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# Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria

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Polymyxins are polycationic antimicrobial peptides that are currently the last-resort antibiotics for the treatment of multidrug-resistant, Gram-negative bacterial infections. The reintroduction of polymyxins for antimicrobial therapy has been followed by an increase in reports of resistance among Gram-negative bacteria. Some bacteria, such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, develop resistance to polymyxins in a process referred to as acquired resistance, whereas other bacteria, such as *Proteus* spp., *Serratia* spp., and *Burkholderia* spp., are naturally resistant to these drugs. Reports of polymyxin resistance in clinical isolates have recently increased, including acquired and intrinsically resistant pathogens. This increase is considered a serious issue, prompting concern due to the low number of currently available effective antibiotics. This review summarizes current knowledge concerning the different strategies bacteria employ to resist the activities of polymyxins. Gram-negative bacteria employ several strategies to protect themselves from polymyxin antibiotics (polymyxin B and colistin), including a variety of lipopolysaccharide (LPS) modifications, such as modifications of lipid A with phosphoethanolamine and 4-amino-4-deoxy-L-arabinose, in addition to the use of efflux pumps, the formation of capsules and overexpression of the outer membrane protein OprH, which are all effectively regulated at the molecular level. The increased understanding of these mechanisms is extremely vital and timely to facilitate studies of antimicrobial peptides and find new potential drugs targeting clinically relevant Gram-negative bacteria.

**Keywords:** lipopolysaccharides, lipid A, Enterobacteriaceae, non-fermentative bacilli, mutation, two-component systems, antibiotic resistance

## INTRODUCTION

Recently, the rise in infections caused by multidrug-resistant (MDR) Gram-negative bacteria, especially the resistance to carbapenems most importantly observed in *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Escherichia coli*, has led to the resuscitation of polymyxins (polymyxin B and colistin) worldwide as a last-resort treatment option (Stein and Raoult, 2002; Falagas and Michalopoulos, 2006; Biswas et al., 2012). Resistance to polymyxins by bacteria that are normally susceptible to these drugs has been reported (Johansen et al., 2008; Arduino et al., 2012; Mammina et al., 2012). There are also reports of increases in infections caused by naturally polymyxin-resistant bacteria, such as *Proteus*, *Providencia*, *Morganella*, and *Serratia* (Hayakawa et al., 2012; Merkier et al., 2013; Samonis et al., 2014).

Bacteria employ several means to protect themselves from adverse environmental stimuli, including exposure to cationic antimicrobial peptides, such as polymyxin B and colistin. These strategies include alterations of their lipopolysaccharides (LPSs), which have overall negative charges and are the initial targets of polymyxins (Moffatt et al., 2010). Such alterations can be

achieved by covalent modifications of the lipid A moiety of LPS through the addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N), deacylation, hydroxylation and palmitoylation by *pagP* (palmitoylation does not contribute to polymyxin resistance) (Ernst et al., 2001; Raetz et al., 2007). Other strategies include the utilization of an efflux pump and capsule formation (Campos et al., 2004; Padilla et al., 2010).

The most common LPS modification is the cationic substitution of the phosphate groups by L-Ara4N, which decreases the net negative charge of lipid A to 0, and the second most common is the PEtN modification, which decreases the net charge from  $-1.5$  to  $-1$  (Nikaido, 2003). The L-Ara4N modification is the most effective of the two modifications due to the nature of the charge alteration. The resultant net positive charge of the modified LPS reduces its binding to polymyxins, leading to resistance.

The activation of two-component systems (TCSs) involving PhoP/PhoQ and PmrA/PmrB is triggered by environmental stimuli and specific mutations within the TCSs that result in their constitutive activation and subsequent overexpression of LPS-modifying genes (Gunn and Miller, 1996; Gunn et al., 2000; Trent et al., 2001b; Abraham and Kwon, 2009; Barrow and Kwon,

2009; Miller et al., 2011). The activation of the PmrA/PmrB TCS leads to the upregulation of the *pmrCAB* and *arnBCADTEF-pmrE* (also called *pmrHFIJKLM-ugd*) operons that mediate the synthesis and transfer of PETn and L-Ara4N, respectively, to lipid A (Gunn, 2001; Raetz et al., 2007; Yan et al., 2007). The PhoP/PhoQ TCS is known to contribute to polymyxin resistance by indirectly activating the PmrA/PmrB TCS via PmrD, except in various bacteria, such as *E. coli* (Kox et al., 2000; Kato et al., 2003; Winfield and Groisman, 2004). Once activated, the phosphorylated PmrA binds to the promoter region of the *arnBCADTEF* operon, increasing the recognition and binding of RNA polymerase and resulting in the upregulation of the operon (Wosten and Groisman, 1999).

There have been reports summarizing the mechanisms of resistance to polymyxins (Nation and Li, 2009; Falagas et al., 2010; Lim et al., 2010; Loutet and Valvano, 2011; Biswas et al., 2012; Cai et al., 2012); however, the lack of updated and comprehensive reports on the different mechanisms mediating or contributing to polymyxin resistance including among the intrinsically-resistant bacteria has further necessitated this work.

## ACQUIRED RESISTANCE TO POLYMYXINS IN ENTEROBACTERIACEAE

### ROLE OF LPS MODIFICATIONS

#### *PmrA/PmrB* and *PhoP/PhoQ* two-component system-mediated LPS modifications

***Salmonella enterica*.** *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) has been used as a model bacterium for most studies aimed at elucidating the mechanisms of resistance to cationic antimicrobial peptides (CAMPs), including polymyxins. Vaara et al. have observed that the LPSs of a polymyxin-resistant *pmrA* mutant of an *S. Typhimurium* strain contains 4–6 times more L-Ara4N in lipid A than the parent strain in addition to an increased level of PETn (Vaara et al., 1981; Trent et al., 2001b). In polymyxin-resistant *Salmonella*, one principal mechanism underlying this modification, apart from external stimuli such as  $Mg^{2+}$ , involves mutations in the PmrA/PmrB and PhoP/PhoQ TCSs. Mutations in these systems can cause their constitutive overexpression, resulting in the activation of the *arnBCADTEF* and *pmrCAB* operons and the modification of lipid A by L-Ara4N and PETn, respectively (Figure 1) (Roland et al., 1993; Guo et al., 1997; Gunn et al., 1998; Zhou et al., 2001; Lee et al., 2004). Several mutations that activate the PmrA/PmrB TCS have been characterized in *in vitro*-selected mutant polymyxin-resistant *S. Typhimurium* (Table 1). Roland et al. have reported a missense mutation in *pmrA*, resulting in an amino acid substitution of R81H (Roland et al., 1993). An extensive genetic analysis of *pmrA/pmrB* of spontaneous colistin-resistant mutants has further identified numerous mutations, including a total of 27 independent missense mutations in the *pmrA* and *pmrB* genes that all result in the elevated expression of *arnB* (*pmrH*) (Sun et al., 2009). The minimum inhibitory concentrations (MICs) of colistin for the spontaneous mutants reported range from 0.25 to 4.4 mg/L, with most mutants displaying a 20–30-fold MIC increase. For PmrA, all mutations occurred in the phosphate receiver domain, while mutations occurred in 4 out of the 6 predicted domains in PmrB (Figure 2A). The histidine kinase gene *pmrB* seems to be

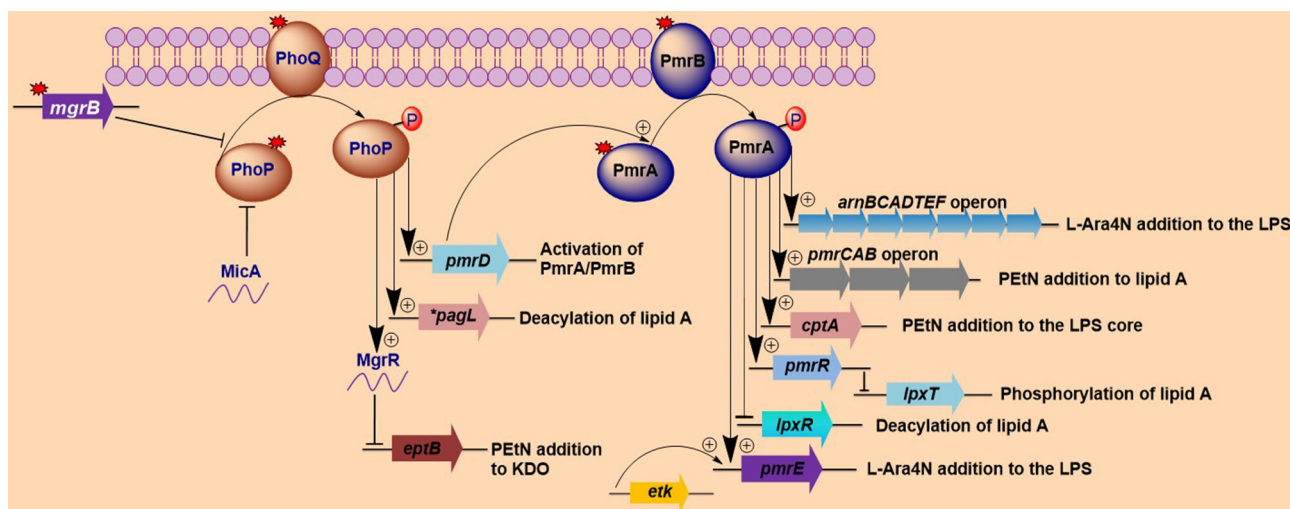
the more common site for bacterial mutations compared to the response regulator gene *pmrA*. However, not all missense mutations in *pmrA/pmrB* result in their constitutive activation and polymyxin resistance. Some non-polymyxin resistance-causing missense mutations have been observed in the *pmrA/pmrB* of various *Salmonella* serotypes (Agerso et al., 2012).

