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Significance of Pseudomonas *aeruginosa* efflux pump system in antibiotic resistance

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Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen with a high morbidity and mortality rate in immunocompromised and cystic fibrosis patients. The treatment of *P. aeruginosa* infections is becoming increasingly difficult due to the bacteria's capacity to tolerate numerous antimicrobial agent. One of the key mechanisms of antimicrobial resistance (AMR) in *P. aeruginosa* that contributes to both innate and acquired resistance is the efflux pump system. Understanding the structure, function, and control of the main efflux pump system, specifically the resistance nodulation division superfamily, is critical. This could provide baseline data for the development of a new antibacterial that targets this crucial resistance pathway in *P. aeruginosa*, thereby halting or slowing morbidity and death associated with AMR.

Keywords: Efflux pump, Resistance nodulation division family, *P. aeruginosa*

1.0 Introduction

Efflux pumps are protein transporters that are found in the cytoplasmic membrane of all types of cells (Rahbar *et al*., 2021). They are active transporters, which means that, to function, they need a source of chemical energy. Others are secondary active transporters in which transport is coupled to an electrochemical potential difference produced by pumping hydrogen or sodium ions into the cell. Some are primary active transporters that use adenosine triphosphate hydrolysis as a source of energy (Rahbar *et al*., 2021). Antibiotics, heavy metals, organic pollutants, substances produced by plants, quorum-sensing signals, bacterial metabolites, and neurotransmitters are just a few of the hazardous substances that can be actively efflux out of cells by Efflux pumps (Puzari and Chetia, 2017). This active efflux mechanism has been attributed to be responsible for various types of resistance to bacterial pathogens within bacterial species - the most concerning being antibiotic resistance because microorganisms can have adapted efflux pumps to divert toxins out of the cytoplasm into extracellular media (Blanco *et al*., 2016). Efflux pumps are found in almost all bacterial species, and genes encoding this class of proteins can be located on chromosomes or plasmids (Sun *et al*., 2014). In addition to their essential role in the resistance of *P. aeruginosa* to several classes of antimicrobial agents, efflux pumps play more expanded and potential physiological functions in the virulence, host-pathogen interactions, pathogenesis, quorum sensing, and biofilm formation (Hirakata *et al*., 2002; Baugh *et al*., 2014; Sun *et al* .,2014; Alav *et al*., 2018 Colclough *et al*., 2020).

1.1 Families of efflux pumps

Based to their composition, amino acid sequence, several transmembrane spanning regions, and energy sources used to export their substrates and substrates, bacterial multidrug efflux systems are classified into six families (Blair *et al*., 2014) as follows:

1.1.1. ATP-binding cassette superfamily

ATP hydrolysis for transportation provides energy for this family. P-glycoprotein, which has two hydrophobic transmembrane domains (TMD1 and TMD2) and two nucleotide-binding

domains (NBD1 and NBD2), is the most significant efflux pump in the family. These efflux pump subgroups are the primary active transporters (Colclough and Colleagues, 2020).

1.2.2. The major facilitator superfamily (MFS)
Both intrinsic and acquired antibiotic resistance are encoded by MFS, which makes up most of the antimicrobial efflux pumps in both types of bacteria. For their energy needs, members of this superfamily use the proton gradient (Colclough *et al*., 2020).

1.1.3. Resistance-nodulation-division (RND) family
Members of this family are prominent in GNB, providing intrinsic and acquired antibiotic resistance. They have tripartite organizations causing the expulsion of a broad spectrum of antibiotics. The RND family efflux pumps are proton-dependent and are secondary active transporters (Singh *et al.,* 2020).

1.1.4. Small multidrug resistance (SMR) family.
A member of this family is made up of four transmembrane helices and tiny proteins with roughly 150 conserved amino acids. Secondary active transporters include these efflux pumps (Colclough *et al*., 2020).

