Regulation of *Francisella tularensis* virulence

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Francisella tularensis is one of the most virulent bacteria known and a Centers for Disease Control and Prevention Category A select agent. It is able to infect a variety of animals and insects and can persist in the environment, thus *Francisella* spp. must be able to survive in diverse environmental niches. However, *F. tularensis* has a surprising dearth of sensory and regulatory factors. Recent advancements in the field have identified new functions of encoded transcription factors and greatly expanded our understanding of virulence gene regulation. Here we review the current knowledge of environmental adaptation by *F. tularensis*, its transcriptional regulators and their relationship to animal virulence.

Keywords: *Francisella*, transcriptional regulators, *Francisella* pathogenicity island, two-component regulatory systems

INTRODUCTION

Francisella tularensis is a Gram-negative non-motile intracellular pathogen and the causative agent of the zoonotic human disease tularemia. Tularemia, also known as rabbit fever, is usually transmitted to humans by arthropod bites, oral consumption of contaminated food or water, or handling of infected animal carcasses (Evans et al., 1985; Thomas and Schaffner, 2010).

Francisella tularensis is divided into several subspecies (Staples et al., 2006; Kugeler et al., 2009). These subspecies are associated with important differences in geographic distribution. *F. tularensis* subsp. *tularensis* (Type A) is found exclusively in North America. It is highly virulent, with less than 10 bacteria causing disease in humans via the respiratory route, and if untreated, demonstrates a high mortality rate of 30–60% (Dienst, 1963). *F. tularensis* subsp. *holarctica* (Type B) is less virulent in humans and is predominant in Europe and Japan, but also found in North America. *F. tularensis* subsp. *mediasiatica* is found primarily in Asia and Russia, and is rarely associated with disease in humans. *F. novicida* is normally considered as the fourth subspecies of *F. tularensis*; however, recent genome-wide polymorphism analysis suggests that it is an independent species highly virulent in mice but not in humans (Johansson et al., 2010). An attenuated strain of *F. tularensis* subsp. *holarctica*, the live vaccine strain (LVS), was developed and is used as a live-attenuated vaccine in the former Soviet Union and in some US laboratory workers (Tigertt, 1962; Burke, 1977). Because *F. tularensis* is able to infect through the respiratory route and cause disease with a very small dose, can be easily disseminated, results in a high mortality rate, and has the potential to cause panic among the public, *F. tularensis* is given the highest priority classification by the Centers for Disease Control and Prevention as a Category A select agent and is a potential bioweapon.

Completed genome sequencing for different subspecies has revealed that despite high nucleotide identity, there are many DNA rearrangements, gene acquisitions, and gene losses between and even within different subspecies (Titball and Petrosino, 2007; Champion et al., 2009; Nalbantoglu et al., 2010; Sjodin et al., 2010). These differences are thought to reflect the environmental adaptation of these subspecies and are associated with their differences in virulence. Most of our current understanding about the genetics of *F. tularensis* pathogenesis have been acquired with *F. novicida*, as this subspecies can be genetically manipulated with relative ease and causes a disease in mice similar to that caused by the human virulent *F. tularensis* subsp. *tularensis*. However, due to the genetic, phenotypic, and host range variations between the species, care should be taken in extrapolating data from one subspecies to another. With the recently developed genetic tools for the human virulent subspecies *tularensis* (LoVullo et al., 2006, 2009), it is now possible and necessary to carry out studies in these strains, which is required gain a full understanding the molecular basis of *Francisella* pathogenesis. Nevertheless, the studies with *F. novicida* and LVS have provided and will continue to provide valuable information on the molecular pathogenesis of tularemia, and the results have been generally applied to *F. tularensis*.

Francisella tularensis exists naturally in a variety of environments. It can survive in water, wet soil, and animal carcasses for several weeks. *F. tularensis* has also been found in a broad range of hosts, including mammals, insects, arthropods, and fresh water protozoans (Ellis et al., 2002). Not only does *F. tularensis* infect different hosts, as an intracellular pathogen, *F. tularensis* also goes through various microenvironments within host cells, including the macrophage phagosome and the host cell cytosol after phagosomal escape (Santic et al., 2010). Essential to the success of *F. tularensis*

as an intracellular pathogen is its ability to adapt to a wide variety of environments and host cell types. This is achieved via timely activation/repression of dedicated patterns of gene expression. Most of the virulence genes of *F. tularensis* identified so far localize to the *Francisella* pathogenicity island (FPI). This is a cluster of 17 genes that is critical for the intracellular survival and virulence of *F. tularensis* (Nano and Schmerk, 2007). FPI genes were the first identified *Francisella* virulence factors and are affected by essentially all environmental cues and transcriptional virulence regulators identified thus far, demonstrating their importance in *F. tularensis* virulence.