It has been observed that up to 88% of the 4'-phosphate of lipid A can be extensively modified with L-Ara4N in *pmrA* mutants of *S. Typhimurium* (Helander et al., 1994). Generally, L-Ara4N modifies the 4'-phosphate, while PETn modifies the 1-phosphate; however, in some cases, either one or two L-Ara4N and/or PETn can be added to the 4'-phosphate and 1-phosphate of lipid A (Zhou et al., 2001; Lee et al., 2004). These two modifications are mediated by the *arnBCADTEF* operon and *pmrC* (also called *eptA*), respectively. Additionally, the phosphorylated heptose-I residue (LPS core) can be further modified with PETn by the *cptA* gene (Figure 1) (Tamayo et al., 2005a). *arnBCADTEF* (also referred to as the *pmrF* or *arnT* operon), *pmrC* and *cptA* are all regulated by *pmrA* in *Salmonella* (Tamayo et al., 2005b). The cationic modifications of these phosphate groups in LPSs are responsible for their reduced binding to polymyxins.

With regard to polymyxin resistance, the modification of LPSs by L-Ara4N confers a higher level of resistance than PETn modifications (Tamayo et al., 2005a). Therefore, in terms of LPS modification-mediated polymyxin resistance in *S. Typhimurium*, the *arnT* operon is the most important, followed by *pmrC* and *cptA*.

Furthermore, the 1-phosphate of lipid A can be phosphorylated to form 1-diphosphate (1-PP) by LpxT (Jones et al., 2008; Touze et al., 2008). This alteration increases the net negative charge of lipid A and reduces the efficiency of the PETn modification (Herrera et al., 2010). These characteristic processes can increase the overall sensitivity of *S. Typhimurium* to polymyxins. However, LpxT activity is inhibited by the activation of *pmrA* (Herrera et al., 2010) via PmrR (Pmr regulator) in *S. Typhimurium*, which further enhances resistance to polymyxins (Figure 1) (Kato et al., 2012). The biology of these genes (*lpxT* and *pmrR*) with respect to polymyxin resistance has yet to be fully elucidated, but it is highly probable that they may have a direct impact on this resistance considering their involvement in altering the overall charges of LPSs.

In *Salmonella*, the R-3-hydroxymyristate at position 3 of lipid A can be removed (deacylated) by *pagL*, which itself is activated by PhoP (Trent et al., 2001a). *PagL* is normally latent due to its inhibition by L-Ara4N and PETn modifications of lipid A (Kawasaki et al., 2005, 2007), but the *PagL*-mediated deacylation of lipid A can occur in strains that are unable to modify this lipid A with either L-Ara4N or PETn. Consequently, in such strains, the *PagL*-dependent deacylation of this lipid A increases polymyxin resistance (Kawasaki et al., 2007). This finding implies that the various forms of lipid A modifications, particularly L-Ara4N and PETn modifications and deacylation, may be compensatory to each other in certain bacteria, such as *Salmonella*. This relationship further depicts the complexity and intricacy of the interactions among the various mechanisms that mediate bacterial resistance to antimicrobial peptides, including polymyxins.



**FIGURE 1 | Activation of lipopolysaccharide-modifying genes involved in polymyxin resistance in Gram-negative bacteria.** Both MgrB and MicA (in *Escherichia coli*) exert negative feedback on the *phoP/phoQ* regulatory system, while mutations (denoted by red-colored star symbols) in *mgrB* or *phoP/phoQ* typically lead to the constitutive induction of the *phoP/phoQ* two-component system. The activation of this two-component system (*phoP/phoQ*) activates *pagL* (which deacylates lipid A in *Salmonella*) and *pmrD* (which in turn activates *pmrA*) and represses *eptB* via the activation of MgrR, with the resultant lipopolysaccharide (LPS) modifications all participating in the mediation of polymyxin resistance. Additionally, the *phoP/phoQ* regulatory system can directly activates the *arnBCADTEF* operon in some bacteria such as *Klebsiella pneumoniae*. The repression of *eptB*

prevents the modification of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) with phosphoethanolamine (PETN). The *pmrA/pmrB* two-component system is activated via *pmrD* (which is activated by *phoP*) or through mutations in the *pmrA/pmrB* genes. Once induced, the phosphorylated *pmrA* activates the *arnBCADTEF* and *pmrE* genes, which collectively modify LPSs with 4-amino-4-deoxy-L-arabinose (L-Ara4N). The lipid A and the LPS core are further modified with PETN by the *pmrCAB* operon and *cptA*, respectively. Additional *pmrA*-activated genes include *pmrR*, which represses *lpxT* (that phosphorylates lipid A) upon activation and *lpxR* gene (which deacylates lipid A). Lastly, *etk* can additionally phosphorylate the *pmrE* gene. The findings illustrated here are limited to modifications that have been shown to affect sensitivity to polymyxins. \**pagL* has only been found in *Salmonella*.

Some genes that have been implicated to be involved in polymyxin resistance and are independent of the PmrA/PmrB and PhoP/PhoQ genes include *rpoN*, which is an alternative sigma factor known to activate the transcription of several genes (Kazmierczak et al., 2005). *S. Typhimurium* with inactivated *rpoN* displayed polymyxin resistance by about two-fold, independent of *pmrA*, possibly due to the downregulation of polymyxin resistance-related gene(s) that are *rpoN*-regulated (Barchiesi et al., 2009).

The clinical relevance of polymyxin resistance in *Salmonella* currently seems to be non-existent, which is possibly because polymyxins are not currently used for treating infections caused by this bacterium. However, *in vivo* colistin resistance has been observed in animals such as pigs and poultry birds (EMEA, 2002; de et al., 2012; Morales et al., 2012; Kempf et al., 2013; Quesada et al., 2014), and such strains can be horizontally transmitted to humans.

***Klebsiella pneumoniae*.** There have been a considerable number of studies aiming to elucidate polymyxin resistance in *K. pneumoniae*. In a genetically uncharacterized polymyxin-resistant strain of *K. pneumoniae*, the phosphate groups of lipid A have been observed to contain five times more L-Ara4N than the susceptible strain (Helander et al., 1996). This altered outer membrane composition is known to lower the negative charge of the outer membrane of *K. pneumoniae* (Velkov et al., 2013a), leading to the reduced interaction of this membrane with

polymyxins. A molecular characterization of the structural alterations of LPSs in *K. pneumoniae* with regard to polymyxin resistance has similarly shown the involvement of *phoP/phoQ* and *pmrA/pmrB* (Cheng et al., 2010). It has been observed that the *phoP/phoQ* and *pmrA/pmrB* systems are upregulated in *K. pneumoniae* exposed to polymyxins (Kim et al., 2014a), indicating that these systems are involved in polymyxin resistance in this bacterium. The constitutive activation of the *pmrA/pmrB* system can also be caused by missense mutations in *pmrA* or *pmrB*, leading to the subsequent upregulation of *pmrC* and the *arnBCADTEF* operon, resulting in the synthesis and addition of PETN and L-Ara4N, respectively, to lipid A, as shown in Figure 1. Recently, various occurrences of such mutations have been identified in both the *pmrA* and *pmrB* genes of clinical and non-clinical isolates of colistin-resistant *K. pneumoniae*, as shown in Table 1 and Figure 2B (Cannatelli et al., 2014b; Jayol et al., 2014; Olaitan et al., 2014b). In all the studies, the MIC range of colistin has been reported to be 3–16 mg/L as shown by an Etest. Interestingly, similar mutations in the same loci have also been observed in colistin-resistant *Salmonella* and *Enterobacter aerogenes* (with a colistin MIC of 32 mg/L) (Sun et al., 2009; Diene et al., 2013). However, several synonymous mutations that are not responsible for resistance have also been observed in *pmrA* and *pmrB*. Similarly, possible mutations in *phoQ* genes that result in resistance have also been observed in colistin-resistant *K. pneumoniae* (Olaitan et al., 2014b).

**Table 1 | Mutations in two-component systems that result in their constitutive activations.**

| Bacteria                       | Gene        | Mutation in aa   | References                              |
|--------------------------------|-------------|--|---|
| <i>Salmonella enterica</i>     | <i>pmrA</i> | R81H, R81C   | Roland et al., 1993; Sun et al., 2009   |
|                                | <i>pmrB</i> | G15R<br>G53E, G53R<br>L14S, L14F<br>L22P<br>S29R<br>T92A<br>P94Q<br>E121A<br>S124P<br>N130Y<br>T147P<br>R155P<br>T156P, T156M<br>V161M, V161L,<br>V161G<br>E166K<br>M186I<br>G206W, G206R<br>S305R | Sun et al., 2009                        |
| <i>Klebsiella pneumoniae</i>   | <i>pmrA</i> | G53C   | Olaitan et al., 2014b                   |
|                                | <i>pmrB</i> | L82R   | Cannatelli et al., 2014b                |
|                                |             | T157P  | Jayol et al., 2014                      |
|                                |             | S85R<br>T140P  | Olaitan et al., 2014b                   |
|                                |             | ΔR14<br>ΔY209<br>T157P<br>S208N  | Choi and Ko, 2014                       |
| <i>Enterobacter aerogenes</i>  | <i>pmrA</i> | G53C   | Diene et al., 2013                      |
| <i>Acinetobacter baumannii</i> | <i>pmrA</i> | M12I   | Arroyo et al., 2011                     |
|                                | <i>pmrB</i> | S119T  |   |
|                                |             | E8D  | Lesho et al., 2013; Rolain et al., 2013 |
|                                |             | P102H<br>T13N<br>A227V<br>P233S, P233T<br>A262P  | Adams et al., 2009                      |
|                                |             | I121F<br>A183T   | Park et al., 2011                       |

(Continued)

