Xian-Zhi Li · Christopher A. Elkins
Helen I. Zgurskaya *Editors*

Efflux-Mediated Antimicrobial Resistance in Bacteria

Mechanisms, Regulation and Clinical Implications

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Foreword

One of the greatest threats to the control of infectious diseases is universal drug resistance; this is an increasing and apparently irreversible phenomenon. The disastrous failure of physicians, government, pharmaceutical companies, and the general population to control the uses and misuses of antimicrobial agents is one of the most tragic events of the twentieth century. Antibiotic resistance is now a worldwide plague that shows no evidence of declining.

It is essential that increased funding and commitment are devoted to studying the origins of the biochemical mechanisms of drug resistance and their genetic dissemination. Resistance takes many forms and is often a complex process, as in the case of multidrug resistance. However, the primary response of most pathogens to antibiotic exposure is drug efflux, and a better understanding of this process with more extensive studies would lead to improved therapeutic applications.

This collection of reviews on the above topic, edited by Drs. Xian-Zhi Li, Christopher A. Elkins, and Helen I. Zgurskaya, is without question the "must-have" book. Herein an expert group of specialists have covered all aspects of efflux-mediated drug resistance. Nothing has been left out, and notably, the last three chapters of this book discuss the absolute necessity for encouraging novel approaches to the prevention of drug efflux processes in bacteria. Insufficient attention has been paid to this essential aspect in the past, and more effort must be devoted to this vital topic.

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February 2016

Preface

Antibiotics are vital to modern medicine. However, bacterial resistance constitutes a growing threat to effective antimicrobial therapy worldwide. Indeed, antimicrobial resistance continues to be one of this century's major public health threats exemplified by intra- and intergovernmental efforts and task forces. In 2014 and 2015, Canada published its Federal Action Plan and Framework on Antimicrobial Resistance and Use, while an executive order was released in 2014 by the president of the United States to establish a National Strategy to Combat Antibiotic-Resistant Bacteria. The World Health Assembly also endorsed a global plan to tackle antimicrobial resistance in May 2015. Resistance takes many forms, and bacterial drug efflux pumps constitute a major mechanism for both natural and acquired resistance to a diverse range of clinically used antibiotics and other toxic agents including biocides. The latter insinuates that infection control may inadvertently link with multidrug resistance through efflux mechanisms. Drug efflux pumps are ubiquitously distributed in bacteria, and their role as a key mechanism of resistance and other functions, including pathogenicity, cannot be overstated.

Initially recognized in the late 1970s for their role in drug-specific resistance, bacterial efflux pumps were further investigated in the early 1990s for their significant contribution to multidrug resistance. Over the last two decades, a large number of bacterial drug exporters have been characterized. In particular, in-depth studies on prototypical pumps of various transporter families have greatly enhanced our understanding of multidrug transporter structures and transport dynamics, as well as their expression, regulation, and clinical ramifications. Efflux-based phenomena also interact synergistically with other resistance mechanisms including the membrane permeability barrier, drug inactivation, and drug target alterations to enhance resistance levels and cause clinically resistant profiles. These characteristics pose major challenges to antimicrobial development and therapy. Efflux pumps are candidate drug targets for therapeutic interventions and open the potential for combinatory products that may reinvigorate the current arsenal of decreasingly effective drugs. This book describes our current understanding of the above advancements.

The contributors of this book hail from various leading international scientific groups. We are sincerely grateful to them for their efforts in the preparation of this

outstanding work divided into four sections with a total of 30 chapters. The insights of the authors on various aspects of drug efflux-mediated resistance will be beneficial to the future of research in this field and provide a strong scientific argument in promoting antimicrobial stewardship to minimize resistance threats. We also wish to express our immense gratitude to Dr. Hiroshi Nikaido, not only for the opportunity to work with him on the drug efflux phenomena but also for his encouragement on this book. We are also honored to have the support from Dr. Julian E. Davies in writing the Foreword of this book. Xian-Zhi Li acknowledges the support from Daniel Chaput. Finally, we especially thank Dene Peters at Springer for initiating and guiding this project. We also deeply appreciate the help from other staff from the publisher, Carole Pearson, Mahalakshmi Sethish Babu, Kalpana Venkataramani, and Cameron Wright, during this entire process.

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Part II Bacterial Drug Efflux Pumps in Antimicrobial Resistance: Substrates, Regulation and Clinical Significance

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Part I
Bacterial Drug Efflux Pumps: Structures and Transport Mechanisms

Chapter 1
Structures and Transport Mechanisms of RND Efflux Pumps

Satoshi Murakami

Abstract Multidrug efflux transporters of the resistance-nodulation-cell division (RND) superfamily are one of the main causes of multidrug resistance (MDR) in Gram-negative bacteria. These proton motive force-dependent transporters generally occur as tripartite efflux complexes that span the cytoplasmic and outer membranes, thus providing a mechanism to directly extrude antimicrobial substrates out of the cell. RND transporters can accommodate an incredibly wide range of antimicrobial agents and play an important role in clinically relevant antimicrobial resistance. The first crystal structure of a multidrug efflux transporter was solved in 2002 for the AcrB RND transporter of *Escherichia coli*. Subsequently over the last decade, several structures of RND transporters have been successfully crystallized both in the presence and absence of their antimicrobial substrates, providing a fundamental understanding of the structural basis for functional transport mechanisms. This chapter describes the current knowledge of the structural and functional characteristics of RND efflux pumps based on these crystal structures.

Keywords Multidrug resistance • Transporters • Structure • Transport mechanism • RND efflux pumps • *Escherichia coli* • *Pseudomonas aeruginosa* • AcrB • MexB

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1.1 Introduction

Emerging and reemerging infectious diseases are still one of the major global health problems. Meanwhile, antimicrobial resistance of microorganisms and pathogens has rapidly evolved, posing a challenge for chemotherapy of these diseases. Nowadays, antimicrobial-resistant pathogens are widely seen in hospital- and community-acquired infections, including life-threatening diseases. Many resistant microbes possess simultaneous resistance to multiple structurally unrelated antimicrobial agents, a phenotype which is referred as multidrug resistance (MDR). Several frequently reported Gram-negative pathogens, such as *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa*, are prevalent with MDR phenotypes and greatly contribute to the major threats from antimicrobial resistance, thus rendering many antimicrobial drugs ineffective [1].

A common structural feature of Gram-negative bacteria is their cell envelope consisting of two cell membranes – the cytoplasmic (inner) membrane and outer membrane [2]. It is well known that Gram-negative bacteria are much less susceptible to many antimicrobial agents that are active against Gram-positive bacteria. This phenomenon is attributable to both the outer membrane permeability barrier [3–5] and the presence of drug efflux systems [6–8]. The latter can actively export a wide range of antimicrobial agents (such as conventional antibiotics, antiseptics, and many cytotoxic chemicals), thus reducing the intracellular concentration of these agents. In bacteria, drug efflux transporters generally belong to one of five transporter superfamilies: (i) ATP-binding cassette (ABC) superfamily; (ii) The major facilitator superfamily (MFS) [9]; (iii) The drug/metabolite transporter (DMT) superfamily (which contains the small multidrug resistance [SMR] family) [10]; (iv) The multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (which includes the multidrug and toxic compound extrusion [MATE] family) [11]; (v) The resistance-nodulation-cell division (RND) superfamily [12]. Recent studies have described novel drug transporter families including antimetabolite transporters (the AbgT family) [13] and the proteobacterial antimicrobial compound efflux transporters (the PACE family) [14].

Of the known multidrug transporters, those within the RND superfamily are typically described as tripartite molecular complexes that span both cell membranes [15]. RND transporters are further divided into seven families that include the hydrophobic/amphiphilic efflux (HAE) family, the heavy metal efflux (HME) family, and the SecDF protein-secretion accessory protein (SecDF) family [12] that are to be discussed in this chapter. Transporters of the HAE subfamily, such as AcrB of *Escherichia coli*, are featured by the broadest substrate profiles. They accommodate drug molecules that not only have cytosolic targets (such as macrolides, quinolones, and tetracyclines) but also target the outer leaflet of the cytoplasmic membrane (i.e., the periplasmic side) (and include agents such as β-lactams). RND pumps export these agents out of the cell across the outer membrane [15]. Thus, they are thought to be the major efflux transporters conferring drug resistance. In fact, RND pumps

are recognized as one of the most effective systems for host defense since detrimental substances are actively extruded out of the cell. Genes encoding RND transporters are found in all Gram-negative bacteria [12]. It is important to note that certain Gram-positive bacteria (such as mycobacteria, *Bacillus subtilis*, and *Staphylococcus aureus*) also possess RND pumps [16–19].

AcrB from *E. coli* and MexB of *P. aeruginosa* were the earliest reported RND pumps. In *E. coli*, the *acr* locus influencing drug susceptibility was first discovered by Nakamura et al. in the 1960s and 1970s [20, 21], although its role for drug efflux remained unknown until in 1993 when Ma et al. [22] reported the molecular characterization of the *acrAE* (i.e., *acrAB*) genes and their involvement in drug efflux. For *P. aeruginosa*, Lei et al. [23] showed in 1991 the elevated ofloxacin extrusion by *in vitro*-selected ofloxacin-resistant mutants. Multidrug efflux mediated by the *mexAB-oprM* (initially named *mexAB-oprK*) operon of *P. aeruginosa* was reported in 1993 by Poole et al. [24], which was substantiated by studies from Li et al. [25–27] that demonstrated the predominant role of drug efflux pumps in the intrinsic resistance (including resistance to β-lactams) of *P. aeruginosa*. However, despite various additional molecular studies revealing the clinical importance of efflux pumps in Gram-negative bacteria in the 1990s [28–30], structural information for these transporters remained unknown until 2002, when the crystal structure of AcrB was reported by Murakami et al. [31]. Subsequently, various structural analyses, protein engineering, and molecular dynamics (MD) simulation studies of RND pumps have been conducted based on these crystal structures. Indeed, RND transporters are one of the most studied transporter superfamilies, with much in-depth knowledge generated for the overall structural and functional basis of transport mechanisms. In this chapter, crystal structures of RND transporters and their functional relevance to transport mechanisms are discussed.

1.2 Crystal Structures of the RND Multidrug Efflux Transporters

The first crystal structure of an RND transporter was described for the AcrB pump of the *E. coli* AcrAB-TolC efflux system in 2002 [31]. Not only is this the first crystal structure of a multidrug efflux transporter but is also the first structure of secondary active transporters driven by the proton motive force across the membrane. Following this accomplishment, various groups have determined crystal structures for several transporters of all five major families including ABC-type Sav1866 [32], MFS-type EmrD [33], SMR-type EmrE [34], and MATE-type NorM [35] (Fig. 1.1). As for the RND superfamily, in addition to crystallized AcrB (both with and without drug substrates) [31, 36], multidrug efflux transporters MexB of *P. aeruginosa* [36, 37] and MtrD of *N. gonorrhoeae* [38] and two heavy metal ion efflux transporters, CusA of *E. coli* [39] and ZneA from *Cupriavidus metallidurans* [40], were reported in the last decade (Fig. 1.2). SecDF, with a pseudosymmetric, 12-helix

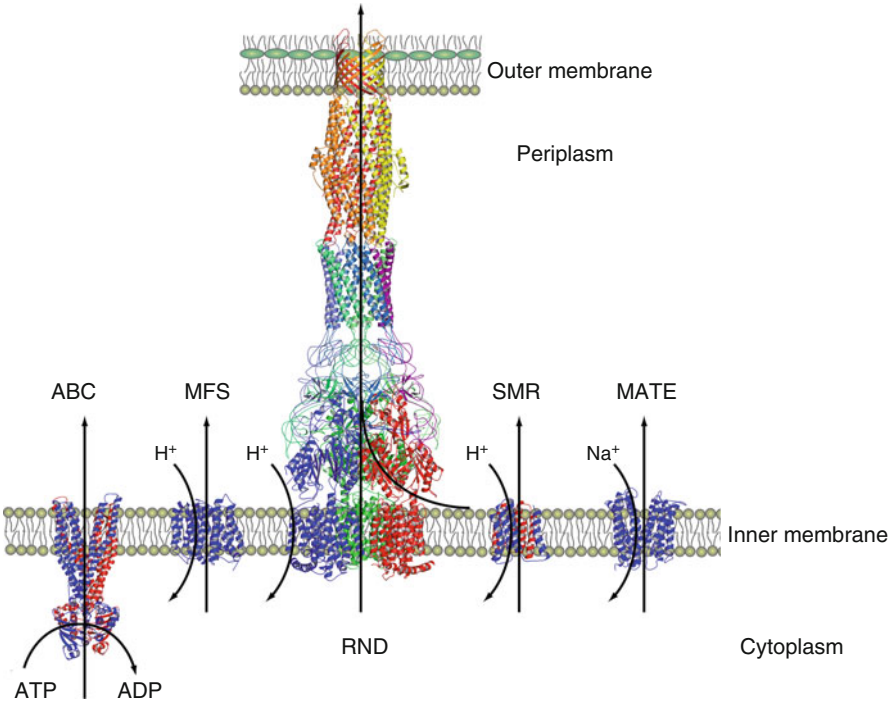


Fig. 1.1 The first reported crystal structures of five major drug efflux transporter superfamilies/families. From *left* to *right*, Sav1866 from *S. aureus* (*ABC*), EmrD (*MFS*), AcrB (*RND*; together with the periplasmic membrane fusion protein AcrA and the outer membrane channel protein TolC), EmrE (*SMR*) from *E. coli*, and NorM of *Vibrio cholerae* (*MATE*) are shown

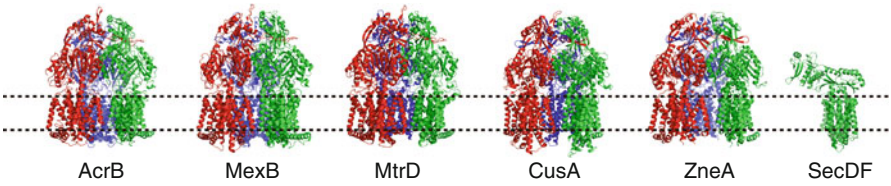


Fig. 1.2 Crystal structures of six RND transporters. AcrB of *E. coli* (PDB code 1IWG), MexB of *P. aeruginosa* (2V50), MrtD of *N. gonorrhoeae* (4MT1), CusA of *E. coli* (3KO7), ZneA of *Cupriavidus metallidurans* (4K0J), and SecDF from *Thermus thermophilus* (3AQP) are shown in the ribbon representation

transmembrane domain belonging to the RND superfamily (Fig. 1.2) [41], mediates protein translocation across the cytoplasmic membrane when in complex with SecYGE as a protein secretion system. A role of SecDF in antimicrobial resistance was demonstrated in *S. aureus* [19]. As expected, the crystal structures of these pumps are not the same for the pumps from different bacterial species with varied substrate specificities. However, they do share very high structural similarity between each other. In this section, fundamental structural features of RND transporters are first introduced for AcrB, the prototypical representative of the RND superfamily, and then described for several other RND transporters.

1.2.1 AcrB from E. coli

The crystal structure of AcrB (Protein Data Bank [PDB] codes 1IWG and 2DHH) was solved at 3.5 Å resolution (Fig. 1.3) [31]. In this analysis, AcrB monomers comprise tight trimeric conformation with domain swapping by mutual insertion of their long hairpin loop in the periplasmic domain (Figs. 1.3 and 1.4). This trimeric conformation has threefold symmetry owing the threefold crystallographic symmetry of the crystal used in this crystallographic analysis. Thus, the structure of

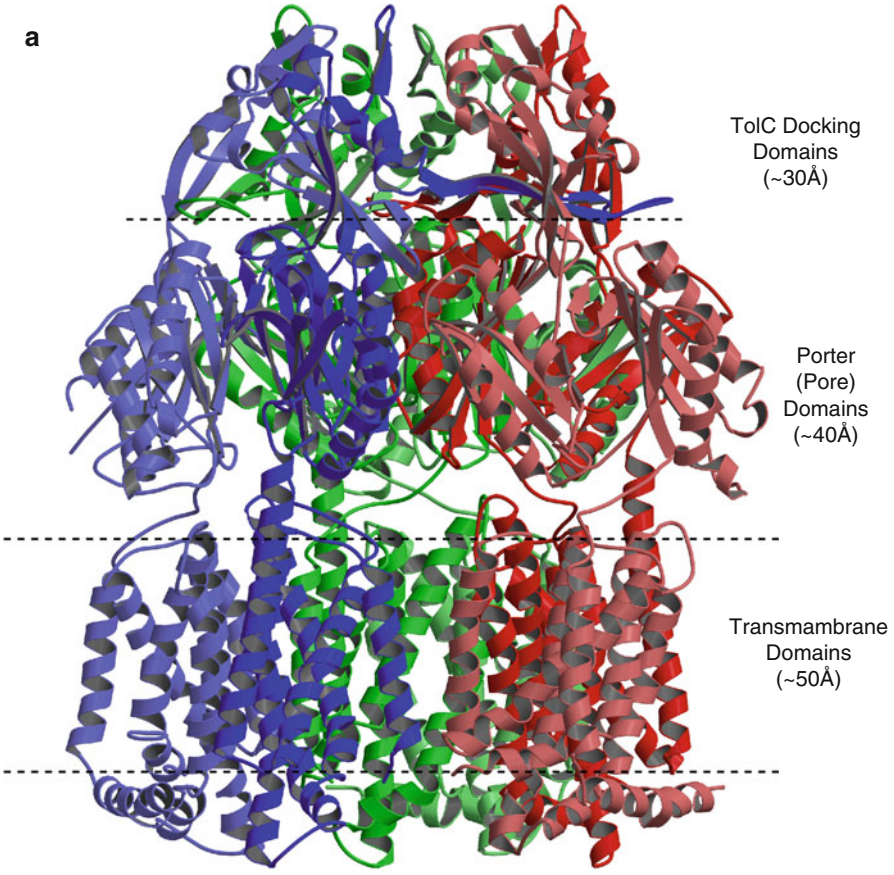


Fig. 1.3 Crystal structure of AcrB. (**a**) Side view, parallel to the membrane plane. Three protomers are individually colored (*blue*, *green*, and *red*). The N-terminal and C-terminal halves of the protomers are depicted as dark and pale colors, respectively. The periplasmic region (TolC-docking domains and pore domains) is at the *top*, and the transmembrane region is at the *bottom*. (**b**) *Top view*. The protomers are individually colored as in A. (**c**) Structure within a slab (23 Å) of the transmembrane domain parallel to the membrane plane near the periplasmic surface. The protomers are individually colored as in A and B. Threefold and pseudo-twofold rotation axes are indicated. The label numbers indicate the transmembrane helix numbers (TMx in **c**)

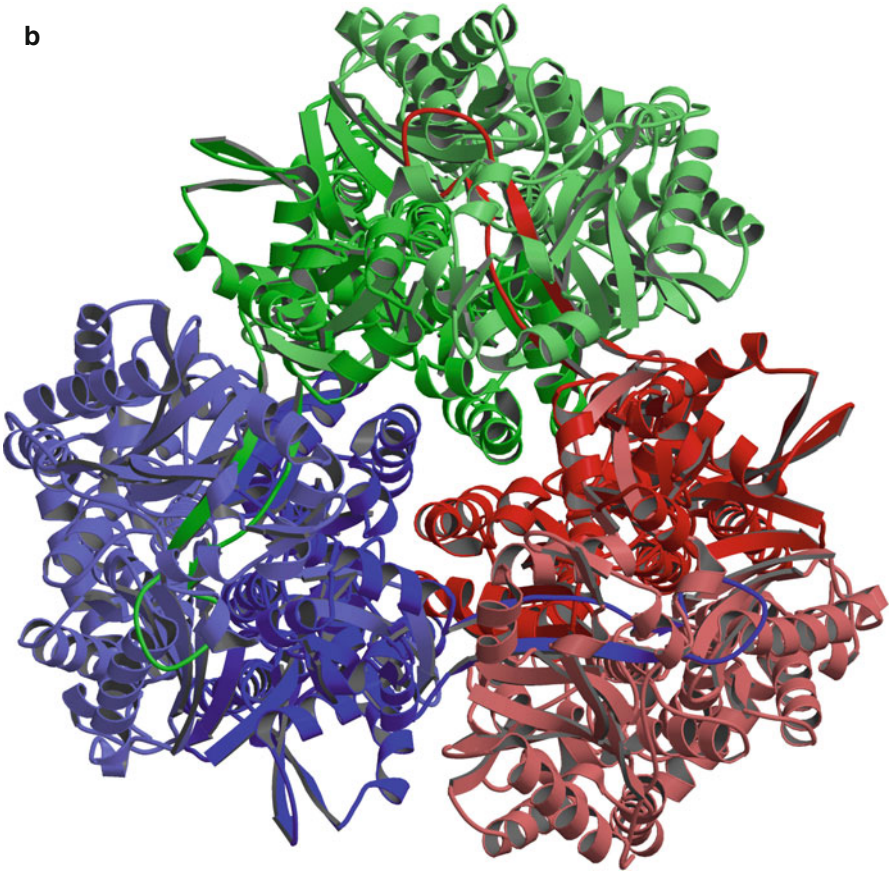
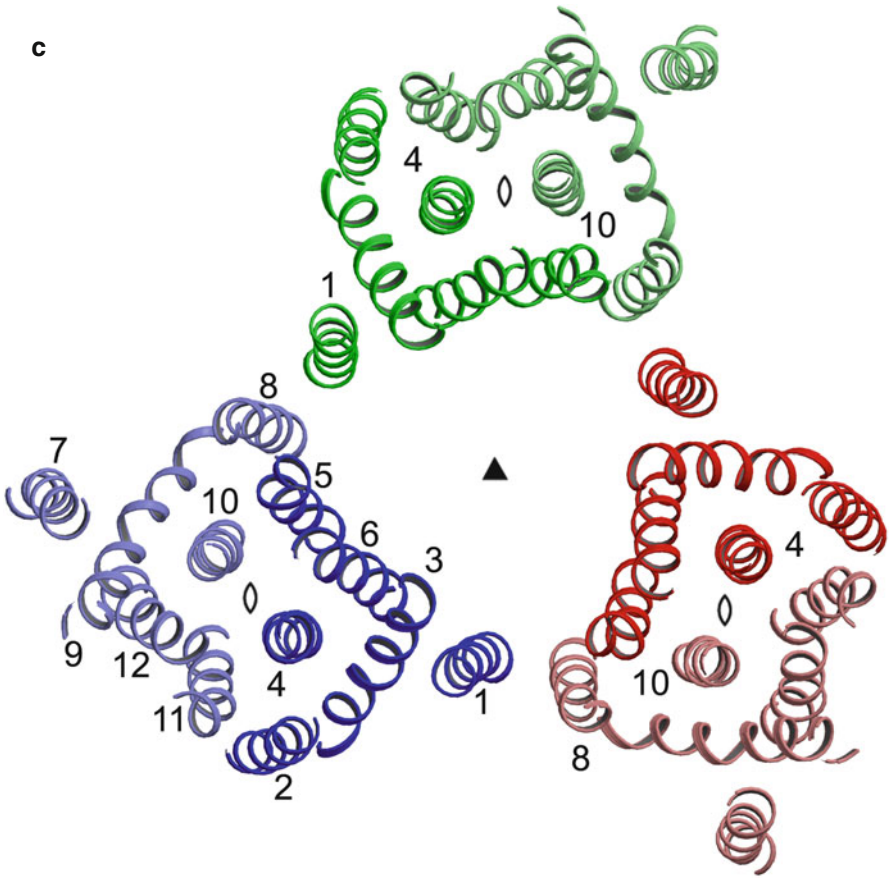


Fig. 1.3 (continued)



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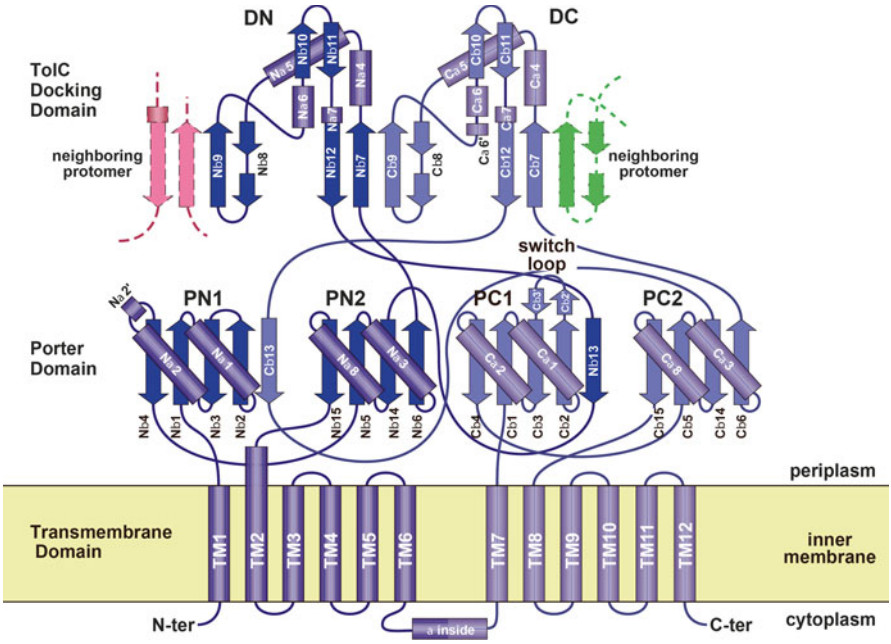

Fig. 1.4 Topology diagram of AcrB monomer. Secondary structure elements are indicated: TM, transmembrane helices; Nα and Nβ, helices and strands, respectively, in the N-terminal half of the headpiece; Cα and Cβ, helices and strands, respectively, in the C-terminal half of the headpiece; lα, a helix attached to the cytoplasmic surface of the membrane. N- and C-terminal halves are depicted in dark and pale blue, respectively. The porter domain is divided into four subdomains; PN1, PN2, PC1, and PC2. The TolC-docking domain is divided into two subdomains, DN and DC. The hairpin structures for mutual insertion between TolC-docking domains of neighboring protomers are depicted in *green* and *pink*

each protomer included in this trimer is averaged and identical by the crystallographic symmetry. In each protomer, there is a pseudo-twofold symmetry (Fig. 1.4). It is not only found at structural level but also at the primary sequence level. Moreover, it has been hypothesized that gene duplication and fusion events evolutionally drove this pseudo-twofold property of RND transporters. A similar pseudo-twofold symmetry can also be found in other transporter families, like the MFS, SMR, and ABC families [32–34].

In 2006, two groups [42, 43], and in 2007, one group [44] reported new crystal structures of the same AcrB from *E. coli*. These independent studies used different crystal forms belonging to differing space groups. All crystals used by each group did not have threefold crystallographic symmetry in their crystallographic analysis. Thus, these groups solved essentially consistent asymmetric structures of AcrB. At the same time, the Murakami group also solved the AcrB structure with bound substrates (doxorubicin and minocycline) with a lower symmetry conformation and showed that only one substrate molecule is bound asymmetrically to one of the protomers of the AcrB trimer (Fig. 1.5). This protomer has a distinct conformation suitable to accommodate substrates inside the molecule. All three groups concluded

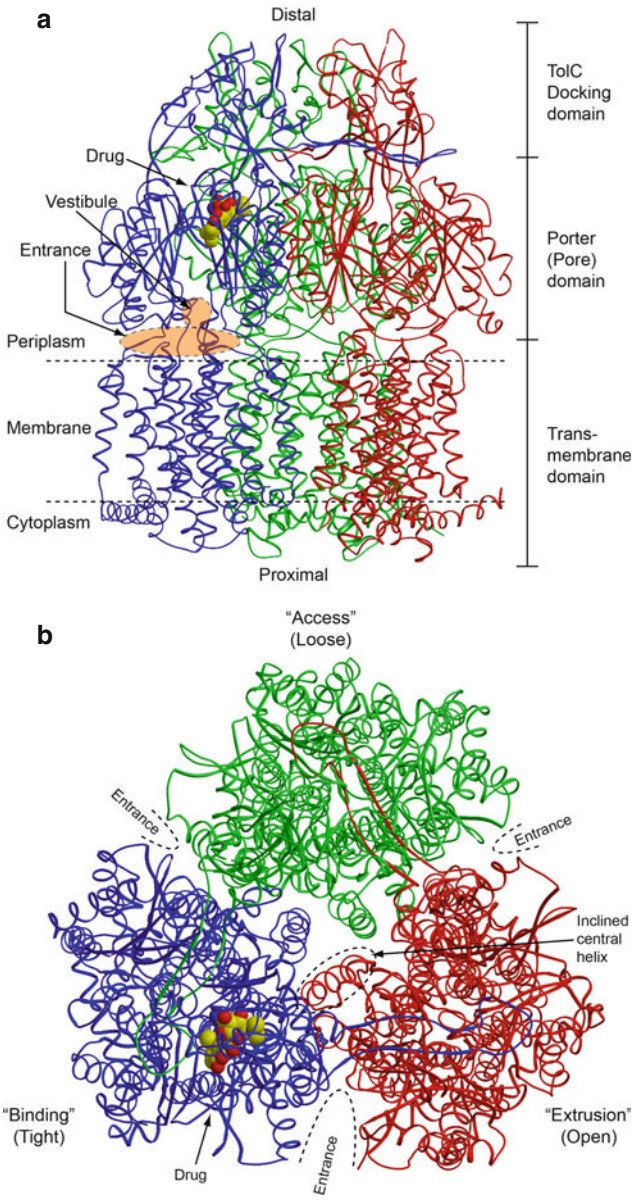

Fig. 1.5 Structure of the AcrB-minocycline complex in the asymmetric conformation. Three protomers are individually colored as same as those in Fig. 1.2. The minocycline molecule is shown in CPK (Corey-Pauling-Koltun) representation, with the position of the C, N, and O atoms indicated by *yellow*, *blue*, and *red* balls, respectively. (**a**) *Side view*, parallel to the membrane plane. (**b**) *Top view*. (**c**) Cut view of the porter domain from the outside of the cell. Phenylalanine residues, including those in the distal binding pocket, are shown in the ball-and-stick representation. The difference Fourier map of bound minocycline is depicted by an orange-colored cage. *Arrows* indicate the relative movements of the subdomains in comparison with those in the former state (The figure is from Murakami et al. [42])

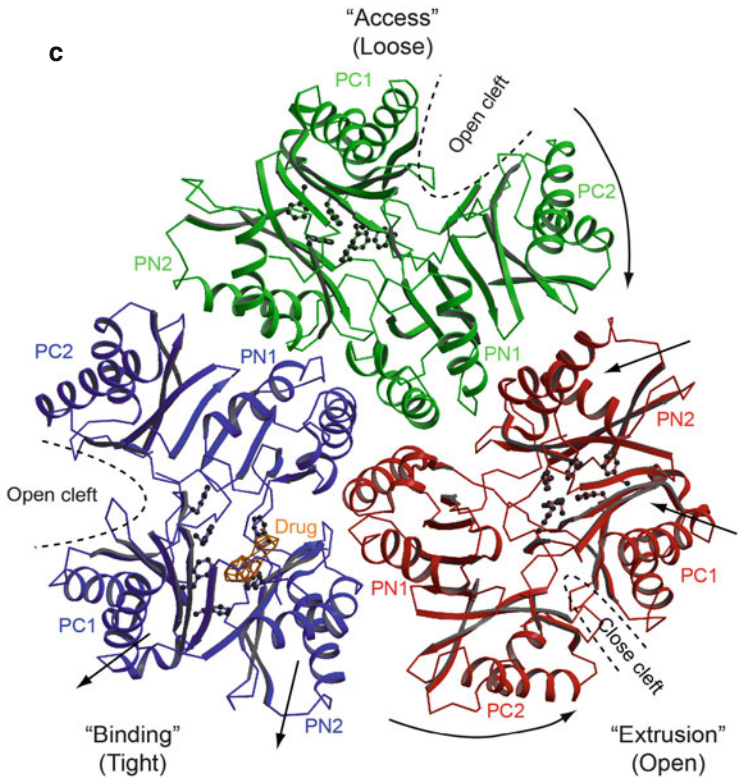

Fig. 1.5 (continued)

that this asymmetric conformation is likely the physiological relevant form and proposed either a functionally rotating mechanism or a peristaltic pump mechanism (Fig. 1.6) [42, 43]. This idea is conceived from the analogy of the rotational catalysis proposed and proved for F_1F_0-ATPase [45].