1.1.5. Multidrug and toxic compound extrusion (MATE) family
This family's transporters are found in plants, people, and animals. They are a subset of the new superfamily. A gradient of Na, H, or both serves as the energy source for these transporters. Genes for MATE efflux pump have been cloned from numerous diseases but have not been examined in the species from whence they originated. Secondary active transporters include these families of efflux pumps (Singh et al., 2020).

1.1.6. Proteobacterial antimicrobial compound efflux (PACE) family
This new set of efflux pumps in certain GNB has been discovered. Although there is little information on PACE's energy source for drug delivery and resistance mechanism, it is known that they impart resistance to a variety of biocides (Hassan *et al*., 2015). Efflux pump genes of PACE family members are conserved in the core of the bacterial genome with highly conserved amino acids.

Except for the RND superfamily, which is only found in Gram-negative bacteria, efflux systems of the other four families: MFS, ABC, SMR and MATE, are widely distributed in both Gram-positive and negative bacteria.

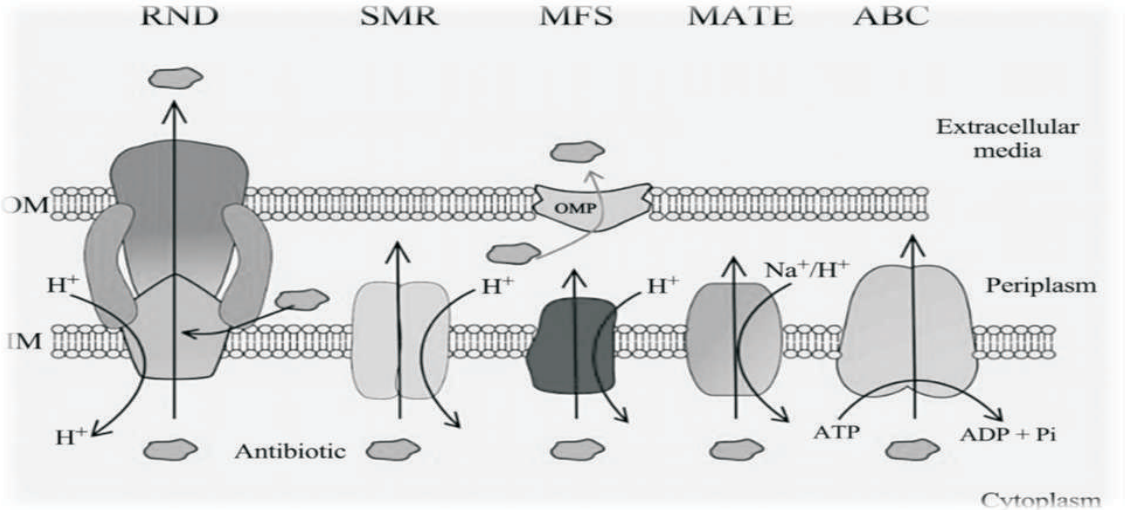

Fig. 1.0 Schematic illustration of the five prominent families of efflux transporters: the RND: resistance- nodulation-division family; SMR: the small multidrug resistance family; MFS: the major facilitator superfamily; MATE: the multidrug and toxic compound extrusion; ABC: family and the adenosine triphosphate (ATP)-binding cassette superfamily. IM: Inner membrane. OM: Outer membrane. OMP: Outer membrane protein (Source: Blanco *et al.,* 2016).

2.0 Efflux pump system in *Pseudomonas aeruginosa*

The most clinically relevant efflux systems in Pseudomonas aeruginosa belong to the (RND) family. Four efflux pump systems have been extensively studied and have been associated with antimicrobial resistance in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM are significant determinants for resistance to various drugs (Rocha *et al*., 2019; Colclough *et al*., 2020). The cytoplasmic and periplasmic components of *P. aeruginosa* RND pumps are named multidrug efflux (Mex) along with a letter. The outer membrane porin is named Opr and a letter (Pang *et al*., 2019). These efflux pumps operate by capturing antimicrobial compounds within the periplasm, cytoplasmic space, or plasmalemma and ejecting them outward into the extracellular matrix through a three-protein conduit. Efflux pumps are energy-dependent using a proton motive driving force. Although these efflux pumps have broad substrate ranges, the greatest is seen with MexAB-OprM; not all compounds are susceptible to the activity of each pump (Colclough *et al*., 2020).