**Table 1 | Continued**

| Bacteria | Gene        | Mutation in aa   | References                                   |
|----------|-------------|--|--|
|          |             | A184V<br>P190S<br>T192I<br>Q228P   |  |
|          |             | S14L<br>L87F<br>M145K<br>A227V<br>P233S<br>N353Y<br>F387Y<br>S403F   | Beceiro et al., 2011                         |
|          |             | P170L<br>P233S   | Pournaras et al., 2014                       |
|          |             | ΔA32-E35<br>D64V<br>A80V<br>ΔL160<br>P170Q, P170L<br>L208F<br>A226V<br>R231L<br>P233S<br>T235I<br>N256I<br>R263P, R263C<br>Q277H<br>G315D<br>P377L | Arroyo et al., 2011                          |
|          |             | S17R<br>Y116H<br>T232I<br>R263L  | Lesho et al., 2013                           |
|          |             | A227V<br>P233S<br>FrF26  | Kim et al., 2014b                            |
|          | <i>pmrA</i> | L157Q  | Lee and Ko, 2014                             |
|          | <i>pmrB</i> | M292T  | Abraham and Kwon, 2009                       |
|          |             | L243Q  | Moskowitz et al., 2004                       |
|          |             | A248V  |  |
|          |             | ΔD45   | Schurek et al., 2009; Moskowitz et al., 2012 |
|          |             | L14P<br>A54V   | Moskowitz et al., 2012                       |

(Continued)

Table 1 | Continued

| Bacteria             | Gene        | Mutation in aa  | References                                    |
|----------------------|-------------|---|---|
|                      |             | R57H<br>R79H<br>R135Q<br>G188D<br>A248T<br>S257N<br>R259H<br>M292I<br>P456S   |   |
|                      |             | V15I<br>M48L<br>A67T<br>D70N<br>L167P<br>H340R<br>T343A   | Lee and Ko, 2014                              |
|                      |             | A247T<br>M292T<br>Y345H   | Owusu-Anim and Kwon, 2012                     |
|                      |             | V281I<br>F237L  | Choi and Ko, 2014                             |
| <i>K. pneumoniae</i> | <i>phoP</i> | G385S<br>L26Q   | Olaitan et al., 2014b                         |
|                      | <i>phoQ</i> | L96P<br>L348Q<br>S174N  | Choi and Ko, 2014                             |
| <i>P. aeruginosa</i> | <i>phoQ</i> | V260G<br>H223R<br>V152 trunc.<br>A143V<br>K123Q   | Owusu-Anim and Kwon, 2012<br>Lee and Ko, 2014 |
|                      |             | Fr I421-428,X<br>429<br>I421X <sup>d</sup><br>D433X <sup>d</sup><br>ΔL364-G365<br>ΔL364-G365,<br>R444C<br>R6C,<br>ΔL364-G365<br>ΔV57-Q332 | Miller et al., 2011                           |
|                      |             | K123E<br>R214H<br>V184G<br>Q133E<br>A207R   | Choi and Ko, 2014                             |

(Continued)

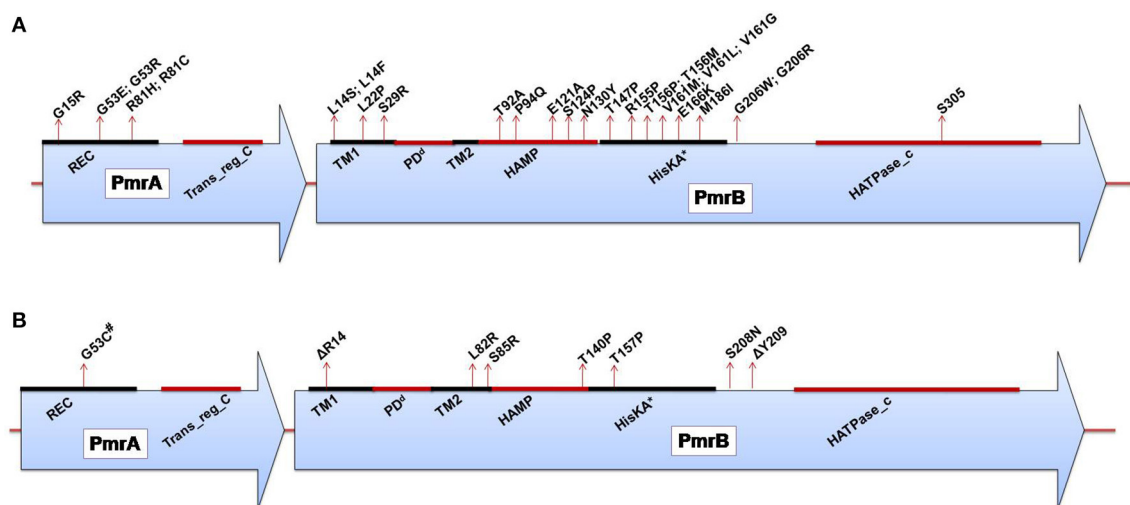
Table 1 | Continued

| Bacteria | Gene                       | Mutation in aa                                    | References          |
|----------|----------------------------|---|---------------------|
|          |                            | N104I   |                     |
|          | <i>parR</i>                | N24S<br>L18I<br>S24N                              | Choi and Ko, 2014   |
|          | <i>parS</i>                | M59I<br>E156K<br>L14Q<br>V101M<br>L137P           | Muller et al., 2011 |
|          |                            | Q232E<br>G361R                                    | Choi and Ko, 2014   |
|          |                            | V295L <sup>†</sup><br>A296P <sup>†</sup><br>H398R | Lee et al., 2014a   |
|          | <i>colR</i>                | D32N  | Muller et al., 2011 |
|          | <i>colS</i><br><i>cprS</i> | A106V<br>R241C                                    | Gutu et al., 2013   |

aa, amino acid; Δ, deletion; trunc., truncation; Fr, frameshift mutation; X<sup>d</sup>, premature termination of amino acid; <sup>†</sup>predicted not to affect protein's function according to SIFT (Sorting Intolerant From Tolerant) analysis.

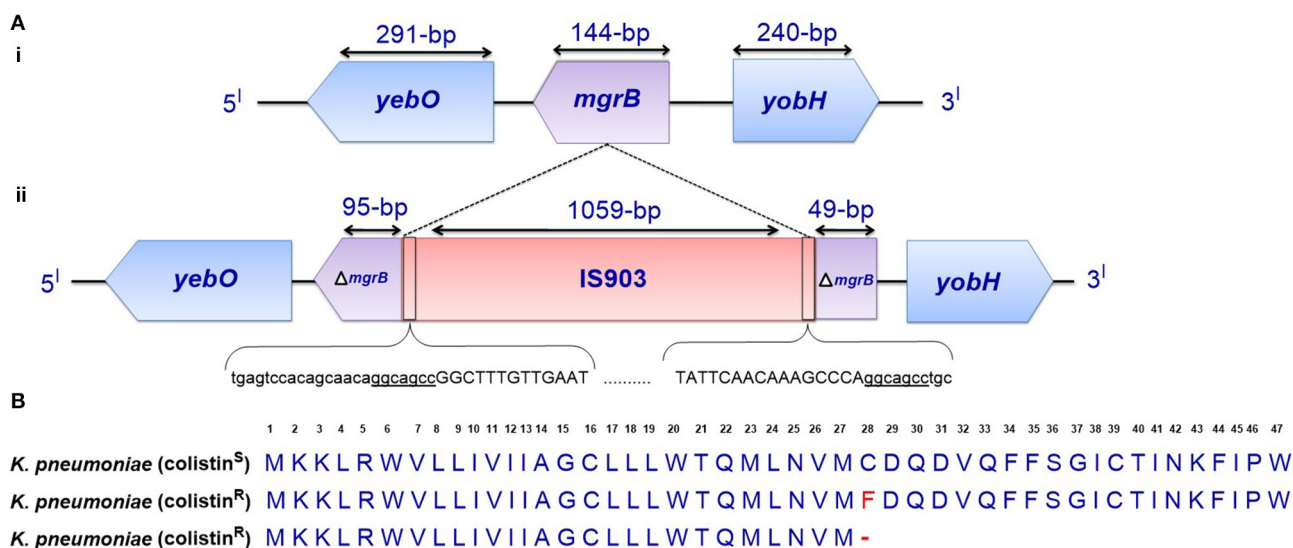
One profound molecular mechanism that leads to the emergence of colistin resistance in *K. pneumoniae* that has recently been discovered is the mutation/inactivation of the *mgrB* gene (Cannatelli et al., 2013), which is a conserved gene of 141 nucleotides in length (the length varies in naturally colistin-resistant Enterobacteriaceae) encoding a short, 47-amino acid transmembrane protein that exerts negative feedback on the PhoP/PhoQ regulatory system. It has been proposed that MgrB accomplishes this feedback by inhibiting the kinase activity of PhoQ and/or stimulating its phosphatase activity, which subsequently suppresses PhoP phosphorylation, leading to the repression of PhoP-regulated genes (Figure 1) (Lippa and Goulian, 2009). In accordance with this proposed mechanism, the deletion of *mgrB* in *E. coli* has been observed to result in the upregulation of PhoP-regulated genes (Lippa and Goulian, 2009).