In asymmetric crystal structures of the AcrB trimer, there are three distinct conformations: "access" ("loose" as in another explanation by Seeger et al. [43]), "binding" ("tight"), and "extrusion" ("open") conformations [42] (Fig. 1.6). These conformations are considered to be the three major functional states during the substrate translocation cycle. Even though AcrB is a homo-trimeric complex, each protomer included within this trimer exhibits asymmetrically different conformations.

Each protomer is divided into three layers: the cytoplasmic domain (~10 Å), the transmembrane domain (~40 Å), and the periplasmic domain (~70 Å) (which protrudes into the periplasmic space) (Fig. 1.3). Within the cytoplasmic domain, there is an α-helix almost parallel to the membrane plane, attaching inner leaflet of the cytoplasmic membrane from the cytoplasmic side (Figs. 1.3 and 1.4). This helix is regarded to play an important role as a stabilizer float of AcrB. Similar parallel

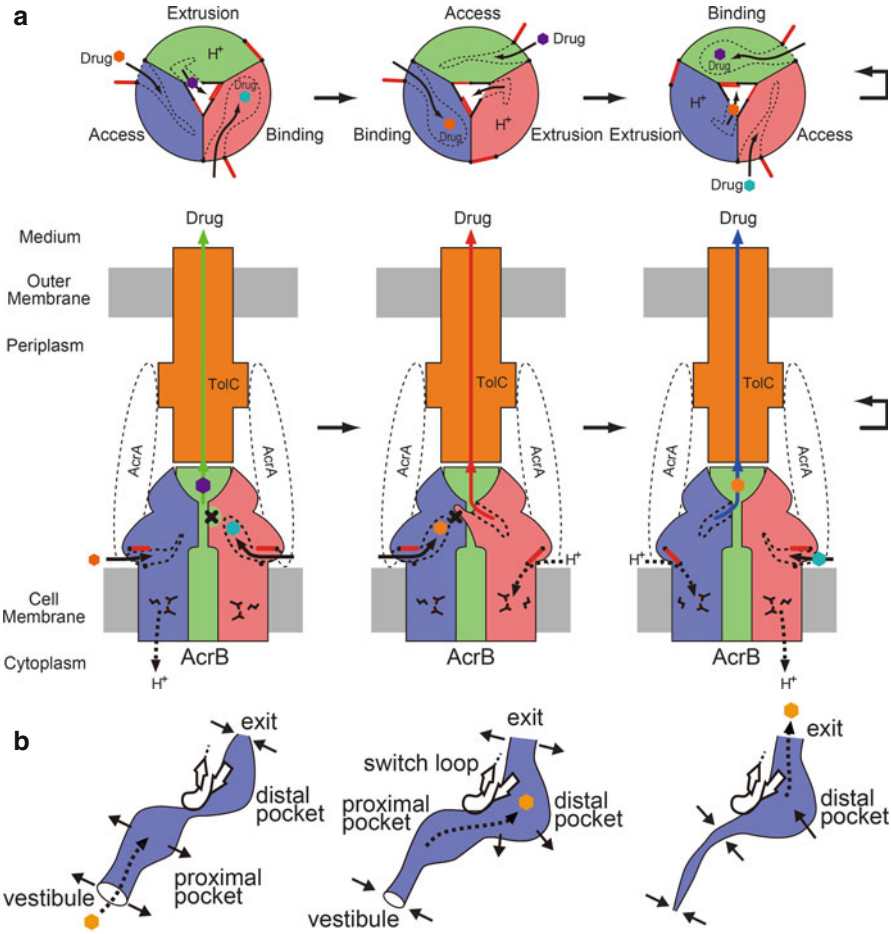

Fig. 1.6 Schematic illustration of the functionally rotating mechanism with the peristaltic pump-like motion in the substrate translocation pathway mediated by AcrB. The top view from the outside of the cell (*upper*). The view from the side parallel to the membrane plane (*middle*). Schematic drawing of the substrate translocation pathway in the protomer colored in blue, and its peristaltic movement during the substrate extrusion cycle ("*left*" → "*center*" → "*right*" corresponds "access"→ "binding"→ "extrusion"). Substrate is transferred from the vestibule through the proximal and distal pockets to the exit toward the TolC outer membrane channel. *Arrows* indicate in the bottom figures the relative peristaltic movement of the translocation pathway during the substrate extrusion cycle. *Dotted arrows* refer to the substrate movement. The indicated "switch loop" separates the proximal and distal binding pockets. *Orange hexagon* represents substrate molecule

helix can be observed not only in other transporters but also in many other membrane proteins [46].

The membrane-embedded region consists of 12 α-helix bundles from each protomer. The bundles of each protomer are inserted circularly into the membrane (Fig. 1.3), and these three bundles create a central spatial hole that is approximately

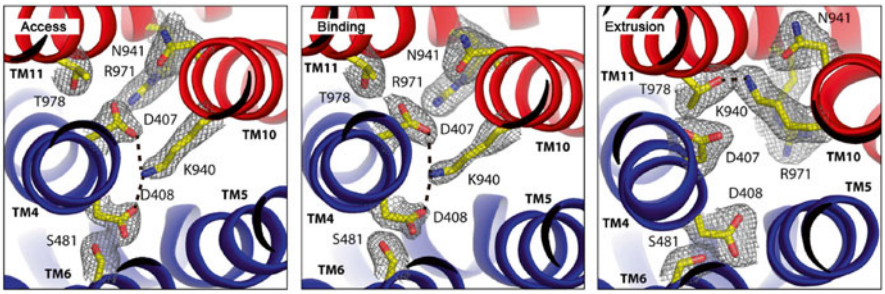

Fig. 1.7 Switch-like conformational change by proton conduction via important titratable residues, D407, D408, and K940, in the TM4 and TM10. A cut view of the transmembrane region viewed from the periplasm. *Dotted lines* show interaction between atoms (The figure is from Eicher et al. [50] with slight modifications)

40 Å in diameter. This hole, surrounded by the helix bundles, is thought to be filled with phospholipid molecules. The transmembrane helices TM4 and TM10 form a central pair in this helix bundle (Fig. 1.3). In these helices, there are functionally important charged residues, two acidic residues (Asp407 and Asp408), and one basic residue (Lys970). These titratable residues produce charge pairs (Fig. 1.7). The protonation and deprotonation events occurring in these residues trigger molecular motion; this motion is transmitted to the periplasmic domain by remote conformational changes for substrate translocation, which mainly occurs in the periplasmic region [47–50]. Even though the greatest resolution of the AcrB crystal structure is at 1.9 Å, protons are far beyond the observation by means of X-ray crystallography. Thus, the protonation state of the aspartates including individual states has not been elucidated so far, but by the MD calculation based on the crystal structure, we can hypothesize a reasonable structure for these residues [49].

It is still unclear whether the substrate translocation pathway across the cytoplasmic membrane exists in the transmembrane region. Only in the case of CusA (see the subsection on CusA below) was substrate (Cu^+) binding observed in this domain [39]. However, there is no other indication of substrate translocation through this transmembrane domain for the other transporters, especially drug exporters. Hence, the transmembrane domain is thought to be a power generator for these types of transporters by exchanging proton conduction into structural changes in this domain. Intriguingly, it was found that SecD, the transmembrane subunit of the SecDF complex, shares structural similarity to the transmembrane region of AcrB [41]. This complex likely stimulates protein translocation through the Sec translocon by proton motive force. The overall configuration of SecD and the transmembrane region of RND transporters are similar, yielding a root-mean-square deviation of around 2.7 Å for the Cα atoms of this region, in spite of their dissimilarity in their primary sequences (~15 %) [41]. SecD also has equivalent charged residues in its middle transmembrane region. Although SecD is very different in function, it shares this structural module to capture the proton motive force and convert this energy into movement of the protein molecule (Fig. 1.2).

In comparison with the transmembrane regions, the periplasmic regions produce a tighter interaction between the protomers and thus affect each other. These regions can be divided in two layers as the lower porter domain and the upper TolC-docking domain. Furthermore, the porter domain can be split into four subdomains (two in N-terminus and two in C-terminus): PN1, PN2, PC1, and PC2 (Fig. 1.4). In the three individual protomers, these four subdomains have different configurations. For instance, the subdomain interactions are looser in the "binding" state to create greater space between the subdomains; the resulting protomer forms a voluminous pocket in the porter region to accommodate substrate binding. On the other hand, the interactions between the subdomains are tighter in the "extrusion" state. In this case, the pocket is shrunken without enough space to accommodate potential substrates. These two different pocket states (i.e., the expanded and shrunken binding pockets) are to control the binding affinity of substrates to the binding pocket. Subdomains in the "access" state also have different conformations; the pocket configuration is not as clearly open as the "binding" state.

The TolC-docking domain, which lies in the upper periplasmic region, includes two subdomains dubbed DN and DC. Two subdomains from each of the three protomers (thus a total of six subdomains in this region) form the rim of a funnel-like conformation. This structure can fit the connecting AcrA subunit in the tripartite efflux complex. At the bottom of the funnel, three exits of the substrate translocation pathways from the porter domain of each protomer are connected together to this funnel-like opening that is linked to TolC directly or indirectly. Another remarkable feature of this region is its domain swapping characteristic by mutual insertion of their long hairpin loop in the periplasmic domain. This long hairpin structure from the DN subdomain inserts into the DC subdomain of the neighboring protomer, thus forming a four-stranded β-sheet. For the pseudo-twofold symmetry of the molecule, the DC subdomain also has a hairpin, but is shorter. This hairpin is inserted into the DN subdomain of the same protomer, yielding another four-stranded β-sheet as in the case of the DN subdomain. Domain swapping within each protomer and between neighboring protomers tightens the trimeric structure. Although molecular movement in this region is rarely observed, however, structural changes in this region may occur upon moving of whole tripartite complex. The outer membrane protein, TolC, is also thought to have open/close mechanism in its helix bundle that protrudes into the periplasm. Therefore, TolC-docking domain may also change its conformation cooperatively with the membrane fusion protein (periplasmic adaptor proteins) (i.e., AcrA) and TolC.

1.2.2 MexB from P. aeruginosa

The crystal structure of the MexB pump of the MexAB-OprM efflux system from *P. aeruginosa* (PDB code 2V50) was solved at 3.0 Å resolution (Fig. 1.2) [37]. MexB is a close homolog of AcrB, as their overall structures are basically similar, with a comparably high primary sequence similarity (70 % identity and 92 % similarity).

However, some of the secondary structural elements in the loop regions between the transmembrane helices are different. The major difference is found around the vestibule, the location of drug entrance in the "access" state. In AcrB, the vestibule is widely open and thus accessible to the substrates at this opening. In comparison, the MexB vestibule in its "access" state is rather closed [37]. Flexibility in the loop structure around the vestibule is highlighted with MexB crystal structure.

1.2.3 MtrD from N. gonorrhoeae

The MtrD crystal structure of the MtrCDE efflux system from human pathogen *N. gonorrhoeae* (PDB code 4MT1) was solved at 3.53 Å resolution (Fig. 1.2) [38]. In this structural analysis by Bolla et al. [38], crystals having threefold crystallographic symmetry were also used as same as the analysis for AcrB in 2002 [31], and thus resulting structure also has threefold crystallographic symmetry. Interestingly, only one protomer of the MtrD structure is included in the asymmetric unit. Similar to the symmetric structure of AcrB [31], MtrD shares a similar "access" conformation shown in the asymmetric structure of AcrB. Bolla et al. [38] successfully solved MtrD structure by using the "access" protomer in the asymmetric AcrB (PDB code 2DHH) as the search model to solve the phase problem of the crystallographic analysis by the molecular replacement method. Yet, in the case of MtrD, the superposition of these two structures shows a significant difference with a high root-mean-square deviation around 7 Å, suggesting that the structures of AcrB and MtrD are quite distinct. An interesting structural feature of MtrD has been observed in its transmembrane region. Its TM9 extends into the periplasm and contributes to the formation of the periplasmic domain. This elongation reaches around the PC1 and PC2 subdomains, the vestibule for the transporter incorporation. The elongated transmembrane helix is proposed to assist substrate incorporation from the outer leaflet of the membrane to the substrate translocation pathway in the periplasmic domain [38].

1.2.4 CusA from E. coli

The crystal structure of CusA of the CusCBA efflux system from *E. coli* (PDB code 3KO7) was solved at 3.52 Å resolution (Fig. 1.2) [39]. CusA is not a multidrug efflux transporter, but instead belongs to the HME subfamily of the RND transporters. It can export copper (I) and silver (I) and confer resistance to these toxic heavy metal ions [51]. CusA cooperates with the membrane fusion protein CusB and the outer membrane protein CusC, producing a tripartite complex similar to the AcrAB-TolC system. Interestingly, the complex crystal structure of CusBA was also solved by the same group that solved the CusA structure. This structure is the only available example for the crystallographic analysis of a complex structure of the components including the tripartite transporter complexes [52]. Overall, the configuration of

CusA is similar to that of other multidrug transporters such as AcrB and MexB. Nevertheless, in CusA, the substrates are coordinated by three methionine residues in the periplasmic and transmembrane regions [39]. In contrast to multidrug transporters, the substrates of the HME subfamily are ions and not hydrophobic compounds. Hence, the substrate binding sites of HME transporters are significantly different from those of the multidrug efflux transporters. Substrate (metal) binding sites of the HME family are composed of ion coordination residues (e.g., methionine residues) and not the hydrophobic residues in the multidrug exporters. Secondary structure elements can also differ from multidrug efflux transporters. Notably, the substrate translocation pathway in the transmembrane region was also observed for CusA, but was not obvious for multidrug exporters. However, the titratable residues in the TM4 and TM10 and the proton translocation pathway with these residues seem to have a similarity between HME transporters and multidrug exporters.

The CusBA complex structure was solved at 2.9 Å resolution [52]. This structure is the only example of a complex crystal structure available for a tripartite RND transporter. Although the correct stoichiometry of tripartite complex components remains under debates, in the case of CusBA, two CusB molecules attach to the periplasmic region of one CusA molecule, and a hexameric CusB seems to form a continuous channel on the top of CusA. This channel also connects to trimeric CusC, the outer membrane component of the CusCBA system. Long et al. [52] have proposed two possible arrangements of CusB and CusC docking: (i) direct docking of CusC on the top funnel of CusA with the hexameric CusB surrounding this complex and (ii) indirect docking in which the CusB channel may connect to the CusC channel.

1.2.5 ZneA from C. metallidurans

The crystal structure of ZneA from the metal-enduring bacterium, *C. metallidurans* (PDB code 4K0J), was solved at 3.0 Å resolution (Fig. 1.2) [40]. ZneA also falls into the HME family (similar to CusA). It exports zinc (II) ion and confers zinc resistance. ZneA is also comprised as a trimer, and its overall conformation is basically similar to other RND transporters. However, the conformation and composition of the amino acid residues in the substrate binding site are fairly different from those of CusA. In CusA, the sulfur atoms of methionine residues coordinate the binding and exporting of monovalent metal ions like copper (I) and silver (I); on the other hand, in the case of ZneA, carboxyl groups of aspartate and glutamate residues coordinate zinc (II) ions. The positions of the binding site remain within the periplasmic substrate translocation pathway (similar to what is observed among many other RND transporters), yet they are not identical to those of CusA. Crystals free from threefold crystallographic symmetry were used for ZneA crystal structure analysis. Structural differences between each protomer were observed in different substrate translocation states, and a unique substrate transport mechanism on this transporter was proposed [40]. No zinc binding in the transmembrane region of

ZneA was observed. However, there are three titratable residues along the TM4 region that may provide a path for protons across the membrane.

Additionally, substrate sizes for the HAE and HME transporter families differ greatly, and thus the sizes of the substrate binding pockets of these transporters are also different. However, similar substrate translocation (through peristaltic or serial switching mechanisms) may share the same evolutionary origin in these RND families.

1.3 Drug Recognition Mechanisms

Crystal structures of the AcrB transporter in complex with substrates in the asymmetric conformation were solved for doxorubicin, erythromycin, minocycline, and rifampicin (Fig. 1.8) [42, 48, 53]. These substrates are observed in various positions along the substrate translocation pathway from the entrance (or vestibule) to the exit where there are two major substrate binding pockets – "proximal (access)" pocket and "distal (deep)" binding pocket [48, 53]. These binding pockets are named by their proximity to the cell membrane and the entrance. These two pockets are separated by a characteristic "switch loop" (Figs. 1.4 and 1.6) [48]. Because of the glycine residues, this loop has a flexible property and is proposed to play an important role in drug efflux activity [48].

Binding of doxorubicin and minocycline was observed in the distal binding pocket in the "binding" state [42, 48]. Within this pocket, there are a few of phenylalanine residues and hydrophobic residues responsible for the substrate binding by hydrophobic interaction or π electron stacking interaction. Accumulation of hydrophobic residues is commonly observed in the core region of the protein molecules, especially for globular soluble proteins or hydrophilic part of the membrane proteins [54]. In the case of the HAE transporters, the accumulating hydrophobic residues are relocated upon the substrate translocation cycle, which is powered by proton conduction across the membrane. Expansive shifting of the subdomains, accompanied with aromatic and hydrophobic residues, produces a voluminous and hydrophobic binding pocket. Binding of doxorubicin, erythromycin, and rifampicin was observed in the proximal binding pocket in the "access" state [48, 53]. This pocket is thought to be the binding site for the substrates which have comparably larger molecular masses.

AcrB is known to export more than 30 structurally unrelated chemical compounds. However, there are only a very limited number of drugs that have been successfully co-crystallized with AcrB. As for other substrates, binding characteristics are predicted by means of *in silico* docking simulation analysis, as co-crystallization of these compounds have not yet succeeded [55, 56]. Studies by the other methods, including protein engineering (including chimeric proteins) and mutagenesis, have elucidated certain mechanisms of the substrate recognition and identified key residues related to substrate recognition [57–62]. Naturally, these important residues are located around the substrate translocation pathway from the vestibule to the exit. In the case of β-lactam recognition, key residues can be found around the proximal binding pocket, but their complex structures are not successfully determined [62].

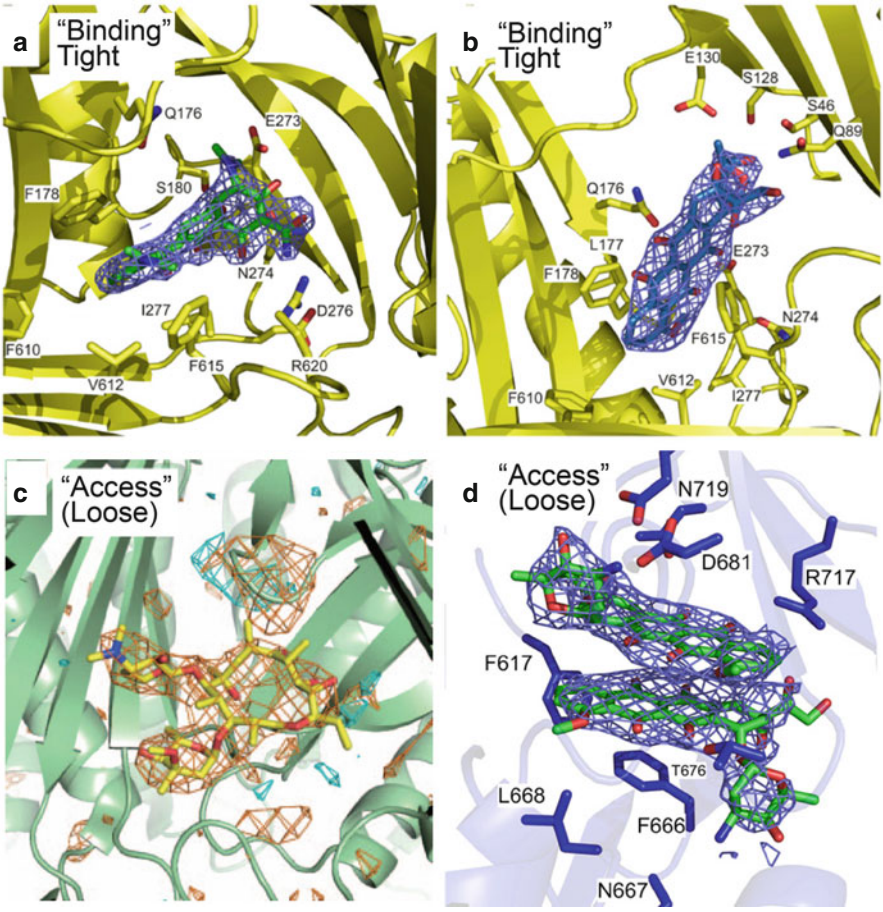

Fig. 1.8 Substrate binding pockets with multidrug binding and their difference Fourier maps. Distal binding pocket with minocycline binding (**a**) and doxorubicin binding (**b**) in the "binding" state of the AcrB trimer. Proximal binding pocket with doxorubicin dimer (**c**) and erythromycin binding (**d**) in the "access" state of the AcrB trimer (Figures **a**–**c** are from Eicher et al. [48], and **d** is from Nakashima et al. [53] with slight modifications)

1.4 Proton Translocation Pathway and Energy Coupling Mechanism

Even before the crystal structures of RND transporters were solved, it was recognized that three charged residues in the TM4 and TM10 regions are highly conserved and aided the prediction of their functional importance. Site-directed mutagenesis of these residues severely impairs the functionality of the drug efflux systems [60, 63–69]. These residues are located triangularly and form ion pair in the middle of the transmembrane helix bundle. In high-resolution structural analysis, differences of interaction between these charged residues are clearly observed upon

protonation/deprotonation of these residues using a switch-like mechanism (Fig. 1.7) [48, 50].

Despite the advances in structural and biochemical studies of the RND transporters, there remain inevitable obstacles that hinder our understanding of the proton translocation pathway for RND transporters. Firstly, protons are virtually invisible in protein X-ray crystallographic analysis. Secondly, it is impossible to control the protonation states of each individual protomer within these crystals. Soaking of crystals into acidic or basic buffer solution during the experimental process influences all the titratable residues in an uncontrollable manner. Thirdly, for a tripartite transporter, it is somewhat useless to reconstitute of a solo RND transporter into proteoliposomes for conventional biochemical analysis. All three components including the membrane fusion protein and outer membrane protein components are likely required to assess the full function of an RND transporter system. It is easy to imagine that reconstitution of all three components, which includes two different membrane proteins, can be difficult to achieve by common biochemical techniques. Accordingly, only a few studies were successful in quantitatively analyzing RND transporters through authentic biochemical methods [69, 70].

For this reason, MD simulation attracts much interest as a complementary method in structural biology [56, 71–75]. This methodology incorporates hypothetical models with protonation or deprotonation states of individual residues for MD calculation. These models are then assessed for their accuracy following the MD calculation. MD simulation has helped propose models ranging from proton utility and generation of molecular movement in the transporters to transmission of the movement to the porter domain via a remote conformational change [49, 72, 76–78].

1.5 Substrate Translocation Pathways

Among the RND transporters with known crystal structures, drug exporters like AcrB and heavy metal ion exporters like CusA have different substrate translocation pathways. For drug exporters, the substrate translocation pathway has only been found in the periplasmic domains (Fig. 1.6). Thus, substrates may be taken up from the periplasm or from just above the outer leaflet of the cytoplasmic membrane. In other words, a transmembrane translocation pathway may not exist, even though many classes of substrates exert their action in the cytosol. In these cases, the substrates might be transferred from the cytosol to the periplasm (or at least the outer leaflet of the cytoplasmic membrane) by diffusion. The result of the substrates taken by RND pumps via these pathways would shift the equilibrium toward efflux of the substrates. Consequently, there would be no positive substrate translocation pathway across the membrane for drug efflux transporters. In contrast, for CusA, binding substrates are observed in the transmembrane region. Thus, there may exist a substrate translocation pathway not only in the porter domain like drug exporters but also in their transmembrane region.

The first substrate translocation pathway (i.e., through the central pore helices at the center of the trimer) was mistakenly proposed by a simplistic interpretation of the structure solved in 2002 [31]. The first physiological pathway was proposed by Lomovskaya and Totrov [79] through a model calculation and took into consideration experimental observations made by Aires and Nikaido [80]. At the same time, the asymmetric structure of AcrB with bound substrate (doxorubicin and minocycline) was solved, and the previous model was corrected according to the crystal structural analysis [42].

The substrate translocation pathway in the periplasmic part of AcrB starts from the vestibule or entrance opening to the proximal part of periplasm and/or just above the outer leaflet of the cytoplasmic membrane. These locations are some entrances that were proposed by structural features and/or by MD calculation [53, 71, 73]. These pathways from several entrances merge into the proximal binding pocket for substrates and continue together. A distinct loop structure divides the middle of this pathway. Following the loop, in the latter part of this pathway, lies the distal pocket (another substrate binding). This pocket has an exit formed by a gap between two protomers, the N-terminal porter subdomains PN1 and PN2. Each exit connects to the funnel-like opening in the TolC-docking domain. Simply put, substrates are translocated between the subdomains in the periplasmic domain from the vestibule to this exit. All along this pathway, substrates are recognized and bound elsewhere in this long pathway. This vast extent of the pathway provides many sites that can accommodate a wide variety of chemical compounds as substrates.

1.6 Peristaltic Pump Mechanism in Multidrug Efflux

In the asymmetric crystal structures, asymmetric configuration of the subdomains including in the porter domain of each protomer is interpreted as consequent of their cooperative movement. In the porter domain, molecular movement of the subdomains is converted to the sequential movement of the substrate translocation pathway. Initially, this movement is triggered by the proton conduction through the transmembrane region and then transmitted by the remote conformational change. The molecular movement in the porter domain includes opening and closing of the vestibule, proximal and distal substrate binding pockets, and the exit gate (Fig. 1.6). The opening of the entrance and the proximal half of the translocation pathway allow the substrates to gain access through the pathway in the “access” state (Fig. 1.6, left). Some parts of this pathway (i.e., the proximal binding pocket) are expanded. In the subsequent “binding” state, the opening of the latter half of the pathway makes the distal binding pocket accessible to the substrates through the loop region that separates the two pockets (Fig. 1.6, middle). In this case, PN2 and PC1 move away from the trimeric center to expand the distal binding pocket, resulting in a gap between the PN1 and PN2 porter subdomains to serve as the exit of the pathway (Fig. 1.5c). Following this event, an α-helix belonging to the PN1 subdomain of the neighboring protomer closes this exit by inserting the α-helix.

Consequently, the expanded distal binding pocket closes the exit, thus keeping the substrates inside the pocket. The helix bundle which is comprised of the α-helix from each protomer is the so-called pore helix, which plays a crucial role for drug export [42]. In this case, the inserted helix is greatly inclined in comparison with the other equivalent helices belonging to the other protomers. In the final "extrusion" state, the PN2 and PC1 subdomains move back toward the center of the trimer (Fig. 1.5c). PC2 moves closer to PC1 to shrink the expanded distal binding pocket in the "binding" state and constrict the pocket and forces the bound substrates out of the pocket (Fig. 1.6, right). During the "extrusion" state, the vestibule closes its conformation by changing the secondary structure around that region. The proximal half of the translocation pathway also gets shrunken. The helix is inclined toward the expanded pocket in the "binding" protomer and contributes to shutting the exit gate (Fig. 1.6, right). However, at the same time, the inclination widely opens the exit gate for the "extrusion" state itself. As a result, the protomer in the "extrusion" state has a closed entrance and opens the exit with a shrunken distal pocket. Accordingly, it forms a suitable conformation for substrate extrusion from the distal pocket to the exit. Next, as PC2 approaches PC1, it cycles back to the "access" state. Drugs are exported through this three-step functionally rotating mechanism in which substrates undergo an ordered binding change. This cyclical process is analogous to the rotational catalysis of ATP synthesis by the F_1F_0-ATPase [45]. The current understanding of this transport cycle is mainly derived from our molecular comprehension of pump crystal structures [42–44]. Although intermediate steps might exist among the "access," "binding," and "extrusion" states, they remain to be elucidated.

1.7 Structure of the Tripartite Efflux Pump Complex

Crystal structures of tripartite RND transporters have only been solved through their components separately, though the one exception, CusBA crystal structure, has been successfully solved at 3.2 Å resolution [52]. In the latter CusBA study, six membrane fusion proteins were demonstrated to be bound to the trimeric RND transporter. It is also thought to connect one trimer of the outer membrane protein to this complex, thus yielding a tripartite efflux complex comprised of three transporter, six membrane fusion protein, and three outer membrane protein molecules. Difficulties in the sample preparation for the whole tripartite complex hinder the identification of a complete tripartite efflux structure. As a result, there have been no solved crystal structures to date. In 2014, the Luisi group [81] successfully prepared a stable tripartite efflux complex by employing a protein fusion technique. This group was able to connect the transporter, the membrane fusion protein, and the outer membrane protein with peptide linkers. A potent tripartite transporter complex was obtained *in vivo* and was then used for structural determination in subatomic resolution under cryo-electron microscopy. The observed structure was noted to be essentially the same as the computer-generated model. However, more

detailed information was obtained from this study. In particular, the distance between the RND transporter (i.e., AcrB) and the outer membrane protein (i.e., TolC) was experimentally determined from these methods, revealing that there is less potential for direct interactions between the RND transporter and the outer membrane protein component (Fig. 1.1) [82]. A recent study using the reconstitution of native AcrAB-TolC and MexAB-OprM systems in a lipid nanodisc system also showed no physical interaction between the cytoplasmic and outer membrane efflux components [83]. To note, an earlier report had potentiated a direct interaction between AcrB and TolC by means of a cross-linking study [84]. The interaction between RND proteins and outer membrane protein components remains to be further investigated; however, the distance between the outer and cytoplasmic membranes is expected to be flexible. Thus, both direct and indirect interactions may be possible for the tripartite RND efflux complex [52].

