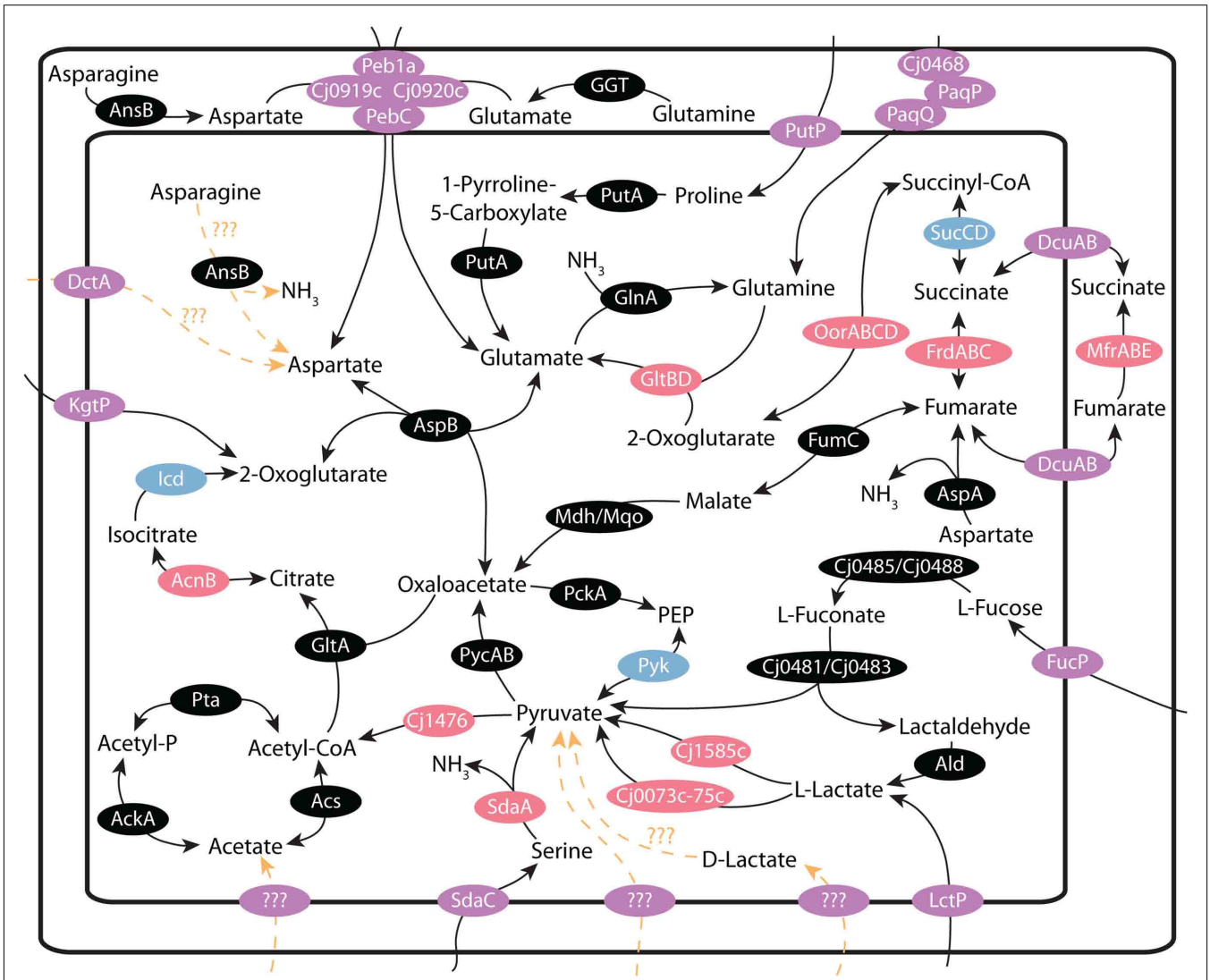

FIGURE 1 | Central metabolism in *C. jejuni*: schematic of the transporters and anaplerotic reactions that make up the central metabolic pathways of *C. jejuni*. The function and experimental evidence for each reaction is explained in the text. Note the central role that pyruvate plays in *C. jejuni* central metabolism; as serine, lactate, and l-fucose metabolism results in the production of pyruvate. Glutamate and aspartate also play key roles in the production of citric acid cycle intermediates. Enzymes or enzyme complexes containing iron–sulfur complexes are indicated in pink, magnesium containing enzymes are indicated in blue, transporters are denoted as purple and hypothetical reactions are indicated in orange.

was too minimal to allow for growth utilizing L-aspartate or L-glutamate as a sole carbon source (del Rocio Leon-Kempis et al., 2006).

Once glutamate has been taken up into the cell, the enzyme AspB (Cj0762c), allows for the transamination of glutamate to aspartate. One molecule of glutamate and oxaloacetate are used to produce aspartate and 2-oxoglutarate in a reversible reaction (Guccione et al., 2008). The aspartate can then be deaminated by the aspartase AspA (Cj0087) into fumarate, which can be fed into the citric acid cycle (Guccione et al., 2008).

Proline

Proline has been found to be a less preferred amino acid compared to serine, glutamate, and aspartate, but is still utilized by most strains of *C. jejuni* (Wright et al., 2009). As a major constituent of mucins, along with L-serine and L-threonine, it is a plentiful nutrient source within the intestine. Proline uptake and metabolism has been linked to the proteins PutP and PutA. PutP (Cj1502c) acts as a sodium/proline symporter, while PutA (Cj1503c) is a bifunctional proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase. PutA works to consume L-proline and FAD, producing 1-pyrroline-5-carboxylate, while reducing the electron acceptor FAD to $FADH_2$. PutA subsequently catalyzes the second step of the pathway, by acting as a 1-pyrroline-5-carboxylate dehydrogenase, and oxidizes 1-pyrroline-5-carboxylate to glutamate with the concomitant reduction of NAD to NADH. Glutamate can then be further metabolized to aspartate and subsequently fumarate as described above. Although neither PutP nor PutA has been

specifically studied in *C. jejuni,* they are closely homologous to the well-studied PutA and PutP proteins in *E. coli* (Tanner, 2008; Zhou et al., 2008) and *Helicobacter* spp. (Krishnan et al., 2008; Nakajima et al., 2008). In *Helicobacter*, PutA has been found to be a key factor for stomach colonization and swarming motility (Nakajima et al., 2008). Whether PutA may have similar importance in *C. jejuni* has yet to be described.

Asparagine

While most strains of *C. jejuni* possess an asparaginase gene (*ansB*), *C. jejuni* does not possess any identifiable asparagine transporters, and indeed many strains that possess *ansB* (cj0029) are unable to grow on asparagine as a primary carbon source. Nevertheless, certain strains, including 81–176, possess an *ansB* gene that contains an additional ∼40 bp sequence that encodes for a Sec-dependent secretion signal peptide (Hofreuter et al., 2008). The AnsB protein is normally found exclusively in the cytoplasm, however with this secretion signal peptide, AnsB is transported to the periplasm, where it would be capable of deaminating periplasmic asparagine to aspartate. Thus strains that lack an asparagine transporter can utilize asparagine since the aspartate formed by AnsB can be transported to the cytoplasm by the Peb1 complex and fed to the citric acid cycle (CAC) by AspA (Hofreuter et al., 2008).

Interestingly, it was found that there are two alternate transcriptional start sites in the *ansB* gene of 81–176, allowing it to produce both the cytoplasmic and periplasmic versions of AnsB. It has been hypothesized by Hofreuter et al. (2008) that the cytoplasmic AnsB might metabolize asparagine originating from small peptides. Although little research has been done to identify small-peptide transporters in *C. jejuni*, *cstA* (Cj0917c) has been annotated as a small-peptide transporter and could possibly serve this purpose. Mutants in the *asnB* gene and/or its signal peptide are still capable of colonizing the intestine in a comparable fashion to the wild-type strain, however, it was found that strains lacking the periplasmic AnsB suffered a significant defect for liver colonization in infected mice. This suggests a tissue specific value for asparagine metabolism (Hofreuter et al., 2008).

Glutamine

The ability of *C. jejuni* to utilize glutamine has remained somewhat controversial. Based on gene annotations and experimental evidence, *C. jejuni* contains a functional glutamine uptake system, encoded by the genes *cj0467* (*paqP*), *cj0468*, and *cj0469* (*paqQ*; Lin et al., 2009). Additionally, *C. jejuni* contains genes encoding for a glutamate dehydrogenase (*gltBD*), capable of converting glutamine and 2-oxoglutarate into two molecules of glutamate. Finally, *C. jejuni* also contains GlnA for the reverse reaction of converting glutamate to glutamine in an ATP dependant manner. This reaction also serves as a means to incorporate free ammonia, a major route of nitrogen assimilation for *C. jejuni*. However, despite having the biochemical means of transporting and converting glutamine to glutamate via glutamate dehydrogenase, the *C. jejuni* NCTC11168 strain has never been shown to be able to utilize glutamine as a primary carbon source (Hofreuter et al., 2008), unlike the other previously discussed amino acids. However other *C. jejuni* strains, such as 81–176 and 81116, contain γ-glutamyl transpeptidase (GGT) and are readily able to use glutamine as a primary carbon source (Hofreuter et al., 2006). GGT is secreted to the periplasm, and acts to hydrolyze either glutamine to glutamate and ammonia, or glutathione to glutamine and a γ-glutamylcysteine. This whole process takes place within the periplasm of the cell. While *C. jejuni* has not been shown to grow on γ-glutamylcysteine, the Peb1 complex readily takes up the glutamate produced from glutathione or glutamine, where it can be metabolized by AspA and AspB as previously discussed (Hofreuter et al., 2008).

The presence of the GGT enzyme in certain strains appears to have a significant effect on colonization. Knockouts of the GGT enzyme (Cjj_0067) in the 81–176 strain impaired its colonization of MyD88$^{-/-}$ deficient mice (Hofreuter et al., 2008), while a GGT knockout (C8j_0033) in the 81116 strain was defective for chick colonization (Barnes et al., 2007). Although both of these knockouts showed considerable colonization disadvantages relative to their wild-type strains, other strains of *C. jejuni* lacking GGT are perfectly capable of colonizing these two hosts (Barnes et al., 2007; Hofreuter et al., 2008). This suggests that individual strains may have varying metabolic requirements, making certain pathways dispensable for them. The lack of GGT in a strain such as NCTC11168 may be compensated for the presence of other pathways, such as the recently discovered L-fucose acquisition and metabolic pathway (Stahl et al., 2011). Overall, the diversity of available mechanisms for amino acid acquisition and utilization between strains may allow different *C. jejuni* strains to co-colonize and to colonize multiple niches or different hosts, thereby expanding the versatility of the species.

SHORT CHAIN FATTY ACIDS

Within the ecosystem of the gut, large amounts of short chain fatty acids (SCFA) are produced as metabolic byproducts from certain bacteria. The host or other bacteria subsequently metabolize these SCFA. One example of this is the SCFA butyrate, which is produced as a byproduct of acetate metabolism in certain bacteria (Duncan et al., 2004). Butyrate is readily taken up by colonocytes and serves as a primary energy source for these cells (Louis et al., 2010). The most significant SCFA found within the gut is acetate (Duncan et al., 2004), with smaller amounts of lactate, propionate, and butyrate also present (Belenguer et al., 2011). Although there are bacteria that will make use of all of these molecules, known pathways and experimental evidence in *C. jejuni* suggest that it only has the capacity to transport and metabolize acetate and lactate (Wright et al., 2009; Thomas et al., 2011).

Acetate

Acetate is initially produced and secreted as a byproduct in several of *C. jejuni's* primary metabolic pathways such as L-serine catabolism. However, in later stages of growth, when more preferable sources are exhausted, acetate can also be used as a carbon source for *C. jejuni*. Although a transporter for acetate has not been identified in *C. jejuni*, with the exception of an annotated acetate permease (*actP*) in *C. jejuni* subsp. *doylei* (Parker et al., 2007), it has been experimentally demonstrated *in vitro* that *C. jejuni* has the ability to use acetate as a primary carbon source (Wright et al., 2009).

The production and excretion of acetate comes as the result of the need to recycle CoA. The enzyme Pta (Cj0688) produces acetyl-phosphate from acetyl-CoA, freeing the CoA for reuse. The enzyme acetate kinase [AckA (Cj0689)] then produces acetate from acetyl-phosphate, which is excreted and builds up in the surrounding medium. As other sources of nutrients become scarcer, a switch in the direction of the pathway allows for the uptake of acetate into the cell, and the synthesis of acetyl-CoA from acetate by Acetyl-CoA synthase [Acs (Cj1537c)], or by reversing the direction of the AckA–Pta pathway (Velayudhan and Kelly, 2002; Wright et al., 2009).

Lactate

Lactate is also produced in large quantities in the colon as a byproduct of carbohydrate fermentation by numerous varieties of bacteria commonly found in the gut. However, despite its rapid production, overall levels of lactate within the gut remain relatively low, as high rates of production are matched by rapid consumption of lactate by other bacteria (Belenguer et al., 2011). A recent study by Thomas et al. (2011) has well documented the usage of lactate by *C. jejuni*, although several points still require further research.

