Review

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Regulating polymyxin resistance in Gram-negative bacteria: roles of two-component systems PhoPQ and PmrAB

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Polymyxins (polymyxin B and colistin) are last-line antibiotics against multidrug-resistant Gram-negative pathogens. Polymyxin resistance is increasing worldwide, with resistance most commonly regulated by two-component systems such as PmrAB and PhoPQ. This review discusses the regulatory mechanisms of PhoPQ and PmrAB in mediating polymyxin resistance, from receiving an external stimulus through to activation of genes responsible for lipid A modifications. By analyzing the reported nonsynonymous substitutions in each two-component system, we identified the domains that are critical for polymyxin resistance. Notably, for PmrB 71% of resistance-conferring nonsynonymous mutations occurred in the HAMP (present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatase) linker and DHp (dimerization and histidine phosphotransfer) domains. These results enhance our understanding of the regulatory mechanisms underpinning polymyxin resistance and may assist with the development of new strategies to minimize resistance emergence.

First draft submitted: 22 November 2019; Accepted for publication: 28 February 2020; Published online: 6 April 2020

Keywords: lipid A modification • PhoPQ • PmrAB • polymyxin resistance • two-component system

Multidrug-resistant Gram-negative pathogens are a significant threat to human health globally [1]. The WHO prioritized *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae as 'critical' pathogens that urgently require novel antimicrobial treatments [1,2]. Polymyxins (i.e., polymyxin B and colistin) are the last-line antibiotics used to treat infections caused by these Gram-negative 'superbugs' [3]. Polymyxins are a group of cationic lipopeptides that act via initial electrostatic and hydrophobic interactions with lipid A of lipopolysaccharides (LPS) in the Gram-negative bacterial outer membrane (OM) [4]. Specifically, the positively charged L-α,γ-diaminobutyric acid residues of polymyxins bind to the negatively charged phosphate groups of lipid A [5], followed by the replacement of cationic ions (e.g., Ca^{2+} and Mg^{2+}) that bridge and stabilize the neighboring LPS molecules in the outer leaflet of the OM. The fatty acyl tails of polymyxins then insert into the destabilized LPS leaflet, resulting in OM disorganization and eventually cell death [6]. However, the exact mechanisms by which polymyxins kill bacteria remain unknown.

Gram-negative bacteria develop polymyxin resistance via multifaceted mechanisms, including lipid A modifications [3,7,8], LPS loss [9], efflux pump [3] and capsule formation [10]. Lipid A modifications with positively charged moieties reduce the negative charge on the bacterial surface, thereby decreasing the ability of polymyxins to bind and disorganize the OM [3]. These modifications include the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N, mediated by *arnBCADTEF-ugd* operon), phosphoethanolamine (pEtN, mediated by chromosomally encoded *eptA* or plasmid-borne *mcr*) and/or galactosamine (*naxD*) [7,11,12]. Additionally, in *A. baumannii* LPS

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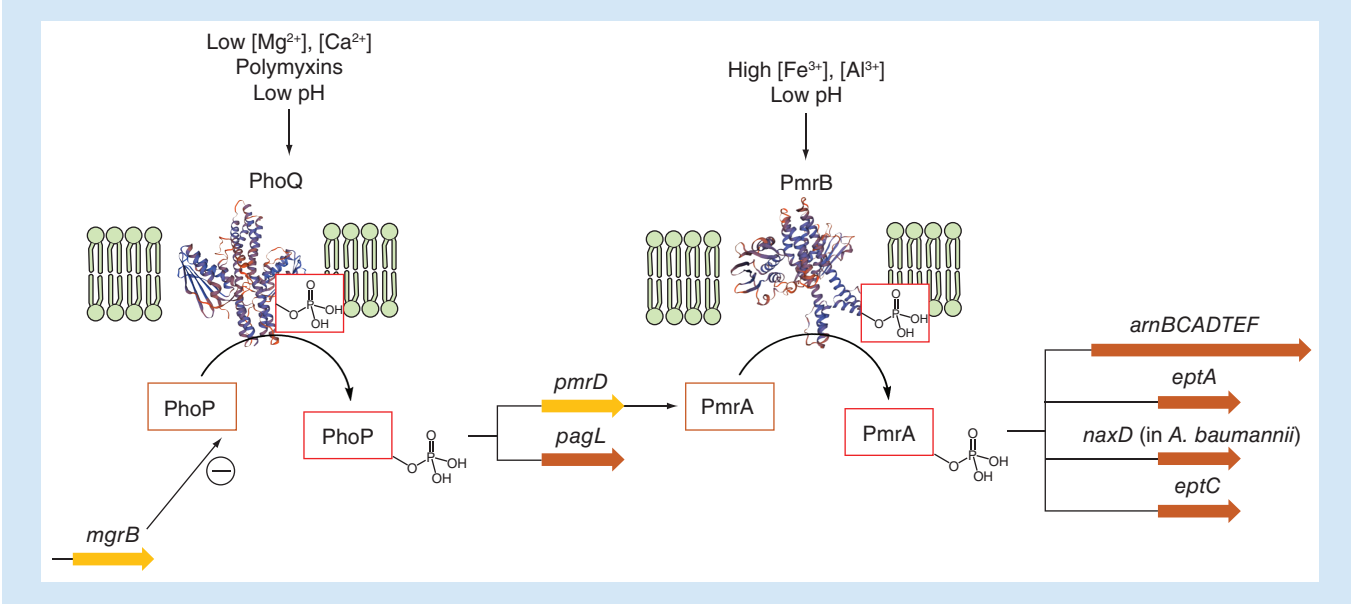