Environmental changes are often sensed and relayed into bacteria through two-component regulatory systems (TCS). These systems are conserved and ubiquitous in bacteria, typically composed of a histidine sensor kinase, regulated by environmental stimuli, and a response regulator that activates downstream responses (Stock et al., 2000). In contrast to the variety of environments *F. tularensis* naturally encounters and opposite to most Gram-negative pathogens, which have numerous TCS, *F. novicida* has very few TCS encoded in the genome, and other subspecies of *F. tularensis*, such as subsp. *tularensis* lack any classically arranged (adjacent and co-transcribed) TCS. Orphaned TCS members KdpD, QseC, and PmrA are the only TCS factors discovered so far that affect virulence/virulence factor regulation (Mohapatra et al., 2007; Bell et al., 2010). In addition, *F. tularensis*, unlike many other bacteria, encodes only one alternative sigma factor (Fuller et al., 2009). This makes *F. tularensis* a unique pathogen with relatively few easily identifiable regulatory factors to survive in diverse environments. It is possible that some regulators exist that do not show homology to know regulators in the database or that alternative means of regulation (e.g., sRNA, post-transcriptional) may predominate in the *Francisella*.

ENVIRONMENTAL STIMULI

To survive in different environments, *F. tularensis* has to be able to sense and respond to signals from its surroundings to precisely regulate virulence gene expression. In fact, the protein profiles of *F. tularensis* (LVS) grown in broth versus inside macrophages revealed the altered expression of several proteins (Golovliov et al., 1997). In addition, global transcriptional profiling of *F. tularensis* Type A strain Schu S4 within infected macrophages confirmed that the expression of 658 genes was significantly changed, among which 298 were up-regulated and 360 were down-regulated, depending on the stage of the infection (Wehrly et al., 2009). These observations support the notion that *F. tularensis* is able to sense and respond to changing environments during the course of infection. Although the molecular mechanisms that underlie gene regulation are not fully understood, studies thus far have suggested that environmental stimuli including temperature, limited iron source, oxidative stress, and host intramacrophage components, lead to altered expression profiles.

TEMPERATURE

A number of pathogenic bacteria that encounter a temperature shift during their life cycle have the ability to respond to this environmental cue with enhanced virulence. *Shigella flexneri* increases *virB* expression in response to elevated temperature

(Tobe et al., 1991, 1995). *Borrelia burgdorferi* and *Yersinia pestis* have also adapted ways to change the expression profile of host-specific virulence genes according to an alteration in temperature (Konkel and Tilly, 2000). As a pathogen with different hosts, *F. tularensis* will go through a temperature shift upon entering a warm-blooded animal. When growing at 25 versus 37°C, *F. novicida* changes its outer surface by modifying the lipid A of lipopolysaccharide (Shaffer et al., 2007). Also, a recent transcriptomic study compared the global expression profiles of *F. tularensis* LVS cultivated in defined media at 26°C (environmental temperature) versus 37°C (mammalian body temperature; Horzempa et al., 2008). Approximately 11% of the genes in the entire LVS genome had significant changes in expression. Notably, around 40% of the genes that were up-regulated were previously identified or predicted to be important for intracellular growth and/or virulence (Horzempa et al., 2008). Another temperature change proteomic study with LVS and the Type A strain Schu S4 confirmed a significant increase in the level of FPI-encoded IglC, IglD, and PdpC, when the temperature shifted from 25 to 37°C (Lenco et al., 2009).

The regulatory mechanisms that underlie these temperature-dependent changes are not yet clear, but a few studies have suggested the involvement of an alternative sigma factor and its induction of heat-shock proteins, which is a mechanism used by many other bacteria (Ericsson et al., 1994; Meibom et al., 2008; Grall et al., 2009). However, unlike most other bacteria that have multiple sigma factors, *F. tularensis* encodes only one alternative sigma factor, RpoH, which is a sigma factor σ^{32} (Grall et al., 2009). Transcription profiling under heat-stress conditions in *F. tularensis* LVS showed that a number of heat-shock proteins and proteins involved in virulence were up-regulated. However, among all the genes that were regulated upon heat-stress, only 13 of them, which did not include FPI genes, had a putative σ^{32} binding site in their promoter region (Grall et al., 2009). This suggested that σ^{32} might not be acting directly on FPI genes.

IRON

Iron acquisition is a critical requirement for the survival of bacteria as it is involved in many metabolic processes. As one of the most common elements in the Earth's crust, iron is easily accessible for bacteria in the environment. However, for intracellular pathogens, their ability to acquire iron is critical because of the limited nature of freely available iron in mammalian host cells. Iron availability has a great impact on both virulence of bacteria pathogens and the host immune response (Wang and Cherayil, 2009; Skaar, 2010).

