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Review

Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations

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ABSTRACT

Multidrug resistance (MDR) refers to the capability of bacterial pathogens to withstand lethal doses of structurally diverse drugs which are capable of eradicating non-resistant strains. MDR has been identified as a major threat to the public health of human being by the World Health Organization (WHO). Among the four general mechanisms that cause antibiotic resistance including target alteration, drug inactivation, decreased permeability and increased efflux, drug extrusion by the multidrug efflux pumps serves as an important mechanism of MDR. Efflux pumps not only can expel a broad range of antibiotics owing to their poly-substrate specificity, but also drive the acquisition of additional resistance mechanisms by lowering intracellular antibiotic concentration and promoting mutation accumulation. Over-expression of multidrug efflux pumps have been increasingly found to be associated with clinically relevant drug resistance. On the other hand, accumulating evidence has suggested that efflux pumps also have physiological functions in bacteria and their expression is subject tight regulation in response to various of environmental and physiological signals. A comprehensive understanding of the mechanisms of drug extrusion, and regulation and physiological functions of efflux pumps is essential for the development of anti-resistance interventions. In this review, we summarize the development of these research areas in the recent decades and present the pharmacological exploitation of efflux pump inhibitors as a promising anti-drug resistance intervention.

Contents

Abbreviations: $3OC_6$-HSL, 3-oxohexanoyl homoserine lactone; $3OC_{12}$-HSL, N-(3-oxododecanoyl)-L-homoserine lactone; ABC, the ATP (adenosine triphosphate)-binding cassette superfamily; ABI-PP, AcrAB/MexAB-specific inhibitor of pyridopyrimidine derivative; AHL, *N*-acylhomoserine lactones; BRC, BmrR C terminus; C_4-HSL, N-butyryl homoserine lactone; CCCP, carbonylcyanide-3-chlorophenylhydrazone; CTD, C-terminal domain; DARPin, designed ankyrin repeat protein; DBD, DNA binding domain; DDM, n-dodecyl-D-maltoside; DMT, drug/metabolite transporter superfamily; DNP, 2,4-dinitrophenol; EPI, efflux pump inhibitor; Eb, ethidium bromide; EMSA, electrophoretic mobility shift assay; Et, ethidium; MATE, the multidrug and toxic compound extrusion family; MDCK, Madin–Darby canine kidney; MDR, multidrug resistance; MFS, the major facilitator superfamily; MIC, minimum inhibitory concentration; NMP, naphthylpiperazines; NP, nature product; Pf, proflavin; PAβN, phenyl-arginine beta-naphthylamide; PQS, 2-heptyl-3-hydroxy-4-quinolone; QS, quorum sensing; RND, the resistance-nodulation-division family; SMR, the small multidrug resistance family; TCS, two component system; TPP, tetraphenylphosphonium.

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1. Introduction: drug efflux transporters and their clinical relevance

Efflux pumps are found in almost all bacterial species and genes encoding this class of proteins can be located on chromosomes or plasmids [1,2]. According to their composition, number of transmembrane spanning regions, energy sources and substrates, bacterial efflux pumps are classified into five families: the resistance-nodulation-division (RND) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family [a member of the much larger drug/metabolite transporter (DMT) superfamily], and the multidrug and toxic compound extrusion (MATE) family [1–3]. 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 [4]. Depending on the specific classes they belong to, efflux pumps are either single-component transporters or multiple-component systems containing not only an inner membrane transporter, but also an outer membrane channel and a periplasmic adaptor protein, such as the RND type efflux pumps [5]. Owing to their tripartite composition which allows direct extrusion of various drugs from cytosol or periplasmic space to the outside of bacterial cells, RND family pumps have been found to be associated extensively with clinically significant antibiotic resistance, such as AcrB in *Escherichia coli* and *Salmonella typhimurium* and MexB in *Pseudomonas aeruginosa*. In Gram-positive bacteria, the clinically significant efflux pumps are members of the MFS family, for example NorA in *Staphylococcus aureus* and PmrA in *Streptococcus pneumoniae* [6].

In recent decades, with the development of various molecular approaches [5], such as reverse transcription quantitative PCR (RT-qPCR) and immunoblotting, association of efflux pump overexpression with clinically relevant levels of MDR has been increasingly reported [7]. For instance, a recent screening of 50 clinical *E. coli* strains isolated from human clinical samples and dog feces in Sapporo, Japan, revealed a strong correlation of overexpression of the AcrAB efflux pumps with the high-level fluoroquinolone resistance in all 20 multi-resistant strains [8]. In another screening of 52 *Klebsiella pneumoniae* strains isolated from burn patients hospitalized in Shahid Motahari Hospital, Tehran, all 40 isolates which displayed resistance to ciprofloxacin, tetracycline, ceftazidime and gentamicin were found to express high levels of the AcrAB efflux pump particularly in ciprofloxacin resistant strains [9]. In addition, clinical resistance caused by overexpression of more than one efflux pumps was also identified. For instance, simultaneous overexpression of the MexAB–OprM and MexXY efflux systems was demonstrated to account for the multi-resistance phenotype of a collection of 12 *P. aeruginosa* clinical isolates identified in a hospital in France [10]. A clinical isolate of *Stenotrophomonas maltophilia* strain with high minimum inhibitory concentration (MIC) of several antibiotics was found to coordinately hyper-express the RND family efflux pumps SmeZ and SmeJK [11]. In addition to those encoded on the chromosomes of bacteria, plasmid-encoded efflux pumps, such as OqxAB, which confer resistance to olaquindox, was also found to cause drug resistance in *E. coli* clinical isolates [12]. Association of efflux pump overexpression with clinically relevant MDR in Gram-positive bacteria was also reported. Among several hundred clinical isolates of *S. aureus* studied by Christos Kosmidis et al. it was found that strains overexpressing efflux pump genes were common and were widely distributed geographically. These strains were mainly resistant to methicillin and the resistance was clonally related with *norA* and *mepA* overexpression [13].

2. Mechanisms of drug extrusion by the efflux pumps

Efflux pumps are prominent in terms of both their high efficiency of drug extrusion and broad substrate specificities, underlying their roles in multidrug resistance. Substrate profile of the *E. coli* housekeeping efflux system AcrAB-TolC has been studied and it was shown to include chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid and β-lactam antibiotics [2]. Similar to that in *E. coli*, the AcrAB-TolC efflux system in *S. typhimurium* was also found to be able to expel different classes of antimicrobial agents such as quinolones, chloramphenicol, tetracycline and nalidixic acid [1,14]. In *P. aeruginosa*, two RND efflux pumps, MexAB–OprM which is the homolog of the *E. coli* AcrAB-TolC system and MexXY-OprM, are constitutively expressed and both of the systems can actively export fluoroquinolones, tetracycline and chloramphenicol. In addition to these common substrates, MexAB–OprM system can also export novobiocin and β-lactams, such as carbenicilline, and MexXY system can also export aminoglycosides [15]. Substrate profiles of other clinically relevant pathogens are reviewed elsewhere [6,14].