Figure 1. Schematic overview of PmrAB and PhoPQ regulons related to polymyxin resistance.

loss due to nonsynonymous mutations or transposon insertions in lipid A biosynthesis genes *lpxACD* can lead to polymyxin resistance [9]. In *Klebsiella pneumoniae*, polymyxin activity can be attenuated due to interaction with the anionic capsule polysaccharides [10].

Two major two-component systems (TCSs), PmrAB and PhoPQ, play crucial roles in regulating the expression of genes for lipid A modifications in Gram-negative bacteria (Figure 1) [13]. A prototypical TCS comprises a histidine kinase (HK) and a cognate response regulator (RR), and responds to environmental stimuli generally through five steps [14,15]: detection of environmental stimuli by HK; autophosphorylation of HK; phosphorylation of RR catalyzed by HK; altered transcription of RR-regulated genes; dephosphorylation of RR. Previous studies have identified a large number of indels and nonsynonymous substitutions in PmrAB or PhoPQ resulting in the constitutive expression of lipid A modification genes and, consequently, polymyxin resistance [16]. However, not all nonsynonymous substitutions could alter bacterial susceptibility to polymyxins. Here, we conducted comprehensive bioinformatics analyses to infer the domain preference of resistance-conferring mutations in PmrAB and PhoPQ. Our results provide important mechanistic information for better understanding polymyxin resistance.

PmrAB

PmrAB is one of the major regulators of lipid A modifications in *Escherichia coli*, *Salmonella enterica*, *K. pneumoniae*, *Yersinia pestis, Citrobacter rodentium, P. aeruginosa* and *A. baumannii* [17]. In general, external signals (e.g., high Fe^{3+}, high Al^{3+} and low pH) trigger the autophosphorylation of PmrB at a conserved histidine residue (e.g., His152 in *E. coli* MG1655) in its cytoplasmic domain, followed by the transfer of the phosphoryl group to a conserved aspartate residue of PmrA (e.g., Asp51 in *E. coli* MG1655). The active form, activated PmrA (PmrA-P), then binds to the promoter regions of the lipid A modification genes (e.g., *arnBCADTEF-ugd*, *eptA* and *naxD*), and induce their transcription [18,19].

PmrB

Sequence conservation of PmrB across different bacterial species

The available tertiary structures of PmrAB were collected from the Protein Data Bank (Table 1) [20]. Due to a lack of PmrB structure, we used Simple Modular Architecture Research Tool as a structural analysis surrogate to identify the four major domains of PmrB in *E. coli* MG1655 (Figure 2) [21]: a transmembrane sensor domain (15–88 amino acids [aa]), an HAMP (present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatase) linker domain (89–141 aa), a DHp (dimerization and histidine phosphotransfer) domain (142–202 aa) and a

Table 1. Currently available structures of PhoPQ and PmrAB.

Protein	Domain	Strain	Method	Resolution (Å)	PDB ID	Ref.
PhoQ	Sensor domain	*E. coli* K12	X-ray diffraction	2.5	3BQ8	[22]
	Sensor domain	*S. typhimurium* LT2	X-ray diffraction	2.4	1YAX	[23]
	Periplasmic domain	*S. typhimurium* LT2	X-ray diffraction	1.9	4UEY	[24]
	Catalytic domain	*S. typhimurium* LT2	X-ray diffraction	1.9	3CGZ	[25]
	Catalytic domain	*E. coli* K12	X-ray diffraction	1.6	1ID0	[26]
PhoP	Receiver domain	*E. coli* K12	X-ray diffraction	2.54	2PKX	[27]
PmrB	No available protein structure					
PmrA	Full length with DNA complex	*K. pneumoniae* JM45	X-ray diffraction	3.2	4S04	[28]
	Receiver domain	*K. pneumoniae*†	X-ray diffraction	1.7	3W9S	[29]

†Strain name was not provided in [29].