Pathogens respond to iron-restriction conditions by up-regulation of not only iron acquisition machinery but also virulence determinants (Ratledge and Dover, 2000; Skaar, 2010). A general mechanism for iron sensing is controlled by the ferric uptake regulator (Fur). Fur forms a complex with ferrous iron and represses gene expression by binding to the Fur box upstream of iron-regulated genes, which is then derepressed under iron-limiting conditions. In *F. tularensis*, an iron acquisition operon (*fsl* for *F*t siderophore *locus* or *fig* for *F*t *iron genes*) involved in siderophore production is located downstream of *fur*, and is induced under iron-restriction conditions in a Fur-dependent

manner (Deng et al., 2006; Sullivan et al., 2006). A Fur box is also found upstream of *iglC* and *pdpB*, two FPI genes (Deng et al., 2006). Microarray analysis found that approximately 80 genes, including FPI genes, are differentially expressed in iron-starved *F. tularensis* LVS (Deng et al., 2006). This was confirmed by a proteomics analysis in *F. tularensis* LVS, which showed that the levels of a significant number of proteins change under iron-limiting conditions, which again included those in the FPI (Lenco et al., 2007). Although it did not appear in these two previously mentioned studies, the FPI-encoded PdpA is also up-regulated under iron-limiting conditions (Schmerk et al., 2009). Moreover, gallium, a transition metal that competes with iron for uptake and/or utilization, inhibits *F. tularensis* LVS and *F. novicida* growth in broth and in macrophages, and inhibits *F. novicida* virulence in a mouse model of pneumonic tularemia (Olakanmi et al., 2010), providing further evidence for an important role of iron in regulating *F. tularensis* virulence factors.

OXIDATIVE STRESS

One of the essential host cell innate immune defenses against invading pathogens is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS; Fang, 2004). Many bacterial pathogens are able to sense the oxidative stress inside host cells and have ways to combat it. *F. tularensis* is in this group of pathogens as it is able to inactivate the phagocyte NADPH oxidase through a mechanism likely involving dephosphorylation of host kinases and *phox* components by bacterial enzymes/bacterial-mediated host signaling cascades (McCaffrey and Allen, 2006; Schulert et al., 2009; McCaffrey et al., 2010; Mohapatra et al., 2010). A screen for *F. tularensis* LVS proteins responding to heat and hydrogen peroxide identified chaperones DnaK, GroEL, and GroES (Ericsson et al., 1994). In addition to these proteins, Lenco et al. (2005) detected approximately 20 proteins that were up-regulated upon exposure to oxidative stress in *F. tularensis* LVS. In searching for the molecular mechanisms that underlie the response to oxidative stress, a proteomics assay was carried out comparing the protein profiles of wild-type *F. novicida* and an *mglA* (macrophage growth locus) mutant. In addition to virulence factors, homologs of oxidative, and general stress response proteins were identified in this study, including the previously identified chaperons, suggesting a role for MglA, a regulator of *F. tularensis* virulence, in the oxidative response (Guina et al., 2007).

OTHER ENVIRONMENTAL CUES

In addition to reactive oxygen and nitrogen species, other components of the host environment may also be signals sensed by *F. tularensis*. A recent study identified spermine, a polyamine produced only in eukaryotic cells, as an intramacrophage component sensed by *F. tularensis* (Carlson et al., 2009). Infection with *F. tularensis* LVS or Schu S4 cultured in the presence or absence of spermine induced different patterns of cytokine production in macrophages. A genome-wide microarray assay indicated that the presence of spermine affected a significant portion of the *F. tularensis* transcriptome. This is in part mediated by the induction of the *Francisella* insertion sequence (IS) elements IS*Ftu1* and IS*Ftu2*, which is sufficient to induce the expression

of downstream genes in response to spermine (Carlson et al., 2009). This observation suggested a model of gene regulation in which intramacrophage compounds elicit substantial changes in *Francisella* gene expression, and result in low cytokine production and immune evasion.

As *F. tularensis* resides temporally in phagosomal vacuoles after entering the host cell, the decrease in pH inside the phagosome may play a role in the induction of virulence factors. In support of this, the expression of RipA, a cytoplasmic membrane protein involves in *F. tularensis* intracellular replication and host immune evasion, is regulated by pH with increased expression at neutral rather than acidic environment (Fuller et al., 2008, 2009; Huang et al., 2010). However, the early acidification of the phagosome is not singularly involved in FPI gene induction as virulence protein expression is increased even upon inhibition of phagosome acidification (Chong et al., 2008).

Another environmental change that might play an important role in the induction of *F. tularensis* virulence is stress/starvation. The host cell phagosome/cytosol is known to be a nutrient poor and stress-inducing environment for bacteria. Several of the *F. tularensis* virulence regulators have been implicated in the stringent response, i.e., a microbial reaction to starvation. For example, SspA is a stringent starvation transcriptional regulator that is highly related to and physically interacts with MglA in *F. tularensis* LVS and *F. novicida* (Charity et al., 2007). In addition, guanosine pentaphosphate (ppGpp), an alarmone that is the global regulator of gene expression during the stringent response, is also involved in *F. tularensis* virulence gene expression by promoting the interaction of FevR/PigR with the MglA–SspA–RNA polymerase (RNAP) complex (Charity et al., 2009). Moreover, RelA, the enzyme involved in ppGpp production, is also involved in *F. tularensis* LVS virulence and intracellular survival (Dean et al., 2009). These findings suggest that starvation plays a critical role in the induction of *F. tularensis* LVS virulence.