2.1. Structures of RND efflux pumps

Owing to their prominent roles in MDR, various of biophysical and biochemical characterization of bacterial efflux pumps, especially the *E. coli* AcrAB-TolC system, have been conducted [16–21]. In recent decade, elucidation of crystal structures of several drug efflux pumps and those complexed with the substrates or inhibitors has greatly accelerated our understanding of the fundamental mechanism of drug export and the characteristics of their multisubstrate specificities. The first crystal structure of drug efflux pump was that of the *E. coli* AcrB protein which was resolved at 3.5 Å resolution by Murakami et al. [22]. The crystal was grown in the trigonal space group R32, implying a symmetric AcrB trimer. The trimeric complex is comprised by a large portion of the periplasmic headpiece and a transmembrane region. The upper part of the headpiece forms the TolC docking domain and the center of the headpiece comprises the pore domain. Crystal structure of AcrB with its substrate, minocycline or doxorubicin [23] was resolved subsequently by the same research group. Findings from this co-crystal showed that only one of the three protomers bound with the substrate minocycline or doxorubicin (Fig. 1A). This, combined with the asymmetric structure of AcrB revealed by the X-ray crystal structure obtained independently by other two groups [24,25], led to the proposal of the asymmetric configuration of AcrB

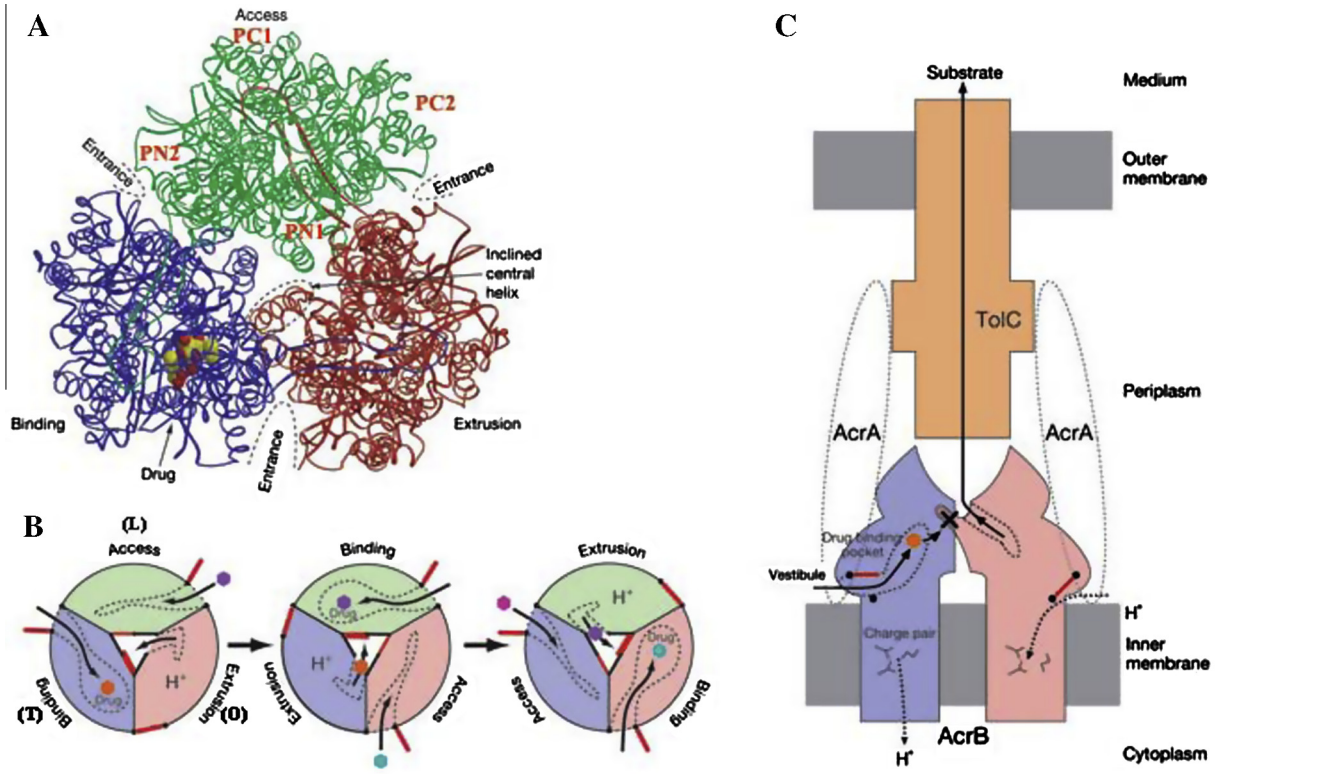

Fig. 1. Structure of AcrB and the functional rotation mechanism of the drug extrusion by the RND efflux system AcrAB-TolC. The three protomers are colored as green, blue and red, respectively. (A) Top view of a ribbon representation with a drug (yellow and red) in the binding protomer. (B) Top view from the distal side of the cell. Drugs are illustrated as hexagons. Red lines represent the entrance and exit sites of each protomer. The drug binding pocket and translocation pathway are in dotted lines. (C) Side view of the AcrAB-TolC efflux system parallel to the membrane plane. Drugs are illustrated as hexagons. Red lines represent the entrance and exit sites of each protomer. The route of substrate access, binding, and extrusion is shown. Figure is adopted and modified based on that by Marakarm et al. [23].

trimer both in vitro and in vivo and a working mechanism of functional rotation in which the three protomers display different conformation. The protomer bound with the substrate in the crystal structure is indicated as the binding protomer which exists as the tight (T) conformation. The substrate binding pocket in this protomer is located in the porter domain, which is consisted of four subdomains, PN1, PN2, PC1 and PC2 (Fig. 1A). Examination of the substrate binding pocket revealed that it is enriched with aromatic amino-acid residues: Phe 136, Phe 178 (PN2), Phe 610, Phe 615, Phe 617, and Phe 628 (PC1), which are proposed to interact with the substrate by hydrophobic or aromatic–aromatic interactions [23]. Both minocycline and doxorubicin interact with Phe 615. In addition, minocycline also interacts with Phe 178 and Asn 274, and doxorubicin also interacts with Gln 176 and Phe 617 [23]. It is suggested that these may provide an explanation on the wide spectrum of substrate specificities of AcrB since different residues are used for binding of different substrates. The second protomer is designated as the extrusion protomer which exists as the open (O) conformation, because it represents the configuration after substrates extrusion. In this protomer, the pore-forming helix of PN1 is inclined and blocks the potential exit from the substrate binding pocket (Fig. 1A red). The third protomer is defined as the access protomer which exists as the loose (L) conformation, because it possesses a vacant binding site, waiting for the binding of the second substrate. Based on these information, the three-step functional rotation mechanism of drug export was proposed [23]. In the first step or the access (or loose, L) state (Fig. 1B and C), a "vestibule" near the entrance is open to the periplasm and allows potential substrates to access [23]. In the binding (or tight, T) state, the binding pocket expands to accommodate the substrate. Hence, drugs enter into the vestibule from the surface of the cytoplasmic membrane, move through the uptake channel, and bind to the different sites in the binding pocket. At this stage, the exit from the binding site is blocked by the central helix inclined from the extrusion protomer. In the extrusion (or open, O) state, the exit is opened because the central helix is inclined away, while the vestibule is closed [23]. The bound drug is pushed out into the top funnel by shrinking of the binding pocket. These changes were expected to be coupled to proton translocation across the membrane. The protonation and deprotonation of charged amino acid residues within the transmembrane domains would affect the accessibility or influence the binding or extrusion of the substrates. This functional rotating mechanism was further confirmed by the study of Takatsuka et al. using covalently linked AcrB trimer which showed that inactivation of one of the three protomers led to dysfunction of the entire trimer, an essential feature of the functional rotating mechanism [26].

Building upon the discovery of multiple phenylalanine residues in the substrate binding pocket of AcrB, Bohnert et al. [27] conducted site-directed Phe mutations to study the role of hydrophobic residues in AcrB. Their results showed that mutation of F610A caused significant decrease of the substrate MICs of oxacillin, doxorubicin, novobiocin and clindamycin suggesting that the F610 residue has a special role in the substrate extrusion process. However, the molecular details of the recognition and transportation of other substrates by AcrB were still unclear, since only minocycline and doxorubicin have been co-crystallized within the binding pocket. To better understand the recognition mechanism of other compounds, computer docking to predict the binding of 30 compounds with the binding pocket of the binding protomer was conducted [28]. Both computer docking and experimental results confirmed that minocycline binds to the upper portion of the binding

