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Polymyxin: Alternative Mechanisms of Action and Resistance

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Antibiotic resistance among pathogenic bacteria is an ever-increasing issue worldwide. Unfortunately, very little has been achieved in the pharmaceutical industry to combat this problem. This has led researchers and the medical field to revisit past drugs that were deemed too toxic for clinical use. In particular, the cyclic cationic peptides polymyxin B and colistin, which are specific for Gram-negative bacteria, have been used as "last resort" antimicrobials. Before the 1980s, these drugs were known for their renal and neural toxicities; however, new clinical practices and possibly improved manufacturing have made them safer to use. Previously suggested to primarily attack the membranes of Gram-negative bacteria and to not easily select for resistant mutants, recent research exploring resistance and mechanisms of action has provided new perspectives. This review focuses primarily on the proposed alternative mechanisms of action, known resistance mechanisms, and how these support the alternative mechanisms of action.

Public news outlets, academic articles (primary literature and reviews), government documents, and countless other sources echo the alarm regarding multiple antibiotic resistance development in nosocomial pathogens (Fernández et al. 2011; CDC 2013; Taylor et al. 2014; Berendonk et al. 2015). Unfortunately, recent antibiotic research has encountered numerous obstacles, notably limited discovery of new antibiotic compounds. This relates to the poor economic incentives for pharmaceutical companies to invest in antibiotic development given pharmacoeconomic considerations (a combination of poor success rates, limited markets because of the large number of different antibiotics on the market, and the fact that antibiotics are generally used acutely and, thus, do not have "repeat customers") and limited funding for academic laboratories working in the field. Even though occasional breakthroughs are made, for example, the antibiotic teixobactin (Ling et al. 2015), it is noteworthy that while chemically novel, this compound addresses a known target and has yet to be examined clinically. The medical community has recognized the critical importance of combating bacterial resistance, and this has led to the revival of disfavored antibacterials such as the cyclic peptide

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polymyxin B and its relatives (Falagas and Kasiakou 2006; Landman et al. 2008; Falagas et al. 2010).

Polymyxin was first isolated in 1947 from the Gram-positive soil bacterium *Bacillus polymyxa*, which was reclassified as *Paenibacillus polymyxa* in 1993 (Storm et al. 1977; Ash et al. 1994). Fifteen different molecular variations are known and are produced by *P. polymyxa* subspecies, including polymyxin E (colistin) from ssp. *Colistinus* and polymyxin M (mattacin) from *Paenibacillus kobensis* (Storm et al. 1977; Martin et al. 2003; Choi et al. 2009b; Tambadou et al. 2015). The polymyxins are small lipopeptide molecules of ~1200 Da in mass and are characterized by a polycationic peptide ring with a short protruding peptide attached to a hydrophobic fatty acid tail (Fig. 1A) (Newton 1956; Evans 1999; Nation et al. 2014). The clinically used polymyxins, polymyxin B (a mixture of composed of polymyxins B3, B6, and minor components B1, B1-I, B2) and colistin (containing two major components colistin A and B and ~30 minor components) (Orwa et al. 2001), differ primarily by a single D-phenylalanine replaced by a D-leucine within the peptide ring (Landman et al. 2008; Yu et al. 2015). The cationic ring makes these drugs soluble in aqueous environments, whereas the hydrophobic acyl chain facilitates insertion into bacterial membranes (Evans 1999; Nation et al. 2014).

All polymyxins are nonribosomally produced (i.e., produced by large enzymes called nonribosomal peptide synthetases) (Finking and Marahiel 2004), and although their structures have been known for years, the mechanisms for their in vivo synthesis are still being elucidated. The peptides are formed in steps by enzyme modules with specific domains that govern adenylation, thiolation (peptidyl carrier

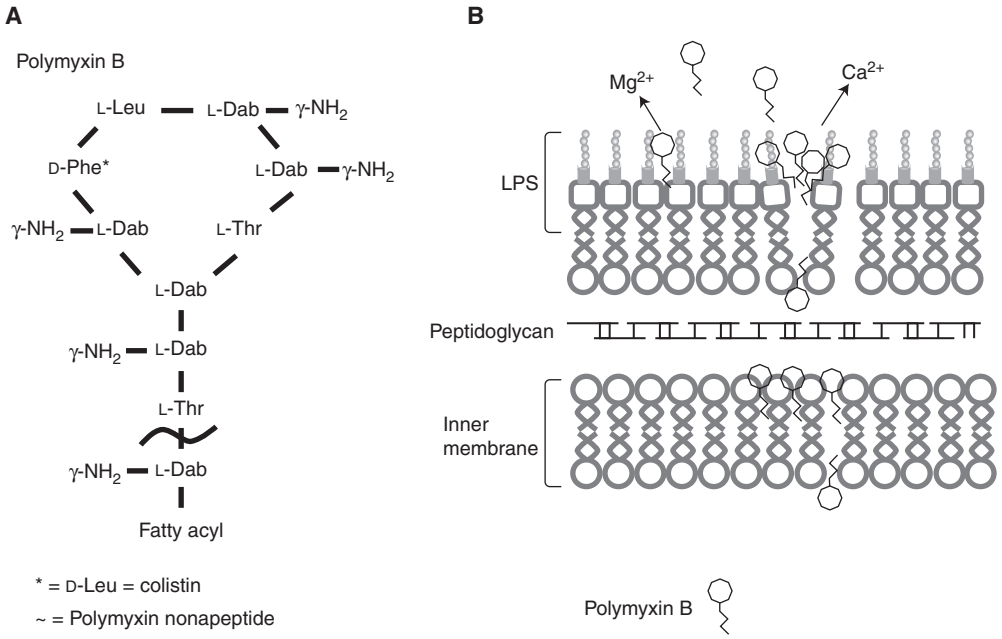

Figure 1. The structure of polymyxin B and likely mode of membrane interaction. (*A*) The general structure of the cyclic cationic peptide polymyxin B. Colistin (polymyxin E) replaces the phenylalanine (D-Phe*) in polymyxin B with a leucine. Attenuated polymyxin nonapeptide has the fatty acyl tail removed adjacent to the threonine outside the ring structure (~). (*B*) Polymyxin B interacts with the lipid A portion of the lipopolysaccharide (LPS) outer membrane. The peptides cross the outer membrane through a "self-promoted uptake" mechanism and then interact with the cytoplasmic membrane to inhibit cellular energization, and possibly cause inhibition of cell division and/or cytoplasmic membrane permeabilization and subsequent cell death.

protein [PCP]), and condensation (Finking and Marahiel 2004; Choi et al. 2009b). To date, the nonribosomal peptide synthetases have been identified for polymyxin M (Martin et al. 2003), polymyxin A (Choi et al. 2009b), variants of polymyxin B (Shaheen et al. 2011), and polymyxin P (Niu et al. 2013).