While lactate is a common byproduct of fermentation in some bacteria, there is no evidence of lactate excretion by *C. jejuni*. Moreover, while *C. jejuni* possesses an annotated L-lactate permease (*lctP*) a mutant into the *lctP* gene (*cj0076c*), was still capable of some L-lactate transport, suggesting the existence of a second, as of yet unidentified lactate permease (Thomas et al., 2011).

Once inside the cell, L-lactate can be catabolized into pyruvate through one of several pathways. The first involves three genes, *cj0073c*, *cj0074c*, and *cj0075c* (*lldEFG/lutABC*). These three genes code for a non-flavin iron–sulfur containing oxidoreductase complex that demonstrate NAD-independent, L-lactate dehydrogenase activity (L-iLDH), thereby converting L-lactate to pyruvate (Thomas et al., 2011). A second, flavin and iron–sulfur containing enzyme Cj1585c (Dld), also demonstrated L-iLDH activity, although the reason for this redundancy is not immediately clear, and indeed, several strains of *C. jejuni* such as the 81–176 and 81116 strains, lack homologs to *cj1585c*. A lactate dehydrogenase (Ldh) was also annotated in the *C. jejuni* genome; however, Thomas et al. were unable to find a role for this enzyme in lactate metabolism. Knocking out *ldh* (*cj1167*) did not impede lactate utilization, while a double knockout in the other two pathways completely eliminated lactate utilization, suggesting that this gene has been either misannotated, or is used under only specific conditions not found in their study (Thomas et al., 2011). *C. jejuni* has also been found to grow on D-lactate, and demonstrated D-iLDH activity. However, D-lactate metabolism was not significantly affected in the *cj0075c* or *cj1585c* mutants and no other transporters or pathways have been identified as responsible for this phenotype (Thomas et al., 2011).

CITRIC ACID CYCLE INTERMEDIATES

Campylobacter jejuni depends heavily on the citric acid cycle for its energy needs. All of the aforementioned pathways produce pyruvate, fumarate, oxaloacetate, or 2-oxoglutarate, each of which is fed directly into the citric acid cycle. In addition to this, *C. jejuni* is capable of transporting several of the citric acid cycle intermediates, and using them directly as nutrient sources. For example 2-oxoglutarate can be transported by the permease KgtP (Cj1619). In addition, succinate, fumarate, and malate can all be transported by the C4-dicarboxylate transporters, DcuA (Cj0088), and DcuB (Cj0671) (Guccione et al., 2008). The putative C4-dicarboxylate transporter DctA may also play a minor role in the uptake of both aspartate and CAC intermediates (del Rocio Leon-Kempis et al., 2006; Guccione et al., 2008); however, this has not been conclusively demonstrated. Additionally, there exists the gene *cj0025c*, annotated as a putative sodium:dicarboxylate family transmembrane symporter (Parkhill et al., 2000). However, no research has been done to characterize this transporter as of yet, so its possible role as a dicarboxylate transporter has yet to be described. Many strains also possess a citrate transporter (Cj0203 in *C. jejuni* NCTC11168), allowing for the acquisition of citrate (Parkhill et al., 2000). *C. jejuni* is known to utilize pyruvate as a primary carbon source while it is present in the growth medium (Velayudhan and Kelly, 2002), however, no pyruvate transporters have been identified or studied in *C. jejuni*, leaving the mechanism by which it transports pyruvate into the cell currently unknown.

The dicarboxylate transporters DcuA and DcuB may play an important role in *C. jejuni* thanks to the prominent role of both fumarate and succinate as electron donors and acceptors respectively. *C. jejuni* possesses the cytoplasmic fumarate reductase FrdABC (Cj0408–Cj0410) that is both capable of oxidizing succinate to fumarate and also catalyzing the reverse reaction. Although *C. jejuni* was initially annotated as possessing succinate dehydrogenase (*sdhABC*/Cj0437–Cj439), it was later found that these genes do not encode for succinate dehydrogenase (Weingarten et al., 2009), but rather for a periplasmic methylmenaquinol:fumarate reductase (*mfrABE*) (Guccione et al., 2010). This complex was found to be in the periplasm, and reduced fumarate, crotonate, and mesaconate to succinate, butyrate, and 2-methylsuccinate respectively under low-oxygen conditions. The cytoplasmic–periplasmic membrane transport of succinate and fumarate necessary for these processes is believed to be facilitated by the DcuA and DcuB transporters (Guccione et al., 2010).

TRANSITION METAL UPTAKE AND UTILIZATION BY *C. jejuni*

In addition to metabolites needed for carbon and energy, the need to have a steady source of micronutrients such as transition metals plays a critical role in the growth of any bacterium, including *C. jejuni*. Metals play vital and varied roles in proteins and metabolic processes. While some provide purely structural roles in stabilizing protein conformations, many others are the key elements of enzyme catalytic centers. These catalytic centers include the iron–sulfur complexes present within the active sites of numerous enzymes previously mentioned above and in **Figure 1**. In addition to these previous examples, iron and other metals are critical for the proper functioning of the electron transport chain. Various metals, such as iron and copper, are used as electron donors/acceptors to facilitate electron flow between proteins.

A complete understanding of the roles played by transition metals is still in its infancy in *C. jejuni*. However the information gleaned thus far indicates that transition metal uptake and utilization are important factors for *C. jejuni* growth, colonization, and virulence.

IRON

The importance of iron acquisition to the cell cannot be understated. In the absence of sufficient iron sources, the growth of *C. jejuni* is significantly reduced, if not completely eliminated (Palyada et al., 2004). It is this importance that has led *C. jejuni* and other bacteria to develop a wide variety of mechanisms for iron acquisition; the details of which have been extensively discussed elsewhere and will only be summarized in this review (Stintzi et al., 2008; Miller et al., 2009; Butcher et al., 2010).

One of the key roles for iron within *C. jejuni* is as part of iron–sulfur complexes. These complexes exist in the active sites of several key enzymes within the metabolic networks outlined in **Figure 1**. These include SdaA, AcnB, Cj1585c, Cj0074c, FrdB, MfrB, GltD, OorD, and Cj1476c, plus many more not discussed in this review. It is important to note, that these proteins occupy key points within *C. jejuni* metabolism, and involve the electron transport chain, serine metabolism, L-lactate metabolism, as well as the citric acid cycle. Without these proteins, many of *C. jejuni's* key metabolic pathways would cease to function, explaining why iron acquisition is critical to *C. jejuni* colonization and growth. The presence of enzymes containing iron–sulfur complexes at key points in *C. jejuni's* metabolic pathways also contributes to *C. jejuni* sensitivity to high levels of oxygen, since the iron–sulfur complexes are sensitive to damage by both molecular oxygen, and reactive oxygen species (Flint et al., 1993).

The *C. jejuni* FeoB (Cj1398) specifically imports ferrous ions across the inner membrane of the cell; and ferrous ion uptake is an important factor for colonization of the chick gastrointestinal tract. In fact, a Δ*feoB* mutant in the NCTC11168 strain showed more than a 10^4 fold reduction in chick colonization (Naikare et al., 2006). *C. jejuni* also contains a FeoA homolog (Cj1397), but its role in ferrous ion import is currently undefined (van Vliet et al., 2002). A small fraction of ferrous ion import could also traffic through ZupT (Cj0263), a transporter primarily responsible for zinc uptake (**Figure 2**). The *E. coli* ZupT homolog has been shown to possess a broad substrate affinity for divalent cations including ferrous ions in *E. coli*, however, it has yet to be determined if this remains true in the case of *C. jejuni* (Grass et al., 2005). It should however be noted that iron will generally not be found in a free form inside a living organism, even in the low-oxygen environment of the intestinal tract. Iron will generally be bound in complex with various molecules such as organic acids (citric, succinic, malic acids) and amino acids (asparagine, glycine; Butcher et al., 2010). Iron can also be present in prosthetic groups such as heme. Hosts also restrict the amount of iron available to the microbiota by secreting iron-binding proteins such as transferrin or lactoferrin. In response to this host restriction, the microbiota secretes siderophores (e.g., enterobactin) to acquire the iron needed for proper growth. Thus pathogens such as *C. jejuni* typically possess pathways for importing several of these diverse iron sources. *C. jejuni* has pathways for the import of iron containing complexes such as ferric–enterobactin (CeuBCDE/CfrA/CfrB; Palyada et al., 2004; Xu et al., 2010), heme (ChuABCDZ; Ridley et al., 2006), ferric–rhodotorulic acid (p19/Cj1658-63; Miller et al., 2009; Butcher et al., 2010), and ferric–transferrin/lactoferrin (CfbpABC/CtuA; Miller et al., 2008; **Figure 2**).

Ferric–rhodotorulic acid is imported into the periplasm though an unidentified outer membrane transporter. Subsequent interactions with P19 (Cj1659), Cj1658 and Cj1660–1663, and other unidentified proteins are required for import into the cytosol and release of the bound iron. It is unclear whether the entire ferric–rhodorulic acid complex enters the cytosol or if iron release occurs in the periplasm. Ferric–enterobactin is imported through either CfrA(Cj755)/CfrB(Cj0444/Cjj81176_0471) and enters the cytosol via the CeuBCDE (Cj1352–55) system (note that CeuE is a lipoprotein). The ferric–enterobactin is subsequently hydrolyzed by an unidentified protein. To note, the ferric–enterobactin complex could also be hydrolyzed within the periplasm and released iron transported through other means. Lactoferrin/Transferrin iron uptake is accomplished though CtuA (Cj0177) via an unidentified mechanism. Released iron is then transported thought the CfbpABC (Cj0173c–Cj0175c) system into the cytosol. Heme is imported through the outer membrane by ChuA (Cj1614) and transported into the cytosol via the ChuBCD (Cj1615–1617) system. ChuZ (Cj1613c) hydrolyzes the porphyrin ring to release the bound iron. It is currently unclear whether ChaN (Cj0177) functions in transferrin/lactoferrin or heme transport. Previous work has shown that ChaN forms dimers that bind heme, but the *chaN* gene is located directly upstream the lactoferrin/transferrin transporter (CtuA; Chan et al., 2006). Iron levels are regulated by the transcriptional regulator Fur by its binding of ferrous ions. There is also one *C. jejuni* strain that may be able to uptake ferrichrome as well, as it contains homologs of the *E. coli* FhuABD system (Galindo et al., 2001). However this pathway is not present in any of the sequenced *C. jejuni* strains (Miller et al., 2009).

The presence of ferric–enterobactin import in *C. jejuni* is particularly intriguing. Enterobactin is the prototypical bacterial siderophore that is produced to scavenge iron from host proteins such as lactoferrin or transferrin. *C. jejuni* does not produce enterobactin and instead takes advantage of enterobactin production from the host microbiota (Miller et al., 2009; Butcher et al., 2010). CfrA and CfrB are the two characterized enterobactin transporters in *Campylobacter* (Palyada et al., 2004; Carswell et al., 2008; Zeng et al., 2009; Xu et al., 2010). Curiously *C. coli* species harbor both proteins; while *C. jejuni* species only express one protein of the pair, with the other existing as a pseudogene (Xu et al., 2010). CfrB appears to be the dominant enterobactin transporter in *C. coli* with CfrA playing a relatively minor role. The situation in *C. jejuni* is relatively more complex. Many *C. jejuni* strains that express CfrB are not able to utilize ferric–enterobactin as a sole iron source; however CfrB is an important colonization factor for those strains that do possess a functional ferric–enterobactin pathway (Xu et al., 2010). Almost all CfrA expressing *C. jejuni* strains can utilize ferric–enterobactin as a sole iron source and CfrA has been demonstrated to be essential for chick colonization in these strains (Palyada et al., 2004; Xu et al., 2010). It is still unclear why *Campylobacter* genomes would contain two different transporters for the same iron source. It is also unknown why some strains that contain either CfrA or CfrB are still unable to utilize enterobactin as a sole iron source, when they appear to contain all the other necessary components (Xu et al., 2010).