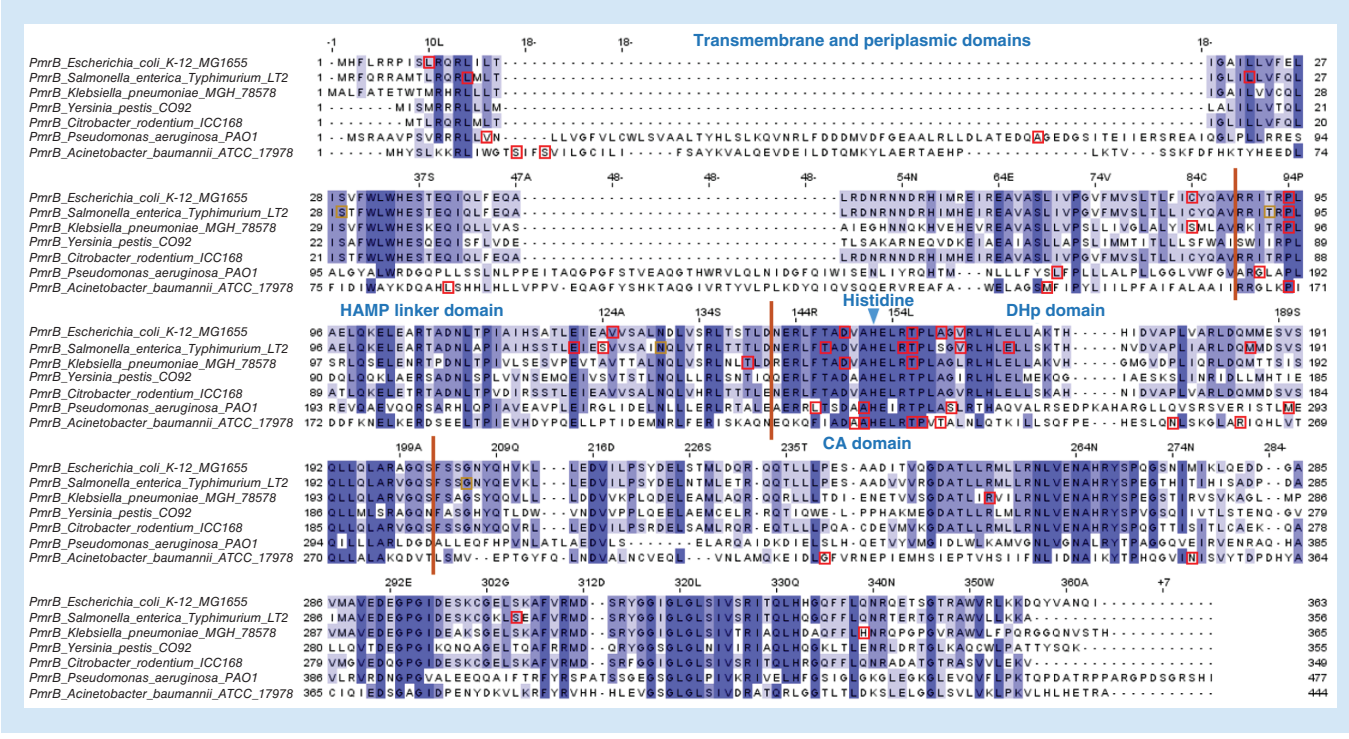

Figure 2. Multiple sequence alignment of PmrB across seven Gram-negative bacteria. The conservation of amino acid residues is indicated by the darkness of the dark blue color. Mutations conferring polymyxin resistance are shown with red boxes and mutations that do not cause polymyxin resistance are shown with yellow boxes.

CA (catalytic and ATP-binding) domain (249–357 aa). The DHp and CA domains are connected by a short, unstructured linker (203–248 aa).

Multiple sequence alignment (MSA) of PmrB sequences from seven key Gram-negative bacteria (one representative strain for each species; Figure 2) showed an identity of 44.4 ± 21.5% using Clustal Omega [30] and SIAS (http://bio.med.ucm.es/Tools/sias.html). Notably, 26 residues (143-ERLFTADVAHELRTPLAGVRLHLELL-168 in *E. coli* MG1655) in the vicinity of the histidine acceptor site (His152 in *E. coli* MG1655) are highly conserved across the seven bacterial species, indicating its critical role in kinase function. The transmembrane sensor domain is relatively less conserved (Figure 2). PmrB from all seven bacterial species share a similar secondary structural element of two transmembrane helices. However, PmrB of *P. aeruginosa* and *A. baumannii* (both belonging to pseu-

domonadales) contain longer periplasmic regions (185 and 174 aa in *P. aeruginosa* and *A. baumannii*, respectively) between the two transmembrane helices compared with other bacteria (e.g., 88 aa in *E. coli*).

PmrB mutations

It has been reported that at least 70 nonsynonymous substitutions in PmrB are related to the acquisition of polymyxin resistance (Table 2 & Figure 2); 50 out of 70 (71%) occurred in the HAMP linker and DHp domains. The HAMP linker domain transduces signals from the transmembrane domain to the CA domain by direct interactions; thus, specific conformational changes (e.g., rotation and helical tilt movements) in the HAMP linker domain can disturb signal transduction, promoting the phosphorylation of the kinase [31–34]. Mutations in the HAMP linker can result in signal transduction through the loss of domain symmetry, subsequently promoting activity of PmrAB and expression of lipid A modification genes, thereby conferring polymyxin resistance [35]. For example, a clinical isolate of *K. pneumoniae* with an increased colistin MIC of 64 mg/l harbored a P95L mutation (Table 2) in the HAMP linker domain of PmrB [36]. The DHp domain constitutes a large portion of the HK dimer interface and has multiple functions including autokinase, phosphotransferase and phosphatase activities [13]. The DHp domain of PmrB also harbors a binding site to interact with the cognate RR PmrA. Mutations in the PmrB DHp domain, therefore, can affect these activities via conformational changes. Abraham *et al.* [37] reported that an M292T substitution in the PmrB DHp domain resulted in a 16-fold increase in polymyxin B MIC (from 0.5 to 8 mg/l) in *P. aeruginosa*. The transmembrane domain senses specific physiological signals (e.g., high Fe^{3+} and high Al^{3+}) and subsequently enhances the phosphorylation of PmrB via conformational changes. Mutations in the transmembrane domain can cause these conformational changes even in the absence of these environmental signals, thereby constitutively promoting the phosphorylation of PmrB [38]. For example, an L10P mutation in PmrB of an *E. coli* clinical isolate resulted in an 83-fold upregulation of the lipid A modification gene *arnT* compared with its wild type, irrespective of polymyxin treatment [39]. Additionally, mutations in the transmembrane and CA domains can also influence activity of PmrB. Additionally, five PmrB mutations conferring polymyxin resistance were reported in the CA domain in *E. coli*, *S. enterica*, *P. aeruginosa* and *A. baumannii* (Table 2). These mutations are assumed to enhance the capture of a phosphate group from ATP [13], thus promoting the autophosphorylation of PmrB and conferring polymyxin resistance.