All of the polymyxin peptides have similar bactericidal activities with efficacy against Gram-negative organisms and a few Gram-positive species (see section ExPortal) (Newton 1956; Storm et al. 1977; Vega and Caparon 2012). Polymyxin and its relatives were commonly used in clinical treatment until the 1980s, when their nephrotoxicity caused a decline in usage (Falagas and Kasiakou 2006; Landman et al. 2008; Falagas et al. 2010). The nephrotoxicity is drug-related acute tubular necrosis leading to acute renal failure and stems from accumulation and persistence in the body, with preferences for kidney and brain tissues (Kunin and Bugg 1971; Evans 1999). Similar to the drug's interaction with the bacterial outer membrane, it permeabilizes eukaryotic membranes leading to swelling and lysis (Berg et al. 1996, 1998; Lewis and Lewis 2004; Falagas and Kasiakou 2006). However, recent studies have indicated that the incidence of nephrotoxicity is less common and severe compared with older studies (Berg et al. 1996, 1998; Lewis and Lewis 2004; Falagas and Kasiakou 2006) and, intriguingly, polymyxin B was found to be less nephrotoxic than the prodrug colistimethate in which the positive charges on colistin are neutralized (Phe et al. 2014). In addition, polymyxins can bind to neurons and block the release of acetylcholine, preventing neurotransmission and affecting muscular activity, although neurotoxic effects are usually mild and resolve on discontinuation of therapy (Falagas and Kasiakou 2006).

After usage was reduced because of toxicity concerns, polymyxins were reserved for treatment of persistent infections in cystic fibrosis patients, ophthalmic conjunctivitis infections, and in over-the-counter topical antibiotic ointments (Hancock and Chapple 1999; Hancock 2000; Falagas et al. 2010). The usage of polymyxins has seen a recent upsurge despite issues with toxicity because of increasing resistance to other drugs. Currently, they are the go-to drug for serious multidrug-resistant (MDR) Gram-negative bacterial infections, particularly those caused by MDR *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, in which they have become the last-line treatment for infections that are resistant to other available antibiotics (Nation et al. 2015). Polymyxin B is administered directly as an antibiotic, whereas colistin is administered as the prodrug colistin methanesulfonate (in which diaminobenzoate residues are derivatized as methane sulphonates), which is hydrolyzed into colistin in vivo (Zavascki et al. 2007; Landman et al. 2008; Nation et al. 2014). As mentioned above, recent examinations into the reported renal and neural toxicities indicate that the severity and incidences of these effects remain high but not nearly as high as previously suggested (Falagas and Kasiakou 2006; Kwa et al. 2008; Landman et al. 2008). Thus, although renal and neural toxicity remain an adverse side effect, our current handling of the drugs has more positive outcomes (Nation et al. 2015). Correct formulations, dosage (lower than past usage), discontinuation on adverse symptoms, avoidance of coadministration with other potential nephro/neurotoxic dugs, and overall better critical care and, possibly, better manufacturing have led to lower incidences of nephro and neural toxicities (Falagas and Kasiakou 2006). Consensus recommendations for their clinical usage were recently published (Nation et al. 2015).

Disruption and/or permeabilization of Gram-negative bacterial cytoplasmic membrane has been suggested as the main mechanism of action by polymyxin B and colistin (Teuber 1974). However, doubt regarding the singular nature of this mechanism has been raised (Zhang et al. 2000). Because of resurgence of their usage, research into the mechanisms of action and resistance of polymyxin B and colistin has intensified. The purpose of this review is to highlight some of the alternative and less characterized mechanisms of action of polymyxin B and colistin as well as the bacterial resistance to these drugs.

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MECHANISMS OF ACTION

Membrane Target and Evidence for Alternative Mechanisms of Action

Since their discovery many years ago, much has been learned regarding the mechanism of action of polymyxins. Newton (1956) originally suggested that the mechanism of polymyxin B involved its "ability to combine with and disorganize structures of the bacterial cell which are responsible for the maintenance of the osmotic equilibrium of the cell." It is generally accepted that the Gram-negative selectivity is mediated by initial interaction with the outer membrane of Gram-negative bacteria. Early studies showed that Gram-negative bacteria pretreated with polymyxin B were more susceptible to lysozyme treatment, indicating that polymyxin B disrupted the outer membrane, forming visible protrusions, thereby exposing the underlying peptidoglycan layer to lysozyme (Warren et al. 1957; Koike et al. 1969). Additionally, treatment of *Escherichia coli* with polymyxin B increases susceptibility to β-lactam antibiotics, which target the peptidoglycan synthesis machinery (Rosenthal and Storm 1977). It was subsequently proposed and shown that the cationic charges in the polypeptide portion of polymyxin electrostatically bind to the negatively charged lipopolysaccharide (LPS) that is the predominant (or only) surface lipid of the outer membrane in Gram-negative bacteria, assisted by the interaction of the lipid tail with the fatty acids of the lipid A moiety of the LPS molecule (Fig. 1B). When it binds to phosphate residues of lipid A, polymyxin displaces the membrane-stabilizing magnesium and calcium ions that cross-bridge adjacent lipid A molecules and stabilize the outer membrane (Moore et al. 1986; Evans 1999; Falagas and Kasiakou 2005; Landman et al. 2008; Fernández et al. 2013). By displacing these divalent cations with the bulkier polycationic polymyxin, the membrane becomes weakened and the permeability barrier is disrupted allowing for uptake of previously nonpermeating or weakly permeating molecules and leakage of periplasmic proteins (Hancock 1984). The displacement of divalent cations and permeabilization allows for self-promoted uptake of the polymyxin molecule itself enabling it to penetrate the periplasm and approach the cytoplasmic membrane (Hancock and Bell 1988; Hancock 1997). Polymyxin B nonapeptide (PMBN), lacking the fatty acyl tail, had virtually no antibiotic activity but was still able to compromise the outer membrane (Vaara and Vaara 1983; Daugelavicius et al. 2000; Zhang et al. 2000; Lu et al. 2014), indicating that outer membrane permeabilization could occur because of charge:charge interactions alone, although larger concentrations of this attenuated polymyxin were required for permeabilization, suggesting that the hydrophobic acyl tail promotes outer membrane permeabilization. The lack of activity of PMBN, however, indicates that the outer membrane is a site of interaction, but it is not the killing target. In addition to binding to LPS, a polymyxin B photoprobe was recently shown to bind to unspecified outer membrane proteins (van der Meijden and Robinson 2015). However, for killing to occur, according to the membrane target model, the cytoplasmic membrane must be compromised, whereas other studies have suggested alternative membrane-associated or cytoplasmic targets (Zhang et al. 2000).