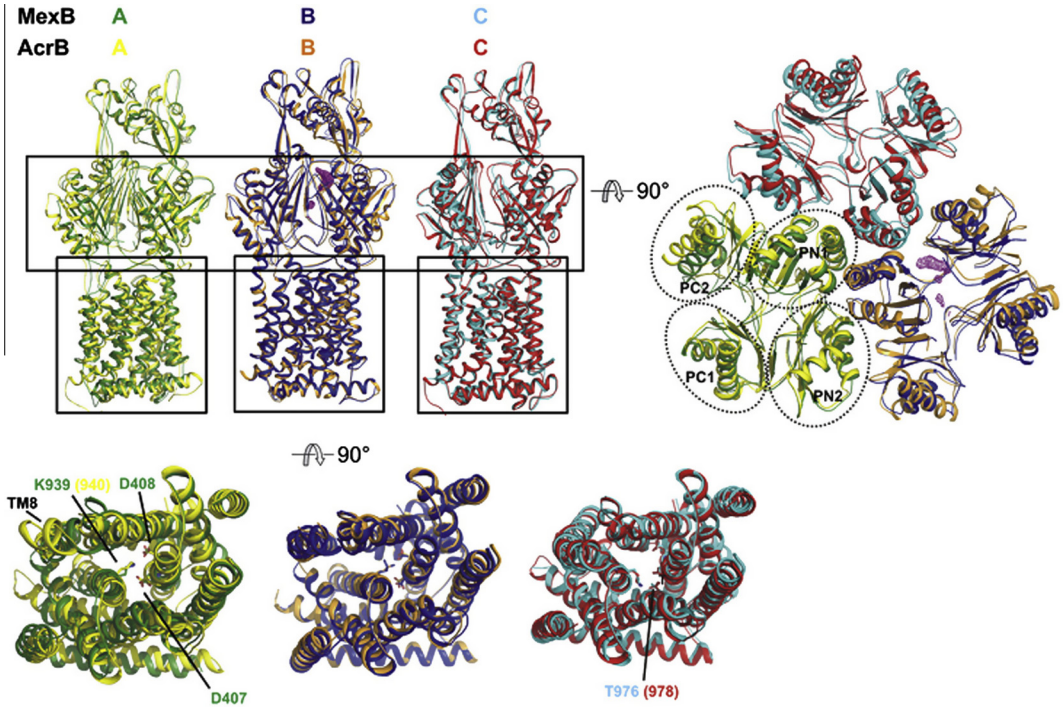

Fig. 2. Comparison of MexB and AcrB structures based on their transmembrane helices. Three subunits of MexB and AcrB are individually represented in the same orientation. The right panel shows the structural differences in the pore domain. The lower panel shows the side-chain conformation in the putative proton translocation site. Figure is adopted from Sennhauser et al. [32].

pocket, which is called "groove binding". Such compounds also include tetracycline, chlortetracycline, doxycycline, novobiocin, erythromycin, nafcillin, rifampin, doxorubicin and levofloxacin. Whereas some other ligands bind to the lower part of the pocket, including chloramphenicol, carbenicillin, and cefamandole, which is called "cave binding." In addition, some compounds seem to use both subdomains, such as cephalothin, cephaloridine, oxacillin, linezolid, and ciprofloxacin. This successive process of binding and transport was further demonstrated by the high resolution structures of AcrB/ankyrin-repeat protein (DARPin, a crystallization chaperone) complexes with bound minocycline or doxorubicin [29].

The opportunistic pathogen *P. aeruginosa* encodes several multidrug efflux pump genes [30]. The MexAB–OprM tripartite system was the first to be identified and was the best characterized in the bacterium [31,32]. The multidrug exporter MexB belongs to the RND family and its constitutive expression confers intrinsic resistance of *P. aeruginosa* to a broad spectrum of antimicrobial agents. MexB is closely related to *E. coli* AcrB. Crystal structures of the tripartite efflux pump components MexA, MexB and OprM have also been reported [30,33,34]. Comparison of the structures of MexB and *E. coli* AcrB showed that the structural similarity between the two proteins is obvious (Fig. 2). Specially, the transmembrane domain of MexB and AcrB was examined because it was likely to be similar in all RND family transporters. Similar to that of AcrB, the structure of the docking domain of MexB is consisted of two subdomains, and one of them forms a long loop that inserts into the docking domain of the neighboring subunit. This may well explain the finding that outer membrane channels and RND efflux pumps interact transiently in a rather low affinity [18]. Moreover, this observation also led to the assumption that the high specificities between MexB and OprM, and AcrB and TolC mostly relies on the associated membrane fusion protein MexA or AcrA [17]. Since crystals of MexB were grown in nonionic detergent n-dodecyl-D-maltoside (DDM), the positive electron density observed in the crystal was interpreted to be part of a DDM molecule that co-crystallized with MexB. Residues interacting with this substrate include Val47, Ser48 and Gln125 of PN1; Val177, Ser180 and Gly179 of PN2; and Arg620 and Gln273 of PC1. The binding site of the DDM to MexB corresponds to the previously reported minocycline and doxorubicin binding sites in AcrB [27]. The fact that all three substrates bind to the binding cavity of the pore domain suggests that the nature of this binding cavity is conserved. Till now, no high-quality co-crystals of MexB with minocycline or a tightly bound DDM molecule was obtained like AcrB, though the substrate specificity of MexB resembles that of AcrB.

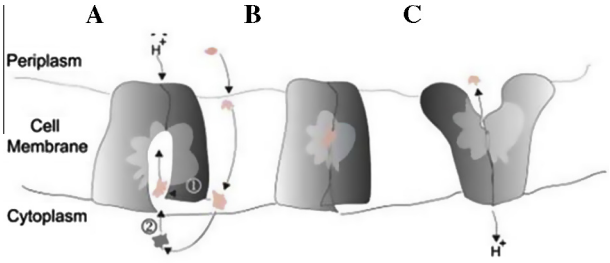

Fig. 3. A potential mechanism for hydrophobic substrate transport by EmrD. (A) Two ways (path 1 and 2) that drug can enter the internal cavity of the transporter. (B) Drug transported through a rocker-switch alternating-access model. (C) Drug transported across the lipid bilayer. Figure is revised from Yin et al. [35].

2.2. Structures of MFS and SMR efflux pumps

In addition to the RND efflux pumps which have attracted intensive research interests owing to their significant roles in clinically relevant drug resistance, contribution of other types of efflux pumps to drug resistance has also been reported and 3D crystal structures of several of these pumps have also been resolved. The crystal structure of a multidrug transporter belonging to the MFS family, EmrD, of *E. coli* has been reported [35]. Its structural

arrangement may reflect a general architecture of MFS transporters, where twelve transmembrane helices form a compact structure with four of the transmembrane helices (H3, H6, H9, and H12) facing away from the interior, and the remaining transmembrane helices forming the internal cavity. Consistent with its function of transporting lipophilic compounds, the internal cavity of EmrD comprises mostly hydrophobic residues. Several of these residues are also conserved in other MFS transporters. This type of hydrophobic core has been previously proposed and also observed in the structure of EmrE, a small multidrug efflux transporter [36,37]. The mechanism of hydrophobic substrate transport by EmrD is proposed (Fig. 3). In this proposed mechanism, the drug can access the cavity either through the cytoplasm or through the inner membrane leaflet, and substrate is transported through a rocker-switch alternating-access model [35].

E. coli EmrE belongs to the small multidrug resistance family (SMR) of the drug transporters. Its X-ray structure shows that it functions as an inverted homodimer [38]. The asymmetric unit contains eight EmrE monomers each composed of four transmembrane helices. The first three helices form a bundle against the other equivalent helices of EmrE molecule, and the fourth helix of each monomer interacts with each other and projects laterally from the main body of the dimer. However, this conformation is not in accordance with biochemical data. One possible reason is that EmrE might be denatured since it was crystallized at pH 4 [38].

2.3. Structures of efflux gene regulators

Since overexpression of efflux pumps causes clinically significant drug resistance, how the over-expression of efflux gene is induced constitutes an important subject in drug resistance studies (see the contents in the section of "regulatory networks of efflux pumps" below). Piddock et al. categorized the mechanisms that lead to increased expression of efflux pumps found in clinical isolates into four groups: (i) mutations in the local repressor gene, (ii) mutations in a global regulatory gene, (iii) mutations in the promoter region of the transporter gene, and (iv) insertion elements upstream of the transporter gene. 3D structures of efflux gene regulators especially those bound with the inducers not only provided structural basis on how the activity of these regulators are mediated by structurally dissimilar antimicrobial agents to regulate efflux gene expression, but also provided insights into the development of novel antimicrobial agents that interfere the induction of efflux pump genes.