Most deletion mutants in iron acquisition pathways show colonization defects. Studies have demonstrated that the deletion of

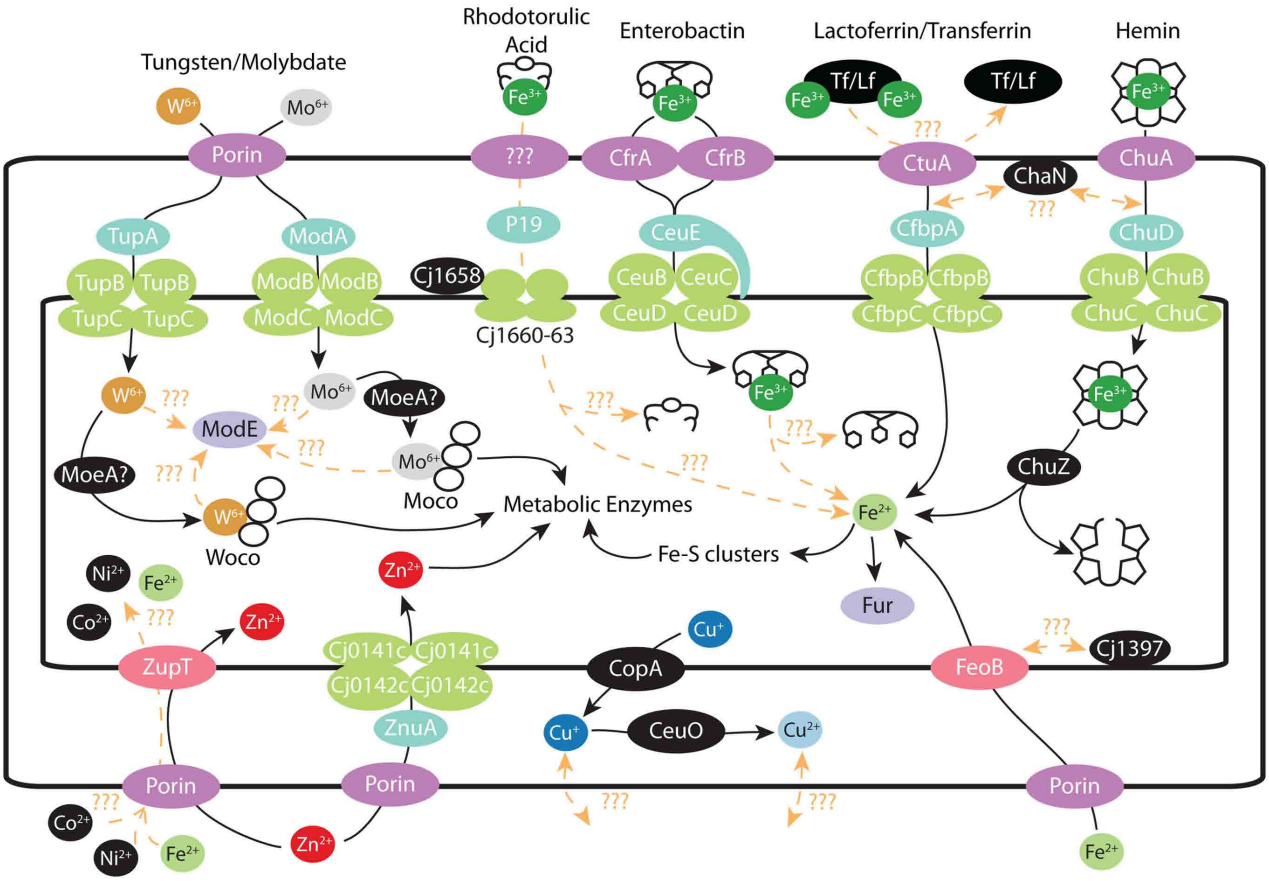

FIGURE 2 | Metal transport in *C. jejuni*: schematic of various metal transport systems present in *C. jejuni*. Where possible, proteins are colored by their function. Outer membrane proteins are denoted by dark purple, periplasmic proteins by turquoise, ABC transporters by green, periplasmic transporters by pink, and transcriptional regulators by light purple. *Iron*: ferric–rhodotorulic acid is imported into the periplasm though an unidentified outer membrane transporter and subsequent interactions with various proteins result in iron release and import into the cytosol. Ferric–enterobactin is imported through either CfrA/CfrB and enters the cytosol via the CeuBCDE system. The ferric–enterobactin is subsequently hydrolyzed by an unidentified protein. Lactoferrin/Transferrin iron uptake is accomplished though CtuA and released iron is then transported thought the CfbpABC system into the cytosol. Heme is imported through the outer membrane by ChuA and transported into the cytosol via the ChuBCD system. Iron release is accomplished by ChuZ hydrolyzing the porphyrin ring. Iron imported as ferrous ions are transported by the high affinity FeoB transporter. Ferrous ions may also be non-specifically imported though the general cation imported ZupT as seen in *E. coli*. Iron levels are regulated by the transcriptional regulator Fur by its binding of ferrous ions. *Tungsten/Molybdate*: tungsten/molybdate presumably cross the outer membrane via porins and are specifically taken up by the TupABC and ModABC transport systems respectively. Tungsten/molybdate are presumably incorporated into Woco and Moco cofactors by separate MoeA enzymes for insertion into metabolic enzymes. ModE senses either free molybdate/tungstate oxyanions or Moco/Woco complexes though unidentified partners. *Zinc/Copper/Others*: zinc crosses the outer membrane and is taken up into the cytosol by either high affinity (ZnuA) or low affinity (ZupT) transporters. Excess cuprous ions are effluxed into the periplasm by CopA, with concomitant oxidization by CeuO into cupric ions. Other metals may cross the outer membrane through porins and imported by ZupT or there may be unidentified specific transport systems for these metals.

the transporters responsible for the ferrous ion (FeoB; Naikare et al., 2006), lactoferrin/transferrin (CtuA; Miller et al., 2008), and enterobactin (CfrA/CfrB; Palyada et al., 2004; Xu et al., 2010) transporters resulted in substantial colonization deficiencies in the chicks (Palyada et al., 2004; Stintzi et al., 2005; Naikare et al., 2006). The *feoB* and *ctuA* mutants are also defective in colonizing pathogenic animal models, as demonstrated using either the colostrum-deprived piglet or the rabbit ileal loop model respectively (Stintzi et al., 2005; Naikare et al., 2006). Curiously, the abrogation of the heme (ChuA; Ridley et al., 2006) or rhodotorulic acid (P19/Cj1658; Stintzi et al., 2008) iron uptake pathways had no effect on *C. jejuni* chick colonization, although these pathways might be important for colonization in other hosts or environments (Butcher et al., 2010).

MOLYBDATE AND TUNGSTATE

Campylobacter jejuni is fairly unique amongst bacteria in that it possesses both molybdenum (Moco) and tungsten (Woco) cofactored proteins. *C. jejuni* formate dehydrogenase, a key enzyme for energy metabolism using formate as an electron donor, has been shown to require Woco for proper function, while nitrate reductase, sulfite oxidase, and SN oxide reductase (Cj0264c)

require Mocos for their activity (Smart et al., 2009; Taveirne et al., 2009). This peculiar metabolic need likely explains the unusual presence in *C. jejuni* of specific high affinity ABC transporters for both metals (**Figure 2**). The ModABC (Cj0300c–Cj0303c) and TupABC (Cj1538c–Cj1540) transporters import either molybdate or tungstate respectively across the inner membrane in an ATP dependant manner, but both are regulated together by a single ModE-like regulatory protein (Cj1507c; Taveirne et al., 2009). Interestingly, the *C. jejuni* ModE protein represses the *mod* operon in the presence of excess molybdate or tungstate while the *tup* operon is only repressed in response to tungsten. Moreover the *C. jejuni* ModE protein does not appear to contain a metal binding domain and instead contains a coiled–coiled domain that is indicative of protein–protein interactions (Taveirne et al., 2009). It is currently unclear what other factor(s) are required to sense excess intracellular molybdate/tungsten in *C. jejuni*. One possibility is that *C. jejuni* contains alternate molybdenum/tungsten sensors that interact with ModE to regulate each operon as needed. It is also possible that ModE senses the concentration of molybdenum and tungsten indirectly though the binding of Moco or Woco respectively.

The presence of both molybdate and tungsten requiring enzymes in *C. jejuni* also creates additional metabolic challenges. Most bacteria, such as *E. coli*, only use Moco cofactored enzymes and these enzymes are normally inactivated if tungsten is misincorporated during cofactor synthesis (Kletzin and Adams, 1996). This is not normally a problem, as these bacteria typically do not have specific high affinity transporters for tungstate. However, *C. jejuni* has specific high affinity transporters for both molybdate and tungstate and also possesses both Moco and Woco dependent enzymes. Thus, *C. jejuni* must contain proteins that can distinguish between molybdate and tungsten for the proper synthesis of these distinct cofactors. *C. jejuni* must also be able to distinguish between the produced Moco and Woco cofactors to correctly insert each cofactor into the correct effector proteins. In fact, Smart et al. (2009) have proposed that these needs are the reason why *C. jejuni* genomes contain two versions of the molydopterin synthesis protein *moeA* (Cj0857c/Cj1519). It remains to be demonstrated if each *moeA* protein is either Moco or Woco specific and how effector proteins ensure that they receive the proper cofactor.

Campylobacter jejuni mutants deficient in either molybdate or tungstate transport have not yet been tested for their ability to colonize *in vivo* models. However, deletion of tungstate acquisition should result in reduced colonization potential, as deletion of the tungsten dependent formate dehydrogenase in *C. jejuni* resulted in chick colonization defects (Weerakoon et al., 2009).

COPPER/ZINC

There is currently very little known about the mechanisms surrounding copper and zinc homeostasis in *C. jejuni*. Copper chelation results in slowed *C. jejuni* growth and this growth inhibition can be partially relieved by deleting non-essential copper containing proteins such as the p19 iron acquisition protein (Chan et al., 2010). *C. jejuni* does not have any annotated homologs of the copper regulators CueR or CusR but does contain CopA (Cj1161) and CeuO (Cj1516) homologs (Hall et al., 2008; **Figure 2**). CopA proteins are transmembrane ATPases that are involved in pumping excess cytosolic copper to the periplasmic space (Osman and Cavet, 2008). Cuprous ions in the periplasmic space are oxidized to the less toxic cupric form by CeuO (Osman and Cavet, 2008). Deleting *C. jejuni copA* results in a severe sensitivity toward excess copper levels (Hall et al., 2008). CeuO deletion also results in a copper sensitivity phenotype (Hall et al., 2008). CopA and CeuO probably function independently from each other since a double deletion mutant in both genes is more sensitive to copper levels then either single deletion mutant (Hall et al., 2008). It is currently unknown whether *C. jejuni* contains specific copper transporters for copper import or export from the cell.

The zinc homeostasis system remains fairly uncharacterized in *C. jejuni*. There are no obvious candidates for the regulation of zinc uptake or efflux despite the almost universal presence of these proteins in other bacteria (Hantke, 2001). *C. jejuni* does possess a homolog of the high efficiency *E. coli* ZnuABC zinc uptake system (Cj0141c–Cj0143c) that is also present in many bacteria (Hantke, 2001). Recent work by Davis et al. (2009) has demonstrated that this system is functional in *C. jejuni* and is required for both survival in low zinc environments and for successful chick colonization. *C. jejuni* also contains a homolog of the low affinity zinc transporter ZupT, however, there has not as of yet been any studies conducted to demonstrate how ZupT may function in *C. jejuni*. As previously mentioned, in *E. coli,* this protein can also transport various other divalent cations including Fe^{2+}, Co^{2+}, and Mn^{2+} (Grass et al., 2005).

OTHER METALS

Bacteria such as *C. jejuni* also make use of several additional metals for either enzymatic or structural purposes, such as cobalt and nickel. While there are no obvious transporters for these metals in the *C. jejuni* genome, there are several examples where these metals are required for proper protein function. For instance, the *C. jejuni* NeuB3 (Cj1317) has been shown to rely on cobalt for full enzymatic activity (Chou et al., 2005). Another example is the *C. jejuni* hydrogenase complex (Cj1263c–1265c), which is predicted to be nickel dependent (Parkhill et al., 2000; Weerakoon et al., 2009). The acquisition of these metals may be accomplished through general cation transporters (e.g., ZupT) or accomplished through uncharacterized metal specific transporters.

CONCLUSION

While the *C. jejuni* metabolic networks and nutrient acquisition systems have remained to a significant extent a mystery, even after the sequencing of the genome, researchers are gradually piecing together a better picture of how this organism functions. Once thought to be completely asaccharolytic, we now know that it possesses a L-fucose metabolic pathway. Its usage of amino acids and CAC intermediates are coming into focus, and we are gaining a better understanding of its complex metal acquisition pathways, especially those involving iron, molybdate, and tungsten. Although

a great deal still remains obscure, the understanding of these pathways is contributing substantially to our understanding of this common gastrointestinal pathogen.

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Campylobacter concisus – a new player in intestinal disease

Nadeem Omar Kaakoush and Hazel Marjory Mitchell*

School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW, Australia

Edited by:
D. Scott Merrell, Uniformed Services University, USA

Reviewed by:
Terry Kwok, Monash University, Australia
Georgina Hold, University of Aberdeen, UK
Albert Joseph Lastovica, University of the Western Cape, South Africa

****Correspondence:***
Hazel Marjory Mitchell, School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW 2052, Australia.
e-mail: h.mitchell@unsw.edu.au

Over the last decade *Campylobacter concisus*, a highly fastidious member of the *Campylobacter* genus has been described as an emergent pathogen of the human intestinal tract. Historically, *C. concisus* was associated with the human oral cavity and has been linked with periodontal lesions, including gingivitis and periodontitis, although currently its role as an oral pathogen remains contentious. Evidence to support the role of *C. concisus* in acute intestinal disease has come from studies that have detected or isolated *C. concisus* as sole pathogen in fecal samples from diarrheic patients. *C. concisus* has also been associated with chronic intestinal disease, its prevalence being significantly higher in children with newly diagnosed Crohn's disease (CD) and adults with ulcerative colitis than in controls. Further *C. concisus* has been isolated from biopsy specimens of patients with CD. While such studies support the role of *C. concisus* as an intestinal pathogen, its isolation from healthy individuals, and failure of some studies to show a significant difference in *C. concisus* prevalence in subjects with diarrhea and healthy controls has raised contention as to its role in intestinal disease. Such findings could argue against the role of *C. concisus* in intestinal disease, however, the fact that *C. concisus* strains are genetically diverse raises the possibility that differences exist in their pathogenic potential. Evidence to support this view comes from studies showing strain specific differences in the ability of *C. concisus* to attach to and invade cells and produce virulence factors, including toxins and hemolytic phospholipase A. Further, sequencing of the genome of a *C. concisus* strain isolated from a child with CD (UNSWCD) and comparison of this with the only other fully sequenced strain (BAA-1457) would suggest that major differences exist in the genetic make-up of this species which could explain different outcomes of *C. concisus* infection.

