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Review

Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions

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Infections caused by multi-drug resistant (MDR) bacterial pathogens are a leading cause of mortality and morbidity across the world. Indiscriminate use of broad-spectrum antibiotics has seriously affected this situation. With the diminishing discovery of novel antibiotics, new treatment methods are urgently required to combat MDR pathogens. Polymyxins, the cationic lipopeptide antibiotics, discovered more than half a century ago, are considered to be the last-line of antibiotics available at the moment. This antibiotic shows a great bactericidal effect against Gram-negative bacteria. Polymyxins primarily target the bacterial membrane and disrupt them, causing lethality. Because of their membrane interacting mode of action, polymyxins cause nephrotoxicity and neurotoxicity in humans, limiting their usability. However, recent modifications in their chemical structure have been able to reduce the toxic effects. The development of better dosing regimens has also helped in getting better clinical outcomes in the infections caused by MDR pathogens. Since the mid-1990s the use of polymyxins has increased manifold in clinical settings, resulting in the emergence of polymyxin-resistant strains. The risk posed by the polymyxin-resistant nosocomial pathogens such as the *Enterobacteriaceae* group, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii,* etc. is very serious considering these pathogens are resistant to almost all available antibacterial drugs. In this review article, the mode of action of the polymyxins and the genetic regulatory mechanism responsible for the emergence of resistance are discussed. Specifically, this review aims to update our current understanding in the field and suggest possible solutions that can be pursued for future antibiotic development. As polymyxins primarily target the bacterial membranes, resistance to polymyxins arises primarily by the modification of the lipopolysaccharides (LPS) in the outer membrane (OM). The LPS modification pathways are largely regulated by the bacterial two-component signal transduction (TCS) systems. Therefore, targeting or modulating the TCS signalling mechanisms can be pursued as an alternative to treat the infections caused by polymyxin-resistant MDR pathogens. In this review article, this aspect is also highlighted.

Keywords. LPS modification; PhoPQ; PmrAB; polymyxin; two-component system

1. Introduction

Infectious diseases caused by bacterial pathogens are one of the leading causes of death in developing nations; moreover, emerging and re-emerging bacterial pathogens are creating a substantial burden on the healthcare sector. The success of these emerging pathogens can be attributed to their survival in the host, and their increased adaptation and resistance to the available antibacterial drugs. The emergence of pathogens that are resistant to multiple antibiotics, including the last-resort ones, is increasingly being reported from several parts of the world. As per the current estimates, globally 700,000 people die annually due to infections caused by drug-resistant bacterial infections (Gandra *et al.* 2020; Limmathurotsakul *et al.* 2019). According to some estimates, by the year 2050, 10 million people would die annually because of the infections caused by drug-resistant pathogens, a significant number to this list would be contributed by the

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low- and middle-income countries (LMICs) (de Kraker *et al.* 2016; O'Neill 2016). This has far-reaching consequences not only in the healthcare sector but also on the global economy, because of the loss of manpower. Considering the unprecedented global threat posed by antimicrobial resistance (AMR), World Health Organization (WHO) in 2015, developed a Global Action Plan to tackle AMR (WHO 2015). An integral part of this framework the "One Health" approach takes into consideration- human health, which is largely influenced by that of the animals and the shared environment.

Though the global organizations and policymakers in different countries have recognized the threat of AMR and have taken several policy initiatives to create awareness, develop stewardship, increase funding in the research to tackle the AMR problem, the situation on the ground remains grim. The reason for such a situation is that the development of novel antibacterial drugs has not been able to keep pace with the emergence of novel drug-resistant pathogens. In the preceding three decades no new antibiotics have been developed. The low discovery rate of new drugs, and the rapid emergence of resistance to them, has often discouraged the pharmaceutical industries to invest in this area of research. Several reasons can be attributed to the rapid emergence of AMR in the clinical settings, the more common of which is the lack of quick diagnostic methods to determine the resistant pathogens in the primary health care facilities, often leading to the prescription of broad-spectrum antibiotics. This indiscriminate use of broad-spectrum antibiotics has reduced our arsenal to fight against novel drug-resistant pathogens. The current situation is such that most bacterial pathogens are resistant to the frontline drugs like carbapenems, imipenem, and vancomycin, etc. The ongoing global pandemic due to SARS-CoV-2 has also dramatically enhanced the use of several antibiotics to treat the secondary bacterial infections among the patients (Rawson *et al.* 2020a, b), the impact of which on the AMR will be required to be assessed in due course (Knight *et al.* 2021; Lai *et al.* 2021; Rawson *et al.* 2020a).

While novel antibiotics are increasingly being required to treat drug-resistant bacterial pathogens, the WHO has recommended a priority list of pathogens against which novel antibiotics are urgently required. This list includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (ESKAPE) (De Oliveira *et al.* 2020). While there is an urgent requirement of novel antibiotics against ESKAPE pathogens, MDR *Mycobacterium tuberculosis*, and other emerging pathogens, there is also a renewed interest in the polymyxins, a group of antibiotics that were discovered almost seventy years back and whose use had been discontinued considering their significant toxicity in the humans.

In this review article, we discuss the general mechanisms of antibiotic resistance with particular emphasis on our current understanding of the polymyxin group of antibiotics. Specifically, the mode of action of the polymyxins, the mechanism of resistance to them, and the possible methods to intervene in the polymyxin resistance development are highlighted. This article has been updated with information available in the literature till July 2021, concerning polymyxin mode of action and resistance mechanisms.

2. Antimicrobial resistance

Antimicrobial resistance (AMR) is defined as the continuation of the growth of a microorganism in presence of an antimicrobial substance at a concentration that is expected to inhibit its growth. In other words, the bacterium that was unable to grow in presence of an antibiotic at a certain concentration can do so by developing novel characteristics. Antibiotic resistance can be an intrinsic feature of some groups of bacteria that can be attributed to the presence of certain genes enabling them to survive in presence of the antibiotic (Balaban *et al.* 2019; Blair *et al.* 2015; Schrader *et al.* 2020). However, a large number of bacteria can acquire the antibiotic-resistant phenotype by incorporating certain mutations or by capturing already existing antibiotic resistance genes via horizontal gene transfer (HGT) mechanisms (Allen *et al.* 2010; Blair *et al.* 2015). While rampant HGT events can help in acquiring antibiotic resistance in a single-step evolutionary process, the acquisition of mutations in the genome may take several generations to develop at a population level. Nevertheless, considering the natural rate of mutations at one per million bases, the rate of growth of the bacterial culture or a short generation time, and the vast number of cells in a culture vessel, resistance to any antibiotic develops in a very short time.

