



# The Effect of Colistin Resistance-Associated Mutations on the Fitness of *Acinetobacter baumannii*

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*Acinetobacter baumannii* had emerged as an important nosocomial and opportunistic pathogen worldwide. To assess the evolution of colistin resistance in *A. baumannii* and its effect on bacterial fitness, we exposed five independent colonies of *A. baumannii* ATCC 17978 to increasing concentrations of colistin in agar (4/5) and liquid media (1/5). Stable resistant isolates were analyzed using whole genome sequencing. All strains were colistin resistant after exposure to colistin. In addition to the previously reported *lpxCAD* and *pmrAB* mutations, we identified four novel putative colistin resistance genes: *A1S\_1983*, *hepA*, *A1S\_3026*, and *rsfS*. Lipopolysaccharide (LPS) loss mutants exhibited higher fitness costs than those of the *pmrB* mutant in nutrient-rich medium. The colistin-resistant mutants had a higher inhibition ratio in the serum growth experiment than that of the wild type strain in 100% serum. Minimum inhibitory concentration (MIC) results showed that the LPS-deficient but not the *pmrB* mutant had an altered antibiotic resistance profile. The compensatory mutations partially or completely rescued the LPS-deficient's fitness, suggesting that compensatory mutations play an important role in the emergence and spread of colistin resistance in *A. baumannii*.

**Keywords:** *Acinetobacter baumannii*, colistin, resistance mechanism, fitness cost, whole genome sequencing

## INTRODUCTION

*Acinetobacter baumannii* had emerged as an important nosocomial and opportunistic pathogen worldwide, especially in intensive care units (Peleg et al., 2008). *A. baumannii* treatment is difficult because the clinical strains generally show resistance to multiple antibiotics (Peleg et al., 2008). Because the extensively drug-resistant isolates demonstrated sensitivity only to colistin, colistin has become the only clinically useful agent against these pathogens (Falagas et al., 2005; Giske et al., 2008; Magiorakos et al., 2012). However, due to its low efficacy, colistin should be used in combination antibiotic therapy for the treatment of XDR *A. baumannii* (Durante-Mangoni et al., 2014).

Colistin is a polycationic antimicrobial peptide that targets the polyanionic bacterial lipopolysaccharide (LPS) of Gram-negative bacteria. There are two different colistin resistance mechanisms (Beceiro et al., 2014). The first involves the inactivation of the lipid A biosynthesis pathway and the complete loss of surface LPS. This loss might be caused by mutations in *lpxC*, *lpxA*, or *lpxD*. Isolates harboring these mutations showed high colistin minimum inhibitory concentrations (MICs), although their fitness cost was also high (Beceiro et al., 2014; Wand et al., 2015). The second resistance mechanism is mediated by the *pmrAB* two-component system. Mutations in *pmrA* and *pmrB* induce the activation of *pmrC*, which adds phosphoethanolamine (PEtn) to the hepta-acylated form of lipid A (Beceiro et al., 2011). The *pmrB* mutation did not result in growth retardation (Durante-Mangoni et al., 2015; Wand et al., 2015).

Antimicrobial pressure drives the evolution of antimicrobial resistance in bacteria (Baker et al., 2013), and resistance is often associated with reduced bacterial fitness. Epistasis can compensate for the fitness cost of these mutants (Andersson and Hughes, 2010). Epistasis occurs not only within the same resistance pathway but also in different resistance pathways. In fluoroquinolone resistance, the *parC* mutation had the greatest effect on increase fitness of low fitness strains that harbored mutations in *gyrA* and *marR* (Marcusson et al., 2009). Moreover, the cost of multiple resistance was smaller than expected during the evolution of multi-drug resistance (Trindade et al., 2009). Understanding the fitness cost of the evolution of resistance is important to control the spread of resistant bacteria (Baker et al., 2013).

To assess the evolution of *A. baumannii* colistin resistance, we evolved *A. baumannii* through continuous exposure to colistin in agar plates and liquid media. Four novel putative colistin resistance genes were identified in addition to *lpxCAD* and *pmrAB*. Fitness and resistance compensatory mutations appeared in the bacteria exposed to colistin.