The membrane-active mechanism of action for polymyxins has been well studied, but it is unclear how polymyxin interacts with and disrupts the cytoplasmic membrane (Landman et al. 2008; Falagas et al. 2010; Yu et al. 2015). Early experiments showed that high concentrations of polymyxin caused the release of cytoplasmic material (Cerny and Teuber 1971; Schindler and Teuber 1975; Dixon and Chopra 1986; Landman et al. 2008). It was shown that polymyxin B caused lipid exchange between outer and inner membrane leaflet binding in experiments with simulated Gram-negative membrane (Clausell et al. 2007). Similarly, Berglund et al. (2015) used computer modeling to propose that polymyxin B initially aggregates at the LPS surface by burying their acyl tails into micelle/pore-like structures. Conversely, polymyxin B did not form aggregates in the inner membrane model, suggesting that the membrane interactions are dependent on the membrane architecture of the inner and outer membranes in Gram-negative bacteria.

Despite this evidence and overall assumption for the membrane as the sole target for the polymyxins, there is evidence for alternative mechanisms of action. An early study investigating how different environmental ions affected polymyxin killing of *P. aeruginosa* (Klemperer et al. 1979) showed that lysis is not required for cell death. In *P. aeruginosa*, at concentrations greater than the minimum inhibitory concentration (MIC), polymyxin led to substantial cell death while only marginally increasing cytoplasmic membrane permeability, as assessed by reduction in the transmembrane potential gradient (Zhang et al. 2000). Similarly, the cytoplasmic membrane of polymyxin-treated *E. coli* was permeabilized, as determined by the measured release of ions, only at concentrations well above the minimal bactericidal range (Daugelavicius et al. 2000). This high cell death at concentrations showing little increase in cytoplasmic membrane permeability indicates that alternative or additional mechanisms of action are likely to contribute to the antibacterial activity of polymyxins. Studies with a modified, fluorescently labeled polymyxin B that retained activity showed that it coalesced at the outer membrane before penetration into the periplasm, cytoplasmic membrane, and, finally, the cytoplasm in *K. pneumoniae* (Deris et al. 2014), indicating a plethora of potential sites of action. Some of the alternative mechanisms of activity that have been proposed are described below.

Ribosome Binding

In early investigations of the mechanism of action of polymyxins and colistin, it was discovered that polymyxin could precipitate *E. coli* ribosomes (Nakajima and Kawamata 1966; Teuber 1967), similar to other polycationic antibiotics. Despite these seminal papers, there was no follow-up for ~50 years. Recently, McCoy et al. (2013) reestablished this link by examining the relationship of polymyxin to aminoglycosides, which are cationic saccharide bactericidal antibiotics that also bind to ribosomes. They posited that the shared cationic nature, nephrotoxicity, and self-promoted uptake (Hancock 1997) needed further exploration. Specifically, they examined the ability of polymyxin to bind to bacterial 16S ribosomal RNA using a modified fluorescence resonance energy transfer assay. Polymyxin showed moderate binding to the 16S A-site of *E. coli* ribosomes, 10 times weaker than kanamycin yet 10 times stronger than the negative control, an inactive colistin derivative with sulfomethylated (charge-neutralized) amino groups. Intriguingly, an analog of polymyxin, with a biphenyl acyl tail and a D-octylglycine at the phenylalanine position, showed similar binding affinity to kanamycin. Also, PMBN showed two times better binding than intact polymyxin B. This is interesting because, in vivo, the nonapeptide apparently cannot pass the outer membrane (Vaara and Vaara 1983). Despite these abilities to bind to the ribosome, bacterial translation was not affected; however, in contrast, eukaryotic translation was negatively affected. Nevertheless, we deduce that ribosome binding is not an alternative or additional mechanism because there were apparently no obvious consequences of ribosome binding on prokaryotic translation nor evidence that polymyxin penetrates sufficiently into the cytoplasm to inhibit the large number of ribosomes in bacterial cells.

Bacterial Respiration

Strictly speaking, membrane permeabilization has many cellular consequences beyond a compromised cell structure or lysis. Indeed, disruption of cytoplasmic membrane integrity can impact cellular energetics, cell division, and cell wall biosynthesis. Bacterial respiration, for instance, requires an intact membrane to function properly (Storm et al. 1977). Consistent with this concept, sublethal concentrations of polymyxin B inhibited oxygen consumption in *A. baumannii* without penetrating the cytoplasmic membrane (Saugar et al. 2002).

Despite intrinsic resistance of Gram-positive bacteria to polymyxin, Tochikubo et al. (1986) examined nicotinamide adenine dinucleotide (NADH) oxidase activity following germination of *Bacillus subtilis* spores. They determined that polymyxin B inhibited NADH oxidase activity as well as NADH cytochrome *c*

reductase activity, but not NADH dehydrogenase activity. It was proposed that the spore pericortex prevents direct interaction with the inner membrane, but somehow the activity of polymyxin still inhibited respiration (Tochikubo et al. 1986). Another group has shown similar effects on bacterial respiration machinery as polymyxin B inhibited the activity of the alternative NADH dehydrogenase and the malate: quinone oxidoreductase in *Mycobacterium smegmatis* (Mogi et al. 2009).

