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Development of Efflux Pumps and Inhibitors (EPIs) in *A. baumanii*

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Abstract

Over the past three decades, *Acinetobacter baumannii* has emerged as an important nosocomial pathogen worldwide. Certain strains of *A baumannii* are now resistant to many common antimicrobial agents, including fluoroquinolone, and multidrug resistance (MDR) is often responsible for the failure of antibiotic therapy. Reasearchers have paid close attention to the emergency of *MDR-AB*, and *PDR* (pandrug-resistance)-*AB*, even *XDR* (extreme-drug resistance)-*AB* isolated from clinical patients, mediated primarily through the production of β-lactamases, aminoglycoside-modifying enzymes (*AMEs*), mutation in gene for DNA gyrase subunit A (*gyrA*) and DNA topoisomerase (parC), as well as efflux pumps. In this article, we discussed the roles of various efflux pumps expressed in *Acinetobacter baumanii* and efflux pump inhibitors (EPIs), aimed at providing an overview of the mechanism to antibiotics in *Acinetobacter baumanii*.

Keywords: *Acinetobacter baumanii*; Efflux Pumps; *RND; adeABC*; Efflux Pump Inhibitors (EPIs)

Introduction

Acinetobacter baumanii is a Gram-negative, non-fermenting, oxidase-negative and non-motile coccobacillus, [1]. It can survive in water, soil, surface of skin and hospital environment. It cause a series of opportunistic infections such as epidemic pneumonia, skin and soft tissue infections, bacteraemia, urinary tract infections and septicemia app:addword:septicemia in immunocompromised patients, especially in the intensive care unit [2]. With the overuse of broad-spectrum antibiotics, *A. baumannii* has become the mayor pathogen in nosocomial infections [3]. To make matters worse, therapeutic outcomes in patients are severely affected due to the emergency of acquired multidrug resistance. These multidrug resistant strains of *A. baumannii* employ resistant mechanisms such as the use of chromosomally encoded cephalosporinase, degradation of enzymes, modification or protection of the drug target sites, decreased permeability of the membrane and the active efflux of antibiotics [3,4]. Resistant mechanisms in *A. baumannii* have aroused researchers' widespread attention in last decades.

Recent studies show that over-expression of efflux system is an efficient mechanism for drug resistance. Various chromosomally encoded efflux systems, both intrinsic and acquired, have been characterized as contributing to *MDR* in *A. baumannii*. The best characterized are the *AdeABC*, first identified in an *MDR* clinical isolate *BM4454* [5], and *adeDE* in *Acinetobacter* Genomic DNA group 3 [6]. This review is focused on efflux mediated resistance in *A. baumannii*, especially on the resistance-nodulation-cell division (*RND*) family and EPIs.

Efflux Superfamilies in *Acinetobacter baumannii*

Efflux system, composed of an inner membrane protein (IM, such as RND efflux) linked by a major fusion protein (MFP) to an outer membrane factor (OMF), are able to extrude a wide range of substrates often unrelated in structure [7], these substrates include aminoglycosides, quinolones, tetracycline, and minocyline. According to the homology of amino acid sequence, five superfamilies of efflux systems are closely related to drug resistance: *RND* family, the major facilitator superfamily (*MFS*), multidrug and toxic compound extrusion (*MATE*) families, the small multidrug resistance (*SMR*) and the ATP-binding cassette (*ABC*) transporters [8] (Table 1). Among the efflux systems, *RND* pumps are the most prevalent systems in gram-negative bacteria [9-11].

RND superfamilies

Efflux pumps of the *RND* superfamily play a significant role in producing multidrug resistance in gram-negative bacteria [12], such as *AcrB* in *Escherichia coli*, *MexB* in *Pseudomonas aeruginosa* [3], and *AdeABC*, *AdeDE*, *AdeIJK*, *AdeFGH* in *Acinetobacter baumannii* [6,7,13-16]. Some efflux pumps of the *RND* family have been shown to play an important role in the colonization and persistence of bacteria in the host thereby maintaining the bacterium cell in steady state as well as expelling toxic compounds from the bacterium cell [17].