In the conventional antibiotic drug development process, the drugs are targeted against the vital metabolic processes in the bacterial cell, therefore, creating an evolutionary selection pressure that hastens the emergence of the resistant phenotype. The most important pathways targeted are the synthesis of the

bacterial cell wall, protein synthesis, DNA replication, and transcription processes. Consequently, the resistance mechanisms developed by the bacterial cells are specifically targeted to these pathways. Broadly, the antibiotic resistance mechanisms in the bacterial cell can be categorized into the following four groups: (a) modification of the antibiotic target site, (b) enzymatic inactivation of the antibiotic, (c) prevention of antibiotic access to the target, and (d) modification of the metabolic pathways (Blair *et al.* 2015). Though the details of the mechanisms are understood to a great extent, the discussion about the mechanisms is beyond the scope of this article.

Genetic mechanisms are largely attributed for the development of antibiotic resistance, however, recent developments in the area of single-cell biology, population-level studies involving microfluidic devices, and microscopy, has increased our understanding of a primarily phenotypic concept called persistence among the bacteria where the bacteria even though not genetically resistant to an antibiotic, can still survive in its presence by modulating its growth pattern. Persistent bacteria are a sub-population of the cells that have stopped growing in presence of the antibiotic. Once the antibiotic treatment is withdrawn, they can regrow normally. With the persistent population, the MIC of a particular antibiotic remains unchanged, therefore it is not a heritable phenomenon and hence not genetic, however, it has a lot of clinical significance that contributes to the persistent infections and clinical failure of antibiotic treatments (Balaban 2004; Balaban *et al.* 2019; Harms *et al.* 2016; Schrader *et al.* 2020).

As mentioned earlier, traditional antibiotic targets are essential pathways in the bacterial cell leading to the selection of resistant phenotypes. However, recently there has been increasing interest to find drugs that are "evolution proof" to which resistance would not develop quickly (Bell and MacLean 2018). There is also a growing interest to develop drugs that are anti-virulence in nature, meaning, they would not target any essential pathways in the bacterial cell, rather the virulence factors would be targeted to reduce the overall disease outcome (Carabajal *et al.* 2019; Curtis *et al.* 2014; Defoirdt 2018; El-Halfawy *et al.* 2020; Ogawara 2021). Also, novel drug combination therapies are being tested against pathogens to design the best treatment regimens (Barbosa *et al.* 2018; Brennan-Krohn *et al.* 2018; Lin *et al.* 2019; Olsson *et al.* 2020). In this context, the role of polymyxin drugs is very interesting considering their mode of action and the development of resistance.

3. Polymyxins: The last-resort antibiotics

The emergence of novel MDR bacterial pathogens has necessitated looking into developing new treatment strategies including the use of old drugs that have been discontinued because of toxicity issues (Biswas *et al.* 2012; Trimble *et al.* 2016). The polymyxin group of antibiotics discovered in 1947 from the bacterium *Bacillus polymyxa* are one such candidate. Till now, more than fifteen varieties of polymyxins have been isolated and characterized, among which the most prominent ones are polymyxin B and polymyxin E (also known as colistin) (Mmatli *et al.* 2020; Nang *et al.* 2021; Poirel *et al.* 2017a; Trimble *et al.* 2016). The polymyxins exhibit significant inhibitory effect against the Gram-negative bacterial pathogens, therefore, it has emerged as an ultimate choice to target such pathogens that have already become resistant to the frontline antibiotics such as β-lactams, aminoglycosides, and the fluoroquinolones (Biswas *et al.* 2012; Landman *et al.* 2008; Mmatli *et al.* 2020; Nang *et al.* 2021; Poirel *et al.* 2017a). Hence polymyxins are considered to be the last-resort antibiotic to be used against MDR Gram-negative pathogens.

At the structural level, polymyxins are very similar to the cationic antimicrobial peptides (CAMPs) produced by the eukaryotes as a first line of defence against pathogens. These are lipopeptides in nature with an approximate mass of 1.2 kDa. Polymyxins have a typical ring and tail structure (figure 1A), where the ring is made up of a polycationic peptide and the tail made up of tripeptide chain attched with fatty acids (Nation *et al.* 2014; Newton 1956; Poirel *et al.* 2017a; Trimble *et al.* 2016; Yu *et al.* 2015). The polymyxin B and colistin differ at a single amino acid residue in the peptide ring where D-phenylalanine is replaced by a D-leucine residue in colistin (Landman *et al.* 2008; Yu *et al.* 2015). While the cationic peptide ring provides a hydrophilic nature to the polymyxins, the fatty acyl chain is hydrophobic. This structural feature is generally attributed to the ability of the polymyxins to insert into the cell membranes leading to its disintegration (Nang *et al.* 2021; Nation *et al.* 2014; Trimble *et al.* 2016). This typical feature is also responsible for the observed nephrotoxicity in humans. The polymyxins are synthesized by the non-ribosomal peptide synthetases and are therefore referred to as the non-ribosomal peptides.

Polymyxin B and colistin have similar antibacterial properties against the Gram-negative bacterial species and are specifically used against infections caused by the MDR pathogens such as *K. pneumoniae*, *P.*