A screen for polymyxin B–resistant mutants in the susceptible Gram-positive *Streptococcus pyogenes*, identified the fatty acid biosynthesis master regulator, *fabT* (Dalebroux and Swanson 2012; Port et al. 2014). Fatty acid biosynthesis is known to activate the stringent response. Similarly, polymyxin treated cells show an increase in ppGpp, but not pppGpp (Cortay and Cozzone 1983b; de la Fuente-Núñez et al. 2014). This increase was independent of RelA, which is the normal producer of (p)ppGpp (Cortay and Cozzone 1983a). It was suspected that the increase in ppGpp was because of a lack of cellular ATP powering the enzymatic degradation by SpoT (Cortay and Cozzone 1983b). Indeed, inhibition of respiration by polymyxin leads to a decrease in the ATP pool of the cell (Storm et al. 1977). These reports provide compelling evidence that respiration inhibition is a target of polymyxin beyond membrane disruption. Similarly, other bactericidal cyclic cationic peptides are known to permeabilize the outer membrane and cause inhibition of respiration at their minimal effective doses (Skerlavaj et al. 1990; Wu and Hancock 1999; Spindler et al. 2011). Nevertheless, inhibition of respiration has not been shown to be a bactericidal target so it is likely that it contributes to, rather than causes, cell killing.

Cell Division

Bacterial cell division is an essential process that depends on cytoplasmic membrane–associated machinery and the membrane potential (Strahl and Hamoen 2010). For example, it is well established that many third-generation cephalosporin antibiotics act directly on penicillin binding protein 3 to inhibit correct cell division leading the formation of long filamentous bodies in which individual cells have grown but not separated. A recent paper using atomic force microscopy (AFM) on colistin-treated *P. aeruginosa* provided evidence that colistin also affects cells division (Mortensen et al. 2009). A significant decrease in dividing cells was observed when cells were treated with sublethal concentrations of colistin in addition to a slight decrease in colony-forming units, but no significant killing (Mortensen et al. 2009). The investigators also observed an increase in cellular rigidity and speculated that colistin binding to peptidoglycan stiffens the bacterial cell wall and speculated that this prevented proper cell division (Mortensen et al. 2009). This conclusion, however, contrasts to another AFM study that showed reduced membrane integrity (Lu et al. 2014). Thus, an alternative possibility might be that polymyxin inserts into the cytoplasmic membrane and interferes with the cell division machinery. A cell division defect was also reported in *Mycobacterium aurum* in which sub-MIC doses of colistin reversibly arrested cell division without killing cells (David and Rastogi 1985). It was proposed that growth arrest arose because of membrane perturbation that disrupted genome attachment and inhibited DNA replication (David and Rastogi 1985), a process that has also been postulated to explain the bactericidal activity of the cationic aminoglycosides (Hancock 1981).

Other cationic amphipathic peptides have also been proposed to affect cell division. The cationic antimicrobial peptide indolicidin has been shown to induce filamentation in *E. coli* (Subbalakshmi and Sitaram 1998), although the investigators proposed that filamentation is a secondary effect because of inhibition of DNA synthesis by indolicidin. Conversely, it was shown that the mouse cathelicidin antimicrobial peptide (CRAMP) caused filamentation in *B. subtilis* by inhibiting FtsZ-mediated ring formation, which is a critical step in cell division (Handler et al. 2008).

At this stage, we cannot conclude that cell division is a direct target of the polymyxins. However, cell division is an extremely complex process, with many moving parts, and much of the cell division machinery lies within the inner

membrane (Margolin 2005; Egan and Vollmer 2015; Gray et al. 2015). Therefore, it is important to further examine whether polymyxin may directly (e.g., through FtsZ) or indirectly (through antagonism of energetics) affect cell division as part of its overall mechanism of action.

ExPortal

The precise mechanism for Gram-positive bacteria resistance to polymyxin and colistin is largely unknown (Storm et al. 1977). It is believed that the large peptidoglycan layer prevents interaction with the cytoplasmic membrane, because *B. subtilis* and *Staphylococcus aureus* protoplasts are susceptible to polymyxin (LaPorte et al. 1977; Xiong et al. 2005). Interestingly, there are a few noted instances of Gram-positives showing polymyxin susceptibility. For example, *S. pyogenes* is susceptible to polymyxin B, likely because of the absence of the *mprF* gene, which encodes an enzyme that modifies the negative charge of phosphatidylglycerol on its capsular polysaccharide (Vega and Caparon 2012). It was observed that polymyxin B targeted the ExPortal structure of *S. pyogenes* (Vega and Caparon 2012). The ExPortal is a microdomain for the Sec secretion system (Rosch and Caparon 2005). Each cell has only one ExPortal found adjacent to the future division site (Rosch and Caparon 2005). Sublethal concentrations of polymyxin B disrupted the lipid structure around the ExPortal and impeded the secretion of the cysteine protease, SpeB, and the streptolysin O cytolysin (Rosch et al. 2007; Vega and Caparon 2012). Interestingly, despite the disruption in the lipids by sublethal doses, membrane integrity was not compromised (Vega and Caparon 2012), supporting the idea of an alternative mechanism of action different from direct membrane disruption. It was hypothesized that the defect in the secretion mechanism and/or another unknown internal target leads to the demise of the cells (Vega and Caparon 2012).

Reactive Oxygen Species

Recently, a highly controversial mode of bacterial cell death by bactericidal antibiotics was proposed. Some groups suggested that these antibiotics, including polymyxin B, colistin, and kanamycin kill through oxidative stress and reactive oxygen species (ROS) generation (Dwyer et al. 2007, 2009; Brochmann et al. 2014; Dong et al. 2015). However, these overall conclusions regarding this proposed common mechanism were disputed by several investigators, in particular, by showing that antibiotic killing was identical under aerobic and anaerobic conditions indicating independence of oxygen and hydroxyl radicals (Kindrachuk et al. 2011; Keren et al. 2013; Liu and Imlay 2013; Paulander et al. 2014). Similarly, ROS independent killing by colistin has been shown (Brochmann et al. 2014). Conversely, Dong et al. (2015) showed that polymyxin B induced transcription in *E. coli* of the oxidative stress response gene *sox*S, and caused an increase in ROS, although they did not imply that this explained the mechanism of killing. A variety of reports have discussed potential mechanisms of resistance mediated by oxidative enzymes but alternative explanations should be sought (Antonic et al. 2013; Heindorf et al. 2014; Pournaras et al. 2014). Despite all the studies and evidence for mechanisms of killing, clearly the exact mechanism of polymyxin action remains to be determined.

