Molecular mechanisms of polymyxin resistance and detection of *mcr* genes

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Antibiotic resistance is an ever-increasing global problem. Major commercial antibiotics often fail to fight common bacteria, and some pathogens have become multi-resistant. Polymyxins are potent bactericidal antibiotics against gram-negative bacteria. Known resistance to polymyxin includes intrinsic, mutational and adaptive mechanisms, with the recently described horizontally acquired resistance mechanisms. In this review, we present several strategies for bacteria to develop enhanced resistance to polymyxins, focusing on changes in the outer membrane, efflux and other resistance determinants. Better understanding of the genes involved in polymyxin resistance may pave the way for the development of new and effective antimicrobial agents. We also report novel in silico tested primers for PCR assay that may be able distinguish colistin-resistant isolates carrying the plasmid-encoded *mcr* genes and will assist in combating the spread of colistin resistance in bacteria.

Key words: polymyxin, colistin, resistance, LPS

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INTRODUCTION

Constantly increasing antibiotic resistance is a global health problem. In particular, serious infections caused by multi-resistant bacteria, especially carbapenem-resistant bacteria, as well as the lack of new antibiotics against gram-negative pathogens, have led to the revival of older antibiotics. In this context, the use of colistin has been reintroduced, especially in infections caused by multidrug-resistant gram-negative bacteria. Unfortunately, increased and disproportionate use of colistin has led to the emergence of colistin-resistant bacteria worldwide. However, the presence of resistant bacteria to colistin may also occur without any prior exposure to colistin[1]. Polymyxins represent a family of antimicrobial cyclic oligopeptides produced by the gram-positive organism *Bacillus polymyxa*. However, only polymyxin B and polymyxin E (colistin) are suitable for clinical use. They act both on the outer and on the cytoplasmic membrane, resulting in loss of integrity in the membrane. Recently, alternative and less characterized mechanisms of action of polymyxins, as well as some bacterial resistance to these antibiotics have been described[2]. The reviewed literature shows a number of published studies on polymyxin resistance. In this review, we cover current knowledge on polymyxin resistance mechanisms in bacteria with regard to changes in the outer membrane and efflux. However, we also focus on other polymyxin resistance determinants with unclear and unknown function which may prove to be important components of resistance. Further, we tried to design primers for the detection of all previously described colistin-resistance genes (*mcr*-1 to -7) and their variants.

Overview of polymyxin resistance

Known resistance mechanisms include intrinsic, mutational and adaptive, but recently horizontally acquired resistance has also been described[3-5]. The major polymyxin resistance mechanisms include (i) alteration of the lipopolysaccharide (LPS) moiety, resulting in a reduction of the net LPS negative charge; (ii) mutations in genes; (iii) increased drug efflux; (iv) reduced porin pathway, (v) formation of capsules and (vi) enzymatic inactivation of antibiotic (colistin). However, other mechanisms of polymyxin resistance have also been described (e.g. antioxidative defense mechanisms, hyper-vesiculation). In the following section we will try to summarize the current state of knowledge concerning polymyxin resistance.

Electrostatic repulsion of polymyxins by modification of cell surface

The most common polymyxin resistance mechanisms in different pathogenic bacteria are associated with modification the phosphate groups of lipid A with amine substituents, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtn), which are regulated by the two-component systems (TCSs) PhoP/PhoQ and PmrA/PmrB regulatory system. The addition of L-Ara4N and PEtn reduces the net negative charge of bacterial surface and limits its interaction with polymyxins, which ultimately results in increased resistance to polymyxins[6,7]. It has been reported that cross-talk between the PhoPQ and PmrAB systems exists in *Salmonella enterica* and is mediated by the protein PmrD which is induced by phosphorylated PhoP (ref.[8]). Recently, it was observed that missense mutations in CrrB is linked to colistin resistance in *Klebsiella pneumoniae* isolates. In this context, CrrC was required to activate PmrAB and thereby connecting

Table 1. Sequences of primers used for PCR for detection of *mcr* genes and their variants.

Primer name	Sequence (5' to 3' direction)[a]	Target	Length (bases)	Amplicon size	Tm (°C)	Ref.
MCR-1F	GTCGATACCGCCAAATACC	MCR-1/-2/-6	19	559 bp	52	This study
MCR-1R	GTTCTTTTGGTGCAAAGGC		19			
MCR-2F	TGCATGTTYTCCAAYATGGGG	MCR-3/-7	21	416 bp	54	This study
MCR-2R	CGRATGGTRTTGTCATAGGTGTT		23			
MCR-3F	GATCCGAAGCTGTGTTCTG	MCR-4	19	380 bp	52	This study
MCR-3R	GGCGCACCATGTAAATACA		19			
MCR-4F	GCTGYAAAGGCGTCTGYGA	MCR-3/-5	19	382 bp	54	This study
MCR-4R	TGATCGGARACGTASAGCA		19			

[a]For degenerate primers: R = A or G; S = G or C; Y = C or T.

crrAB and *pmrAB* TCSs (ref.[9]). Further, other TCSs like ParRS, CprRS and ColRS are also implicated in the regulation of lipid A modification enzymes[4,10-12]. The spectrum of genes controlled by these TCSs are shown in Table 1 and Figure 1. Another mechanism for repulsion of cationic polymyxins is D-Ala modification of lipoteichoic acid. Briefly, GraXSR-promoted polymyxin B resistance in *Staphylococcus aureus* is related to the increased net positive surface charge due to the induction of genes *dltABCD* associated with addition of D-Ala to teichoic acids[13]. The same mechanism of resistance to polymyxin B involving surface modification through the incorporation of D-alanine into an outer membrane (OM) has also been described in *Bordetella pertussis*, *Streptococcus gordonii* and *Listeria monocytogenes* by regulating the *dra* and *dlt* operon[14-16]. Additionally, in the case of *S. gordonii* and *Streptococcus agalactiae*, involvement of two-component regulatory system response regulators LiaSR and CiaR in polymyxin B and to the cationic antimicrobial peptides (CAMP) resistance respectively have been found, and their effect on the expression of *dlt* has been demonstrated[16-18].