Keywords: *Campylobacter concisus*, oral, intestine, enteritis, Crohn's disease, pathogenesis, reservoirs of infection, antibiotic susceptibility

INTRODUCTION

Members of the *Campylobacter* genus are fastidious Gram-negative spiral or curved shaped rods, which grow under microaerobic or anaerobic conditions. The majority of species are motile, having a single polar flagellum at one or both ends of the cell (Vandamme et al., 2005; Man, 2011). The most well-studied species of the *Campylobacter* genus is the intestinal pathogen *Campylobacter jejuni*, a bacterium currently recognized as the leading cause of acute diarrhea and gastroenteritis worldwide, accounting for 400 million cases of gastroenteritis in adults and children each year (Blaser et al., 1983; Tauxe et al., 1987; Allos, 2001). Additionally in a small number of those acutely infected with *C. jejuni* a number of serious sequelae can occur including Guillain–Barré syndrome and reactive arthritis (Altekruse et al., 1999). In addition to *C. jejuni*, *C. coli* has also been recognized as an important cause of gastroenteritis accounting for 5–18.6% of cases of gastroenteritis caused by *Campylobacter* spp. (Friedman et al., 2004; Valenza et al., 2010). While from a clinical perspective *C. jejuni* and *C. coli* are major foci of attention worldwide, over the last decade mounting evidence has accrued that would suggest that other members of the *Campylobacter* genus including *C. concisus*, *C. gracilis*, *C. upsaliensis*, and *C. lari* may also play a role in intestinal disease. While evidence to support the role of each of these four non-*jejuni/coli Campylobacter* species in intestinal disease is increasing (a recent review by Man, 2011 provides a comprehensive reference source for the clinical importance of emerging *Campylobacter* species), evidence for the role of *C. concisus* is perhaps the most contentious.

Campylobacter concisus was first isolated and named by Tanner et al. (1981). In a study, which compared 46 Gram-negative, asaccharolytic, rod shaped bacteria isolated from humans with periodontal disease with 10 reference strains including *Eikenella corrodens*, *Vibrio succinogenes* (now known as *Wolinella succinogenes*), *Bacteroides ureolyticus* (now known as *C. ureolyticus*), and *Campylobacter* species, Tanner et al. (1981) identified six isolates from patients with gingivitis and periodontitis that were described as non-corroding, microaerophilic, Gram-negative, rods predominantly curved in shape, with deoxyribonucleic acid guanine-plus-cytosine contents of 34–38%, which they proposed should be named "*Campylobacter concisus* sp. nov." (type strain, ATCC 33237). Although it is now 30 years since Tanner and colleagues first named *C. concisus*, advances in our understanding of the required growth conditions, phenotypic characteristics (**Table 1**; Tanner et al., 1981; Vandamme et al., 2005), genetic make-up, and

Table 1 | Phenotypic characteristics of *Campylobacter concisus*.

Phenotypic characteristic	Reaction*		
	Bergey's manual (Vandamme et al., 2005)	**Tanner et al. (1981)**	**Others**
Active motility	A	A	A
Growth on minimal media	N	–	N
Growth in air+CO_2	–	N	N
Growth stimulated by formate and fumarate	–	A	A
Growth stimulated by nitrate	–	N	–
Growth at 25°C	N	–	–
Growth at 42°C	M	–	–
GROWTH IN THE PRESENCE OF			
Sodium fluoride	M	A	–
Oxgall	F	A	–
Sodium deoxycholate	–	A	–
Phenol	–	N	–
Janus green	–	A	–
Basic fuchsin	–	A	–
Crystal violet	–	A	–
Safranine	F	A	–
Alizarine red S	–	A	–
Azure II	–	A	–
Potassium cyanide	–	M	–
Methyl orange	–	A	–
$KMnO_4$	N	–	–
END PRODUCTS			
H_2S	N[#]	A	–
Formate	–	N	–
Acetate	–	F	–
Lactate	–	N	–
Succinate	–	A	–
Pyruvate	–	N	–
Hydrogen	–	A	–
Nitrous oxide	–	N	–
REDUCTION OF			
Nitrite	–	A	–
Nitrate	F	–	–
Selenite	F	–	–
Benzyl viologen	–	A	–
Neutral red	–	A	–
ACTIVITY			
Oxidase	M	A	–
Urease	N	N	–
Catalase	N	N	–
Benzidine	–	A	–
Lysine and ornithine decarboxylase	–	N	–
Indoxyl acetate hydrolysis	N	–	–
Hippurate hydrolysis	N	–	–
Arylsulfatase	–	–	A
Pyrazinamidase	–	–	A

**A, 95–100% positive; M, 60–93% positive; F, 14–50% positive; N, 0–11% positive; not available, [#]Trace quantities.*

pathogenic potential of *C. concisus* have been relatively slow, and somewhat sporadic, with only approximately 80 papers relating to *C. concisus* being published over the last 30 years.

While initially studies focused upon the role of *C. concisus* in periodontal disease, over the last 20 years the major research focus has predominantly been investigation of the role of *C. concisus* in

intestinal disease, including enteritis and more recently inflammatory bowel diseases (IBD). Although a relatively large number of studies have examined the relationship between *C. concisus* and intestinal disease there still remains no consensus as to its role. By enlarge, this relates to the fact that in many studies no significant difference was detected between the prevalence of *C. concisus* in those with intestinal disease and healthy controls (Van Etterijck et al., 1996; Lawson et al., 1998; Inglis et al., 2011). While these findings *per se* would argue against such a link, the fact that there is an extremely high degree of genetic heterogeneity among *C. concisus* strains raises the possibility that only some *C. concisus* strains have the ability to cause intestinal disease. Given this, a number of studies have attempted to link specific genomospecies of *C. concisus* with disease outcome, however, although some progress has been made in this area no specific *C. concisus* genomospecies has been associated with disease outcome. This review aims to provide an overview of our current understanding of the association between *C. concisus* and oral and intestinal disease, the importance of genetic diversity and its association with disease outcome, the pathogenic potential of *C. concisus*, as well as potential reservoirs of infection and susceptibility to antibiotics.

ASSOCIATION WITH DISEASE

ORAL DISEASES

As described above *C. concisus* was first recognized and named as a member of the microflora of the oral cavity by Tanner et al. (1981). The isolation of six *C. concisus* strains from three subjects with gingivitis and three with periodontitis raised the possibility that an association may exist between *C. concisus* and oral diseases (Tanner et al., 1981, 1987). Following Tanner's paper further reports of the isolation of *C. concisus* from the oral cavity of small numbers of subjects were published. For example, Tyrrell et al. (2003) isolated *C. concisus* from a patient with oral malodor, while Haffajee et al. (1984) reported the level of *C. concisus* to be associated with a gain in tooth attachment in a subject with periodontitis.

In one of the few studies to examine the immune response to *C. concisus* in periodontal disease and controls, Ebersole et al. investigated the longitudinal effects on systemic antibody levels to a range of oral microorganisms known to exist in subjects with different periodontal diseases. This showed that in adult patients with periodontitis undergoing subgingival scaling, antibodies to *C. concisus* were elevated as compared with that in the normal population (Ebersole et al., 1985). This association was supported by a study by Taubman et al. (1992) who also found higher antibody levels to *C. concisus* in periodontally diseased subjects when compared to healthy subjects.

Moore et al. (1987) compared the subgingival bacterial flora cultured from children and adults with naturally occurring gingivitis with that in healthy controls and subjects with moderate, severe, and juvenile periodontitis. This showed that in both children and adults with naturally occurring gingivitis, *C. concisus* was more numerous than in healthy controls or those with periodontitis. Based on their findings Moore et al. suggested that the composition of the subgingival microflora represented a transition between that associated with health and periodontitis. In a similar study Macuch and Tanner (2000) compared the presence of *Campylobacter* spp. in healthy subjects with that in subjects with gingivitis, initial periodontitis, and established periodontitis. While *C. concisus* was more frequently isolated from subjects with initial periodontitis (approximately 68%) than healthy individuals (35%), the isolation rates from those with gingivitis (32%), or established periodontitis (32%) did not differ from that in controls (Macuch and Tanner, 2000). Interestingly, the proportion of *C. concisus* as a percentage of all *Campylobacter* species was higher in shallow pocket sites (19.7%) as compared with deep pocket sites (6.6%) and healthy controls.

In a further study that examined the composition of the subgingival microbiota of children, Kamma et al. isolated 45 microbial species from subgingival plaque of both permanent and deciduous teeth of systemically healthy children. While *C. concisus* was not one of the most frequently detected species, its detection rate in permanent teeth was significantly higher than that in deciduous teeth ($P < 0.001$). Furthermore the presence of *C. concisus* was significantly associated with the bleeding index in permanent teeth (Kamma et al., 2000a,b), a finding that was in line with a previous study by Kamma et al. that showed *C. concisus* to be associated with bleeding in young adults with rapidly progressive periodontitis (Kamma et al., 1994; Nakou et al., 1998). Furthermore, Kamma et al. found *C. concisus* to be present in significantly higher numbers, and more frequently, in smokers as compared with non-smokers (Kamma and Nakou, 1997; Kamma et al., 1999).

Given reports that the enzyme aspartate aminotransferase (AST) is markedly elevated in gingival crevicular fluid (GCF) from sites with severe gingival inflammation and progressive attachment loss, Kamma et al. (2001) determined the association between AST activity in GCF and the subgingival microbiota in periodontal sites of individuals with early onset periodontitis. This showed the isolation frequency of seven bacterial species, including *C. concisus*, to be significantly higher at AST positive sites than at AST negative sites. Furthermore, *C. concisus* had the fourth strongest positive association with AST activity (Odds Ratio 3.9; 95% CI: 1.91–22.36; Kamma et al., 2001).

While there have been further sporadic reports of the detection and isolation of *C. concisus* from subjects with periodontal disease over the last 10 years, there is currently limited evidence to support a role for *C. concisus* in oral disease. Given that many of the studies reported only the isolation of *C. concisus* from sites of oral disease, rather than comparing this with that in healthy controls, assessment of the role of *C. concisus* in oral disease is problematic. Thus, currently it remains unclear whether *C. concisus* is an oral pathogen, an opportunistic pathogen in inflamed areas or is simply a commensal of the oral cavity.

ENTERITIS

Johnson and Finegold (1987) published the first evidence that *C. concisus* did not exclusively colonize the oral cavity. While this finding was of significant interest, it was a study by Vandamme et al. (1989) 2 years later that provided the first convincing evidence that *C. concisus* was present in the gastrointestinal tract (GIT) of humans. In their study Vandamme et al. (1989) used protein analysis, immunotyping, DNA hybridization, and DNA base analysis to determine the species identity of 14 *Campylobacter* species previously isolated from a blood culture, two antral biopsies, a duodenal aspirate, two esophageal biopsies and the feces

of eight patients with persistent diarrhea, which had previously, based on a small number of phenotypic tests and immune-typing, been provisionally grouped and named EF-group 22. Based on their testing of these isolates as well as a range of *Campylobacter* reference strains, all 14 were shown to be *C. concisus* a finding that led Vandamme et al. (1989) to conclude that fecal carriage of *C. concisus* may be common, and might be associated with gastrointestinal disease.

Further evidence that *C. concisus* could be isolated from fecal samples of subjects with diarrhea came from a prospective study of Belgian children and adults conducted by Lauwers et al. In this study, *C. concisus* was isolated from the stools of 2.4% of children ($n = 3165$) and 1.5% of adults ($n = 1265$) with diarrhea (Lauwers et al., 1991). Of those positive for *C. concisus*, 20% of children, and 10.5% of adults harbored another enteropathogen. Interestingly, 54% of the *C. concisus*-positive children were <1 year of age while only 9% were positive in the age group >5 years. Of the 78 children in whom only *C. concisus* was isolated, 72% had enteritis symptoms (62% diarrhea and 22% vomiting) while 89.4% of adults had diarrhea. Lauwers et al. (1991) concluded that although *C. concisus* may be commonly found in the feces of children and adults, further studies were necessary to determine its pathogenic role in the human intestinal tract. In contrast, in a study comparing the isolation rates of *Campylobacter* species from stool samples of 867 adults with diarrhea and 1077 children (<10 years; 90% with diarrhea), Lindblom et al. (1995) failed to isolate *C. concisus* from any adult stool sample, although stool samples from four children with diarrhea were positive, which represented 4% of all *Campylobacter* species isolated. Moreover, following the detection of *C. concisus* from fecal samples of healthy individuals (3/20; 15%), Lawson et al. (1998) also suggested that *C. concisus* might only be transiently present within the GIT.