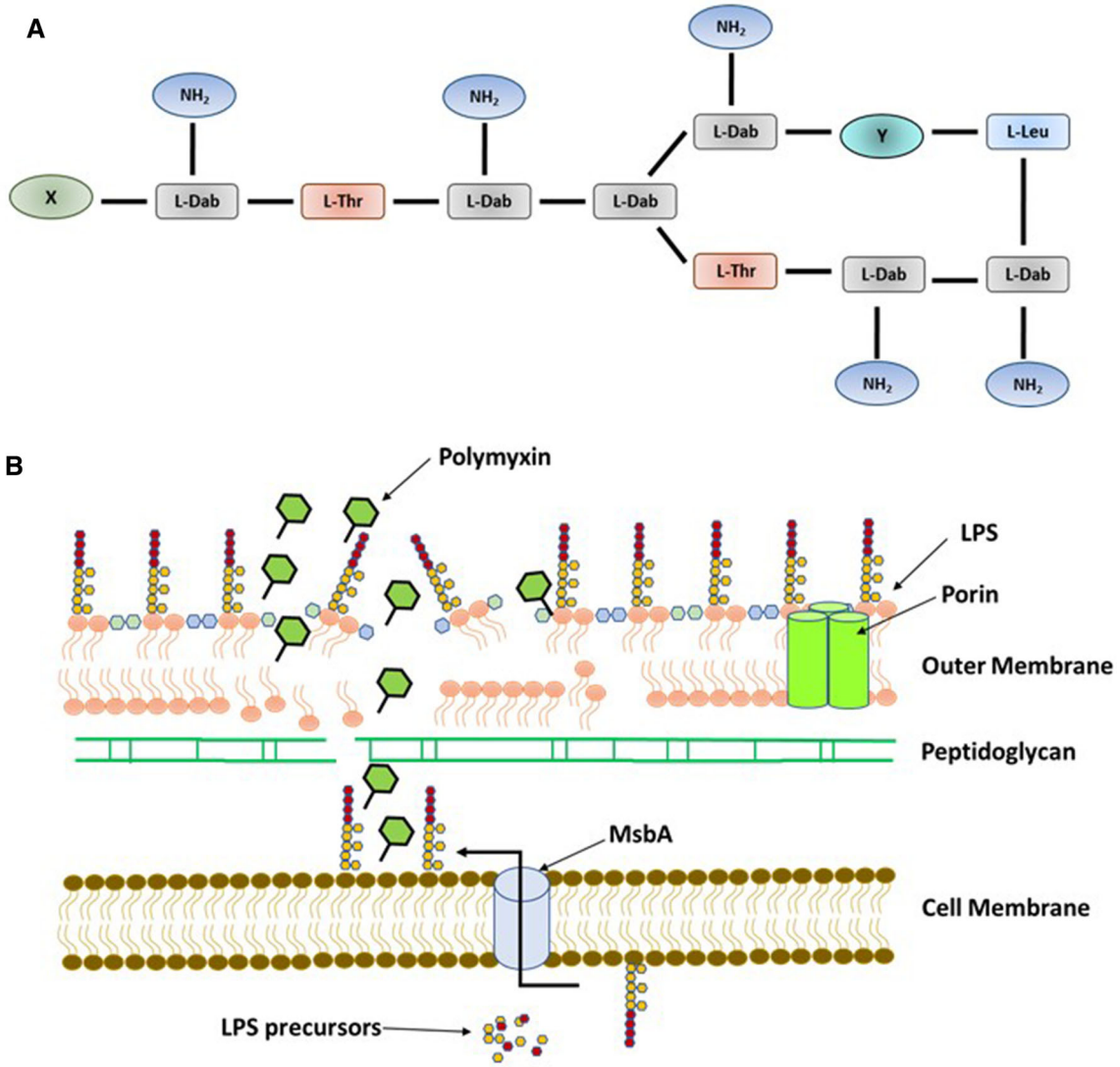

Figure 1. The structure of polymyxins and the possible mode of action. (**A**) Structure of the polymyxin B molecule. In colistin, a D-leucine residue replaces the D-Phe residue (denoted as Y) in the cyclic peptide region of the polymyxin backbone. X- fatty acid chain, Dab- diamino-butyric acid. (**B**) The model of the polymyxin- bacterial membrane interaction. Polymyxin molecules (shown as a hexagon with a tail) when interacting electrostatically with the bacterial outer membrane lipopolysaccharide the Mg^{2+} and Ca^{2+} ions (small hexagons attached to the LPS) that stabilize the membrane structure are displaced leading to the insertion of the polymyxin molecule into the membrane. This process destabilizes the membrane integrity. After this event, the polymyxin can also interfere in the assembly of the nascent LPS molecules at the cell membrane and their transport to the outer membrane. The overall process helps in the disintegration of the bacterial membrane leading to cell lysis and death (see the text for details).

aeruginosa, and *A. baumannii* as the last option (Nang *et al.* 2021; Nation *et al.* 2014; Trimble *et al.* 2016). As a primarily membrane-targeting antibiotic, polymyxins show limited activity against Gram-positive pathogens considering the presence of a thick peptidoglycan layer and the absence of the outer membrane (OM) (Yin *et al.* 2020). Polymyxins cause cell damage leading to cell death by disrupting the membrane integrity. This mechanism is also at play when the polymyxins interact with the membranes of the human cells, disintegrating them leading to toxicity. Primarily, toxicity is observed in the kidney and brain tissues, though more severity is observed in the renal tissues, thereby, leading to the prolonged discontinuity in their use till the mid-1990s (Nang *et al.* 2021). Recent chemical modifications in the drug moiety have been attempted to reduce toxicity. Also, changes in the dosage, modifications in the formulations have reduced the toxicity level to a great extent. Polymyxin B is shown to be less toxic than its prodrug colistimethate, therefore can be

administered directly. However, colistin is generally administered as prodrug colistin methanesulfonate that undergoes hydrolysis to produce the colistin (Nang *et al.* 2021; Nation *et al.* 2014; Poirel *et al.* 2017a; Trimble *et al.* 2016).

3.1 *Mode of action*

Considering the presence of the cationic peptide ring and the hydrophobic fatty acyl chain, the polymyxins act by disrupting the membrane integrity leading to the permeabilization of the bacterial cell. Though this mode of action is plausible because of the typical structure of the drug, however, there are several hypotheses/models about multiple modes of activity of the drug responsible for its bactericidal activity (Nang *et al.* 2021; Poirel *et al.* 2017a; Trimble *et al.* 2016; Yu *et al.* 2015). The most important pathways of polymyxin activity are as follows.

3.1.1 *Membrane lysis pathway:* Since the discovery and the initial uses of polymyxins, the membrane lysis pathway has been proposed as the most important mechanism by which polymyxins can exert their effect. The ability of the polymyxins to be associated with the membranes and thereby disrupting the osmotic balance of the cell is considered to be the primary mechanism (Newton 1956). The selective targeting of the Gram-negative bacteria by polymyxins can be attributed to the presence of the outer membrane (OM). Also, investigations have shown that Gram-negative bacterial cells pre-treated with polymyxins became more susceptible to the treatment of lysozyme and the peptidoglycan targeting β-lactam antibiotics, indicating the role of the polymyxin-membrane interaction preceding peptidoglycan lysis (Brennan-Krohn *et al.* 2018; MacNair *et al.* 2018).

In the Gram-negative bacteria, as per the current understanding of the polymyxin cell membrane interaction, the cationic peptide region binds electrostatically to the negatively charged lipopolysaccharide (LPS) molecules of the bacterial OM. At the same time, the hydrophobic fatty acid chain of polymyxin interacts with the lipid A of the LPS. These interactions lead to the displacement of the membrane-stabilizing cations such as Ca^{2+} and Mg^{2+} and the "self-promoted uptake" of the polymyxin into the OM (Ayoub Moubareck 2020; Evans *et al.* 1999; Fernández *et al.* 2013; Landman *et al.* 2008; Trimble *et al.* 2016). At this point, the OM becomes weakened and the permeability barrier is breached leading to the release of the periplasmic proteins and the breakage of the membrane (figure 1B) (Hancock 1984; Trimble *et al.* 2016; Yu *et al.* 2015). In the subsequent step, polymyxin interacts with the cell membrane (CM), though there is no consensus about the mechanism of action at the CM level (Landman *et al.* 2008; Yu *et al.* 2015). Previous studies have indicated that polymyxin B treatment results in lipid exchange between the outer and inner membranes of the Gram-negative bacteria, leading to the loss of specificity in the phospholipid composition (Berglund *et al.* 2015; Clausell *et al.* 2007; Yu *et al.* 2015). The inner leaflet of the OM and the CM, largely made up of the anionic phospholipids, when interacting with the cationic polymyxin, undergo disruption causing osmotic imbalance leading to cell lysis (Yu *et al.* 2015).