It should be noted that mutations in PmrB do not always cause resistance to polymyxins (Table 3). For example, an R231L substitution in DHp domain of PmrB was identified in a susceptible *A. baumannii* strain with a polymyxin B MIC of 2 mg/l (the MIC of the wild type is 0.5 mg/l) [63], suggesting that PmrB mutations do not necessarily lead to polymyxin resistance (MIC $\geq$2 mg/l as defined by the EUCAST guideline [70]). The lack of structural information of PmrB poses a significant challenge for mechanistic interpretations of polymyxin resistance. Hence, comprehensive characterization of conformational differences between the mutant and wild-type PmrB is essential for better understanding the role of PmrB in polymyxin resistance.

PmrA

Sequence conservation of PmrA across different bacterial species

PmrA has two major domains: an *N*-terminal receiver domain and a *C*-terminal DNA-binding domain [28]. The receiver domain is responsible for sensing the activation of PmrB and promoting the DNA-binding domain residues to recognize the DNA-binding site, thereby bind to and activate the transcription of the targeted genes [29]. Our MSA results demonstrated that PmrA sequences are generally conserved with a pairwise identity of 56.6 $\pm$ 18.9% across seven bacteria. The aspartate residue of PmrA (e.g., Asp51 in *E. coli* MG1655) was particularly well conserved across the seven species examined.

PmrA mutations

Hitherto, ten nonsynonymous mutations that confer resistance to polymyxins have been identified in PmrA of *S. enterica*, *K. pneumoniae* and *A. baumannii* (Table 2 & Figure 3); all occurred in the receiver domain. The conformational changes caused by mutations in the receiver domain likely augment the phosphorylation of PmrA and contribute to the enhanced DNA-binding capacity and upregulation of the targeted genes.

Stimuli of PmrAB

Environmental stimuli affecting PmrAB have mostly been studied in *Salmonella*. In *S. enterica*, PmrB senses high concentrations of Fe^{3+} (e.g., 100 μM) and Al^{3+} (e.g., 100 μM) [73] or low pH (e.g., pH 5.8) [74]. The ferric ions

Table 2. Nonsynonymous substitutions of PhoPQ and PmrAB in polymyxin-resistant bacteria.

Species	MIC (mg/l)	Nonsynonymous substitution				Ref.
		PmrB	PmrA	PhoQ	PhoP	
Escherichia coli	4	C84Y				[40]
	8	D149Y				
	4	L10P				[39]
	4	V161G				[41]
	>2	T156K				[42]
	>2	A159V				
	>64	P94L				[43]
	64	V125E				
	64	A159V				
Salmonella enterica	3.5	L14S				[44]
	3.5	L14F				
	3.5	L22P				
	2.5	P94Q				
	4	E121A				
	2.3	S124P				
	2.5	T147P				
	3.5	R155P				
	4.4	T156P				
	3.5	T156M				
	4	V161M				
	3	V161G				
	3	E166K				
	2.8	M186I				
	2.7	S305R				
	2.7		G15R			
	3		G53E			
	3		G53R			
	4		R81C			
	3		R81H			
Klebsiella pneumoniae	>128	T157P				[45]
	>2	S85R				[46]
	>2	T140P				
	128	S85R				[47]
	>256	H340R				
	64	P95L				[36]
	64	D150Y				
	64	T157P				
	3–6	T157P				[48]
	4–32	R256G				[49]
	32	T157P				[50]
	128	P95L				[51]
	64		G53C			[36]
	16			A21S		[52]
	32			L26P		[53]
	>2			L96P		[46]
	>2			L348Q		
	64			G385S		
	128			S174N		[45]
	64			T244N		[36]

Table 2. Nonsynonymous substitutions of PhoPQ and PmrAB in polymyxin-resistant bacteria (cont.).