Figura et al. (1993) reported the isolation of *C. mucosalis* from the feces of two children with enteritis (NCTC 12408 and NCTC 12407) from Sienna, Italy. The classification of these isolates as *C. mucosalis* was however later challenged by Lastovica et al. (1993) due to the limited number of phenotypic tests used by Figura et al. to classify them. Subsequently, based on rigorous phenotypic analysis, consisting of 64 individual phenotypic tests, On (1994) confirmed NCTC 12408 (NCTC 12407 was no longer extant) to be *C. concisus*. In a large study also conducted in the Sienna/Tuscany region of Italy, Musmanno et al. investigated the presence of unusual *Campylobacter* species in fecal samples of 288 Italian children with enteritis. Based on a filtration technique *C. jejuni* subsp. *jejuni* was isolated from 6.9% of children, *C. coli* from 2%, and each of *C. jejuni* subsp. *doylei*, *C. upsaliensis*, and *C. concisus* from 0.7% of children (Musmanno et al., 1998). While the prevalence of *C. concisus* in these Italian children was low (0.7%), in the same year a significantly higher isolation rate of *C. concisus* was reported in South African children (Lastovica, 2006). In his study, which used the Cape Town Protocol to determine the distribution of *Campylobacter* and related species in diarrheic stools collected from children attending the Red Cross Children's Hospital in Cape Town, Lastovica reported *C. concisus* to represent the third most common of the *Campylobacter* and related species isolated (399/2351; 17.0%), the most common being *C. jejuni* (33.4%) followed by *C. upsaliensis* (23.7%). Unfortunately, as this study did not include a healthy control group, the role of *C. concisus* in diarrheal disease could not be assessed.

The importance of *Campylobacter* species other than *C. jejuni* and *C. coli* in diarrheal disease was also investigated by Aabenhus et al. (2002b) who determined the prevalence of *C. concisus* infection in 11,550 stool samples obtained from patients with enteric disease, using a filter isolation method. Of the 224 *Campylobacter* isolates cultured, 110 (49%) were shown to be *C. concisus*, with concomitant infection, with an established gastrointestinal pathogen being found in 27% of the 103 patients from whom these isolates were cultured. No conclusive association was observed between any specific age group and *C. concisus* infection. Interestingly, of the infected patients 73 (70.9%) were immunocompromised the majority of whom were adults (84%). Based on these findings, Aabenhus et al. (2002b) concluded that the prevalence of *C. concisus* was likely to be significantly underestimated, and that although *C. concisus* could be isolated from both immunocompetent and immunocompromised adult patients, immunocompromised patients appeared to be at a higher risk of infection.

Maher et al. (2003) employed both culture and a 16S/23S PCR/DNA probe assay to detect *Campylobacter* species in 320 consecutive liquid and semi-solid fecal samples from subjects with acute infectious gastroenteritis, and found *Campylobacter* DNA, other than *C. jejuni* or *C. coli*, to be present in 30% of fecal specimens (including *C. curvus*, *C. concisus*, and *C. gracilis*). Furthermore, Kulkarni et al. (2002) reported the isolation of *C. concisus* using membrane filtration and selective culture from only 1 of 343 stool samples collected from patients with gastroenteritis.

In a recent epidemiological study conducted over a 22-month period commencing January 2009, Nielsen et al. (2011a) which investigated the prevalence of *Campylobacter* species and other enteric bacterial pathogens in 10,388 stool samples using a filter isolation technique, found the most prevalent enteric pathogen isolated from these stools was *C. jejuni* (456 patients) followed by *C. concisus* (378 patients). Interestingly, *C. concisus* was found to be most frequently isolated in young children and elderly patients, a finding consistent with that of Engberg et al. (2000). Moreover, in the Nielsen study elderly patients with *C. concisus* tended to present with a more long-lasting diarrhea.

In the first of a limited number of studies to investigate the pathogenic role of *C. concisus* in intestinal disease, Van Etterijck et al. (1996) used a filter isolation technique to isolate *C. concisus* from children with enteritis ($n = 174$) and a control population ($n = 958$) with no diarrhea. This study showed that while the isolation rate of *C. concisus* in children with diarrhea (13%) was higher than that in the control children (9%) this did not reach significance ($P = 0.15$), which led Van Etterijck et al. to conclude that *C. concisus* should not be considered a primary pathogen associated with gastrointestinal disease. In a further study which used a range of culture techniques to isolate *Campylobacter* species from the feces of Danish children and adults with diarrhea, Engberg et al. (2000) reported the isolation of *C. concisus* from a large number of diarrheal cases, particularly from those at the extremes of age (0–9 and >60 years). However, similar to the findings of Van Etterijck, when the isolation rates of *C. concisus* in patients with diarrhea were compared with healthy controls, isolation rates in

those with diarrhea (5.6%) were not significantly different to that in controls (2.8%).

More recently, Inglis et al. (2011) investigated the presence of *Campylobacter* species in stools from diarrheic ($n = 442$) and healthy ($n = 58$) humans living in southwestern Alberta using PCR. In contrast to other studies, in this population the prevalence of *C. concisus* DNA as detected by two primer sets was significantly higher ($P < 0.001$) in healthy subjects (52%) as compared with subjects with diarrhea (34.8%).

Although overall these findings support the presence of *C. concisus* in the intestinal tract of healthy humans and those with diarrheal disease, evidence to support a role for *C. concisus* in acute enteritis, although increasing, remains tenuous.

INFLAMMATORY BOWEL DISEASES

Inflammatory bowel diseases are chronic inflammatory conditions of the GIT, with currently unknown etiology and rising global incidence (Economou and Pappas, 2008). The two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Both forms have distinct phenotypical characteristics: CD inflammation may occur in any location along the GIT, with transmural lesions that may extend through the mucosal surface and into the underlying serosa, whilst UC is characterized by continuous sub-mucosal inflammation occurring only in the colon (Colletti, 2004). A histopathological hallmark of CD is the presence of compact granulomas, composed of macrophages, giant cells, and epithelioid cells. The prominent histological features of UC include the presence of an elevated number of polymorphonuclear cells within the lamina propria as well as in the intestinal crypts, in which microabscesses form (Colletti, 2004). Research indicates that the etiopathogenesis of CD and UC are to some extent different; yet, the general hypothesis regarding their etiology is that gastrointestinal microbes or their by-products, in association with a disrupted gastrointestinal epithelium and/or environmental trigger, propagate a dysregulated immune response leading to chronic inflammation in genetically predisposed individuals (Man et al., 2011).

The possible association between *Campylobacter* species and IBD was first investigated in 1984. While initial studies examining a possible link between *C. jejuni* and IBD failed to provide evidence for an association (Blaser et al., 1984; Weber et al., 1992; Boyanova et al., 2004), more recent studies would suggest that *C. jejuni* infection may be associated with increased IBD risk and flare ups of IBD (Gradel et al., 2009).

Interestingly, Aabenhus et al. (2002a) reported the isolation of *C. concisus* from fecal samples of IBD patients. However, it was not until 2009 that Zhang et al. (2009) compared the prevalence of *C. concisus* in patients with CD and controls. In this study which investigated a possible association between *C. concisus* and newly diagnosed pediatric CD, a significantly higher prevalence of *C. concisus* DNA ($P < 0.0001$) as well as *C. concisus* specific IgG antibody levels ($P < 0.001$) were detected in children with newly diagnosed CD (51%; 0.991 ± 0.447) as compared with controls (2%; 0.329 ± 0.303; Zhang et al., 2009). Additionally, *C. concisus* UNSWCD was successfully isolated from an intestinal biopsy of a child with CD (Zhang et al., 2009), indicating that viable bacteria were present within the patient's intestine. These findings were later confirmed in a larger cohort of children where detection of *C. concisus* DNA from fecal samples showed that 65% (35/54) of CD patients were positive, which was significantly higher than that in healthy (33%, 11/33) and non-IBD controls (37%, 10/27; Man et al., 2010b). Recently, Hansen et al. (2011) also reported the detection rate of *C. concisus* DNA in intestinal biopsies of pediatric patients with IBD (14/24; 58.3%), specifically CD (8/12; 66.7%), was higher than that in controls (11/26; 42.3%), however in their study the difference was not significant, which most likely relates to the relatively small number of subjects in their study.

These findings in children have recently been supported by studies in adult CD patients, where a significantly higher prevalence of *C. concisus* DNA was detected in colonic biopsies of CD patients (53%) as compared with controls (18%, $P < 0.05$; Mahendran et al., 2011). Further indirect evidence of a possible association between *C. concisus* and CD comes from the finding that rifaximin, an antibiotic with good activity against *Campylobacter* species, is effective for adults with active CD, and when combined with long-term administration of probiotics can induce and maintain remission of CD (Guslandi et al., 2009).

In a recent study, Kovach et al. (2011) investigated *C. concisus* immunoreactive proteins in the sera of *C. concisus*-positive children with CD, and identified 37 proteins of which flagellin B, ATP synthase F1 alpha subunit and outer membrane protein 18 were predominant antigens recognized by all CD patients. At least six of the identified proteins functioned in adhesion to the host cell, suggesting that this bacterium can cross the mucus layer and attach to the intestinal epithelium (Kovach et al., 2011).

Of significant interest is the recent finding by Mukhopadhya et al. (2011) that *C. concisus* may also be associated with UC. In their study the prevalence of *C. concisus* DNA in biopsy specimens from adults with UC was shown to be significantly increased ($P = 0.0019$) in UC patients (33.3%; 23/69) as compared with controls (10.8%; 7/65). This finding is supported by the study of Mahendran et al. (2011) who not only isolated *C. concisus* from patient biopsies, but also determined that *C. concisus* positivity in UC patients (77%) was significantly higher than that in controls (36%, $P < 0.05$). In a study to investigate the presence of *Campylobacter* species in IBD, Tankovic et al. (2009) employed real-time PCR to detect *Campylobacter* species in the stools of 8 CD patients, 11 UC patients and 11 symptomatic controls. In addition stools were cultured for 10 days on Skirrow-supplemented blood agar plates in a microaerobic atmosphere. While detection of *Campylobacter* DNA was higher in IBD patients (21%) than control patients (9%), this did not reach significance ($P = 0.46$). Further analysis of the *Campylobacter* PCR positive isolates using species-specific primers showed four of these to be *C. concisus*. Of these four PCR positive patients, *C. concisus* was isolated from the stools of two patients. Based on the finding that *C. concisus* was only present in the stools of IBD patients, the authors suggested that *C. concisus* may be implicated in IBD (Tankovic et al., 2009).

Finally, attempts to associate oral *C. concisus* with IBD showed no significant difference to be present in the detection of *C. concisus* DNA in saliva samples of IBD patients (100%; CD, $n = 13$, UC, $n = 5$) and healthy controls (97%, 57/59; Zhang et al., 2010).

Overall, these findings provide considerable evidence that some *C. concisus* strains may be involved in IBD (**Table 2**), either as a direct trigger or as an opportunistic pathogen.

Table 2 | Detection rates of *Campylobacter concisus* in inflammatory bowel disease patients and controls.

Study	Disease	Population	Sample type	Detection rate (%)		Significant
				Patients	Controls	
Zhang et al. (2009)	Crohn's disease	Pediatric	Intestinal biopsy	53.0	2.0	Yes
Man et al. (2010b)	Crohn's disease	Pediatric	Fecal	65.0	35.0	Yes
Hansen et al. (2011)	Crohn's disease	Pediatric	Intestinal biopsy	66.7	42.3	No
Mahendran et al. (2011)	Crohn's disease	Adult	Intestinal biopsy	53.0	18.0	Yes
Mukhopadhya et al. (2011)	Ulcerative colitis	Adult	Intestinal biopsy	33.3	10.8	Yes
Mahendran et al. (2011)	Ulcerative colitis	Adult	Intestinal biopsy	77.0	36.0	Yes
Tankovic et al. (2009)	Inflammatory bowel disease	Adult	Fecal	21.0	9.0	No
Zhang et al. (2010)	Crohn's disease	Mixed	Saliva	100.0	97.0	No

OTHER DISEASES

The majority of research associating *C. concisus* with human disease has focused on periodontal disease, enteritis, and IBD, however, several studies have associated *C. concisus* with other human diseases. The most significant of these is the association of this bacterium with Barrett's esophagus (BE; Macfarlane et al., 2007), a condition in which gastroduodenal reflux leads to squamous epithelial cells lining the esophagus being replaced with columnar epithelial cells. In this study by Macfarlane et al. (2007), high levels of *C. concisus* and *C. rectus* were found in four of seven patients (57%) with BE compared with none of the controls. *C. concisus* was isolated from both the aspirate and mucosal samples, and was the most prevalent bacterium. As a result of their study Macfarlane et al. hypothesized that pathogenic and putative toxin producing *Campylobacter* species could be involved in the initiation, maintenance, or exacerbation of esophageal disease.