2.1 Structure and mode of action of MexAB-OprM RND efflux pump.

These pumps are systems with three components. These pumps consist of three components. The first component is a protein located in the cytoplasmic membrane known as the transporter protein (MexB, MexD, MexF and MexY), which utilizes an energy-dependent pump with broad substrate specificity. The second component is an outer membrane protein, referred to as the outer membrane porin protein (OprM, OprJ and OprN). The third protein is linker protein (MexA, MexC, MexE and MexX) (Rocha *et al*., 2019). It is now confirmed that RND pumps remove antibacterial agents from the periplasm either during entry into the bacterium or after removal from the cytoplasm by a transporter. An outer membrane protein, a moderate periplasmic protein, an inner membrane protein, and a transmembrane duct are the main components of MexAB-OprM Efflux pumps. The cell's outer membrane contains the transmembrane duct (Shi *et al*., 2019). A periplasmic membrane protein and an integral membrane transporter are two more proteins linked to the duct. The system's inner membrane protein and periplasmic membrane protein are connected to regulate the duct's opening and shutting (channel). The inner membrane proteins initiate a metabolic cascade when a toxin binds to them, signaling the periplasmic membrane protein and outer membrane protein to open the channel and release the poison from the cell. An energy-dependent, protein-protein interaction is utilized in this method. (Ughachukwu and Unekwe, 2012; Wang *et al*., 2017; Shi, *et al*., 2019).

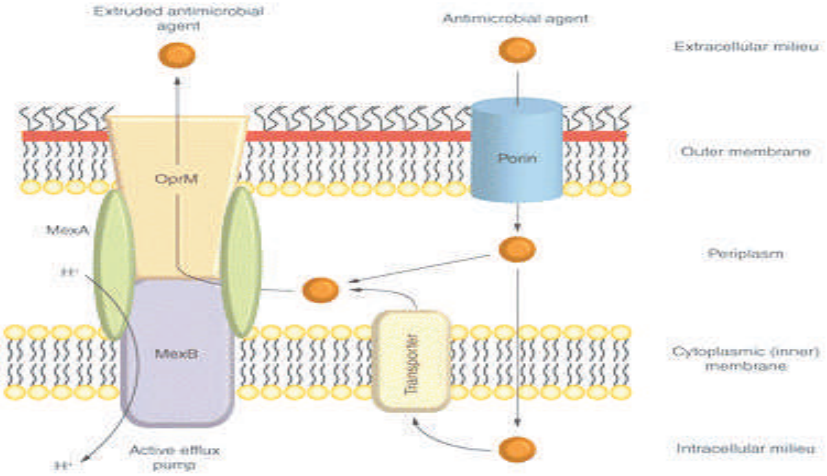

Figure 2.0 A schematic illustration of the MexAB-OprM efflux pump in *Pseudomonas aeruginosa* . These efflux systems span the entire cell envelope and consist of the transporter, or the pump located in the cytoplasmic membrane (MexB), which physically contacts the outer membrane channel protein (OprM). The linker protein (MexA) plays a vital role in the assembly and function of the efflux system.

Table 1. The summary of major efflux pumps of *P. aeruginosa*.

System	Regulatory gene	Cytoplasmic membrane transporter protein	Membrane fusion protein	Outer membrane porin protein	Mutation causing upregulation	Substrates
MexAB-OprM	mexR (repressor)	MexB	MexA	oprM	nalB (affects *mexR*) and *nalC* (lies outside *mexR*)	BL, FQ, CM, TC, NV, TP, SM, ML,
MexCD-oprJ	*nfxB* (repressor)	MexD	MexC	oprJ	*nfxB*	BL, FQ, CM, TC, NV, TP,
MexEF-oprN	*mexT* (activator) MexS (repressor)	MexF	MexE	oprN	*nfxC*	FQ, CM, TP,
MexXY-oprM	*mexZ* (repressor)	MexY	MexX	OprM	ParRS§	FQ, AG, TC, ER

Key: AG: Aminoglycosides; BL: β-lactams; CM: Chloramphenicol; ER: Erythromycin; FQ: Fluoroquinolones; ML: Macrolides; NV: Novobiocin; SM: Sulphonamides; TC: Tetracycline; TP: Trimethoprim.