Even though several studies indicate that polymyxin interaction with the bacterial membranes leading to their lysis is the primary mechanism by which polymyxin acts, reasonable gaps exist in our understanding if mere interaction with the membrane is sufficient to cause cell death (Trimble *et al.* 2016; Velkov *et al.* 2010). A recent study (Sabnis *et al.* 2021) indicated that colistin can easily permeabilize the bacterial CM that can lead to cell death. Using *E. coli* expressing the plasmid-encoded *mcr-1*, this study showed that colistin can easily penetrate the OM, however, the CM can resist the onslaught of colistin. In contrast, *E. coli* carrying the empty plasmid can easily be breached leading to cell death (Sabnis *et al.* 2021). This work also unequivocally demonstrated that the LPS of both OM and CM are the targets of colistin. However, the LPS modified with phosphoethanolamine (PEtN) in the CM is more protective towards colistin treatment. This also proves the previous finding that in comparison to the OM, the CM contains a hundred times fewer LPS (Osborn *et al.* 1972; Sabnis *et al.* 2021), therefore, significantly a smaller number of colistin targets at the CM. These authors using murepavadin, a drug that inhibits the transport of LPS from the CM to OM, have shown that the *P. aeruginosa* can be re-sensitized to colistin treatment (Sabnis *et al.* 2021), suggesting that the accumulation of nascent LPS at the CM makes it more susceptible to colistin action.

Notwithstanding the above, it has been shown previously in *P. aeruginosa* and *E. coli* treated with polymyxin at concertation greater than the MIC, the proportion of cell death increases drastically, whereas the amount of cell membrane disruption increases marginally (Daugelavicius *et al.* 2000; Zhang *et al.* 2000). These data suggest the existence of multiple

alternative mechanisms of polymyxin activity responsible for the death of the bacterial cell.

Some of the alternative mechanisms suggested for the polymyxin mode of action are as follows.

3.1.2 *Inhibition of bacterial respiration:* Polymyxin is shown to inhibit bacterial respiration in some of the models studied. Disruption of the cell membrane caused by the polymyxins can affect cellular respiration adversely, as an intact cell membrane is a prerequisite for this physiological process in the bacteria (Ayoub Moubareck 2020; Nang *et al.* 2021; Storm *et al.* 1977; Trimble *et al.* 2016). Interestingly, polymyxins have also been shown to affect the normal respiratory process in the Gram-positive bacterium *Bacillus subtilis* during the sporulation process. Specifically, polymyxin inhibits the NADH oxidase and the NADH cytochrome c reductase enzymes (Tochikubo *et al.* 1986). In *Mycobacterium smegmatis* too, polymyxin inhibits NADH dehydrogenase ultimately affecting respiration (Mogi *et al.* 2009).

Bacterium *Streptococcus pyogenes* is known to be susceptible to polymyxin unlike many other Gram-positive bacterial species (Olaitan *et al.* 2014; Trimble *et al.* 2016). Polymyxin exerts its inhibitory effect in *S. pyogenes* by targeting the fatty acid biosynthetic pathway leading to the activation of the stringent response. Interestingly, polymyxin treated *S. pyogenes* cells show an increase in the concentration of the guanosine tetraphosphate (ppGpp) alarmone, whose accumulation in the cell can be attributed to the inactivation of the SpoT enzyme, because of the decrease in the cellular ATP level (Cortay and Cozzone 1983a,, b; Storm *et al.* 1977; Trimble *et al.* 2016). This mechanism of inhibition of cellular respiration is consistent with the effect exerted by other cationic antimicrobial peptides (Hancock and Chapple 1999; Spindler *et al.* 2011). Though inhibition of cellular respiration is not primarily responsible for cell death, polymyxin contributes to it by affecting cellular energetics.

3.1.3 *Generation of reactive oxygen species (ROS):* Polymyxin treatment can also lead to the generation of reactive oxygen species (ROS) as suggested by several recent studies. ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) can lead to the oxidative damage of the cellular components such as lipids, DNA, and proteins resulting in cell death (Ayoub Moubareck 2020; Imlay 2015; Kohanski *et al.* 2007; Yu *et al.* 2015). However, this mechanism is not fully accepted yet considering experimental observation of ROS independent killing of cells by colistin (Brochmann *et al.* 2014). All these conflicting results and hypotheses need reconciliation by more comprehensive studies to find out the role of ROS in the overall cell growth inhibition and death.

3.1.4 *Ribosome binding:* Polymyxins have been shown to precipitate the ribosomes in the *E. coli* cells (Nakajima and Kawamata 1966; Teuber 1967) indicating their interaction. This is similar to what has been observed using cationic aminoglycoside antibiotics that bind to the ribosomes (McCoy *et al.* 2013). Fluorescence resonance energy transfer (FRET) assay demonstrated that polymyxin indeed interacts with the 16S rRNA in the A-site of the *E. coli* ribosome, albeit with a lower affinity than the kanamycin for the above site (Hancock 1997). However, the significance of this interaction is not fully elucidated, and no conclusive evidence is available to suggest any influence on protein synthesis in the bacterial system. Interestingly, eukaryotic translation is known to be inhibited by polymyxin as observed *in vitro* (McCoy *et al.* 2013; Trimble *et al.* 2016).

3.1.5 *Effect on cell division:* Cell division is not the primary cellular function that is affected by the polymyxins, however, experimental evidence suggests that treatment with a sublethal concentration of colistin in *P. aeruginosa* reduces the number of dividing cells significantly resulting in a reduction in the number of colony-forming units (Mortensen *et al.* 2009). Though this does not result in the significant killing of the cells, it resulted in increased rigidity of the cell. Therefore, it may be argued that colistin binds to the peptidoglycan affecting the cell division. The bacterial cell division is a very dynamic process involving a plethora of different factors functioning at a spatial and temporal dimension. Alternatively, polymyxin may bind with some of these factors resulting in the arrest of cell division. It has also been proposed that polymyxin and cell membrane interaction can negatively impact the segregation of the duplicated chromosome before the cytokinesis (David and Rastogi 1985). Though these results suggest some leads, our understanding of polymyxin affecting the cell division remains patchy at the moment and the details are inconclusive.

3.1.6 *Gram-positive secretion system:* Gram-positive bacterial species though are largely unaffected by the polymyxin, *S. pyogenes* is one species that shows polymyxin sensitivity, which can be attributed to the absence of the gene *mprF* responsible for modifying the negative charge of phosphatidylglycerol on the

capsular polysaccharide (Vega and Caparon 2012). It has also been observed that the *S. pyogenes* ExPortal system, a part of the Sec secretion system located close to the site of cell division, is targeted by the polymyxins (Vega and Caparon 2012). The sublethal concentration of polymyxin inhibited the secretion of cysteine protease SpeB, and the streptolysin O, by disrupting the lipid structure around the ExPortal (Rosch *et al.* 2007; Vega and Caparon 2012). Interestingly, this experiment did not detect any membrane damage leading to the conclusion that this could be one of the several types of secondary effects the polymyxins have on the cell.