In addition to the above study there have been a number of sporadic reports of the isolation of *C. concisus* from a range of body sites. For example, Johnson and Finegold (1987) reported the isolation of *C. concisus* from a diabetic foot ulcer of a patient with underlying osteomyelitis. Further, in a report on *Campylobacter* species isolated from extra-oro-intestinal abscesses, *C. concisus* was one member of a polymicrobial flora isolated from a brain abscess of a patient who had previously undergone craniotomy for maxillary sinus carcinoma (de Vries et al., 2008). Despite antibiotic therapy the patient later died from an intracerebral infection due to chronic sinusitis (de Vries et al., 2008). In this case it is likely that *C. concisus* was an opportunistic pathogen, an interpretation that may also explain the isolation of *C. concisus* from human blood samples (Lastovica, 2009). Further, following a survey of pediatric records Lastovica reported the isolation of *C. concisus* from stool specimens of nine liver transplant patients, six patients with biliary atresia and three with renal transplants or renal failure (Lastovica, 2009). While a role for *C. concisus* in these diseases cannot be confirmed, these results in combination with the finding of micro-abscesses in the livers of two mice infected with *C. concisus* (Aabenhus et al., 2008), raises the possibility of an association between this bacterium and hepatobiliary disease.

GENETIC TYPING AND DIVERSITY

While the above studies support the role of *C. concisus* as a human pathogen, its isolation from healthy individuals, and the failure of some studies to show a significant difference in *C. concisus* prevalence in subjects with disease and healthy controls has raised contention as to its role in disease. The existence of high genetic diversity among *C. concisus* strains has been proposed as a possible justification for this inconsistency.

An early study conducted by Vandamme et al. (1989) that used DNA–DNA hybridization to investigate diarrheal isolates that fitted the phenotypic description of *C. concisus*, first raised the possibility that *C. concisus* was genetically diverse. In this study phenotypically confirmed *C. concisus* strains were shown to exhibit only 42–50% DNA–DNA hybridization values with the type and reference strains of oral origin, leading Vandamme et al. to conclude that *C. concisus* was likely to be genetically diverse. Given this, On and Harrington (2000) suggested that, based on current taxonomic guidelines, diarrheal isolates and oral strains should actually be distinct genomic species, however, as they could not be differentiated phenotypically they should be regarded as a "complex species comprising at least two genomospecies."

Further evidence that considerable genetic diversity existed within *C. concisus* isolates of fecal origin came from a study by Van Etterijck et al. (1996) who used randomly amplified polymorphic DNA (RAPD) analysis to compare *C. concisus* strains isolated from fecal samples of 37 children with diarrhea attending the same Belgian day care center. This showed that *C. concisus* strains isolated from 35 of the 37 children (94.6%) had distinct RAPD profiles, further confirming the genetic heterogeneity of *C. concisus* strains.

The considerable genetic diversity between oral and fecal isolates of *C. concisus* was further confirmed by Aabenhus et al. (2002b) who examined the protein profiles of *C. concisus* strains isolated from Danish subjects with a range of symptoms including diarrhea and dyspepsia using SDS-PAGE. This showed, based on major protein band composition, that 85% of *C. concisus* fecal strains differed from the oral reference type strain *C. concisus* ATCC 33237. Given this, Aabenhus et al. assigned the *C. concisus* isolates into two broad groups, Group 1 which included *C. concisus* strains resembling the oral *C. concisus* ATCC 33237 type strain, and Group 2 which comprised *C. concisus* strains whose protein profiles differed from that of the *C. concisus* type strain. Interestingly Group 2, which included 83 of the 98 (85%) *C. concisus* isolates tested, predominantly comprised *C. concisus* strains isolated from patients with diarrhea, as well as all *C. concisus* strains isolated from

children and immunocompetent patients. In the same year, Matsheka et al. (2002) used pulsed field gel electrophoresis (PFGE), with the restriction enzyme *Not*I to determine the genetic diversity of 53 *C. concisus* strains isolated from fecal samples of children with diarrhea. This showed that of the 53 isolates examined, 51 had distinct *Not*I macrorestriction fragments, while 2 strains were resistant to *Not*I digestion. The patterns comprised between 1 and 14 restriction fragments, with type and reference strains of two well-defined genomospecies of oral and fecal origin containing 6 and 12 fragments, respectively. Based on these results Matsheka et al. (2002) concluded that *C. concisus* was genetically diverse and that the species was likely to be "a taxonomic continuum comprised of several genomospecies." In a later study, Matsheka et al. (2006) typed 100 *C. concisus* isolates obtained from 98 children with diarrhea and 2 dental isolates from adult patients by DNA fingerprinting. RAPD analysis of the 100 isolates showed 86% of isolates to be genotypically diverse. Of these heterogeneous isolates, 25 had previously been shown by the same group to have unique profiles using PFGE (Matsheka et al., 2002). The remaining 14 strains were shown to have 5 RAPD profiles. The high level of heterogeneity observed in these *C. concisus* strains was in line with this group's previous study.

This high level of diversity is consistent with a study by Pruckler et al. (2002) who examined the genetic diversity of 73 phenotypically and genotypically proven *C. concisus* strains including six ATCC/NCTC reference strains (two of which represented two established genomospecies; Aabenhus et al., 2002b) using PFGE and the restriction enzyme *Not*I. This showed all strains to have an unique *Not*I PFGE pattern. Further, composite analysis revealed five clusters, with the reference strains representing the two established genomospecies being clearly separated into different clusters. Moreover, in the same year, Aabenhus et al. (2002a) employed a lectin typing system, which used a panel of 4 lectins, to type 44 clinical isolates of *C. concisus* obtained from patients presenting with malignancies, HIV, IBD, and other conditions, who had suffered diarrhea and upper gastrointestinal dyspepsia, as well as the type strain ATCC 33237 of oral origin. This typing system grouped the 45 strains into 13 lectin reaction patterns, however, when the authors attempted to correlate the reaction patterns with the clinical category of the patients, no association was observed.

To determine the clinical relevance of *C. concisus* in gastrointestinal disease, Engberg et al. (2005) investigated the genotypic characteristics of 39 *C. concisus* isolates from Danish patients with diarrhea, three from healthy individuals and the type strain CCUG 13144. Protein profiling and PCR amplification of the 23S ribosomal RNA (rRNA) gene showed the isolates to cluster into two distinct, but discordant groups. Furthermore, automated ribotyping showed 34 of the 43 isolates to have distinct patterns. Additional analysis of 37 isolates using RAPD showed these to have 37 unique profiles. Interestingly, two strains from the feces of healthy human carriers were distinct from the majority of strains of diarrheal origin. Based on these studies Engberg et al. concluded that *C. concisus* consists of at least two genomospecies with extensive genetic diversity, however, no clear genotypic differences were observed between isolates from patients with diarrhea and isolates from healthy carriers. In a further study published in the same year, Aabenhus et al. (2005a) investigated the genotype of 62 *C. concisus* clinical isolates (56 diarrheal strains, 4 oral strains, and the oral CCUG 13114 and intestinal CCUG 19995 type strains) using amplified length fragment polymorphism (AFLP) analysis and correlated the results to clinical data. All strains examined were shown to have unique profiles, however, following numerical analysis of the AFLP profiles, relationships at the taxonomic level were revealed that allowed the strains to be grouped into four distinct clusters. AFLP cluster 1 contained the type strain of oral origin (CCUG 13144) as well as 22 other clinical isolates, 27% of which were isolated from immunocompetent (IC) patients. Cluster 2 contained the reference strain CCUG 19995 originally isolated from a human diarrhea sample, as well as 32 other clinical isolates, 59% of which were isolated from IC patients. Interestingly, five of the seven *C. concisus* strains isolated from IBD patients were present in cluster 2. AFLP cluster 3 consisted of a single diarrheal isolate from an IC patient, whereas cluster 4 contained five strains all of which were isolated from severely immunodeficient patients, a finding that led the authors to suggest that the strains in AFLP cluster 4 may be less invasive. As result of their study, Aabenhus et al. (2005a) concluded that *C. concisus* contains at least four distinct genomospecies that may vary in their pathogenic potential.

In a recent study, Kalischuk and Inglis (2011) compared the genotypes of *C. concisus* fecal isolates from diarrheic and asymptomatic healthy individuals using AFLP analysis and a genomospecies-specific 23S rRNA gene PCR, that grouped isolates into genomospecies A or B. Of the 22 isolates examined, six were assigned to genomospecies A and 12 to genomospecies B, while three isolates generated PCR products with both the genomospecies A and B primer sets, and were designated genomospecies A/B. One isolate failed to amplify with either primer set. Consistent with previous observations the type strain, LMG7788, was assigned to genomospecies A. Based on AFLP analysis the *C. concisus* isolates were shown to cluster into two phylotypes, distinguished from each other at a 34% similarity level. All isolates assigned to AFLP cluster 1 belonged to genomospecies A and included the type strain LMG7788, four isolates from healthy controls and one isolate from a patient with diarrhea. Of the 17 *C. concisus* isolates assigned to AFLP cluster 2, 12 (70.6%) belonged to genomospecies B, 3 to genomospecies A/B (17.6%), 1 to genomospecies A (5.9%), and the final untypable isolate (5.9%). While these results are in contrast to the findings of Aabenhus et al. (2005a) who based on AFLP identified four clusters among 62 *C. concisus* isolates, it should be noted that the majority of isolates (90.3%) in that study formed two main clusters.

In studies conducted in our own laboratory we recently sequenced the genome of *C. concisus* strain UNSWCD, isolated from a patient with CD and found it to have a 1.8-Mb genome that is substantially smaller than the available 2.1 Mb *C. concisus* BAA-1457 genome (Deshpande et al., 2011). While 1593 genes were conserved across UNSWCD and BAA-1457, 138 genes (7.8%) from UNSWCD and 281 (13.98%) from BAA-1457 were unique when compared against the other, and substantial functional differences were observed between the two strains. When cutoff values of 70% identity plus at least 85% gene length coverage were employed, searches between *C. concisus* UNSWCD and BAA-1457 showed that 76% of genes were homologs, whereas

those between *C. jejuni* strains showed 90–91% to be homologs, indicating substantial variation exists within these two *C. concisus* genomes (Kaakoush et al., 2011a). Furthermore, average percentage identities for all homologs revealed that *C. concisus* strains had higher variation when compared to the phylogenetically related species *C. jejuni* (96 vs. 98%, respectively). We investigated the protein profiles of these two strains and a further six *C. concisus* strains isolated from different disease states, and found BAA-1457 was highly divergent from the other *C. concisus* strains (Kaakoush et al., 2011a). Interestingly, the healthy control strain ATCC 51561 was divergent from the other strains isolated from acute and chronic gastroenteritis and CD. Attempts to discover markers for the detection of *C. concisus* through the identification of proteins found in all our strains but not in another bacterial species, found two candidates that may be good taxon-specific markers for this species.

PATHOGENESIS

Until recently, the pathogenic potential of *C. concisus* has remained relatively unexplored with comparatively few studies dedicated to elucidating the pathogenic mechanisms within this bacterium. However, following the association of *C. concisus* with enteritis and IBD, studies have increased significantly.

MOTILITY AND CHEMOTAXIS

Owing to its single polar flagellum (Man et al., 2010a), *C. concisus* has the ability to swim through protective host barriers such as saliva or the intestinal mucus layer, allowing it to achieve close contact with epithelial layers, and thus, a greater chance of triggering disease. One of the first studies to investigate *C. concisus* pathogenesis was that of Paster and Gibbons (1986) who in an attempt to understand the role of this bacterium in oral disease studied its chemotactic response to a range of compounds. While this showed that *C. concisus* exhibited a chemotactic response to formate, it was not chemoattracted to many sugars, inorganic salts, amino acids and their derivatives, purines and pyrimidines, fatty acids, or natural mixtures such as saliva, serum, and crevicular fluid (Paster and Gibbons, 1986). Intriguingly, unlike *C. jejuni* strains, *C. concisus* strain 288 was not chemoattracted to mucin (Paster and Gibbons, 1986), a finding that may relate to the oral origin of this strain. In contrast, the aggregation and possible attraction of *C. concisus* strains isolated from human intestinal biopsies to mucin has been observed through scanning electron microscopy (SEM; Kaakoush et al., 2011b).

PRODUCTION OF TOXINS AND OTHER VIRULENCE FACTORS

Campylobacter concisus strains have been shown to produce several toxins and virulence factors. Istivan et al. (1998) reported 20 *C. concisus* strains isolated from children with enteritis, and one from a healthy control to have hemolytic activity associated with lysis of human and animal erythrocytes. The same group later showed that *C. concisus* strains had membrane-bound hemolytic phospholipase A_2 activity that caused stable vacuolating and cytolytic effects on Chinese hamster ovary (CHO) cells (Istivan et al., 2004), and that this activity was encoded by the *pldA* gene (Istivan et al., 2008). Additionally, Istivan et al. (2008) detected secreted hemolytic activities in *C. concisus* strains isolated from children with enteritis. These findings are in accordance with Musmanno et al. (1998) who showed that a *C. concisus* strain isolated from a child with enteritis produced a cytotoxic-like effect on CHO cells and induced intracytoplasmic vacuole formation, and Kalischuk and Inglis (2011) who also showed that *C. concisus* isolates had hemolytic activity against sheep red blood cells. Moreover, Engberg et al. (2005) reported strain specific differences in the ability of *C. concisus* strains to induce cytolethal distending toxin-like effects on monkey kidney (Vero) cells, however, no association with disease outcome was found.