Other mechanisms have been described as associated with modification of the cell surface leading to electrostatic repulsion of antimicrobial peptides. This refers to (1) deacylation of lipid A, (2) phosphorylation and dephosphorylation of LPS, (3) glycylation of lipid A, (4) activation of LPS-modifying operon by mutations in two-component system, (5) repression of PhoPQ system, (6) L-Lys modification of cell membrane phoshpatidylglycerol, (7) addition of amide-linked acyl chains in the lipid A and (8) glucosamine modification. All these findings are carefully described in Trimble et al.[2]. It is also worth mentioning that a ferrous iron-binding protein, outer membrane protein (Omb) with unclear biological role, caused alterations in the bacterial surface charge that decreased affinity to antimicrobial peptides and increased resistance to polymyxin B in *S. enterica*[19].

Recently, plasmid-mediated resistance to polymyxin has been reported in a number of animal and human isolates of *E. coli* and *K. pneumoniae*, and the gene responsible for this phenotype was identified as *mcr-1* (ref.[5]). For more detail, please see reference[2]. Up to now, at least seven different *mcr* genes and their variants have been identified. In response to the growing number of *mcr* genes and their variants, we have tried to design the primers used for the detection of all previously described types of the respective gene. The nucleotide sequences of *mcr* genes were downloaded from the GenBank database (accession numbers CP010516, KP347127, KU886144, KY807920, KY807921, KY924928, LT598652, MF176240, MG267386, MF176239, MF176240, MF495680, MF543359, MF598076, MF598077, MF598078, MF598079, MF598080, MF598564, MG459156, MG459157, MG581979, MG822663, MG822664, MG822665, NQCO01000074) and were aligned (data not shown) using Geneious Pro 8 (Biomatters) to identify highly homologous regions suitable for designing primers. The proposed but untested primers may be used for detection of *mcr-1* to *-7* genes and their variants. The primer sequences and calculated lengths of the corresponding amplicons are listed in Table 1.

Membrane fluidity/permeability changes

Other mechanisms related to surface structural changes, acylation and deacylation of lipid A, influences resistance to different types of antimicrobial peptides. These changes have been shown to alter the properties of the outer-membrane permeability barrier. It was shown that activation of PhoPQ system in *K. pneumoniae* with polymyxin B stimulates PagP (*crcA* in *E. coli*) involved in acylation of lipid A (ref.[20]). In addition, PagP-like gene, *rcp* in *Legionella pneumophila*, also conferred resistance to polymyxin B (ref.[21]). In *Vibrio cholerae*, *K. pneumoniae*, *Escherichia coli* and *Salmonella* Typhimurium, the acylation of lipid A has been shown to be regulated by *lpxM* (formally *msbB* or *waaN*). Inactivation of these genes resulted in a lack of L-Ara4N modification and in a significant decrease in polymyxin resistance[22-25]. Furthermore, other genes such as Bmul_2133 and Bmul_2134 have been shown to contribute to polymyxin B resistance in *Burkholderia multivorans* through alterations in the OM permeability[26]. It has been proposed that Bmul proteins appear to confer polymyxin B resistance by the mechanism of hopanoid (analogues of eukaryotic sterols) biosynthesis involved in maintaining membrane fluidity and permeability[27]. Moreover, it was observed that genes involved in staphyloxanthin (virulence factor) biosynthesis confer resistance to polymyxin B in *S. aureus*. The resistance to polymyxin B in this case is derived from its sta-

Table 2. Strategies used by bacteria to achieve resistance to polymyxins.