POLYMYXIN RESISTANCE

It has been observed that, compared with many other antibiotics, resistance to polymyxins is difficult to attain in the laboratory. Because polymyxins are being used as a last line of defense in treating MDR bacterial infections, it is of considerable interest to examine mechanisms of resistance that have arisen in the clinic or laboratory (Falagas et al. 2010; Fernández et al. 2011; Olaitan et al. 2014). Known resistance mechanisms include intrinsic, mutational, and adaptive, although there are no known horizontally acquired resistance mechanisms. The majority of known mechanisms address the first point of attack by polymyxin, namely, the outer membrane LPS. Overall resistance mechanisms include alterations to reduce the net negative charge or fluidity of LPS, increase in drug efflux, reduced porin pathway, capsule

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formation, and hypervesiculation. For brevity, this section will focus on the most studied mechanisms of resistance and ones related to alternative mechanisms of action.

LPS–Lipid A Modifications

In most Gram-negative bacteria, the two-component PhoP/Q and PmrA/B (response regulator/sensor kinase) regulatory systems mediate polymyxin resistance by governing the mechanisms that modify LPS (Fig. 2; Table 1). These systems regulate cationic antimicrobial resistance in response to low environmental Mg^{2+} and Ca^{2+} as well as cationic antimicrobial peptides (CAMPs) and other inducers, such as low pH, excess Fe^{3+}, excess Al^{3+}, and macrophage phagosomes (Brown and Melling 1969; McPhee et al. 2003; Nishino et al. 2006; Prost et al. 2007; Gunn 2008; Gooderham et al. 2009). In *Salmonella enterica* serovar Typhimurium, PhoP activates the expression of *pmrD* whose product was shown to activate the PmrA/B system (Kox et al. 2000; Kato et al. 2003), although PmrD is absent in *P. aeruginosa* and instead PhoP directly interacts with the *pmrAB* promoter while the two response regulators PhoP and PmrA bind to and maybe compete for binding to the same promoter site(s) on the LPS modification operon. Other two-component systems like ParR/S, CprR/S, and ColR/S in *P. aeruginosa* have been also implicated in the regulation of lipid A modification enzymes (McPhee et al. 2003; Moon and Gottesman 2009; Fernández et al. 2010; Muller et al. 2011; Fernández et al. 2012; Gutu et al. 2013). In addition, *P. aeruginosa* has a two-component sensor kinase *cbrA* that responds to carbon source/catabolite repression and adaptively affects polymyxin B resistance by altering the transcriptional expression of the *oprH-phoPQ* operon, the *pmrAB* operon, and the LPS modification *arn* operon (Yeung et al. 2011). The most likely physiological adaptive resistance mechanism is because of the direct re-

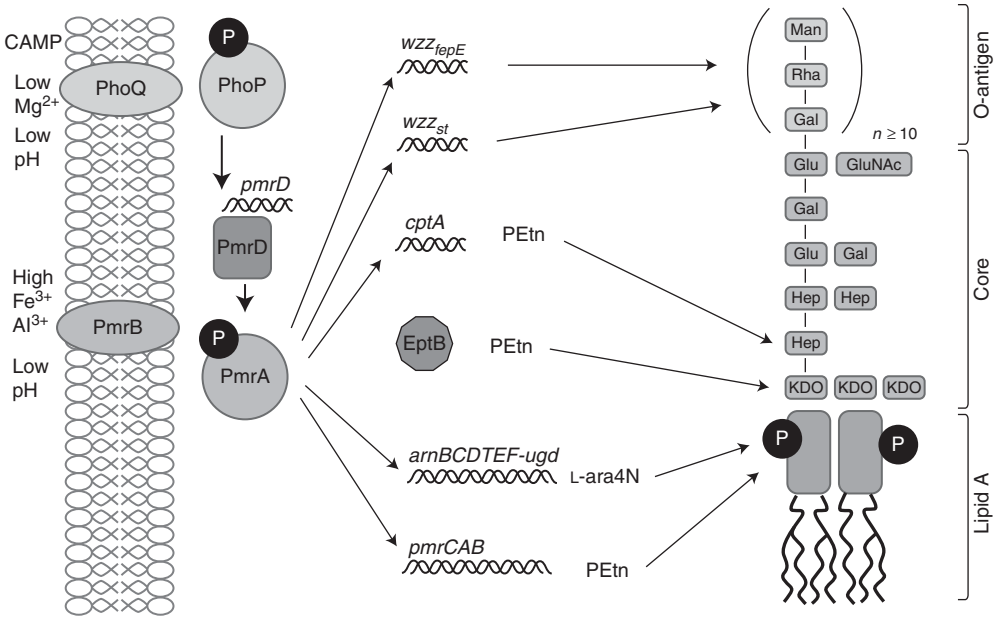

Figure 2. General mechanisms of resistance to polymyxin by lipopolysaccharide (LPS) modifications in *Salmonella* (mechanisms differ in *Pseudomonas*). The two-component system PhoQP, activates expression of *pmrD*. PmrD activates PmrA, which activates *cptA* and the *pmr* and *arn* operons. Along with EptB, CptA modifies the LPS core. The products *pmr* and *arn* substitute the phosphates the lipid A portion of the LPS for PEtn and L-Ara4N, respectively. Collectively, the net charge of the outer membrane changes, resulting in repulsion of polymyxin.

Table 1. Lipopolysaccharide (LPS) modifications leading to polymyxin resistance or supersusceptibility