Further evidence of the cytotoxicity of *C. concisus* isolates was provided more recently by Nielsen et al. (2011b) who observed an elevated level of lactate dehydrogenase release in cells exposed to this bacterium. In contrast, Kalischuk and Inglis (2011) reported that none of their *C. concisus* isolates caused significant epithelial cytotoxicity (also measured through lactate dehydrogenase release). However, these authors also reported that 64.3% (9 out of 14) of their *C. concisus* isolates induced epithelial DNA fragmentation and this was correlated with an increase in host cell metabolic activity (Kalischuk and Inglis, 2011).

In studies conducted in our own laboratory we recently identified an invasin InvA, a hemolysin TlyA and zonula occludens toxin Zot within the genome of *C. concisus* BAA-1457, isolated from a patient with acute enteritis (Kaakoush et al., 2010). A complete list of virulence factors and their putative functions is provided in Kaakoush et al. (2010). Zot, a protein used by virulent pathogens to increase tissue permeability was not a common feature among our *C. concisus* strains (one out of eight; Kaakoush et al., 2011a). In contrast, Kalischuk and Inglis (2011) detected *zot* in 42.8% (6 out of 14) of their *C. concisus* isolates, which may suggest that these six isolates were genetically related to the BAA-1457 strain. We also analyzed the secretome of *C. concisus* strain UNSWCD isolated from an intestinal biopsy of a patient with CD, and identified a S-layer RTX protein and an outer membrane fibronectin-binding protein CadF (Kaakoush et al., 2010). RTX proteins are pore-forming toxins synthesized by a diverse group of Gram-negative pathogens, whereas CadF is known to be involved in adhesion to the host cell. Analysis of the genome of *C. concisus* UNSWCD revealed an O-antigen ligase that may play a role in the aggregation and adherence onto host cells, and an Acr protein (α-crystallin) that may contribute to persistent infection within the host and may influence the host response to infection (Kaakoush et al., 2011a), as has been observed in *Mycobacterium tuberculosis* (Stewart et al., 2005). Furthermore, we identified variations within the flagellin glycosylation pathways of *C. concisus* strains (Kaakoush et al., 2011a), a factor that may contribute to the differences observed in their pathogenic potential (Man et al., 2010a; Kaakoush et al., 2011b).

ADHERENCE AND INVASION INTO HOST CELLS

Russell and Ward (1998) investigated the ability of *C. concisus* strains isolated from children with diarrhea to adhere to and invade HEp-2 cells, and found rates 4- to 100-fold higher than the pathogenic and invasive *Campylobacter* species *C. jejuni* and *C. coli*. In contrast, in a recent study Kalischuk and Inglis (2011) found that *C. concisus* isolates exhibited comparable epithelial

adherence, invasion, and translocation abilities to that of *C. jejuni* 81–176. Moreover, the authors did not observe any differences in mean adherence, invasion, or translocation between isolates from diarrheic and healthy humans (Kalischuk and Inglis, 2011). In a recent study, Nielsen et al. (2011b) examined six oral and eight fecal *C. concisus* isolates and found that all strains invaded confluent host cells and impaired epithelial barrier function. The authors concluded that epithelial barrier dysfunction by *C. concisus* strains could mainly be assigned to apoptotic leaks together with moderate tight junction changes (Nielsen et al., 2011b).

In recent studies we have investigated the invasive phenotype of *C. concisus* in more detail through the analysis of isolates from different disease states (Man et al., 2010a). These studies showed that the percentage invasion of *C. concisus* strain UNSWCD, isolated from a child with newly diagnosed CD, was significantly higher than that of *C. concisus* strains isolated from patients with acute enteritis and a healthy control. Interestingly, *C. concisus* ATCC 51561 isolated from a healthy subject, showed no evidence of invasion. These findings were confirmed with SEM (**Figure 1**; Man et al., 2010a). To investigate the possible involvement of host microtubules and microfilaments on the *C. concisus* UNSWCD invasion process, we pre-treated Caco-2 cells with colchicine or cytochalasin D, and this showed the level of invasion by *C. concisus* UNSWCD to be significantly attenuated in the presence of both colchicine and cytochalasin D (Man et al., 2010a). We also showed that *C. concisus* UNSWCD preferentially attached to intercellular junctional spaces (**Figure 2**) and that this spatial distribution was concomitantly associated with a loss of membrane-associated ZO-1 and occludin. Given that *C. concisus* UNSWCD was isolated from a patient with CD, we also investigated the potential effect of pre-existing inflammation (driven by TNF-α and IFN-γ) on *C. concisus* UNSWCD invasion. This showed that the ability of *C. concisus* UNSWCD to invade Caco-2 cells treated with TNF-α or IFN-γ was significantly increased, suggesting that the presence of inflammation increases the invasive ability of *C. concisus* (Man et al., 2010a).

In a more recent study, we investigated the invasive potential of eight *C. concisus* strains isolated from different disease states (three CD, one chronic enteritis, three acute enteritis, and one healthy subject), and found that the percentage invasion of *C. concisus* strains isolated from chronic intestinal diseases were more than 500-fold higher than that of *C. concisus* strains isolated from acute intestinal diseases and a healthy control (Kaakoush et al., 2011b). As was previously reported by Russell and Ward (1998), the levels of invasion observed for *C. concisus* strains in our study were higher than that reported for *C. jejuni* (Kaakoush et al., 2011b). Importantly, we detected a plasmid harboring virulence determinants from several pathogenic organisms only in the highly invasive strains isolated from chronic intestinal diseases, thus, elucidating the feature that may be responsible for the heterogeneity in the invasive potential of *C. concisus* (Kaakoush et al., 2011b).

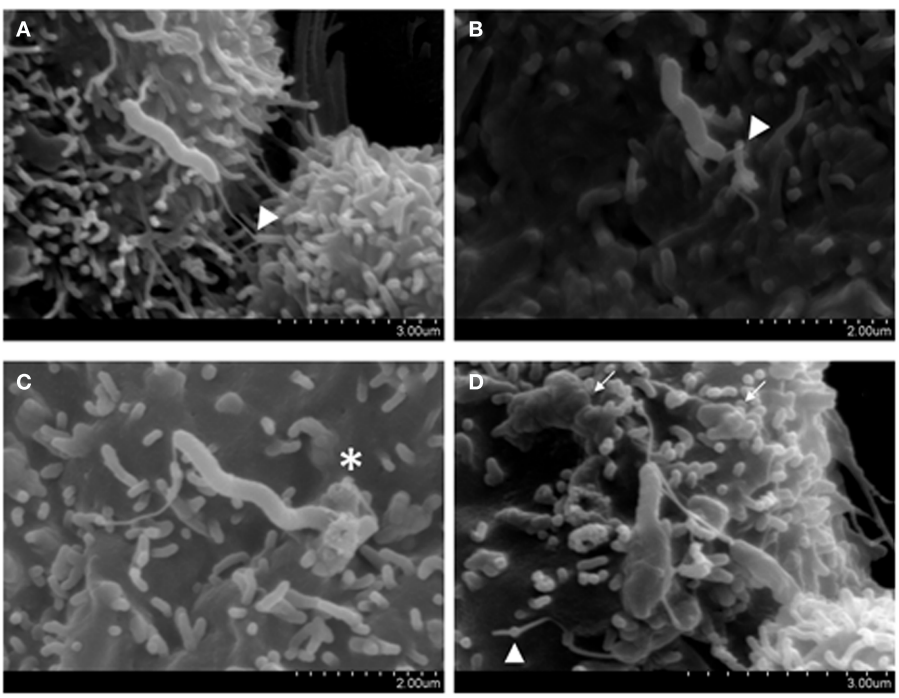

FIGURE 1 | Host attachment and invasion by *Campylobacter concisus* UNSWCD (adapted from Man et al., 2010a). Caco-2 cells were infected with bacteria at a MOI of 200 for 6 h. **(A)** The polar flagellum of *C. concisus* UNSWCD mediated attachment to the microvillus tip (*triangles*). **(B)** The flagellum appears to fold around the microvillus (*triangle*). **(C)** *C. concisus* induced a membrane ruffling-like effect (*). **(D)** *C. concisus* is observed half internalized in the host cell, resulting in a surface protrusion on the host cell membrane, and the flagellated half remains externally exposed. A host cell infected with multiple bacteria displays cell membrane irregularities and uneven texture because of bacteria-induced protrusions (*arrows*).

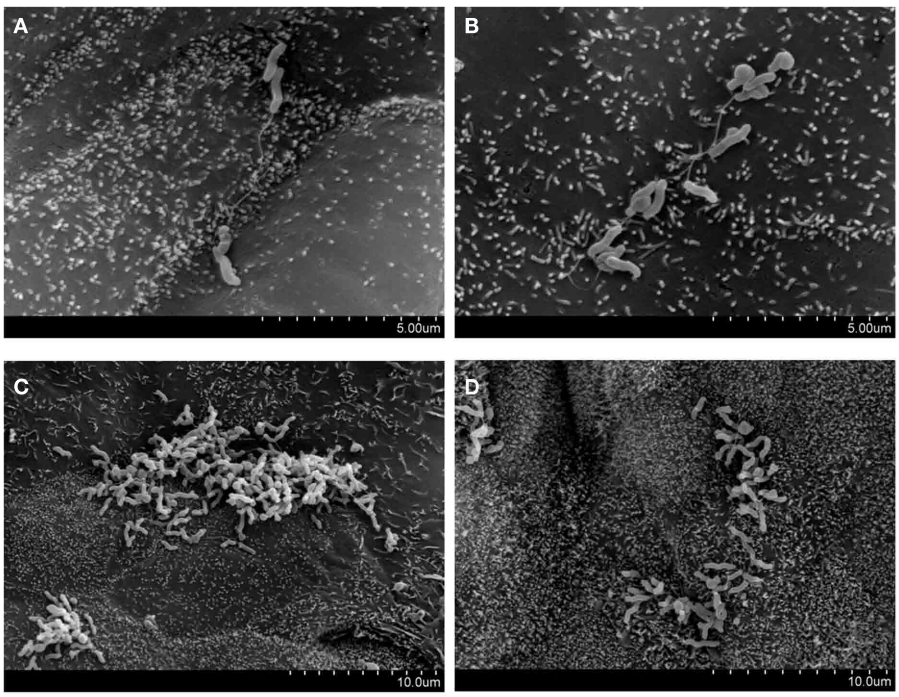

FIGURE 2 | Preferential attachment of *Campylobacter concisus* UNSWCD to intercellular junctional spaces of host cells (adapted from Man et al., 2010a). HT-29 cells were infected with bacteria at a MOI of 200 for 6 h. **(A–D)** *C. concisus* UNSWCD preferentially attached to areas resembling the intercellular junctional space.

HOST IMMUNE RESPONSE TO *CAMPYLOBACTER CONCISUS*

Several studies have attempted to elucidate the host immune response to *C. concisus* infection. In our own studies we have shown that following infection of intestinal cells, *C. concisus* UNSWCD stimulated significantly higher levels of IL-8 as compared with *Escherichia coli* K-12 (Man et al., 2010a), a finding that is supported by Kalischuk and Inglis (2011) who also reported the infection of intestinal cells with *C. concisus* strains to result in increased expression of IL-8 by more than twofold. Interestingly, in our study the levels of IL-8 stimulated with heat-killed *C. concisus* were found to be comparable to that of their viable counterparts. Furthermore, in an examination of the THP-1 monocytic cell response to bacterial stimulation we observed that *C. concisus* UNSWCD induced significantly elevated levels of IL-8 and TNF-α, but not IL-1β when compared with unstimulated controls (Man et al., 2010a). When primary human macrophages were infected with *C. concisus* UNSWCD a significantly higher level of TNF-α was observed as compared with the unstimulated control, while relatively low levels of IL-1β were produced. Whilst *C. concisus* UNSWCD induced increased levels of IL-8 in primary human macrophages, the levels observed were not significantly higher than that in the unstimulated control (Man et al., 2010a). Comparison of the results of *C. concisus* UNSWCD to that of other *C. concisus* strains, showed strain dependency in their ability to stimulate IL-8 and TNF-α in the different types of host cells examined (Man et al., 2010a).

More recently, we showed that host cells infected with any of our *C. concisus* strains (CD, chronic enteritis, acute enteritis, and a healthy subject) produced high amounts of IL-12, however, only the *C. concisus* strains capable of internalizing into host cells induced a significantly increased quantity of IFN-γ with respect to controls (Kaakoush et al., 2011b). These findings, along with the regulation of proteasomal subunits, ubiquitination pathways, and NF-κB inhibitors, led us to hypothesize that invasive *C. concisus* strains can activate the NF-κB pathway (Kaakoush et al., 2011b).