AdeABC

AdeABC, the major and the first identified efflux pump in *RND*, it is a kind of ATP-dependent multidrug transporter. In this efflux pumps, antimicrobials can be expelled from cells by utilizing the proton motive force. [4]. The *adeABC* operon encodes *AdeA* (Membrane Fusion Proteins, MFP), *AdeB* (Inner Membrane Efflux Transporters, IM) and *AdeC* (Outer Membrane Proteins, OMP). These three genes are contiguous in the genome. Adjacent to them are two-component regulatory systems, *adeR* and *adeS*, which specify proteins homologous to sensors and regulators of two-component systems, respectively (Figure 1). *AdeB*, which consist of 12 transmembrane fragments, has

Figure 1: Structure of adeABC system.

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Efflux Pumps		Energy resource	Substrates
RND	adeABC	Proton motive force	Aminoglycosides, tetracyclines, erythromycins, fluoroquinolones, β-lactams, chloramphenicol, trimethoprim, cefotaxime, minocyline,some toxicant and dyes such as ethidium bromide
	adeDE		Ceftazidime, amikacin, ciprofloxacin, chloramphenicol, erythromycin, ethidium bromide, meropenem, rifomycin, tetracycline
	adeIJK		Similar to adeABC, include β-lactams, tetracyclines, chloramphenicol, erythromycins, fluoroquinolones, trimethoprim, and sodium dodecyl sulfate (SDS) but not ethidium bromide
	adeFGH		Chloramphenicol, clindamycin, tigecycline, fluoroquinolones, trimethoprim, sulfamethoxazole and varies dyes such as ethidium bromide, sarranine
	adeXYZ		β-Lactams, ciprofloxacin, tetracycline, rifampin, chloramphenicol
MFS	Tet	Proton motive force	TetA-tetracycline only; TetB- tetracycline and minocycline; TetM- tetracyclines
	cmlA		Chloramphenicol
	CraA		Chloramphenicol
	AmvA		Mainly dyes, disinfectants, detergents; and only antibiotic-erythromycin
	SmvA		Erythromycin, methyl viologen, quaternary ammonium salt
MATE	abeM	Proton motive force	Fluoroquinolones, aminoglycosides, chloramphenicol, acriflavine,daunorubicin, rhodamine6G, ethidium bromide and so on
SMR	abeS	Proton motive force	Dyes, detergents; chloramphenicol, fluoroquinolones, erythromycin, and novobiocin
ABC	ABCB1	ATP hydrolysis	Cancer cells

Table 1: Efflux pump and its substrates.

the capacity to recognize antibiotics and extrude them out of cell, play a significant role in the efflux pump. Thus the *adeB* has been proposed to be an epidemiological tool for detecting clinical *MDR* strains. It has been shown that *adeC* is not essential for the *MDR* strains conferred by the pump, because an mutant displays similar resistance to the substrates of *AdeABC* to that of the parental strain when *adeC* is inactivated [18], but *AdeC* can expel antibiotics out of cell, because it is located in the epicyte and has porin as well. *AdeA*, which stabilize OMP, allows antibiotics to pass the inner and the outer membranes of the bacteria without accumulating within the periplasm [13].

AdeABC is very common in both resistant and susceptible *A. baumannii* strains. Alexander et al. [19], show the *adeA*, *adeB*, *adeR* and *adeS* are present in 80% of 116 strains of *Acinetobacter*, while *adeC* is present in 40% [19,20] also found that 34 carbapenem resistant *A. baumannii* expressed all the 5 genes, whereas other 2 strains are defective of *adeC* [20]. Expression of *adeABC* is tightly regulated by the two-component regulatory system, *adeR-adeS.* These *adeS* and *adeR* are sensor kinase and response regulator, respectively [10,18]. They are located upstream of *adeABC* and are transcribed in the opposite direction [8]. Mutations in *AdeRS* have been shown confer to overexpression of *AdeABC*, whereas inactivation of *adeR-adeS* is responsible for suscepbility in *A. baumannii* [18], it is known that *adeR-S* acts as a transcriptional activator in the pump. Mutation in *adeR* (Pro116Leu) and adeS (Thr153Met or Gly30Asp) will cause overexpression of *AdeABC* [18], and an insertion of an *ISAba1* upstream from the operon can also lead to overexpression of *AdeABC*, thereby enhancing efflux effect [14,21]. It has been reported that the *AdeABC*-overexpressing strains confer resistance to aminoglycosides, and decrease susceptibility to fluoroquinolones, cefotaxime, tetracycline, chloramphenicol, erythromycin, trimethoprim, minocyline [13] and tigecycline [14,22]. Recent data suggest that this system decreased netilmicin susceptibility of *A. baumannii* [19]. However, the role of *AdeABC* in carbapenem resistance strains raises many controversies. According to [23], overexpression of *AdeABC* leads to significantly high-level resistant to carbapenems, especially to imipenem, meropenem, and other class D carbapenemases [23]. However, in *OXA-23*-producing strains, overexpression of *AdeABC* is associated with resistant to meropenem but not to imipenem following inactivation of the pump [20]. Moreover, the overexpression of the *AdeABC* efflux pump is suggested to confer high-level resistance to carbapenems in conjunction with carbapenem-hydrolyzing oxacillinases in clinical isolates [24,25]. Thus, researchers agreed that overexpression of *AdeABC* contributes to carbapenem resistance, but other efflux mechanisms may probably also be involved. In addition, NaCl, in physiological concentrations, can induce significant tolerance to aminoglycosides, carbapenems, quinolones, and colistin among isolated clinical strains of *MDR A. baumannii*, resulting in broad-spectrum tolerance to antibiotics [26]. Several reports further suggest that *AdeABC* exhibits a wide substrate range that includes biocides, dyes and disinfectants, and other chemical substrates in *A. baumannii* [5,13,18].