Resistance mechanisms		Genes/determinants involved	Bacteria	Ref.
Electrostatic repulsion	L-Ara4N and PEtn modification of lipid A	*pmrAB*, *pmrD*, *phoPQ*, *parRS, mcr*	*Escherichia coli, Salmonella enterica, Pseudomonas aeruginosa*	4-8, 10-12
	Activation of LPS-modifying operon by mutations in two-component system	*crrB* (*crrC*)	*Klebsiella pneumoniae*	9
	D-Ala modification of lipoteichoic acid	*graXSR*, *dra*|*dlt* operon, *liaSR*, *ciaR*	*Staphylococcus aureus, Bordetella pertussis, Streptococcus gordonii, Listeria monocytogenes, Group B Streptococcus*	13-18
	Unclear	outer membrane protein (Omb)	*Salmonella enterica*	19
Membrane fluidity/ permeability	Acylation of lipid A	*pagP*, *lpxM*, *rcp*	*Vibrio cholerae, Klebsiella pneumoniae, Escherichia coli, Salmonella enterica, Legionella pneumophila*	20-25
	putative hopanoid and staphyloxanthin biosynthesis	Bmul_2133/Bmul_2134, genes involved in staphyloxanthin biosynthesis	*Pseudomonas aeruginosa, Burkholderia multivorans, Staphylococcus aureus*	26, 28
Surface and membrane remodelling	Capsule production	*siaD*, *cps* operon, *ompA*, *kpnEF, phoPQ, rcs*	*Neisseria meningitidis, Klebsiella pneumoniae, Salmonella enterica*	20, 29-32, 34
	Alterations in membrane composition	*virB*, *suhB*$_{Bc}$, *bvrRS*, *epsC-N*, *cgh*, *vacJ*, *waaL, rfbA*, *ompW*, *micF*, *pilMNOPQ* operon, *parRS*, *rsmA*, *bveA*, *ydeI* (*omdA*), *ompD (nmpC)*, *ygiW* (*visP*), *ompF*, *rcs*	*Brucella ovis, Salmonella enterica, Brucella melitensis, Burkholderia cenocepacia, Vibrio cholerae, Brucella abortus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Neisseria meningitidis, Brucella melitensis*	10, 12, 33, 34, 37-47, 49, 50, 52-54
	Alterations of membrane integrity	*cas9*, *tracrRNA*, *scaRNA*, Lol, TolQRA	*Francisella novicida, Acinetobacter baumannii*	55, 56
	LOS and LPS modifications	*spgM*, *pgm*, *hldA*, *hldD*, *oprH*, *cj1136*, *waaF*, *lgtF*, *galT*, *cstII*, *galU*, *lic1*, *lic2A*, *lpsA*, *lgtF*, *opsX*, *firA*, *lpxO*	*Stenotrophomonas maltophilia, Vibrio fischeri, Burkholderia cenocepacia, Eschericha coli, Pseudomonas aeruginosa, Salmonella Typhimurium, Campylobacter jejuni, Haemophilus influenzae*	57-65, 68, 69, 71
	Loss of LPS	*lpxACD, lptD*	*Acinetobacter baumannii*	72, 73
Efflux and transport	Efflux	Mex pumps, AdeABC, HlyD	*Pseudomonas aeruginosa, Acinetobacter baumannii*	74, 55
	Transport	*trkA*	*Vibrio vulnificus*	75
Other polymyxin resistance determinants with known, unclear and unknown function		*bacA, sip*, *pbs*, type IV pili, *vc genes*, *yieM*, *sodC*, *sodB*, *katA, pilB, yjdB*|*pmrC*|*pagB*|*pmrF*, PA1199, PA2583, PA5548, PA2928*, eraR*, *wbpZ*, PA4541, PA1938, *pgm*, *surA*, *tolB*, *gnd*, PA0401, *pyrB*, *pdxB*, *sucC*, *tpiA*, *aroB*, *pyrD*, *mpl*, hypothetical protein (*rmlD* homolog), *ampR*, *lptC*, *amgS*, *galU*, *lptC*, *wapR, ssg, cgt*, *kdnA*|*kdnB*, *fopC, pstA/C/S, ebsA, topA, ftsH, gdpP, fabT, yfmH, agaS, manL/N, clpX, deoB, hpt, tilS, gmk, nanH, guaA, nupP, ptsI, luxR, fba, ugtL, virK, mig-14, pgtE, asmA, pbgP, mglB, glpQ-like, feoC, hflc, pitA, rpoE, BCAL2831, mucD, lytB, hpnJ,* genes encoding putrescine*, yceI, yejABEF operon*, *spy*, *zraP*, *cpxP*, *micA*, *rybB*, *pgmA*, *grxD*, low pH, extracellular DNA, lack of magnesium/ phosphate/iron ions, JSG945, JSG946, JSG948, YPTB0331–0332–0333 and other genes	*Brucella ovis, Thermus thermophilus, Acinetobacter baumannii, Klebsiella pneumoniae, Saccharomyces cerevisiae, Pseudomonas aeruginosa, Vibrio cholerae, Escherichia coli, Staphylococcus aureus, Burkholderia cenocepacia, Salmonella enterica*, Group B *Streptococcus, Brucella suis, Helicobacter pylori, Shewanella oneidensis, Francisella novicida, Streptococcus pyogenes, Yersinia pestis, Proteus mirabilis, Yersinia pseudotuberculosis, Yersinia enterocolitica*	3, 26, 28, 38, 43, 49, 61, 77-87, 89, 90, 92, 93, 95, 96, 98-104, 106-111, 113-117