## MATERIALS AND METHODS

### Bacterial Isolates and Antimicrobial Susceptibility Testing

All bacteria used in this study were the ATCC 17978 strain and its laboratory-evolved mutants of *A. baumannii*. The MICs were determined by broth dilution using Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton (MH) agar. The results were interpreted according to the CLSI or EUCAST breakpoints (Clinical and Laboratory Standards Institute, 2014; European Committee on Antimicrobial Susceptibility Testing, 2015). The bacteria were cultured in Luria-Bertani (LB) medium at 37°C.

### Laboratory-Evolved Colistin Resistant Mutants

Four independent single colonies of *A. baumannii* ATCC 17978 were grown overnight at 37°C. The *in vitro* serial passage

experiments were performed using three methods. First, the bacteria were streaked on LB agar plates containing 10 mg/L colistin once each day for 15 days and then incubated at 37°C. Then, the bacteria were serially passaged for another 15 days in the presence of 50, 100, and 200 mg/L colistin. Second, the bacteria were streaked on LB agar plates containing 10 mg/L colistin once each day for 60 days and then incubated at 37°C. Third, the cultures were exposed to serially increasing concentrations of colistin, starting from 1/2 the MIC and doubling every day for 12 days. The overnight cultures were stored at -80°C prior to the experiments and analysis.

### Whole Genome DNA Sequencing and Analysis

Bacteria from a single colony were cultured overnight at 37°C in MH broth. The genomic DNA was extracted using a QIAamp DNA minikit (Qiagen, Valencia, CA, USA) following the protocol of the manufacturer (Hua et al., 2012). Agarose gel electrophoresis and a NanoDrop spectrophotometer (ND-1000, ThermoFisher, Waltham, MA, USA) were used to determine the quality and quantity of the extracted genomic DNA. The 300 bp library used for Illumina paired-end sequencing was constructed from 5 µg of genomic DNA purified from the laboratory-evolved mutants. Mapping and SNP detection were performed using breseq (Deatherage and Barrick, 2014). The regions containing the detected SNPs were amplified by PCR. The PCR products were sent to Biosune (Biosune, Hangzhou, China) for Sanger sequencing. Duplications were detected using the CLC genomic workbench (8.02.2) and were verified by real-time PCR (Supplementary Table S2).

### Fitness Cost and Serum Resistance Measurement

Four independent cultures per strain were grown overnight in MH (Mueller-Hinton), diluted 1:1000 [MH or fetal bovine serum (FBS)] and aliquoted into a flat-bottom 96-well plate in four replicates. The plate was agitated at 37°C. The OD<sub>600</sub> of each culture was determined every 5 min for 16 h using a the BioTEK Synergy plate reader (Biotek, Winooski, VT, USA). The growth rate was estimated by an R script based on the OD<sub>600</sub> curves (Li et al., 2015). Fitness data are shown in the Supplementary Table S1. Statistical analyses were performed using R 3.2.1 (R Development Core Team, 2015). Two-way analysis of variance (ANOVA) and TukeyHSD analysis were used to assess differences between the means, with a significant probability at a *P* value of ≤0.05.

## RESULTS

### Mutations in Laboratory-Evolved Colistin Resistant Strains

To investigate the colistin resistance mechanism in *A. baumannii*, we evolved *A. baumannii* ATCC 17978 in agar plates and liquid media with increasing or constant concentrations of

colistin. A strain description and the MICs for colistin of the laboratory-evolved strains are listed in Supplementary Table S1. The MIC of ATCC 17978 for colistin was 0.19 mg/L. The MICs of the laboratory-evolved strains ranged from 6 mg/L to > 256 mg/L. The final isolates from each colony were sent for whole genome sequencing (WGS) to identify mutations. The mutations in the laboratory-evolved strains were detected via breseq, which mapped the sequence reads onto the ATCC 17978 reference genome. Then, the generation time each mutation appeared during the experiment was determined by PCR and sanger sequencing.

The mutations that occurred in the early stage of evolution were found in *pmrB*, *lpxC*, *lpxA*, and *lpxD* (Figure 1). Additional mutations were detected in *AIS\_1983*, *hepA* (*AIS\_2462*), *AIS\_3026*, *rsfS* (*AIS\_0570*), and *adeS* (*AIS\_1754*) (Supplementary Tables S3 and S4).