AdeDE

The *adeDE* efflux pump has been reported to confer resistance to ceftazidime, amikacin, ciprofloxacin, chloramphenicol, erythromycin, rifampin, meropenem, tetracycline in *Acinetobacter genomic DNA group 3(GDG3)*[6,27] and imipenem in *A. baumannii* [28]. It however differs from *AdeABC*, *AdeDE* increases the host resistance to ceftazidime and rifampicin while *AdeABC* protects the host from cefotaxime. Unlike what was found for *AdeABC* efflux systems, the structural gene for the outer membrane proteins (OMP) is not found in downstream of the *adeDE* gene cluster [6].

Chu proved that 70% out of 83 *GDG3* isolates were positive for *adeE*, while all the 59 *GDG2* (*A. baumannii*) isolates were negative, and found that *adeE* was not only expressed in *GDG3*, but also exist in *GDG13TU* and *GDG17* isolates [27]. Lin found that *adeE* was only detected in *adeSR-adeABC/adeIJK/int1-negative* isolates that were relatively susceptible to some antibiotics, and also proved that *adeE* did not coexist with *adeIJK* and *adeABC*. In addition, mRNA expression of *adeE* was not observed in strains that were positive for adeE [29]. Thus, different distribution of *RND* genes may likely be used as indicators for differentiating *Acinetobacter* species. But recent studies suggest that *adeE* coexists with *adeB* in a small proportion of *A. baumannii* isolates [28].

AdeIJK

AdeIJK is the second identified efflux pump in *RND* families. *AdeIJK* encode a three-component *RND* efflux system consisting of *AdeI*, *AdeJ* and *AdeK*, which is similar to MFPs, inner membrane efflux transporters: *RND* proteins and OMFs, as in the *adeABC* efflux [30]. As opposed to *AdeABC*, *AdeIJK* is intrinsic present in all strains of A. *baumannii.* It contributes to resistance to β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, pyronine, safranin,

imipenem and sodium dodecyl sulfate (SDS) but not to ethidium bromide [28,30]. Several reports have shown that the levels of *adeIJK* overexpression are lower than those of *adeABC*, suggesting a threshold for toxicity for the host and that *adeIJK* is tightly regulated [8,16,31].

There are no ORFs coding for regulatory proteins in the vicinity of the *adeIJK* genes, therefore expression of *adeIJK* seems not to be specifically regulated but controlled at integrated level. Recent study showed that *AdeN*, a TetR-Type regulator, regulates the expression of *AdeIJK* in *A. baumannii* and also represses expression of the *adeIJK* (16). Moreover, some strains can express *adeIJK*, *adeABC* and *int1* simultaneously in *MDR-AB* [29].

AdeFGH

AdeFGH, encoded by the *adeFGH* operon, also confers multidrug resistance when overexpressed [15]. Expression of *adeFGH* is regulated by LysR-type transcriptional regulator (*LTTR*), named *adeL*, which locate upstream from the operon and transcribed in the opposite direction [8]. Mutation in *adeL* may lead to the overexpression of *adeFGH* [32]. It is responsible for high-level resistance to fluoroquinolones, tetracyclines, tigecycline, chloramphenicol, trimethoprim, sulfamethoxazole and various dyes such as EB, sarranine but has little effect when compared to β-lactams, erythromycin, rifampin and aminoglycosides [15,33].