1.8 Concluding Remarks

RND efflux transporters are one of the most studied among the major transporter families. Based on identified crystal structures (especially asymmetric conformations with bound substrates), plausible functional mechanisms are proposed and proved by many molecular biological analyses [15, 47, 70, 85–87] and MD simulations [55, 56, 72, 77]. High-resolution X-ray crystallographic analysis is a reliable technique to elucidate protein structure, as diffraction data is a direct output of structural averaging between the billions of molecules included in the crystals. At the same time, however, it is still important to note that the dynamic aspects of these molecules within crystals may also be averaged; resulting crystal structures are merely static snapshots of the protein molecules. Additionally, the factor of temperature for the peptide chains within protein molecules may contain information regarding structural flexibility, but dynamic aspects of the molecules are mostly lost. In order to observe live images of protein molecules, other methodologies like MD simulation can produce supplemental information for their dynamic aspect. MD calculation for macromolecules is an established methodology that has already been applied in structural biology [88, 89]. To note, precise MD simulation first requires a definite high-resolution crystal structure, and therefore, crystallographic analysis and MD calculation are mutually complementary. Moreover, to continue MD calculation in an appropriate period of time in each biological system, especially for large molecules like RND transporters and their tripartite complexes, supercomputer-assisted techniques with reliable high-resolution starting model and reasonable force field for the MD calculation will be critical for elucidating the structural and functional basis of the drug efflux systems. Overall, additional structures (especially those with great complexity), more quantitative biochemical analyses (especially involving kinetics), and more precise long-term MD simulations are required for an in-depth understanding of RND drug efflux complexes to facilitate our efforts in tackling the efflux-mediated multidrug resistance problem.

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Chapter 2
Structural and Functional Landscape of MFS and MATE Efflux Pumps

Asha V. Nair, Kenneth W. Lee, and Hendrik W. van Veen

Abstract Multidrug transporters play a crucial role in causing drug resistance in infectious microorganisms and tumors. They are integral membrane proteins that exhibit an exceptionally broad specificity for unrelated molecules including antibiotics and anticancer agents. By mediating export of toxic pharmaceuticals from the cell's interior, multidrug transporters reduce the concentration of these agents in the cell to a level where toxicity is lost. In spite of intense efforts, it is still not clear exactly how multidrug transporters work. In this chapter, we discuss some of the recent advances for multidrug transporters in two important families, the major facilitator superfamily (MFS) and multidrug and toxic compound extrusion (MATE) family.

Keywords Multidrug resistance • Efflux transporter • Structure • Transport • MFS • MATE

2.1 Introduction

Multidrug resistance transporters are present in all the kingdoms of life and can have several important biological functions. Among these, they can extrude a broad range of structurally unrelated organic compounds from the cell and thus provide resistance to cytotoxic agents in both prokaryotes and eukaryotes. They are generally subdivided into six families/superfamilies [1, 2], the primary-active ATP-binding cassette (ABC) transporter superfamily, the members of which utilize energy provided by ATP binding and hydrolysis to export substrates, and five

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families of H^+ and/or Na^+-coupled secondary-active antiporters: the resistance-nodulation cell division (RND) superfamily, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the recently discovered proteobacterial antimicrobial compound efflux family (PACE), and the multidrug and toxic compound extrusion (MATE) family. This chapter provides an overview of the structural and molecular characteristics of the MFS and MATE drug transporters, in particular the features of MATE pumps.

2.2 MFS

In 1993, Saier and coworkers provided the first evidence for the existence of a large family of structurally related secondary-active transporters, termed the MFS [3]. Since the time of its establishment, the MFS has rapidly expanded and is currently composed of 74 families [4–6]. The members of this superfamily transport a range of related substrates including simple monosaccharides, oligosaccharides (carbohydrates), amino acids, peptides, vitamins, enzyme cofactors, drugs, chromophores, nucleobases, nucleosides, nucleotides, iron chelates, organic and inorganic anions and cations, and other small molecules [7–10]. Well-known multidrug transporter members of the MFS are *Escherichia coli* QacA [11], EmrB of the EmrAB-TolC system [12, 13], EmrD [14, 15], MdfA [16, 17], and *Lactococcus lactis* LmrP [18].

Hydropathy analysis of MFS protein sequences and topological studies with gene fusion constructs predicted that almost all MFS proteins possess a uniform topology of 12 transmembrane α-helices (TMs) connected by hydrophilic loops, with both their N- and C-termini located in the cytoplasm [4, 5, 19]. Interestingly, the N-terminal half of the protein (TM1–TM6) displays weak sequence homology to the C-terminal half (TM7–TM12), suggesting that the molecule may have arisen from a gene duplication or fusion event [20, 21]. Exceptions to the 12 TM rule do exist – a few MFS families have 14 TMs, one representative only 6, and yet another 24 [4]. The extra two helices in the 14 TM members probably arose via insertion of the central cytoplasmic loop into the membrane, whereas the 24 TM members are likely a consequence of a gene fusion event [19]; one suspects that the example with six TMs functions as a homodimer.

Most MFS transporters consist of 400–600 residues in with the core helices arranged into two pseudosymmetrical six-helix bundles [4–6]. At the level of the superfamily, individual MFS members share low sequence identity or similarity, have distinct substrate specificities, and are united only by a limited number of conserved signature sequences [1, 20, 22]. However, the X-ray crystallographic models and homology models of MFS transporters reveal the existence of a common MFS fold in which important residues for catalysis are located at the apex of a deep central cavity formed by two pseudosymmetrical six-helix bundles. The two bundles contain two inverted-topology repeats of three-helix bundles each and are thought to form rigid bodies that move during transport relative to each other to achieve alternating access by a rocker switch mechanism [23, 24]. In general, alternating

access models represent a uniform concept for secondary-active transport. In these models, a transporter undergoes distinct conformational changes for the binding and release of substrate(s) across the lipid bilayer, in which the bound substrate(s) are never simultaneously exposed to both sides of the membrane. The conformational changes associated with alternating access provide a molecular basis for the coupled translocation of substrate and ions [25].

The multidrug transporter LmrP from *Lactococcus lactis* is a 408-amino acid transporter that is predicted to contain 12 TMs. Like MdfA, this system is typical for other MFS members in pathogenic bacteria. Bolhuis and coworkers first cloned the *lmrP* gene in genetic screens based on its ability to confer resistance on *E. coli* to high concentrations of the monovalent amphiphilic ethidium cation [26]. LmrP can utilize both the membrane potential ($\Delta\psi$, interior negative) and the chemical proton gradient (ΔpH, interior alkaline) of the proton motive force (Δp) to mediate the efflux of amphiphilic substrates from cells. LmrP mediates electrogenic antiport, where monovalent cations like ethidium and tetraphenylphosphonium move in exchange of two or more protons [27, 28]. For this purpose, LmrP contains a number of carboxyl residues which, along with polar and aromatic residues, forms two clusters on the surface of a large interior chamber. In an inward-facing three-dimensional homology model of LmrP [29], the N- and C-terminal membrane domains are suggested to form an interior chamber containing three carboxyl residues (Asp142, Asp235, and Glu327) that are surrounded by polar and/or aromatic residues and that are organized into two catalytic clusters, one containing Asp235 and Glu327 in the C-terminal domain (Cluster 1) and the other containing Asp142 in the N-terminal domain (Cluster 2). The observed stoichiometry for the electrogenic antiport of divalent cationic propidium ($3H^+$/propidium^{2+}) in LmrP and effect of carboxyl replacements on this stoichiometry suggest that in the propidium transport reaction, Asp235 and Glu327 (Cluster 1) are important for the interaction of LmrP with two protons and substrate, whereas Asp142 (Cluster 2) is dedicated to the interaction with one proton only. Although each of the two clusters interacts with 1 proton in the ethidium transport reaction, none of the three catalytic carboxylates is critical for the interaction with this monovalent cation [28]. Redundancy in catalytic carboxylates has also been observed in the 410-amino acid MFS transporter MdfA from *E. coli* [16, 30]. Transport studies show that MdfA is a drug/proton antiporter driven by the proton electrochemical gradient [31, 32], with orthologs in several pathogenic bacterial species, *Shigella flexneri* [33], *Salmonella enterica* serovar Typhi [34], and *Yersinia pestis* [35]. Whereas the transport of neutral substrates by MdfA is electrogenic, the transport of monovalent cationic substrates is electroneutral and therefore driven only by the ΔpH [32]. However, MdfA can be easily converted into a divalent cationic drug/$2H^+$-antiporter, either by random mutagenesis or by rational design, by inclusion of an additional acidic residue in the multidrug binding pocket [36]. Recently, Heng and coworkers [17] reported three substrate-bound crystal structures of wild-type MdfA, all in the inward-open conformation. Based on these structures, the substrate-binding site is predicted to be located near the conserved acidic residue Asp34 [17]. The importance of this carboxylate was also identified in previous investigations. Together with Glu26, Asp34 was found to

be essential for active transport of cationic substrates. However, for electrically neutral substrates, the carboxylates may compensate for one another's deficiencies [37, 38]. Similar to the observation on LmrP, these elegant studies on MdfA illustrate the exceptional promiscuous capabilities of this type of multidrug transporter.

When the multidrug transporter NorM was first characterized, it was initially thought to be a member of the MFS. Based on conserved amino acid sequence motifs, Brown et al. [39] suggested that NorM and YdhE (from *E. coli*) are not members of the MFS family. Thus a new family was created which was termed the MATE family [39].

2.3 MATE Family

The members of the MATE family are part of the multidrug/oligosaccharidyl-lipid/polysaccharide [MOP] transporter superfamily [40] and are involved in a variety of important biological functions across all kingdoms of life. The MATE family has been allocated over 1,000 proteins, which are divided into three large subfamilies consisting of 14 smaller subgroups. Family 1 consists of bacterial MATE transporters and includes *Vibrio parahaemolyticus* NorM, a prototypical MATE transporter. Family 2 comprises eukaryotic MATE transporters and is further divided into four subfamilies: 2A, comprising yeast and fungal MATEs; 2B, comprising plant MATEs; 2C, comprising animal MATEs; and 2D, comprising protozoan MATEs. Family 3 contains bacterial and archaebacterial MATEs. Functional studies on a limited number of MATE transporters in intact cells and membrane vesicles suggested that they mediate substrate efflux by catalyzing a substrate-coupling ion antiport reaction [39–42]. The length of proteins in the MATE family ranges from 400 to 700 amino acid residues with most members consisting of 400–550 residues. Although there is no apparent consensus sequence conserved among MATE proteins, they share about 40 % sequence homology [41].

2.3.1 MATE Transporters in Eukarya

MATE transporters are highly dominant in plants, with 58 paralogs found in *Arabidopsis thaliana*. Plant MATEs are involved in the detoxification of endogenous secondary metabolites and xenobiotics along with the secretion of a wide range of secondary metabolites and provide a defense mechanism against herbivores and microbial pathogens [40, 42]. The *Arabidopsis* gene *tt12* has been proposed to be involved in the vacuolar accumulation of anthocyanin in the seed coat endothelium [43]. A similar transporter AMT1 has also been identified in tomato [44]. The MATE gene *eds5* in *Arabidopsis* was identified as an essential component of the salicylic acid-dependent signaling pathway for the disease resistance and exposure to UV light [45]. Berberine from *Rhizoma coptidis*, an oral hypoglycemic agent with

anti-dyslipidemia and anti-obesity activities [46], is accumulated in the vacuole through an H^+-coupled antiport mechanism [47]. This transporter might be a MATE transporter as the *Arabidopsis* MATE transporter DTX1 mediates the extrusion of toxic compounds such as berberine and tetraethylammonium [48, 49]. Furthermore, plant MATE transporters play a significant role in the tolerance toward aluminum [50, 51], maintenance of iron homeostasis [52, 53], alkaloid accumulation [54], plant development [55], and the vacuolar accumulation of flavonoids [56, 57].

In fungi, MATE-type transporter of the yeast *Saccharomyces cerevisiae*, Erc1, has been characterized partially to date [58]. This protein confers fungal resistance to ethionine (methionine analogue) and seems to be involved in the accumulation of *S*-adenosyl methionine in vacuoles.

In mammals, MATE transporters are involved in the extrusion of a diverse array of xenobiotic cations in the liver and kidney, affecting the concentrations of many drugs in plasma and thereby reducing their therapeutic efficacy [39, 59]. As MATE transporters are bidirectional antiporters, high levels of expression in areas of low extracellular pH exemplifies the role of drug/proton antiporters as efflux pumps in humans. This low extracellular pH is present in urine for renal MATE transporters, bile for hepatic MATE transporters, and lactic acid-rich extracellular fluid for MATE transporters in skeletal muscle. Hence, the localization of MATE transporters appears to be directly correlated to the existence of transmembrane chemical H^+ gradient in these locations. MATE1 and MATE2-K (a splice variant of MATE2) are located at the brush border of renal proximal tubules where they are responsible for excretion of a variety of drugs such as cimetidine and metformin [39, 60, 61]. Located on the apical membrane, MATE1 and MATE2-K work in tandem with human organic cation transporter OCT2 located on the basolateral membrane of renal proximal tubule cells to mediate the excretion of various drugs and substances from the bloodstream into urine [62]. The disruption of this system has severe pharmacological consequences like cisplatin-induced nephrotoxicity. The liver utilizes a similar system as the kidney to excrete toxins and drugs from the bloodstream into bile, although the transporters involved here are MATE1 and OCT1. OCT1 mediates transport of drugs from the blood across the basolateral membrane of the hepatocyte, and these drugs are then transported by MATE1 across the apical membrane into bile [62]. Thus, impairment of MATE transporters may adversely affect both hepatic and renal excretion. Such effects can be seen during therapy with metformin, a drug commonly used for treatment of type 2 diabetes. The dysfunction of MATEs (SLC47A1/MATE1 and SLC47A2) synergistically increases the metformin accumulation in the liver and blood lactate levels resulting in the development of lactic acidosis.

2.3.2 MATE Transporters in Eubacteria

Bacterial MATE transporters primarily function as efflux pumps for xenobiotics. With respect to multidrug resistance, 17 MATE proteins from 11 species have been characterized. The potential role of MATE transporters in drug resistance is

evidenced by the extensive range of substrates that are transported. MATE-type transporters confer drug resistance to cationic drugs such as acriflavine, berberine, ethidium bromide, methylene blue, tetraphenylphosphonium, ciprofloxacin, norfloxacin, doxorubicin, daunomycin, streptomycin, and trimethoprim. Among the characterized MATE transporters, MepA from *Staphylococcus aureus* is clinically important as the overexpression of MepA confers resistance to tigecycline, a glycylcycline antibiotic that was developed to overcome methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus* strains [63–65]. Being a unicellular organism, bacterial transport of drugs may occur in any direction from the cytoplasm, and this allows MATE transporters to function in a much simpler system than in humans/eukaryotes.

MATE transporters have a role beyond the efflux of antimicrobial agents. It has been discovered recently that the endogenous expression of the MATE transporters NorM and DinF in *E. coli* are protective against reactive oxygen species, particularly in the absence of the DNA mismatch repair gene *mutT* [66, 67]. When cells are exposed to hydrogen peroxide, the overexpression of NorM or DinF increases the survival rate of *mutT*-deficient cells, thus matching the survival rate of wild-type cells. In enterobacteria, the genes producing DinF and LexA are present in a *dinF-lexA* operon, which is the major coordinator of the DNA damage-induced SOS response in bacteria. A functional implication of this is that DinF may be protective against bile salts, which are known oxidants in the gastrointestinal tract. The transporter might thus contribute to the survival of enterobacteria in this hostile environment [67]. As multidrug efflux pumps, including MATE transporters, may confer a selective advantage for specific bacteria in their natural environment, a positive selection for multidrug-resistant bacteria might therefore arise even without the exposure to antibiotics.

MATE transporters might also be important in quorum sensing, which is a form of intercellular signaling between unicellular microorganisms to impact gene regulation in control of bioluminescence, virulence factor expression, biofilm formation, entry into stationary phase, conjugal transfer of plasmid DNA, spore formation, and transformation competence [68, 69]. Quorum sensing uses chemical messengers, known as quorum-sensing signals, which are released from a cell. These signals subsequently bind to receptors on a different cell and induce an alternate gene transcription in that cell. The rate of efflux of quorum-sensing signals by membrane transporters would be an important determinant of the time required to reach a threshold concentration above which gene transcription in the population is altered.

Quorum sensing work on *E. coli* by Yang et al. [70] showed that the MATE transporter NorE is involved in the efflux of such quorum-sensing signals. *E. coli* cells lacking NorE have the same growth rate as wild-type cells in the exponential phase but grow to higher cell density in stationary phase due to a slower buildup of the quorum signal concentration in the growth medium. On the other hand, the enhanced expression of MATE transporters such as NorE reduces the cell density that can be achieved due to a faster rise in this concentration and results in the formation of antimicrobial-resistant biofilms as well as increased virulence at lower cell densities. These biofilms allow microorganisms to be in close proximity, thus creating a

niche where the prevailing antimicrobial concentration remains at sub-toxic concentrations. At the very low growth rates of cells in biofilms, the exposure to these low doses of antimicrobials leads to the development of drug tolerance. Thus, as secretion systems for quorum-sensing signals, microbial MATE transporters and other multidrug efflux pumps might be interesting targets for the development of new anti-infective agents or agents that can alter the flux of metabolites through physiological pathways in a biotechnological setting.

2.3.3 Basic Structures of MATE Transporters

Crystal structures of several MATE transporters have been elucidated in recent years. These include structures of NorM in *Vibrio cholerae* [71], NorM in *Neisseria gonorrhoeae* [72], PfMATE in *Pyrococcus furiosus* [73], and DinF (DNA damage-inducible protein F) in *Bacillus halodurans* [74]. Along with molecular dynamics simulations [75, 76], these advances have revealed many details about how MATE proteins are able to function as multidrug transporters.

The core structure of the prokaryotic and plant members of the MATE family of transport proteins is generally considered to involve 12 TMs [7, 40]. Currently solved structures of bacterial MATE transporters indeed contain 12 transmembrane domains, though hydropathy analysis has predicted the presence of 13 TMs. It was shown by Zhang et al. [77] that an epitope-tagged construct of rabbit MATE1 has an extracellular COOH terminus, consistent with the presence of the putative 13th TM and an extracellular C-terminus [77, 78].

Chang and coworkers [71] solved the first structure of a MATE transporter in *V. cholerae* (NorM-VC). This first crystal structure was in an outward-facing conformation, in a Cs^+ or Rb^+ (a more electron-dense Na^+ analogue)-bound, drug-free state [71]. Multiple sequence alignments indicated that the highly conserved regions in the NorM-containing cluster of the MATE family are located near TM1 and TM7, in the extracellular loops between TM1 and TM2 and TM7 and TM8, in the cytoplasmic loops between TM1 and TM3 and TM8 and TM9, and in the loops between TM4 and TM5 and TM10 and TM11. The symmetric repetition of the conserved regions in the N- and C-terminal halves suggests that MATE proteins evolved from a common ancestral gene that underwent gene duplication. A cytoplasmic loop (residues 218–232 between TMs 6 and 7) connects the two halves, as is also seen in transporters of the MFS, while the initial helix of each half (TM1 and TM7) is preceded by a helical extension (residues 2–18 and 233–247) from the inner membrane leaflet side. An additional helix (residues 450–461) following TM12 is nestled under the cytoplasmic side of TM11 [71].

Nureki and coworkers [73] presented the structure of H^+-coupled PfMATE, which consists of an N-terminal lobe (TM1–TM6) and C-terminal lobe (TM7–TM12) that are related by a pseudo-twofold symmetry axis. Similar to NorM-VC, the PfMATE structure adopts a V-shaped conformation, with the central cleft open toward the extracellular side, thus representing an outward-open state. PfMATE

shares approximately 22 % sequence identity with NorM-VC. A large, hydrophobic central cleft, formed between the N- and C-lobes, can be divided into two cavities, the N- and C-lobe cavities, with the N-lobe cavity being larger than the C-lobe cavity. It was also found that the TM1 in PfMATE adopts two distinct conformations, "bent" and "straight." In the straight conformation, TM1 forms a single, straight helix, whereas in the bent conformation it is kinked at Pro26 and Gly30 and bent toward the TM2 side [73]. Following the protonation of Asp41, its side chain becomes sequestered within the hydrophobic environment, thereby inducing the bending of TM1 at Pro26, which collapses the N-lobe cavity and extrudes the bound drug substrate into the extracellular space. A drastic rearrangement of the side-chain interactions was also observed between the straight and bent conformations, near the N-lobe cavity [73]. One of the features of this model is that substrate binding from the interior of the cell and dissociation of substrate to the exterior could both occur at the outward-facing PfMATE conformation. One of the questions of this transport model is how substrate is prevented from rebinding to this state after its dissociation. In the MFS transport models, alternating access of the substrate-binding pocket ensures that rebinding of substrate to the outward-facing state is physically hindered by conversion of the outward-facing conformation to an inward-facing conformation, in which the binding site is physically inaccessible to the external environment. Although an inward-facing MATE transporter has not yet been crystallized and, hence, the structural evidence for this state is missing at present time, it is possible that a MFS-type alternating access mechanism is also relevant for MATE transporters.

Recent co-crystal structures of Na^+-coupled NorM and a protonation-mimetic mutant of H^+-coupled DinF with bound verapamil provide insights in the inhibition of the MATE drug transporters by verapamil [79]. This known inhibitor of mammalian and bacterial multidrug transporters [80–82] can adopt different conformations to fit into the multidrug binding site of these MATE pumps and displays structural flexibility that enables verapamil to preoccupy the substrate-binding site and thus to prevent the binding of drug substrates to the pumps [79].

2.3.4 Conserved Amino Acids

MATE transporters share a number of amino acid residues that help us to understand the mechanics of how these transporters function in the membrane. The conserved amino acids in MATE transporters are generally required as part of either cation-binding residues or the substrate-binding site or have supporting roles in maintaining protein structure or in mediating conformational changes. X-ray crystallography analysis and mutational and biochemical studies have been carried out to identify these residues.

Na^+-coupled transporters like NorM-VP and NorM-VC have three well-conserved acidic residues, Asp32, Glu251, and Asp367 in NorM-VP and corresponding Asp36, Glu255, and Asp371 in NorM-VC [71, 83]. In the medium

resolution, outward-facing crystal structure of NorM-VC, these carboxylates are arranged asymmetrically. Glu255 and Asp371 are located in the C-terminal helical bundle facing the internal cavity, whereas Asp36 is located in the N-terminal helical bundle. This asymmetric distribution was further confirmed in the recent crystal structures of NorM from *N. gonorrhoeae* (NorM-NG) in outward-facing and drug-bound states [72]. In the H^+-coupled MATE transporters, X-ray crystallography analysis and functional studies on mutant proteins suggest that Asp40 (DinF-BH) or Asp41 (PfMATE) have a role in H^+ binding and that these carboxylates are also part of the substrate-binding cavity such that H^+ and cationic substrate compete for binding at this residue [73, 74].

2.3.5 MATE Transporters and Ion Coupling

It is believed that MATE transporters are coupled to either Na^+ or H^+ for transport based on structural evidence (Fig. 2.1) and that this property forms the basis for their functional classification. However, recent studies on the energetics of NorM-VC have shown that this MATE transporter may be coupled to both Na^+ and H^+ cycling. The effects of these ion gradients on the transport rate are additive. The sigmoidal dependence of the rate of ethidium transport on the Na^+ concentration suggests that Na^+ binding is cooperative and involves at least two Na^+-binding sites. It has also been shown that NorM-VC exhibits a very high affinity for Na^+. NorM-VC mediates active ethidium with a transport constant *Kt* value for Na^+ of just 4.7 μM Na^+, suggesting that small traces of Na^+ in cell suspensions are sufficient to activate NorM-VC and, thus, that Na^+ dependence can easily be missed [84]. These recent studies urge a rethink about the roles of some of the conserved acidic residues. Crystal structures published till date have suggested that Glu255 and Asp371 form a Na^+-binding site [71, 72]. However, functional experiments with NorM-VC in proteoliposomes have shown that replacement of Asp371 by asparagine residue completely abrogated dependence of ethidium transport on the transmembrane chemical proton gradient and membrane potential, but not the chemical Na^+ gradient dependence. Furthermore, whereas ethidium binding causes proton release from wild-type protein, this phenomenon was not observed for the transport active Asp371Asn mutant. These results highlight the importance of Asp371 in proton coupling in NorM-VC and point to the absence of proton in the antiport reaction catalyzed by the Asp371Asn mutant. The observations that ethidium binding to NorM-VC is associated with H^+ release but is stimulated by Na^+ binding point to different roles of these coupling ions in the ethidium transport reaction. As the Asp371Asn mutation does not affect the interaction of NorM-VC with ethidium, ethidium binding-induced proton release is most likely based on indirect competition between these cations for binding by NorM-VC [84]. Based on structural studies, the MATE transporters NorM-NG (Na^+-coupled) and DinF (H^+-coupled) were proposed to transport substrate in antiport with cations (either Na^+ or H^+) at a 1:1 ratio [72, 74]. The recent studies by Jin et al. [84] point to electroneutral $1Na^+$/ethidium$^+$ for the Asp371Asn mutant versus electrogenic

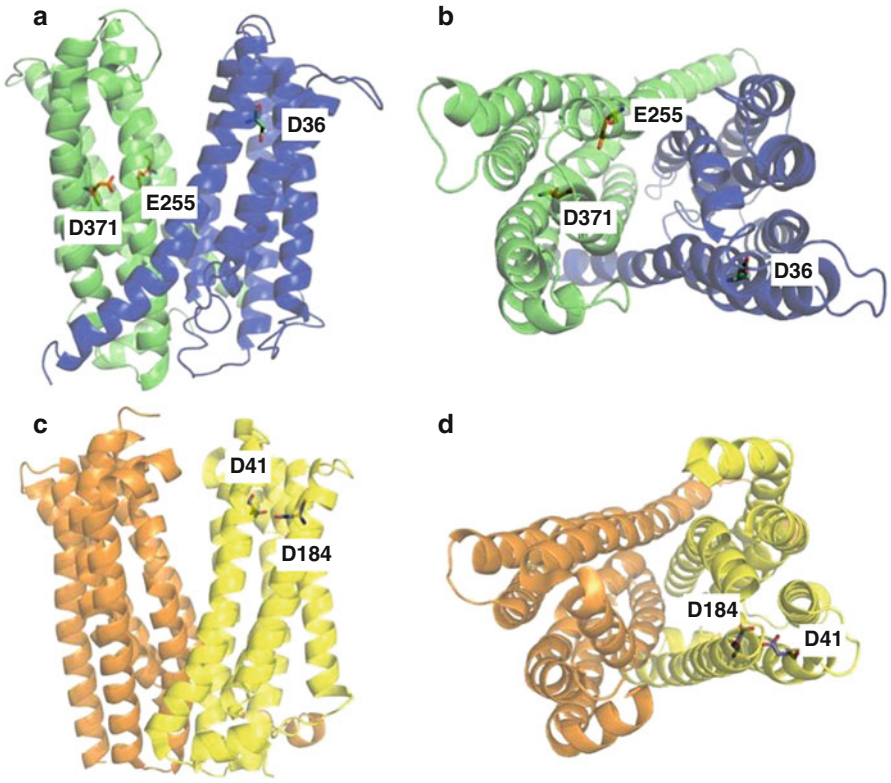

Fig. 2.1 Structural comparison of NorM-VC (Na^+-coupled MATE transporter) and PfMATE (H^+-coupled MATE transporter). (**a**, **b**) Structure of NorM-VC (PDB code 3MKT) viewed from the membrane plane (**a**) and the periplasm (**b**). Catalytic residues D36, E255, and D371 are shown as *sticks*. (**c**, **d**) Structure of PfMATE (PDB code 3VVN) as viewed from the membrane plane (**c**) and the periplasm (**d**). Catalytic residues D41 and D184 are shown as *sticks*

($1Na^+$, $1H^+$)/ethidium$^+$ for wild-type NorM-VC and demonstrate that ion coupling stoichiometries can only be determined when the energetics and coupling ion dependence of complete transport reactions are analyzed rather than their binding in crystallized states of the transporter.

Due to difficulties in detecting Na^+ with X-ray crystallography, Cs^+ ions were used in NorM-NG to elucidate Na^+-binding sites. The location of Cs^+ suggests Na^+ binding near Glu255. Molecular dynamics simulations also showed that Na^+ ions are first transiently bound to Asp36 before binding to Glu255 or Asp371. It has been suggested that Na^+ can perhaps bind in the Glu255 and Asp371 region in two distinct modes depending on the relative distance between these residues [75]. In the first mode, binding of Na^+ between Glu255 and Asp371 is established with spontaneous switching between the two conformations, whereas in the second mode, the two residues approach each other leading to a shortening of the distance between Glu255 and Asp371, thus enabling the simultaneous binding of Na^+ ion to

both the residues at the same time [75]. In contrast to these previous experiments implying a single Na^+-binding site, further work with molecular models shows that there may in fact be an additional Na^+-binding site at Asp371 that was inaccessible to Cs^+ ions [76]. Experimental evidence has also shown that a single Na^+ ion is not sufficient for transport, as site-directed mutagenesis of Asp371 in NorM-VP interrupts the transport activity. These findings also suggest that at least two Na^+ ions are required for transport [76, 83].

2.4 Concluding Remarks

MATE transporters are still relatively new in the field; they were first classified as a family separate from the MFS only 15 years ago. To date, research has focused more on high-resolution crystal structures, which have provided very useful insights into the question how MATE transporters operate. However, functional and biochemical studies are much needed, as it is their complementarity with the structural approaches that will provide a new impetus to the elucidation of the mechanisms of MATE transporters. Given the important roles of MATE transporters in all living cells, research on these transporters promises to be useful and exciting in the context of the development of new drugs and therapies.

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Chapter 3
Small Multidrug Resistance Efflux Pumps

Denice C. Bay and Raymond J. Turner

Abstract Small multidrug resistance (SMR) transporters confer resistance to a variety of quaternary cation compound antimicrobials. These secondary active transporters are the smallest known transporters and have been demonstrated to function within the membrane. The focus of this chapter explores and updates SMR family diversity and reviews current structural and functional knowledge of these members. This chapter also provides an update of known SMR pump-mediated resistance to antimicrobial substrates (including naturally synthesized quaternary cation compounds) and their clinical significance.

Keywords Small multidrug resistance • Multidrug resistance • Efflux • Dual topology • Antiseptic • Quaternary ammonium compound • Quaternary cation compound • EmrE • SugE • Paired SMR • AbeS • EbrAB • YkkCD • YvdRS

3.1 Introduction

Small multidrug resistance (SMR) family proteins confer resistance to a diverse assortment of antiseptics and a limited range of antibiotics. They are the smallest known multidrug resistance (MDR) transporters found in prokaryotes and transport toxic quaternary cation compounds (QCCs) (also known as quaternary ammonium compounds [QACs]) using proton motive force [1, 2]. The SMR family is one of the 14 phylogenetically distinct secondary active transporter families that belong to the

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drug/metabolite transporter (DMT) superfamily [3]. As their name implies, SMR family proteins are short in length (100–170 amino acids) and span the plasma membrane as four hydrophobic α-helical transmembrane strands (TMS). The SMR protein active site of H^+/drug binding is centered at a single highly conserved Glu residue located in the first TMS of SMR proteins [2, 4, 5].

SMR family proteins have been studied for many reasons. Their wide distribution within bacterial species [6] and presence on conserved regions of mobile genetic elements [7] make them clinically significant targets to combat the spread of antiseptic resistance [8]. The small size and novel dual-topology dimer orientation also makes the SMR family evolutionarily significant since they are hypothesized to serve as progenitors of larger multidrug transporters [3, 6, 9, 10]. This chapter describes current structural and functional knowledge gathered for this family to explain how these remarkably small proteins are capable of transporting such a diverse array of substrates, with a focus on characterized SMR subclass family members.