In summary, polymyxins primarily target the bacterial membrane. However, this is not sufficient to explain the observed lethality exerted by them. Several secondary mechanisms as discussed above certainly play a role in the overall inhibitory action of the polymyxins.

3.2 *Polymyxin resistance*

As the clinical use of polymyxin was stopped due to the toxicity issues there are not many studies conducted initially to determine the emergence of polymyxin resistance. However, in recent times there is renewed interest to understand the mechanisms of resistance to polymyxins as this remains our last hope antibiotic. The primary way by which bacteria become resistant to polymyxins is by modifying the membrane structure to reduce the interaction with the polymyxins. This is primarily accomplished by modifying the charge on the LPS moiety of the membrane to repel the cationic polymyxins. Other mechanisms can also contribute to the development of resistance such as drug efflux, decreased uptake, and production of capsules, etc.

Polymyxin resistance among the bacterial species can be looked at from two perspectives- intrinsic resistance and acquired resistance that are discussed in the following sections.

3.2.1 *Intrinsic resistance to polymyxins:* There are a few Gram-negative bacterial species known to be naturally resistant to polymyxins such as *Proteus mirabilis*, *Moraxella catarrhalais*, *Helicobacter pylori*, *Serratia marcescens*, *Providencia* spp., *Burkholderia cepacia*, *Edwardsiella tarda,* etc. (Aquilini *et al.* 2014; Biswas *et al.* 2012; Jiang *et al.* 2010; Landman *et al.* 2008; Lin *et al.* 2014). Primarily the addition of the cationic groups such as phosphoethanolamine (PEtN) and the 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the LPS moiety of the outer membrane contributes towards the resistance to polymyxins in these organisms (Raetz *et al.* 2007; Simpson and Trent 2019). This results in reducing the overall negative charges of the membrane that can no more bind to the polymyxin leading to resistance (Olaitan *et al.* 2014; Poirel *et al.* 2017a; Trimble *et al.* 2016). In these organisms, the operon *arnBCADTEF* and the gene *eptB* are constitutively expressed leading to the addition of PEtN and L-Ara4N to the LPS (Poirel *et al.* 2017a). This pathway is discussed in the following sections.

3.2.2 *Acquired resistance to polymyxins:* The acquired resistance towards the polymyxins generally involves the modification of the LPS to reduce the polymyxin-outer membrane interaction. Resistance to polymyxins can emerge by the modulation of genes involved in the LPS modification pathways. These pathways are generally regulated by the bacterial sensory modules such as two-component signal transduction systems (TCSs). Therefore, after sensing polymyxins the TCSs systems can direct the modification of the LPS leading to resistance. Resistance can also emerge by the acquisition of plasmids (mobile colistin resistance or *mcr*) encoding genes for polymyxin resistance, by horizontal gene transfer events. These mechanisms are addressed in the following sections.

3.2.3 *Two-component signal transduction system (TCS) mediated resistance to polymyxins:* Bacterial TCSs were first reported almost three decades ago highlighting their role in sensing the environmental cues and responding to the changes by modulating the expression of several genes (Stock *et al.* 2000). Almost all bacterial species possess several of these signal transduction systems in their genome; their numbers are related to the genome size and the range of environmental niches the organism lives (Bem *et al.* 2015; Bhagirath *et al.* 2019; Tierney and Rather 2019). Bacterial TCSs consisting of a sensor histidine kinase (HK) get autophosphorylated after sensing an external signal, which relays the signal to the response regulator (RR) by phosphorylating it at a conserved aspartic acid site (Stock *et al.* 2000). The activated response regulator acts as a transcription factor and affects the transcription of several genes. There are several TCSs involved in the polymyxin resistance pathway specifically in modifying the LPS (Table 1) (Huang *et al.* 2020; Simpson and Trent 2019). In our discussion, two TCSs, PhoPQ and PmrAB, which are studied in great detail will be addressed. PhoPQ and PmrAB TCSs are

Table 1.. Two-component signal transduction systems (TCSs) that are involved in the polymyxin resistance pathways in different Gram-negative bacterial pathogens

Two-component systems (TCSs)	Pathways regulated	Bacterial species	References
PhoPQ	Lipid A modification via the addition of L-Ara4N and PEtN	*E. coli*	Moon and Gottesman 2009
		S. Typhimurium	Gunn *et al.* 1998a
		P. aeruginosa	Macfarlane *et al.* 2000
		A. hydrophila[*]	Liu *et al.* 2021
PmrAB	Lipid A modification via addition of L-Ara4N and PEtN	*P. aeruginosa*	McPhee *et al.* 2003
		S. Typhimurium	Gunn *et al.* 1998b
		A. baumannii	Arroyo *et al.* 2011
		K. pneumoniae	Choi and Ko 2014
		E. coli	Trent *et al.* 2001
ParRS	Lipid A modification via addition of L-Ara4N and PEtN	*P. aeruginosa*	Fernández *et al.* 2010
CprRS	Lipid A modification via addition of L-Ara4N and PEtN	*P. aeruginosa*	Fernández *et al.* 2012
ColRS	Lipid A modification via addition of L-Ara4N and PEtN	*P. aeruginosa*	Gutu *et al.* 2013
CbrAB	Lipid A modification via addition of L-Ara4N and PEtN	*P. aeruginosa*	Yeung *et al.* 2011
CrrAB	Modulation of PmrAB	*K. pneumoniae*	Wright *et al.* 2015
Rcs system	Lipid A modification via addition of L-Ara4N and PEtN	*S.* Typhimurium	Mouslim and Groisman 2003
VprAB	Glycosylation of lipid A	*V. cholerae*	Herrera *et al.* 2014
CarRS	Glycosylation of lipid A	*V. cholerae*	Bilecen *et al.* 2015
EnvZ/OmpR	Lipid A modification via addition of L-Ara4N and modulation of PhoPQ	*A. hydrophila*	Liu *et al.* 2021

[*] In *A. hydrophila* PhoPQ is not primarily involved in the colistin resistance pathway. However, in the absence of EnvZ/ OmpR, PhoPQ system is activated.

present in most Gram-negative pathogens. Though at the molecular level there are few functional differences, for simplicity we will present a general picture for a better understanding of their role.