TRANSCRIPTIONAL REGULATORS

Bacteria typically utilize specialized transcription factors that bind non-specifically or specifically to DNA recognition sequences. The location and nucleotide composition of transcription factor binding sites in part determines whether a transcription factor represses or activates the expression of a certain gene. An interesting feature of *Francisella* is that very few transcription factors have been identified by experimentation or homology searches (**Table 1**), but these few factors likely control the virulence cascades of this pathogen. While translational or post-translational regulation may play a role, the majority of evidence points to regulation at the level of transcription/mRNA transcripts. The FPI is the primary site of virulence-related genes; thus the remainder of this review will focus on transcription factors or regulators that have been identified to play a role in FPI gene regulation.

RNA POLYMERASE

Bacteria react to various environmental conditions by employing different modes of regulation. *F. tularensis* can replicate in a wide range of hosts and cell types, which suggests an extensive ability to adapt to different niches through variable gene

Table 1 | Transcription factors of *Francisella tularensis* identified by protein relatedness to known transcription factors/transcription factor motifs.

FTT# (Type A; Schu S4)	Gene	Description	Conservation in other *Francisella*					Experimentally studied in:		
			W96-3418 (Type A)	FSC198 (Type A)	OSU18 (Type B)	LVS (Type B)	U112 (*F. novicida*)	Schu S4 (Type A)	LVS (Type B)	U112 (*F. novicida*)
30	*fur*	Fe transcriptional regulator	+	+	+	+	+	Ramakrishnan et al. (2008)	Deng et al. (2006)	Kiss et al. (2008)
94	*qseC*	Sensor histidine kinase	+	+	+	+	+	Rasko et al. (2008)		Weiss et al. (2007), Durham-Colleran et al. (2010)
112		Homolog of Bvg acc. factor	+	+	+	+	+			
383	*fevR/ pigR*	Transcriptional regulator	+	+	+	+	+	Wehrly et al. (2009)	Charity et al. (2007, 2009)	Brotcke and Monack (2008)
458	*sspA*	Stringent starvation protein A regulator	+	+	+	+	+		Charity et al. (2007, 2009)	
492	*lysR*	Homolog of LysR	+	+	+	+	+			
556	*oxyR*	Oxidative stress regulator	+	+	+	+	+			Moule et al. (2010)
748		Homolog of TtgV	+	+	+	+	+	Mortensen et al. (2010)	Mortensen et al. (2010)	Weiss et al. (2007), Mortensen et al. (2010)
864		LysR family regulator	+	+	–	–	+			
932		ROK family regulator	–	+	–	+	+			
970		Transcriptional repressor	+	+	+	+	+			
977		MarR-like regulator	+	–	–	–	–			
981		Sensor kinase	+	+	+	+	+			
985		Transcriptional regulator	+	+	+	+	+			
1010		Cro family regulator	–	–	–	–	–			
1035	*rpoD*	Sigma factor	+	+	+	+	+		Grall et al. (2009)	
1076	*hipA*	Homolog of HipA	–	+	+	+	–			
1112	*rpoH*	Sigma factor	+	+	+	+	+		Grall et al. (2009)	
1119		LysR family regulator	+	+	+	+	+			
1172		Cold shock protein	+	+	–	+	–			
1202		LysR family regulator	+	+	–	+	+			

(Continued)

Table 1 | Continued

FTT# (Type A; Schu S4)	Gene	Description	Conservation in other *Francisella*					Experimentally studied in:		
			W96-3418 (Type A)	FSC198 (Type A)	OSU18 (Type B)	LVS (Type B)	U112 (*F. novicida*)	Schu S4 (Type A)	LVS (Type B)	U112 (*F. novicida*)
1255		AraC family regulator	+	+	+	+	+			
1267		LysR family regulator	+	+	+	+	+			
1275	*mglA*	Macrophage growth locus regulator	+	+	+	+	+		Bonquist et al. (2008), Charity et al. (2007, 2009)	Baron and Nano (1998), Brotcke et al. (2006), Guina et al. (2007)
1281	*rpoN*	Sigma factor	−	−	−	−	−			
1285		LysR family regulator	+	+	−	+	+			
1392		Homolog of Bvg acc. factor	+	+	+	+	+			
1401		LexA homolog	+	+	+	+	+			
1543		Response regulator	+	+	−	−	+			
1557c	*pmrA*	Response regulator	+	+	+	+	+		Sammons-Jackson et al. (2008)	Bell et al. (2010), Mohapatra et al. (2007)
1594		LysR family regulator	+	+	+	+	−			
1652		LysR family regulator	+	+	+	+	+			
1613		Homolog of BolA	−	−	−	−	−			
1684		Transcriptional regulator	+	+	−	+	+			
1736	*kdpD*	Sensor kinase KdpD	+	+	−	+	+			Bell et al. (2010), Weiss et al. (2007