3.2 SMR Family Diversity

SMR family members are encoded chromosomally and/or on mobile genetic elements in conserved 3′ regions of class 1 integrons [7, 8] and on MDR plasmids [11] in archaea and bacteria [6]. Members of this family have been subdivided into three subclasses based on function, isogenicity, and phylogenetic relatedness [6, 12]: small multidrug pumps (SMPs), suppressor of *groEL* mutation proteins (SUG), and paired small multidrug resistance (PSMR) pumps. A brief summary of experimentally characterized SMR subclass members is provided in the following sections (Table 3.1).

3.2.1 The SMP Subclass

SMR members belonging to the SMP subclass are characterized by their ability to confer isogenic resistance to a broad range of toxic lipophilic QCC [13–17] and phylogenetic association to the γ-proteobacterial *Escherichia coli* ethidium MDR protein E (Eco-EmrE) [6, 9]. Eco-EmrE is the archetypical member of both the SMR family and the SMP subclass due to the extensive functional and high-resolution structural characterization of this protein (as reviewed in [12, 65, 66]). These biophysical studies have resulted in cryoelectron microscopy [67–70], X-ray diffraction [71], and nuclear magnetic resonance (NMR) (solution-state [72–74] and solid-state [75, 76]) structures of Eco-EmrE. Although the majority of studies have focused on the Gram-negative Eco-EmrE, experimental structure-function characterization of other closely related SMP subclass members has also been performed on γ-proteobacterial *Acinetobacter baumannii* AbeS [19, 20] and *Pseudomonas aeruginosa* EmrE (Pae-EmrE) [21, 77], β-proteobacterial *Bordetella*

Table 3.1 Characterized SMR protein family members and their substrate profiles

SMR subfamily/member	Examined species[a]	Location[b]	Known substrates[c]	References
Small multidrug pumps (*SMP*)				
EmrE/MvrC/Ebr	Eco	C	ACR, BAC, BET, CHL, CHN, EB, ERY, MV, PY, TET, TMP, TPP, VAN	[13–18]
AbeS	Aba	C	ACR, BAC, CHL, CIP, DAPI, DOC, ERY, EB, NOV, SDS, TPP	[19, 20]
Pae-EmrE/Pasmr	Pae	C	MV, TPP	[21, 22]
Bpe-EmrE/BPsmr	Bpe	C	MV, TPP	[21]
EmrE	Lmo	C	BAC	[23]
Mtu-Smr/TBsmr/Mmr	Msm, Mtu	C	ACR, EB, ERY, PY, SFO, TPP	[21, 24]
Hsmr/EbrB	Hsa	C	ACR, EB, MV	[25, 26]
Smr/Ebr/QacC/QacD	Eco, Pae, Sep, Sma, Spa, Swa	C, P, In	EB, ERY, GH, HQ, TET, TMP, TPP	[27–34]
QacE	Eae, Kae	P, In	ACR, APG, BAC,BEN, CTA, CV, EB, PY, R6G, SFO, TPA	[7, 35]
QacEΔ1	Eco, Sau, Sen	P, In, Tn	ACR, APG, BAC, BEN, CTA, CTP, CV, EB, PY, R6G, SFO, TPA	[7, 35–38]
QacF	Eae, Eco, Vch	P, In, Tn	AMP, BAC, CTA	[39–41]
QacG	Sau	P, In	ACR, BAC, CTA, EB	[33, 34, 42–44]
QacH	Eco, Lmo, Sau, Sin, Spa, Ssi, Vch	P, In	ACR, BAC, CTA, EB	[42, 43, 45, 46]
QacJ	Sau, Sha	P	BAC, CTA	[43]
QacZ/EFA0010	Efa	C	BAC	[47]
Suppressor of groEL mutation proteins (*SUG*)				
SugES/SugE	Eco	C	CET, CTP	[48–50]
SugE	Amo	C	TBT	[51]
SugE	Cfr	C	CHL, EB	[52]
SugE	Ecl	C	BAC, EB, CTA, CTP, SDS, TPP	[53]
SugE/Sug	Cfr, Eco, Kox	P, In	BAC, CET, CTP, EB, SDS, TPP	[37, 52, 54, 55]
Smr-2	Sau	In		[56]

(continued)

Table 3.1 (continued)

SMR subfamily/member	Examined species[a]	Location[b]	Known substrates[c]	References
Paired small multidrug resistance (PSMR) pumps				
EbrAB	Bsu	C	ACR, BAC, CHL, CTA, CTP, CV, EB, ERY, PY, R6G, PY, TPA, TPP	[57–59]
MdtIJ/YdgEF	Eco	C	ACR, BAC, CHL, CTP, CV, EB, ERY, R6G, SDS, SPE, TET, TMP, VAN	[15, 60]
NepAB	Ani	P	MAM	[61]
PsmrAB	Hda	C		[62]
YkkCD	Bsu	C	BAC, CET, CTP, CV, EB, PY, TPA	[9, 63]
YvdRS	Bsu	C		[9, 64]
YvaE (YvaD)	Bsu	C	BAC, CET, CTP, CV, EB, PY, TPA	[9]

[a]Species abbreviations: *Aba Acinetobacter baumannii*, *Amo Aeromonas molluscorum*, *Ani Arthrobacter nicotinovorans*, *Bpe Bordetella pertussis*, *Bsu Bacillus subtilis*, *Cfr Citrobacter freundii*, *Eae Enterobacter aerogenes*, *Ecl Enterobacter cloacae*, *Eco Escherichia coli*, *Efa Enterococcus faecalis*, *Esp Enterococcus* sp., *Hda Halobacillus dabanensis*, *Kae Klebsiella aerogenes*, *Kox Klebsiella oxytoca*, *Lmo Listeria monocytogenes*, *Mtu Mycobacterium tuberculosis*, *Msm Mycobacterium smegmatis*, *Pae Pseudomonas aeruginosa*, *Sau Staphylococcus aureus*, *Sep Staphylococcus epidermidis*, *Sha Staphylococcus haemolyticus*, *Sin Staphylococcus intermedius*, *Sma Stenotrophomonas maltophilia*, *Spa Staphylococcus pasteuri*, *Ssi Staphylococcus simulans*, *Swa Staphylococcus warneri*, *Vch Vibrio cholerae*

[b]Location abbreviations: *C* chromosome, *In* integron, *P* plasmid, *Tn* transposon

[c]Substrate abbreviations: *ACR* acriflavine, *AMP* ampicillin, *APG* alkylpolyaminoethylglycine, *BAC* benzalkonium chloride, *BEN* benzethonium, *BET* betaine, *CET* cetrimide, *CHL* chloramphenicol, *CHN* choline, *CIP* ciprofloxacin, *CTA* cetyltrimethylammonium, *CTP* cetylpyridinium, *CV* crystal violet, *DAPI* 4′,6-diamidine-2-phenylindole, *DOC* deoxycholate, *EB* ethidium bromide, *ERY* erythromycin, *GH* guanidine hydrochloride, *HQ* 8-hydroxyquinoline, *MAM* methylamine, *MV* methyl viologen (paraquat), *NOV* novobiocin, *PY* pyronin Y, *R6G* rhodamine 6G, *SDS* sodium dodecyl sulfate, *SFO* safranin O, *SPE* spermidine, *TBT* tributyltin, *TET* tetracycline, *TMP* trimethoprim, *TPA* tetraphenylarsonium, *TPP* tetraphenylphosphonium, *VAN* vancomycin

pertussis (Bpe-Smr) [21], Gram-positive firmicutes *Staphylococcus aureus* (Sau-Smr) [27, 28, 78], and actinobacterial *Mycobacterium tuberculosis* (Mtu-Smr) [21, 24, 79–81]. The consensus from these studies indicates that bacterial SMP members form isogenic functional homooligomers that confer broad polyspecific drug resistance profiles similar to Eco-EmrE despite their variable sequence identity (32–54 % Eco-EmrE). As observed for bacterial SMP members, experimental characterization of the archaeal *Halobacterium salinarum* (Hsa-Smr) [25] also suggests that archaeal SMP homologs adopt similar structural and functional features, despite their high overall content of Ala and Val residues (40 % total of 112 Hsa-Smr residues) by comparison to Eco-EmrE (13 % of total 110 residues).

Members of the SMP subclass have a diverse distribution within prokaryotes and have been identified from chromosomes, from a variety of MDR plasmids, and within the 3′ conserved gene cassette region of various class 1 integrons and transposons (Table 3.1). SMP members encoded on mobile genetic elements are typically designated as Qac efflux pumps based on their ability to confer resistance to these toxic compounds. The SMP subclass also possesses the greatest diversity of laterally transferred members: QacC, QacE, QacEΔ1, QacF, QacG, QacH, QacJ, and QacZ (Table 3.1 and Fig. 3.1). QacE and the semi-functional QacEΔ1 (which lacks 16 C-terminal residues from the QacE sequence [35]) were identified as conserved genes in the 3′ region of class 1 integrons [7] isolated from Gram-negative and Gram-positive bacteria [35, 36, 83]. The remaining Qac members, QacF, QacG, QacH, QacJ, and QacZ, have been identified from integrons, transposons, and/or MDR plasmids (Table 3.1 and Fig. 3.1). QacF shares a close homology with QacE (68 % identity) and is frequently detected on class 1 integrons and various MDR plasmids in Gram-negatives [39, 40, 84]. QacG, QacH, and QacJ frequently identified on Gram-positive staphylococcal MDR plasmids and share closer homology to Sau-Smr (69–83 % identity) than Eco-EmrE (41–62 %). The most recent addition to this group, QacZ (74 % identity to Sau-Smr), was identified from a Gram-positive enterococcal plasmid (pTEF1) and conferred resistance to benzalkonium chloride but not ethidium or chlorhexidine, indicating that some Qac members may provide selective QCC resistance [47].

3.2.2 The SUG Subclass

Similar to the SMP subclass, members of the SUG subclass also confer resistance when they are expressed as a single gene but only to a limited range of QCCs [48]. SUG members have been identified on chromosomes and within mobile genetic elements (Table 3.1) indicating that they also share a diverse heritability similar to SMP subclass members. These members are homologous to *E. coli* suppressor of *groEL* mutation protein E (Eco-SugE) [6] which was named according to its initial identification in an experiment involving the suppression of *groEL* chaperonin mutations [49]. Confirmation of Eco-SugE involvement suggests that *groEL* suppression was caused by a cloning artifact, since Eco-SugE was located adjacent to the *groES* locus [85]. Despite this study, SugE proteins have been suggested to confer some chaperone-like activity [9, 86, 87] making it uncertain what role this subclass plays in bacterial protein folding processes.

The most well-characterized SUG member is Eco-SugE [48, 50, 88–91], and relatively few studies have examined this protein in comparison to Eco-EmrE, possibly due to its selective resistance profile. Eco-SugE serves as the representative SUG member and confers resistance to a limited range of detergent-like QCCs [48] (Table 3.1). Mutational analysis of Eco-SugE has shown that alteration of specific residues can alter substrate transport from an exporter to an importer [52]. Characterization of other SUG members has focused solely on Gram-negative

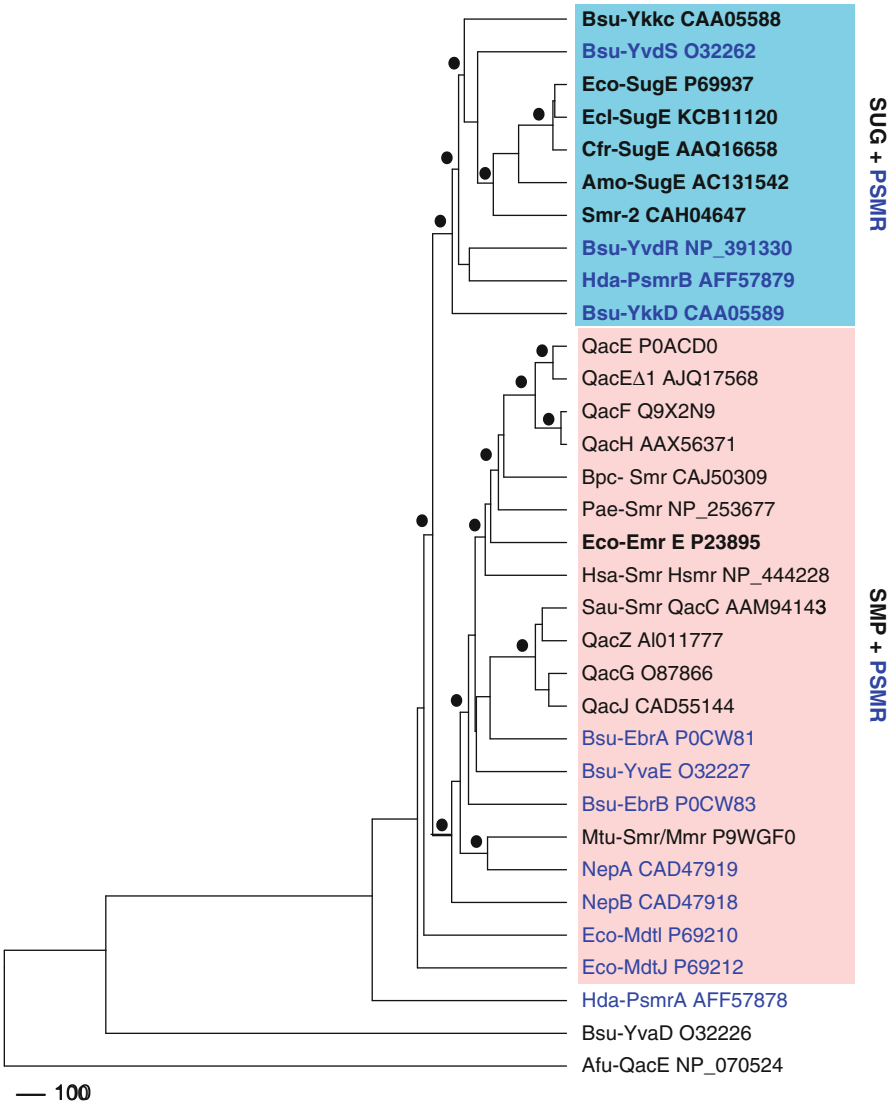

Fig. 3.1 A rooted neighbor joining (NJ) phylogenetic tree of experimentally characterized SMR protein family members. NJ distance analysis was performed with a ClustalW [82] multiple sequence alignment of 32 characterized SMR family protein sequences listed in Table 3.1. The outgroup for this analysis was the *Archaeoglobus fulgidus* QacE (Afu-QacE) based on a previous phylogenetic study [6]. The NJ tree represents a consensus of 100 bootstrap replicates, and nodes with 75 % or more confidence are indicated by *black filled circles*

proteobacterial homologs from *Aeromonas molluscorum* (Amo-SugE) [51], *Citrobacter freundii* (Cfr-SugE) [52], and *Enterobacter cloacae* (Ecl-SugE) [53]. It should be noted that functional analysis of Ecl-SugE [53] and Amo-SugE [51]

has demonstrated that other SUG members may confer resistance to a broader range of substrates that include ethidium and tetraphenylphosphonium (Table 3.1). SUG members are most frequently identified from chromosomes and more widely distributed within archaeal and bacterial species than SMP members. In contrast to the SMP subclass, SUG homolog diversity and distribution on mobile genetic elements are low, and many SUG sequences are closely related to or identical to Cfr-SugE [54]. Laterally transferred SUG members are most frequently identified within class 1 integrons and transposons [92] as well as MDR plasmids [37, 54, 55] from Gram-negative proteobacteria and less frequently from Gram-positive species [93] (Table 3.1 and Fig. 3.1).

3.2.3 PSMR Subclass Members

Unlike the SMP and SUG subclasses, members of PSMR subclass require simultaneous expression of two SMR gene copies located within the same operon/locus to produce drug resistance [57]. PSMR members were originally predicted and identified from sequenced genome surveys [9, 87] and now include a variety of characterized members from both Gram-positive and Gram-negative bacteria (Table 3.1). PSMR diversity within bacteria has been shown to be greater in Gram-positive species (in *Bacillus subtilis* EbrAB, YkkCD, YvaDE, and YvdRS) as compared to Gram-negative species (*E. coli* MdtIJ) [6] (Table 3.1 and Fig. 3.1). Phylogenetic analysis of SMR subclass members from taxonomically diverse bacteria has indicated that PSMR members recently evolved from gene duplication events and demonstrated that PSMR members MdtIJ, EbrAB, and YvaDE originated from SMP members, while PSMR members YkkCD and YvdRS evolved from SUG subclass members [6] (Fig. 3.1).

The most well-characterized PSMR members are from *B. subtilis* EbrAB (Bsu-EbrAB) [57–59, 63, 94, 95] and *E. coli* MdtIJ/YdgEF (Eco-MdtIJ) [15, 60, 84, 96, 97]. Structural analysis of Bsu-EbrAB has demonstrated that the pair forms a heterooligomer [58, 59, 94]. Studies of Bsu-EbrAB and Eco-MdtIJ demonstrated that PSMR members adopt an opposite insertion orientation from each other in the membrane [94, 97, 98]. It is important to note that drug resistance from the overexpression of a single PSMR gene, specifically Bsu-EbrB [95] and Bsu-YvaE [9], has demonstrated that overexpression of both proteins may not be required to confer resistance in *E. coli* expression systems. Additionally, one protein of the PSMR pair is generally longer (Bsu-EbrA 105 aa versus Bsu-EbrB 117 aa; Eco-MdtI 109 aa versus Eco-MdtJ 121 aa) which result in loop (loops 1 and 3) and C-terminus lengthening. Mutational analysis of Bsu-EbrA and Bsu-EbrB which removed the loops and C-termini from each protein resulted in a PSMR drug resistant protein when expressed as a single gene [58] indicating that loops and termini enhanced PSMR multimerization. The remaining chromosomally encoded PSMR members YvaDE and YvdRS appear to be the only subclass members with an unknown substrate profile [9]. Studies of the Bsu-YvdRS homolog, PsmrAB from the halophilic

Halobacillus dabanensis (Hda-PsmrAB) revealed that this protein functions as a Na^+/H^+ antiporter [62] suggesting that YvdRS homologs may not confer drug resistance but function solely in osmotic regulation. The identification of PSMR members on mobile genetic elements is relatively low and appears to be present only on plasmids that confer specialized cell functions, as observed for the toxic methylamine efflux pump NepAB (a homolog of EbrAB [6]; Fig. 3.1) from Gram-positive *Arthrobacter nicotinovorans* [61]. Taken altogether, PSMR subclass distribution and diversity appears to be evolving toward specialized substrate transport that in some cases maintain antimicrobial transport.

3.3 SMR Transporter Structure Analysis

Structural analysis of SMR family members has primarily focused on Eco-EmrE. Over the past two decades, many high-resolution biophysical techniques, cryoelectron microscopy (EM), X-ray crystal diffraction, and solution-/solid-state NMR, have been performed on Eco-EmrE protein. Early EmrE structural analyses of two-dimensional crystals using cryo-EM [68, 99] provided a low-resolution (7.0–7.5 Å) projection structure (protein database [PDB] code 2I68) [69] that supported an asymmetrically arranged EmrE dimer. The three-dimensional (3D) projection structure also demonstrated tetraphenylphosphonium binding occurring in TMS1–TMS3 regions of each monomer and supported an antiparallel arrangement of each protein monomer within the dimer [69]. Controversy ensued when X-ray crystal structures of EmrE were published that failed to agree with the available biochemical and biophysical data [100, 101] and were later retracted due to software calculation errors [102, 103]. Re-examination of EmrE X-ray diffraction crystals resulted in a 3.8 Å (PDB 3B5D) 3D structure of an EmrE dimer with bound tetraphenylphosphonium [71] (Fig. 3.2b). Analysis of the X-ray structure also resulted in two additional 3D homology structures that provided an apo-EmrE form at 4.5 Å resolution (PDB 3B61) and an EmrE dimer bound to tetraphenylphosphonium at 4.4 Å (PDB 3B62) [71]. The revised EmrE X-ray structures appear to be in greater agreement with previous cryo-EM structures, by confirming an asymmetrical arrangement of each protein monomer in an antiparallel orientation. Other biophysical techniques such as systematic spin-labeling electron paramagnetic resonance (EPR) [105], solution-state NMR [72, 89, 106, 107], and solid-state [75, 76, 90] NMR studies of EmrE in bicelles and liposomes also support an asymmetrical antiparallel EmrE dimer. Altogether, these biophysical structural studies are beginning to support biochemical analyses that indicate EmrE forms a functional antiparallel dimer (as reviewed in [70]).

Currently, high-resolution structural analyses are not available for other SMR subclass members, but attempts have been made to examine other SMP members Mtu-Smr [79–81] and SUG members [89–91] by NMR techniques. Acquiring high-resolution structures of other SMR members would be invaluable for comparing the

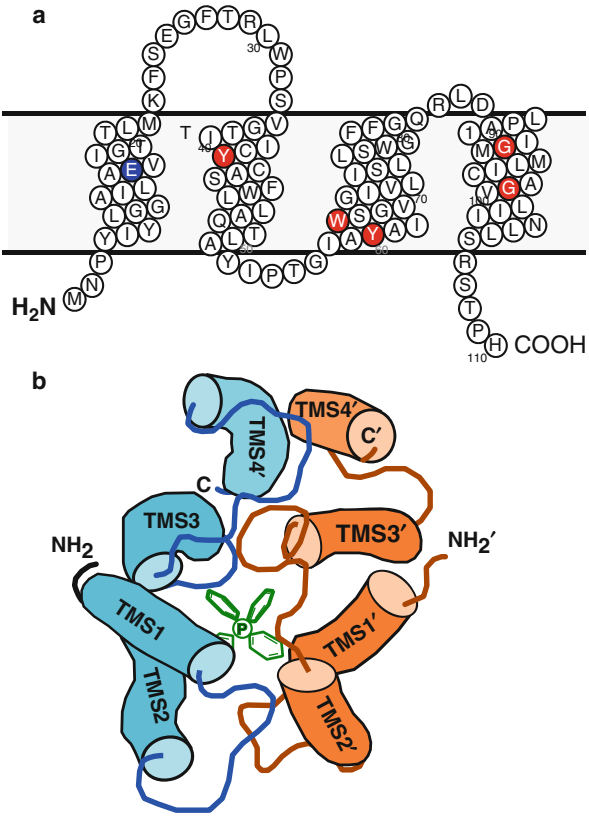

Fig. 3.2 Cartoon diagrams of Eco-EmrE structures. (**a**) A secondary structure and topology map of the Eco-EmrE protein (PDB P23895) generated using the online Protter program version 1.0 [104]. Amino acid residues are shown as circles, where E14 (*blue*), W63, Y40, Y60, G90, and G97 (*red*) are highlighted. The membrane bilayer is represented as horizontal lines. (**b**) The 3D X-ray diffraction structural model of Eco-EmrE (PDB code 3B5D) bound to the ligand tetraphenylphosphonium [71]. A top down view of all four TMSs (*cylinders*) and loops (*thick lines*) in each EmrE monomer (*light blue*, monomer A; *orange*, monomer B). The bound ligand tetraphenylphosphonium (*green*) is shown as a *stick* chemical diagram where the phosphorous atom is in a *circle*, and each of the four aromatic rings is shown as *hexagons*

plasticity, drug selectivity, and structural conservation of Eco-EmrE which can aid the development of efflux pump inhibitors and improve antimicrobial development.

3.3.1 SMR Transporter Topology

The topological orientation of SMR family members has been a source of considerable controversy over the last decade [100, 101, 108–110]. According to the positive-inside rule, the orientation of a TMS is directed by the number of positively

charged (K and R) residues located within loops and termini of the membrane protein [111–113]. The topology or "KR" bias of a membrane protein can be reliably estimated by summing the difference in net positive charges in oppositely facing loops and termini, where loops/termini with the greatest positive charge will orient to face the cell cytoplasm [98]. Interestingly, SMR family members from SMP and SUG subclasses have KR bias values at or close to zero indicating that their insertion orientation is neutral or random [98, 114]. In contrast, heterooligomeric PSMR subclass members are predicted to orient in a fixed but opposite topology from each other to form an antiparallel heterodimer [9, 97]. The topology of SMP member Eco-EmrE has been extensively studied, and evidence supporting antiparallel insertion of EmrE has been demonstrated using reporter tag fusions [98, 114–116], alteration of positively charged residues [98, 116–118], cysteine accessibility and cross-linking [95, 119, 120], tandem genetic fusions [95, 121], NMR [72, 107, 122, 123], and high-resolution X-ray crystal diffraction techniques [71]. It should be noted that experiments have demonstrated that EmrE adopts functional fixed parallel orientations as tandem fusions [95] and as cross-linked monomers [124, 125]; the emerging consensus appears to support a functional antiparallel topology for EmrE due to its reoccurrence in high-resolution structural analyses [70, 72]. Antiparallel PSMR pair insertion orientations have been reported for Bsu-EbrAB [94] and Eco-MdtIJ [97, 98, 114] providing further support that SMR family members can adopt a functional antiparallel topology.

3.3.2 SMR Transporter Multimerization

All SMR proteins are expected to function as oligomers, where SMP members form homooligomers (as reviewed in [12]) and PSMR proteins form heterooligomers [59]. Multimerization studies of SUG members currently indicate these proteins predominate as monomers *in vitro* [50, 88], but due to their sequence similarity to SMP members, SUG homologs may also function as multimers. Extensive examination of SMP subclass member, Eco-EmrE, has revealed that the protein can adopt a variety of states: monomeric [89, 126–129], dimeric [66, 67, 71, 73, 99, 120, 128, 130–132], trimeric [5, 17, 128, 133], and higher multimeric [5, 17, 67, 68, 99, 126, 134] states, depending on experimental conditions. The overall consensus from these biochemical and structural approaches shows that the minimal functional unit is a dimer (as reviewed in [135]). Although the arrangement of monomers within the dimer is still contested, growing support for an antiparallel arrangement appears to be emerging [72, 120, 121] (refer to discussion in Sect. 3.3.1). Closer examination of Eco-EmrE and another SMP homolog Hsa-Smr has revealed the importance of the fourth TMS (TMS4) for multimer stability and transport [26, 136], and mutagenesis of moderate to highly conserved Gly residues in Eco-EmrE TMS4 has identified a Gly90-X_6-Gly97 motif (Fig. 3.2) [75, 137]. Studies of PSMR Bsu-EbrAB protein variants lacking regions within loops 1 and 3 in addition to the C-terminus resulted in drug resistance from either EbrA or EbrB when expressed individually

[58] supporting their involvement in multimerization. Therefore, a variety of SMR regions, TMS4, loops 1–3, and C-terminus, are currently known to participate in SMR multimerization.

The variation in SMR multimerization may be explained by its small size and diverse topology and its plasticity may be linked to modifications and conditions used to isolate these proteins. Most studies of Eco-EmrE have involved the addition of an affinity purification tag and the most commonly used tag adds a C-terminal *myc*-epitope linker with a hexahistidine tag [5, 138], and this approach has yielded preparations with mixed monomeric and dimeric states [131]. Due to its extreme hydrophobicity, untagged Eco-EmrE purification approaches that involve organic solvent extraction have also been performed and shown to yield predominately monomeric protein with a low occurrence of dimers [128, 139]. The choice of membrane mimetics, such as different detergents (*N*-dodecyl-β-D-maltoside is the most commonly used as reviewed in [12]), bicelles [72, 73, 76, 78, 106, 107, 123], nanodiscs [91], and liposomes [2, 73, 106, 140–143] used to isolate these proteins, may also influence multimeric diversity and stability.

3.3.3 SMR Transporter Lipid Dependence

The influence of the membrane mimetic environment on the structure and function of SMR proteins has been gaining interest but has also underscored the importance of the membrane environment used to study these proteins. Membrane composition is known to influence the folding and function of bacterial transporters *in vitro* [144] and *in vivo* [145, 146]. The surfactant and membrane disrupting mechanisms of action caused by SMR antimicrobial substrates are also known to significantly alter lipid domain organization in the membrane [147]. Therefore, studies of SMR family members have also highlighted the importance of considering not only the protein and its modifications but also the membrane mimetic systems used for their characterization.

Studies of SMP and SUG protein folding and reconstitution in different detergents have revealed differences in multimerization and ligand binding affinities by proteins [126, 128]. Analysis of Eco-EmrE purified in the detergent *N*-dodecyl-β-D-maltoside has shown that multimer formation and protein stability alter depending on the concentration of detergent added [128, 131]. Comparisons of Eco-EmrE dimer stability have also been performed in NMR experiments and determined that dimer affinity increased when the protein was reconstituted from *N*-dodecyl-β-D-maltoside detergent micelles into bicelles composed of dilauroylphosphatidylcholine [73, 106]. These findings indicate that SMR protein multimerization and folding stability vastly improve when membrane mimetics that resemble the native lipid environment of SMR proteins are used for *in vitro* characterization.

Many *in vitro* studies examining SMR protein folding and transport activity in liposomes have also been performed on SMP members. The advantage of these self-contained artificial phospholipid bilayer vesicles is the ability to determine transport

activities in contrast to using detergent micelles and bicelles/nanodisc systems. Examination of Eco-EmrE transport, folding, and insertion into liposomes composed of derivatized phosphatidylcholine (PC) (a nonnative phospholipid in *E. coli*) and phosphatidylethanolamine (PE) (the dominant phospholipid [70–75 %] in *E. coli* membranes [148]) has demonstrated that as the ratio of PE increased, the rate of protein insertion decreased, but the drug transport activity and folding of inserted proteins improved [138, 140, 142]. The addition of derivatized anionic lipids, like phosphatidylglycerol (present at 15–18 % in *E. coli* membranes [148]), to PE/PC liposomes increased Eco-EmrE drug transport [80]. In the same study, the addition of the derivatized anionic phospholipid, phosphatidylinositol (present at 12.5 % in *M. tuberculosis* membranes [148]), to PE/PC liposomes containing Mtu-Smr also increased drug transport [80]. Studies of Eco-EmrE protein reconstituted into lipid monolayers also identified that EmrE clustering was significantly altered in the presence of the anionic lipid, cardiolipin, and long unsaturated fatty acid chains [149]. Brewster angle microscopy experiments with Eco-EmrE reconstitution into lipid monolayers have demonstrated preferential lipid domain sorting around EmrE clusters [149, 150]. Bioinformatic analysis of SMR homologs from diverse Gram-positive and Gram-negative bacteria revealed that the conservation and abundance of positively charged residues that determine dual topology were correlated to the total anionic phospholipid abundance [151]. When considered altogether, these findings highlight the importance and influence of anionic phospholipid content on SMR protein structural stability, topology, and transport activity.

3.3.4 SMR Transporter Ligand Binding

Site-directed mutagenesis studies of SMR members Sau-Smr and Eco-EmrE determined that the antiport of drug and H^+ was associated with a single highly conserved and negatively charged glutamate residue (TMS1 Glu14 of Eco-EmrE; TMS1 Glu13 of Sau-Smr) in its membrane-spanning segments [2, 4, 5, 16]. In Eco-EmrE, replacement of Glu14 with Cys or Ala resulted in a complete loss of drug resistance [2, 4, 152] and replacement with Asp resulted in reduced or selective drug resistance compared to wild-type proteins [4, 5]. Analysis of all SMR subclass members indicates that these members possess a glutamate residue within the first TMS [6] and replacement of this conserved residue in mutagenesis studies of PSMR subclass members from *B. subtilis* Bsu-EbrAB [59, 94], Bsu-YkkCD [63] YvaE [9], and *E. coli* MdtIJ [60] reduced or eliminated their ability to confer drug resistance.