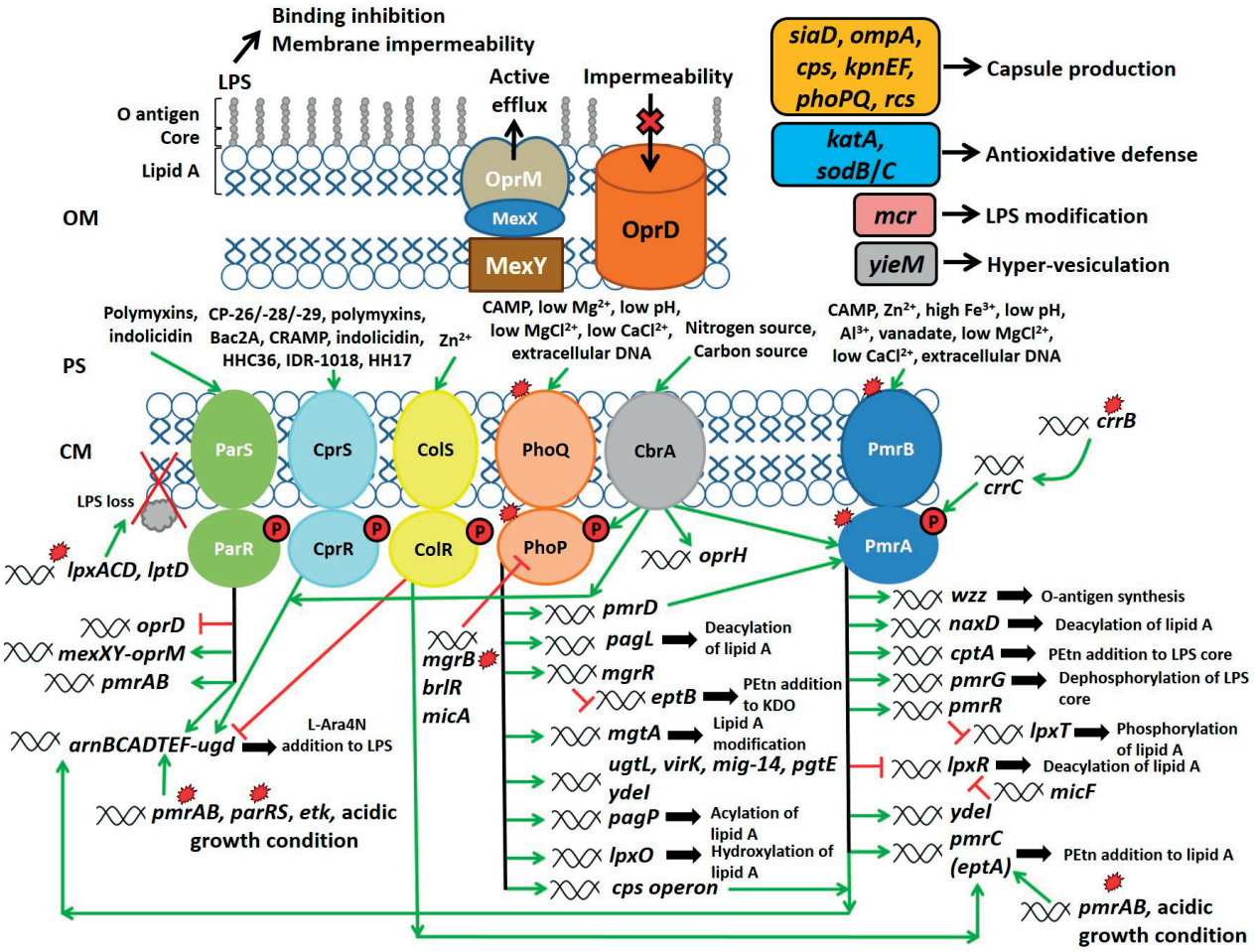

Fig. 1. Proposed polymyxin resistance mechanisms in bacteria. PhoP-PhoQ, PmrA-PmrB, ColR-ColS, CprS-CprR, ParS-ParR and CbrA-CbrB two-component systems confer bacterial resistance to polymyxin. Diagram of gram-negative cellular envelope, which shows typical inner and outer double layer separated by a periplasm (OM, outer membrane; PS, periplasmic space; CM, cytoplasmic membrane). The outer layer of the outer membrane contains a lipopolysaccharide (LPS), which is anchored to the membrane by the LPS lipid A. The inner layer of the outer membrane and also the entire inner membrane are composed only of phospholipids and the two bilayers can contain a variety of different types of membrane proteins. The figure shows different stimuli that induce various membrane and cytoplasmic proteins which then positively or negatively regulate other proteins. PhoPQ two-component system is activated (green arrow) by cationic antimicrobial peptides (CAMP), low Mg^{2+}, low pH, low $MgCl^{2+}$, low $CaCl^{2+}$, extracellular DNA and mutations (denoted by red-colored star symbols) in *phoP*/*phoQ*. PhoPQ controls many genes required for LPS modification and alteration of the cell surface. These include the *pmrD*, *pagL*, *mgrR*, *mgtA, ugtL, virK, mig-14, pgtE, ydeI, pagP, lpxO* and *cps* operon. The activation of this two-component system mediates acylation, deacylation and hydroxylation of lipid A regulated by genes encoding the enzymes Pag, PagL and LpxO, respectively. The expression of EptB (phosphoethanolamine transferase) is suppressed (red line) with MgrR, while EptB is associated with the phosphoethanolamine addition to 3-deoxy-D-manno-oct-2-ulosonic acid (KDO). The lipid A is further modified by *mgtA*. Transcription of the genes *ugtL*, *virK*, *mig-14* and *pgtE* participate in the mediation of polymyxin resistance and have functions associated with the alteration of the bacterial envelope. PhoPQ, as well as PmrAB, are required for the induction of *ydeI* which contributes to polymyxin resistance through its interaction with an outer membrane porin OmpD/NmpC. Additional *cps* genes are required for synthesis of polysaccharide capsule and be involved in the phosphorylation of Ugd. By contrast, MgrB, BrlR and MicA apply negative feedback to the PhoPQ regulatory system. With the help of PmrD, CbrA and in the presence of CAMP, Zn^{2+}, high Fe^{3+}, low pH, Al^{3+}, vanadate, low $MgCl^{2+}$, low $CaCl^{2+}$ and extracellular DNA, transcription of PmrA-activated gene is induced by PmrAB system. PmrA-P activates transcription of LPS modification loci (i.e., *wzz*, *naxD*, *cptA*, *pmrG*, *pmrR*, *ydeI*, *pmrC*, and *arnBCADTEF-ugd*), except for *lpxR*, which is downregulated. Synthesis of the O-antigen is controlled by the *wzz* gene products. The NaxD and LpxR proteins are responsible for the deacylation of lipid A. However, MicF causes downregulation of LpxR synthesis, which is associated with deacylation of lipid A. The initiation of transcription of *lpxR* may play a role in resistance to polymyxin, but its effect on the resistance has not yet been demonstrated. Further, the CptA protein and PmrG regulate the phosphorylation modification of heptose-I and heptose-II residues on the LPS core, respectively. Likewise, PmrR inhibits the activity of *lpxT*, which phosphorylates lipid A. Additionally, phosphorylated PmrA activates the *arnBCADTEF-ugd* and *pmrC* genes, which modify LPS with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and lipid A with PEtn, respectively. Moreover, also mutations in *pmrA*, *pmrB* and *crrB* genes through CrrC result in the activation of PmrA, which subsequently upregulates *pmrC* and *arnBCADTEF-ugd*. Alternatively, *pmrC* (*eptA*) can be activated by the ColRS two-component system, mutations in pmrAB or acidic growth conditions. ParR can be phosphorylated and