PmrAB and PhoPQ mediated LPS modification has been studied thoroughly in the Gram-negative bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (Chen and Groisman 2013; Groisman *et al.* 2021; Huang *et al.* 2020). Therefore, this bacterium serves as a model to understand the mechanism of resistance to cationic antimicrobial peptides (CAMPs) including polymyxins. The overall mechanism of signal transduction in the PhoPQ and PmrAB and the gene regulation leading to polymyxin resistance is depicted in figure 2. Briefly, the PhoPQ consisting of the membrane located PhoQ sensor kinase senses the environmental signals such as low magnesium (Mg^{2+}), low pH (pH 5.5), CAMPs, and polymyxins, etc., and gets autophosphorylated that in turn activates the PhoP response regulator. The activated PhoP upregulates the expression of the gene *pagL* that deacylates lipid A leading to increased hydrophobicity of the LPS resulting in less penetration of polymyxins (Han *et al.* 2018). Phosphorylated PhoP inhibits the expression of *eptB* via a small RNA regulator *mgrR* that is responsible for adding the PEtN to the Kdo (3-deoxy-D-manno-octulosonic acid) residue in the LPS (Olaitan *et al.* 2014; Poirel *et al.* 2017a). The significance of this modification is not completely understood from the polymyxin resistance point of view. The evidence though exists for the presence of other sRNAs such as MicA that may have a role in the overall regulation of the PhoPQ system (Olaitan *et al.* 2014). The phosphorylated PhoP also activates the second TCS PmrAB via a connector *pmrD*. The PmrD via protein-protein interaction stabilizes the phosphorylated PmrA by inhibiting its dephosphorylation. The PmrA response regulator activates several operons involved in the LPS modification pathways such as *arnBCADTEF* (L-Ara4N addition to LPS), *pmrCAB* (PEtN addition to lipid A), *cptA* (PEtN addition to LPS core), *pmrR* (phosphorylation of lipid A) (figure 2)

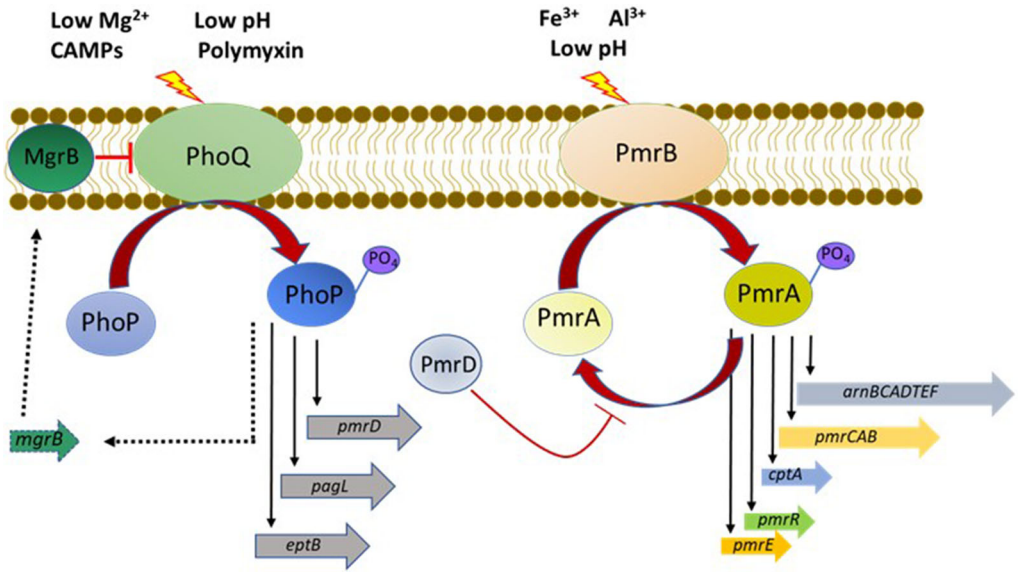

Figure 2. Bacterial two-component signal transduction system (TCS) mediated lipopolysaccharide (LPS) modification pathway leading to polymyxin resistance. The PhoPQ gets activated after sensing the environmental cues (low Mg^{2+}, Ca^{2+}, CAMPS, and polymyxin, etc.) and in turn, activates the PmrA response regulator of the PmrAB TCS via PmrD protein. The PmrA activates several genes and operons that modify the LPS by adding positively charged PEtN and L-Ara4N. This modification ultimately restricts the interaction of the membrane with the polymyxin thereby avoiding its toxic effects. The PmrB sensor kinase also gets activated sensing cations such as Fe^{3+} and Al^{3+} as well as low pH conditions and can activate the cognate response regulator PmrA. This is a general mechanism of LPS modification via bacterial TCS that is reported in *E. coli* and *S.* Typhimurium. Variations to this general mechanism are observed in the case of other Gram-negative pathogens. As shown here in the case of *K. pneumoniae*, the PhoP also modulates the expression (shown as dashed arrows) of a membrane located small protein MgrB that can inhibit the activity of PhoQ sensor kinase. This negative feedback mechanism helps in the reset process of the whole pathway (see the text for details).

(Table 1) (Nang *et al.* 2021; Olaitan *et al.* 2014). The activation of all these operons and genes converges in the LPS making it more and more positively charged resulting in the polymyxin resistance phenotype.

Though the TCS-mediated LPS modification pathway looks quite straightforward, several variations in different organisms can be found that can be attributed to their presence in different host environmental niches, pathogenesis mechanisms, and the host innate immune response. Interestingly, polymyxin-resistant bacterial pathogens exhibit mutations in the genes encoding PhoPQ, PmrAB, and the operons they regulate, leading to their constitutive expression and LPS modification. There are hundreds of mutations documented in different organisms responsible for LPS modification leading to polymyxin resistance (Huang *et al.* 2020; Liu *et al.* 2021; Olaitan *et al.* 2014; Poirel *et al.* 2017a).

Apart from these two TCSs, many bacteria possess multiple TCSs that possibly crosstalk and ultimately coordinate in the LPS modification. For example, in the *P. aeruginosa,* six TCS modules are involved in the polymyxin resistance pathways such as PhoPQ, PmrAB, ColRS, CprRS, ParRS, and CbrAB (Fernández *et al.* 2010, 2012; Gutu *et al.* 2013; Macfarlane *et al.* 2000; McPhee *et al.* 2003; Yeung *et al.* 2011). There is quite a possibility that these TCSs sense different environmental cues and get activated in different circumstances, however, they all respond similarly by activating the LPS modifying genes (Table 1) (Fernandez and Hancock 2012; Gutu *et al.* 2013; Olaitan *et al.* 2014). Similarly, in the case of *K. pneumoniae* an additional TCS CrrAB has been reported to be involved in the polymyxin resistance pathway (McConville *et al.* 2020; Wright *et al.* 2015) along with the PmrAB and PhoPQ systems. Interestingly, the CrrAB TCS operates via modulating the PmrAB system (Cheng *et al.* 2016). *K. pneumoniae* also possesses a negative regulator (MgrB) of PhoQ kinase, inactivation of which can lead to upregulation of the PhoPQ system (figure 2) (Cannatelli *et al.* 2014; Poirel *et al.* 2015). It can be argued that these complex regulatory architecture among the bacteria has evolved to integrate

a diverse range of environmental stimuli to a common response by the modification of the outer membrane.