AdeFGH is also very popular in *A. baumannii*. PAβN is found to inhibit the ability of the *AdeFGH* pump to efflux chloramphenicol, trimethoprim and clindamycin in the *A. baumannii* strains [34].

AdeXYZ

AdeXYZ, chromosomally encoded efflux systems, shares more than 97% identity with *AdeIJK* [30]. *AdeXYZ* is found in *Acinetobacter GDG3*, *Acinetobacter GDG13TU* and *Acinetobacter GDG 17* [27]. *AdeX*, *adeY*, *adeZ* are similar to MFP, RND, OMP in *RND* efflux system. The identity of the theoretical peptides to *AdeX*, *AdeY* and *AdeZ* are 80, 89 and 87, respectively [27]. In addition, *AdeXYZ* and *AdeDE* are predominantly in *GDG3* while *AdeABC* efflux system is specific for *GDG2* [27]. Until now, the function of *adeXYZ* in antimicrobial resistance is not well understood.

MFS

MFS belong to prevalent efflux system. *Tet*, *cmlA*, *CraA*, *AmvA* and *SmvA* are the most clinically relevant pumps in *MFS* superfamily in *A. baumannii*. *TetA* confers resistance to tetracycline alone while *TetB* is involved in tetracycline and minocycline resistance, but these two efflux pumps have no effect on tigecycline [35]. Researchers have found no strain carry *TetA* and *TetB* simultaneously in 79 tetracycline-resistant *A. baumannii* clones [35]. *TetA*, *TetB* and *TetM* were all resisitant to tetracyclines [36]. Another *MFS* resistant gene *cmlA*, found in *MDR A. baumannii* strain *AYE*, which is resistant to β-lactams (except imipenem, piperacillin-tazobactam, ticarcillin-clavulanate), aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, and rifampin, extrude chloramphenicol [37]. *CraA* is also reported to display intrinsic resistance to chloramphenicol [38]. Together with other efflux genes, *CraA* is overexpressed in response to NaCl exposure [26]. *AmvA*, a member of the *MFS* efflux pumps, mediates dyes, disinfectants and detergents resistance in *A. baumannii* [8,39]. Moreover, *SmvA* confers resistance in *A. baumannii* with erythromycin, methyl viologen and quaternary ammonium compounds [3].

MATE

MATE families have two energy sources: the PMF (proton motive force) and the sodium ion gradient [40]. A member of the *MATE* family, *AbeM*, a H+-coupled multidrug efflux pump extrudes fluoroquinolones, aminoglycosides, chloramphenicol, trimethoprim, erythrocin, ethidium bromide, and dyes [41]. *AbeM* protein, which consists of 448 amino acid residues, contains numerous hydrophobic amino acid residues and 12 hydrophobic regions. The substrates for the *AbeM* efflux pump are not widely known as compared to *adeABC* [41]. Some studies have shown that expression of *abeM* is not related to resistance to cephalosporins or aztreonam [42]. To date the contribution to antimicrobial resistance of this system in clinical isolates remains unknown.

SMR

abeS, a chromosomally encoded *SMR* efflux pump, exhibits efflux effect in relation to chloramphenicol, fluoroquinolones, erythromycin, novobiocin, dyes and detergents in *A. baumannii* [43]. *abeS*, a 330-bp gene, encodes a 109-amino-acid protein, and displays 100% identity to the product of ORF *ABAYE1181*. It exhibits 51.8% identity to *E. coli EmrE* found in the *A. baumannii AYE* genome [37]. Subsequently, *abeS* is confirmed to be an energy-dependent efflux pump, and its extruding energy source is proton (H+) [43].

ABC

ATP-binding cassette (*ABC*) transporters mediate multidrug resistance though ATP-dependent drug efflux pumps. It can energize the transport of variety of compounds across biological membranes via ATP hydrolysis and confer cellular resistance to a broad spectrum of drug substrates [44]. *ABCB1*, is a 170 kD transmembrane glycoprotein from the super family of ATP-binding cassette and one of the most widely studied transporters that enable cancer cells to develop drug resistance [45]. The major mechanism of efflux is dependent on energy generated for protein transport through the hydrolysis of ATP [46]. *ABCB1*, also known as P-glycoprotein (P-gp) or multidrug resistance protein 1 (*MDR1*), serves as an ATP-dependent efflux pump for a variety of chemicals, including many antimicrobial agents [45].