Species	MIC (mg/l)	Nonsynonymous substitution				Ref.
		PmrB	PmrA	PhoQ	PhoP	
	>64			L348Q		
	4			L173P		[50]
	64			T244N		
	16			S260N		
	>2			T281M		[54]
	>2			G385C		
	>2				L26Q	[46]
Pseudomonas aeruginosa	32	L243Q				[55]
	32	A248V				
	4	V15I				[56]
	>64	L167P				
	>64	V15I				[43]
	64	A67T				
	>64	L167P				
	16	M292T				[37]
	>512	G188D				[57]
	>512	A248T				
	>512	S257N				
	8			H223R		[58]
	8			V260G		
	128			N104I		[45]
	128			V184G		
	128			A207R		
	128			R214H		
	8				N188Y	[59]
	8				N188H	
Acinetobacter baumannii	128	P233S				[60]
	8	P233S				[61]
	64	S17R				[62]
	64	T235I				
	64	A226V				[63]
	8	T235I				
	16	N256I				
	4	G315D				
	>128	P233T				[64]
	64	A227V				
	16	L87F				[65]
	4	M145K				
	32	A227V				
	16	P233S				
	16	N353Y				
	32	P170L				[66]
	128	P233S				
	>64	H263R				[43]
	≥4	S17R				[67]
	16	T232I				
	≥4	R263L				
	6	A227V				[68]

Table 2. Nonsynonymous substitutions of PhoPQ and PmrAB in polymyxin-resistant bacteria (cont.).

Species	MIC (mg/l)	Nonsynonymous substitution				Ref.
		PmrB	PmrA	PhoQ	PhoP	
	16	P233S				
	64		M12R			[43]
	>2		E8D			[69]
	4		M12I			[63]
	4		E8D			[67]

Table 3. Nonsynonymous substitutions of PhoPQ and PmrAB in polymyxin-susceptible bacteria.

Species	MIC (mg/l)	Mutation in			Ref.
		PmrB	PhoQ	PhoP	
Salmonella enterica	0.5	S29R			[44]
	0.25	T92A			
	2	N130Y			
	2	V161L			
	2	G206W			
	2	G206R			
Klebsiella pneumoniae	1	R256G			[53]
	1		D150G		
	1		V258F		
	1		D434N		[71]
	1			V3F	[53]
	1			S86L	
Pseudomonas aeruginosa	<2		E72G		[72]
Acinetobacterbaumannii	2	R231L			[63]

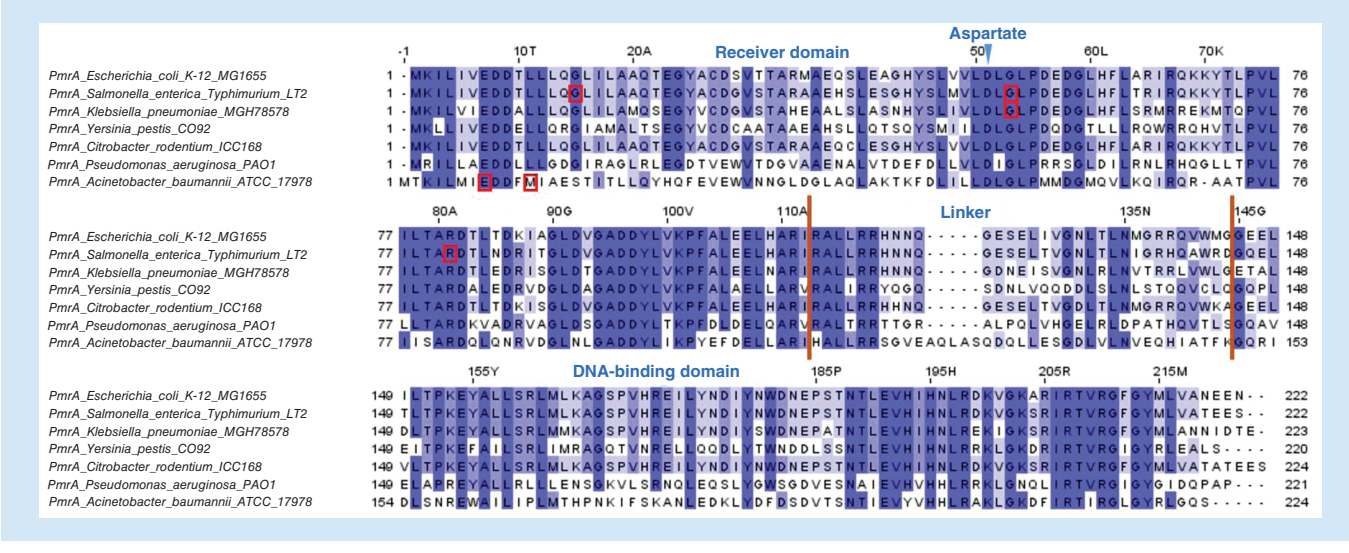

Figure 3. Multiple sequence alignment of PmrA across seven Gram-negative bacteria. The conservation of amino acid residues is indicated by the darkness of the dark blue color. Mutations conferring polymyxin resistance are shown with red boxes.

directly bind to the periplasmic domain of PmrB which harbors two copies of the ExxE motif [73,75]. The ExxE motif is also necessary for the response to high concentrations of Al^{3+} [73], although the detailed mechanisms that underpin aluminum signaling are unknown [76]. The direct sensing of environmental mild acid (i.e., pH 5.8) by PmrB in *Salmonella* requires the single histidine residue and the four glutamate residues (i.e., H35, E36, E39, E61