The expression of *E. coli acrB* efflux gene is controlled by the local transcriptional repressor AcrR, which can be inactivated by a variety of structurally unrelated antimicrobial agents [39]. Crystal structure of AcrR has been reported and it was revealed that AcrR is constituted largely of α-helices and functions as a dimer [39]. Each subunit of AcrR in the dimer contains nine α-helices which are divided into two domains, the N-terminal domain (α1–α3) and the C-terminal domain (α4–α9). In the homologs regulators TetR, QacR and CmeR, the C-terminal domains are ligand-binding domains [40–42]. However, the C-terminal α-helical bundle of AcrR forms an internal cavity, which overlaps with the substrate binding pocket of QacR, thus, it has been predicted to be a drug-binding pocket. Uniquely, the ligand-binding pocket of AcrR possesses three openings, one is located at the dimer interface and the other two are located at the front and side surfaces of the monomer. There are 14 residues in the inner wall of the ligand-binding pocket, and most of these residues are hydrophobic in nature [39]. The inner surface of the pocket displays a negative potential owing to the presence of an acidic residue E67. This suggests that AcrR mainly binds neutral and positively charged ligands, which is consistent with the fact that most of the AcrR ligands are indeed positively charged [39].

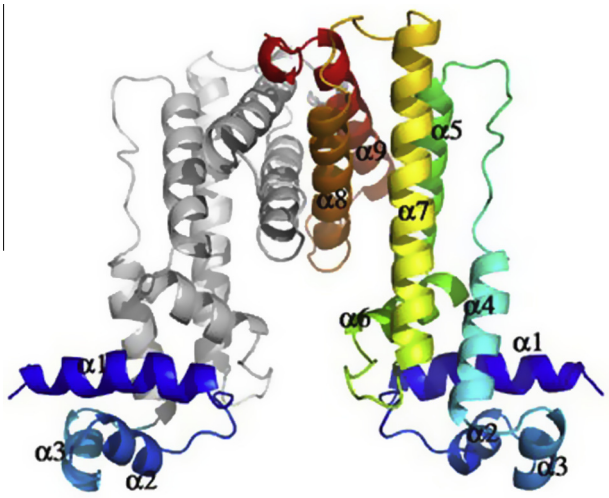

Fig. 4. Crystal structure of MexZ. Shown is the ribbon diagram of MexZ dimer. In each monomer, the N-terminal is shown as blue and the C-terminal is shown as red. Figure is adopted from Alguel et al. [44].

The transcription regulator MexR is the primary repressor of the MexAB–OprM efflux system in *P. aeruginosa* which belongs to the MarR transcription regulator superfamily [43]. The crystal structure of MexR has been resolved. It showed that MexR is mainly consisted of α-helices and forms a triangular dimer which contains two DNA binding domains. Each of the domains is connected via two long helices to the dimerization domain made up of the N- and C-terminal regions from the two monomers [43]. The dimer exhibits a conformation in which the C-terminal tail of a monomer inserts into the DNA binding domains of the neighboring monomer. In the absence of an effector or effector-like ligand, the positively charged side chains repulse each other to maintain the crevice between the DNA binding domains, allowing the DNA binding conformation of the MexR dimer. Consequently, it was proposed that the MexR acts through an effector-induced conformational change which reduces the spacing of DNA binding domains and prevent its binding of DNA [43].

The TetR family transcription regulator MexZ is the negative regulator of *mexXY* efflux gene in *P. aeruginosa*. Its repression is relieved in the presence of inducing antibiotics [44]. The clinical importance of MexZ has been strikingly highlighted [45], because *mexZ* was found to be the most frequently mutated gene in clinical isolates of *P. aeruginosa* from cystic fibrosis patients lungs. However, unlike many other TetR family transcription regulators, such as TetR, QacR, and TtgR, which activity is modulated by the same substrates as the efflux pumps they control, MexZ does not bind those substrates directly and its activity is regulated by an anti-repressor PA5471/ArmZ [46]. X-ray structure of MexZ is similar to that of a typical TetR family protein and the overall structure of MexZ is composed of nine α helices (Fig. 4). Among them, α1–α3 forms the DNA binding domain with α3 being the DNA recognition helix. The rest α helices form the C-terminal domain (CTD). Within the CTD, α5, α8, and α9 and the C terminal half of α7 form a helical bundle with hydrophobic residues at the core. MexZ exists as a dimer in solution, and the dimer has excessively widely separated DBD domains with the average distance between the two recognition helices as 60 Å, significantly larger than the distance between the major grooves of B-DNA (34 Å). This suggested that the X-ray crystal structure of MexZ represented that of the activated form of MexZ that is released from the DNA. Analysis of MexZ mutations isolated from clinical strains showed that

some mutations are located in the DNA recognition helix of the protein which may affect the DNA binding ability of MexZ through perturbing its interactions with the *mexXY* promoter DNA.

2.4. Structures of Gram-positive bacterial efflux gene regulators

Transcription regulators of clinically important Gram-positive bacteria, such as *S. aureus* and *Bacillus subtilis* have also been reported [41,47,48]. BmrR is a transcription regulator of the multidrug transporter Bmr in *B. subtilis*. BmrR activates the transcription of *bmr* in response to lipophilic cationic drugs. X-ray crystal structure of BmrR complexed with the inducing drug tetraphenylphosphonium (TPP) and the promoter DNA of *bmr*, BmrR-TPP-*bmr* promoter, has been resolved [47]. The BmrR monomer contains three domains: the N-terminal DNA-binding domain, the linker that connects the N- and C-terminal domains, and the C-terminal drug binding domain (or BRC). The most striking feature of the complex is that of the *bmr* promoter DNA. It was shown that the base pair between the forward and lag strands in the promoter region was broken because of the BmrR-TPP complex, which led the unpaired adenine and thymine to slide towards the 3′ direction [47]. As a result, the nearby adenine and thymine also undergoes displacement but still forms a distorted Watson–Crick base pair. These evidence reveals a three step mechanism for transcription activation, that is, localized base-pair breaking, base sliding, and realignment. This pattern is different from that of many DNA-binding proteins which bend and kink their operators.

Another well studied regulator of Gram-positive bacteria efflux genes is QacR. QacR is the transcription repressor of the gene encoding the multidrug transporter QacA. QacR binds to the IR1 operator site of *qacA* efflux pump gene in the absence of inducing drugs and represses *qacA* expression. QacR can bind to various structurally dissimilar compounds that are the substrates of QacA efflux transporter, such as rhodamine 6G, ethidium and crystal violet [49]. Structure of QacR bound simultaneously with two or more different drugs such as ethidium (Et) and proflavin (Pf) has been resolved, and it reveals the regulatory mechanism of QacR in response to structurally different compounds. Surprisingly, it was demonstrated that no additional global structural changes were caused by dual drug binding to QacR [48], and the volume of the multidrug-binding pocket of the QacR–Pf–Et ternary complex did not need to expand to accommodate both drugs. An interesting observation is that while the Pf binding site remains identical in the QacR–Pf–Et ternary structure in comparison with that in the binary complex of QacR–Pf, the Et molecule in the ternary structure has shifted considerably in the pocket compared to its location in the QacR–Et binary structure. Based on this, an uncompetitive binding mode was proposed because the exact Et-binding site found in the ternary complex was not occupied in the absence of Pf.

3. Physiological roles of MDR efflux pumps

The fact that all bacterial genomics contain efflux pump genes and their expression is subject to tight regulation by various of local and global transcriptional regulators has led to the proposal that drug efflux pumps have physiological functions, especially during the stress adaptation, development, and pathogenesis and virulence of bacteria. There has been accumulating evidence demonstrating that drug efflux pumps indeed play a general role of detoxification in various of bacterial physiological processes. Exploring the regulation of efflux pump genes as well as their specific physiological functions will advance our understanding of the naturally or physiologically originated drug resistance which occurs frequently in nature.