expression. Transcription is the process of transcribing DNA into RNA and is performed primarily by a complex of proteins that form the RNAP. RNAP binding involves the alpha subunit recognizing the upstream sequences (40 to −70 base pairs) of the gene, as well as the σ factor recognizing the −10 to −35 regions of the promoter. *Francisella* RNAP is unique, as it contains two distinct forms of the alpha subunit. Two separate genes encode these proteins and they are both incorporated into the enzyme (Charity et al., 2007). Bacterial σ factors recognize different promoter elements upstream of genes allowing the cell to respond to various environmental conditions by directing the RNAP to unique sets of genes. However, in contrast to *Escherichia coli* and *Bacillus subtilis* which have six to eight alternative sigma factors that are used under various circumstances, as mentioned previously, *F. tularensis* has only two (the housekeeping σ^{70} and the stress-related σ^{32}).

MglA AND SspA

Baron et al. was the first to identify genes that are required for the replication of *F. novicida* within macrophages, where they described *mglA* and *mglB* (Baron and Nano, 1998). As discussed previously, MglA is highly up-regulated upon infection of macrophages. An *mglA* mutant of *F. novicida* is unable to escape from macrophage phagosomes and is highly attenuated in the mouse model but does not provide protection against homolgous or heterologous *Francisella* subsp. challenge (Lauriano et al., 2004; Santic et al., 2005; Brotcke et al., 2006). MglA has been characterized as a transcription factor and is responsible for regulating ~100 genes including those of the FPI (Lauriano et al., 2004; Brotcke et al., 2006; Guina et al., 2007), while proteomic analysis indicates that the abundance of ~350 proteins is altered in the absence of MglA. In addition to the FPI, other virulence factors such as the metalloprotease PepO are regulated by MglA (Guina

et al., 2007; West et al., 2008). MglA is similar to SspA (Baron and Nano, 1998; Brotcke et al., 2006), a transcription factor in *E. coli* that responds to nutrient limitation and is involved in regulation of genes contributing to the stringent starvation response. SspA of *Francisella* is important for the regulation of virulence genes, and the regulons of MglA and SspA are overlapping, including those genes within the FPI (Charity et al., 2007). MglA and SspA physically interact and both bind *Francisella* RNAP in a heterodimer, which is required for FPI gene activation (Charity et al., 2007).

FevR/PigR

An additional regulator of the FPI that is essential for intramacrophage replication and virulence in the mouse model has been named *Francisella* effector of virulence regulation or FevR (also called PigR; Buchan et al., 2008; Charity et al., 2009). Microarray analysis comparing *fevR/pigR* and *mglA* mutant strains of *F. novicida* to their parental strains revealed identical regulons for these two transcription factors (Brotcke and Monack, 2008), suggesting that FevR/PigR works in parallel with MglA and SspA to modulate gene expression. FevR/PigR has poor homology to DNA binding proteins, has not been demonstrated to bind to any specific DNA sequences, and appears to act upstream of MglA, SspA, and RNAP binding (Brotcke and Monack, 2008). Studies have varied on whether FevR/PigR, MglA, and SspA physically interact, but recently Charity et al. (2009) demonstrated that ectopic expression of *fevR/pigR* in LVS restored expression of the MglA/SspA-regulated genes to wild-type levels in a *fevR/pigR* mutant strain, failed to restore expression of the MglA/SspA-regulated genes in a *mglA* mutant strain, and partially restored expression of the MglA/SspA-regulated genes in a Δ*relA* Δ*spoT* mutant strain, which suggested that PigR/FevR functions together with the MglA–SspA complex downstream of ppGpp (Charity et al., 2009). Detailed studies of ppGpp and RelA/SpoT gene regulation under stress conditions are described in a later part of this review.

MigR/CaiC

Another regulator, macrophage intracellular growth regulator (MigR, also called CaiC), is a large protein with a putative AMP-binding domain and homology with acyl-transferase/ligase proteins. This regulator was identified from a random pool of LVS mutagenized with the Tn5 transposon as one that regulates the *iglABCD* and *fslABCD* operons (Buchan et al., 2009). In *F. tularensis* LVS, the mutation of *migR* demonstrated a defect of growth and intracellular trafficking in macrophages (Buchan et al., 2009). However, mutation of *migR* in the Schu S4 strain retained its regulatory effect but growth inside macrophages was unaffected. MigR positively regulates *fevR/pigR*, and controls the expression of the FPI genes, *iglABCD*. The lack of a DNA binding domain in MigR calls into question its role as a transcriptional regulator, instead it seems likely that the regulatory effect of MigR on *fevR/pigR* is indirect. MigR activity could affect RelA or SpoT (stringent response regulators) to induce the synthesis of (p)ppGpp, which has been demonstrated to contribute to the expression of *fevR*, and *iglABCD*, and thus is involved in the pathogenesis of *F. tularensis* (Charity et al., 2009).