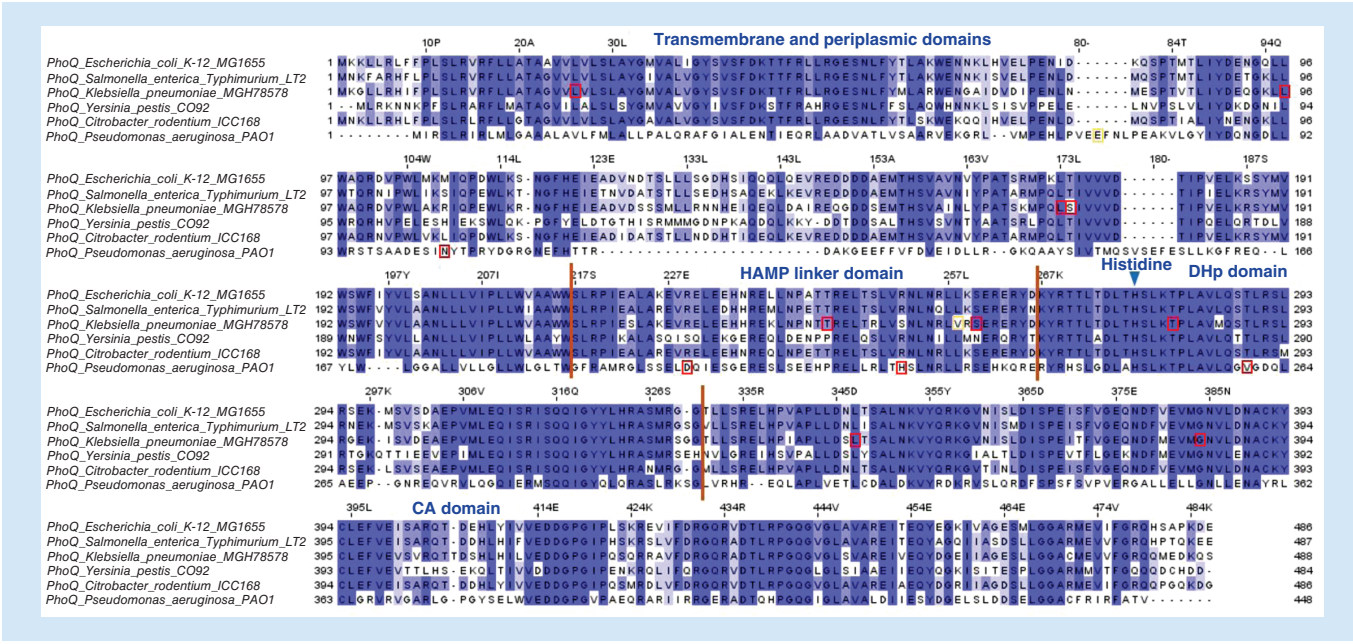

Figure 4. Multiple sequence alignment of PhoQ across six Gram-negative bacteria. The conservation of amino acid residues is indicated by the darkness of the dark blue color. Mutations conferring polymyxin resistance are shown with red boxes and mutations that do not cause polymyxin resistance are shown with yellow boxes.

and E64) in the periplasmic domain [77]. PmrA-regulated genes *eptA* and *arnBCADTEF-ugd* were activated when the medium pH fell from 7.7 to 5.8 [78]. Wild-type *S. enterica* 14028s grown at pH 5.8 was >100,000-fold more resistant to polymyxin B when compared with those grown at pH 7.7 [77]. Besides the direct regulation by the aforementioned signals, PmrAB can also be indirectly activated by PhoPQ (see section PhoPQ).

PmrA regulon-associated lipid A modifications

The known PmrA regulated genes include *arnBCADTEF-ugd*, *eptA*, *eptC* and *naxD* [79]. *arnBCADTEF-ugd* encode a series of enzymes catalyzing the synthesis of L-Ara4N from UDP-*N*-acetylglucosamine and transfer of L-Ara4N to lipid A. Specifically, *arnT* encodes a glycosyltransferase catalyzing the transfer of L-Ara4N from an undecaprenyl phosphate-α-L-Ara4N donor to a phosphate group of lipid A [80]. *eptA* and *eptC* encode pEtN transferases catalyzing the addition of pEtN to the phosphate groups of lipid A and the heptose-I phosphoryl group of LPS inner core oligosaccharide, respectively [81]. In *A. baumannii naxD* encodes a deacetylase that deacetylates *N*-acetylgalactosamine to galactosamine, a step required for the subsequent addition of galactosamine to lipid A [7]. Overexpression of these genes may confer polymyxin resistance in Gram-negative bacteria [82].

PhoPQ

PhoPQ plays a critical role in virulence and LPS remodeling in Gram-negative bacteria by regulating over 200 genes [83–85]. Consisting of an HK PhoQ and a cognate RR PhoP, PhoPQ senses the presence of specific environmental stimuli (e.g., Mg^{2+}, Ca^{2+} and cationic antimicrobial peptides) and activates the transcription of a set of PhoP-regulated genes (e.g., *pagL* and *pmrD*) [86]. Similar to PmrAB, PhoQ contains four major domains: a transmembrane sensor domain, an HAMP domain, a DHp domain and a catalytic domain [87,88]; PhoP has an *N*-terminal receiver domain and a *C*-terminal effector domain (i.e., DNA-binding domain) [27]. Interestingly, *phoPQ* is absent in *A. baumannii* genomes [89], indicating a unique regulation of polymyxin resistance in this problematic 'superbug'. Compared with PmrAB (80 mutations reported), far fewer mutations that confer resistance to polymyxins have been reported for PhoPQ (22 mutations reported).