3.1. Roles in bacterial pathogenicity and virulence

Following the recognition that the house-keeping efflux pump AcrAB-TolC serves as an important antibiotic resistance determinant and plays a major role in the MDR phenotype of *E. coli* clinical isolates [50,51], it was soon reported that the efflux system can also pump toxic bile salts out of the cells and consequently promote the adaptation of the bacterium in the animal intestinal tract [52]. Subsequently, similar roles of AcrAB homologs in various other species, such as *P. aeruginosa* [31,53], *Neisseria gonorrhoeae* [54] and *S. typhimurium* [55] were also reported. These observations led to the proposal that bacterial efflux pumps have the capacity to extrude various host-derived antimicrobial compounds and facilitate the adaptation and survival of bacteria in their ecological and physiological niches. Indeed, this notion is supported by increasing evidence demonstrating that efflux pump defective mutations caused reduced virulence of several pathogens. Buckley et al. studied the role of efflux pumps on virulence of *S. typhimurium* using efflux pump defect mutants in a chicken model, and found that mutants deficient in either *acrB* or *tolC* genes colonized poorly and did not persist in the avian gut, indicating that AcrAB-TolC system is essential for the colonization of *S. typhimurium* in chickens [56]. Utilizing similar approaches Nishino et al. showed that *S. typhimurium* lacking the *macAB* efflux pump genes displayed significantly attenuated virulence in a mouse model and a strain lacking all drug efflux systems became completely avirulent [57]. In *N. gonorrhoeae*, a bacterial pathogen of the human genital mucosae, deletion of *mtrD* or *mtrE* gene which product constitutes the MtrCDE efflux system caused poor colonization of the bacteria in genito-urinary tract of female mice [58]. More recently, studies have shown that Δ*acrB* in *K. pneumoniae* and Δ*acrA* or Δ*tolC* in *Enterobacter cloacae* led to a reduced capability of the pathogens to cause infection in a mouse model, indicating that AcrAB-TolC is essential for the virulence of *K. pneumoniae* and *E. cloacae* [59,60]. In addition to causing defect in animal models, in plant pathogen an *acrB*-deficient *Erwinia amylovora* was reported to have impaired virulence on apple rootstock and fail to colonize its host plant [61]. Similarly, deletion of either *acrA* or *dinF* pump gene in *Ralstonia solanacearum* caused reduced virulence on the tomato plant, while complementary expression of these two genes in plasmid restored its virulence to nearly wild type level [62].

In addition to their roles in host-pathogen interaction, multidrug efflux pumps may also play a direct role in bacterial pathogenesis. Hirakata et al. [63] examined the contribution of the four best-studied *P. aeruginosa* multidrug efflux pumps (MexAB–OprM, MexCD–OprJ, MexEF–OprM and MexXY–OprM) to the virulence of the bacterium by testing the ability of efflux pump mutants to invade epithelial cells (Madin–Darby canine kidney (MDCK) cells), and showed that except for the *mexCD-OprJ* system, deletion of other efflux systems caused a significantly reduced ability of the bacterium to invade MDCK cells. Mutants lacking the MexAB–OprM system could not invade MDCK cells and that invasion could be restored by complementation of the strain with plasmid encoded *mexA–oprM* or by supplementation with culture supernatant obtained from MDCK cells infected with wild-type *P. aeruginosa*. These studies suggested that efflux systems can directly export virulent determinants and contribute to bacterial pathogenesis. In some other cases, efflux pump activity affects bacterial virulence in a more indirect manner, such as through altering quorum sensing responses. Those circumstances will be discussed as below.

3.2. Roles in cell-to-cell communication

Intercellular communication among bacteria plays an important role in bacterial stress response and community behaviors.

One of the most important cell-to-cell communication mechanisms is the quorum sensing system. Quorum sensing (QS) bacteria produce and release chemical signaling molecules called autoinducers which increase in concentration as a function of cell-population density. As the accumulated autoinducers reach a minimal threshold stimulatory concentration, these small molecules bind to specific receptors which act as transcription regulators and consequently alters the gene expression profile in bacteria [64]. QS controls many cellular functions, including biosynthesis of antimicrobial peptides, metabolic switch, motility, polysaccharide synthesis and activation of many virulence factors.

In order to be sensed by other bacteria, autoinducers synthesized in vivo need to be exported and released into the extracellular space. Therefore transportation of the autoinducers is a key aspect for the regulatory function of QS. Autoinducers differ in their capability to diffuse across bacterial membranes. For instance, two *N*-acylhomoserine lactones (AHL) autoinducers from *Vibrio fischeri* and *P. aeruginosa*, 3-oxohexanoyl homoserine lactone ($3OC_6$-HSL) and N-butyryl homoserine lactone (C_4-HSL, also called PAI-2) respectively, are freely diffusible [65,66]. However, N-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL, also called PAI-1) and 2-heptyl-3-hydroxy-4-quinolone (PQS) autoinducers of *P. aeruginosa* are not readily diffusible across the membrane, either due to their lager size or hydrophobic nature [66,67]. Under these circumstances, additional membrane vesicles or membrane transporters are required to assist the cross membrane transportation of those autoinducers. Herein we discuss the roles of bacterial MDR efflux pumps in autoinducers traffic and QS response.

The involvement of MDR efflux pumps in antoinducers traffic was first reported in *P. aeruginosa*, of which the QS system has been intensively studied. Poole et al. reported that hyper expression of MexAB–OprM efflux system in a *P. aeruginosa* isolate produced less AHL autoinducer $3OC_{12}$-HSL and displayed diminished QS response as indicated by the reduced production of several QS controlled extracellular virulence factors [68]. It was proposed that active efflux of $3OC_{12}$-HSL by MexAB–OprM efflux pump limited the intracellular concentration of the autoinducer and consequently its dependent activation of the autoinducer producing gene *lasI* and genes encoding those virulence factors. The capability of efflux pumps to export QS autoinducers was also supported by the studies of Pearson et al. which demonstrated that Δ*mexAB-oprM P. aeruginosa* display reduced diffusion of the [^{3}H]$3OC_{12}$-HSL autoinducer to the extracellular environment and accumulation of the intracellular [^{3}H]$3OC_{12}$-HSL, confirming that MexAB–OprM plays a role in the active efflux of $3OC_{12}$-HSL [66]. The MexEF–OprN and MexGHI–OpmD efflux system has also been shown to play a role in QS response, but in an indirect manner [69,70]. Expression of C_4-HSL autoinducer synthase gene *rhlI* in a MexEF–OprN hyper expression mutant was found to be at only half level of that in wild type strain which led to decreased production of extracellular virulence factors controlled by QS [69]. Another efflux pump MexGHI-OpmD was found to export anthranilate, a toxic metabolite and PQS autoinducer precursor [70]. Consequently, deletion of *mexG* or *opmD* led to the failure of the synthesis of $3OC_{12}$-HSL and PQS owing to the accumulated anthranilate precursor inside the cells [70].

The involvement of MDR efflux pumps in QS has also been reported in other bacterial species. For instance, it was reported that overexpression of the QS regulator SdiA in *E. coli* had increased AcrA and AcrB protein levels, suggesting a potential role of the AcrAB efflux pump in QS [71]. *E. coli* AcrAB and MdtK (NorE, a MATE type pump) efflux pumps were also proposed to export growth cessation QS signals and consequently mutation of these two pumps affected the adaptation of the bacterium to its stationary growth [72]. In *Bacteroides fragilis*, a predominant anaerobic opportunistic pathogen in gastrointestinal infection, it has been shown that the expression of the BmeB efflux pump is controlled by QS, and it was proposed that BmeB can efflux AHL autoinducers outside of cells and modulate the intracellular AHL concentrations [73]. In *Burkholderia pseudomallei*, MDR efflux pump BpeAB–OprB was shown to be essential for the extracellular secretion of six AHL autoinducers [74,75], although a more recent study by another group demonstrated that this specific function of BpeAB-OprB pump is absent in a distinct *B. pseudomallei* strain [76].

On the other hand, Martinez et al. proposed that efflux pumps facilitate the shut-down of QS response in bacteria by increasing the efflux of autoinducers and/or autoinducer precursors, thus enabling the bacteria to quickly respond to environmental changes [77]. This notion is supported by the observation that QS null mutants are frequently isolated from environmental and clinical samples [78], which suggesting that lacking of this cell-to-cell communication system may be advantageous to *P. aeruginosa* at least in certain situations. This notion also seems reasonable at an economic aspect of view, as it has been calculated that QS response consumes at least 5% of the total energy supply in *P. aeruginosa*, whereas production of autoinducers requires only 0.01% of the total energy supply [79], underlying the important roles of efflux pumps in mediating the response of QS. Although export of autoinducers might not be the main physiological function of MDR efflux pumps, their involvement in this physiological process is clearly illustrated. Considering the central role of QS in virulence regulation in many pathogens, these efflux pumps dependent QS systems provide an alternative target for the development of antimicrobial interventions.