In the pathogen *A. baumannii*, a very interesting phenomenon is observed concerning the polymyxin resistance, where the complete LPS is lost from the bacterial cell making it resistant to polymyxins. This happens because of the insertional inactivation by the ISAba11 element in the lipid A biosynthetic cluster involving the genes *lpxA*, *lpxC*, and *lpxD* (Moffatt *et al.* 2011, 2019, 2010). As the lipid A synthesis is stopped, the polymyxin target is no more available in the *A. baumannii* cells leading to a very high level of resistance. Interestingly, in *A. baumannii* PmrAB is the only TCS that is involved in the polymyxin resistance pathway (Nang *et al.* 2021).

In the aquatic bacterial pathogen *Aeromonas hydrophila*, resistance to polymyxins is not so prevalent. However, with the indiscriminate use of polymyxins in aquaculture, resistance is increasingly being reported (Gonzalez-Avila *et al.* 2021). In *A. hydrophila*, the primary way by which colistin resistance develops is by increased expression of the TCS EnvZ/OmpR that regulates the LPS modification pathway via the addition of L-Ara4N moiety to the lipid A (Liu *et al.* 2021). It has also been observed that EnvZ/OmpR regulates the expression of an outer membrane protein (encoded by the gene 3832) which is predicted to be an autotransporter possibly involved in colistin uptake. Expression of this autotransporter is upregulated in presence of colistin, whereas deletion of the EnvZ/OmpR TCS abolishes its expression. Another significant difference concerning *A. hydrophila* colistin resistance is that the TCS PhoPQ does not play a primary role in colistin resistance. However, in the absence of the EnvZ/OmpR, PhoPQ gets activated and modify the LPS via PEtN modification (Liu *et al.* 2021). It remains to be discovered how EnvZ/OmpR system is connected to PhoPQ at the molecular level, though the existence of a protein connector between the two TCSs cannot be ruled out.

Apart from the TCSs some bacteria also employ various other strategies to mount resistance to polymyxins. Important among them is the formation of biofilms at the site of infections, in the medical devices, and their environmental niche. Biofilms are considered to be a response to the stress and the biofilm architecture is such that it makes the biofilm residents recalcitrant to very high concentrations of antibiotics including polymyxins. In *P. aeruginosa*, *psrA,* and *cbrA,* and in *Vibrio cholerae* the *carR* genes are found to be regulating the biofilm formation as well as developing polymyxin resistance (Bilecen *et al.* 2015; Gooderham *et al.* 2008). *K. pneumoniae* is known to produce a lot of capsular polysaccharides that can use them to trap antimicrobial peptides including polymyxins reducing their access to the outer membrane leading to resistance development (Llobet *et al.* 2008), though this mechanism is still not fully understood.

Bacterial efflux pumps are very important to expel the accumulated noxious substances inside the cell. Many of these efflux pumps also contribute to the polymyxin resistance pathway. The efflux pumps such as AcrAB-TolC, MtrC-MtrD-MtrE, RosAB, KpnEF, and VexAB, etc. are shown to be responsible for developing tolerance to polymyxins in several bacterial pathogens (Bengoechea and Skurnik 2000; Bina *et al.* 2008; Fehlner-Gardiner and Valvano 2002; Padilla *et al.* 2010; Srinivasan *et al.* 2014; Tsai *et al.* 2012; Tzeng *et al.* 2005; Warner and Levy 2010). In polymyxin B resistant *E. coli* strains the *marRAB* operon has been shown to upregulate the AcrAB-TolC efflux pump (Warner and Levy 2010). Apart from the efflux systems, the outer membrane porins (OMPs) are also shown to be involved in polymyxin resistance most likely by limiting the uptake (Mathur and Waldor 2004).

3.2.4 *Plasmid-mediated polymyxin resistance:* For quite a long time, it was accepted that bacteria become resistant to polymyxins via different adaptation mechanisms including the LPS modification pathways. However, a plasmid-mediated gene *mcr-1* (mobilized colistin resistance gene) was detected in the *E. coli* and *K. pneumoniae* isolates from China during the years 2011-2014 (Liu *et al.* 2016). The *mcr-1* gene encodes a protein that is homologous to the phosphoethanolamine (PEtN) transferase enzyme involved in the LPS modification pathway. This finding of the plasmid-borne gene *mcr-1* is quite significant considering that it could be acquired horizontally leading to the rapid development of polymyxin resistance (Ayoub Moubareck 2020; Mmatli *et al.* 2020; Poirel *et al.* 2017b). As *mcr-1* expression alone increases the MIC of polymyxin by 4- to 8-fold, a single step of acquisition of the plasmid can make the susceptible bacteria resistant.

Since the initial discovery of the *mcr-1* in China, this gene has been reported from across the world. There is strong evidence that the emergence of the plasmid encoding *mcr-1* gene correlates with the widespread use of polymyxins in farm animals as a growth supplement several years ago. Therefore, it is quite possible for the rapid transmission of the plasmid along with the food supply. This gene is associated with the

Enterobacteriaceae isolated from the environment, food, meat products, animals, and humans (Poirel *et al.* 2017a). Since the first report of *mcr-1* nine more *mcr* genes (*mcr-2* to *mcr-9*) encoded on different plasmids have been reported (Ayoub Moubareck 2020; Carroll *et al.* 2019; Gonzalez-Avila *et al.* 2021; Mmatli *et al.* 2020). The various *mcr* genes act similarly and are classified based on their sequence divergence from each other. The *mcr-1* is the most prevalent type in the environment. Considering its widespread occurrence around the world, it could make polymyxins ineffective if urgent steps are not taken to curb the use of polymyxins in animal farming.

3.3 *Potential solutions to polymyxin resistance*

The emergence of resistance to different drugs including polymyxin is inevitable considering it is a normal physiological and evolutionary process. Therefore, to keep our last resort antibiotic useful for times to come, its use must be rationalized to prolong the development and spread of resistance. Meanwhile, there have been several novel methods that can be considered to develop new drugs, as well as the use of combinatorial treatment approaches must be adopted. Some of these approaches are described as follows.

3.3.1 *Bacterial TCS as novel drug targets:* Studies in several bacterial species have identified dedicated TCS modules that are implicated in the regulation of genes involved in antibiotic resistance pathways. In the case of *S. aureus*, TCSs such as BraSR, VraSR, GraSR, VanSR are responsible for developing resistance against bacitracin, vancomycin, and several other CAMPs (Bem *et al.* 2015; Depardieu *et al.* 2007; Fridman *et al.* 2013; Hiron *et al.* 2011). In *M. tuberculosis* the TCS MtrAB is implicated in the multidrug resistance phenotype (Nguyen *et al.* 2010). Similarly, in the cases of *Enterobacteriaceae*, TCSs such as BaeSR, ArcBA, PhoPQ, PmrAB, PhoBR, CpxAR, LysR, etc. have been found to contribute to the drug resistance against various classes of antibiotics such as β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, polymyxins, and several CAMPs (Bem *et al.* 2015; Doddangoudar *et al.* 2011; Fernandez and Hancock 2012; Fernández *et al.* 2012; Guerrero *et al.* 2013; Gunn 2008; Kawada-Matsuo *et al.* 2013; Nishino 2018). Specific TCS knockout strains have shown a significant decrease in the emergence of resistance. Apart from the TCSs, which are directly implicated in the emergence of drug resistance, some of them have been found to modulate the expression of drug efflux pumps, membrane transporter proteins that actively pump out the drugs from the bacterial cells leading to resistance. Most importantly, pathogens such as *K. pneumoniae* and *A. baumannii* possess several of these efflux systems that get signals from the TCS and lead to drug resistance (Laub and Goulian 2007; Stock *et al.* 2000).