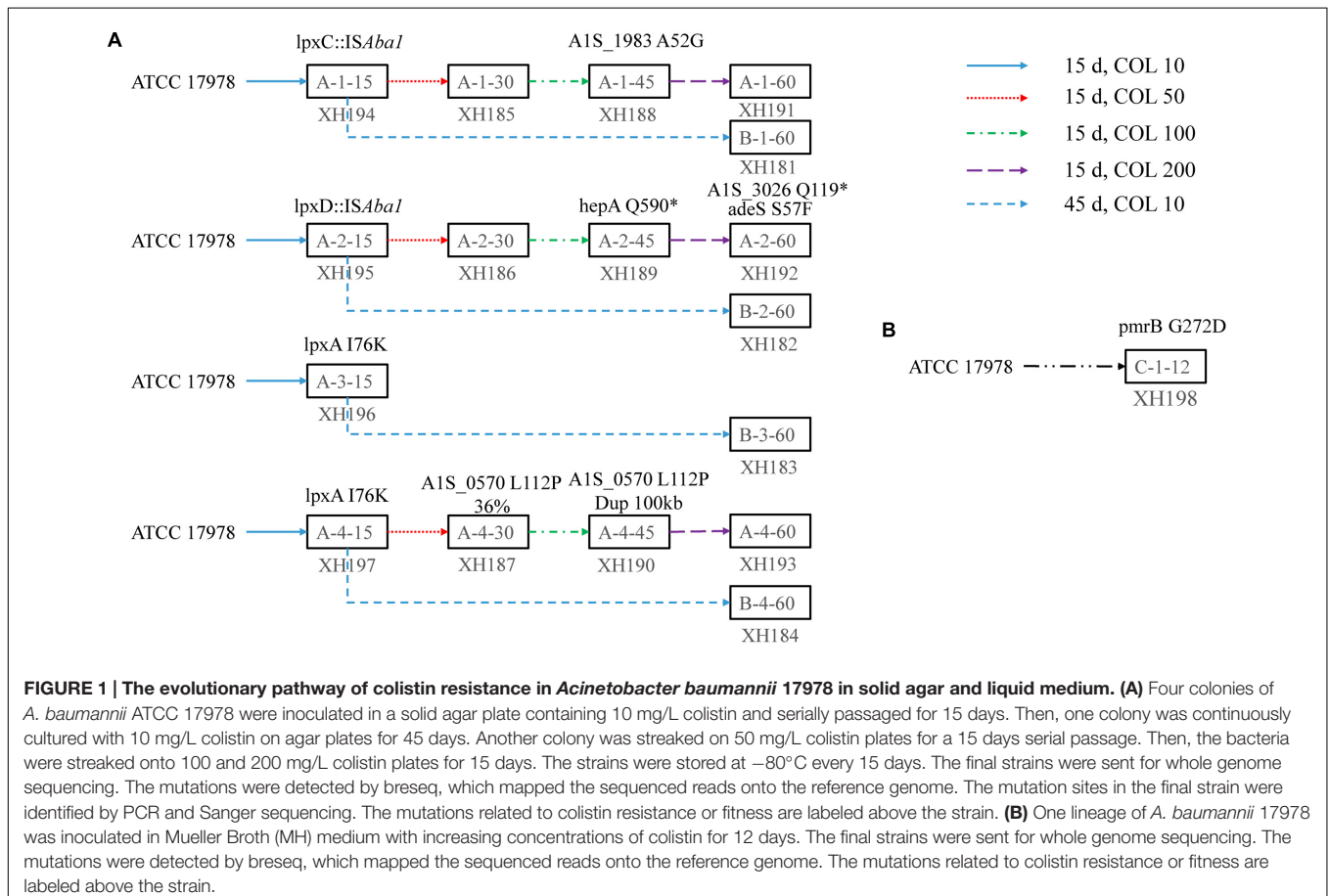
### Colistin Resistance had a Fitness Cost