3.3. Roles in biofilm formation

Most chronic and persistent bacterial infections are associated with biofilm growth, a strategy that has accelerated the emergence and rapid spread of multidrug resistant bacteria. It has been known for decades that biofilm associated bacteria is much more difficult to be eradicated by bactericidal antimicrobials than planktonic cells [80]. There seem to be multiple mechanisms operating simultaneously to contribute to this phenomenon. However, our understanding regarding the detailed underlying mechanisms is still very limited except for a few cases [81,82].

Recently, several studies have provided evidence to show that defect in efflux activity impairs biofilm formation, which linked the physiological function of efflux pump to biofilm formation. Kvist et al. [83] reported that inhibition of efflux activities by efflux pump inhibitors (EPIs) reduced biofilm formation in both *E. coli* and *Klebsiella* strains, while simultaneous treatment with different EPIs abolished biofilm formation completely. A screening of 22 efflux pump mutants for reduced biofilm formation has led to the identification of six efflux pumps that contribute to biofilm formation in *E.* coli K-12 strain [84]. Baugh et al. demonstrated that in *Salmonella enterica serovar Typhimurium*, genetic inactivation of any efflux pump or chemical inhibition of the efflux activity (EPIs treatment) results in compromised ability of *Salmonella* to form biofilm [85]. Further studies showed that defect of biofilm formation in efflux pump mutants was resulted from transcriptional repression of curli biosynthesis genes and consequently inhibition of its production, but was not associated with altered aggregative ability or export of any biofilm-promoting factor [86]. Their study also showed that efflux pump inhibitors that known to be active against *Salmonella* also effectively prevented biofilm formation in other species, including *E. coli*, *P. aeruginosa* and *S. aureus*, at a concentration lower than the growth inhibition concentration [86]. The impaired biofilm formation resulted from efflux pump inactivation has also been reported in *B. pseudomallei* [76]. The growing body of evidence which unveils the role of MDR efflux pump

systems in biofilm formation has provided a promising antibiofilm strategy via inhibition of efflux activity.

4. Regulatory network of efflux pumps

Although efflux genes are ubiquitously distributed in bacterial genomes, except for the few housekeeping efflux systems, expression of the majority of them is subject to tight control by various transcription regulators, underlying their roles in facilitating the adaptation of bacteria to specific stimuli. Recent observation that overexpression of SmeDEF efflux pump in *S. maltophilia* impairs the fitness and virulence of this bacterium supported this notion [87,88]. Considering the capacity of efflux pump to extrude a wide range of structurally unrelated chemicals, it is reasonable to speculate that improper overexpression of efflux pumps may cause unwanted efflux of metabolites or other signaling molecules, resulting in deleterious effects on cell physiology. Therefore, expression of efflux pumps is usually well tuned and is only expressed at a low, basal level under the ordinary laboratory growth condition. Although the composition and functions of MDR efflux pumps are relatively conserved in different species, their regulatory mechanisms vary significantly. For instance, *S. typhimurium*, a member of the closest known genus to *E. coli*, employs a distinct regulatory network of efflux pump gene expression from that of *E. coli*. On the other hand, despite the divergence between species, different bacteria still share some common patterns in regulating the expression of efflux pump genes. A summary of the regulatory network of efflux pump gene expression in the model organisms *E. coli* and *Salmonella* has been reviewed by Nishino et al. [14]. Here, we focus on the findings in recent years and in a broader range of bacterial species. We categorize them into different regulatory patterns.

4.1. Regulation by local repressors

A glance of the currently known regulators of MDR efflux pumps in various species (Table 1) shows that majority of the regulators belong to the TetR, MarR, or MerR family, which usually acts as the transcriptional repressors [89–91]. For most of the efflux pumps, especially the RND type efflux pumps, a local repressor gene is usually found adjacent to the structural genes of efflux pumps [92]. Mutation in the local repressor gene is frequently observed in clinical isolates that display MDR phenotypes, suggesting that the purpose of this local repressor might be to avoid the excessive production of efflux pumps.

The simplest pattern of efflux pumps activation is through derepression of the local repressor mediated directly by specific effector molecules (e.g., bile salts, antibiotics etc.). A typical example is the activation of *emrAB* expression via the de-repression of the active form of EmrR, a MarR family repressor. In this system, *emrR* gene is located immediately upstream of the *emrAB* genes and encodes EmrR protein. EmrR binds directly to the promoter region of *emrRAB* operon and blocks the transcript of *emrAB* under non-inducing conditions [93]. When exposed to toxic chemicals, such as carbonylcyanide-3-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol (DNP) and ethidium bromide (Eb), binding of these drugs to EmrR causes conformational changes in the protein which attenuates its binding to the DNA promoter, resulting in the relief of the repression of *emrAB* [94]. Similar regulatory mechanism is also found in the case of the Bmr pump in *B. subtilis* which expression is mediated by the local repressor BmrR and binding of BmrR to DNA is destabilized by rhodamine 6G and tetraphenylphosphonium (TPP) [95,96]. Similar systems also include the expression of *cmeABC* pump in *Campylobacter jejuni* [97] and *mtrABC* pump in *N. gonorrhoeae* [98], which expression is repressed by the regulator CmeR and MtrR respectively and both repressors can interact with bile salts and consequently be released from DNA binding. Other examples include the BpeR–BpeAB system in *B. pseudomallei* [74], MexT–MexEF system in *P. aeruginosa* [99,100], TtgR–TtgABC system in *P. putida* [101], MepR–MepA [102] and QacR–QacA systems [41,103,104] *in S. aureus*, and the SmeT-SmeDEF system in *S. maltophilia*. In all these cases, the repressor can be released from its bound DNA promoter upon the binding of their corresponding ligands or effector molecules. One explanation for the universal distribution of this negative regulation pattern might be that this mechanism does not require additional time to synthesize the activator and thus enables the corresponding bacterium to act more rapidly to environmental changes, e.g. toxic chemical exposure.

4.2. Regulation by global response regulators

Another common pattern is that the expression of efflux pumps is regulated by global regulators, in the presence or absence of a local repressor. One example is the intensively studied AcrAB housekeeping efflux pump in *E. coli*, which is conditionally activated by three XylS/AraC family regulators, MarA, Rob, and SoxS. These three regulators show high homology to each other and can bind to the same DNA sequence called marbox on the promoter of *acrAB* [105]. These three regulators can activate *acrAB* expression in response to different environmental signals. For instance, AcrAB is activated by salicylate, a plant hormone, through the MarA regulator. Binding of salicylate to MarR, a local repressor of *marRAB* operon, causes conformational changes in the protein which led to disassociation of MarR from the *marRAB* promoter [106–108]. Consequently, expression of MarA is de-repressed, which in turn activates the expression of *acrAB*. When *E. coli* is exposed to decanoate and unconjugated bile salts, chemicals commonly found in intestinal tract, expression of *acrAB* is activated by the Rob transcription regulator [109]. The inducers specifically bind to the non-DNA-binding domain of Rob, and since in this case activation of *acrAB* expression is not due to the increased expression level of Rob, it was proposed that expression of *acrAB* is activated by conformational changes of pre-existing Rob [109,110]. During oxidative stress (e.g., superoxide generated by redox-cycling agents), expression of *acrAB* is induced in a SoxRS dependent manner. Under this circumstance, SoxR is first activated via the oxidation of its [2Fe–2S] cluster by superoxide species. It then oxidizes SoxS into its active form which then binds to the maxbox on *acrAB* promoter and induces *acrAB* expression [111].