Modification	Function	Genes[a]	Bacterial spp.	References
L-Ara4N or PEtn modification of lipid A	Two-component system (TCS) kinase/response regulator (K/RR)	*phoP/phoQ*	*E. coli*	Moon and Gottesman 2009
			S. Typhimurium	Gunn et al. 1998
			P. aeruginosa	Macfarlane et al. 2000
		pmrA/pmrB	*P. aeruginosa*	McPhee et al. 2003
			S. Typhimurium	Gunn et al. 1998
			A. baumannii	Arroyo et al. 2011
			K. pneumoniae	Choi and Ko 2014
			E. coli	Trent et al. 2001
		parR/parS	*P. aeruginosa*	Fernández et al. 2010
		cprR/cprS	*P. aeruginosa*	Fernández et al. 2012
		colR/colS	*P. aeruginosa*	Gutu et al. 2013
		Rcs system	*S.* Typhimurium	Mouslim and Groisman 2003
		cbrAB	*P. aeruginosa*	Yeung et al. 2011
	Proposed response regulator	*rppA*	*P. mirabilis*	Wang et al. 2008
	Activates *pmrAB*	*pmrD*	*S.* Typhimurium	Kato et al. 2003
	UDP-glucose dehydrogenase	*pmrE* (*pagA* or *ugd*)	*P. mirabilis*	Jiang et al. 2010a
			S. Typhimurium	Mouslim and Groisman 2003
				Gunn et al. 1998
		arnBCADTEF	*P. aeruginosa*	Muller et al. 2011
		(*pmrHFIJKLMpbgP*)	*E. coli*	Yan et al. 2007
			S. Typhimurium	Gunn et al. 2000
			P. mirabilis	Lee et al. 2004
			Y. pseudotuberculosis	Jiang et al. 2010b
			K. pneumoniae	Marceau et al. 2004
				Cheng et al. 2010
	L-Ara4N transferase	*arnT*	*E. coli*	Subashchandrabose et al. 2013
		(*pmrK*)	*S.* Typhimurium	Trent et al. 2001
		(*pqaB*)	*S. typhi*	Baker et al. 1999

Continued

Table 1. *Continued*

Modification	Function	Genes[a]	Bacterial spp.	References
	Mannosyltransferase	*pbgE1*	*P. luminescens*	Bennett and Clarke 2005
	UDP-glucose dehydrogenase	*ugdBCAL2946*	*B. cenocepacia*	Loutet et al. 2009
	Ugd phosphorylation	*etk*	*E. coli*	Lacour et al. 2008
	Oxidoreductase	*DsbA3*, *DsbA2*, and *DsbA3*	*N. meningitidis*	Piek et al. 2014
PEtn modification of LOS/LPS core	TCS (kinase/response regulator)	*misS/misR*	*N. meningitidis*	Tzeng et al. 2004
	Transferase	*eptB*	*E. coli*	Reynolds et al. 2005
			S. Typhimurium	Gibbons et al. 2008
	PEtn transferase	*pmrC* (*eptA* and *lptA*)	*S.* Typhimurium	Lee et al. 2004
			A. baumannii	Arroyo et al. 2011
			E. coli	Herrera et al. 2010
	Phosphotransferase	*cptA*	*S.* Typhimurium	Tamayo et al. 2005a
	sRNA	*mgrR*	*E. coli*	Moon and Gottesman 2009
	Transferase (also affects PEtn modification of flagella and glycans	*eptC*	*C. jejuni*	Cullen et al. 2012, 2013; Scott et al. 2012
Deacylation of lipid A	Deacetylase	*naxD*	*F. tularensis*	Llewellyn et al. 2012
	Deacetylase	*pagL*	*S. enterica*	Kawasaki et al. 2007
	Deacetylase	*yfbH* (*pmrJ*)	*E. coli*	Subashchandrabose et al. 2013
	sRNA	*lpxR*	*S.* Typhimurium	Reynolds et al. 2006
Repressor of *phoPQ* expression	Transmembrane regulator	*mgrB* (*yobG*)	*K. pneumoniae*	Lopez-Camacho et al. 2014
	MerR-like regulator	*brlR*	*P. aeruginosa*	Chambers and Sauer 2013
	sRNA	*micA*	*E. coli*	Coornaert et al. 2010
L-Lys modification of cell membrane phosphatidylglycerol	Transferase	*mprF* (*lpiA*)	*S. aureus*, *E. faecalis*, and *R. tropici*	Yang et al. 2012 Bao et al. 2012 Sohlenkamp et al. 2007
	Lysinylation	*lysX*	*M. tuberculosis*	Maloney et al. 2009

Continued

Table 1. *Continued*

Modification	Function	Genes[a]	Bacterial spp.	References
Phosphorylation of lipid A	Phosphotransferase	*lpxT*	*S.* Typhimurium	Herrera et al. 2010
			E. coli	Touze et al. 2008
		waaP (*rfaP*)	*E. coli*	Yethon et al. 2000
			S. Typhimurium	Tsai et al. 2012
			P. aeruginosa	Delucia et al. 2011
Dephosphorylation of lipid A	Phosphatase	*lpxE* and *lpxF*	*H. pylori*,	Tran et al. 2006
			F. novicida, and *R. etli*	Wang et al. 2006
				Ingram et al. 2010
	Phosphatase	*PG1587* and *PG1773*	*P. gingivalis*	Coats et al. 2009
	Transcriptional activator	*slyA*	*D. dadantii*	Haque et al. 2009
			S. Typhimurium	Shi et al. 2004b
		ugtL	*S.* Typhimurium	Shi et al. 2004a,b
Glycylation of lipid A	TCS (K/RR)	*vprA/vprB*	*V. cholerae*	Herrera et al. 2014
	TCS (K/RR)	*carR/carS*	*V. cholerae*	Bilecen et al. 2015
		almEFG	*V. cholerae*	Hankins et al. 2012
Addition of amide-linked acyl chains in lipid A	Dehydrogenase and transaminase	*gnnA* and *gnnB*	*C. jejuni*	van Mourik et al. 2010
Glucosamine modification		*lgmABC*	*B. pertussis*	Shah et al. 2014
		pgaABCD	*E. coli*	Amini et al. 2009
			A. baumannii	Choi et al. 2009a

[a]For brevity, not all genes are mentioned in the text. Gene contained within parentheses are alternative names or homologous genes.

sponse of the sensor kinases of two component regulators to the presence of peptides, both natural host defense (antimicrobial) peptides and polymyxins, which interact directly to activate these sensor kinases (Bader et al. 2005). In *Salmonella*, the peptide-specific sensor kinase is PhoQ, whereas in *Pseudomonas*, a pair of sensor kinases, ParS and CprS, respond to polymyxin and/or peptides (Fernández et al. 2010, 2012). Thus, adaptive resistance can be a result of both self-induced resistance during polymyxin therapy and the antimicrobial peptide component of host defense responses to infections.