Disruption of the *mgrB* gene in *K. pneumoniae* has been identified to play a prominent role in polymyxin resistance in this bacterium. Various disruptions in *mgrB* have recently been described in diverse clinical and non-clinical isolates of colistin-resistant *K. pneumoniae* and *K. oxytoca*, including insertional inactivation by an IS5-like element and other insertion sequences (Figure 3A) (Cannatelli et al., 2013; López-Camacho et al., 2013; Gaibani et al., 2014; Olaitan et al., 2014b; Poirel et al., 2014). Additional alterations that have been reported in *mgrB* include a non-sense mutation leading to the premature termination of the MgrB transmembrane protein and missense mutations resulting in amino acid substitutions (Figure 3B) (Cannatelli et al., 2014a; Olaitan et al., 2014b; Poirel et al., 2014). The range of MICs for colistin that has been reported in these isolates is



**FIGURE 2 | (A)** Domains of the PmrA/PmrB two-component system and positions of all mutations conferring polymyxin resistance to *Salmonella enterica* serovar Typhimurium. *PmrA* domains, cheY-homologous receiver domain [REC]; aa 1–112. Transcriptional regulatory protein, C-terminal domain [Trans\_reg\_C]; aa 145–216. *PmrB* domains, First transmembrane domain [TM1]; aa 13–35. <sup>d</sup>Periplasmic domain [PD]; aa 35–67. Second transmembrane domain [TM2]; aa 67–89. Histidine kinases, adenyl cyclases, methyl-binding proteins, and phosphatases [HAMP domain]; aa 89–141. Histidine kinase A (phosphoacceptor) domain [HisKA]; aa 142–202. Histidine kinase-like ATPases [HATPase\_c]; aa 249–356. \*HisKA, with the active site at H148 in PmrB of *Salmonella enterica* subsp. enterica serovar Typhimurium str. LT2 (GenBank accession no. AE006468). <sup>d</sup>Periplasmic domain was not predicted in SMART but was assumed to be between the TM1 and TM2; aa, amino acid. **(B)** Domains of the PmrA/PmrB

two-component system and positions of all mutations conferring polymyxin resistance to *Klebsiella pneumoniae*. *PmrA* domains, cheY-homologous receiver domain [REC]; aa 1–112. Transcriptional regulatory protein, C-terminal domain [Trans\_reg\_C]; aa 145–216. *PmrB* domains, First transmembrane domain [TM1]; aa 13–35. <sup>d</sup>Periplasmic domain [PD]; aa 35–67. Second transmembrane domain [TM2]; aa 67–89. Histidine kinases, adenyl cyclases, methyl-binding proteins, and phosphatases [HAMP domain]; aa 89–141. Histidine kinase A (phosphoacceptor) domain [HisKA]; aa 142–202. Histidine kinase-like ATPases [HATPase\_c]; aa 250–358. \*HisKA, with the active site at H153 in PmrB of *K. pneumoniae* subsp. *pneumoniae* MGH 78578 (GenBank accession no. CP000647). #Same mutation as that reported for colistin-resistant *Enterobacter aerogenes*. <sup>d</sup>Periplasmic domain was not predicted in SMART but was assumed to be between TM1 and TM2; aa, amino acid; Δ, deletion.



**FIGURE 3 | (A)** Genetic representation of the *phoP/phoQ* negative regulator, *mgrB*. (i) *K. pneumoniae* with intact *mgrB* (colistin-susceptible), and (ii) *K. pneumoniae* with *mgrB* truncated by an insertion sequence (colistin-resistant). **(B)** Alignment of

unmutated MgrB from colistin-susceptible *K. pneumoniae* and mutated MgrB from a colistin-resistant strain with a missense mutation and premature termination of MgrB. Premature termination (-).

4–64 mg/L, as shown by Etest colistin strips. Several insertion sequences, such as those of the IS5-like, IS903B, IS1F-like and ISKpn14 elements belonging to several IS families (especially the IS5 family), have been observed to lead to the truncation of *mgrB*. An IS5D-like element initially localized on the plasmid has been observed to transposed to the chromosome and inactivate *mgrB* (Cannatelli et al., 2014a; Olaitan et al., 2014b). Likewise, several amino acid substitutions, such as C28F (which has already been reported to affect PhoQ activity in *E. coli* (Lippa and Goulian, 2012), have been described in colistin-resistant *K. pneumoniae* (Cannatelli et al., 2014a; Olaitan et al., 2014b). Furthermore, a small deletion or the complete deletion of the *mgrB* locus has been reported in various colistin-resistant strains (Cannatelli et al., 2014a; Olaitan et al., 2014b). Complementation studies carried out on some of these *mgrB* mutant strains have resulted in the restoration of colistin sensitivity (Cannatelli et al., 2013, 2014a). This disruption of *mgrB* can result in the upregulation of the *arnBCADTEF* operon, which adds L-Ara4N to lipid A (Cannatelli et al., 2013). The upregulation of the *arnBCADTEF* operon is attributed to phosphorylated PmrA, which is activated by PmrD, which is in turn activated by the phosphorylation of PhoP resulting from the disruption of *MgrB* (Cheng et al., 2010; Kim et al., 2014a). Moreover, it has been demonstrated that activated PhoP can also directly activate the *arnBCADTEF* operon in *K. pneumoniae* independent of the PmrD- and PmrA-activated proteins in a phenomenon termed the feedforward connector loop (FCL) (Mitrophanov et al., 2008). We have recently observed that the mutation/inactivation of *mgrB* accounts for greater colistin resistance among resistant *K. pneumoniae* compared with that resulting from TCS mutations (*pmrA/pmrB* or *phoP/phoQ*) (Olaitan et al., 2014b). This observation demonstrates that *mgrB* plays an important role in polymyxin resistance in *K. pneumoniae*.

A cross-regulatory interaction between PhoP/PhoQ and the regulator of the capsule synthesis (Rcs) phosphorelay system has been demonstrated in *K. pneumoniae*. Mutant *rscB* upregulates the expression of *phoP/phoQ*, implying that the Rcs system normally downregulates the *phoP/phoQ* system (Llobet et al., 2011). In light of this crosstalk between the two regulatory systems, it is plausible that a mutation in *rscB* may play a role in polymyxin resistance via the upregulation of the *phoP/phoQ* system in *K. pneumoniae*, although this mechanism remains to be demonstrated in polymyxin-resistant isolates.

***Escherichia coli*.** In polymyxin-resistant *E. coli*, lipid A is typically modified with 2-aminoethanol and also with L-Ara4N (Nummala et al., 1995). The 2-aminoethanol molecule is added to the glycosidically linked diphosphate, whereas L-Ara4N is attached to the 4'-phosphate (Nummala et al., 1995). The importance of the L-Ara4N modification for polymyxin resistance in *E. coli* has been further demonstrated in other studies (Yan et al., 2007).

As depicted in **Figure 1**, in addition to the phosphorylation of Ugd (or PmrE) and the *arnT* operon by the phosphorylated PmrA from the PmrA/PmrB TCS (Aguirre et al., 2000), Etk (a tyrosine-kinase) has been observed to phosphorylate Ugd, thereby participating in polymyxin resistance in *E. coli* (Lacour et al., 2006); the mutant *E. coli* strain displayed a reduced survival rate in the presence of polymyxin B. The phosphorylation of the

Ugd protein (a UDP-glucose dehydrogenase) by Etk increases Ugd dehydrogenase activity. This elevated activity leads to an increase in the synthesis of UDP-glucuronic acid, which is the starting material for L-Ara4N synthesis by the *arnT* operon (Lacour et al., 2008). Interestingly, *etk* is expressed under conditions also known to activate the PmrA/PmrB system (Lacour et al., 2008). This finding indicates that, apart from the PmrA/PmrB system, polymyxin resistance via the modification of LPSs with L-Ara4N may be partially regulated by *etk*. Some studies have reported that Ugd is not directly activated by PmrA (Wosten and Groisman, 1999), but others have provided contrasting results (Aguirre et al., 2000). However, it is possible that *etk* is a principal activator of Ugd.

The 1-phosphate group of lipid A can be further modified by PETN, albeit to a lesser extent (Kim et al., 2006). The addition of PETN is mediated by *pmrC* (also called *eptA*) and regulated by PmrA (Raetz et al., 2007). The lipid A of *E. coli* O157:H7 has been found to be distinctly modified by PETN, most likely due to the increased activity of *pmrC* in this serotype. This modification confers slight resistance to the cationic antimicrobial peptide PMBN (Kim et al., 2006). These findings demonstrate that alterations with L-Ara4N confer increased resistance to polymyxins compared with that of PETN.

*mgrR* is another genetic determinant that has been shown to mediate polymyxin resistance in *E. coli*. It is an Hfq-dependent sRNA that is one of the regulons known to be regulated by the PhoP/PhoQ regulatory system in *E. coli*. *mgrR* is conserved in other enteric bacterial genera, such as *Salmonella*, *Citrobacter*, *Enterobacter*, and *Klebsiella*. It has been described to negatively regulate *eptB*, which mediates the modification of the outer Kdo (3-deoxy-D-manno-octulosonic acid) residues of LPSs with PETN (**Figure 1**) (Reynolds et al., 2005; Raetz et al., 2007). *eptB* is indirectly influenced by the PhoP/PhoQ system (Moon and Gottesman, 2009). The modification of Kdo with PETN reduces the net negative charges of LPSs, eventually leading to polymyxin resistance (Moon and Gottesman, 2009). It has been observed that the deletion of *mgrR* in *E. coli* increases resistance to polymyxin B (Moon and Gottesman, 2009) as a result of the increased PETN modification of Kdo. The expression of the PhoP/PhoQ TCS itself has been observed to be regulated by an Hfq-dependent sRNA termed MicA in *E. coli* and has been shown to repress the expression of PhoP (Coornaert et al., 2010). This process controls several PhoP-regulated genes, including *MgrR*. Thus, it is possible that MicA can also influence polymyxin resistance, although this influence has yet to be demonstrated.

It has recently been reported that cross-talk between QseB/QseC (quorum-sensing regulatory proteins) and PmrA/PmrB exists in *E. coli*. PmrB activates QseB in *E. coli* lacking QseC, and PmrA also directly regulates the transcription of QseB/QseC (Guckes et al., 2013). This interaction raises the possibility of cross-talk among other TCSs, which may influence the PhoP/PhoQ and/or PmrA/PmrB TCSs with respect to bacterial resistance to cationic antimicrobial peptides.