Similarly, the expression of *acrAB* efflux pump in *Salmonella* can be induced by paraquat, a superoxide generator, also in a SoxS dependent manner [112]. In *E. coli*, indole induces the expression of multiple efflux pumps but not AcrAB. However, indole can induce AcrAB expression in*Salmonella* [113]. It was shown that indole activated expression of *acrAB* in *Salmonella* is mediate by RamA, an AraC family transcriptional regulator [114]. This *Salmonella* specific regulator constitutes a distinct regulatory network which differs from that of *E. coli*. It was reported that bile salts induced *acrAB* expression is also dependent on RamA [114]. Shift of the intrinsic tryptophan spectrum of RamA upon adding of bile salts suggested direct binding of bile salts to this regulator, indicating a similar role of RamA in *Salmonella* as that of Rob in *E. coli*. Following study by Abouzeed et al. showed that RamA is regulated by its local repressor, RamR, a TetR family transcriptional regulator [115].

In *B. subtilis*, both Bmr and Blt efflux pumps are positively regulated by the global regulator Mta [116]. This MerR family regulator binds directly to *bmr* and *blt* promoters and activates their expression [116], yet the inducing signal of Mta is still unknown. In *N. gonorrhoeae*, it was reported that induction of the MtrCDE pumps by detergents is dependent on MtrA, an AraC family

Table 1
A summary of the known regulators of MDR efflux pumps.

Efflux pump	Pump type	Regulator	Regulator family	Inducible signal	References
Acinetobacter baumannii					
AdeABC	RND	AdeRS	TCS	?	[131]
Agrobacterium tumefaciens					
AmeABC	RND	AmeR	TetR	?	[154]
Burkholderia pseudomallei					
AmrAB	RND	AmrR putative	TetR	?	[155]
BpeAB	RND	BpeR	TetR	Erythromycin, stationary phase growth	[74]
Campylobacter jejuni					
CmeABC	RND	CmeR	TetR	Bile salts	[97]
		CosR	OmpR	Paraquat	[119]
Bacillus subtilis					
Bmr	MFS	BmrR	MerR	Rhodamine 6G, TPP	[95,96]
		Mta	MerR	?	[116]
Blt	MFS	BltR	MerR	?	[156]
		Mta	MerR	?	[116]
Escherichia coli					
AcrAB	RND	AcrR	TetR	?	[157]
		AcrS	TetR	?	[158]
		MarA	AraC	Salicylate, DNP	[111]
		MarR	MarR	Salicylate, DNP	[106]
		SoxS	AraC	Paraquat	[111]
		Rob	AraC	Bile salts, fatty acids	[111]
		SdiA	LysR	?	[71]
AcrD	RND	BaeSR	TCS	Indole	[122]
		CpxAR	TCS	Indole	[122]
AcrEF	RND	H-NS	Histone-like protein	?	[159]
CusCFBA	RND	CusRS	TCS	Copper, silver, anaerobic amino acid limitation	[160]
EmrAB	MFS	EmrR	MarR	CCCP, DNP, Eb, salicylate	[93]
EmrKY	MFS	EvgSA	TCS	?	[124]
		H-NS	Histone-like protein	?	[159]
MdtABC	RND	BaeSR	TCS	Indole	[123]
		CpxAR	TCS	Indole	[122]
MdtEF	RND	ArcAB	TCS	Anaerobic condition	[126]
		CRP	CRP-FNR	N-Acetyl-D-glucosamine	
		EvgSA	TCS	?	[125]
		H-NS	Histone-like protein	?	[126,159]
		GadE		Acid stress	[161]
		GadX		Indole	[162]
Neisseria gonorrhoeae					
FarAB	RND	FarR	MarR	?	[118]
MtrCDE	RND	MtrR	TetR	Fatty acids, bile salts	[98]
		MtrA	AraC	TX-100 (detergent)	[117]
Pseudomonas aeruginosa					
MexAB	RND	MexR	MarR	Superoxide stress	[163,164],
		NalD	TetR	?	[165]
MexCD	RND	NfxB	LacI/GalR	Biocide chlorhexidine	[53]
MexEF	RND	MexT	LysR	Chloramphenicol, GSNO (nitrosative stress)	[99,100]
MexXY	RND	MexZ	TetR	Tetracycline, erythromycin, gentamicin	[166,167,168]
Pseudomonas putida					
ArpAB	RND	AprR	TetR	Organic solvents	[169]
SrpABC	RND	SrpR	TetR	?	[170]
		SrpS	IclR	?	[170]
TtgABC	RND	TtgR	TetR	Chloramphenicol, tetracycline	[101]
TtgDEF	RND	TtgT		Organic solvents	[171]
TtgGHI	RND	TtgV	IclR	Organic solvents	[172]
Salmonella typhimurium					
AcrAB	RND	AcrR	TetR	?	[173]
		MarA	AraC	?	[174]
		RamA	AraC	Indole, bile salts	[114]
		RamR	TetR	?	[115]
		SoxS	AraC	?	[112,174]
AcrD	RND	BaeSR	TCS	Indole, zinc, copper	[130]
		CpxAR	TCS	Indole, zinc, copper	[130]
AcrEF	RND	AcrS	TetR	?	[175]
MacAB	ABC	PhoQP	TCS	Magnesium	[57]
MdsABC	RND	GolS	MerR	Gold	
MdtABC	RND	BaeSR	TCS	Indole, zinc, copper	[130]
		CpxAR	TCS	Indole, zinc, copper	[130]

Table 1 (*continued*)

Efflux pump	Pump type	Regulator	Regulator family	Inducible signal	References
Staphylococcus aureus					
MepA	MATE	MepR		Chlorhexidine, cetrimide, dequalinium,	[102]
QacA	MFS	QacR	TetR	Rhodamine 6G, TPP	[41,103,104]
Stenotrophomonas maltophilia					
SmeABC	RND	SmeRS	TCS	?	[132]
SmeDEF	RND	SmeT	TetR	Triclosan	[176,177]

transcriptional regulator [117]. However, there is no direct evidence to demonstrate that MtrA binds to the *mtrCDE* promoter. FarAB, another RND type efflux pump in *N. gonorrhoeae*, was reported to be negatively regulated by FarR, a MarR family regulator, through direct binding to the *farAB* promoter [118]. In *C. jejuni*, the major MDR efflux pump CmeABC is negatively regulated by the global regulator, CosR, in response to paraquat [119]. CosR is an OmpR family regulator, and can directly bind to the *cmeABC* promoter as confirmed by both electrophoretic mobility shift assay (EMSA) and DNase I footprinting assays.

4.3. Regulation by two component systems

Two component system (TCS) is widely distributed in prokaryotes, which allows bacteria to sense and respond to various environmental changes. TCS is typically consisted of a histidine kinase and a response regulator [120]. The Membrane associated histidine kinase can sense and transduce different environmental stimuli through phosphorylation of the response regulator, which acts as the transcriptional regulator and alters the expression of downstream genes [120]. Based on homology analysis, it was suggested that there are total of 30 sensors and 34 response regulators in *E. coli* gnome [121].

A comprehensive investigation of the relationship between TCSs and efflux pumps revealed that 17 response regulators are involved in drug resistance in *E. coli* [122]. Using efflux pump gene deletion strains, it was found that the increased resistance in TCS overexpression background was attributed to the expression of several efflux pumps [122]. Among them, BaeSR system activated the expression of *mdtABC* and *acrD* in response to indole [113,123], CpxAR system activated the expression of *mdtABC* and *acrD* probably in response the envelop stress [122], and EvgSA system activated the expression of *mdtEF* and *emrKY* in response to yet unknown signals [124,125]. Recently, our group found that ArcAB TCS activated the expression of *mdtEF* efflux pump in M9 glucose medium under anaerobic condition by directly binding to multiple regions on the promoter of *gadE-mdtEF* operon [126,127].

In *Salmonella*, the expression of RND type MacAB pump is regulated by the PhoPQ TCS, the master regulator of the virulence of *Salmonella*, which senses and responds to low magnesium level and low pH condition [128,129]. Protection of *macA* promoter by purified PhoP protein in DNase I footprinting suggested that PhoP directly controls the expression of *macAB* [57]. Similar as in *E. coli*, the *Samonella* TCS BaeSR system also activates the expression of *mdtABC* and *acrD*, but in response to copper or zinc. EMSA assay confirmed the direct binding of BaeR to the promoter of *mdtABC* and *acrD* [130].