The Development of Efflux Pump Inhibitors (EPIs)

Resistant to antibiotics is increasingly becoming a worldwide severe problem in recent decades. Efflux system plays significant roles in antibiotic resistance in *A. baumannii* and many other Gram-negative bacteria. Subsequently, efflux pump inhibitors were developed to decrease the resistant effects employed by efflux system. PAβN and CCCP were the best studied synthetic inhibitors while reserpine and verapamil are the most used EPIs in clinical practice in recent years. EPIs can increase the intracellular concentration of antibiotics expelled by efflux pumps, and then maintain the initial sensitivity to antibiotics associated with overexpression of efflux pumps [47] and decrease the frequency of resistant mutant strains [48]. EPIs can hinder efflux channel, block-up energy resources of efflux pumps and make bacterium cells sensitive to the antibiotics.

PAβN

PAβN (phenylalanine-arginine β-naphthylamide, also called MC-207 110), is a well studied EPI and has been described as a broad-spectrum efflux pump inhibitor. PAβN has no effect on bacteria cells itself, unless used synergistically with β-lactam [49], fluoroquinolones and macrocyclic antibiotics. It is known to reverse resistance in *Pseudomonas aeruqinosa, Staphylococcus aureus, Enterococcus* and Gram-negative bacteria through increasing the level of accumulation of efflux pump substrates within the cell [50,51]. Many studies suggest that PAβN reduce the minimal inhibitory concentrations (MICs) of several β-lactam antibiotics such as chloramphenicol, rifaximin, tigecycline,

clarithromycin against *E. coli*, **E. aerogenes,** or **P. aeruginosa** [49,52-56]. Again, PaβN is able to enhance the permeability of cell membrane to increase antibiotic levels in the bacterium cells [51].

CCCP

CCCP (Carbonyl cyanide-m-chlorophenylhydrazone) is a strong uncoupler that disturbs electrochemistry gradient to inhibit *RND*, *MATE*, *MFS* and *SMR*, whose driving force come from proton motive [57]. Thereby increasing intracellular concentrations of antibiotics. CCCP has become a necessary tool for studying efflux systems MICs. It is known that with CCCP, MICs of fluoroquinolone, chloramphenicol, gentamicin, amikacin, ceftazidime, cefoperazone/sulbactam and imipenem in *A. baumannii* decreases relatively as compared to without CCCP [58-64].

Reserpine and verapamil

Reserpine and verapamil can block-up substrates' channel to play its role. Alkaloid reserpine is the first identified inhibitor of *NorA*, but it is documented to be toxic to the host cell if used to inhibit the activity of *NorA* [65]. Compared to CCCP, reserpine decreases the MICs of nalidixic acid, ciprofloxacin and norfloxacin, and the reserpine affects isolates which are not affected by PAβN. This therefore suggests that the two inhibitors may possibly act on different efflux pumps [66].

Others

NMP(1-(1-naphthylmethyl)-piperazine), can partially reverse *MDR* in *A. baumannii* [58], it has stronger effect than PAβN when higher concentration (100 mg/L) was used, but its effect is limited at a lower concentration (25 mg/L) [54]. In addition, NMP is capable of inhibiting the overexpression of *RND* pumps in *Escherichia* [54]. Omeprazole and lansoprazole are proton pump inhibitors, which can suppress the activity of H^+/K^+-ATP enzyme. Omeprazole is effective in *Helicobator pylori* and *Staphylococcus aureus* [67], but it's function in *A. baumannii* is not clearly been documented.

Interestingly, researchers have found a natural EPI extracted from a plant which is used in Chinese medicine, named *5′-methoxyhydnocarpin (5′-MHC)*, which inhibits the *NorA MDR* pump of a human pathogenic *Staphylococcus aureus* when combined with antibiotics [68].

Conclusion

The positive rate of multidrug resistance *A. baumannii* is dramatically increased in recent decades all over the world, and the mechanism of resistance is complicated. Together with antibiotics, EPIs provide a new horizon to reverse bacterial resistance, but the studies are in preliminary stages. Its usage is known to be restricted to *in vitro* because the effective inhibitory concentration is much higher than the concentration used in clinical application, so it could be toxic if applied to patients. Investigators are recommended to research into these mechanisms explored by *A. baumannii* in other to produce effective and safe EPIs for clinical use.

Competing Financial Interests

The authors have declared that no competing interests exist.

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