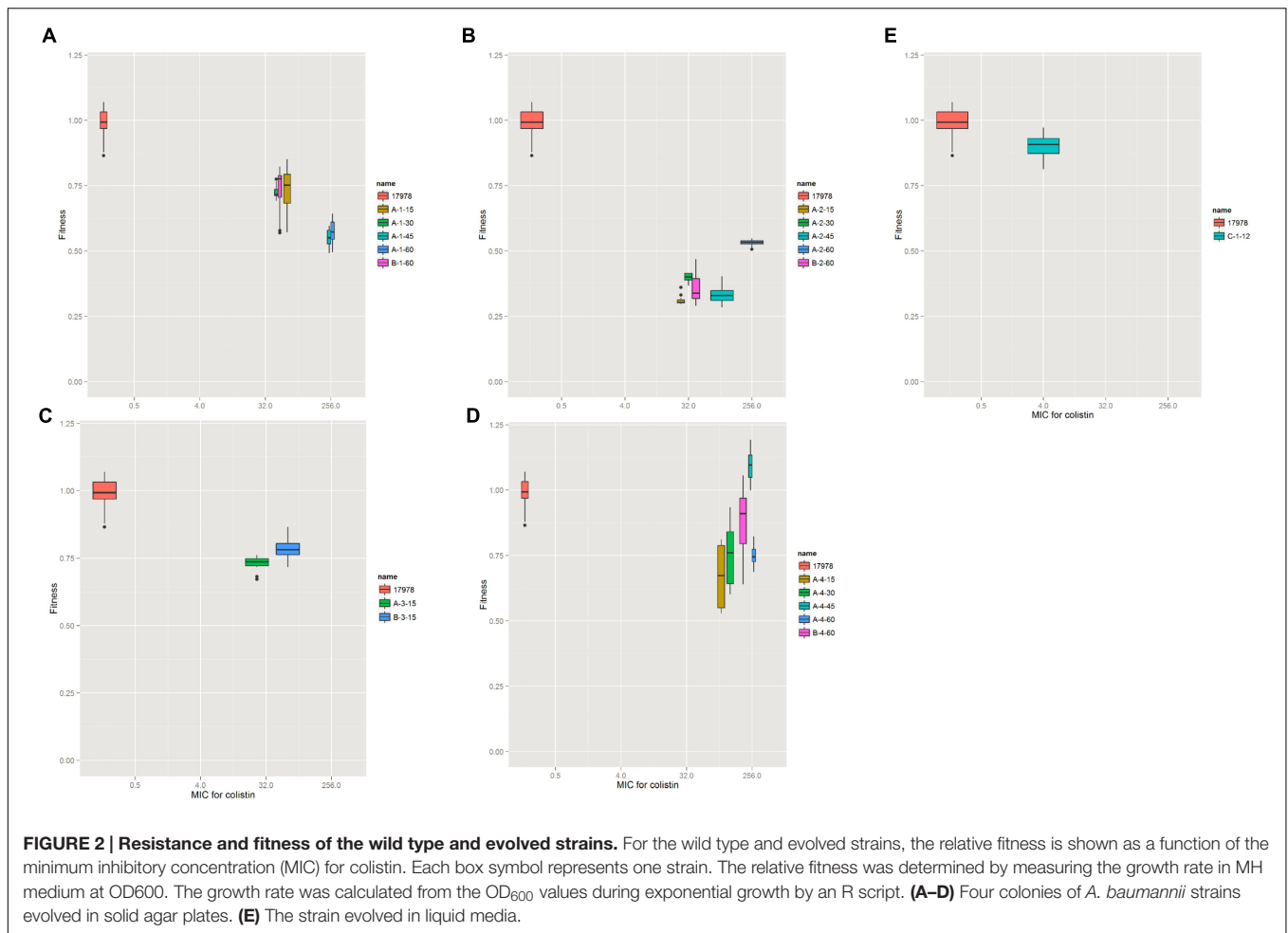
Among the mutations detected, *AIS\_1983*, *hepA*, *AIS\_3026*, and *rsfS* were hypothesized to be involved in colistin resistance. Conversely, the *adeS* mutation was not involved in colistin resistance (data not shown). The ratio of the *AIS\_0570* mutation in A-4-30 (XH187) was 36%, and this ratio increased to 100% in A-4-45 (XH190).