The involvement of TCS in the regulation of efflux pumps has also been reported in other species. It was reported that in *Acinetobacter baumannii*, expression of RND type efflux pump AdeABC is controlled by the AdeRS TCS encoded by *adeRS* genes located upstream of *adeABC* genes [131]. Inactivation of AdeR or AdeS led to susceptibility of *A. baumannii* to aminoglycosides which are the substrates of this pump, indicating the essential role of AdeRS in *adeABC* expression. However, the nature of the inducing signal and the mechanism of AdeRS activation remains unclear. The RND type efflux pump SmeABC in *S. maltophilia* is also reported to be controlled by a TCS called SmeRS, located upstream of the efflux pump genes. Studies have found that inactivation of SmeR response regulator resulted in decreased resistance to several antibiotics and overexpression of *smeR* can increase the transcript of *smeABC* [132].

5. Efflux pump inhibitors

Overexpression of multidrug efflux pumps has been frequently found in clinical isolates that have increased MICs to antibiotics. Recent studies suggested that multidrug transporters have become a major determinant for the efficacy of both new and old antibiotics [133]. As a consequence, antibiotic drug discovery and development in recent years have to take into consideration the overcome of antibiotic resistance in common Gram-positive and Gram-negative pathogens. Studies on the structures of multidrug efflux pumps not only provided essential evidence for the mechanisms of multidrug binding and extrusion, but also have shed light on the structure-based approach to discover efflux pump inhibitors (EPIs). To date, at least two classes of broad-spectrum EPI, such as peptidomimetics and pyridopyrimidines, have been extensively characterized [133].

Phenyl-arginine beta-naphthylamide (PAβN) [MC-207,110] was the first identified EPI that inhibits all four clinically relevant *P. aeruginosa* efflux systems, MexAB–OprM, MexCD–OprJ, MexEF–OprN, and MexXY–OprM [134]. It successfully reduced the emergence of levofloxacin-resistance in *P. aeruginosa* strain PAO1 treated with levofloxacin [135]. PAβN also has an activity against the AcrAB-TolC in a variety of Gram-negative pathogens, such as *E. coli*, *S. typhimurium* and *K. pneumoniae* [134]. It's inhibition mechanism was proposed to be acting as an RND substrate that occupies an affinity site in the large substrate-binding pocket different from that occupied by a given antibiotic [134]. However, the general use of this molecule remains questionable for several reasons. The most important reason is that high concentration of PAβN affects the membrane integrity of bacteria which may lead to LPS modification [135] and consequently the induction of undesirable resistance profiles by reducing drug penetration. PAβN derivatives have been explored in order to improve the efficacy of this class of peptidomimetic EPI. One derived compound, MC-04,124, is more stable than the original PAβN molecule in biological fluids, exhibits less toxicity, and has stronger activity against *P. aeruginosa* strains that overexpress efflux pumps [136].

Considerable efforts have been made to further modify this chemical series which led to the development of pyridopyrimidine compounds. However, the first generation of pyridopyrimidine derivatives had the problems of low solubility, high serum protein binding as well as lack of efficacy in vivo. Following the approaches of scaffolds re-design [137,138], addition of hydrophilic chains, as well as introduction of quaternary ammonium salt side chains [139,140], the compound [[2-({[((3*R*)-1-{8-{[(4-*tert*-butyl-1,3-thiazol-2-yl)amino]carbonyl}-4-oxo-3-[(*E*)-2-(1H-tetrazol-5-yl)vinyl]-4*H*-*pyrido*[1,2-a]pyrimidin-2-yl}piperidin-3-yl)oxy]carbonyl}amino) ethyl]

(dimethyl) ammonio] acetate [D13-9001] [141], which was referred to as AcrAB/MexAB-specific inhibitor of pyridopyrimidine derivative (ABI-PP) by Nakashima et al. [142], was developed. ABI-PP exhibited potent inhibitor efficacy in vivo, high solubility, and a good safety profile in an acute toxicity assay [141]. Recently, the X-ray crystal structures of ABI-PP bound with AcrB and MexB have been obtained and the structure shows that the pyridopyrimidine derivative (ABI-PP, mentioned as D13-9001priverously) binds to a narrow pit located in the distal pocket and hinders the rotation of the pump [142]. These structural details provided essential evidence for the structure-based development of universal inhibitors that inhibit both MexB and MexY.

Another group of EPIs is called the quinoline derivatives, because of their structural similarity with quinolones [143]. These compounds include chloroquinolone, alkoxyquinolone, alkylaminoquinolone, pyrridoquinolone, and thioalkoxyquinolone [136]. Some of them have been shown to be able to reduce the MICs of quinolones and cyclines. Moreover, these derivatives can increase intracellular concentration of radiolabelled norfloxacin or chloramphenicol [144,145] and increase antibiotic susceptibility for various strains including the clinical isolates of *Enterobacter aerogenes* and *K. pneumoniae* [145]. An advantage of this series of derivatives is that they do not destroy the membrane integrity of bacteria as measured by the potassium leakage and periplasmic activity tests [145].

In addition to the structurally modified synthetic compounds that display efflux pump inhibition activities, high-throughput assays have also used to screen compounds that might be putative EPIs. In a high-throughput screening for putative inhibitor of *E. coli* in the presence of novobiocin, a 3-arylpiperidine derivative was identified to be able to increase the intracellular concentration of norobiocin and another antibiotic linezolid [146]. Another screening of an N-heterocyclic organic compound library was conducted to identify putative EPIs that can reverse multidrug resistance in *E. coli* that over-expresses AcrAB and AcrEF efflux pumps [147]. Among the compounds tested, naphthylpiperazines (NMP) was the most potent arylpiperazines that has been shown to increase the intracellular accumulation of several antibiotics, such as fluoroquinolones, chloramphenicol, and linezolid. However, these compounds seem to be too toxic for clinical usage because of "serotonin agonist" properties [136]. Natural products (NP) that have been implicated in efflux inhibition have also attracted intensive attention since these compounds are often less toxic than synthetic compounds. Lee et al. [148] discovered two compounds, EA-371α and EA-371δ, in microbial fermentation extracts to be specific inhibitors of the MexAB–OprM pump in *P. aeruginosa*. In addition, pheophorbide *a* extracted from *Berberis aetnensis* was shown to lower the MIC of ciprofloxacin against *E. coli* and *P. aeruginosa* [149,150].

In Gram-positive bacteria, EPIs against the NorA system in *S. aureus* has been intensively studied. A large number of both synthetic and natural compounds have been found to be EPIs against *S. aureus* NorA, especially those of natural origins such as genistein isolated from *Lupinus argenteus*, spinosan A isolated from *Dalea spinosa* and Tiliroside isolated from *Herissantia tiubae* [151–153].

Though considerable efforts have been made to the development of EPIs, none of these EPIs is used in clinics currently. One main reason is that the mechanisms of most EPIs remain unknown, except the extensively investigated PAβN. But very recently, with the availability of the first inhibitor-bound structures of AcrB and MexB [142], our understanding of the detailed mechanism of EPIs and the development of clinically useful efflux pump inhibitors have been advanced significantly. Future research will focus on the development of extensive biological assays towards the application of EPIs in clinics, such as fitness and in vivo modeling studies [134]. In addition, expanded EPI efficacy assays beyond the several model microorganisms, such as *P. aeruginosa* and *E. coli* should also be developed.

In summary, significant development on the mechanism, regulation, and physiological functions of multidrug efflux pumps have been achieved in the past decades. The growing numbers of the X-ray crystal structures of efflux pumps as well as those with bound substrates or inhibitors continue to advance our understanding of this important determinant of MDR and guide the development of efflux pump inhibitors. EPIs combining with antibiotics presented as a promising intervention to combat infections caused by drug resistant pathogens. Approaches to prevent the over-expression of efflux genes by targeting to their transcription regulators provide an alternative emerging strategy. However, to facilitate their clinical applications, future endeavors to lower their cytotoxicity, improve solubility, as well as search for the candidates that can inhibit different classes of efflux pumps is necessary.

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