#### **Underacylation of lipid A and its effect on polymyxin resistance**

***Salmonella enterica*.** The importance of the myristoylation of lipid A (a late secondary acylation) with regard to polymyxin resistance has been highlighted in *Salmonella*. *Salmonella mshB*

strains (*lpxM* mutants) that produce predominantly penta-acylated lipid A (underacylated) and are unable to add the myristoyl group to this lipid A display sensitivity to polymyxin (Tran et al., 2005; Murray et al., 2007). This sensitivity occurs because the myristoylation of lipid A is essential for the L-Ara4N modification of the phosphate groups of lipid A (Tran et al., 2005). Nonetheless, a mutation in *pmrA* can still confer polymyxin resistance to a *Salmonella lpxM* mutant as a result of an alternative lipid A modification, such as that involving PEtN, although this resistance is lower than that conferred by the addition of L-Ara4N. Therefore, the secondary acylation of lipid A is important for the addition of L-Ara4N (but not PEtN) to the phosphate groups of this lipid and for resistance to polymyxins.

***Klebsiella pneumoniae*.** In *K. pneumoniae*, the *lpxM* gene also encodes the enzyme involved in the addition of the myristoyl group to lipid A, which results in the formation of hexa-acylated lipid A. An *lpxM* mutant of *K. pneumoniae* that produces predominantly penta-acylated lipid A has been found to be 8- to 16-fold more sensitive to both polymyxin B and colistin than the wild type with hexa-acylated lipid A (Clements et al., 2007). Velkov et al. have shown that the LPSs of the *K. pneumoniae lpxM* mutant strain with a colistin MIC of 0.25 mg/L display a four-fold higher binding affinity to polymyxins compared to the wild-type strain, with an MIC of 4 mg/L (Velkov et al., 2013b).

Similarly, a greater degree of acylation has been observed in polymyxin-resistant *K. pneumoniae* by Helander et al. (1996). Similar to *Salmonella*, underacylation has been reported to play an important role in polymyxin resistance in *K. pneumoniae*, likely due to the inability of underacylated *K. pneumoniae* to perform the L-Ara4N modification of lipid A, as has been noted in other enteric bacteria (Tran et al., 2005). It has been alternatively proposed that the increased susceptibility of underacylated *K. pneumoniae*, which results in fewer acyl chains, leads to the enhanced insertion of the polar heads or the fatty acid tails of polymyxins into membranes and thus a higher binding affinity of lipid A to these antibiotics (Clements et al., 2007; Velkov et al., 2013b).

***Escherichia coli*.** The myristoylation of lipid A by *lpxM* resulting in a hexa-acylated lipid A is likewise important for the addition of L-Ara4N to the lipid A of *E. coli*. Therefore, a loss of lipid A myristoylation in an *lpxM* mutant results in a lack of L-Ara4N modification and a subsequent decrease in polymyxin resistance (Tran et al., 2005).

#### Other LPS modifications mediating polymyxin resistance

The removal of the 3'-acyloxyacyl residue from lipid A has been observed in *S. Typhimurium*. This removal is mediated by *lpxR*, which is a hydrolase gene that encodes 3'-O-deacylase and has been shown to be present in other Gram-negative bacteria. *lpxR* is usually latent because its expression is inhibited by PmrA/PmrB-regulated lipid A modifications (Reynolds et al., 2006). The removal of 3'-acyloxyacyl results in the attachment of a reduced number of acyl groups to lipid A, and this alteration has been demonstrated to contribute to polymyxin resistance (Helander et al., 1996; Tran et al., 2005; Clements et al., 2007; Murray et al.,

2007). The activation of *lpxR* may play a role in polymyxin resistance, but the contribution of *lpxR* to this resistance has not yet been demonstrated.

Recently, genomic analysis of colistin-resistant *K. pneumoniae* isolates has revealed that these isolates harbor eight distinct mutations compared to susceptible isolates that have been found in both non-coding and coding regions. Mutations have been detected in genes encoding a microcin transporter, a putative membrane protein, a putative transport protein and the methyl viologen-resistance protein SmvA (Snitkin et al., 2012), and the isolates display a colistin MIC range of 4–128 mg/L. Another study involving a comparative genomic analysis of two colistin-resistant *K. pneumoniae* strains identified non-synonymous mutations in the *waaL*, *rfaA*, and *vacJ* genes. All three of these genes are known to be involved in the biosynthesis of outer membrane proteins (Sassera et al., 2013). However, it is not yet known if these genes or their mutations contribute to colistin resistance. Furthermore, *Salmonella enterica* with a mutation in *waaP* (a gene encoding a protein involved in the phosphorylation of the heptose residue of the LPS inner core) has been shown to display a higher susceptibility to polymyxins (Yethon et al., 2000).

Overall, the activities of most of these genes are outer membrane-related, and any gene that acts to undermine the integrity of the bacterial outer membrane may play a role in polymyxin resistance (Snitkin et al., 2012). Therefore, some of these aforementioned genes need to be further scrutinized.

#### ROLE OF CAPSULE IN POLYMYXIN RESISTANCE

It has been reported that *K. pneumoniae* is able to shed capsular polysaccharides (CPSs) from its surface. The released CPSs are able to trap or bind to polymyxins, thereby reducing the quantity of drug that reaches the bacterial cell surface, leading to increased polymyxin resistance (Llobet et al., 2008). The underlying mechanism is attributed to electrostatic interactions between the cationic polymyxins and anionic CPSs.

It has been further observed that CPSs play a direct role in resistance to antimicrobial peptides, including polymyxin B, by reducing their interactions with the bacterial outer membrane, mainly through the upregulation of capsular biosynthesis genes. CPS genes are further dependent on a certain CPS biosynthesis threshold for resistance (Campos et al., 2004). However, the role of the capsule in mediating polymyxin resistance remains under debate. Various studies have reported that the capsule does not contribute to resistance against antimicrobial peptides, including polymyxin B (Weiss et al., 1982; Clements et al., 2007). Interestingly, the *ugd* gene plays a dual role in CPS and L-Ara4N biosyntheses (Lacour et al., 2006), and its phosphorylation serves as a bridge between capsular synthesis and polymyxin resistance (Lacour et al., 2008).

#### ROLE OF EFFLUX PUMP IN POLYMYXIN RESISTANCE

A few studies have shown that efflux pumps can also contribute to polymyxin resistance. The efflux pumps that have been studied include AcrAB and KpnEF. It has been observed that *K. pneumoniae* with a mutation in *acrB* is significantly more susceptible to polymyxin B than the wild-type strain and that

polymyxin B is pumped out of *K. pneumoniae* in an energy-dependent manner (Padilla et al., 2010). Another study has reported that mutant KpnEF (a member of the small MDR efflux pump family) is more susceptible to several antibiotics, including colistin, compared with wild-type *K. pneumoniae* and that this mutant displays a two-fold reduction in the colistin MIC compared with the wild-type strain (Srinivasan and Rajamohan, 2013). Interestingly, the same *kpnEF* mutant also displays an impairment in capsular synthesis (Srinivasan and Rajamohan, 2013).

## ACQUIRED RESISTANCE TO POLYMYXINS IN NON-FERMENTATIVE BACILLI

### *Acinetobacter baumannii*

Two primary mechanisms that provide colistin resistance have been described in *A. baumannii* to date. The first is the modification of the lipid A moiety of LPS with PEtN as a result of mutations in the *pmrA/pmrB* TCS. The second is the complete loss of LPSs caused by either mutations or the insertional inactivation of lipid A biosynthesis genes (Moffatt et al., 2010, 2011).

#### *PmrCAB-mediated LPS modifications*

*A. baumannii* does not possess the genetic machinery required for L-Ara4N biosynthesis; however, it contains an ortholog of the *pmrCAB* operon that mediates the addition of PEtN to its lipid A (Adams et al., 2009). Similar to enteric bacteria, the PmrA/PmrB TCS has been shown to mediate resistance to colistin in this bacterium. Mutations in *pmrA* and/or *pmrB* have been widely observed to induce the constitutive expression of *pmrA* (the cognate response regulator) and the subsequent autoregulation of the promoter region of the *pmrCAB* operon, which leads to the modification of the 4'-phosphate or 1-phosphate group of lipid A with PEtN. Missense or small indel mutations in the *pmr* locus (primarily in *pmrB*) in both colistin-resistant clinical isolates (*in vivo*) and *in vitro*-selected isolates have been shown to result in colistin resistance in *A. baumannii* (Table 1) (Arroyo et al., 2011; Beceiro et al., 2011; Lesho et al., 2013; Rolain et al., 2013; Snitkin et al., 2013). Mutations have been reported in different domains of both PmrA and PmrB (Figure 4A) (Arroyo et al., 2011; Beceiro et al., 2011; Lesho et al., 2013; Rolain et al., 2013). Additionally, *pmrB* appears to be the most commonly mutated gene in *A. baumannii*, similar to enteric bacteria (Figure 4A). In most of these studies, the MICs of colistin have ranged from 4 to 256 mg/L and from 4 to 128 mg/L as shown by Etest and broth microdilution techniques, respectively.