In addition to Glu14 within TMS1, biochemical and mutagenesis studies targeting residue replacements within the first three TMS domains of Eco-EmrE have identified that a number of aromatic residues, such as conserved residues Tyr40, Tyr60, and Trp63, also contribute to drug binding and resistance within the membrane-spanning domains [153–155]. Charged residue replacement within loop1 (Lys22, Glu24, and Arg29) and loop3 (Arg82 and Asp84) of Eco-EmrE has demonstrated reductions in drug transport [4].

X-ray [71], cryo-EM [69], and NMR [72, 105] structural analyses of the SMR archetype Eco-EmrE all indicate that ligand binding occurs within TMS1–TMS3. Cysteine-scanning mutagenesis of Eco-EmrE determined that the TMS1 residues Ala10, Ile11, and Thr18 all located on the same α-helical face as Glu14 participated in the substrate binding pocket [130, 152, 156]. TMS2 has been implicated as a hydrophobic pathway [156], and alterations of conserved residues in this helix were tolerated to greater extents than other TMSs, suggesting that TMS2 plays a role in determining SMR drug polyspecificity [157]. Eco-EmrE TMS3 flexibility [105] and the structured loop between TMS3 and TMS4 [75] have been shown to alter ligand binding within the dimer. Therefore, a number of key residues located within TMS1–TMS3 and the positioning of these helices relative to TMS4 all appear to influence drug binding interactions.

Due to the chemical diversity of substrates recognized and transported by SMR family proteins, drug binding studies have endeavored to identify additional residues responsible for polyspecificity by these proteins. A recent study exploring SMP protein specificity to methyl viologen identified that Eco-EmrE residue Ser43 was specifically involved in methyl viologen resistance [158]. Alteration of this residue located at the same position in SMP proteins that lack methyl viologen transport ability, Bpe-Smr (Ala43Ser) and Mtu-Smr (Ala42Ser), to Ser conferred resistance to methyl viologen [158]. Arrangement dynamics determined for Eco-EmrE TMS1–TMS3 have also been proposed to contribute to polysubstrate recognition by SMR proteins [105, 157]. A recent study compared the substrate specificity of *A. baumannii* AbeS with that of EmrE, and several AbeS variants (with Ala16Gly, Tyr3Ala, and/or Ala42Ser substitution) produced a substrate-dependent phenotype, providing the molecular basis of polyspecificity of AbeS pump [20]. Further exploration of conserved and variable residues in SMR family proteins using these approaches will likely identify other residues responsible for specific drug recognition and transport.

The stoichiometry of H^+/SMR binding has been demonstrated to be variable, where H^+/protein binding was shown to be 1:1 [143, 159], 1:2 [67, 71], and 2:3 [5, 160]. High-resolution structural models currently favor a $1H^+/2$SMR stoichiometry. Ligand/SMR binding was shown to be much more variable at 1:1 [88, 129], 1:2 [67, 71, 131], 1:3 [5, 67, 160, 161], and 1:5 [67]. The affinity of ligand binding to SMR proteins in these experiments was also shown to range from μM to nM concentrations. These variations may reflect differences in ligand properties, such as differences in cationic charge (methyl viologen +2 versus tetraphenylphosphonium +1), aromatic versus acyl chain composition of the ligand tested, oligomerization, and the membrane mimetics used to reconstitute the protein (as reviewed in [12]). Based on the structural plasticity, dual-topology, and potential lipid dependence of SMR proteins, it is not surprising that SMR/ligand interactions also appear to be dynamic and condition dependent. It is clear that SMR proteins bind and transport a variety of structurally diverse lipophilic cation compounds as well as other potentially lipophilic or transiently charged compounds. It seems likely that the plasticity of SMR proteins is essential to recognize diverse substrates and may be intrinsically tied to their broad substrate recognition [128, 134, 162].

3.4 Transport Mechanisms of SMR Efflux Pumps

Numerous transport mechanisms have been proposed to explain Eco-EmrE efflux (as reviewed in [12]). Transport mechanisms have been proposed to account for specific multimers such as the trimeric model of EmrE protein [5, 160] or variable multimeric states [139, 163] during H^+/drug transport. The remaining mechanisms involve variable H^+ binding by EmrE dimers [159] and differ based on the involvement of particular TMS [68, 71, 134] and/or their movements [69, 75]. Recent NMR analyses support the involvement of symmetrical inward and outward conformation transitions of the asymmetric dimer during H^+/ligand transport [66, 72]. Based on current studies, further exploration by NMR analysis may provide more detail into EmrE transport dynamics and clarify its transport mechanism. A recent study demonstrated asymmetric protonation of EmrE by focusing on the pKa values of the active-site residue of Glu14 with ^{1}H-^{15}N transverse relaxation optimized spectroscopy-heteronuclear single quantum coherence spectra [164]. Protonation of the membrane-embedded Glu14 was shown to modulate the dynamics of EmrE in an allosteric fashion [165, 166]. This protonation leads to extensive rotation and tilt of TMS1–TMS3 in conjunction with repacking of loops, at this point conformational changes alter the coordination of the bound substrate and modulate its access to the binding site from the lipid bilayer [166]. Additionally, using EmrE as the model transporter, a novel liposome method, termed fluorosomes, was developed to study the interaction of antimicrobial substrates and single efflux transporters [167].

Another question that has concerned SMR transport is how QCCs transported by these proteins are completely expelled from Gram-negative systems. Studies of other MDR transporters such as AcrAB and EmrAB identified the involvement of an outer membrane protein TolC forming a multipartite complex spanning both membranes to completely efflux substrates from the cell (as reviewed in [168, 169]). Studies of Eco-EmrE and other SMP members failed to demonstrate any requirement for TolC [29, 170, 171]. A recent study involving an osmotic growth phenotype and screening of overexpressed Eco-EmrE in outer membrane protein gene deletion mutants identified that OmpW participates with EmrE in drug and osmoprotectant efflux in *E. coli* [18]. It is uncertain if OmpW forms a multipartite dual membrane-spanning complex with EmrE, but it does support the involvement of outer membrane protein(s), such as OmpW, in substrate efflux by other SMR members and potentially for other TolC-independent MDR transporters in Gram-negative bacteria.

3.5 SMR Efflux Pumps in Antimicrobial Resistance

SMR proteins confer resistance to a variety of toxic lipophilic QCCs used as industrial surfactants (tetraphenylphosphonium and tetraphenylarsonium), membrane-disrupting detergents (alkylpolyaminoethylglycine cetylpyridinium and

cetyltrimethylammonium), antiseptics (benzalkonium chloride, cetrimide, and 8-hydroxyquinoline), DNA-intercalating (acriflavine and ethidium bromide) and toxic dyes (crystal violet, rhodamine 6G, and safranin O), and reactive oxygen-generating compounds (methyl viologen) (Table 3.1). QCCs represent a structurally diverse group of chemicals that possess one or more cationic atoms (most commonly nitrogen and phosphorous) bound to three to four R groups that consist of acyl chain or aromatic hydrocarbons. SMR members have also demonstrated low to moderate resistance to antibiotics such as chloramphenicol, erythromycin, fluoroquinolones, and tetracyclines (Table 3.1) [42, 51, 62, 172] by comparison to other larger MDR transporters [15, 170]. Curiously, reports have also shown that SMR proteins can confer resistance to sodium dodecyl sulfate [15, 42, 53] (Table 3.1). Based on the negative charge of the conserved glutamate residue shown to bind both drugs and protons and the lack of conserved positively charged residues in membrane-spanning segments in SMR proteins [6], it is difficult to understand how anionic sodium dodecyl sulfate can be transported by SMR proteins. It is more likely that tolerance to this detergent is enhanced in bacterial strains overexpressing SMR members due to their affinity for anionic lipids as discussed in Sect. 3.3. In general, it appears that the lipophilicity and cationic properties of a drug determines its potential as an SMR substrate.

Studies of SMR family member drug resistance have also demonstrated SMR members belonging to each subclass appear to differ in their conferred drug resistance profiles suggesting that different SMR subclass members have evolved to accommodate more specific substrates [6]. Supporting evidence of this can be observed when comparing the substrate diversity of SUG and PSMR members to SMP drug resistance profiles as well as comparisons between chromosomally encoded SMR genes and those present on mobile genetic elements (Table 3.1).

3.5.1 Natural SMR Substrates and Potential Functions

SMR proteins confer resistance to a variety of anthropogenically derived QCC antimicrobials. Naturally synthesized QCCs can also build up in cells as metabolic intermediates that serve as osmoprotectants and/or toxic by-products like polyamines during amino acid catabolism. Recent studies involving the efflux of biologically produced QCCs have identified the involvement of many SMR members (Table 3.1). A study assessing growth phenotype changes in *E. coli* cells grown in media with high osmolarities identified the Eco-EmrE involvement in osmoprotectant (betaine and choline) export and its participation in cellular osmoregulation [18]. PSMR members Eco-MdtIJ were shown to transport the polyamine spermidine, a toxic metabolite that builds up during amino acid degradation [60]. Mutagenic analysis of Eco-EmrE has demonstrated that a single residue mutation of conserved Trp63Gly converts the protein into a polyamine exporter [173]. These findings agree with evolutionary studies demonstrating that Eco-MdtJI has recently evolved from EmrE homologs in Gram-negative species [60]. The PSMR member Ani-NepAB encoded

by a plasmid of a Gram-positive aerobe has shown transport of the toxic nicotinamide degradation intermediate methylamine [61]. Interestingly, the PSMR member Hda-PsmrAB, a homolog of Bsu-YvdRS that fails to confer drug resistance, was recently shown to function as a Na^+/H^+ antiporter indicating its involvement in cell osmoregulation [62]. Altogether, SMR subclass diversity and their selective transport of natural substrates and ions may also explain why significant differences in drug resistance profiles occur within subclasses and some of driving forces influencing their phylogenetic distinctions [6]. Selective transport of particular biological substrates may also explain the redundancy of SMR family proteins and other MDR transporters that confer resistance to similar drugs (as discussed by [171]).

3.5.2 Clinical Significance and Pathogenicity

Improving our understanding of the structure, function, and regulation of SMR family proteins is essential to combat the emerging problem of antiseptic resistance. Exposure to QCCs is increasing as these antiseptics are added to commonly used commercial products such as soaps, detergents, mouthwashes, toothpastes, and cosmetics. Large quantities of QCCs are also used in industrial surfactants and in medical/agricultural sterilization resulting in QCC-polluted environments (as reviewed in [8]). SMR family members transmitted via mobile genetic elements and plasmids are frequently associated with QCC-polluted environments [174–176]. The pressure to maintain SMR genes within these mobile elements also appears to be driven by QCC and antibiotic exposure [11, 177] indicating that QCC contamination is a major factor driving SMR-mediated resistance and transmission.

The clinical relevance of SMR-mediated QCC resistance may be associated with bacterial growth states. Enhanced QCC resistance associated with SMR efflux genes has been demonstrated for bacterial cultures grown as sessile surface-attached biofilms [178, 179] and as free-living planktonic cultures [170]. Hence, SMR members may influence the biofilm formation and virulence similar to other MDR transporters [179]. Recent studies identifying SMR family member involvement in the efflux of osmoprotectants, polyamines, and other metabolites (as discussed above) have also shown that similar to other MDR transporters, SMR proteins may confer added benefits and improve cell fitness by removing potentially toxic natural substrates (as reviewed in [180]). Overall, this suggests that the bacterial lifestyle and physiology play an important role in determining the extent of virulence associated with SMR activity and QCC resistance.

Efforts to thwart SMR-mediated QCC resistance have focused on the design and use of TMS-like peptide inhibitors that disrupt multimer formation in the Hsa-Smr complex and its QCC resistance [26, 136]. This inhibition strategy relies on fundamental structural knowledge gained from SMR structural and functional analyses and underscores their importance for novel SMR efflux pump inhibitor design. The initial success of this peptide-based inhibition may provide a future therapeutic strategy that could be applied to selectively inhibit SMR and potentially other MDR efflux systems.

3.6 Concluding Remarks

After two decades of research examining SMR family protein structure and function, many insights have been gained into how its members confer drug resistance and have provided a number of high-resolution structures. It has also fueled a number of controversies surrounding SMR topology and multimerization which have helped drive and focus structural exploration of Eco-EmrE and other members. Further examination of Eco-EmrE and SMR subclass members, specifically those encoded on mobile genetic elements and SUG subclass members, will reveal more insights into the structure, function, clinical significance, and evolution of these remarkable SMR proteins.

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Chapter 4
Structures and Transport Mechanisms of the ABC Efflux Pumps

Cédric Orelle and Jean-Michel Jault

Abstract The ATP-binding cassette (ABC) transporters form one of the largest families of proteins in living organisms. They are overrepresented in bacteria where they are involved in the influx or efflux of various molecules. Although bacterial drug efflux transporters were initially discovered as ion-motive-driven pumps, evidence has accumulated since the mid-1990s that members of the ABC superfamily can play a prominent role in drug resistance mechanisms. Yet, the implication of drug efflux ABC transporters in clinical settings is still lagging behind for most bacterial pathogens. Thanks to the accumulation of three-dimensional structures, our knowledge of the functioning mechanisms of drug efflux transporters has progressed tremendously in the recent years, but many questions still remain. In this chapter, we will summarize the current view of the structures and transport mechanisms of drug efflux ABC transporters with an emphasis on multidrug bacterial efflux pumps. Unsolved mysteries about these fascinating transporters will also be mentioned.

Keywords ABC transporters • Multidrug resistance • P-Glycoprotein • ATP switch model • Constant contact model • Drug binding • BmrA • LmrA • MacAB • MsbA • PatAB • Sav1866

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4.1 Introduction

The ATP-binding cassette (ABC) transporters form a large superfamily of proteins that use the energy of ATP binding and hydrolysis to translocate a wide variety of solutes across biological membranes [1]. (Although this chapter is focused on bacterial transporters, some references to eukaryotic transporters will also be included when relevant to this topic.) Apart from a few exceptions [2], the importers are only present in prokaryotes, while exporters are found in all organisms. The minimal functional unit contains two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) [3, 4]. These domains can be synthesized as four separate polypeptides or as various combinations of three, two, or a single polypeptide. The NBDs energize the transporter by binding and hydrolyzing ATP, while the TMDs are responsible for the specificity and translocation pathway of the substrates. Therefore the NBD primary sequences are fairly well conserved, while the TMD sequences and topology, in particular the number of transmembrane helices, are highly variable depending on the solute transported. In addition, the majority of the importers require an extracellular substrate-binding protein that delivers the substrate to the transporter [5].

4.2 Multidrug Transporters

4.2.1 Homodimers

The first bacterial multidrug resistance (MDR) ABC transporter was discovered in *Lactococcus lactis* and was named LmrA (*Lactococcus* multidrug resistance ATP) [6]. LmrA functions as a homodimer [7], each monomer being made of one TMD containing six predicted transmembrane helices and one ABC domain. LmrA was initially chosen for investigation based on its homology with the human MDR transporter P-glycoprotein. It was later shown to complement the P-glycoprotein human gene in eukaryotic cells [8]. The overexpression of LmrA in a drug-hypersusceptible strain of *Escherichia coli* induced a resistance phenotype to several structurally unrelated compounds, i.e., ethidium, daunorubicin, rhodamine 6G, and tetraphenylphosphonium [6]. In addition, the accumulation of daunorubicin in inverted membrane vesicles was dependent on ATP hydrolysis and inhibited by the other substrates and reserpine, a classical inhibitor of drug efflux pumps.

A *Bacillus subtilis* transporter homologous to LmrA was shown to transport a variety of drugs (e.g., Hoechst 33342, doxorubicin, and 7-aminoactinomycin D) when overexpressed in inverted *E. coli* membrane vesicles. Originally known as YvcC [9], this homodimeric transporter [10] was renamed BmrA (*Bacillus* multidrug resistance ATP) [11]. Later, a *B. subtilis* strain resistant to the antibiotic cervimycin C was isolated and shown to strongly upregulate the expression of *bmrA* due to a promoter mutation [12].

Two other drug transporters, Sav1866 from *Staphylococcus aureus* and MsbA from various bacteria, will be described in more details below.

4.2.2 Heterodimers

While LmrA and BmrA are typical homodimers, other drug transporters are heterodimers. The latter transporters include LmrCD in *L. lactis* [13], BmrCD in *B. subtilis* [14], PatAB in *Streptococcus pneumoniae* [15], and SmdAB in *Serratia marcescens* [16]. In these heterodimers, one of the ATP-binding sites is degenerated [17, 18] with conserved residues such as the glutamate adjacent to the Walker B motif, the histidine of the H-loop, and/or some residues in the signature motif of the opposite NBD being naturally substituted by non-consensual residues. Consequently, the functioning mechanism of these heterodimers is asymmetric with the degenerated NDB being poorly active in ATP hydrolysis. A similar scenario occurs in eukaryotic transporters that bear two nonequivalent NBDs (e.g., multidrug resistance protein MRP1, cystic fibrosis transmembrane conductance regulator CFTR, or antigen peptide transporter TAP1/TAP2) [19, 20].

LmrCD was shown to be a major MDR transporter in *L. lactis* [21, 22] and its expression is under the control of the transcriptional repressor LmrR [23, 24]. Binding of drugs to LmrR reduces its affinity for LmrCD promoter thereby inducing the expression of the MDR transporter [25–27].

BmrCD is a *B. subtilis* transporter whose expression is stimulated by various antimicrobial agents [14], especially protein synthesis inhibitors. The latter drugs were recently shown to induce the expression of BmrCD through a ribosome-mediated transcriptional attenuation mechanism [28]. When overexpressed in *E. coli* membranes, BmrCD transports several drugs such as Hoechst 33342, doxorubicin, and mitoxantrone [14]. Its efficient expression and purification was exploited for several structural and functional studies [29–31].

The implication of the *S. pneumoniae* transporter PatAB in MDR was first demonstrated when inactivation of its genes induced an increased susceptibility to several antimicrobial agents: acriflavine, berberine, ethidium bromide, and norfloxacin [32]. After exposing a laboratory strain to ciprofloxacin, a multidrug-resistant strain was isolated in which PatA and PatB genes were upregulated [33]. Such upregulation by fluoroquinolones was also found in clinical isolates [34–36]. Importantly, disruption of PatA and PatB genes overexpressed in many clinical isolates restored drug susceptibility, either completely for ethidium bromide or partially for fluoroquinolones [35]. Several mechanisms were described for PatAB upregulation: disruption of a transcriptional attenuator [37], gene duplication [38], or promoter region and internal mutations [39]. Studies of the transporter overexpressed in *E. coli* show that only the heterodimer is functional for drug efflux [40].

Other transporters whose function is less characterized were shown to have drug transport capabilities, such as TmrAB in *Thermus thermophilus* [18] or TM287/

TM288 in *Thermotoga maritima* [41]. A recent study suggests that TmrAB may be a glycolipid flippase analogous to the transporter MsbA [42].

4.2.3 Other Drug ABC Exporters with a Different Topology

DrrAB from *Streptomyces peucetius* exports the anticancer antibiotics daunorubicin and doxorubicin that this microbe produces. It was long thought to be a narrow-spectrum drug transporter until a recent biochemical characterization indicated its ability to also transport the Hoechst 33342 and ethidium bromide [43]. In contrast to LmrA and BmrA, where each monomer is a TMD fused to an NBD, DrrA is a single NBD subunit, while DrrB is a TMD subunit predicted to contain eight transmembrane helices [44].

MacAB-TolC from *E. coli* was characterized as a macrolide-specific tripartite efflux pump [45]. Homologues are present in various Gram-negative bacteria. MacB is an ABC transporter from the cytoplasmic membrane with an inverted topology: an *N*-terminal NBD is fused to a *C*-terminal TMD containing four predicted transmembrane helices. TolC is an outer membrane channel protein, while MacA is a membrane fusion protein that interacts with both partner proteins. Nanomolar affinity interactions occur between TolC and MacA and between MacA and MacB [46]. MacB is a dimer [47] whose ATPase activity is strongly stimulated by MacA [48]. Several drug-unrelated physiological functions have been proposed, the latest being protoporphyrin efflux [49].

4.3 Structure of the Nucleotide-Binding Domains, Consensus Motifs, and the ATP Sandwich Dimer

The NBDs of ABC transporters are well conserved, both in sequence with several motifs and in structure [50]. HisP, which is the ATPase subunit from a bacterial histidine importer, was the first NBD crystallized [51]. The domain had an L shape and is made of three subdomains (Fig. 4.1) [52]. One is a RecA-like subdomain present in many ATPases and that carries the Walker A and Walker B motifs [53, 54] as originally described in numerous ATPases (e.g., the Fo-F1) [55]. The former, also known as the P-loop, has the consensus sequence GX_2GXGKT/S (where X is any residue; see Fig. 4.1). Some backbone amino groups within this motif, and especially the ε-amino group of the conserved lysine, stabilize the bound nucleotide by making hydrogen bonds with the β- and γ-phosphate oxygen atoms. The Walker B motif is usually made of four hydrophobic residues that form a β-strand and is terminated by a conserved Asp. This Asp residue coordinates the catalytic Mg^{2+} cofactor by hydrogen bonding via a water molecule.

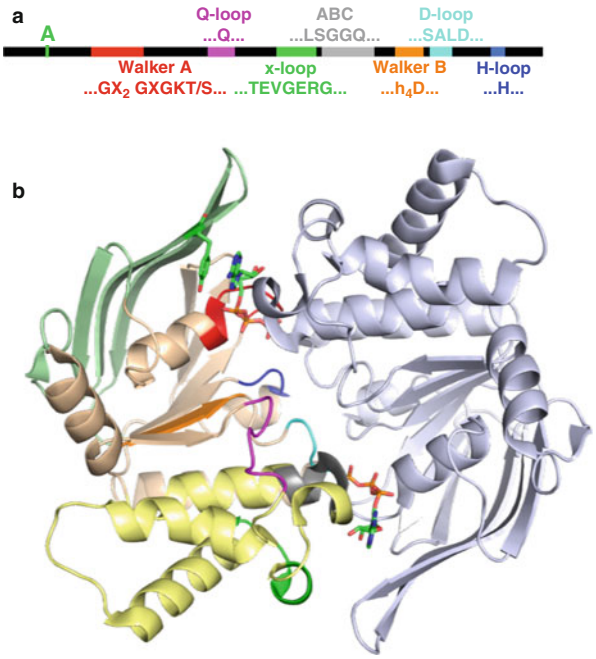

Fig. 4.1 Structure of the nucleotide-binding domains. (**a**) Schematic view of the conserved elements present in the NBD of exporters (see the text for details). The color-coding is the same as in (**b**). (**b**) The dimer of NBDs trapped in a transient ATP-bound state. The two identical NBDs of LolD (PDB code MJ0796) are shown here, and one is colored in gray and the other is colored in pale green, wheat, and pale yellow for the β-, RecA-, and α-helical subdomains, respectively. The conserved motifs are shown for one monomer in *red* (Walker A), *orange* (Walker B), *blue* (H-loop), *magenta* (Q-loop), *gray* (ABC signature), *green* (x-loop), and *cyan* (D-loop). The tyrosine which is part of the A-loop and stacks the adenine ring of ATP is shown in stick representation, like the two ATP molecules trapped at the NBD interface, and colored by elements (C, *green*; O, *red*; N, *blue*; and P, *orange*). An ATPase inactive mutant was used here (Glu171Gln) allowing the stabilization of this transient ATP-bound conformation. This figure was made with PyMOL using the PDB code 1L2T [65]

In ABC transporters with NBDs bearing consensual signatures motifs, the Walker B motif is immediately followed by an invariant Glu residue whose position in the three-dimensional (3D) structure is reminiscent of the catalytic Glu found in other ATPases [53, 56, 57]. The involvement of this residue as a catalytic base, as initially proposed based on mutagenesis and kinetic studies [58], has been later substantiated by the 3D structure of the maltose transporter [59], and this seemed to nail down the original controversy about this residue [60]. Additional motifs are present in the RecA-like subdomain including: (i) The Q-loop, a stretch of ~ eight amino acids starting with a conserved Gln and joining the RecA-like and α-helical subdomains, makes part of the interface with the TMDs. A conformational switch of the Gln residue during the

catalytic cycle, engaging the MgATP and moving away after ATP hydrolysis, may be involved in transmitting conformational changes between NBDs and TMDs. (ii) The H-loop contains a conserved His that acts as a linchpin in ATP hydrolysis by interacting with the γ-phosphate of ATP and the catalytic Glu [61]. (iii) The D-loop carries usually the conserved sequence "SALD," a distinctive feature of ABC proteins located downstream of the Walker B motif. When the NBDs are sufficiently close to each other, the D-loop establishes a complex hydrogen bond and electrostatic network with the Walker A motif and H-loop of the opposite NBD. Because of its position at the dimer interface, the "D-loop" originally referred to "dimer" [62], while it was later alluded to the invariant aspartic acid of the motif. In the NBD dimers, the D-loops also connect and stabilize the catalytic Glu and attacking water [59, 63]. By contacting the ATP-binding sites both in *cis* and in *trans*, the D-loops are likely to play a major role in the communication between the active sites, the control of ATP hydrolysis, and also the directionality and energy of the transport as shown recently [64].

The two other subdomains are specific to the ABC family. One is the α-helical subdomain, which contains the family signature motif. Its sequence usually starts with LSGGQ and belongs to a loop located at the N-terminus of an α-helix. The role of this motif had remained elusive for a long time until the 3D structures of ABC dimers were solved [62, 65]. Hence, in all ABC family members, the NBDs associate transiently in a head-to-tail dimer in which the ATP molecules are sandwiched between the Walker A motif of one domain and the signature motif of the other domain. The LSGGQ sequence makes extensive hydrogen bonds with the ATP and is required for ATP hydrolysis. The role of this motif is likely similar to the arginine finger present in some P-loop GTPases, which stabilize the active site of the opposite domain (see Fig. 4.1). Another motif present in this subdomain is the x-loop, defined as TEVGERG sequence in Sav1866 (see below). It is only present in exporters and precedes the signature motif in the α-helical subdomain [66]. Its name refers to the fact that it interacts with both intracellular loops. Based on its proximity with the signature motif, it has been hypothesized to transmit conformational changes between the ATP-binding site and the TMDs [67–70].

The other subdomain is called the β-subdomain. It encompasses the A-loop which is located upstream of the Walker A motif and bears a conserved aromatic (A) residue that stacks against the adenine ring of the nucleotide, helping to stabilize it [53, 71]. While providing extremely valuable insights into the mechanism of ABC transporters, the structures of isolated NBDs lead to flawed interpretations of catalytic mechanisms since the TMDs impose some structural constraints and alter the geometry of the catalytic sites [59, 72, 73].

4.4 Structures of Whole Drug Exporters

Crystallized exporters were captured in mainly two opposite conformational states: outward facing and inward facing (Fig. 4.2).

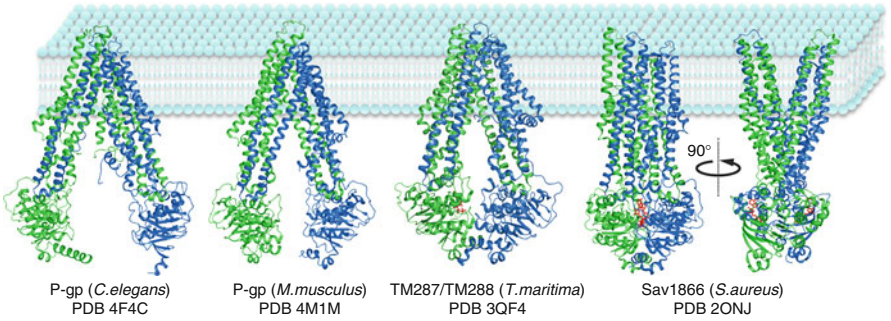

Fig. 4.2 3D structures of selected drug exporters from ABC family. The N-terminal half of P-glycoprotein (P-gp), TM287, and one monomer of Sav1866 are colored in *green*, while the C-terminal half of P-gp, TM288, and the other monomer of Sav1866 are shown in *blue*. When present, AMP-PNP is shown in *red stick* representation

Some structures originally contained major errors and were later corrected [74, 75]. Sav1866 is a multidrug transporter from *S. aureus* [76], and its crystal structure caused the retraction of erroneous MsbA structures [77] and thus revealed for the first time the correct architecture of an ABC exporter [66]. Sav1866 is a homodimer, and each protomer is made of six transmembrane helices located at the *N*-terminal side of the transporter and one *C*-terminal NBD (Fig. 4.2). Sav1866 was crystallized in an outward-facing conformation in which the NBDs are in close contact (closed state). Although this conformation was observed with ADP bound in the catalytic sites, this state most likely represents an ATP-bound state. Accordingly, a second structure with AMP-PNP bound instead was virtually identical [78]. The two monomers exhibit an extensive twist, and the domains of each monomer significantly contact those of the other monomer (Fig. 4.2). In this state, a central cavity was formed at the interface of the two TMDs. This cavity was shielded from the cytoplasm and the inner leaflet of the lipid bilayer, but accessible from the outer leaflet and the extracellular space (outward-facing conformation). The transmembrane helices are connected via short extracellular loops and long intracellular loops that protrude and extend the helical transmembrane bundles (Fig. 4.2). Consequently, the NBDs are located 25 Å away from the membrane. The TMD interface with the NBDs mostly involves the so-called coupling helices of the intracellular domains ICD1 and ICD2. The coupling helix 1 of ICD1 is located between transmembrane helices 2 and 3 and interacts mostly with the NBD of its own monomer. The coupling helix 2 of ICD2 located between transmembrane helices 4 and 5 interacts only with the opposite monomer (Fig. 4.3). This *trans* interaction of ICD2 is the trademark of the ABC exporters. Yet, the coupling helix 2 is rather similar to the coupling helix of the importers since it docks into a groove at the interface between the RecA-like and α-helical subdomains of the NBDs (Fig. 4.3). In this

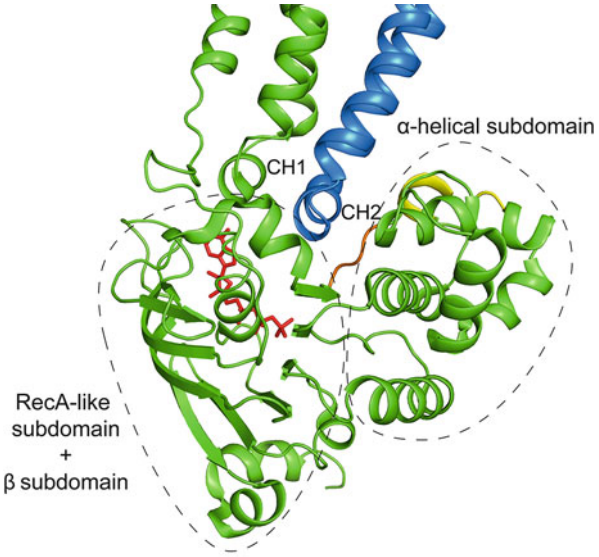

Fig. 4.3 Close view of the interface NBD/TMD in Sav1866 (PDB code 2ONJ). One monomer is shown in *green*, while transmembrane helices 4–5 and coupling helix 2 (CH2) from the other monomer are colored in *blue*. The Q-loop is colored in *orange*, while the x-loop is shown in *yellow*. AMP-PNP is visible in *red* sticks

closed outward-facing conformation, the interaction of the two NBDs is similar to the transient head-to-tail conformation of isolated NBDs.