To investigate the effect of the mutations on *A. baumannii* fitness, we measured the growth rate of the laboratory-evolved strains as a proxy for fitness. The strains selected in the agar plates showed a high fitness cost in the first 15 selected generations, while increased resistance and fitness compensation were observed later in the later selection process (Figure 2). The evolution in colistin resistance and fitness was diverse among the four lineages. In lineage 1, no fitness compensation was observed while resistance to colistin was increased (Figure 2A). In lineage 2, the fitness was partially recovered with a mutation in *AIS\_3026*, and the resistance to colistin increased (Figure 2B). Due to contamination, lineage 3 did not have enough isolates for analysis. In lineage 4, the fitness was completely recovered with a mutation in *AIS\_0570*, while the resistance increased (Figure 2D). A 100 kb duplication occurred in A-4-45(XH193) and A-4-60(XH190; Figure 3), which showed a higher fitness compared to that of A-4-30(XH187). These results indicated that the duplication of the long chromosome fragment may play a role in the bacterial fitness. In addition, the growth of strains harboring the *pmrB* mutation was slightly retarded (Figure 2E).

### Lipopolysaccharide Affects Serum Resistance

To investigate the effect of LPS on the serum resistance of *A. baumannii*, we measured the growth rate of the wild type strain





(ATCC 17978), the LPS-deficient mutant (A-4-60, XH193), and the LPS-modified mutant (C-1-12, XH198) in FBS. There was significant difference in the growth rate of the wild type strain and LPS-deficient mutants in MH (Figure 4A). However, both the LPS-deficient mutant and LPS-modified mutant showed higher inhibition in FBS compared to that of the wild type strain. Both the LPS-deficient mutant and LPS-modified mutant exhibited a decrease in their final biomass (Figures 4B,C).

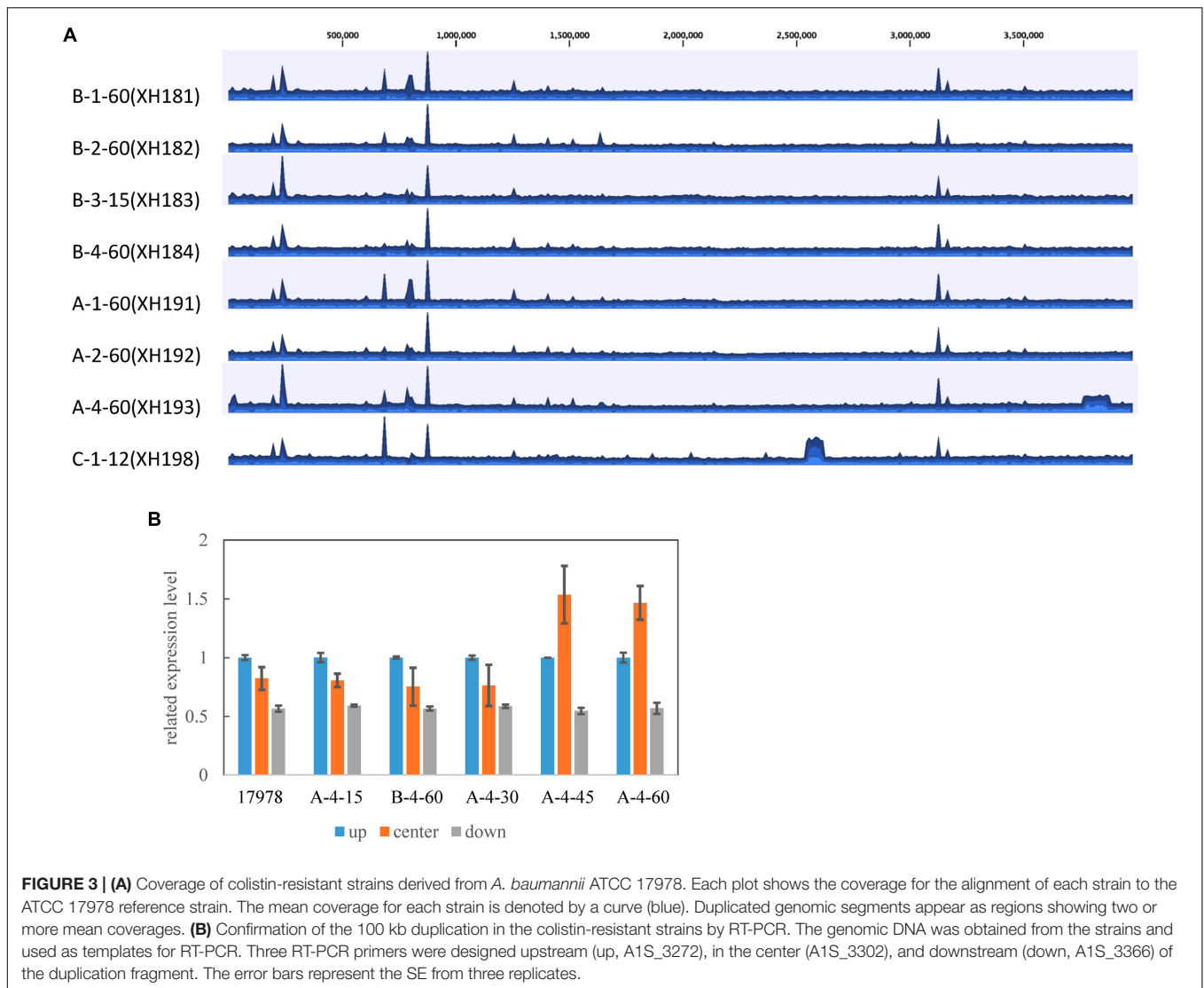
### Colistin-Resistant Mutation Alters the Antibiotic Resistance Profiles