(p)ppGpp

Under nutrient starvation conditions, certain bacterial species resist environmental stress by the production of hyperphosphorylated guanosine diphosphate and triphosphate analogs, collectively termed (p)ppGpp. During this process, uncharged tRNA molecules bind to the ribosome, which results in ribosome stalling, activation of ribosome-associated RelA, and production of (p)ppGpp, which is subsequently converted to ppGpp. These ppGpp small molecules bind to RNAP to affect expression of a wide range of physiological systems. In some bacteria, inactivation of both *relA* and *spoT* is required to completely abolish (p)ppGpp synthesis; SpoT is a bifunctional enzyme capable of both synthesis and degradation of the signal molecule, thereby preventing uncontrolled accumulation of (p)ppGpp (Jain et al., 2006). It has been proposed that the RelA-dependent response is linked to amino acid starvation and that the SpoT-dependent response is linked to fatty acid metabolism in bacteria (Battesti and Bouveret, 2009). In some pathogens, regulation of (p)ppGpp synthesis is dependent on SpoT, and virulence in *Legionella pneumophila* is critically dependent on the degradation of (p)ppGpp by SpoT (Dalebroux et al., 2009). Both *relA* and *spoT* genes have been annotated in the *F. novicida* U112 (Rohmer et al., 2006) and *F. tularensis* Schu S4 (Larsson et al., 2005; Dean et al., 2009) genome sequences. Inactivation of the *relA* gene in *F. novicida* rendered it defective for (p)ppGpp production, and showed increased biofilm formation and reduced resistance to *in vitro* stress (Dean et al., 2009). The mutant was attenuated in the J774.1 macrophage cell line and in the mouse model, and induced a protective immune response against homologous challenge. The double deletion of *relA* and *spoT* in *F. tularensis* LVS resulted in complete disappearance of (p)ppGpp production and increased the robustness of the *relA* mutant phenotypes. ppGpp promotes interaction of FevR/PigR with the MglA–SspA complex and RNAP by an unknown mechanism (Charity et al., 2009).

PmrA AND KdpD

Bacteria have evolved sensory systems including two-component systems to sense and respond to their immediate surroundings. TCS typically consist of a membrane-bound sensor kinase and a cytoplasmic response regulator whose genes are usually adjacent in a single transcription unit. The communication between the sensor kinase and response regulator involve phosphotransfer at conserved residues.

The *F. tularensis* genome encodes only two such systems, neither of which is chromosomally paired (Mohapatra et al., 2007). One of these unpaired response regulators is homologous to PmrA in *Salmonella enterica*, where it is primarily involved in the induction of genes that mediate LPS modification (Gunn, 2008). Inactivation of this gene in *F. novicida* led to increased bacterial susceptibility to killing by antimicrobial peptides, decreased intramacrophage growth, lack of phagosomal escape in macrophages and attenuation in the mouse model while providing protection against homologous wild-type challenge (Mohapatra et al., 2007). Microarray analysis of this mutant revealed a change in expression of 65 genes when compared to wild-type bacteria grown under the same conditions (Mohapatra et al., 2007). PmrA

is a DNA binding protein that is phosphorylated at Asp51 primarily by the unlinked putative histidine kinase KdpD, forming the first TCS described for *F. tularensis* (Bell et al., 2010). Phosphorylation of PmrA is complicated in *F. novicida* by the presence of a secondary KdpD target, KdpE, and an additional kinase, FTN1453, both of which negatively affect the phosphorylation state of PmrA. However, neither FTN1453 or KdpE is present/functional in the *F. tularensis* Schu4 strain, resulting in increased net phosphorylation of PmrA (Bell et al., 2010).

Coimmunoprecipitation assays suggested that PmrA, MglA, and SspA are a part of the same protein complex and likely physically interact (Bell et al., 2010). The *pdpD* gene is regulated by PmrA, MglA, and SspA; however, *pmrA* is positively autoregulated by PmrA but not affected by MglA or SspA (Bell et al., 2010). Phosphorylation aids PmrA binding to regulated promoters (Bell et al., 2010). A strain expressing PmrA D51A retains some DNA binding, but exhibits reduced expression of the PmrA-regulon, a deficiency in intramacrophage replication and attenuation in the mouse model. Thus, *Francisella* gene regulation includes a TCS consisting of KdpD and PmrA. Once phosphorylated, PmrA binds to regulated gene promoters recruiting free or RNAP-bound MglA and SspA to initiate FPI gene transcription. Interestingly, recent published and unpublished data suggest that the gene regulation by PmrA varies between *Francisella* subsp. and even within different isolates of the same subspecies (Sammons-Jackson et al., 2008). The external signals that are required for KdpD and PmrA two-component system activation remain to be identified but are a focus of current investigation.