To date, 19 PhoQ single aa substitutions (13 in *K. pneumoniae* and six in *P. aeruginosa*) that increase resistance to polymyxins have been reported (Table 2). Generally, these mutations are distributed in all four domains without any obvious preference (Figure 4). Our MSA result revealed that PhoQ proteins across the six selected species share a mean pairwise identity of 61.3 ± 21.3%. PhoQ in *Y. pestis* and *P. aeruginosa* had the lowest sequence similarities

compared with those in *E. coli*, *S. enterica*, *K. pneumoniae* and *C. rodentium*. Five papers have reported on truncated structures of PhoQ domains *from E. coli* or *S. enterica* solved by X-ray diffraction (resolution from 1.6 to 3.2Å), although no full-length structure has yet been reported (Table 1).

To date, only three PhoP mutations that confer polymyxin resistance have been reported in Gram-negative bacteria. These are L26Q (receiver domain) in *K. pneumoniae* [46] and N188Y/N188H (DNA-binding domain) in *P. aeruginosa* [59].

Stimuli of PhoPQ

In *E. coli* and *S. enterica*, PhoQ can sense the presence of low concentrations (e.g., 10 μM) of divalent cations (e.g., Mg^{2+} and Ca^{2+}), cationic antimicrobial peptides and low pH, subsequently activating the transcription of PhoPQ [90]. Environmental Mg^{2+} and Ca^{2+} are detected by the periplasmic domain of PhoQ via direct binding [91]. Véscovi *et al.* [92] demonstrated in *S. enterica* serovar *Typhimurium* that an amino acid substitution (T48I) in the periplasmic domain of the PhoQ reduced its affinity for millimolar concentrations of Ca^{2+} and attenuated virulence. PhoPQ is also activated by cationic antimicrobial peptides including polymyxins, indolicidin and LL-37 [93,94]. Exposure of *S. enterica* to subinhibitory concentrations of cationic antimicrobial peptides resulted in the activation of PhoPQ-regulated gene expression [95]. Low pH (pH 5.5) also promotes PhoPQ expression via the periplasmic domain of PhoQ [96]. It is of interest that both PhoPQ and PmrAB respond to acidic pH and affect the structure of the bacterial OM [76]. The expression of PhoPQ can also be promoted by macrophage phagolysosomes and other host tissues and cell vacuoles [90], indicating the complex interplay between host immunity and bacterial defense systems.

PhoP regulon-associated lipid A modifications

In *E. coli*, PhoPQ regulates the expression of hundreds of genes directly (e.g., *pagP* and *pagL*) and indirectly (e.g. *pmrAB*) [97]. PagP is an OM palmitoyltransferase that catalyzes palmitoylation at the hydroxyl group of the R-3-hydroxymyristate chain at position two of lipid A [98]. Mutants with *pagP* deletion display increased membrane permeability, which is directly activated by PhoPQ, and susceptibility to an antimicrobial peptide C18G [99]. The 3-*O*-deacylase PagL in the OM mediates deacylation at the C3 of lipid A in *Salmonella* and *Pseudomonas*, which increases the hydrophobicity of lipid A [100,101]. Han *et al.* [101] showed that even highly polymyxin-resistant *P. aeruginosa* (e.g., MIC = 16 mg/l) responded to polymyxin treatment by PagL-mediated lipid A deacylation. In this case, exposure to polymyxin B affected OM packing and hydrophobicity, decreasing polymyxin penetration.

Modulators & regulators of PmrAB & PhoPQ

Apart from external signals, the expression of PhoPQ or PmrAB can be influenced by some modulators (e.g., PmrD) or regulators (e.g., MgrB). PmrD is a small connector protein (85 aa in *S. enterica*) and modulates the interaction between PmrAB and PhoPQ (Figure 1) [102]. PhoP activates the expression of *pmrD*, while PmrD in turn alters the activity of PmrA-P by inhibiting the dephosphorylation of PmrA and prolonging its phosphorylation state [103]. PmrA-P also represses the transcription of *pmrD* by binding to the *pmrD* promoter [104]. When challenged by polymyxin B, a *pmrD*-inactivated mutant of *E. coli* W3110 had dramatically reduced survival compared with the wild-type strain [103]. This connector loop PmrPQ–PmrD–PmrAB has also been reported in *S. enterica* and *K. pneumoniae*, but not in *P. aeruginosa*, *A. baumannii* or *Y. pestis* [104,105].

MgrB is a small transmembrane repressor (47 aa in *K. pneumoniae*) of PhoPQ in *E. coli*, *S. enterica* and *K. pneumoniae* [16]. MgrB spans the inner membrane and represses the expression of PhoPQ by directly binding to the periplasmic domain of PhoQ [106]. Polymyxin resistance due to the inactivation of *mgrB* (via IS element insertion, indels and nonsynonymous mutations) has commonly been reported in clinical isolates of *K. pneumoniae* [51,107–110].