The reversion from a resistant to a susceptible phenotype has been observed in some strains in which the mutant *pmr* strain acquires an additional mutation (a compensatory mutation) elsewhere in the *pmr* locus that counteracts the hyper-activation of the TCS caused by the first mutation (Adams et al., 2009; Snitkin et al., 2013). It is also possible that the mutant *pmr* gene may revert back to its unmutated form, thereby reversing the resistance phenotype (Snitkin et al., 2013). However, compensatory mutations have not been observed in all revertants, with some strains maintaining the mutation in *pmrB*. This finding indicates the possible involvement of other unknown gene(s) in addition to the

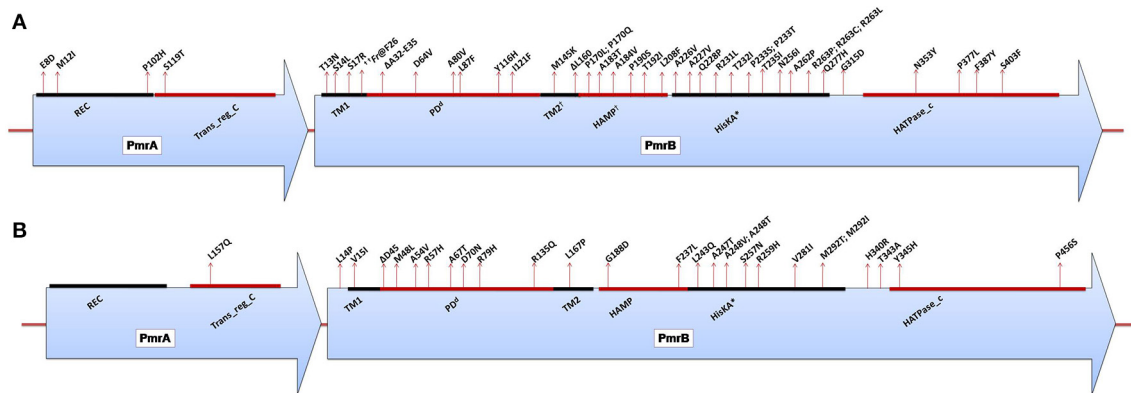
*pmrA/pmrB* locus in the mediation of colistin resistance and its reversion in some colistin-resistant strains. This idea is supported by the findings of Park et al., who did not detect any mutations in the *pmrA/pmrB* of six colistin-resistant *A. baumannii* clones out of 30 resistant isolates analyzed (Park et al., 2011), indicating the existence of other possible candidate gene(s). The identification of these genes is crucial for improving current knowledge of polymyxin resistance.

Colistin resistance in *A. baumannii* can emerge independently in a susceptible strain, and some strains may have a higher tendency of becoming resistant (Lesho et al., 2013). In some cases, *A. baumannii* possesses an auxiliary *pmrC*-like gene (named *eptA*), and some strains can have more than one copy of this gene (Arroyo et al., 2011; Lesho et al., 2013). It has been shown that the expression of this additional *pmrC*-like gene markedly increases in colistin-resistant strains. Interestingly, it has also been found to be located closer to mobile elements (prophage or integrase), suggesting the possibility of its horizontal acquisition (Lesho et al., 2013). The role of this auxiliary phosphoethanolamine transferase-encoding gene in colistin resistance in *A. baumannii* is still unknown.

**Other modifications.** Phosphorylation with PEtN usually occurs at the 4'-phosphate or 1-phosphate group of lipid A in *A. baumannii* colistin-resistant strains. In addition to this modification, a unique glycosylation of lipid A with galactosamine (hexosamine) at the 1-phosphate group has been recently reported in resistant strains (Pelletier et al., 2013). This unique modification is similar to the L-Ara4N modification in colistin-resistant enteric bacteria. Clinical isolates with both PEtN and galactosamine modifications have been reported, displaying colistin MICs of 1.5–48 mg/L as shown by a colistin Etest (Pelletier et al., 2013). The genetic basis underlying this modification remains to be determined. Furthermore, a predominance of hepta-acylated lipid A has been observed in colistin-resistant *A. baumannii* by Beceiro et al. (2011). This increase in lipid A acylation appears to be significant to colistin resistance, and the unique pattern of modifications (phosphorylation and glycosylation) observed in colistin-resistant *A. baumannii* is similar to that reported in enteric bacteria (Tran et al., 2005).

#### *Loss of LPS-mediated colistin resistance*

In colistin-resistant *A. baumannii*, mutations have been reported that involve a nucleotide substitution or deletion and an insertional inactivation with the insertion of ISAbal1 element in the first three lipid A biosynthesis genes, namely *lpxA*, *lpxC*, and *lpxD*, resulting in the complete loss of LPSs (Moffatt et al., 2010, 2011). Resistant strains harboring such mutations display a >128 mg/L colistin MIC with broth microdilution, and they lack LPSs because of their inability to synthesize lipid A. Recent analysis of polymyxin B-resistant *A. baumannii* isolates has further shown the presence of unique mutations in the *lpxC* and *lpxD* genes (Lean et al., 2014) in addition to the mutations found in the *lpsB* gene encoding a glycosyltransferase (involved in the biosynthesis of the LPS core) that have also been implicated in colistin resistance in *A. baumannii* (Hood et al., 2013; Lean et al., 2014). Because lipid A is the initial target of colistin, its absence results



**FIGURE 4 | (A)** Domains of the PmrA/PmrB two-component system and positions of all mutations conferring polymyxin resistance to *Acinetobacter baumannii*. *PmrA* domains, cheY-homologous receiver domain [REC]; aa 2–112. Transcriptional regulatory protein, C-terminal domain [Trans\_reg\_C]; aa 150–221. *PmrB* domains, First transmembrane domain [TM1]; aa 10–29. <sup>†</sup>Periplasmic domain [PD]; aa 29–142. <sup>‡</sup>Second transmembrane domain [TM2]; aa 142–164. <sup>§</sup>Histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases [HAMP domain]; aa 145–214. Histidine kinase A (phosphoacceptor) domain [HisKA]; aa 218–280. Histidine kinase-like ATPases [HATPase\_c]; aa 326–437. <sup>\*</sup>HisKA, with the active site at H228 in PmrB of *Acinetobacter baumannii* ATCC17978 (GenBank accession no. CP000521). <sup>†</sup>Periplasmic domain was not predicted in SMART but was assumed to be between TM1 and TM2. <sup>‡</sup>TM2 and HAMP overlapped based on SMART

prediction. <sup>††</sup>Fr denotes frameshift mutation; aa, amino acid. **(B)** Domains of the PmrA/PmrB two-component system and positions of all mutations conferring polymyxin resistance to *Pseudomonas aeruginosa*. *PmrA* domains, cheY-homologous receiver domain [REC]; aa 1–112. Transcriptional regulatory protein, C-terminal domain [Trans\_reg\_C]; aa 145–216. *PmrB* domains, First transmembrane domain [TM1]; aa 15–37. <sup>†</sup>Periplasmic domain [PD]; aa 38–160. Second transmembrane domain [TM2]; aa 161–183. Histidine kinases, adenylyl cyclases, methyl-binding proteins, and phosphatases [HAMP domain]; aa 186–238. Histidine kinase A (phosphoacceptor) domain [HisKA]; aa 239–304. Histidine kinase-like ATPases [HATPase\_c]; aa 348–459. <sup>\*</sup>HisKA, with the active site at H249 in PmrB of *Pseudomonas aeruginosa* PAO1 (GenBank accession no. AE004091). <sup>†</sup>Periplasmic domain was not predicted in SMART but was assumed to be between TM1 and TM2; aa, amino acid.

in the loss of the colistin target site and a high level of resistance in *A. baumannii*.

Finally, transposon mutagenesis performed to elucidate the mechanisms underlying adaptive resistance to colistin has identified over 20 genes, most of which participate in processes that protect bacteria from osmotic stress, which likely occurs due to colistin exposure (Hood et al., 2013). Therefore, the expression of these genes stimulated by the syntheses of compatible solutes and the expression of proteases can attenuate such stress.

**Colistin heteroresistance in *A. baumannii*.** Colistin heteroresistance has been widely observed among *A. baumannii* clinical isolates at levels as high as 90% (Li et al., 2006; Barin et al., 2013). These resistant subpopulations have been reported to significantly contribute to both the regrowth and increased development of colistin resistance (Li et al., 2006). However, the underlying molecular mechanism involved in this phenomenon remains to be elucidated, and its understanding is critical due to the clinical significance of colistin heteroresistance.

**Fitness cost of colistin resistance in *A. baumannii*.** Development of colistin resistance in *A. baumannii* may incur fitness costs to this microorganism. Such costs have been observed in colistin-resistant isolates both *in vitro* and *in vivo* and include growth retardation, impaired virulence, and substantially reduced clinical invasiveness (Fernandez-Reyes et al., 2009; Lopez-Rojas et al., 2011; Rolain et al., 2011; Pournaras et al., 2014). Furthermore, Hraiech et al. have reported that colistin-resistant *A. baumannii* possesses a reduced ability to cause infections, including systemic

dissemination and lung damage, in a rat model with pneumonia (Hraiech et al., 2013). Some of these biological costs, such as the impairment of virulence, have been linked with the reduced expression of metabolic proteins and of the OmpA porin (involved in the virulence of *Acinetobacter*) (Lopez-Rojas et al., 2011). Proteomic analysis of *in vitro*-selected colistin-resistant *A. baumannii* has revealed the differential expression of 35 proteins, the majority of which are downregulated in the resistant strain. These proteins include outer membrane proteins, chaperones, translation factors, and enzymes involved in metabolism (Fernandez-Reyes et al., 2009). Interestingly, some of these isolates incurring biological cost also harbor mutations in the *pmrA/pmrB* TCS (Fernandez-Reyes et al., 2009; Lopez-Rojas et al., 2011; Rolain et al., 2011; Hraiech et al., 2013; Pournaras et al., 2014). Beceiro et al. have recently demonstrated significant biological costs (in virulence and fitness) in colistin-resistant *A. baumannii* *lpx* mutants (lacking LPSs) compared to colistin-resistant *A. baumannii* *pmrA/pmrB* mutants (PETN-modified LPSs) (Beceiro et al., 2014).