MsbA is a lipid A flippase [79], but it also has the ability to transport some drugs [80, 81]. Depending on its origin and the crystallization conditions, three different conformations were obtained for this exporter: one which is very similar to the Sav1866 structure (closed AMP-PNP bound or ADP-Vi state, MsbA of *Salmonella enterica* serovar Typhimurium), one where the two NBDs are close to each other but not yet engaged in a tight interaction (closed apo state, MsbA of *Vibrio cholerae*), and finally one with the two NBDs widely separated in the so-called open state with an inward-facing conformation (MsbA of *E. coli* [EcMsbA]) [75]. In all the conformations, the transmembrane helices 4–5 and the associated coupling helix 2 cross over the homodimer interface and contact the opposite subunit. However, intracellular loop 1 loses contact with the opposite subunit in the open apo state. The open conformation has been subjected to controversy, but similar conformations were obtained for the mouse or *Caenorhabditis elegans* P-glycoproteins [82, 83], yet not as widely open as in the structure of EcMsbA (i.e., the NBDs are separated by ~ 25–30 Å in the P-glycoprotein *vs.* ~ 50 Å in EcMsbA). Interestingly, a structure of a flippase was recently solved in three different conformations: two open with various separations of the NBDs (44 Å and 30 Å) and one closed in an ADP-bound outward-occluded conformation [84]. Again, the two open inward-facing structures were suspected to be biased by the presence of detergent or crystal lattice contacts. However, the 3D structure of BmrA obtained in a lipidic environment was consistent with the open structures of P-glycoproteins [85], and this suggests that the presence of detergent in the X-ray crystallography experiments was not forcing the structure of the transporters in abnormal conformations.

The structure of an antimicrobial peptide exporter, McjD, offers presumably the first view of an intermediate conformation of the catalytic cycle of exporters, in an outward-occluded state [86]. While the two NBDs of McjD are still engaged in an ATP-bound conformation similar to that found in Sav1866, the TMD moiety shows a different organization of the transmembrane helices. This creates an internal cavity not accessible to either side of the membrane and that could possibly accommodate the transported molecule, i.e., the microcin J25.

The first structure of a heterodimer was obtained for TM287/TM288 [41]. It showed an inward-facing conformation at the membrane level, but the NBDs were only partially disengaged with significant contact being maintained at the degenerate ATP-binding site where an AMP-PNP molecule was still bound. However, even in the apo form of TM287/TM288, its two NBDs keep the same interaction/orientation at the degenerate site [87]. Hence, the authors raised the possibility that AMP-PNP is a poor ATP analogue for heterodimeric ABC transporters. Of note, AMP-PNP and AMP-PCP also failed to generate a closed NBD conformation in the homodimer of ABCB10 [88].

In addition to the crystal structures, the structure of the heterodimer TmrAB in a nucleotide-free state was obtained at a subnanometric scale by cryo-EM. It revealed an inward-facing conformation, yet a contact was maintained between the two NBDs at the level of the two *C*t-helices, one in each NBD [89]. Therefore, it is possible that a full physical separation between the two NBDs, in the nucleotide-free state, is a prerogative of homodimers or full-length transporters bearing two consensual ATP-binding sites.

4.5 An Alternating Access Mechanism

An alternating access mechanism seems the prevailing process in ABC transporters. It involves switching between two opposite conformations in which the substrate-binding site is alternatively accessible to one side of the membrane [75, 90–92]. Substrate binding on the inner or outer membrane leaflet and release on the opposite side are coordinated by the catalytic events, i.e., ATP binding, hydrolysis, and product release. Several studies on drug transporters suggest a lower affinity for drugs in the outward-facing conformation thereby explaining their release outside the cell [7, 93, 94]. The different conformational structures of MsbA lead to a transition model [75] in which pivoting of transmembrane helices 4–5 around the extracellular loops 2 and 3 brings the NBDs near each other; in this configuration, the NBDs are not properly aligned since the two Walker A motifs are facing each other, and a sliding movement of the NBDs along the interface would be required to align each Walker A motif with each LSGGQ motif, thereby pulling transmembrane helices 3–6 away from transmembrane helices 1–2. The newly formed outward opening is created between transmembrane helices 1 and 3, whereas the inward opening was formed between transmembrane helices 4 and 6. Recent molecular dynamic studies suggest twisting of the NBDs during the catalytic cycle of MsbA [95] and P-glycoprotein

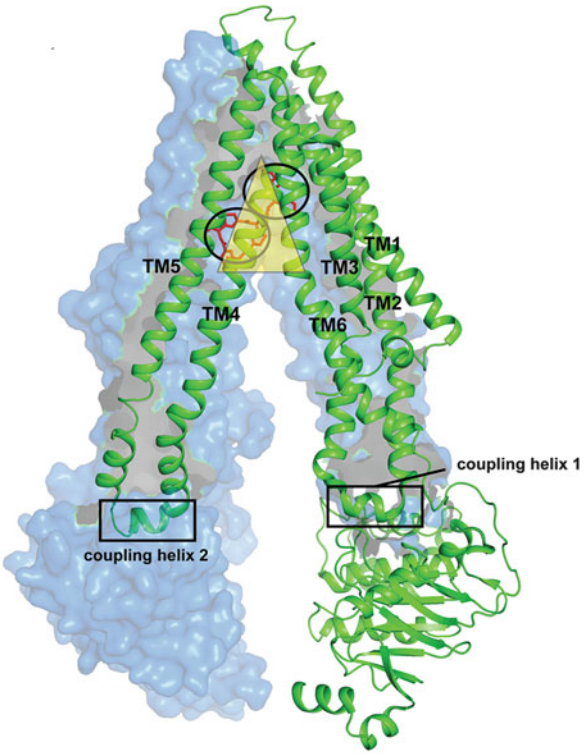

Fig. 4.4 Structure of P-glycoprotein in complex with two QZ59-SSS molecules (PDB 4M2T). The N-terminal half of P-glycoprotein is shown in *green* cartoon representation, and the C-terminal half is shown in *transparent blue* surface (*TM* transmembrane helix). The two drugs are colored in *red* and *circled* by *black spheres*. The putative drug-binding cavity is indicated with the *yellow triangle*

[96]. Furthermore, a misalignment of the NBDs was also observed in the crystal structures of ABCB10 [88], a putative transporter of heme precursors [97, 98]. In contrast, the NBDs in the heterodimer TM287/TM288 are partially interacting and are correctly aligned for canonical dimer formation [87]. Further experimental validation will be required to determine whether these conformational differences are physiologically relevant and truly reflect mechanistic differences between transporters. A variation of the classical alternating access mechanism has been recently proposed for the lipid-linked oligosaccharide flippase PglK of *Campylobacter jejuni* [84]. In this model, although the transporter can adopt inward- and outward-facing conformations, the substrate directly binds the outward-facing state and is flipped upon ATP hydrolysis.

4.6 Drug-Binding Sites

The most remarkable feature of MDR pumps is their ability to transport a wide variety of structurally dissimilar drugs. X-ray structures of murine P-glycoprotein revealed a large internal cavity open to both the cytoplasm and the membrane inner

leaflet, with a wide separation between the two NBDs [74, 82]. This configuration generates the presence of two portals at the level of the inner membrane leaflet. The first is located between transmembrane helices 4 and 6 on one side and the second between transmembrane helices 10 and 12 on the other side (Fig. 4.4). Of note, an *N*-terminal helical hairpin occludes one of these portals in the crystal structure of the *C. elegans* P-glycoprotein, but the overall shape of the two proteins is otherwise similar [83]. Drugs could reach access to the transport pathway from the aqueous phase [99–101] or through these portals within the membrane. Because many drug substrates partition and concentrate in the membranes [102], it is likely that drugs usually enter the transporter through the membrane inner leaflet. Consistent with this, Jin et al. observed a 100- to 4,000-fold increase in drug apparent affinity when studying the drug-stimulated ATPase activity of P-glycoprotein in membranes as compared to detergent [83].

The identification of the drug-binding site(s) in P-glycoprotein has been the goal of many studies (see a review in [103]). It was early recognized that a drug-binding site was localized within the TMDs [104]. Binding and kinetic analysis suggested the presence of several drug-binding sites [105–107]. Based on kinetic studies, Shapiro and collaborators proposed the existence of three drug-binding sites in P-glycoprotein, named H (Hoechst), R (rhodamine), and P (prazosin and progesterone) sites [108, 109]. Both the H and R sites are competent for transport, while the P site is an allosteric site. The R site preferentially binds rhodamine 123 and anthracyclines; the H site preferentially binds Hoechst 33342, quercetin, and colchicine; the P site binds preferentially prazosin and progesterone. The existence of two different H and R sites in P-glycoprotein was also evidenced by Förster resonance energy transfer (FRET) studies from Sharom's laboratory [110, 111]. A positive cooperative effect between the R and H sites was observed: the addition of a small concentration of a drug that binds to one site stimulates the transport of the substrate bound to the other site. Such reciprocal drug transport stimulation was also later observed with LmrA [7] and BmrA [11]. Shapiro and Ling also reported that other drugs such as vinblastine, etoposide, and actinomycin D compete with both H and R sites. Cysteine-scanning mutagenesis and thiol-reactive drugs such as dibromobimane, methanethiosulfonate-rhodamine, and methanethiosulfonate-verapamil were extensively employed to localize drug-binding sites in P-glycoprotein [112]. A common drug-binding pocket was found at the interface between the TMDs that can accommodate at least two drugs [113].

Murine P-glycoprotein was co-crystallized with two stereoisomers of cyclic hexapeptide inhibitors, cyclic-tris-(R)-valineselenazole (QZ59-RRR) and cyclic-tris-(S)-valineselenazole (QZ59-SSS). Either one molecule of QZ59-RRR or two molecules of QZ59-SSS were found in the central cavity of P-glycoprotein (Fig. 4.4) [74, 82].

The drug-binding cavity contains nine aromatic residues that are identical in human and murine P-glycoprotein but not conserved in *C. elegans* P-glycoprotein. There are no charged residues in the drug-binding pocket of mammalian P-glycoprotein structures, in contrast to *C. elegans* P-glycoprotein and MsbA, which has 16 charged residues pointing directly toward the substrate translocation

pathway [74]. Knowing the position of the two cyclic peptides QZ59-RRR and QZ59-SSS in the central cavity of the mouse P-glycoprotein, Martinez et al. sought to localize the H and R sites by assessing whether these peptides compete with the transport of Hoechst 33342 and daunorubicin and by performing molecular docking simulations [114]. They proposed the location of the H site along the central cavity and the QZ59-SSS molecule closer to the center of the membrane, with the R site at a deeper position in the cavity, overlapping the location of the QZ59-SSS molecule most embedded in the structure (see also [103]). Another group proposed similar locations for H and R sites, and the potent inhibitors tariquidar and elacridar bind to P-glycoprotein sites that coincide or overlap with these sites [115]. Importantly, the suggested R site is consistent with the cross-linking studies with methanethiosulfonate-rhodamine [112]. However, daunorubicin-binding site in MsbA was mapped at a different location, closer to the inner leaflet of the membrane [116]. Since the physiological substrate of MsbA is lipid A, it is conceivable that drugs opportunistically accommodate to the binding pocket of the transporter, possibly in a different location from typical multidrug transporters [117].

4.7 Basal ATPase Activity in Multidrug Transporters

Multidrug transporters typically display a high basal ATPase activity, which for bacterial transporters is often moderately stimulated by drugs [11, 30, 118, 119]. This behavior contrasts for instance with the well-coupled peptide exporter complex TAP [120] or some ABC importers [121–123]. Nevertheless, several lines of evidence suggest that drugs binding facilitate the dimerization of the NBDs thereby stimulating ATP hydrolysis [124, 125]. These observations are reminiscent of the mechanism of ATPase stimulation in other ABC transporters by allocrites [126], partner proteins [127], or proteins delivering the solute to importers [128–130].

Several plausible explanations could account for this seemingly “futile” ATPase activity in drug transporters. First, it might be due to nonoptimal conditions of purification or reconstitution procedures; given that this behavior is widespread among differently purified multidrug transporters, this seems unlikely. Second, it might result from the transport of lipids that could stimulate the ATP hydrolysis of the transporters [42, 80, 131, 132]. Third, it might be an intrinsic property of drug transporters, as suggested by a thermodynamic analysis of P-glycoprotein activity [133]. Ernst, Schmitt, and collaborators have proposed an elegant hypothesis: the kinetic substrate selection model [134, 135]. The basal ATPase activity may have the advantage of maximizing the number of transporters competent for substrate binding in inward-facing conformations. This model proposes that the time spent in the inward- or outward-facing states affects substrate selection and explains how two substrates with identical affinities, but dissimilar k_{on} and k_{off}, can be transported with different efficiencies. MDR transporters have the unique ability to recognize a huge variety of structurally dissimilar substrates. If the ATPase activities of these transporters were tightly coupled to the drug extrusion process, then only the substrates

capable of stimulating the ATPase activity would be transported. Possibly, a tight coupling would only be achieved at the expense of substrate diversity. Being capable to switch rapidly between the two opposite conformations, inward facing (i.e., in a conformation allowing to capture a noxious compound if present) and outward facing in an ATPase active conformation, even in the absence of a drug, might be the price to pay to make sure that any bound drug will be rapidly expelled out of the cell before being released from the transporter in an on and off process. Given the apparently relatively low affinity for many drugs, a fast rate of ATPase activity (coupled or not with the transport process) might overcome the rate constant of the drug release (k_{off}). Thus, wasting some energy in the absence of a drug might ensure the polyspecificity for many unwanted molecules and their efficient efflux once captured by the transporter.

4.8 Transport Mechanisms and Structural Flexibility of Multidrug Transporters

During the catalytic cycle, the two NBDs of ABC transporters engage and disengage with each other [129, 136]. Because the two ATP-binding sites are localized at the interface of the two monomers, ATP binding promotes the formation of a closed conformation [137, 138]. Although the interface of dimerization was a matter of debate for some time [139], the head-to-tail orientation first envisioned by Jones and George [140] was observed in the crystal structure of the ABC protein Rad50 involved in DNA double-strand break repair [62]. Later, this arrangement was validated with the crystal structure of an NBD dimer stabilized by the mutation of the catalytic glutamate [65] and the photocleavage of both the Walker A and LSGGQ motifs by the transition state analogue orthovanadate in the maltose transporter [141]. The latter observation also indicated that ATP hydrolysis occurs only in the closed conformation. Consistent with this, mutations in the LSGGQ motif strongly alter the ATPase activity of ABC transporters [142, 143]. The release of Pi and/or ADP destabilizes the dimer such that the NBDs move apart from each other. In addition to the interdomain movement, the RecA-like and α-helical subdomains within each NBD rotate toward each other upon ATP binding and move outward in the post-hydrolysis stage [52, 139, 144]. Hence, the energy of ATP binding and hydrolysis is coupled to conformational changes in the TMD thereby mediating alternating access of the substrate-binding site to each side of the membrane.

Several inward-facing conformations of P-glycoprotein exhibiting different degrees of domain separation were crystallized [74, 83, 145] hence suggesting a highly flexible protein. The distance between the α-carbons of the Walker A cysteines in the mouse or *C. elegans* P-glycoprotein varied between 38 and 53 Å [103]. These observations are consistent with the flexibility reported in the apo states of LmrA [146] and BmrA [147] in detergent. This flexibility evidenced by high rates

of H/D exchange in the apo state of BmrA is presumably caused by multiple conformations of the two ICDs, thereby allowing some freedom of rotation of the NBD [147]. In line with this, Cys-Cys cross-links were obtained for BmrA between the NBD and ICD1 that suggests the existence of additional, possibly transient, conformations of BmrA in the resting state [148]. Additionally, it was possible to cross-link two Cys residues, one in each Walker A motif of the P-glycoprotein suggesting that the two NBDs adopt alternate orientations in the resting state [149]. P-Glycoprotein structures have been suspected to exhibit nonphysiological conformations due to the absence of lipid bilayer and nucleotides. However, Wen et al. recently showed that, in intact lipid bilayers and in the presence or absence of nucleotides, P-glycoprotein adopts a wider range of conformations (both longer and shorter) compared to the original mouse P-glycoprotein crystal structure [150]. The authors suggested that this flexibility might originate from a high number of Gly and Pro residues thereby causing kinking and/or unwinding within the TMDs. Such flexibility may be advantageous to accommodate substrates of various sizes and chemical properties.

The mode and extent of NBD separation in MDR transporters is, however, still under debate [151], and two main hypotheses describing the mechanism of action of ABC transporters have been proposed: the ATP switch model [152] and the constant contact model [153].

4.8.1 The ATP Switch Model

In the switch model [152], which is also referred as the processive clamp model [154], the NBDs are proposed to dimerize upon ATP binding, sequentially hydrolyze ATP, and completely separate upon release of Pi and/or ADP. The ATP-dependent dimerization generates the outward-facing state, during which the drugs are translocated from the inner to the outer membrane leaflet, while ATP hydrolysis and release of hydrolysis products reset the transporter to the inward-facing conformation. This model largely relies on the available structures of ABC transporters in nucleotide-free or nucleotide-bound conformations. Many experimental data support this model. ATP binding promotes association of isolated NBDs [155] and ATP hydrolysis induces their dissociation [156]. In the context of intact transporters, biophysical and cross-linking studies suggest large-scale movements in MsbA [136, 157–159]. Moreover, ATP binding promotes large conformational changes in LmrA [146] and BmrA [160]. The main concern regarding this model is that apo states, as studied in biochemical experiments, may not be physiologically relevant. Given the prevalence of the nucleotide in cells, it has been proposed that transporters will likely have ATP bound *in vivo* [161]. Yet, cells and microorganisms in particular have to face stressful conditions that will strongly deplete ATP concentrations (see the discussion in [85]). Moreover, even in optimal laboratory conditions and for fast-growing *E. coli* bacteria, the ATP level can vary greatly among a bacterial population that originates

from a single clone [162]. Two other points are worth considering. First, in which conformational state ADP is released from the transporter? If ADP is released in the open state, then the transporter will return to this state before ATP can bind again, regardless of its concentration in the cell. Second, it is not the ATP concentration itself that really matters but rather the k_{on} of ATP binding. If this rate is slow as compared to the rate of transition between the closed state (just after ATP hydrolysis) and the open state, then the transporter will be able to return to the open state before ATP binds again. Considering all these parameters, the apo state should not be so infrequent for multidrug transporters, in particular in bacteria. In order to test the presence of the inward-facing conformation of P-glycoprotein in mammalian cells, Loo and Clarke [163] placed reporter cysteines in extracellular loops close enough to form a disulfide bond in this conformation but widely separated in the outward-facing conformation. Spontaneous cross-linking strongly suggested the existence, at least transiently in cells, of the inward-facing conformation in which the NBDs are open.

4.8.2 The Constant Contact Model

An alternative model has been proposed by Jones and George [153], in which the NBDs remain in contact throughout the catalytic cycle. This model should not be mistaken with a constant peripheral interaction between NBDs, as, for instance, in many ABC importers. In this constant contact model, ATP hydrolysis occurs alternately at each site, with one site able to open and exchange hydrolysis products, while the other ATP-bound site remains closed. Hydrolysis of ATP promotes an opening at that site by an outward rotation of the RecA-like subdomain relative to the helical subdomain [164]. This model built on earlier P-glycoprotein work from Senior and collaborators [165]. They proposed an alternating hydrolysis of the NBDs based notably on the observation that both sites were equally active and that orthovanadate-induced ADP trapping in one catalytic site was sufficient to inhibit ATP hydrolysis in both sites [166]. The occlusion of one nucleotide during the transition state has indeed been observed in several proteins: P-glycoprotein [165], BmrA [58], LmrA [7], and maltose transporter [167]. In contrast, two nucleotides were shown trapped in the heterodimeric TmrAB transporter [18]. The asymmetry observed in structural [168], biochemical [169–171] and molecular dynamic studies [164, 172] is often interpreted in favor of the constant contact model.

Another argument cited in favor of this model is that P-glycoprotein retains an ATPase activity when the NBDs are covalently linked together [173–175]. A single molecule FRET analysis of reconstituted P-glycoprotein rather supports a model where the NBDs do not completely dissociate from one another during steady state catalysis although, given the broad distance distribution recorded in all ligand conditions, full dissociation of the NBDs cannot be entirely excluded and may occur during some of the cycles [176]. This model involves an alternating catalysis in which ATP hydrolysis and Pi release are coupled to drug transport.

Lastly, the NBDs in the crystal structures of TM287/TM288 remained in contact, but with coupling helices separated by 15 Å, which is sufficient to make the substrate-binding cavity accessible from inside without the need for NBD full disengagement [87].

4.9 Concluding Remarks

It should be noted that alternate models are rarely discussed but could be as plausible as the models discussed above. For instance, one can imagine a scenario in which the binding of two ATP molecules generate an outward-facing conformation, as in the switch model, but the hydrolysis of one ATP molecule is sufficient to destabilize the dimer thereby implying a catalytic asymmetry. Whether the two catalytic sites in homodimeric ABC exporters hydrolyze ATP simultaneously, sequentially, in an alternating or stochastic manner has not yet been settled. In the isolated NBDs, MJ0796, the hydrolysis of one molecule of ATP is sufficient to allow the physical disengagement of the two NBDs [156]. Although a stoichiometry of two ATP molecules per substrate transported has been found for the OpuA importer [177], a stoichiometry of one ATP molecule was determined for P-glycoprotein [178]. Owing to the structural and functional diversity of ABC transporters, there might not be a single unified mechanism for all members. For instance, one of two catalytic sites is poorly active in heterodimeric ABC exporters, and such transporters may employ a different catalytic cycle than the homodimeric transporters. Recently, Mchaourab and colleagues proposed that the power stroke for drug export by BmrC/BmrD is the ATP hydrolysis step, in contrast to homodimeric exporters like MsbA where the transport process will occur during the NBD dimerization driven by ATP binding [31].

The recent 3D structure of ABCG5/ABG8, the human sterol exporter exemplifies again the diversity of this family. It shows a unique structure with some traits similar to importers like the lack of cross talk afforded by a coupling helix [179].

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Chapter 5
Multidrug Efflux in the Context of Two-Membrane Cell Envelopes

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Abstract The trans-envelope drug efflux in Gram-negative bacteria demands assembly of specialized protein complexes that in addition to inner membrane transporters include periplasmic membrane fusion proteins and outer membrane channels due to the presence of a double membrane. These complexes are highly versatile and constitute a major antimicrobial resistance mechanism of Gram-negative bacteria. The modular organization of the tripartite assemblies in Gram-negative bacteria allows them to accommodate a wide array of multidrug efflux transporters enabling efflux across both the inner and the outer membranes of the cell envelope. This chapter focuses on the structures and mechanisms of trans-envelope multidrug efflux pumps from Gram-negative bacteria. We summarize the current state of the field and the emerging model for multidrug efflux in the context of two membranes.

Keywords Gram-negative bacteria • Multidrug resistance • Multidrug efflux pump • Cytoplasmic (inner) membrane • Outer membrane • Outer membrane factor • Membrane fusion protein • AcrAB • MacAB • TolC • MexAB-OprM

5.1 Introduction

Functional studies and subsequent phylogenetic analysis demonstrated that bacterial multidrug resistance (MDR) efflux transporters can be organized into several evolutionarily distinct protein families that significantly differ in bioenergetics, structure, and transport mechanism [1]. Most of these efflux transporters are found

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in three large and diverse superfamilies: ATP-binding cassette (ABC) [2], major facilitator superfamily (MFS) [3], and resistance-nodulation cell division (RND) [4]. In addition, some MDR exporters form a core of smaller families: the small multidrug resistance (SMR) family (now part of the drug/metabolite transporter [DMT] superfamily) [5] and the multidrug and toxic compound extrusion (MATE) family (recently joined the multidrug/oligosaccharidyl-lipid/polysaccharide [MOP] superfamily) [6]. More recently, a new family of transporters involved in efflux of chlorhexidine has been identified in *Acinetobacter* spp. – the so-called proteobacterial antimicrobial compound efflux (PACE) transporters [7].

The ABC multidrug efflux pumps are primary active transporters which couple substrate translocation with binding and hydrolysis of ATP [2]. Multidrug transporters in all other superfamilies are secondary active transporters which utilize electrochemical gradients of ions (most frequently protons sometimes sodium) to transport their diverse substrates. Both primary and secondary transporters are ubiquitous in bacteria; however, their relative presence seems to correlate with energy generation: fermentative bacteria tend to rely more on the primary transporters, while aerobic bacteria contain somewhat more secondary transporters in their genomes [8, 9].

At least three steps common to various transporters could be distinguished during transport across the cytoplasmic membrane: (i) binding of a substrate on the cytoplasmic or periplasmic side of the membrane, (ii) conformational changes in a transporter leading to reorientation of the binding site to the other side of the membrane, and (iii) the release of the substrate. The conformational change leading to reorientation of substrate binding sites is an energy-dependent step, which is provided by either ATP hydrolysis (ABC pumps) or by electrochemical gradients such as proton/sodium motive force [10]. The basic mechanism of energization of transporters by ATP and electrochemical gradients is well understood on examples of ABC and MFS transporters [11, 12], and recent advances in the coupling of the proton translocation and drug extrusion have greatly increased our understanding of the mechanisms of RND transporters [13].

The directionality of the transport is defined by binding affinity on either side of the membrane, and the transport reaction is thought to be reversible at least in the case of proton motive force-dependent pumps [14, 15]. However, the polyspecific RND pumps seem to be unidirectional and to transport substrates across the outer membrane [16]. At least some RND pumps, such as the metal efflux pump CusBAC from *Escherichia coli*, also transport substrates across the cytoplasmic (inner) membrane [17, 18]. Although RND transporters are often called “antiporters,” they do not actually exchange their substrates for protons [13].

Among various multidrug exporters, some transporters belonging to the RND, ABC, and MFS superfamilies utilize energy conserved in ATP or in the electrochemical potential of the cytoplasmic membrane to transport drugs across the outer membrane, which is energy deficient. This transduction of energy is possible because of the association between the transporters and two types of accessory proteins: the periplasmic adaptor proteins (PAPs) also historically known as the membrane fusion proteins (MFPs) and the outer membrane factors (OMFs) [10]. Sometimes, the tripartite pumps which utilize RND transporters also contain a

fourth component, which binds the RND transporter itself in the membrane modulating its function, YajC [19] or AcrZ [20]. These small proteins do not appear to be essential for the function of the pump, are entirely α-helical, and bind the transporter within the inner membrane [21]. This chapter focuses on the mechanism of coupling drug transport reactions separated in the cytoplasmic and outer membranes that is facilitated by these two families of proteins working in assembly with inner membrane transporters.

5.2 Diversity and Distribution of Outer Membrane Channels in Gram-Negative Bacteria

Outer membrane channels belonging to the outer membrane factor (OMF) family of proteins provide a path for drugs and other substrates to cross the outer membrane [22]. The important role of TolC, the major OMF from *E. coli*, in cell protection has been known for at least 30 years [23–25]. The early observations that *tolC* mutants are tolerant to colicin E1 and at the same time hypersusceptible to certain drugs, dyes, and detergents led to the conclusion that these mutants have alterations in the cell membrane. Later studies established that TolC and OMFs in other Gram-negative bacteria enable transport of various toxic molecules across the outer membrane [26, 27].

TolC plays a central role in facilitating the efflux of a wide range of substrates in *E. coli*, ranging from antibiotics [28, 29] to peptides such as colicin V [30] and microcin J25 [31] and as a part of the type I secretion systems powered by ABC transporters even large protein cargoes such as α-hemolysin [32]. TolC also is a target receptor for bacteriophages [33] and a number of colicins, from where it takes its name (tolerance to colicin). TolC is thus a highly promiscuous channel, and the broad range of substrates that it transports may reflect its evolutionary history [34], as in *Enterobacteriaceae*, the multidrug efflux pumps have likely evolved to allow endosymbiotic lifestyle and expulsion of detergent-like biological substrates such as bile salts [35, 36], and due to that, TolC also is a conduit for a number of detergents.

The OMFs from different Gram-negative bacteria share little local sequence similarity with each other. The characterized OMFs could be clustered into three clades corresponding to their broadly defined efflux functions: (i) the multidrug efflux, where the best characterized representative is *Pseudomonas aeruginosa* OprM; (ii) the cation efflux with *E. coli* CusC as a typical representative; and (iii) the protein export (type I secretion system) represented by *E. coli* TolC [37]. Perhaps as a result of such functional specialization, genomes of Gram-negative bacteria usually contain several OMF genes. Due to this divergence, OMFs do not present an easily identifiable sequence motif for the family. Only when combining the multiple sequence alignment analysis with hydrophobicity and structural feature predictions did the features of the family become apparent [38], allowing the identification of two tandem common motifs, suggesting an early gene duplication

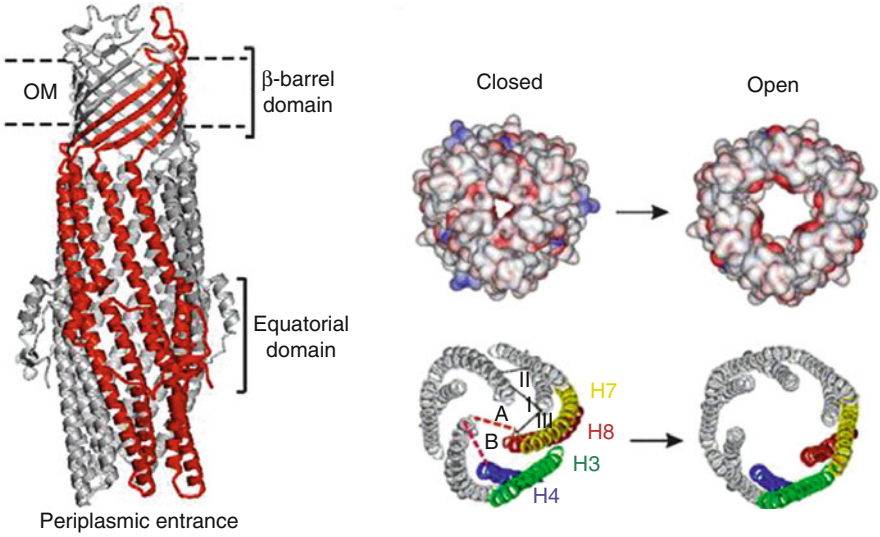

Fig. 5.1 Structure and mechanism of TolC. (**a**) Ribbon representation of TolC homotrimer (*side view*) with key domains indicated. A monomer of TolC is highlight in *red*. (**b**) The proposed mechanism of TolC transition into the open state. Space-filled (*upper*) and ribbon (*lower*) depictions of the closed and modeled open states of the tunnel entrance, viewed from the periplasm. The coiled coils H3/4 and H7/H8 of one protomer are colored and show the constraining intramonomer (I and II) and intermonomer (III) links (Reproduced from [22])

event. These motifs reflect the structural signature of OMFs – their coiled coil regions, but also uniquely suggested a presence of porin-like transmembrane β-strands (Fig. 5.1) [39].