hfq AND SMALL RNA

It is increasingly apparent that in most living organisms, small non-coding RNAs (sRNA) are responsible for wide range of changes in gene expression (Gottesman and Storz, 2010). The regulatory mechanism of these sRNAs is based on pairing with complementary sequences within mRNA messages (Papenfort and Vogel, 2010), though in some cases the regulatory RNA directly affects protein function (Yang et al., 1996). The sRNA–mRNA interaction impacts mRNA stability and translational efficiency. Sometimes sRNA activity is dependent upon interaction with a specific protein, which can aid in stabilization of the regulatory RNA or in targeting to a specific mRNA (Thomas and Schaffner, 2010). One such protein, host factor for bacteriophage Q β replication (Hfq), has been identified in many bacterial species, including *F. tularensis*, and often is involved in the regulation of a wide range of genes (Meibom et al., 2009). Not surprisingly, mutation of *hfq* in *F. tularensis* LVS resulted in pleiotropic effects including an increased sensitivity to elevated salt concentrations, detergents, and high temperatures (Meibom et al., 2009). In addition to these *in vitro* phenotypes, the *hfq* mutant was attenuated for growth in several types of macrophages and demonstrated reduced virulence in mice. Microarray and qRT-PCR analysis of the mutant revealed an effect on the expression of 104 genes, including 10 genes within the FPI. Strikingly, Hfq negatively regulates only one of two divergently expressed putative operons in the FPI, in contrast to the other known regulators that appear to affect all FPI-encoded genes. Because of the large size of the Hfq regulon,

it is difficult to attribute specific phenotypes of the mutant to specific genes or to assess the impact of reduced FPI gene expression on virulence in this mutant.

A recent study identified several sRNAs in the *F. tularensis* (LVS) genome (Postic et al., 2010) by *in silico* predictions and experimental assays. These non-coding regulatory RNAs, include members of well conserved families of structural and housekeeping RNAs as well as less conserved RNAs that may have novel functions/regulate novel targets in *Francisella*. Thus, the combined work on Hfq and the discovery of these sRNAs suggest a non-protein based means of gene regulation in the *Francisella* that may somewhat explain the paucity of protein regulatory factors.