#### ***Pseudomonas aeruginosa***

The mode of polymyxin resistance in *P. aeruginosa* is very similar to that observed in enteric bacteria. Unlike *A. baumannii*, *P. aeruginosa* has both the *pmrA/pmrB* and *phoP/phoQ* TCSs, each of which can separately regulate the *arnBCADTEF* operon (McPhee et al., 2003). To date, five TCSs have been described to play a role in polymyxin resistance in *P. aeruginosa* as follows: PmrA/PmrB (McPhee et al., 2003; Moskowitz et al., 2004, 2012; Abraham and Kwon, 2009), PhoP/PhoQ (Macfarlane et al., 1999; Barrow and Kwon, 2009; Schurek et al., 2009; Miller et al.,

2011), ParR/ParS (Fernández et al., 2010; Fernandez et al., 2012), ColR/ColS and CprR/CprS (Gutu et al., 2013).

### **PmrA/PmrB and PhoP/PhoQ two-component system-mediated LPS modifications**

Several studies have shown that *P. aeruginosa* can develop resistance to polymyxins via the constitutive modification of its LPSs with L-Ara4N, which is stimulated by the *pmrA/pmrB* and *phoP/phoQ* TCSs (Macfarlane et al., 1999, 2000; McPhee et al., 2003). The constitutive stimulation of these regulatory systems is typically (but not always) induced by mutations (Moskowitz et al., 2004, 2012). The synthesis and transfer of L-Ara4N to the lipid A moiety of LPSs are accomplished by the *arnBCADTEF* operon in *P. aeruginosa*, as has been observed in enteric bacteria.

For the *pmrA/pmrB* TCS, several polymyxin-resistant *P. aeruginosa* strains harboring mutations in *pmrB* have been reported (Table 1). These genetic alterations are known to lead to the modification of lipid A with L-Ara4N. Moreover, various resistant isolates harbor double mutations in *pmrB*, and these mutant strains tend to display high levels of resistance to colistin (Moskowitz et al., 2012). For *P. aeruginosa*, only one study has reported a mutation in *pmrA* that may be responsible for resistance to date (Lee and Ko, 2014), while the rest of the mutations have been mainly localized to PmrB or generally distributed within the cognate regulators of TCSs (Figure 4B; Table 1). Mutations have been observed in different portions of the PmrB domain (Moskowitz et al., 2012; Lee and Ko, 2014), and mutations in the periplasmic and DHP (dimerization and phosphoacceptor) domains, particularly in the histidine box motif, appear to be more significant (Moskowitz et al., 2012), i.e., those close to the putative active histidine residue (H249) tend to evoke greater polymyxin resistance than those distant from this residue (Abraham and Kwon, 2009). In most of the *P. aeruginosa pmrA/pmrB* mutants studied, the MICs of colistin have commonly ranged from 4 to >512 mg/L, mostly performed by the broth microdilution method.

In terms of lipid A modifications in resistant bacteria, there appears to be an absolute relationship of colistin resistance and L-Ara4N addition as well as a significant relationship of this resistance and the loss of secondary lauroyl chain hydroxylation (Miller et al., 2011; Moskowitz et al., 2012). There seems to be wide structural variation in lipid A in polymyxin-resistant *P. aeruginosa* in terms of the presence of either penta- or hexa-acylated lipid A (Moskowitz et al., 2012) in contrast to enteric bacteria, in which a higher degree of acylation is necessary for the addition of L-Ara4N to lipid A (Tran et al., 2005).

Another TCS that has been extensively studied and that also confers resistance in *P. aeruginosa* upon activation is the *phoP/phoQ* TCS. Several mutations resulting in in-frame deletions, frameshifts or truncations of the *phoQ* gene that consequently activate the *arnBCADTEF* operon have been reported in resistant strains (Table 1) (Miller et al., 2011; Lee and Ko, 2014). However, not all non-synonymous mutations in *phoQ* result in the constitutive activation of the system (Miller et al., 2011). In contrast to *Salmonella*, polymyxin resistance caused by

the activation of *phoP/phoQ* is not dependent on *pmrA/pmrB* in *P. aeruginosa* (Miller et al., 2011). The mutations responsible for PhoP/PhoQ activation appear to occur in *phoQ*, whereas mutations in *phoP* tend to act as primary suppressors in susceptible strains possessing mutations in both genes (Miller et al., 2011). In most of the *P. aeruginosa phoP/phoQ* mutants, colistin MICs usually ranged from 8 to >512 mg/L and were mostly performed by the broth dilution technique. It has been recently reported that mutations in both *pmrB* and *phoQ* result in greater resistance to polymyxins compared to a single mutation in either of the two genes (Owusu-Anim and Kwon, 2012).

Reversions from polymyxin-resistant to polymyxin-susceptible phenotypes among TCS (*pmrA/pmrB* or *phoP/phoQ*) mutants have been observed, and they can occur by the following mechanisms: the reversion of mutant *pmrB* or *phoQ* back to the wild-type gene (loss of mutation), an additional mutation in *phoP* or even the truncation of *phoQ* due to a secondary mutation (Miller et al., 2011; Lee and Ko, 2014). However, there is a high possibility that there are other gene(s) that may act as secondary suppressors when altered because some susceptible strains harbor polymyxin-inducing mutations in *phoQ* with wild-type *phoP* (Miller et al., 2011).

### **Other two-component systems**

The ColR/ColS and CprR/CprS TC regulatory systems have been reported to mediate resistance in *P. aeruginosa* either directly or indirectly. Mutations in these two systems have been observed to contribute to high levels of polymyxin resistance in *phoQ* mutant isolates (Gutu et al., 2013). From one viewpoint, the involvement of *colR/colS* and *cprR/cprS* in polymyxin resistance could occur through an interaction with the PhoP/PhoQ regulatory system that enhances *phoQ* activity and influences L-Ara4N modifications, which could lead to high levels of resistance. This interaction could be initiated by the presence of mutations in *colR/colS* and *cprR/cprS* (Table 1). Alternatively, Gutu et al. have suggested that the *colR/colS* and *cprR/cprS* systems may regulate other unknown genes involved in polymyxin resistance in addition to mediating L-Ara4N modifications via mutant *phoQ* (Gutu et al., 2013). This finding indicates that the L-Ara4N modification alone may not be sufficient for the development of polymyxin resistance in *P. aeruginosa*.

Additionally, *cprR/cprS* and another TCS (designated *parR/parS*) have been shown to be involved in adaptive resistance to polymyxins and other antimicrobial peptides. The *cprR/cprS* TCS participates in adaptive resistance by sensing various antimicrobial peptides, such as the synthetic peptide CP28 and polymyxins, and upregulating the *arnBCADTEF* operon (Fernandez et al., 2012). Meanwhile, *parR/parS* can cause the upregulation of the LPS modification operon under sub-inhibitory concentrations of polymyxins and other CAMPs, such as indolicidin but not CP28 (Fernández et al., 2010), demonstrating that the two TCSs are activated by different antimicrobial peptides besides polymyxins. Furthermore, mutations in either ParR or ParS have been observed to cause the constitutive expression of the *arnBCADTEF* operon independent of PmrA/PmrB, resulting in polymyxin resistance. Colistin MIC

of 2 mg/L (by agar dilution method) was reported for the clinical strains of *P. aeruginosa* harboring mutations in *parR/parS* genes. This activity has been observed in addition to the mediation of other antibiotic-related genes in *P. aeruginosa* by ParR/ParS (Muller et al., 2011).

#### Other polymyxin resistance determinants in *P. aeruginosa*

It has been shown that the overexpression of the outer membrane protein OprH in either an Mg<sup>2+</sup>-deficient medium or in a mutant parent strain can result in polymyxin resistance (Young et al., 1992). This is because OprH is a basic protein that binds to divalent cation-binding sites of LPSs, making these sites unavailable for polymyxin binding. However, it has also been suggested that the overexpression of OprH alone is not sufficient for polymyxin resistance in *P. aeruginosa* (Young et al., 1992).

Using transposon mutagenesis, other genes that may play roles in polymyxin resistance have been identified. A total of 17 genes were identified to contribute to intrinsic resistance in *P. aeruginosa* and thus confer supersusceptibility phenotype to polymyxin; the mutant strains showed between 1.5- and 3- fold MIC reduction to polymyxin B compared to the wild-type strain. Notably among these genes, *galU*, *lptC*, *wapR*, and *ssg* participate in LPS biosynthesis-related functions (regulatory functions, metabolism, synthesis and transport) (Fernandez et al., 2013). Disruptions in any of these LPS-mediated genes have been reported to affect the permeability of the outer membrane or to hinder LPS modifications, causing *P. aeruginosa* to be more susceptible to polymyxins (Fernandez et al., 2013).

Other genes that have been recently described to possibly play roles in resistance/susceptibility include those that are able to influence LPS modifications via *pmrA/pmrB* [including a TCS hybrid (PA2583), an arabinose efflux permease-encoding gene (PA5548), a lipoprotein-encoding gene (PA1199), and a hypothetical protein-encoding gene (PA2928)] as well as non-LPS-mediated genes [the response regulator *eraR* (PA1980), the glycosyl transferase *wbpZ* (PA5447) and hypothetical protein-encoding genes (PA4541 and PA1938)]. All of these genes have been mapped against the PAO1 genome (Lee et al., 2014b). The mutant strains display between 0.5- and 4- fold colistin MIC reduction compared to the colistin-resistant wild-type strain with the exception the *pdxB* mutant. The full characterization of these genes would improve the understanding of the complex mechanisms guiding polymyxin resistance in *P. aeruginosa* and help to determine whether these genes are part of the other alternative pathways that have been suggested to mediate colistin resistance in this bacterium (Lee et al., 2014a).

### INTRINSIC RESISTANCE TO POLYMYXINS IN BACTERIA

There are a number of Gram-negative bacteria that are naturally resistant to polymyxins, including but not limited to the following: *Proteus* spp., *Providencia* spp., *Morganella morganii* (all three collectively referred to as the *Proteeae* tribe), *Serratia* spp., *Edwardsiella tarda* and *Burkholderia cepacia* complex (Muyembe et al., 1973; Rozalski et al., 1997; Loutet and Valvano, 2011; Biswas et al., 2012; Samonis et al., 2014). Most of the aforementioned naturally resistant bacteria have LPSs that are modified with L-Ara4N, which may explain their intrinsic

resistance (Basu et al., 1986; Boll et al., 1994; Vinogradov et al., 2006).