The output targets of the two component systems are diverse, but commonly make modifications to the phosphate groups of lipid A in LPS to amine substituents, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N), or phosphoethanolamine (PEtn). The addition of L-Ara4N or PEtn reduces the net negative charge of the bacterial surface and decreases the electrostatic interaction with polycationic peptides, such as the polymyxins, thus inhibiting self-promoted uptake. The *arnBCADTEF* operon, activated by ParR/S and PmrA/B in *P. aeruginosa* and *Salmonella*, etc. (Subashchandrabose et al. 2013) adds an L-Ara4N modification to lipid A (Aguirre et al. 2000; Gunn et al. 2000; Tamayo et al. 2005b; Raetz et al. 2007; Barrow and Kwon 2009; Gooderham et al. 2009; Fernández et al. 2010).

Conversely, in *Salmonella pmrC* (also named *eptA* and *lptA*), which is also activated by PmrA, is involved in PEtn modification of lipid A (Lee et al. 2004). Similarly, the *pmrC* gene encodes a phosphoethanolamine transferase and modifies LPS with PEtn in *A. baumannii* (Arroyo et al. 2011). The LPS core may also be modified with PEtn by the *eptB* (analogous to *pmrC*) and *cptA* gene products. EptB regulates PEtn addition to 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) in *E. coli* (Reynolds et al. 2005) and CptA in *Salmonella* modifies phosphorylated heptose-I residue of the LPS core (Tamayo et al. 2005a). Paradoxically, activation of PhoP/Q system activates the sRNA *mgrR*, which is a repressor of *eptB* (Moon and Gottesman 2009). Various oxidoreductases, such as DsbA1, DsbA2, and DsbA3, contribute to PEtn modifications of lipid A in *Neisseria meningitidis*. Specifically, DsbA3 was shown to be important for the activity of LptA, a PEtn transferase (Piek et al. 2014).

Independent of the PhoP/Q and PmrA/B, the Rcs system (RcsC–YojN–RcsB) in *S. enterica* has been shown to regulate the expression of *ugd*, which encodes a UDP-glucose dehydrogenase, an enzyme important in the synthesis of L-Ara4N (Mouslim and Groisman 2003). Also the tyrosine-kinase *etk* in *E. coli* phosphorylates Ugd independently of the two-component systems (Lacour et al. 2008).

In addition to L-Ara4N or PEtN modifications, deacylation of lipid A occurs to decrease the fluidity of LPS and, thus, the ability of cationic peptides to partition into the outer membrane leading to self-promoted uptake (Olaitan et al. 2014). In *Salmonella*, the activation of PhoP/Q system activates *pagL*, the product of which deacylates lipid A (Kawasaki et al. 2007). Conversely, the *naxD* gene in *Francisella tularensis* has also been shown to be responsible for deacylation of lipid A (Llewellyn et al. 2012). Further, PmrA-activated genes include *lpxR*, which is associated with deacylation of lipid A and is repressed by the sRNA *micF* (Papenfort et al. 2006; Reynolds et al. 2006; Corcoran et al. 2012). Additional modifications to lipid A include phosphorylation, dephosphorylation, glycylation, and glucosylation. It has also been shown that *misR* mutants in *N. meningitidis* lose all of their lipooligosaccharide (LOS) core HepII PEtn decorations leading to an increase in polymyxin B sensitivity (Tzeng et al. 2004). Similar to LPS, LOS is essential for maintaining the integrity of the outer membrane and provides protection from antimicrobial peptides. The *waaP* (*rfaP*) gene product is also involved in LPS core modification by phosphorylation of lipid A and was required for polymyxin resistance in *S. enterica* and *P. aeruginosa* (Yethon et al. 2000; Delucia et al. 2011; Tsai et al. 2012).

Although it is more difficult to obtain polymyxin resistance than resistance to other antibiotics, the increased use of polymyxin has started to lead to clinical resistance. Mutations in *phoQ* genes have been identified in colistin-resistant clinical isolates of *P. aeruginosa* and

K. pneumoniae (Choi and Ko 2014; Olaitan et al. 2014). Further mutations in the *pmrA* or *pmrB* genes resulted in activation of *arnB* (*pmrH*) or other PmrA-activated loci that are required for polymyxin resistance (Sun et al. 2009). Mutations in *arnT* (also known as *pmrK* and *pqaB*) have been shown to affect resistance to polymyxin B in *E. coli* and *S. enterica* (Trent et al. 2001). Conversely, *S. enterica* and *Yersinia pseudotuberculosis* mutants in the *pmrF* gene, which is necessary for L-Ara4N substitution on bactoprenol phosphate, displayed an increased susceptibility to polymyxin B (Gunn et al. 2000; Marceau et al. 2004). The most exotic polymyxin resistance mutation is the complete loss of LPS by clinical isolates of *A. baumannii* (Moffatt et al. 2010, 2011), although many polymyxin resistant isolates in this bacterium have *pmrB* mutations influencing expression of the upstream *pmrC* gene (Arroyo et al. 2011). Another interesting clinical mutant was in the *parR* gene (one of the polymyxin receptors of *Pseudomonas*), which influenced both inducible and constitutive multidrug resistance to four different classes of antibiotics through the activation of three distinct mechanisms (efflux, porin loss, and LPS modification) (Muller et al. 2011).

The *tolA* gene is involved in LPS production and this gene has been shown to be necessary for polymyxin B resistance in *S. typhimurium* (Paterson et al. 2009). Further, LPS modification loci—wzz_{st} and wzz_{fep} activated by PmrA/P, *wbaP* (*rfbP*), *waaG* (*rfaG*), *waaI* (*rfaI*), *rfaH*, *waaJ* (*rfaJ*), *waaL* (*rfaL*), or *wzy* (*rfc*)—are involved in synthesis of the LPS rough core in *S. enterica* and appear to be required for polymyxin B resistance, likely because of effects on phosphorylation and/or acylation of LPS (Delgado et al. 2006; Holzer et al. 2009; Ilg et al. 2009; Kong et al. 2011; Pescaretti et al. 2011). Similar mutations in *waaL* and *rfbA* (*rffH*) contribute to resistance to colistin in *K. pneumoniae* (Sassera et al. 2014). Similarly, in *E. coli*, five lipid A–modifying genes, *ais* (*b2252*), *b2253* (*yfbE*, *orf1*), *b2254* (*yfbF*, *orf2*), *b2256* (*yfbH*, *orf4*), and *crcA* were associated with antimicrobial peptide resistance (Kruse et al. 2009). Overexpression of *K. pneumoniae* RamA, which regulates the *lpxC*, *lpxL-2*, and *lpxO* genes associated with lipid A biosynthesis, resulted in increased polymyxin B resistance because of LPS alterations (De Majumdar et al. 2015). An LPS synthesis gene encoding the glycosyltransferase, *lpsB*, has also been implicated in colistin resistance in *A. baumannii* (Hood et al. 2013). In addition, the *S. typhimurium* PhoP-activated gene, *mgtA*, is also required for resistance to the cationic antibiotic polymyxin B. MgtA controls modification of phosphate residues in the lipid A portion of the LPS (Park and Groisman 2014).