Four OMFs were identified in *E. coli* genome: *tolC*, *mdtP* (*yjcP*), *mdtQ* (*yohG*), and *cusC* (*ylcB*) [40]. In comparison, *P. aeruginosa* genome contains 18 OMFs, which are further subdivided based on phylogenetic analyses into two subgroups, the OprM subfamily comprising 11 highly homologous channels involved in multidrug efflux and the more divergent AprF protein export subfamily, which also includes OpmH and OpmM [41].

E. coli TolC functions with the majority of MFP-dependent transporters encoded in the genome of *E. coli*; an exception is a cation transporter CusBA, which functions with CusC [29]. Similar requirements for TolC were described in *Salmonella*, *Klebsiella*, *Vibrio*, and other species [36, 42–48]. The multifunctionality is also reflected by the fact that the *tolC* gene in these organisms is transcribed independently from the inner membrane counterparts, and its genetic context is conserved only in the closely related species (i.e., all *Enterobacteriaceae*).

On the other side of the OMF spectrum are situated specialized cation efflux and type I secretion OMFs that function exclusively with specific transporters. These OMFs are usually expressed in gene clusters along with the inner membrane components of the complex. However, even these specialized OMFs demonstrate certain promiscuity. For example, *Serratia marcescens* contains several type I secretion

systems, which export proteins such as the lipase LipA, the metalloprotease PrtA, and the heme-binding protein HasA [49–51]. Studies of hybrid transporters demonstrated that OMFs do not possess any substrate specificity, and their involvement in transport reactions is determined by their ability, or the lack of such, to bind an inner membrane complex. In general, the substrate specificity of the complex appears to be determined by the inner membrane component [52]. In enteroaggregative *E. coli*, the AatA OMF is a part of a specialized type I secretion system transporting the dispersin protein Aap across the membrane, which is plasmid encoded and transferrable [53].

5.3 Structure and Assembly of Outer Membrane Channels

The crystal structure of TolC reported by Koronakis et al. [39] in 2000 was followed by high-resolution structures of *E. coli* CusC [54]; *P. aeruginosa* OprM [55, 56], OprJ, and OprN [57]; *Vibrio cholerae* VceC [58]; *Campylobacter jejuni* CmeC [59]; and *Neisseria gonorrhoeae* MtrE [60]. Despite very low sequence identity between these proteins, their structures are very similar and unique among outer membrane proteins [22]. Extending from the extracellular space well into the periplasm, TolC is a 140-Å long, trimeric assembly resembling a cannon shape, with one end forming a 12-stranded, antiparallel β-barrel embedded in the membrane, while an extended, α- barrel domain and a mixed α/β equatorial domain (Fig. 5.1) reside in the periplasmic space [39]. Each protomer reveals an internal duplication, resulting in pseudo-6-fold symmetry of the assembled trimer. The β-barrel of TolC is unique among the outer membrane proteins, as despite the typical porin fold [61], the barrel is not encoded by a single polypeptide but each protomer contributes 4 β-strands to build a 12-stranded barrel. Such barrel architecture is only shared by some trimeric autotansporter proteins. The α-domain is fully unique among the outer membrane proteins and made up similarly of 12 helices with four being contributed by each protomer in the form of two sets of double helical hairpins. Those are organized in a peculiar fashion, with each hairpin pair formed of one continuous helix spanning the full length of the periplasmic domain, while the other helix is interrupted and formed of two shorter helices that stack up end to end to match the length of the first one. Spliced into this interruption is a small alpha-beta extension, which along with a part of the C-terminal tail of the protein forms the so-called "equatorial domain." The helical hairpins of the alpha-barrel domain curve slightly toward the periplasmic end of the channel forming a tight constriction that is prohibitive to transport of even small hydrated ions. This dense packing of the three twin sets of coiled coils is stabilized by a number of ionic bridges, forming the so-called primary "gates" of the channel [62, 63], that have been proposed to keep the periplasmic opening of TolC in its "closed" conformation (Fig. 5.1). Those gates however are not the only restrictions in the OMF channels, and a set of double aspartate rings, which has become known as "secondary gate," is a prominent feature of the TolC subfamily, accounting for the cation selectivity of the channel and making it prone to inhibition by heavy metals and hexaminecobalt compounds. Both sets of gates need to be open for a

productive transport to happen [63–65]. It is notable that the secondary gates can be varied in different types of OMFs and that has implications for the substrate specificity, and in the case of VceC, the channel is locked by a hydrophobic constriction formed of a ring of leucine residues [58]. Still, the cavity of TolC is one of the largest known among protein structures, at 43,000 $Å^3$, and it is capable of allowing passage of particles as large as 160 kDa [66]. Recently solved crystal structure of CmeC shows that it forms an extra gate at the outer membrane surface in the β-barrel domain in addition to the gate at the periplasmic side similar to TolC [59]. It is suggested that both these gates must be opened sequentially to allow drug passage.

A feature of some OMFs, such as OprM, CusC, MtrE, and VceC, is the presence of an N-terminal lipid modification via a conserved cysteine moiety. It was shown that this N-terminal lipid anchor is crucial for the insertion and assembly of CusC [67]. Why an N-terminal lipid modification is required for some but not other OMFs remains unclear. Perhaps, the lipid modification could act as an anchor and aid in the insertion of the β-barrel into the outer membrane [55] or even play a functional role by inducing the channel transition to an open state during drug efflux [67, 68]. On the other hand, the absence of the lipid anchor in TolC and OpmH suggests that a similar structure and function could be possibly achieved through a different mechanism. In addition, the flexible C-termini of OMFs have also been shown to be important for their stability and functionality [69, 70]. More detailed analyses indicated that removal of 20 or more amino acids or disruption of a short motif $_{463}$LeuGlyGlyGly$_{466}$ could affect the stability and functionality of OprM negatively [71]. In the OMF AatA which is a part of a dedicated type I secretion system, the C-terminus was also shown to be essential for cargo transport [53].

5.4 Transport Mechanisms of OMFs

The structures of OMFs suggested a possible mechanism of transport. The large periplasmic extension of outer membrane channel is thought to be a docking site for MFPs and transporters. Since in crystals the periplasmic entrance of TolC and several other OMFs is tightly closed so that even ions cannot easily diffuse through, the association with the inner membrane complex is thought to trigger opening of TolC channel [63, 72, 73]. The proposed allosteric opening mechanism envisages that the inner coiled coil α-helices (H7/H8) undergo an iris-like movement to realign with the outer coils (H3/H4), thereby enlarging the pore in a “twist-to-open” transition (Fig. 5.1). This hypothesis is supported by studies of TolC mutants in which the network of constraining polar interactions have been disrupted [63, 72, 73]. The inter-protomer bonds, including the Asp153-Arg367 salt bridge, tether the inner coiled coil H7/H8 to the outer H3/H4 coiled coils. Substitutions in these residues increase the conductance of TolC channel from the “closed” 80 pS to partially open 205-370 pS (Arg367Ser mutant) and maximal conductance of 800-1,000 pS in the double Arg367Ser Tyr362Phe TolC mutant. The sequential open states were also found in crystal structures of these TolC mutants providing further evidence that destabilization of ionic bonds in the periplasmic tip could dilate the TolC

pore and allow passage of substrates through the channel [63, 73]. This idea was tested *in vivo* by the fluorescein-5-maleimide labeling studies. This labeling of cysteine residues positioned inside of TolC channel, its two constriction bottlenecks, and the periplasmic tip showed that the structural differences between the closed and the open conformers of TolC persist *in vivo* [74]. However, no transitions between the two forms could be detected, presumably because of the highly dynamic nature of TolC interactions with the inner membrane complexes. Opening of the OMF channel can also be detected via induction of sensitivity to vancomycin, a drug that is influx dependent and under normal conditions not permeable, but certain mutants become "leaky" allowing vancomycin in by dilating the OMF channel [75]. Some mutants however were found not to be leaky when OMF channel is expressed by itself, but only become so upon interaction with the MFP partner demonstrating the dependency of OMF gate opening on the MFP [76].

5.5 Membrane Fusion Proteins: Linking the Outer and Inner Membranes

MFPs play important roles in trans-envelope drug efflux: (i) by stimulating the efflux activities of transporters and possibly participating in the presentation of the cargo to the transporters and (ii) a crucial structural role by engaging OMFs and opening them for substrates to be expelled from the cell [10, 77, 78]. *In vitro* reconstitution studies showed that the stimulating activities of an MFP are a common feature of transporters belonging to various protein families. *E. coli* AcrA that functions with the RND pump AcrB and MacA, a subunit of the ABC-type MacB transporter, both stimulate activities of their cognate transporters located in the inner membrane [79–81]. On the other hand, MFPs mediate and stabilize the functional interactions between transporters and OMFs and, in doing so, could possibly transmit energy from the energized transporter to the closed OMF in order to open the channel and allow the diffusion of substrates through the OMF into the extracellular medium. Under some models of assembly, this transfer of energy is thought to be mediated by conformational energy and movement of the MFPs. Conversely, it is also possible that spontaneous interactions between OMFs and MFPs mediate the opening of the channel, but upon conformational changes induced by the transporters, interactions between MFPs and OMFs are destabilized, thus causing the OMF to return to its resting closed state and disassembly of the complex [21, 76, 82].

5.5.1 Structure of MFPs

MFPs are highly modular proteins which are composed of several well-defined domains; however, not all of them are universal among the family [78]. While amino acid conservation among MFPs is relatively low, their structures are

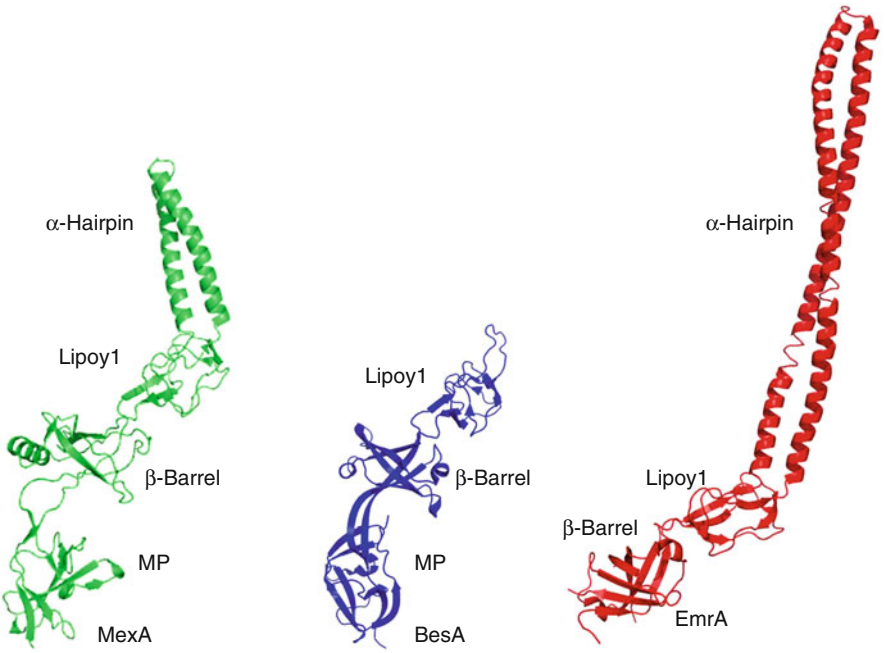

Fig. 5.2 Structures of divergent MFPs. Crystal structures of MexA, BesA, and EmrA are shown to show structural variation of MFPs (PDB codes 2V4D, 4KKS, and 4TKO, respectively). The typical MFP is exemplified by the structure of MexA, which contains all four of the typical domains. BesA is the MFP of the BesABC RND efflux complex of *B. burgdorferi*. The OMF, BesC, does not contain both of the ionic gates which keep the periplasmic aperture in a closed position. Due to this lack of additional gating, it is thought that BesA no longer needs the α-helices because of the weakened gating of the aperture. Conversely, EmrA interacts with the MFS transporter EmrB. EmrB contains little to no periplasmic domain for EmrA to interact with. It is thought that EmrA interacts with EmrB primarily through the N-terminal transmembrane segment which anchors EmrA to the membrane and not through the membrane proximal (MP) domain. Due to the loss of the MP domain, EmrA the α-helical domain has been extended. This extended α-helical domain allows for EmrAB to interact properly with TolC so as to allow efflux of substrates into the extracellular space (Reproduced from Zgurskaya et al. [10])

conserved. Typical MFPs are highly elongated proteins, which are usually anchored in the inner membrane and extend deep into the periplasm to meet the OMFs [83, 84]. Starting at the inner membrane, the characteristic domains are the membrane proximal (MP), β-barrel, lipoyl, and α-helical hairpin domains (Fig. 5.2), with additional specialized domains also present in some members of the family, e.g., involved in metallochaperoning (siderophore) function, transmembrane domains. A peculiar feature of the MFP family is that while these protein domains appear as "beads on a string," this apparent linear structure is in fact contributed by a polypeptide chain folded into itself in a hairpin fashion, with both N- and C-terminal parts of the protein contributing to each of the characteristic domains.

The α-hairpin domain, unlike the other three classical MFP domains, consists of two antiparallel helices forming a coiled coil separated by a single turn. The α-hairpin domain has a pronounced similarity to the coiled coils in the alpha-barrel of the OMFs and hence has been speculated to form an extended helical bundle upon interaction with the OMF [85] in some of the models of assembly (See Sect. 9 below). In isolation, the α-helical hairpins have been shown to oligomerize and assemble into a funnel-like structure with comparable diameter to that of the OMF barrel, and that have been hypothesized to interact with an OMF in a tip-to-tip fashion. The α-hairpin domain is present in all MFPs studied to date apart from the BesA (of *Borrelia burgdorferi* BesABC complex), where it is lacking entirely [86], while in some other MFPs such as CusB, it is observed to fold upon itself [87].

Unlike the α-hairpin domain the MP, β-barrel and the lipoyl domains are mostly β-stranded structures. The lipoyl domain which is located right next to the α-hairpin domain consists of β-sandwich of two interlocking motifs of four β-strands and is sequentially and structurally homologous to biotinyl/lipoyl carrier domains in dehydrogenase enzymes [38]. However, in the MFPs, it lacks the signature "lysine loop" of the classical biotinyl domains, as the hairpin domain is spliced into the structure in that position. The role of the lipoyl domains is not clearly established; however, they appear to play an important role in stabilization of the complex, via interaction with transporter and possibly self-association. Indeed disruption of the lipoyl domains results in loss of hexamerization of the MFP DevB of the glycolipid ABC efflux pump DevBCA [88].

The β-barrel domain is as its name suggests consisting of six antiparallel β-strands forming a barrel capped by a single α-helix and presents a ribokinase nucleotide-binding domain fold [89]. In the electron microscopy (EM) reconstructions of the complete pump [21], as well as in the crystal structures of CusBA complex [17, 87], these domains seem to form a head-to-tail ring assembly, which is remarkably similar to the one observed in the flagellar assembly proteins FlgT and F1-type ATPases β – α domain assemblies with which they also share similar topology [78].

The last of the canonic MFP domains, which however is not universally present, is the MP domain. It has a much more flexible nature, being disordered in many of the structures of the MFPs available, and although its evolutionary connections are not quite clear, some topological connection can be established to the β-barrel domain [85].

MFPs working in conjunction with metal efflux pumps may also present additional N- and C-terminal domains. C-terminal domain of SilB MFP from *Cupriavidus metallidurans* CH34 has been shown to play the role of a metallochaperone [90]. These domains present a unique metal-binding β-barrel fold which is also found in related stand-alone proteins, such as the *E. coli* CusF [91]. The N-terminal domains have an intrinsically disordered structure, which has been difficult to capture crystallographically and become ordered upon interaction with metal ions and other metallochaperone domains [92].

The domains are linked by flexible unstructured regions which give the overall protein its conformational flexibility and allow for a high degree of movement believed to be important for functional interactions with transporters and OMFs.

While the α-hairpin interacts with the OMF, the MP, β-barrel, and possibly the lipoyl domain are thought to be responsible for interactions with the cognate transporter. In addition, some MFPs, such as MacA, contain N-terminal transmembrane domains that interact with transporters within the cytoplasmic membrane. However, in many other MFPs, this transmembrane domain is replaced by a lipid modification (e.g., triacylation or palmitoylation) that anchors the protein into the membrane. This lipidation takes place at an N-terminal cysteine; the polypeptide chain is then cleaved by signal peptidase 2.

As the different families of transporters vary in sizes of domains exposed to the periplasm, MFPs and their domains which interact with specific transporters also vary. There is a clear pattern of domain composition between the transporters and associated MFPs, and the MP domains occur exclusively in MFPs that work in combination with transporters possessing large periplasmic domains (e.g., RND and MacB-type ABC transporters) that pick up their cargo from the periplasmic side of the membrane, which is consistent with their roles in cargo binding and presentation [78]. Correspondingly, the MFPs which work with transporters that pick up their cargo on the cytoplasmic side of the inner membrane (e.g., MFS and type I secretion system ABC transporters such as HlyB) not only do not require MP domains but need to be able to provide a tight seal extending all the way to the outer membrane. This is achieved by rigidifying of the coiled coils of the hairpin domains by helical-extension domains [78], which form extended tubular structures reaching to the OMF such as in the case of EmrA and HlyD [93, 94]. These "extension subdomains" display only limited homology to the remainder of the hairpins. On the other side of the MFP, diversity spectrum is BesA, which lacks an α-hairpin domain all together [86]. The exact reasons for the lack of the α-hairpin remains unclear, but it could be due to the fact that OMF, BesC, lacks the aspartate ring forming the constriction in the distal end of the channel suggesting that BesA (or transporter) binding to the periplasmic tip of BesC could be sufficient to trigger opening of the channel.

5.5.2 Interactions Between MFPs and Other Complex Components

Oligomerization MFPs oligomerize independently of interactions with their cognate inner membrane proteins and OMFs. The first evidence of MFP oligomerization came from *in vivo* cross-linking studies of AcrA and HlyD [32, 95]. More recently, structural studies showed that in crystals MFPs such as *P. aeruginosa* MexA and *E. coli* MacA and AcrA, all have repeating dimer pairs [84, 96, 97]. AcrA is a dimer of dimers, MacA forms a hexameric funnel-like structure, whereas MexA has sets of dimers aligned with one another creating a sheet, which wraps upon itself in an asymmetric fashion. These structures suggested that the MFPs could function as dimers, but the strongest evidence for a dimer as a functional unit of MFPs was collected by biochemical and genetic studies [98–101].

For MFPs which interact with RND transporters (e.g., MexA and AcrA), the MFP oligomerization was treated more as an artifact of crystallization, partly due to the fact that hydrodynamic studies and size exclusion chromatography studies showed only monomers in solution [83, 99]. However, kinetic studies established that MFPs that function with transporters belonging to different protein families all oligomerize, albeit with different affinities [99]. EmrA and MacA, MFPs functioning with MFS and ABC transporters, respectively, form more stable oligomers and with a higher affinity than AcrA. This result suggested that interactions with the periplasmic domains of AcrB stabilize the oligomeric state of AcrA. In contrast, MFPs that function with transporters lacking large periplasmic domains have a strong propensity to self-oligomerization. These studies suggested that oligomerization of MFPs is needed to seal the gap between their cognate transporters and TolC.

Further *in vivo* (construction of genetic fusions [101, 102]) and *in vitro* (binding affinities of stabilized dimers [98]) studies showed that the functional unit of MFPs is a dimer, which assembles into a trimer of dimers when in the complex. Perhaps the most natural evidence of MFP dimers, as functional units, is the *P. aeruginosa* triclosan-specific TriABC-OpmH complex. Mima et al. [100] identified this complex through a screen for increasing triclosan resistance and discovered that TriA and TriB are unique MFPs which are both required for functionality of the complex. Further studies of the TriABC-OpmH complex revealed that a fused dimer of TriAB was functional and that unfused TriA and TriB co-purify together indicating their strong interaction with one another independent of interactions with the transporter or OMF [102].

Interactions between MFPs and transporters Physical interactions between MFPs and transporters remain a subject of intense investigation using such approaches as crystallography, mutagenesis, surface plasmon resonance, isothermal calorimetry, chemical cross-linking, co-purification, and chimeric protein studies [17, 98, 101, 103–105]. Symmons et al. [85] proposed the first model of AcrAB-TolC complex based on crystal structures of individual components, *in vivo* chemical cross-linking, and molecular docking analyses. In this model, the AcrA molecule occupies the AcrB surface between the PN2 and PC2 subdomains, extending up along the DN subdomain. The MP, β-barrel, and lipoyl domains of AcrA are modeled to interface with AcrB. Interestingly, the PC1 and PC2 cleft of AcrB has been proposed to be one of the main entrance tunnels which substrates use to enter into the drug-binding pocket [106]. This further suggested that MFPs could stimulate activities of their cognate transporters by participating in drug binding.

In the co-crystal structure of the metal efflux pump CusBA, the two CusB protomers are positioned substantially higher on the surface of the CusA transporter than in the cross-linking-based model of AcrAB described above [17, 85]. Here, the MP and β-barrel domains of CusB interface primarily with the DN and DC docking subdomains of CusA and only make limited contacts with the top portions of the PN2, PC1, and PC2 subdomains (see Fig. 1.4 for these specific subdomains in the topology of AcrB monomer). The authors were able to define two different types of MFP-transporter interactions. The first CusB molecule primarily engages CusA

through an extensive network of salt bridges. Conversely, the second CusB molecule interacts with CusA primarily through a network of hydrogen bonding. In addition, the two CusB molecules are staggered: one of the two molecules sits slightly higher on the surface of CusA. In this arrangement, the MP domain of CusB molecule 1 fits into a pocket formed between the MP and β-barrel domains of molecule 2. When compared with the cross-linking-based model of AcrAB, CusB molecule 1 is positioned similar to AcrA with the exception that the lipoyl domain of CusB does not interface with the transporter and its MP occupies the position of β-barrel domain of AcrA. This upward shift also tilts the MFPs and gives them a different overall structure; CusB has a sickle shape as observed in crystal structures of other MFPs including MacA, AcrA, and MexA. The recently reported cryo-EM-based structures of the complete AcrAB-TolC complex show similar positioning of AcrA on AcrB transporter [21, 94]. However, only lipoyl domains of AcrA contribute to AcrA oligomerization, and there are no contacts between the MP domains of AcrA.

In various models of MFP-transporter complexes, at least two domains of MFP directly contact a transporter: the MP domain and the β-barrel. Functional importance of these domains in various MFPs has been supported by genetic and biochemical studies [102, 107–109]. Mutagenesis of the C-terminal domains of MacA, AcrA, and TriB identified conserved glycine residues important for the functional interactions between these MFPs and the cognate components of the complexes. A substitution of Gly353 with Cys leads to inactivation of MacA and prevents stimulation of the MacB ATPase activity, but not physical interactions with MacB or TolC [108]. An analogous alteration in AcrA, Gly363Cys, prevented functional but not physical interaction with AcrB and TolC [107]. Mutational analysis of the C-terminal domains of TriA and TriB showed that analogous mutation of $TriA_{Gly350Cys}$ or $TriB_{Gly339Cys}$ (also analogous to $AcrA_{Gly363Cys}$) gave differing results; cells expressing $TriA_{Gly350Cys}$ exhibited no functional defects, whereas cells expressing $TriB_{Gly339Cys}$ were hypersusceptible to triclosan and sodium dodecyl sulfate [102]. Limited proteolysis studies both *in vivo* and *in vitro* showed that these substitutions affect conformations of the MP domains of AcrA and MacA. Although EmrA does not have an MP domain, its C-terminal signature residues of the MFP family of proteins are incorporated into the β-barrel [77]. Chacón et al. [110] utilized X-ray absorption spectroscopy to determine the role of CusB binding of copper and silver ions. In this study, they showed that while CusB is able to bind metal ions, it does not act as the intermediate step between the metallochaperone CusF and the transporter CusA, but instead receives ions from CusF, in turn activating CusA to allow transfer of ions from CusF to CusA. After equilibrium is reached, CusF "turns off" CusB by removing the Cu^+/Ag^+ ion.

5.6 Conformational Changes and Effectors

Early hydrodynamic and electron paramagnetic resonance studies pointed to significant structural flexibility of MFPs [83, 111]. In the presence of magnesium ions, the highly asymmetric shape of AcrA was found to be more compact, whereas

mildly acidic pH restrained significantly conformational dynamics of this protein. The X-ray structure of AcrA suggested that these conformational changes could involve the α-hairpin, which undergoes an ~15° rotation between the two most dissimilar molecules [84]. In addition, the refined full structure of MexA showed that the MP domain is also mobile and that in one of the MexA protomers, this domain is twisted by 85° clockwise relative to the β-barrel domain [85]. Additionally, when the lipoyl domains of MexA were superimposed, the α-hairpins exhibited ~35° of rotation, while the β-barrel and MP domains underwent an additional ~25° of rotation. Molecular dynamics simulations of MexA and AcrA supported the high interdomain flexibility of MFPs [112, 113]. *In vivo* proteolysis studies, however, pointed onto the MP domains of AcrA and MacA that undergo significant conformational changes and that these changes are detected only when all three components of complexes are present and functional [107, 108, 114].

The protonation/deprotonation of His285 was proposed to underlie the pH-regulated conformational dynamics of AcrA by disturbing the local hydrogen bond interactions [113]. Interestingly, in a heavy metal MFP ZneB, the same His residue is a part of the metal-binding site [115]. In MacA, the patch of positively charged residues located at the interface between β-barrel and the MP domains are important for both binding to core lipopolysaccharide and functionality of the pump, suggesting that lipopolysaccharide could be a substrate of MacAB-TolC [116]. Extended X-ray fine structure (EXAFS) spectroscopy combined with mutational analysis of conserved methionine residues indicated that CusB binds Cu^+ and Ag^+ ions specifically via three residues situated at the extreme N-terminus of the protein. Unfortunately, these residues lie within the unstructured portion prior to the MP domain [117, 118]. As stated above, this metal-binding activity of CusB was shown to be important for regulating the ability of CusF to transfer its cargo to CusA, indicating that conformational changes of CusB are able to activate or open the metal-binding site of the transporter [110]. In addition, kinetic experiments showed that AcrA and other MFPs show differential binding affinities to OMF and transporters depending on pH [98, 99]. Hence, the changes of pH in the periplasm accompanying the drug efflux or binding of substrates/effectors could act as a signal to trigger the action of MFPs, which undergo reversible conformational rearrangement.

Several genetic screens with defective complexes were carried out to identify gain-of-function suppressor mutations in MFPs. Surprisingly, most of such suppressors were mapped to the β-barrel domains of MFPs, which do not contact OMFs directly and suppress the defects in complexes through the contact with transporter and long-range conformational changes in the protein. Suppressors of defects in MexB transporter mapped to the β-barrel domain of MexA were found to increase the stability of MexA binding to MexB [119]. An analogous study of an assembly defective TolC mutant also gave rise to gain-of-function suppressor mutations within the β-barrel domain of AcrA [120]. One such suppressor of the defective TolC mutant was $AcrA_{Leu222Gln}$, which is itself labile and subject to degradation if not in a functional complex with AcrB and TolC [103]. It is thought that this mutant protein is in a conformation which AcrA assumes transiently during typical complex assembly and drug efflux; however, when locked into this conformation, the

protein becomes highly sensitized to proteolytic degradation by the periplasmic protease DegP. Other AcrA β-barrel mutants suppressed a TolC mutant defective in functional interactions with AcrA and AcrB presumably by controlling the opening of TolC channel [103] or enabled functioning of a hybrid AcrA-MexB-TolC efflux complex [121].

5.7 Toward Understanding of Full Pump Architecture

As mentioned earlier (also see Chaps. 1, 2 and 4), the structures of all the representative members of the tripartite pump assemblies are currently available (with the small exception of the MacB- and HlyD-families of ABC transporters, for which only partial structures have been solved), allowing to speculate about the general stoichiometry and architecture of the complete assembly.

Early efforts in this direction have been attempted using a priori docking and modeling [55, 89, 122], providing a widely varying estimations of the complex assembly, and in particular the number of MFPs engaged in it ranging from as low as 3:3:3 to 3:12:3 (OMF/MFP/transporter). Several independent studies converged onto a model, in which MFPs bind TolC in stoichiometry 3:6:3 for RND transporters and 3:6:2 for the ABC-based transporters [17, 98, 99, 123, 124]; however, the actual mode of engagement remains a subject of debate. The principal differences between the emerging models, which were initially based on the RND transporter systems such as AcrAB-TolC, were whether or not the hairpin domains of the MFPs contact the OMF channel in a tip-to-tip fashion as a continuous tube of hexamers [101] or in a lateral deep interpenetration fashion with individual hairpins docking into separate grooves on the surface of the OMF channel [85]. The tip-to-tip models also rule out a direct interaction between the OMF and the RND transporters, and hence both the engagement and the opening of the OMF channel are attributed entirely to the MFP hairpin tip, without any contribution from the transporter.

Initially, deep interpenetration models gained popularity due to a wealth of mutagenesis and *in vivo* and *in vitro* cross-linking studies supporting them on the level of OMF-MFP interaction [63, 120, 121, 125–127], as well as on the level of OMF-transporter interaction [128]. Kinetic data from surface plasmon resonance and isothermal calorimetry studies [98, 99, 123] also support the deep interpenetration model and provide an explanation for the differential affinities observed between different MFP-OMF pairs [99, 114]. However, following solution of the X-ray structures of several MFPs in oligomeric state, such as MacA [97] and MexA [124], as well as a string of modified structures based on the MacA scaffold, including MacA-OprM/MacA-MexA chimaeras [124] and AcrBA-fusion/MacA-TolC chimaeras [129], a new model was proposed. In this model, the only contact between the OMF and the MFP is provided by a limited tip-to-tip cogwheel-like intermeshing of the turns of the helices of the TolC and the hairpin domains of the hexamerized MFP.

Direct verification of either model was hindered for many years by the difficulties associated with complex isolation in sufficient quantity and monodispersity for crystallization studies. However, a breakthrough in cryo-EM resolution provided by the next generation of direct electron detectors [130], allowing to greatly extend the resolution of the technique heralded a new era in the research of the full complex. Recently, several major advances in our understanding of the association of the complete pump assemblies have taken place utilizing cryo-EM imaging and provided a major boost in support to the tip-to-tip model. A pivotal work by Du et al. [21] has provided the first snapshot of a fully assembled structure using chimeric concatenated protein constructs AcrBAB/AcrAZ/TolC. The structure resolved to approx. 15 Å provided a strong support to the 3:6:3 stoichiometry and a tip-to-tip association of the hexameric MFP and OMF. Further support to this model has been landed by Jeong et al. [131] who provided a cryo-EM reconstruction of a chimeric protein complex AcrAB-MacA-TolC to 8.2 Å resolution, which at the time of writing this chapter is the highest-resolution structure published. A study by Daury et al. [132] has been so far the only one to provide a non-engineered complex reconstituted using a lipid nanodisc system and single-particle EM reconstruction. Both AcrAB-TolC and MexAB-OprM complexes were found to assemble in a similar fashion, which appears broadly compatible with the lower-resolution structures reported by Du et al. [21], suggesting that at least *in vitro* there is no direct interaction between the RND transporter and the OMF channel.