Considering their role in different physiological processes of the bacterial cell including the development of drug resistance, the TCSs have been explored as novel drug targets (Bhagirath *et al.* 2019; Hirakawa *et al.* 2020; Tierney and Rather 2019; Worthington and Melander 2013). Several investigators have attempted to develop novel drugs by screening small molecules against the bacterial TCSs. A pioneering study in this regard was done in the Sperandio lab where a TCS QseCB in the *E. coli* was targeted as a part of their antivirulence strategy. QseCB is involved in the quorum-sensing pathway leading to the expression of virulence genes. High-throughput screening to search for a QseC kinase inhibitor led to the identification of the compound LED209, which specifically inhibits the autophosphorylation of the QseC kinase (Curtis *et al.* 2014; Rasko *et al.* 2008). An important TCS present among the firmicutes (low G+C Gram-positive) group of bacteria known as WalKR, play an important role in the metabolism, stress response, virulence, and other regulatory pathways (Dubrac *et al.* 2008, 2007; Howden *et al.* 2011; Velikova *et al.* 2016). Because of its role in several physiological processes, WalKR has been used as a drug target and studies from the Utsumi group have shown promising results with the identification of a class of WalK inhibitors named walkmycin (Eguchi *et al.* 2011; Okada *et al.* 2010). These groups of compounds inhibit the formation of biofilm and the development of competence in *Streptococcus mutans*. Similarly, another WalK inhibitor named signermycin was also identified that targets the WalK dimerization (Watanabe *et al.* 2012).

Resistance against vancomycin, considered to be one of the most potent antibiotics, arises because of the VanSR TCS in the *Enterococcus faecalis* and *E. faecium*. Inhibitors of VanS kinases have been found that function by uncoupling the energy required for ATP synthesis. However, such inhibitors were not found to be suitable considering their negative impact on mitochondrial respiration. Nevertheless, such compounds serve as templates to develop more potent and specific inhibitors (Barrett *et al.* 1998; Macielag *et al.* 1998). In the case of *M. tuberculosis,* a TCS named DosRS (also called DevRS) contributes to its survival in the hypoxic

conditions in the macrophages (Honaker *et al.* 2009; Park *et al.* 2003). Small molecule inhibitors have been identified against the DosR response regulator that can bind and inhibit its DNA binding property (Gupta *et al.* 2009; Mai *et al.* 2011). For the TCSs involved in polymyxin resistance pathways, studies have been conducted to find anti PhoPQ compounds. A compound called radicicol, a known Hsp90 inhibitor was found to inhibit the kinase activity of the PhoQ (Guarnieri *et al.* 2008). Similarly, virtual screening based on the putative PhoQ 3D structure of *Shigella flexneri* identified four different compounds that have potential anti-PhoQ kinase activities (Cai *et al.* 2011). These compounds exhibited a binding affinity for the cytoplasmic domain of the PhoQ inhibiting it. Interestingly, the compounds were also found to be inhibiting the *S. flexneri* to invade the HeLa cells in cell culture indicating their antivirulence characteristics (Cai *et al.* 2011).

Most of the drug candidates developed against the TCS work by blocking the signal transduction steps such as – inhibition of the sensor kinase activity, inhibition of the response regulator activity, signal sequestration, etc. (Hirakawa *et al.* 2020). All these studies show the potential of TCS-based antibiotic development, which needs to be seriously pursued.

3.3.2 *Drug combination therapy:* On many occasions, antibiotics are used in combinations for better treatment outcomes, to restrict the emergence of resistance, etc. In this regard, polymyxins seem to work synergistically with several other antibiotics. Carbapenems have been used in combination with polymyxins against the MDR Gram-negative pathogens resulting in impressive therapeutic outcomes (Clancy *et al.* 2013). The polymyxin-doripenem combination was shown to be synergistic with enhanced bactericidal activity against the *A. baumannii* strains (Park *et al.* 2016). Against *P. aeruginosa* this combination has shown increased bactericidal activity including against the colistin heteroresistant subpopulations (Bergen *et al.* 2011; Deris *et al.* 2012). Interestingly, the polymyxin-doripenem combination also exhibited anti-biofilm activity in a dynamic biofilm model, as well as inhibited the emergence of colistin resistance highlighting the therapeutic potential of the combination (Lora-Tamayo *et al.* 2014).

Polymyxin-rifampicin combination showed very good effectiveness against MDR *A. baumannii* and KPC-producing *K. pneumoniae* (Biswas *et al.* 2012; Gaibani *et al.* 2014; Lagerback *et al.* 2016; Liang *et al.* 2011). Colistin in combination with meropenem and tigecycline has shown promising results against carbapenem-resistant *A. baumannii* strains (Liang *et al.* 2011; Sheng *et al.* 2011). Several studies have been conducted using novel combinations of polymyxin with other antibiotics including vancomycin, fosfomycin, aminoglycosides, daptomycin, doxycycline, etc. resulting in various efficacies and treatment outcomes (Nang *et al.* 2021).

In summary, the drug combination therapy could be a good alternative to polymyxin monotherapy considering the increased risk of the emergence of resistance. However, more rigorous studies are warranted to systematically explore the combination therapy approaches.

4. Concluding remarks and future perspectives

The rapid emergence of MDR pathogens has necessitated the urgent development of novel drugs and therapeutic interventions. As we have exhausted our arsenal of antibiotics, very few treatment options are available at the moment. The polymyxins, our last line antibiotics, therefore, must be used judiciously to prevent the onset of resistance. Recent studies using polymyxin have shown promising results clinically that have improved in designing a better dosing regimen. Also, the development of less toxic derivatives of polymyxins has reduced nephrotoxicity and neurotoxicity to a great extent. However, the mode of action of polymyxins at the molecular level is not yet fully understood, and at the same time, significant research effort must be put forth to develop an understanding of the resistance mechanism. These efforts would possibly further improve our treatment strategy in the future. The indiscriminate use of polymyxins must be curbed to preserve its usefulness at least till the time a better alternative drug emerges. The use of polymyxins as a growth supplement in animal farming must also be restricted which is responsible for the global spread of mobile colistin resistance (*mcr*) genes. Meanwhile, investment in novel antibiotic drug development must be encouraged and pursued urgently to delay the arrival of the post-antibiotic era.

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