2.2 Genetic organization of *P. aeruginosa* RND efflux operons.

The genetic organization of RND efflux pump operons comprises three steps. First, the genes regulating the RND transporter and membrane fusion protein are always present. Second, the genes encoding the operon regulators and the outer membrane channel proteins are not always present, considering that some operons do not have regulatory genes or outer membrane regulatory genes linked to the efflux operon. Third, some additional regulatory genes can be present besides the regulatory genes for the efflux transporter and membrane fusion protein, such as in the case of mexGHIopmD, which contains mexG which encodes a membrane protein required for pump function (Askoura *et al*. 2011).

2.3 Regulation of expression of *Pseudomonas aeruginosa* RND efflux pumps.

The transcriptional regulation of RND efflux systems is mediated by local and global regulators acting to fine-tune gene expression in response to a range of signals. Some of these signals, such as increased intracellular antibiotic concentration, lead to temporary increases in RND gene expression. In contrast, mutations permitting increased RND gene expression can confer increased tolerance or resistance to antimicrobials. Mutations in regulators and repressors result in the overexpression of these elements, having a significant role in the acquisition and expression of the MDR phenotype (Olivares *et al*., 2017).

Expression of the MexAB-OprM is governed mainly by regulatory loci such as *mexR, nalC* and *nalD* (Fig. 1a), amongst several others. The *mexR* gene, encoding a repressor protein of MarR family, is located upstream of the *mexAB-oprM* operon (Tian *et al*., 2016). An intergenic region with divergently oriented genes on either side, *mexR* and *mexAB-oprM*, controls their transcription. MexR binds to this intergenic region as a stable homodimer and represses transcription from the *mexAB-oprM* operon. Mutations in *mexR* (*nalB* mutants), resulting in loss of dimerization and binding capacity of

MexR protein led to hyperexpression of MexAB-OprM (Pan *et al*., 2016). MexR protein is also endowed with oxidation- sensing mechanism which regulates virulence and antibiotic resistance in *P. aeruginosa* (Chen *et al*. 2008; Lister *et al*. 2009; Choudhury *et al*. 2016). The *nalC* encodes a protein, NalC, of TetR family, which acts as a repressor of a divergent two-gene operon comprising *PA3720* and *PA3719* (renamed *armR*). ArmR works as an anti-repressor by allosterically inhibiting the dimeric MexR repressor resulting in derepression of *mexAB-oprM* (Cao *et al*. 2004; Braz *et al*. 2016). Another member of the TetR family of transcriptional regulators, NalD, acts as a secondary repressor of the tripartite MexAB-OprM multidrug efflux system by binding to a sequence between *mexAB-oprM* and the *mexR* binding site proximal to the *mexA* promoter. Hence, impairment of NalD in nalD-type mutants leads to MexAB-OprM overexpression (Morita *et al*., 2006; Chen *et al*., 2016).

In contrast to MexAB-OprM, the MexCD-OprJ system does not contribute to the natural resistance of the pathogen to antimicrobials (Jeannot *et al*., 2008). However, the alteration of the *nfxB* gene, whose product strongly represses the *mexCD-oprJ* operon, leads to a dramatic increase in MexCD-OprJ production and a significant cross-resistance to fluoroquinolones, macrolides, novobiocin, tetracyclines, and zwitterionic cephems like cefepime and cefpirome (Jeannot *et al*., 2008; Morita *et al*., 2006).

MexEF–OprN contributes to antibiotic resistance to chloramphenicol, quinolones, trimethoprim, and imipenem (Ko hle *et al*., 1997). This tripartite pump is regulated by MexT, a LysR-like activator whose gene (*mexT*) is located upstream from operon MexEF-OprN (Ko hle *et al*., 1999).