controls its regulatory network in response to polymyxins and indolicidin. The ParRS system controls the expression of the porin (*oprD*), efflux (*mexXY-oprM*) and LPS modifying (*arnBCADTEF-ugd*, *pmrAB* operon). In addition, the *arnBCADTEF-ugd* can be activated via mutations in the *pmrAB and parRS*, *etk* (required for phosphorylation of Ugd), acidic growth conditions and other two-component systems, such as CprRS and CbrAB. However, expression of *arnBCADTEF-ugd* is negatively regulated by ColRS. CprRS is able to sense CP-26/-28/-29, polymyxins, Bac2A, CRAMP, indolicidin, HHC36, IDR-1018 and HH17. Further, Zn^{2+} and nitrogen/carbon sources trigger activation of ColRS and CbrAB, respectively, whereas the CbrA protein is also able to induce the *oprH-phoPQ* operon. Mutations within genes *lpxACD* and *lptD* involved in lipid A biosynthesis and assembly of LPS in the outer leaflet of the outer membrane are associated with LPS loss, rescpectively. These results show that polycationic antibiotics (colistin and polymyxin B) and other factors are capable of promoting the expression of different two-component system and the *mexXY* operon and coordinated downregulation of the *oprD* gene by activation of ParRS, which ultimately leads to multiple resistance. In our model LPS represents the major barrier to binding of polymyxins. Further, *siaD, ompA*, *cps*, *kpnEF*, *phoPQ* and *rcs*, participate in capsule production leading to an increase in polymyxin resistance. Catalase, KatA, and other detoxifying enzymes, SodB and SodC, are also associated with resistance to polymyxin through their antioxidant defense mechanisms, whereas the *yieM* gene can mediate resistance by inducing outer membrane vesiculation. Recently, a mobile phosphoethanolamine transferase gene, *mcr*, has been associated with colistin resistance. It indicates resistance due to modifications of the phosphate groups of lipid A in LPS.

bilization of membrane structure (reduction of membane fluidity) (ref.[28]).

Surface and membrane remodelling

Further cell wall alterations, particularly capsule production, changes in membrane composition, and LOS and LPS modifications, are related with the development of polymyxin resistance. It has been found that the production of capsular polysaccharide or capsule is responsible for resistance to polymyxin B in *Neisseria meningitidis* and *K. pneumoniae*. However, synthesis of polysaccharide capsule was regulated by *siaD*, OmpA and *cps* operon (*wca*) (ref.[29-31]). In this context, further study showed that PhoPQ is necessary for polymyxin B-triggered induction of *cps* operon in *K. pneumoniae*[20]. Interestingly, a multidrug efflux pump *kpnEF* mutant showed a defect in capsular synthesis, indicating the direct involvement of KpnEF in capsule synthesis[32]. In addition, Rcs system (regulator of capsule synthesis) has been described to contribute to polymyxin B resistance in *S. enterica* for its role in regulating the expression of gene *ydeI* (ref.[33,34]). Furthermore, it has been found that the expression of *cps* operon and *ugd* is regulated by the Rcs system[34], whereas the strain with expressed RcsA transcriptional regulator is able to synthesize colanic acid[35]. The effect of the capsules in this case lies in increasing electrostatic interaction between capsule polysaccharides and polymyxins, the binding of the cationic polymyxins to the anionic polysaccharides of the capsule, thereby reducing the amount of peptides reaching the bacterial surface and reducing its bactericidal activity[36].

With respect to the changes in membrane composition, *virB* has been implicated in cationic peptide polymyxin B resistance of *Brucella ovis* and *Brucella melitensis* through a mechanism that involves modification of cell surface which is achieved by the down-regulation of Omp25/Omp31 family and regulation of type IV secretion system[37-39]. Other genes such as $suhB_{Bc}$, *bvrR/S* two-component regulatory system, *epsC-N*, *cgh* (choloylglycine hydrolase), *waaL*, *rfbA*, *vacJ* and *ompW*, have been shown to contribute to polymyxin B or colistin resistance in many pathogens through alterations in the OM composition[37,40-47]. It has been found that various environmental signals such as high temperature, oxidative stress, or salicylate have an effect on the expression of porins through *micF* regulation[48]. MicF expression has been connected with downregulation of OM porin OmpF mRNA, which contributed to polymyxin B resistance in *S. enterica*[49]. In *P. aeruginosa*, it was found that the ParRS system affects polymyxin B and colistin resistance through down-regulation of the porin (OprD) (ref.[10,12]). In this context, it has been demonstrated that oligosaccharide/oligonucleotide binding-fold (OB-fold) proteins YdeI (OmdA) and YgiW (VisP), and porins [OmpD (NmpC in *E. coli*) and OmpF] contribute to polymyxin B resistance in *S. enterica* by cell wall remodelling (interaction between general porins and OB-fold proteins blocks antibiotic entry) or it is also likely that increase antibiotic export[33,50]. However, the main feature of general porins, for example OmpF in *E. coli*, is to create a size-selective defined channel for the diffusion of hydrophilic molecules with a certain priority of molecules with charges which are the opposite of the amino acids that line the channels[51]. It has been also demonstrated that the *pilMNOPQ* operon encoding components of the type IV pilin secretion system in *N. meningitidis* contributes to polymyxin B resistance[52]. In addition, in *P. aeruginosa*, the small RNA-binding protein RsmA is associated with polymyxin B and colistin resistance through its involvement in the type three secretion system (TTSS) (ref.[53]). It is believed that pilin secretion apparatus may be the entry gate for several structurally different antimicrobial agents. Moreover, the type III and IV pilin secretion system is involved in the regulation of the delivery of proteins or DNA through the bacterial cell envelope. Further, a mechanism has been described by which *B. melitensis* maintains a low level of phosphatidylethanolamine in the cell wall by expression of the BveA phospholipase A1 enzyme[54]. This property of the cell envelope contributes to polymyxin resistance as well as to persistence in the infected host. It has been suggested that BveA is important because it is capable of preventing the formation of pore-like structure and the permeabilization of the cytoplasmic membrane by polymyxin.