#### *Proteus mirabilis*

The lipid A and Kdo residue of the LPSs of wild-type *Proteus mirabilis* are known to contain L-Ara4N (Sidorchuk et al., 1983; Boll et al., 1994), which is believed to contribute to the intrinsic resistance of this bacterium to polymyxins. On the other hand, polymyxin-susceptible mutants usually lack L-Ara4N in their LPSs, and those showing reduced resistance contain fewer L-Ara4N substitutions (Kaca et al., 1990; McCoy et al., 2001). Similarly, the genome of *P. mirabilis* has been shown to contain the *eptC* gene, which is involved in the modification of core LPSs with PETN (Aquilini et al., 2014).

Polymyxin-susceptible mutants generated using transposon mutagenesis have been studied to gain insight into the mechanisms underlying polymyxin resistance in *P. mirabilis*. Some putative loci that may either directly or indirectly affect LPS modifications that have been identified to date include the *sap* operon (encoding a transport protein), the ATPase gene and a putative *O*-acetyltransferase gene (most likely involved in either the biosynthesis or transfer of aminoarabinose) (McCoy et al., 2001). The *galU* gene (involved in L-Ara4N biosynthesis) and a TCS termed *rppA/rppB*, which shares close similarity with the *pmrA/pmrB* and *phoP/phoQ* TCSs and is able to activate the *arnBCADTEF* operon, have been recently discovered to mediate polymyxin resistance in *P. mirabilis* (Wang et al., 2008; Jiang et al., 2010a,b). The inactivation of any of these genes in *P. mirabilis* results in a polymyxin-susceptible phenotype (Wang et al., 2008; Jiang et al., 2010a,b). For example, the transposomic inactivations of *PpmrI* (homologous to *arnA* of *S. Typhimurium*) and *rppA* (homologous to both the *pmrA* and *phoP* of *S. Typhimurium*) genes of *P. mirabilis* resulted in 10240- and >160-fold susceptibility to polymyxin B, respectively, compared to the wild-type *P. mirabilis* strain (Wang et al., 2008; Jiang et al., 2010b).

Additionally, genomic analysis of intrinsically colistin-resistant *Morganella morganii*, which is closely related to *Proteus* spp., has revealed that this bacterium possesses an *arnBCADTEF* operon and *eptB* gene known to mediate the modifications of LPSs with L-Ara4N and PETN, respectively (Olaitan et al., 2014a). The genome of *M. morganii* also contains most of the genes that have been implicated in intrinsic polymyxin resistance in *P. mirabilis*, such as the *rppA/rppB* and *phoP/phoQ* TCSs (Olaitan et al., 2014a). It is therefore possible that the *Proteeae* bacteria possess the same underlying mechanisms for polymyxin resistance.

#### *Serratia marcescens*

The inactivation of the *arnB* and *arnC* genes, which are part of the *arnBCADTEF* operon, has been shown to result in polymyxin sensitivity in *Serratia marcescens* mutants (Lin et al., 2014). The *arnB* and *arnC* mutants displayed a reduced susceptibility to polymyxin B with MIC from 2048 to 2 mg/L (a 1024-fold reduction) compared to the wild-type *S. marcescens* strain. This operon has been further reported to be regulated by *phoP*, which is the cognate response regulator of the *phoP/phoQ* TCS (Lin et al.,

2014). Thus, these findings show that the modifications of the LPSs of *S. marcescens* as mediated by the *arnBCADTEF* operon are partly or wholly responsible for the intrinsic resistance of this bacterium to polymyxins.

The *arnBCADTEF* operon appears to be constitutively expressed in intrinsically resistant bacteria in contrast to non-intrinsically resistant bacteria. The principal question that remains to be answered is why this is true for these two sets of bacteria. An understanding of this mechanism will greatly aid in the elucidation of the evolution of polymyxin resistance in Gram-negative bacteria.

### ***Burkholderia* species**

Generally, members of the genus *Burkholderia* comprising the *Burkholderia cepacia* complex (BCC) and many other

*Burkholderia* species are intrinsically resistant to polymyxins (Loutet and Valvano, 2011). L-Ara4N is a predominant part of both the lipid A and Kdo moieties of the LPSs of *Burkholderia* spp., including *B. thailandensis* and *B. pseudomallei* (Isshiki et al., 1998; Silipo et al., 2005; Novem et al., 2009). It is constitutively produced and is essential for these bacteria. A *B. cenocepacia* mutant lacking L-Ara4N has been observed to be highly susceptible to polymyxin B (Ortega et al., 2007). This unique composition together with the contributions of other genes, such as *ispH* (involved in the synthesis of isoprenoids), *rpoE*, *norM* (an efflux pump-encoding gene), and *hpnJ* (encodes hopanoid), play roles in the multifaceted mechanisms that result in high levels of polymyxin resistance in *Burkholderia cepacia* complex (Loutet and Valvano, 2011; Malott et al., 2012). An excellent review by Loutet et al. on the intrinsic resistance of *Burkholderia cepacia*

**Table 2 | Strategies employed by Gram-negative bacteria for achieving resistance to polymyxins.**

| Resistance mechanism  | Genes involved   | Bacteria  | References  |
|---|--|---|---|
| Modification of the lipid A or Kdo with aminoarabinose                                  | <i>arnBCADTEF</i> operon and <i>pmrE</i>                 | <i>Salmonella enterica</i> , <i>Klebsiella pneumoniae</i> , <i>Escherichia coli</i> , <i>Proteaeae</i> bacteria, <i>Serratia marcescens</i> , <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cepacia</i> complex | Vaara et al., 1981; Boll et al., 1994; Nummilla et al., 1995; Helander et al., 1996; Rozalski et al., 1997; Trent et al., 2001b; Moskowitz et al., 2004; Yan et al., 2007; Loutet and Valvano, 2011; Lin et al., 2014 |
| Modification of the lipid A with phosphoethanolamine                                    | <i>pmrC</i>  | <i>S. enterica</i> , <i>K. pneumoniae</i> , <i>E. coli</i> and <i>Acinetobacter baumannii</i>   | Zhou et al., 2001; Lee et al., 2004; Kim et al., 2006; Arroyo et al., 2011; Beceiro et al., 2011; Jayol et al., 2014  |
| Activation of LPS-modifying operon by mutations in TCSs                                 | <i>pmrA/pmrB</i> and or <i>phoP/phoQ</i>                 | <i>S. enterica</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> and <i>A. baumannii</i>  | Roland et al., 1993; Sun et al., 2009; Arroyo et al., 2011; Owusu-Anim and Kwon, 2012; Cannatelli et al., 2014b; Jayol et al., 2014   |
| Inactivation of <i>phoP/phoQ</i> negative feedback regulator                            | <i>mgrB</i>  | <i>K. pneumoniae</i>  | Cannatelli et al., 2013; López-Camacho et al., 2013; Gaibani et al., 2014; Olaitan et al., 2014b  |
| Modification of the Kdo with phosphoethanolamine  | <i>eptB</i> , <i>phoP/phoQ</i> and <i>mgrR</i>           | <i>E. coli</i>  | Reynolds et al., 2005; Moon and Gottesman, 2009   |
| Increased acylation of lipid A enhancing its modification with aminoarabinose           | <i>lpxM</i>  | <i>S. enterica</i> , <i>K. pneumoniae</i> and <i>E. coli</i>  | Tran et al., 2005; Clements et al., 2007; Murray et al., 2007; Velkov et al., 2013b   |
| Trapping of polymyxins by capsule   |  | <i>K. pneumoniae</i> and <i>P. aeruginosa</i>   | Campos et al., 2004; Llobet et al., 2008  |
| Efflux pump   | <i>acrAB</i> and <i>kpnEF</i>                            | <i>K. pneumoniae</i>  | Padilla et al., 2010; Srinivasan and Rajamohan, 2013  |
| Loss of LPS   | <i>lpxA</i> , <i>lpxC</i> and <i>lpxD</i>                | <i>A. baumannii</i>   | Moffatt et al., 2010, 2011  |
| Glycosylation of lipid A with hexosamine  |  | <i>A. baumannii</i>   | Pelletier et al., 2013  |
| Acquired/adaptive resistance to polymyxins through LPS modification with aminoarabinose | <i>colR/colS</i> , <i>cprR/cprS</i> and <i>parR/parS</i> | <i>P. aeruginosa</i>  | Fernández et al., 2010; Muller et al., 2011; Fernandez et al., 2012; Gutu et al., 2013  |
| Overexpression of outer membrane protein OprH   | <i>oprH</i>  | <i>P. aeruginosa</i>  | Young et al., 1992  |

complex to polymyxins explains the roles of some of these genes (Loutet and Valvano, 2011).

## CONCLUSIONS

Resistance to polymyxin antibiotics depends on an intricate mechanism that involves several genes participating in bacterial cell membrane remodeling. However, most of the strategies employed by bacteria target the cell surface, which compromises its integrity or results in its modification, as summarized in **Table 2**. Although a large amount of work aimed at elucidating the mechanisms underlying polymyxin resistance has been performed, a great deal of information remains to be discovered because resistant strains exist for which the mechanisms underlying their resistance are unknown. Several other candidate genes have also been described, but their contributions to polymyxin resistance have not been fully examined. Such findings are of paramount importance given the current significance of polymyxins in clinical practice and the increased bacterial resistance to these drugs.

## AUTHOR CONTRIBUTIONS

Conceived and designed the study: Jean-Marc Rolain and Serge Morand; analyzed the data: Abiola O. Olaitan and Jean-Marc Rolain; wrote the paper: Abiola O. Olaitan and Jean-Marc Rolain.

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