MexXY operon lacks the gene encoding outer membrane protein, in contrast to other operons encoding the MexAB-OprM, MexCDOprJ, and MexEF-OprN systems. OprM, a component of the outer membrane, is predominantly used by the MexXY system, along with probably other outer membrane proteins, including OpmB, OpmG, OpmH, and OpmI, to create a functional tripartite system (Suresh *et al*., 2018). Expression of the *mexXY* operon is negatively regulated by the *mexZ* gene product, located upstream of the operon and transcribed divergently (Fig. 1b). MexZ, another protein which belonging to the TetR family, has a DNA-binding helix-turn-helix motif at its N-terminus. AgrZ, AgrW1 and AgrW2 are the three types of MexXY-overproducing mutants that have been identified. The DNA-binding, dimerization, or other structural domains of the encoded repressor can be altered in the agrZ mutants, which also carry mutations that render the mexZ gene inactive. While agrW2 mutants hyper express MexXY with changes in the sensor ParS or the response regulator ParR of the two-component ParRS system, both are known to be crucial in multidrug resistance, and impaired protein synthesis occurs in agrW1 mutants due to a variety of defects in ribosomal proteins. (Lister *et al*. 2009; Morita *et al*. 2012; Li *et al*. 2015). The MexXY multidrug efflux system is considered a significant determinant of aminoglycoside resistance in clinical strains of *P. aeruginosa*, particularly those isolated from the patients with cystic fibrosis (Morita *et al*. 2012).