Recently, Cheah et al. have described perturbation of the membrane in polymyxin-treated *A. baumannii* through over-expression of protein complexes involved in mem-

brane homeostasis, namely Lol lipoprotein transport complex and the TolQRA transmembrane complex[55]. This supports findings related to the reduced integrity and barrier function of the remodelled OM in *A. baumannii* treated with polymyxin. Further, the genes *cas9*, *tracrRNA* and *scaRNA* in *Francisella novicida* promote enhanced envelope integrity through the regulation of bacterial lipoproteins and were necessary for polymyxin B resistance[56].

In view of LPS and LOS changes, inactivation of the genes (*spgM*, *pgm*, *hldA* and *hldD*) showed changes in LPS and this correlated with increased susceptibility to polymyxin B in *Stenotrophomonas maltophilia*, *Vibrio fischeri*, *Burkholderia cenocepacia*, *E. coli* and *Proteus mirabilis*, respectively[38,57-60]. HldA and HldD gene products have been shown to play a role in the modification of heptose sugars. However, *spgM* and *pgm* (phosphoglucomutase) genes have been demonstrated to play a role in catabolism of galactose and in the promotion of UDP-glucose production in *E. coli*, and LPS and alginate biosynthesis as a homologue of the *algC* gene in *P. aeruginosa*, respectively. Moreover, OM protein OprH has been reported to affect resistance to antimicrobial peptide polymyxin B in *P. aeruginosa*[61,62]. Polymyxin B resistance resulted from LPS alteration (interaction of OprH with divalent cation-binding sites of LPSs). Further, expression of the *cj1136* gene (putative galactosyltransferase), involved in LOS biosynthesis, is associated with in *Campylobacter jejuni* polymyxin B resistance[63]. In this context, insertional inactivation of genes involved in synthesis and extension of LOS, namely *waaF*, *lgtF*, *galT*, c*stII* and *galU* in *C. jejuni*, resulted in decreased resistance to polymyxin B (>15-fold reduction in MIC) (ref.[64,65]). Additionally, it has been described elsewhere that *galU* (involved in L-Ara4N biosynthesis) also in other species such as *P. mirabilis*[66], and *Yersinia pestis*[67] contributes to resistance to polymyxin B. It was further reported that mutations in the genes of *Haemophilus influenzae* (*lic1*, *lic2A*, *lpsA*, *lgtF*, *opsX*) also involved in LOS biosynthesis lead to increased susceptibility to polymyxin B (ref.[68]). FirA in *E. coli* and *S.* Typhimurium has also been shown to be important for lipid A biosynthesis and resistance to polymyxin B (ref.[69]). Interestingly, UDP-3-O-3-hydroxymyristoyl glucosamine N-acyltransferase (LpxD) in higher copy number, a FirA homolog, was found in *Pseudomonas putida* strain HB3267 and may be responsible for higher resistance to polymyxin B than other strains[70]. Furthermore, in one study, it has been reported that other genes regulating, for example, biofilm formation or LPS and LOS modification (ie, *lpxO*) (ref.[71]) correlate with increased resistance towards polymyxins, which are described in more detail elsewhere[2].

It was also found that complete loss of LPS production by mutations in *lpxACD* which are involved in lipid A biosynthesis, exhibited a colistin-resistant phenotype in *Acinetobacter baumannii*[72]. In addition, a mutation in the OM protein, LptD, which allows the final transfer of the newly synthesized LPS, resulted in a complete loss of LPS and decreased susceptibility to polymyxin in *A. baumannii*[73].

Efflux and transport

Several different types of multidrug efflux pumps in different pathogens have been shown to confer tolerance towards polymyxin B. For more informations see[2]. Recently, implication of efflux transporter proteins (AdeABC and HlyD family) in polymyxin resistance in *A. baumannii* has been reported[55]. The AdeABC is homologous to the AcrABC and MexAB-OprM pumps. Further, relationship between TTSS via the RsmA protein and the expression of multidrug efflux (Mex) pumps has also been described in *P. aeruginosa*, whereas increased expression of MexCD-OprJ or MexEF-OprN was associated with decreased expression of the TTSS regulon[74]. Furthermore, it has been reported, that the potassium uptake protein in *Vibrio vulnificus*, TrkA, was responsible for resistance to polymyxin B (ref.[75]). Besides, it has been shown that the reaccumulation of K^+ by protamine-treated cells results in protease expression of PgtE, which in turn degrades protamine, thus preventing the death of bacteria[76].