CONCLUSION

Transcription factors are important regulators of virulence in virtually all known bacterial pathogens. However, virulence gene regulation in *Francisella* spp. is unique with regard to the fact that it has only one alternative sigma factor, no classically arranged TCS, and the involvement of MglA, SspA, PmrA, MigR, Hfq, sRNA, and FevR/PigR in regulatory control of the FPI. The published literature suggests that MglA, SspA, PmrA, MigR, Hfq, and FevR/PigR co-operate with each other to regulate the *Francisella* virulence network. Only one identified two-component system exists in virulent Type A *F. tularensis*, where the sensor kinase KdpD becomes phosphorylated by an unidentified environmental signal and relays that signal to the response regulator PmrA through phosphorylation of D51. PmrA binds to regulated promoters (including those within the FPI) and interacts with the MglA–SspA–RNAP complex to regulate virulence gene transcription (Bell et al., 2010). Microarray data have demonstrated that *fevR/pigR* is transcriptionally regulated by *pmrA*, *mglA*, and *sspA* (**Figure 1**). Thus, several environmental cues are likely integrated to activate *fevR/pigR*, which then plays a key role in FPI-mediated transcription. Additionally, several nutrient starvation and stress response proteins, such as RelA and SpoT (ppGpp), and sRNA species/sRNA carrier proteins are involved in virulence
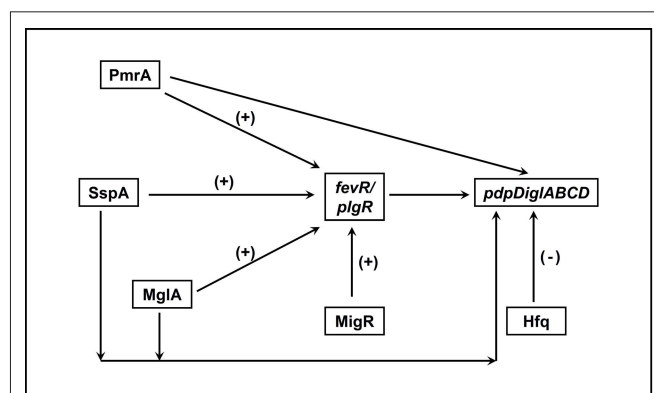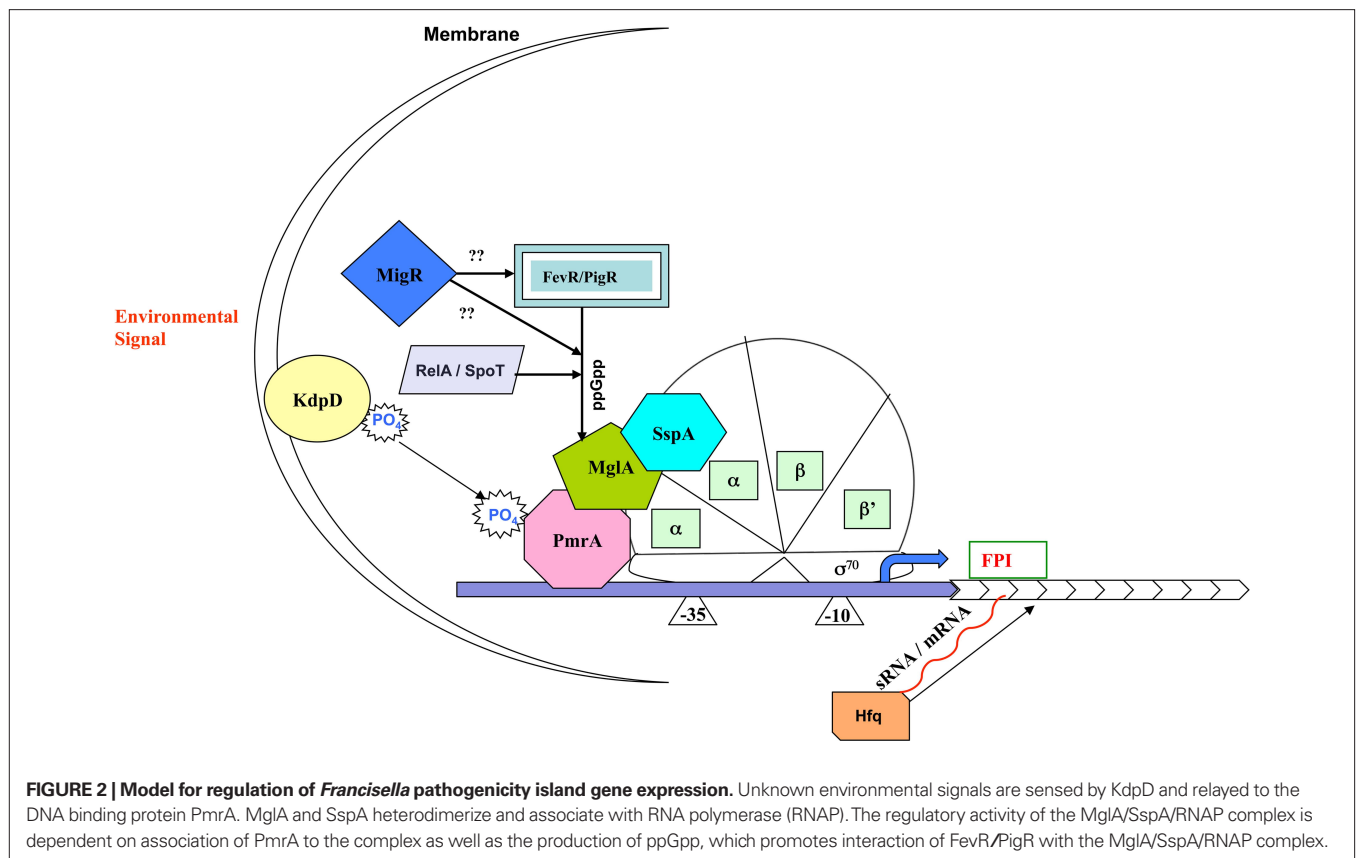

FIGURE 1 | Interactions between transcriptional regulators of *Francisella* that control FPI gene expression. PmrA, SspA, MglA, and MigR all positively regulate expression of *fevR/pigR*. The *fevR/pigR* product then plays a role, in conjunction with all of the other illustrated factors, in FPI gene transcription. PmrA, MglA, and SspA also directly regulate the genes of the FPI (see **Figure 2**). (+), Positive transcriptional regulation. (–), Negative transcriptional regulation.

FIGURE 2 | Model for regulation of *Francisella* pathogenicity island gene expression. Unknown environmental signals are sensed by KdpD and relayed to the DNA binding protein PmrA. MglA and SspA heterodimerize and associate with RNA polymerase (RNAP). The regulatory activity of the MglA/SspA/RNAP complex is dependent on association of PmrA to the complex as well as the production of ppGpp, which promotes interaction of FevR/PigR with the MglA/SspA/RNAP complex.

gene regulation in *Francisella* (see **Figure 2** for model). Present and future studies will investigate the molecular interactions between the known FPI regulators and identify new regulators/DNA binding proteins that aid *Francisella* pathogenesis in various host and non-host niches.

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