Stress Responses

Biofilm formation is thought to be a response to stress (de la Fuente-Núñez et al. 2014). The *P. aeruginosa* genes *psrA* and *cbrA*, and *V. cholerae carR*, regulate biofilm formation and polymyxin B resistance (Gooderham et al. 2008; Bilecen et al. 2015). Interestingly, *psrA* responds to cationic peptides, selected antibiotics (Gooderham et al. 2008) as well as an increase in fatty acids and is also an activator of the stationary phase σ factor, RpoS (Kang et al. 2009). As mentioned above, mutations in FabT, the fatty acid biosynthesis master regulator of *S. pyogenes*, led to an increase in fatty acids and polymyxin resistance (Port et al. 2014). The increase in fatty acids may activate RpoS through PsrA, which in turn activates the stringent response, biofilm formation, and resistance. Additionally, PhoP/Q has been shown to stabilize RpoS in *S. enterica* (Tu et al. 2006). Moreover, biofilm genes of *P. aeruginosa* have been correlated with increased resistance toward colistin because of the complex architecture of biofilms (Folkesson et al. 2008). Interestingly, it was shown that *P. aeruginosa* biofilm cells with high metabolic activity are more resistant to colistin through up-regulation of the *arn* operon (Pamp et al. 2008); in principle, this could be mediated through either PsrA or the carbon stress triggered regulator CbrA.

σ Factor RpoE in *Burkholderia cenocepacia* and other pathogens has been shown to contribute to polymyxin B resistance, likely through the regulation of genes controlling envelope and oxidative stress responses (Sikora

et al. 2009; Loutet and Valvano 2011). Moreover, stationary phase σ factor RpoS regulon genes (*katE*, *osmY*, *wrbA*, *yciF*) are required for polymyxin resistance in *E. coli* and *S. enterica* (Oh et al. 1998, 2000; Bader et al. 2003). In *S. enterica*, an *rpoN* (nitrogen responsive σ factor σ^{54}) mutant increased polymyxin resistance. It was suggested that *rpoN* induced resistance to polymyxin via regulation of PTS transporters (Barchiesi et al. 2009).

Polymyxin Efflux

Multidrug efflux pumps play an important role in antibiotic and polymyxin resistance in bacteria but have only been shown to influence polymyxin resistance in a small number of instances. In different organisms, the MtrC–MtrD–MtrE, RosAB, AcrAB–TolC, NorM, KpnEF, and VexAB efflux pumps have been described to confer tolerance toward polymyxin B (Bengoechea and Skurnik 2000; Fehlner-Gardiner and Valvano 2002; Tzeng et al. 2005; Bina et al. 2008; Padilla et al. 2010; Warner and Levy 2010; Tsai et al. 2012; Srinivasan et al. 2014). Further, *sap* has been implicated in polymyxin B resistance in *E. coli* because of its homology with ABC effluxers that function against antibiotics (Subashchandrabose et al. 2013). The *marRAB* operon (activator of efflux) was associated with polymyxin B resistance in *E. coli* through interactions with Rob and up-regulation of the AcrAB–TolC efflux pump (Warner and Levy 2010). Similarly, overexpression of RamA in *K. pneumoniae* resulted in increased polymyxin B resistance by mechanisms, including modulation of efflux pump genes such as *acrAB*, *oqxAB*, and *yrbB-F* (De Majumdar et al. 2015). Mutation of the outer membrane porins, PorB and OmpU, conferred resistance to polymyxin B in *N. meningitidis* and *V. cholerae*, suggesting a potential role in uptake, perhaps by influencing an active efflux process (Mathur and Waldor 2004; Tzeng et al. 2005).

Plasmid-Mediated Resistance

Until now, polymyxin and colistin resistance was thought to be strictly limited to chromosomal mutations. Very recently, Liu et al. (2016) noted an increase of colistin resistance among food-borne commensal *E. coli* in China. They identified the resistance gene and defined it as *mcr-1*. The gene product, MCR-1, was found to share sequence similarities to phosphoethanolamine transferases suggesting resistance by modifying the phosphate groups of lipid A in LPS. An expanded search identified the *mcr-1* gene in *E. coli* and *K. pneumoniae* from both animal and human isolates. The initial publication of this work online immediately drew the attention of research groups worldwide initiating searches for the *mcr-1* gene in public and local databases. By the time this initial report was published, other instances of *mcr-1* in Gram-negative bacteria had been found in North America, Europe, South America, and Africa (Arcilla et al. 2016; Hu et al. 2016; Olaitan et al. 2016; Tse and Yuen 2016; Webb et al. 2016). The potential for widespread uncontrolled transmission is undeniable, and every precaution should be made to prevent further dissemination of resistance.

CONCLUDING REMARKS

The cyclic cationic peptides polymyxin B and colistin are potent bactericidal agents against Gram-negative bacteria. Despite their traditional record of neural and renal toxicities, they have performed well as the last line of defense against persistent MDR infections. Although neural and renal toxicities still occur, physicians are more knowledgeable and better equipped to manage toxicity (Falagas and Kasiakou 2006), which has led to a resurgence in the interest and use of this class of antibiotics to combat serious infections.

As discussed in this review, the widely accepted mode of action of polymyxins involves targeting the membranes of Gram-negative bacteria, leading to permeabilized outer and inner membranes and resulting in lysis and cell death. However, there are substantial reasons to doubt this mechanism of action as outlined here. Conversely, groups have explored the ability of polymyxins to bind ribosomes, prevent cell division, and inhibit bacterial respiration. It is hoped that

a more profound understanding of the mechanisms of action and resistance will improve our ability to design and develop more potent and less toxic derivatives of polymyxin and colistin. Indeed, some groups are exploring these novel polymyxins (Katsuma et al. 2009; Velkov et al. 2014) and leading the charge in combating bacterial resistance.

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