Other polymyxin resistance determinants with known, unclear and unknown function

In *A. baumannii*, some 35 genes have shown to influence colistin resistance. Identified genes have been shown to play roles in the regulation of OM proteins, chaperones, protein biosynthesis factors, and metabolic enzymes (putative role in loss of biological fitness) (ref.[77]). Furthemore, it was found that additional 30 genes in *A. baumannii* were involved in resistance to colistin. These were identified to be involved in amino acid transport, lipid and phosphate metabolism (pathways and systems associated with osmotolerance), protein folding, and cell envelope biosynthesis[3]. In *K. pneumonia*e, in addition to the new two-component system CrrAB characterized by the regulation of colistin resistance through the activation of PmrAB, other genes were also transcriptionally upregulated [genes of cation transport/membrane integrity/efflux transporters (*macAB*)] and have been linked with LPS modification, cation transport, maintenance of membrane integrity and unknown functions[78]. In *Saccharomyces cerevisiae*, *pbs2* gene was involved in resistance to polymyxin B when overexpressed. However, Pbs2 was found to encode a predicted protein kinase that plays a role in osmoregulation and thus affects the plasma membrane[79]. In addition, it has been found that several genes in *V. cholearae*, including *vc2728* (*gspI*), *vc2731* (*gspF*), *vc2732* (*gspE*), *vc0212* (*lpxN*), *vc0224*, *vc0239*, *vc1981*, associated with type II secretion system, LPS biosynthesis and modification, and unknown functions, were involved in resistance to polymyxin[80]. Also, it has been previously shown that Sip (silica-induced protein) involved in the increase of robustness of the cell surface of *Thermus thermophilus* helps to protect against peptide antibiotics like polymyxin B (ref.[81]). Interestingly, Manning and Kuehn revealed that hyper-vesiculating *yieM* mutant was able to confer polymyxin B and colistin resistance to *E. coli* by induction of OM vesiculation[82]. It is also worth mentioning that pilus structural subunit PilB of *S. agalactiae* has been demonstrated to confer polymyxin B resistance and PilB

contributed to binding of polymyxin B, thereby preventing its interaction with the cell membrane[83]. In this context, type IV pili of *P. aeruginosa* have been shown to be important for resistance to colistin with regulation of motility and development of mushroom caps[84]. Additionally, some PhoP/PhoQ-regulated genes, namely *ugtL*, *virK*, *mig-14* and *pgtE*, have been shown to contribute polymyxin B and/or CAMP resistance in *S. enterica*, based on potential inhibition of polymyxin binding[85-87]. However, UgtL has been also described to be involved in dephosphorylation of lipid A (ref.[88]). So far, it is still unknown whether this protein functions as an enzyme or as a regulator of the reaction. In addition, genes for the synthesis of putrescine and YceI, found to act as infochemicals, have been recently discovered to mediate polymyxin B resistance in *B. cenocepacia*, most likely by sequestering the antibiotic[89]. Another study has reported that mutants *of the yejABEF* operon, genes encoding putative ATP-binding cassette (ABC) transporter, were found to be susceptible to polymyxin B (ref.[90,91]). It has been suggested that the transporter system encoded by the *yej* operon may be involved in virulence regulation in *Brucella* and may also be involved in antimicrobial peptides neutralization, similar to the transporter system encoded by the *Salmonella yej* operon. Furthermore, it was reported that acidic growth conditions were associated with polymyxin resistance and was mediated by transcriptional activity of genes (*yjdB*/*pmrC*/*pagB* and *pmrF*) (ref.[92,93]). In another study, it has been reported that the transfer of bacteria to a mildly acidic environment (pH 5.8) resulted in the decrease of LpxT activity and strong induction of the addition of L-Ara4N and PEtn (ref.[94]). It is also worth mentioning that lack of magnesium, phosphate and iron ions have all been reported to lead to resistance to antimicrobial peptide polymyxin B (ref.[61,95,96]). In this context, it has previously been shown that expression of the *etk* (required for phosphorylation of Ugd) during cultivation of *E. coli* was stimulated with low pH, low concentrations of magnesium and iron ions[97]. In addition, several genes have been implicated in the resistance towards polymyxin B in *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains, namely, *YPTB0331–0332–0333*, likely through the ferric uptake regulation[95]. Interestingly, the presence of extracellular DNA also has been shown to contribute to colistin and polymyxin B resistance in *S. Typhimurium* and *P. aeruginosa* by activation of PhoPQ and PmrAB systems[98,99]. Last but not least, additional determinants associated with polymyxin resistance are described in (ref.[2]).