Other TCSs in Gram-negative bacteria

A number of other TCSs associated with polymyxin resistance in Gram-negative bacteria have been reported including ParRS [111], CprRS [112], ColRS [113], VprAB [114] and CrrAB [71]. Collectively, the large number of TCSs involved in polymyxin resistance highlights the complexity of the regulatory networks in Gram-negative bacteria involved in such resistance. ParRS, ColRS and CprRS have been reported to regulate polymyxin resistance in *P. aeruginosa* [111–113]. ParRS is a newly identified TCS and is required for the activation of the *arnBCADTEF* operon in *P. aeruginosa* [111]. Mutations in either *parR* or *parS* can reduce adaptive resistance to polymyxins, indicating that *parRS* are required for polymyxin resistance in *P. aeruginosa* [111]. *cprRS* and *colRS* mutations may also contribute

to high-level polymyxin resistance in the clinic via interactions with PhoPQ [112]. Deletion of the *cprRS* genes, individually or in tandem, abrogated polymyxin resistance of a *phoQ* deletion mutant, as did individual or tandem deletion of *colRS* [112]. Notably, in *P. aeruginosa* PA14 ColRS specifically induces *eptA* expression and lipid A modification with pEtN in the presence of extracellular zinc ions (2 mM $ZnSO_4$) [113]. VprAB in *Vibrio cholerae* has been shown to induce lipid A modification involved in polymyxin resistance by directly regulating the expression of the *alm* operon, the latter encoding proteins essential for glycine modification of lipid A [114]. Two recent studies revealed that CrrAB is associated with polymyxin resistance in *K. pneumoniae* [71,115]. It is hypothesized that CrrAB induces the expression of a glycosyltransferase-like protein that transfers a sugar to lipid A phosphate [71] and CrrB mutations activate PmrAB through CrrC, inducing elevated expression of *arnBCADTEF-ugd*, *eptA* and leading to polymyxin resistance [115].

Conclusion & future perspective

Over the past decade, use of the polymyxins (polymyxin B and colistin) for the treatment of otherwise untreatable infections caused by Gram-negative 'superbugs' has increased dramatically. At the same time, reports of resistance to polymyxins have also increased, threatening the clinical utility of this important class of antibiotics. The mechanisms underpinning polymyxin resistance are multifaceted and controlled by multiple TCSs. This review discussed the regulatory functions of two key TCSs, PmrAB and PhoPQ, that contribute to polymyxin resistance in Gram-negative bacteria. Of particular importance, polymyxin resistance due to nonsynonymous substitutions in PmrAB and PhoPQ was reviewed with several hotspots in different domains identified by MSA. The findings are of potential significance in the prediction of polymyxin resistance in Gram-negative pathogens. Further elucidation of the protein structures of these TCSs will assist with our understanding of their roles in LPS modification and bacterial pathogenesis. Mechanistic investigations on TCS-mediated polymyxin resistance are also warranted in order to optimize polymyxin use in the clinic and minimize the emergence of resistance.

Executive summary

- Polymyxins are last-line antibiotics against Gram-negative bacteria and resistance is increasingly reported worldwide.
- Polymyxin resistance is mediated by multifaceted mechanisms including lipid A modifications.
- Lipid A modifications are regulated by two-component systems such as PmrAB and PhoPQ, anonymous mutations that confer resistance to polymyxins.
- Seventy nonsynonymous substitutions in PmrB reported to date are related to polymyxin resistance, and 50 of them are in the HAMP (present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatase) linker and DHp (dimerization and histidine phosphotransfer) domains.
- PmrB senses external high concentrations of Fe^{3+} and Al^{3+}, and low pH; while PmrA regulates the expression of *arnBCADTEF-ugd*, *eptA*, *eptC* and *naxD*.
- PhoQ senses external divalent cations (e.g., Mg^{2+} and Ca^{2+}), cationic antimicrobial peptides and low pH, and PhoP regulates the expression of *pagP* and *pagL*.
- The activity of PhoPQ or PmrAB can be influenced by some modulators (e.g., PmrD) or regulators (e.g., MgrB).
- Several other two-component systems ParRS, CprRS, ColRS, VprAB and CrrAB are associated with polymyxin resistance.

Author contributions

J Huang, Y Zhu and J Li conceived the study. J Huang and C Li collected the data. J Huang, C Li, J Song, T Velkov, L Wang and Y Zhu performed the analysis. J Huang, Y Zhu and J Li wrote the manuscript with extensive input from all authors.

Acknowledgments

The authors thank Dr Phillip Bergen for his critical reading of the manuscript.

Financial & competing interests disclosure

J Li and T Velkov are supported by research grants from the National Institute of Allergy and Infectious Diseases of the NIH (R01 AI132154 and AI132681). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. J Li is an Australian National Health and Medical Research Council (NHMRC) Principal Research Fellow and T Velkov is an Australian NHMRC Career Development

Research Fellow. C Li is supported by an NHMRC CJ Martin Early Career Fellowship (1143366). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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