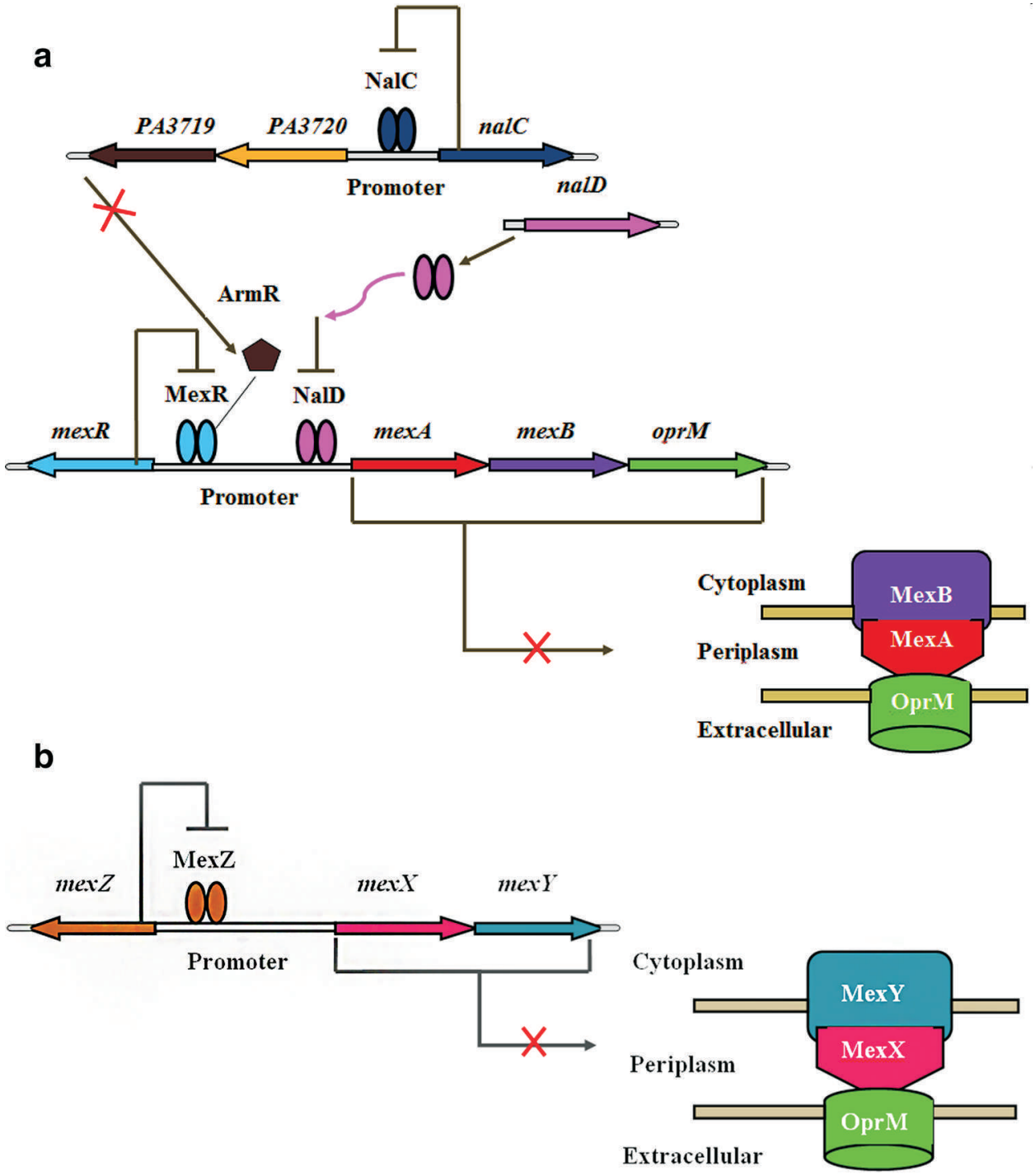

Fig 3.0 Schematic representation of transcriptional regulatory mechanisms controlling MexAB-OprM and MexXY-OprM efflux pumps in *P. aeruginosa*. Transcriptional repression of the *mexAB-oprM* operon is mediated directly by MexR and NalD proteins and indirectly by NalC, which represses ArmR protein, an anti-repressor of MexR. B Transcriptional repression of the *mexXY* operon is mediated directly by MexZ (Suresh *et al*., 2018).

2.4 Significance of *P. aeruginosa* efflux pump system in antibiotic resistance

The significance of the *P. aeruginosa* efflux pump system in antibiotic resistance lies in its ability to pump out a wide range of antibiotics. This means that even when multiple antibiotics are used in combination to treat an infection, the bacteria can still survive if the efflux pumps are overexpressed. Furthermore, the efflux pump system can confer resistance to antibiotics that are not normally effective against *P. aeruginosa*, making it an even more formidable opponent in the fight against infections. Efforts to develop new antibiotics are often hindered by the presence of efflux pumps in bacteria like *P. aeruginosa*. Even when a new antibiotic is discovered, it may not be effective against P. aeruginosa if the bacteria are overexpressing their efflux pumps. Therefore, researchers are looking for ways to target efflux pumps directly, either by inhibiting their function or by blocking their expression.

One promising approach is to develop efflux pump inhibitors (EPIs) that could prevent the pumps from functioning. Several EPIs have been identified that are effective against the efflux pump system in *P. aeruginosa,* including PAβN and phenylalanine-arginine-β-naphthylamide (PAβN). These compounds work by binding to the efflux pumps and preventing them from pumping out antibiotics, making the bacteria more susceptible to these drugs.

Conclusion

Given the importance of efflux pumps in *P. aeruginosa* resistance, understanding their structure and mechanism of action could be used to develop new agents that can interfere with their function by down regulating the expression of efflux pump genes through genetic regulation, redesigning antibiotics that are no longer recognized as substrates, or (iii) inhibiting the assembly of functional efflux pump. This will assist to reduce resistance-related mortality in *P. aeruginosa* infections. The *P. aeruginosa* efflux pump system plays a significant role in antibiotic resistance and is an important target for new drug development. Efflux pumps are a major mechanism for antibiotic resistance in bacteria, and the efflux pump system in *P. aeruginosa* is particularly effective at pumping out a wide range of antibiotics. Efforts to develop new antibiotics are often hindered by the presence of efflux pumps, but the development of efflux pump inhibitors shows promise as a new strategy for combating antibiotic resistance

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Conflict of interest

The authors have no conflict of interest to declare.

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