Regarding genes with unclear function, there are some genes that have been differently regulated in colistin-resistant strains of *P. aeruginosa* including PA1199, PA2583, PA5548, PA2928 probably participating in LPS biosynthesis as well as non-LPS-mediated genes including PA1980 (*eraR*), PA5447 (*wbpZ*), PA4541 and PA1938 (ref.[100]). Further, it has been previously reported that *P. aeruginosa* genes [PA0401, *pyrB*, *pdxB*, *sucC*, *tpiA*, *aroB*, *pyrD*, *mpl*, hypothetical protein (*rmlD* homolog), *ampR*, *lptC*, *amgS*, *galU*, *lptC*, *wapR*, and *ssg*] play a role in polymyxin B resistance given their potential regulatory function, role in metabolic pathways, LPS biosynthesis and unknown functions[101]. Moreover, gene *cgt* has been reported to be associated with colistin resistance in *Helicobacter pylori*, suggesting its potential involvement in lipid A modification[102]. Moreover, DNA sequence analysis of the mutagenized loci, JSG945 (putative O-acetyltransferase, which is essential for the addition of aminoarabinose to lipid A), JSG946, JSG947 (putative ATP synthase), and JSG948 (putative *sap* loci), revealed the role of these genes in conferring resistance to polymyxin B in *P. mirabilis*[103]. It has also been demonstrated in *B. ovis* that resistance to the polymyxin B affects the *bacA* gene, probably by altering the structure of the bacterial envelope[38]. Furthermore, it was found that a putative *pgm* gene (BRA 0348) in *Brucella suis* and *surA*, *tolB*, and *gnd* genes in *S. enterica* were shown to be necessary for polymyxin resistance, probably due to its putative OM modification (LPS structure or from destabilization of the membrane) (ref.[43,104]). In this context, it has been found that periplasmic chaperone SurA in *E. coli* plays an important role in transporting the LptD to the OM assembly site[105]. Gattis et al. have been shown that three genes *kdnA/kdnB* and *fopC* contribute to polymyxin resistance in many pathogens because of its potential role in OM integrity[106,107]. From *E. coli* and *Streptococcus pyogenes* mutant screening, a number of other genes (PstA/C/S, EbsA, TopA, FtsH, GdpP, FabT, YfmH, AgaS, ManL/N, ClpX, DeoB, Hpt, TilS, Gmk, NanH, GuaA, NupP, PtsI, LuxR, Fba) have been identified to be required for resistance to polymyxin. These genes encode proteins with potential role in OM integrity and disruption of ExPortal integrity and thereby contribute to polymyxin resistance[108,109]. In a previous study, it was shown that *asmA*, *pbgP*, *mglB*, *glpQ*-like, *feoC*, *hflc*, *pitA* genes have been implicated in resistance to polymyxin B in *Y. pestis*. This resistance was linked with putative modification of molecules or molecular composition of the bacterial OM (ref.[110]). Further, periplasmic chaperone proteins, Spy, ZraP, CpxP, have been reported to play a role in resistance against polymyxin B in *S. enterica* which could be affected by the envelope stress response regulation, while ZraP has been proposed to act to repress the expression of two-component system ZraSR (ref.[111]). Interestingly, σ^E-controlled small non-coding RNAs, MicA and RybB, were activated in *S. enterica* treated cells with polymyxin B and are likely to facilitate the remodelling of the OM by reduction of the expression OM proteins (MicA represses OmpA synthesis, while RybB inhibits both OmpC and OmpW expression) (ref.[49]). Moreover, MicA has been shown to be a feedback inhibitor of the *phoPQ* system of *E. coli*[112]. Further, the involvement of *sodB* (A1S_2343) and *sodC* genes in colistin resistance have been observed in *A. baumannii*, probably by detoxifying reactive oxygen species[113,114]. In this context, *katA* (catalase) was found to confer resistance to polymyxin B in *S. aureus*. The resistance to polymyxin B was derived from its antioxidative defense mechanisms[28].

Furthermore, other genes, such as *rpoE* (σ^E), *BCAL2831*, *mucD*, $ispH_{BCAL2710}$ (*lytB*), *hpnJ*, have been found to be related to polymyxin B resistance in *B. cenocepacia* through unknown mechanisms, with the exception of mechanisms that do not contribute to weak binding of

polymyxin to *B. cenocepcia* cells or to poor permeabilization of the outer membrane. However, the gene *ispH* (isoprenoid synthesis) increased OM stability and reduced molecular permeability[26,115]. Furthermore, *Y. pestis* resistance to cationic peptide polymyxin B is due to changes in unidentified surface structures mediated by regulation of *pgmA* activity[116]. In addition, *grxD* mutant, a gene with unknown function, showed increased susceptibility to polymyxin B in *P. aeruginosa*[117].

CONCLUSION

Polymyxins are powerful bactericidal antibiotics that are effective against gram-negative bacteria. Despite their possible negative effect on the nerves and the kidneys in humans, they represent the last line of defense against persistent multidrug-resistant infections. Research into polymyxin resistance has led to the elucidation of many mechanisms and pathways that in some way affect resistance itself. In this report, we have tried to approach all the mechanisms of resistance described so far, although there are still many unknown and unresolved mechanisms of resistance. Still, we hope that a deeper understanding of the resistance mechanism will improve the ability to design and develop more efficient and toxic derivatives of polymyxins. It is also worth mentioning the discovery of the plasmid-mediated colistin resistance genes, *mcr*, and therefore we can assume that other plasmid-mediated genes will be described in the near future. The primers described in our study may be used for detection of *mcr* genes and their variants, which could ultimately limit the dissemination of colistin-resistant bacteria.

ABBREVIATIONS

ABC, ATP-binding cassette; CAMP, cationic antimicrobial peptides; L-Ara4N, 4-amino-4-deoxy-L-arabinose; LPS, lipopolysaccharide; PEtn, phosphoethanolamine; OM, outer membrane; TCSs, two-component systems; TTSS, type three secretion system.

Search strategy and selection criteria

We searched Google Scholar for articles published in English between 1970 and December 2018 using the keywords “polymyxin resistance”, “polymyxin B”, “colistin”, “bacteria”, “genes”, “colistin-resistant”, “LPS” and “*mcr*”. Bibliographies of all appropriate studies have been reviewed to identify other eligible studies.

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