To examine the effect of the LPS mutation on antibiotic resistance, we determined the MICs of several antibiotics (including quinolones, aminoglycosides, tetracycline, and  $\beta$ -lactams). Table 1 shows the MICs of different antibiotics for *A. baumannii* ATCC 17978 and its colistin mutants. Overall, the changes in the MIC trends could be divided into two groups. The first group included seven strains that showed increased sensitivity to several types of antibiotics except colistin. All seven strains harbored mutations that disrupted the LPS biosynthesis pathway, leading to LPS loss. The second group contained only one strain: C-1-12 (XH198). This strain was resistant to all antibiotics

except ampicillin and imipenem, and its colistin resistance mechanism involved an LPS modification. Thus, the LPS mutations resulted in differences in the antibiotic resistance profiles.

## DISCUSSION

In this study, we investigated the evolutionary pathway of *A. baumannii* that led to colistin resistance. Mutations in *pmrB*, *lpxC*, *lpxA*, and *lpxD* were detected early in the selection process. These results confirmed the findings of previous reports (Adams et al., 2009; Moffatt et al., 2010; Olaitan et al., 2014). In addition, four putative proteins, *A1S\_1983*, *hepA*, *A1S\_3026*, and *rsfS* were proposed to be involved in colistin resistance in this study. *A1S\_1983* is a putative exported protein. The ATP-dependent helicase HepA (*A1S\_2462*) is a transcriptional regulator that activates transcription under stress conditions. The HepA mutation resulted in reduced exopolysaccharide coverage and increased retention of penicillin G compared to that of the wild type strain in *E. coli* strain; moreover, the mutant was more sensitive to norfloxacin, chloramphenicol, and gentamicin than the wild type (Lynch et al., 2007). *A1S\_3026*