PATHOGENESIS IN ANIMAL MODELS

Only one study has observed the pathogenic potential of *C. concisus in vivo* (Aabenhus et al., 2008). In this study, five clinical *C. concisus* isolates of intestinal origin and the ATCC 33237 type strain of oral origin were inoculated into immunocompetent BALB/cA mice treated with cyclophosphamide to suppress their immune functions. While *C. concisus* was isolated from the liver, ileum, and jejunum of some of these mice, this was not consistent across experiments. Further histological examination did not consistently find signs of inflammation in the gut, but occasionally micro-abscesses were found in the liver of infected animals (Aabenhus et al., 2008). Compared to controls, mice infected with some *C. concisus* strains showed a significant weight loss in the first couple of days, but this effect wore off by the fifth day of infection. Finally, a total of three *C. concisus*-infected mice died during the course of the experiments.

The findings in relation to animal models of *C. concisus* infection are of no surprise given the difficulty faced with developing a good animal model of *C. jejuni* infection. For example, many

murine models of *C. jejuni* infection have resulted in sporadic colonization and/or absence of clinical disease signs (Bereswill et al., 2011), an outcome that is suggested to relate to colonization resistance caused by the murine intestinal flora. The recent development of novel *C. jejuni* infection models by Bereswill et al. (2011) may however now overcome previous problems relating to both poor colonization and the absence of the classical campylobacteriosis picture observed in humans. To overcome colonization resistance due to the murine flora, Bereswill et al. eradicated the intestinal flora of gnotobiotic mice using antibiotic treatment and then investigated the colonization potential of these mice with *C. jejuni*. This showed that *C. jejuni* could colonize all regions of the GIT of these mice. Further, a group of gnotobiotic mice treated with antibiotics were recolonized with human intestinal microflora and following recolonization, were infected with *C. jejuni* and the colonization and inflammatory response investigated. This showed that *C. jejuni* could not only stably colonize the gnotobiotic "humanized" intestinal tract but also exhibited the typical pro-inflammatory features of human campylobacteriosis. Given this, the gnotobiotic and "humanized" mouse models may turn out to be an excellent model of *C. concisus* infection (Bereswill et al., 2011).

RESERVOIRS OF INFECTION

While the source and routes of transmission for the closely related species *C. jejuni* and *C. coli* are well established (Vandamme et al., 2005), little is known about the source and route of transmission of *C. concisus*. Given the importance of birds and animals as a source of *C. jejuni* and *C. coli* in human infection, current studies have focused on detection of *C. concisus* in domestic animals, sheep, cattle, pigs, and chickens. For example, in a study investigating the presence of *C. concisus* DNA in the saliva of domestic pets, Petersen et al. (2007) identified this bacterium in 1 of 8 cats but in none of 12 dogs. In another study, which screened fecal samples from healthy dogs for different *Campylobacter* species, although several *Campylobacter* species were identified, *C. concisus* was not detected (Chaban et al., 2009). Interestingly, in a follow up study by the same group, which screened fecal samples from both healthy ($n = 70$) and diarrheic dogs ($n = 65$), *C. concisus* DNA was detected in six diarrheic dogs but not in healthy dogs ($P < 0.05$; Chaban et al., 2010). In an attempt to determine if farm animals were a reservoir for emerging *Campylobacter* species, Oporto and Hurtado (2011) detected two isolates from healthy sheep feces that were shown to cluster with *C. concisus* strains, despite sharing higher similarity with *C. mucosalis*, however, no *C. concisus* DNA was detected in swine, beef cattle, or dairy cattle. Interestingly in a study which investigated the presence of *C. concisus* in chicken ($n = 185$), pork ($n = 179$), and beef ($n = 186$) samples collected throughout the Republic of Ireland, Lynch et al. (2011) found *C. concisus* to be present in 10 and 3% of chicken and beef samples, respectively. Notably, the detection level of *C. concisus* in chicken meat was similar to that observed for *C. jejuni* (13% of samples; Lynch et al., 2011). While *C. concisus* may be associated with enteritis in dogs (Chaban et al., 2010), collectively current studies would suggest that chickens, and not domestic pets, are a more likely reservoir for *C. concisus*, thus suggesting the possibility of zoonotic transmission. Furthermore, the presence of viable *C. concisus* in the saliva of most humans (described above) may support a person to person route of transmission.

ANTIBIOTIC SUSCEPTIBILITY

The susceptibility of *C. concisus* to a variety of antimicrobial agents has been examined in several studies and these are summarized in **Table 3** (Tanner et al., 1981; Johnson et al., 1986; Aabenhus et al., 2005b; Vandenberg et al., 2006). In 1981, upon designation of six isolates from the oral cavity of humans into the new species *C. concisus*, Tanner et al. (1981) measured the effects of 17 antimicrobial agents against these isolates and found them to be resistant to bacitracin, nalidixic acid, rifampin, and vancomycin (**Table 3**). Five years later, Johnson et al. (1986) observed the effects of 17 antimicrobial agents, 7 of which had been previously investigated by Tanner et al. against a group of five isolates designated "*Wolinella* spp./*C. concisus*" (**Table 3**) and showed that as reported by Tanner et al. isolates were susceptible to chloramphenicol, metronidazole, clindamycin, erythromycin, tetracycline, and penicillin. In contrast to Tanner et al., Johnson et al. (1986) found two of five isolates to be resistant to gentamicin. O'Connor et al. (1990) studied the effect of minocycline on *C. concisus* and as was observed by Tanner et al. (1981), they found the drug to be effective against the bacterium. However, they also observed an increase in the MIC of minocycline for *C. concisus* after the bacterium was exposed to sub-lethal concentrations of the antibiotic for 6–7 weeks, a finding that was not observed in most of the bacteria they tested (O'Connor et al., 1990).

In a more recent study Engberg et al. (2005) reported that all 43 *C. concisus* isolates tested were susceptible to erythromycin, tetracycline, nalidixic acid, streptomycin, ciprofloxacin, gentamicin, colistin, chloramphenicol, sulfamethizole, neomycin, and ampicillin, however, their data was not shown. In a study which characterized and sub-grouped 109 *C. concisus* strains isolated from 98 patients with gastrointestinal disease into two major groups (Group 1, $n = 15$; Group 2, $n = 94$) using protein profiling, Aabenhus et al. (2005b) tested the effects of eight antimicrobial agents against the isolates in each group using a range of techniques (**Table 3**). While resistance to nalidixic acid was detected in 87–90% of strains (API Campy system and disks from Biodisc AB), all strains were resistant to cefazolin (API Campy system). Based on two techniques (Neosensitabs and E-tests), *C. concisus* strains were found to be generally susceptible to ampicillin (93–100%), tetracycline (97–100%), ceftriaxone (89–98%), mecillinam (92–100%), erythromycin (93–97%), and ciprofloxacin (87–95%). In contrast, the API Campy system found all isolates to be resistant to erythromycin (Aabenhus et al., 2005b). The authors concluded that the API Campy system was not a valid tool in the identification of *C. concisus* as they considered the reaction patterns to be too heterogenous (Aabenhus et al., 2005b). In agreement with Aabenhus et al., Vandenberg et al. (2006) reported all 20 *C. concisus* isolates tested to be susceptible to ampicillin and tetracycline, 5% to be resistant to gentamicin, ciprofloxacin, and erythromycin, and 80% to be resistant to nalidixic acid. Strikingly, Aabenhus et al. (2005b) found that the majority of their strains (94%) were susceptible to cephalothin, a finding that contradicts Roop et al. (1985) who stated that *C. mucosalis* strains can be differentiated from *C. concisus* through the latter's resistance to cephalothin. In agreement

Table 3 | Susceptibility* of *Campylobacter concisus* to 30 antimicrobial agents.

Antibiotic	Tanner et al. (1981)	Johnson et al. (1986)	Aabenhus et al. (2005b)#		Vandenberg et al. (2006)	Bergey's (Vandamme et al., 2005)
			Group 1	Group 2		
Amikacin	–	>64	–	–	–	–
Ampicillin	–	–	1.5	0.38	2.0	–
Bacitracin	>128	–	–	–	–	128
Cefazolin	–	>128	–	–	–	–
Cefoperazone	–	>128	–	–	–	–
Cefotaxime	–	4.0	–	–	–	–
Cefoxitin	–	>128	–	–	–	–
Ceftazidime	–	64	–	–	–	–
Ceftriaxone	–	–	2.0	0.5	–	–
Chloramphenicol	4.0	4.0	–	–	–	4.0
Ciprofloxacin	–	≤1.0	0.094	0.125	0.25	–
Clindamycin	2.0–4.0	1.0	–	–	–	24
Colistin	0.5–1.0	–	–	–	–	0.5–1.0
Erythromycin	4.0	2.0	3.0	3.0	4.0	4.0
Gentamicin	2.0–4.0	>32	–	–	1.0	24
Imipenem	–	≤1.0	–	–	–	–
Kanamycin	1.0–2.0	–	–	–	–	12
Mecillinam	–	–	3.0	3.0	–	–
Metronidazole	0.5–2.0	1.0	–	–	–	0.5–2.0
Minocycline	2.0	–	–	–	–	2.0
Moxalactam	–	128	–	–	–	–
Nalidixic acid	64–128	–	–	–	32	64–128
Neomycin	16–32	–	–	–	–	16–32
Penicillin	0.5–4.0	8.0	–	–	–	0.5–4.0
Piperacillin	–	256	–	–	–	–
Polymixin B	≤0.25–1.0	–	–	–	–	0.25–1.0
Rifampin	16–64	–	–	–	–	16–64
Streptomycin	1.0–2.0	–	–	–	–	12
Tetracycline	1.0–2.0	1.0	0.5	0.75	0.5	12
Vancomycin	>128	–	–	–	–	128

**MIC_{90} represents the MIC that completely inhibited visible growth of 90% of the strains. #C. concisus strains were split into two groups based on their protein profiles.*

with Aabenhus et al., Pruckler et al. (2002) reported that only 5% of their 73 *C. concisus* isolates to be resistant to cephalothin. In addition, the authors reported that the overall resistance rates for their 73 *C. concisus* isolates were 93.75% for nalidixic acid, 30% for clindamycin, 7.5% for ciprofloxacin, 3.75% for tetracycline, 2.5% for erythromycin, 1.25% for azithromycin, and 0% for both chloramphenicol and gentamicin (Pruckler et al., 2002).

Notably, Moore et al. (2006) upon examining 457 *C. concisus* isolates collected between 1998 and 2006, reported that resistance to nalidixic acid increased from 40 to 62%, resistance to ciprofloxacin increased from 6.9 to 18%, resistance to erythromycin increased from 4.8 to 21.7%, whereas ceftriaxone resistance remained stable at 2% over the time frame. Moreover, some isolates were resistant to several classes of antibiotic (Moore et al., 2006).

Other studies reporting the effect of antimicrobial agents against *C. concisus* include the finding that this bacterium expresses pyrazinamidase activity (Shingaki et al., 1999), a reaction that converts the pro-drug pyrazinamide to its active form pyrazinoic acid.

Interestingly, a number of studies investigating the effect of antibiotic therapy in patients with CD have reported nitroimidazoles and ciprofloxacin, antibiotics to which *C. concisus* remains sensitive, to have a beneficial effect (Feller et al., 2010). For example, in a recent systematic review and meta-analysis of randomized clinical trials, which investigated the effect of antibiotic therapy on remission in patients with active disease and relapse in patients with inactive disease, three studies using nitroimidazoles (two, metronidazole and one, omidazole), showed a beneficial effect (OR: 3.54; 95% CI: 1.94–6.47). Further, a trial examining treatment with ciprofloxacin for 6 months also showed benefit (OR: 11.3; 95% CI: 2.60–48.8). In contrast, the combined OR (OR: 0.58, 95% CI: 0.29–1.18) obtained from three trials involving treatment with anti-tuberculosis drugs

(including rifamycins) showed no beneficial effect (Feller et al., 2010).

Collectively, these studies show that although several classes of antibiotics that are generally effective against anaerobes remain effective against *C. concisus*, resistance to antimicrobial agents within *C. concisus* isolates is on the rise.

CONCLUSION

Significant advances in our understanding of *C. concisus* have occurred over the last 5 years with over a third of studies on *C. concisus* published within this time period. Current evidence would suggest that likely reservoirs of this bacterium include humans and chickens. In relation to disease, *C. concisus* has been associated with periodontal diseases, acute enteritis, and IBDs, with the strongest evidence relating to acute and chronic intestinal diseases. The finding that *C. concisus* strains can adhere to and invade host cells, translocate across cell monolayers and induce inflammatory immune responses supports the possible role of this bacterium in intestinal diseases. Further studies that compare the prevalence and pathogenic potential of *C. concisus* strains isolated from different disease outcomes with those from healthy controls are required to reinforce the current evidence suggesting an association between *C. concisus* and intestinal diseases. A further important step will be the identification of a suitable animal model in which to investigate the pathogenesis of *C. concisus* infection.

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