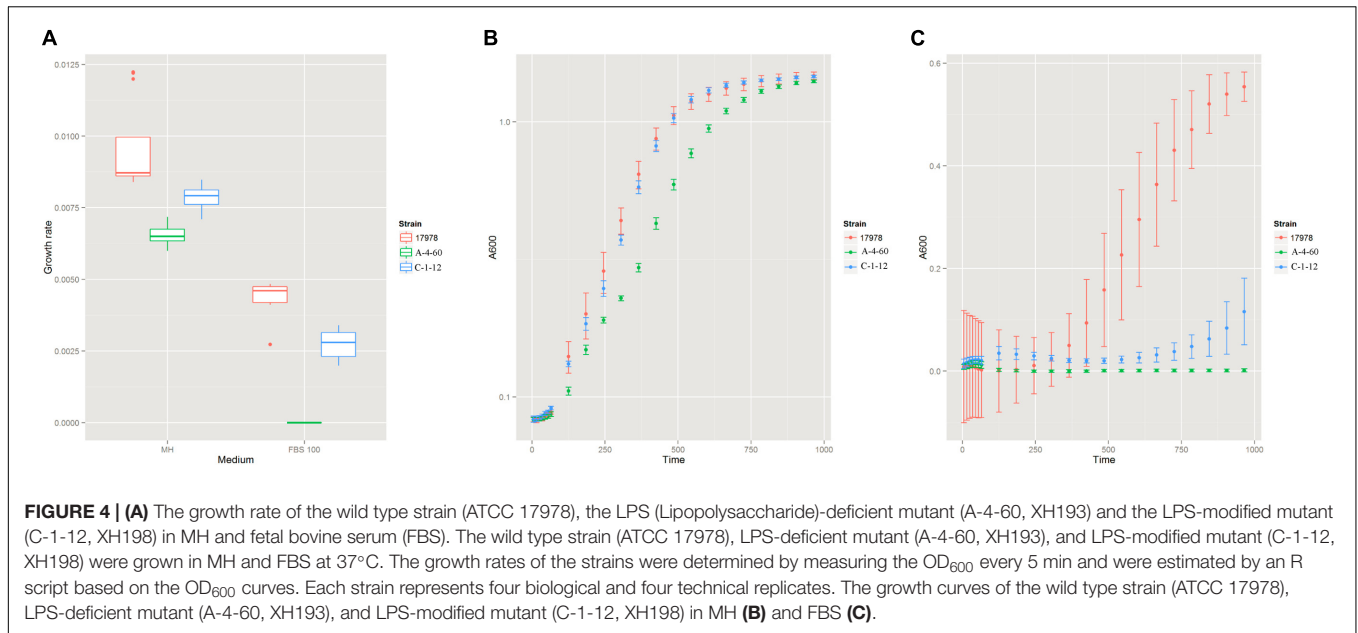


is a ribonuclease T2 family protein, and a mutation in this gene limits *A. baumannii*'s ability to colonize inanimate surfaces and bacterial mobility (Jacobs et al., 2014). RsfS (A1S\_0570) is a ribosomal silencing factor that helps bacteria adapt to slow growth conditions (Hauser et al., 2012). The first protein is a membrane protein; its mutation would influence the integrity of the membrane, possibly leading to colistin resistance. The latter three proteins affected exopolysaccharide production, biofilm formation and bacterial adaptation, indicating new colistin resistance mechanisms. Recently, six genes (*vacJ*, *pldA*, *ttg2C*, *pheS*, and conserved hypothetical protein) were shown to have a role in colistin resistance (Thi Khanh Nhu et al., 2016). We also identified mutation in *vacJ*, but did not link the *vacJ* mutation to colistin resistance. This may be due to different mutations in *vacJ* in Nhu's and our study. Moreover, these result also demonstrated the diversity of novel colistin resistance mechanism.

The LPS loss mutant showed a high fitness cost, which limited its spread in clinical environments. We identified two fitness

compensatory mutations: A1S\_3026 and RsfS in this study. These fitness compensatory mutations increase the fitness of the LPS loss mutant and promote its survival and spread in the clinical environment. However, we confirmed that the LPS-deficient mutants possessed an altered antibiotic resistance profile that was not observed in the LPS modification mutants. Colistin resistance in *A. baumannii* leads to changes in the antibiotic resistance profile (Moffatt et al., 2010; Lopez-Rojas et al., 2011b; Wand et al., 2015). The change in the antibiotic resistance profiles of the LPS-deficient mutants was caused by the increased permeability of the outer membrane to antibiotics (Moffatt et al., 2010). LPS in the outer membrane of *A. baumannii* acts as a highly selective permeability barrier (Nikaido, 2003). LPS loss removed the selective permeability of the barrier for antibiotics, whereas the LPS modification resulted in only minor changes in the permeability of the *A. baumannii* outer membrane. The *pmrB* mutant presented little or no change in susceptibility to these antibiotics (Wand et al., 2015). In this study, the *pmrB* mutant





**TABLE 1 | Antimicrobial susceptibilities of *Acinetobacter baumannii* ATCC 17978 and its colistin mutants.**

Strain	Lineage	Minimum inhibitory concentration (MIC; mg/L) <sup>a</sup>							
		CI	GM	AK	MC	TGC	AM	MP	IP
17978		0.25	0.75	2	0.75	0.38	48	0.38	0.38
XH191	A-1-60	0.125	0.38	0.5	<0.016	0.032	3	0.016	0.094
XH181	B-1-60	0.094	0.25	0.5	<0.016	0.047	2	0.012	0.125
XH192	A-2-60	0.25	1	0.5	0.016	<0.016	1	0.016	<0.002
XH182	B-2-60	0.125	0.094	0.19	0.032	0.064	1	0.008	0.064
XH183	B-3-60	0.094	0.38	0.5	0.032	0.032	3	0.016	0.125
XH193	A-4-60	0.094	0.5	1	0.016	0.094	3	0.032	0.125
XH184	B-4-60	0.125	0.19	0.5	0.032	0.064	1.5	0.008	0.064
XH198	C-1-12	1	2	8	1	3	12	0.5	0.25

<sup>a</sup>CI, ciprofloxacin; GM, gentamicin; AK, amikacin; MC, minocycline; TGC, tigecycline; AM, ampicillin; MP, meropenem; IP, imipenem.

showed increased resistance to antibiotics. This may be due to changes in the permeability of the outer membrane by the *pmrB* mutation.

Previous studies have also demonstrated that the *pmrB* mutation resulted in no or a slight fitness cost (Durante-Mangoni et al., 2015; Wand et al., 2015). Our result confirmed these findings. These results explained why the LPS modification only resulted in a small change in the bacteria compared to the LPS loss. However, we did not determine the effect of colistin resistance-related mutations on the virulence of *A. baumannii* isolates *in vivo*. Colistin resistance was previously associated with lower *in vivo* fitness and reduced virulence in *A. baumannii* (Lopez-Rojas et al., 2011a).

In our study, LPS loss affected the growth rate of *A. baumannii* in nutrient-rich medium and serum. The loss of the O-galactose in *Salmonella typhimurium* was also reported to reduce the resistance to serum but did not affect the growth rate (Marshall and Gunn, 2015). The LPS loss was not shown to

contribute to serum resistance in *A. baumannii* (Moffatt et al., 2013). However, our results did not support that conclusion. LPS modification decreased the growth rate of *A. baumannii* in serum and in nutrient-rich medium. The LPS modified mutation also contributed to serum resistance in *A. baumannii*. The conflict between Moffatt and our results may be caused by the different genetic backgrounds of ATCC 19606 used in Moffatt's study and ATCC 17978 used in our study. Both of the LPS mutants showed a decreased the final biomass, whereas serum resistance was normal following incubation in serum for 30 min (Moffatt et al., 2013). The decreased biomass indicated that the serum resistance assay required further improvements.

Overall, colistin resistance *A. baumannii* rapidly developed. We not only confirmed the existence of a previously reported colistin resistance mechanism, but also identified four novel putative proteins involved in colistin resistance. The compensatory mutations partially or completely recovered the LPS-deficient mutant's fitness, suggesting that compensatory

mutations played an important role in the emergence and spread of colistin resistant *A. baumannii*.

## Nucleotide Sequence Accession Numbers

The whole genome shotgun sequencing results for B-1-60 (XH181), B-2-60(XH182), B-3-15(XH183), B-4-60(XH184), A-1-60(XH191), A-2-60(XH192), A-4-60(XH193), and C-1-12(XH-198) have been deposited at DDBJ/EMBL/GenBank under the accession numbers MDWH00000000, MDWJ00000000, MDWK00000000, MDWL00000000, MDWG00000000, MDWI00000000, MDWF00000000, and MDWM00000000, respectively.

## AUTHOR CONTRIBUTIONS

XH and YY conceived and designed the study. XM, NW, XL, and KS performed the experiments. XH, ZZ, and YY performed data

analysis and drafted the manuscript. All authors reviewed and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2016.01715/full#supplementary-material>

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