Interestingly, an interspecies AcrA-TolC-MexB complex was also successfully assembled, suggesting limited promiscuity of the assembly at least at the level of MFP-RND transporter recognition. This finding is in line with previous observations of the chimeric assembly of AcrA-TolC-MexB by Krishnamoorthy et al. [121] and Stegmeier et al. [126] that reported association, but lack of functionality of the pump, presumably due to a failure to open the OMF channel. Such observations perhaps point toward the tip-to-tip engagement being either an early or terminal step of the assembly process.

While the tip-to-tip models are clearly gaining popularity based on the wealth of EM and cryo-EM structures, their relevance to the actual physiological functional state of the pump remains to be fully established. Indeed, some central issues include the very limited interaction interfaces between OMF and MFPs, which make it difficult to account for both apparent stability and specificity of the complex. A conserved RLS motif has been proposed to play a central role in the recognition of the OMF and possibly being involved in unlocking of the OMF channel [133] in RND and ABC transporters. This idea has then been extended to include type I secretion system MFPs [134]; however, the recent crystal structures of the HlyD from *E. coli* [94] and EmrA from *Aquifex* [93] do not position the motif at the tip of the hairpin. Indeed, the new structure of HlyD [94] reveals a noncanonical tip region, and the authors suggest that the "recognition" sequence is ALD instead of RLS. These results, however, are difficult to reconcile with the apparent OMF-MFP pairing specificity [135] and high-binding affinities required for keeping the complex together. The existence of noncanonic MFPs, with either very short hairpin domains or lacking the hairpin altogether such as BesA [86, 136], also poses

significant questions regarding the validity, or at least universality of the tip-to-tip interactions revealed by the recent cryo-EM structures.

A recent work by Hayashi et al. [137] using concatenated AcrBA chimeric proteins forcing a 3:3:3 stoichiometry of the pump, which is incompatible with a tip-to-tip model due to gaps in the assembly have demonstrated a near wild-type activity of the complex, further raising the question of whether that model is representative of the functional state of the pump. Only high-resolution structures providing side-chain information would resolve these pending conundrums, and there is little doubt that the advances in cryo-EM technology and crystallography will provide the answers in the coming years.

5.8 Dynamics and Energetics of Pump Assembly and Drug Efflux

The dynamics of tripartite efflux complexes *in vivo* and the role of substrates and energy remain under intense investigation. Significant conformational changes in TolC are needed to undergo transition into an open state (Fig. 5.1). It seems that the association with the inner membrane complex or even with MFP alone is sufficient to trigger such transition [76]. Once the complex is assembled, all three export components undergo conformational changes needed to couple the binding of substrate in the transporter to its translocation across the outer membrane.

Early studies showed that binding to TolC changes the conformation of CvaA, an MFP functioning with TolC in export of colicin V. In cells lacking TolC, CvaA is highly unstable and rapidly cleaved by periplasmic proteases [138]. Similar changes in sensitivity to proteases were reported for the hemolysin exporting HlyD [32], multidrug efflux AcrA, and macrolide efflux MacA [74, 107, 108]. In the case of AcrA and MacA, these conformational changes were shown to affect the membrane proximal domain of the proteins, which directly interfaces with the transporter (Fig. 5.2) [98, 108]. These studies also suggested that the TolC-dependent conformational change in the membrane proximal domain of MFP may be required for the complex assembly and activation of the transporter [98, 108].

It is unclear how conformational transitions in TolC are integrated into reaction cycles. One possibility is that the assembly of the transmembrane complex stabilizes the open conformation of TolC, so that the lifetime of the complex and the turnover number of the transporter determine the number of substrate molecules translocated through TolC. In the type I secretion systems, binding of substrate to the inner membrane complex seems to trigger TolC recruitment and stabilization of its open state [32]. However, TolC forms more stable complexes with drug efflux transporters, which are independent of the presence of substrates [139].

Furthermore, kinetic analyses showed that mutational opening of TolC decreases its affinity to AcrAB suggesting that TolC opening could actually trigger the disassembly of the complex [98]. Such mechanism could explain how the low permeability barrier of the outer membrane is maintained during efflux. It is apparent that

the open TolC channel would be a significant breach of the outer membrane permeability barrier. Studies of vancomycin resistance in *E. coli* show that cells containing functional AcrAB-TolC pump are more susceptible to this antibiotic than cells lacking either AcrAB or TolC [63, 74]. Since vancomycin is too large to cross the outer membrane by diffusion, the explanation for this result is that this antibiotic can slip through TolC engaged by AcrAB. One could imagine that to limit the influx of substrates through TolC, the complex is assembled with the closed TolC conformer and that recruitment and the transition into the open state is triggered, for example, by substrate binding, as proposed for CusBAC [140]. In the open state, the complex is unstable and rapidly dissociates relaxing TolC into its closed conformation (Fig. 5.1). This notion found further support in studies of AcrA proteolysis *in vivo*, which demonstrated that TolC mutant mimicking the open state of the channel was structurally defective and did not make same interactions as the closed TolC conformer with AcrA [74].

Further insight into the energetics and dynamics of complexes came from the studies of the *Neisseria gonorrhoeae* MtrCDE efflux pump expressed in *E. coli* [76]. Using the vancomycin assay, the authors demonstrated that the MFP MtrC opens MtrE even in the absence of the MtrD transporter and that the dissociation of MtrCD from the complex with MtrE is energy-dependent event. Additional support for the energy requirement in the dissociation of efflux complexes has been recently provided by an *in vitro* reconstitution study, which showed that liposomes containing MFP-transporter complex (in this case MexAB) pull-down non-biotinylated liposomes containing the OMF (OprM) upon acidification of the buffer, which creates a gradient of protons [82]. The use of a proton-relay-deficient mutant of MexB allows the OprM-containing liposomes to be pulled down both pre- and post-acidification, indicating the energy-dependent nature of dissociation and the absence of pH-induced conformational changes being involved. Extending earlier reconstitution assays utilizing two proteoliposome systems [79], a study using MexAB and OprM proteoliposomes with cargoes that become fluorescent upon transiting from one liposome to another has provided the first direct observation of an energy-dependent transport in the RND-based tripartite assembly *in vitro* [141].

5.9 Proposed Mechanism of Trans-envelope Drug Efflux

Studies discussed above suggest that efflux complexes assembled with transporters belonging to various protein families utilize the same mechanism of transport across the outer membrane and that MFPs play an important role in the functional communication between transporters and OMF. Although molecular details of how ABC, MFS, and RND transporters bind their substrates and transport them to the OMFs differ significantly between members of different families, all these transporters cycle through three states that promote access, binding, and extrusion of substrates (Fig. 5.3) [10]. The function of MFPs is to couple these transitions in transporter to opening of OMFs and transport of substrates across the outer

membrane. Most of the investigations were done on either RND or ABC efflux complexes, but it is likely that the same conclusions apply to MFS complexes. The major findings on the mechanism of MFPs in transport can be summarized as follows:

(i) MFP-transporter interactions are sensitive to conformational changes in transporters. When MacB transporter was trapped in different conformations by mutations or by the presence of nucleotide analogs, the affinity of association between MFP MacA and MacB increased upon ATP binding but remained unchanged during ATP hydrolysis [114]. Similarly, the interactions between the MFP AcrA and the RND transporter AcrB are most stable when AcrB is in its protonated state [76]. These results suggested that MFPs act on the pre-translocation "binding" state of transporters (Fig. 5.3).

(ii) Stimulation of transporters is coupled to a conformational change in the MP domain of MFPs. Reconstitution studies showed that interactions between MFPs and transporters increase the rates of transport and energy consumption [79, 80]. Further studies of MacAB, AcrAB, and TriABC showed that the MP domains of MFPs are critical for their ability to stimulate transporters [102, 107, 108]. These domains of MFPs interface with periplasmic domains of transporters and undergo conformational changes needed for stimulation of transporters. Studies of MacAB-TolC suggested that MFPs stimulate ABC transporters by stabilizing the closed pre-ATP hydrolysis conformation [108]. Assuming conservation of the mechanism, we propose that in the case of RND transporters, MFPs stabilize the binding-to-extrusion transition that precedes the transmembrane proton transfer (Fig. 5.3). Functional importance of the MFP-transporter interaction is further supported by the demonstration that the AcrB-AcrA fusion protein is functional [137].

(iii) Interaction with OMFs is needed for conformational changes in MFPs. The *in vivo* proteolysis approach showed that *in vivo* conformational changes in MFPs occur only in the presence of TolC [107, 108]. The TolC-induced conformational changes in MFPs were further linked to stimulation of activities of transporters by MFPs and to stabilization of tripartite complexes *in vitro*. Taken together, these results support the model that *in vivo* MFPs exist in stable complexes with transporters and that association with OMF triggers the conformational changes in MFPs needed for stimulation of transporters (Fig. 5.3). The results from several more recent studies also emphasized the roles of a MFP as part of exit duct with no physical interactions between the inner and outer membrane components [132].

(iv) Interactions between transporters and OMFs are dynamic. Current models postulate that OMF association with an MFP-transporter complex leads to opening of the channel (Fig. 5.1) [76, 103]. In the docked and cryo-EM models of AcrAB-TolC, TolC is in the open conformation [21, 85, 94, 131]. The dilation of the TolC channel in the assembled complex is apparently driven by energy-dependent conformational changes in MFP-transporter complexes. The open

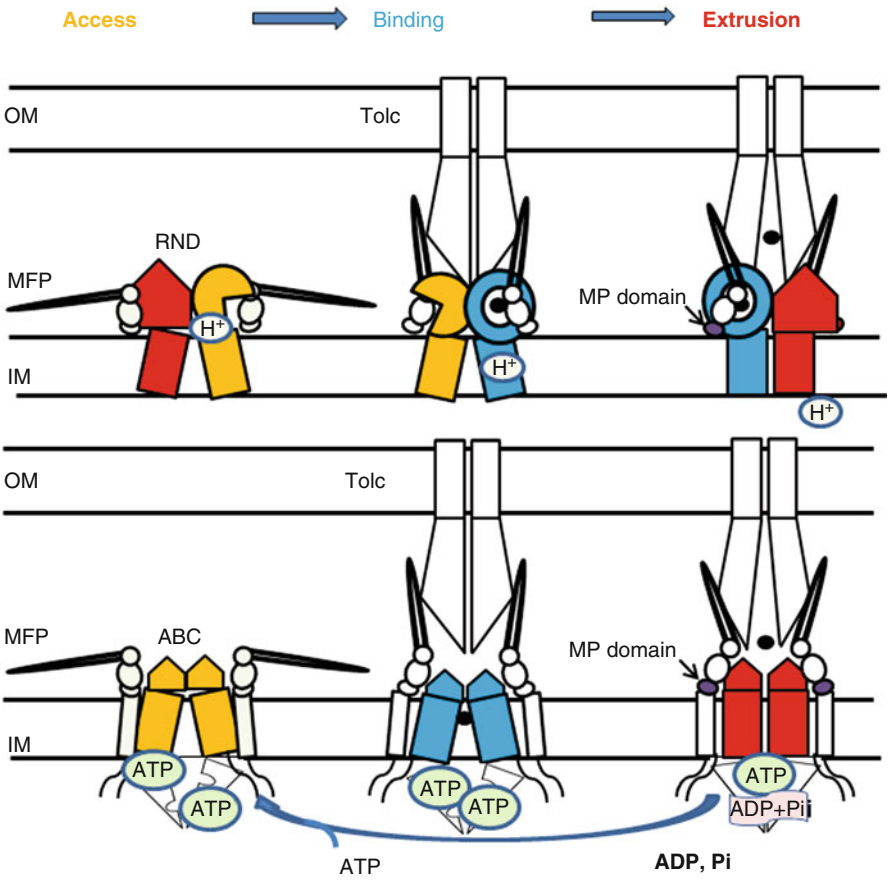

Fig. 5.3 Proposed mechanism of assembly and trans-envelope transport by tripartite efflux pumps across the inner membrane (IM) and outer membrane (OM). Despite different structures and mechanisms, RND and ABC transporters cycle through three states that promote access, binding, and extrusion of substrates. In RND transporters, each protomer cycles through the three conformations. The protomer in the substrate-bound state (*blue*) is likely to be protonated, and its transition into the extrusion state (*red*) is coupled to influx of protons. Dimeric ABC transporters are saturated with ATP, whereas binding of the substrate leads to transition into a semi-closed (*middle* panel) conformation. MFPs stabilize the substrate-bound state of transporters (*blue*) and drive transporters into the closed state needed for ATP hydrolysis. Association with TolC drives conformational changes in the MFP needed for stimulation of transporters (Reproduced from Zgurskaya et al. [10])

and closed conformers of TolC stabilized by mutations can be readily distinguished *in vivo* by drug susceptibility, proteolytic, and thiol-labeling profiles [74]. However, the interactions with TolC are highly dynamic and the lifetime of TolC-containing complexes is very short [98, 99]. Thus, TolC binding to the inner membrane complexes is transient and disassociation is regulated by energy consumption by the transporter.

Based on the data described above, we proposed that reaction cycles of transporters are tightly coupled to the assembly of the trans-envelope drug efflux complexes [10]. Transporters and MFPs exist in the inner membrane as inactive complexes (Fig. 5.3). The activation of complexes is triggered by MFP binding to OMF, which leads to a conformational change in MFP needed for stimulation of transporters. The activated MFP-transporter complex engages an OMF to expel substrates across the outer membrane. The recruitment of an OMF is likely triggered by binding of effectors (substrates) to MFP or MFP-transporter complex. This possibility has been discussed for the *P. aeruginosa* MexJK pump where experimental evidence strongly suggests that the MexJK complex recruits either OprM or OpmH in a substrate-dependent matter [142].

5.10 Asymmetric Efflux Complexes

The above-discussed MFP-transporter complexes have a threefold symmetry befitting the trimeric architecture of an OMF. However, asymmetric assemblies like RND transporters MdtABC or TriABC are also broadly represented in bacterial genomes. The mechanism and the requirement for asymmetry in such complexes remain unclear.

MdtB and MdtC share only 50 % sequence identity with each other. The expression of both MdtB and MdtC together with the MFP MdtA leads to a decreased drug susceptibility of *E. coli* to cloxacillin, novobiocin, bile salts, and sodium dodecyl sulfate [143–145]. The expression of MdtAB does not yield a functional transporter, but expression of MdtAC resulted in partial activity: a drug-resistant phenotype against bile salts. Co-purification experiments and protein fusion studies suggest that MdtBC associates as a B_2C complex [144]. Furthermore, there is convincing evidence that MdtB and MdtC fulfill distinctly different roles for the transporter. Site-directed mutagenesis studies have shown that a mutation in the proton-relay network of MdtB abolishes functionality of the complex to a greater extent than corresponding mutations in MdtC [144]. On the other hand, mutations in the proposed drug-binding pocket and 3D docking studies on homology models indicate that substrates bind primarily to MdtC [145]. The proton-relay network in MdtB is thus thought to provide the energy required for a conformational change in MdtC resulting in removal of substrates. This arrangement differs from the current model of drug expulsion in homotrimeric RND transporters, in which each protomer is functionally identical. MuxABC-OpmB in *P. aeruginosa* similarly includes two RND components in a single operon MuxB and MuxC. In this system, both peptides are necessary in order to observe drug resistance against a range of antibiotics including carbenicillin and novobiocin [146, 147].

In some complexes, the asymmetry arises through hetero-oligomerization of MFPs. TriABC-OpmH from *P. aeruginosa* requires two MFPs TriA and TriB that share 36 % sequence identity with each other [100, 102]. TriABC-OpmH provides resistance to triclosan with both TriA and TriB required for efflux. Weeks et al.

[102] recently showed that TriA and TriB form heterodimers, in which each protomer serves a different role in the functional assembly of the TriABC-OpmH/TolC complex. This analysis revealed that the α-helical domain of TriA is important for stimulating interactions with the OMF, whereas the MP domain of TriB is important for stimulating interactions with the transporter.

The existence of asymmetric complexes implies that current models of the MFP-dependent transport may be incomplete. It is possible that the asymmetry split functional roles of subunits and promotes one of the transition states. What step(s) in the transport could be enhanced by additional subunits? As it was demonstrated on the example TriA and TriB, the two different MFPs could play separate roles in the stimulation of a transporter and the recruitment/opening of OMF [102]. In transporters, this could be an added substrate specificity or a separation of energy transduction and substrate translocation as suggested for MdtBC [144, 148]. It is also possible that the hetero-oligomerization of transporters and MFPs might serve similar roles in the transport cycle such as modulation of transporter affinities to substrates and/or MFPs and OMFs. In this way, asymmetry might increase the specificity of a MFP-transporter complex to an OMF, which in turn could be a way to control the activity of the transporter.

5.11 Concluding Remarks

In recent years, significant progress has been made in understanding how multidrug efflux pumps function in the context of two membranes and how transport across the outer membrane is achieved. The current efforts are focused on molecular details of MFP-transporter interactions and how conformational transitions in proteins are integrated into an effective efflux process. This new level of understanding is expected to facilitate development of new therapeutic approaches for treatment of Gram-negative infections. Efflux pump inhibitors acting on the assembly of the complex or disrupting the conformational cross-talk could be an effective part of such new approaches.

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Part II
Bacterial Drug Efflux Pumps in Antimicrobial Resistance: Substrates, Regulation and Clinical Significance

Chapter 6
Antimicrobial Resistance in Bacteria: An Overview of Mechanisms and Role of Drug Efflux Pumps

Xian-Zhi Li

Abstract Antimicrobial agents target several essential cellular functions in bacteria including biosynthesis of the cell wall, nucleic acids, and proteins, which consequently produce inhibitory and even lethal effects on bacterial survival. In contrast, bacterial cells possess remarkable capacities to counteract the action of antimicrobials, thus contributing to resistance. The mechanisms of resistance predominantly involve the production of drug-inactivating enzymes, alteration of drug targets, and prevention of drug access; the latter mechanism refers to the function of drug influx and efflux. This chapter provides an overview of molecular and biochemical mechanisms of antimicrobial resistance with an emphasis on the role of drug efflux pumps and their relationship with other key resistance mechanisms in clinically relevant intrinsic and acquired resistance.

Keywords Antimicrobials • Antibiotics • Mode of action • Resistance • Resistome • Permeability barrier • Efflux pumps • Enzymatic inactivation • Target modification • Chromosome • Plasmid • Gene transfer

6.1 Introduction

The clinical use of penicillin G in the 1940s was a pivotal event that started the human era of antibiotics. Today these antibiotics (antimicrobials) play vital roles in modern medicine. Unfortunately, resistance to antimicrobials in microbes occurs without any regard to geographical borders and constitutes a growing threat to effective antimicrobial therapy. Indeed, the antimicrobial resistance crisis, which

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initially emerged more than two decades ago [1], continues to be a major global public health risk [2, 3]. The worldwide emergence of resistant pathogens is significant given their potential for dissemination and incredible capacities to survive under antimicrobial selective pressures. Moreover, the nature of this resistance phenomenon is further complicated by both the ancient origins of resistance genes and the diverse and rapid modern evolution of resistance genes (or the "resistome") [4]. This situation is additionally strained by the shortage of new antimicrobial drugs [5, 6]. Once established, resistant bacteria persist and disseminate readily. Mechanisms of resistance take many forms and predominately include prevention of drug access to their targets (i.e., through influx/efflux), enzymatic destruction of drugs, and modification of drug targets. To better appreciate the role of efflux systems in antimicrobial resistance, the author provides an overview of the evolution and the mechanisms of antimicrobial resistance, with an aim to capture its molecular and biochemical basis. This chapter does not presume to be comprehensive but covers the pertinent recent literature.

6.2 Origin and Evolution of Antimicrobial Resistance

Resistance to penicillin G was detected soon after its initial clinical use in the 1940s [7]. Antimicrobial resistance is a natural, inevitable phenomenon that can be intrinsic and further acquired and is a result of the evolutionary arms race to escape the action of antimicrobials [3]. Resistance genes exist on chromosomes as well as mobile genetic elements (such as plasmids). They can be transferred vertically to progeny and horizontally to other bacteria, including those of different species or genera. Remarkable advances in molecular technologies within the past two decades have provided useful means to facilitate the elucidation of resistance origins and evolution. Interestingly, numerous studies in recent years have revealed the ancient origins of resistance genes in environmental isolates independent of the modern era of antibiotics, together highlighting the environmental reservoirs of resistance genes and their linkage to the resistance genes observed in clinical settings [4, 8, 9]. The concept of the "resistome" refers not only to all resistance genes associated with disease-causing pathogens but also resistance genes within the environmental communities of microbes [4], which has no doubt broadened our understanding of resistance. For instance, genes encoding resistance to β-lactams, glycopeptides, and tetracyclines are not only present in ancient DNA molecules [8] but are also prevalent in the microbiomes of isolated regions which have not previously been exposed to human activities [9]. It is also crucial to note that novel mechanisms of resistance continue to be identified [9]. Moreover, the history of the 70-year antibiotic era has clearly revealed the impact of anthropogenic activities, particularly the use of antimicrobial agents, on the rapid emergence of resistance. The heavy selective pressure by antimicrobial use forces microorganisms to evolve for survival.

Table 6.1 shows the 2013 assessment of public health threats from multidrug-resistant bacteria by the U.S. Centers for Disease Control and Prevention [10]. This

Table 6.1 Resistant bacterial pathogens that constitute a major threat to public health

Threat level	Bacterial pathogens
Urgent	*Clostridium difficile*
	Carbapenem-resistant Enterobacteriaceae
	Drug-resistant *Neisseria gonorrhoeae*
Serious	Multidrug-resistant *Acinetobacter*
	Drug-resistant *Campylobacter*
	Extended spectrum β-lactamase producing Enterobacteriaceae
	Vancomycin-resistant *Enterococcus*
	Drug-resistant non-typhoidal *Salmonella*
	Drug-resistant *Salmonella* Typhi
	Drug-resistant *Shigella*
	Methicillin-resistant *Staphylococcus aureus*
	Drug-resistant *Streptococcus pneumoniae*
	Drug-resistant tuberculosis
Concerning	Vancomycin-resistant *Staphylococcus aureus*
	Erythromycin-resistant group A *Streptococcus*
	Clindamycin-resistant group B *Streptococcus*

The table is based on the reference [10] that deals with resistance threats in the USA. However, these resistant pathogens are generally applicable to many countries worldwide based on antimicrobial resistance surveillance data [11]

resistance landscape also accurately reflects the global and undoubtedly challenging situation regarding drug resistance [11]. A common feature of these problematic pathogens is the multidrug resistance (MDR) phenotype which can be attributable to both single and multiple mechanisms of resistance. Mutational changes in chromosomal genes and the incorporation (or concentration) of resistance genetic elements (chromosomal or extrachromosomal, such as plasmids) via various gene transfer pathways equip microbes with the molecular basis to fight the actions of antimicrobials. The emergence of CTX-M extended-spectrum β-lactamases (which hydrolyze many β-lactams including advanced oxyimino-cephalosporins) provides a compelling example that establishes a relationship between the emergence of β-lactamase genes in environmental pathogens and the use of extended-spectrum β-lactam agents [12–15]. Other examples of resistance that emerges under selective pressure are various resistance genomic islands (as observed in *Acinetobacter* and *Salmonella*) [16–18] and resistance gene cassettes showing a cluster of genes conferring resistance to multiple drugs. These genetic elements often include the earliest-discovered antibiotics such as penicillin, streptomycin, and tetracycline as well as newer agents such as fluoroquinolones and the third-generation cephalosporins [19, 20]. The characterization of a large number of plasmid-borne *qnr* genes (conferring quinolone resistance) also demonstrates evolution of resistance under antimicrobial selective pressure [21]. The *qnr* genes also coexist with other resistance genes on plasmids. More recently, the emergence of plasmid-mediated mobile colistin resistance (*mcr-1*) in *Enterobacteriaceae* captured global attention [22].

The *mcr-1* gene encodes a phosphoethanolamine transferase that adds phosphoethanolamine to lipid A of lipopolysaccharide (LPS) and results in clinically relevant resistance to the last resort of antimicrobials, polymyxins [22]. Within only a few months, *mcr-1* was confirmed to already be widespread in isolates of *E. coli* and *Salmonella* spp. of human, food, and animal sources from Asia, Africa, Europe, and North America [23–28]. These observations are not surprising because of (i) rapid natural selection in bacteria; (ii) the huge population of microbes in any given niche; (iii) the various circulating mobile genetic elements; and (iv) the short generation times of microbes. Additionally, under selective pressure, bacteria can change their growth rates (e.g., through the adjustment of lag time) to develop tolerance to antibiotics [29]. A recent review has analyzed the many mechanisms leading to elevated MDR [30]. It is noteworthy to mention that multidrug efflux represents only a single biochemical mechanism conferring MDR; an overall understanding of the molecular evolution pathways is necessary to truly comprehend the forces at work in selection for resistance and thus in turn for tracking the emergence and spread of resistance.

6.3 Molecular and Biochemical Mechanisms of Antimicrobial Resistance

Antimicrobial agents inhibit and/or kill microbes by attacking numerous vital cellular processes; these mechanisms include inhibition of cell wall, nucleic acid and protein biosynthesis, disruption of metabolic pathways and cell membrane integrity, as well as various other as listed in Table 6.2. For instance, β-lactams affect bacterial cell wall assembly, causing it to weaken and burst. Quinolones and coumarins act on DNA gyrase, which is involved in the separation of double-stranded DNA during replication of DNA and transcription of RNA [31]. Many other classes of antimicrobials (e.g., aminoglycosides, macrolides, and tetracyclines) block protein synthesis by interfering with bacterial ribosomal synthesis of proteins [32]. In contrast, bacterial cells have remarkable mechanisms to defend against the action of antimicrobials. Numerous resistance genes provide the molecular basis for resistance, which together compile into the thousands of bacterial genes that make up resistance gene databases [33, 34].

6.3.1 Prevention of Drug Access: Reduced Influx and Increased Efflux

Drug Influx Across the Membrane Permeability Barrier In order to exert actions onto microbes, antimicrobial agents must first access their cellular targets within the bacterial cell at sufficient inhibitory levels, which are ultimately determined by competition between drug influx and efflux (Fig. 6.1) [35–37]. Thus, drug

Table 6.2 Antimicrobial classes: modes of action and mechanisms of resistance

Antimicrobial class	Modes of action: drug targets	Mechanisms of resistance		
		Drug inactivating enzymes	Drug target alteration/protection	Cell envelope membrane permeability/drug efflux pump
β-Lactams	Inhibition of cell wall synthesis by targeting to penicillin-binding proteins	Ambler class A, B, C and D β-lactamases	Mutations in PBP with reduced affinity to β-lactams	OM permeability with reduced porin production; RND pumps
Aminoglycosides	Inhibition of protein synthesis (30S ribosome inhibitors affecting initiation and translocation)	*N*-acetyltransferases, *O*-nucleotidyltransferases, *O*-phosphotransferases	Mutations in ribosome	Lipopolysaccharide alterations; RND pumps
Amphenicols	Inhibition of protein synthesis (50S ribosome inhibitors blocking the peptidyl transferase)	Chloramphenicol acetyltransferase	Mutations in 23S rRNA	RND and MFS pumps
Cationic peptides	Disruption of cell membranes and/or intracellular targets (cell wall, nucleic acid, or protein synthesis)	Proteolytic degradation	Membrane alteration	Cell membrane and lipopolysaccharide alterations; efflux
Coumarins (novobiocin)	Inhibition of RNA synthesis (DNA gyrase B)		Mutations in DNA gyrase B	OM permeability; RND pumps
Ethambutol	Disruption of cell wall synthesis (by inhibiting the polymerization of cell wall arabinan)		Mutations in or overexpression of mycobacterial arabinosyl transferases	Efflux
Fluoroquinolones	Inhibition of DNA synthesis by targeting topoisomerases to block DNA replication and repair	*N*-acetyltransferase AAC(6′)-Ib-cr	Alterations of DNA gyrase and topoisomerase IV; Qnr pentapeptide family proteins	OM permeability with reduced porin production; RND, MFS, and ABC pumps
Glycopeptides (vancomycin)	Disruption of cell wall synthesis (binding to *N*-acyl-D-Ala-D-Ala termini of peptidoglycan precursors)		Acquisition of genes generating alternative peptidoglycan termini (D-Ala-D-lactate and D-Ala-D-Ser)	Cell wall thickening; outer membrane permeability

(continued)

Table 6.2 (continued)

Antimicrobial class	Modes of action: drug targets	Mechanisms of resistance		
		Drug inactivating enzymes	Drug target alteration/protection	Cell envelope membrane permeability/drug efflux pump
Glycylcyclines (tigecycline)	Inhibition of protein synthesis (30S ribosome inhibitors to block tRNA delivery)		Mutations in the ribosomal S10 protein	RND pumps
Isoniazid	Inhibition of fatty acid synthesis	Loss of drug activation (mutations in catalase-peroxidase)	Target modification (InhA)	Efflux
Ketolides	Inhibition of protein synthesis (50S ribosome inhibitors by binding to 23S rRNA to block nascent chain elongation)		Mutations in 23S rRNA and ribosomal L4 protein	OM permeability; efflux
Lincosamides	Inhibition of protein synthesis (50S ribosome inhibitors by binding to 23S rRNA and causing premature dissociation of the peptidyl-tRNA)		Target modification by Erm methylases (mutations in 23S rRNA)	OM permeability; efflux
Lipopeptides (daptomycin)	Disruption of cell membrane	Inactivation by daptomycin hydrolase	Mutations in lysylphosphatidylglycerol synthetase	Cell wall thickening; cell membrane lipid and protein modification; outer membrane permeability
Macrolides	Inhibition of protein synthesis (50S ribosome inhibitors by binding to 23S rRNA to block nascent chain elongation)	Macrolide esterase or phosphotransferase	Target modification by Erm methylases (mutations in 23S rRNA)	OM permeability; efflux
Nitrofurans	Disruption of DNA synthesis (by cross-linking to DNA)	Loss of drug activation (mutations in nitroreductase)		

Nitroimidazole	Disruption of DNA synthesis	Loss of drug activation		Efflux
Oxazolidinones	Inhibition of protein synthesis (50S ribosome inhibitors by binding to the peptidyl transferase center)		Mutations in 23S rRNA; inactivation of methyltransferase (RlmN)	OM permeability; efflux by RND pumps
Pleuromutilins	Inhibition of protein synthesis (50S ribosome inhibitors by binding to the peptidyl transferase center)		Mutations in the 50S ribosome (23S rRNA)	OM permeability; efflux
Polyketides (mupirocin)	Inhibition of protein synthesis (inhibiting the Ile-tRNA synthetases)		Mutations or acquisition of Ile-tRNA synthetases	
Polymyxins	Disruption of outer membrane		Lipopolysaccharide alteration	Lipopolysaccharide alteration; efflux
Pyrazinamide	Inhibition of fatty acid synthase; interference with ATP production	Loss of drug activation (mutations in or lack of pyrazinamidase)	Target protein overexpression (ribosomal protein S1)	Efflux (in non-*M. tuberculosis*)
Rifamycins	Inhibition of RNA synthesis (targeting the DNA-dependent RNA polymerase)	ADP-ribosyltransferases, phospherases, glycosyltransferase	Mutations in the *rpoB* gene	OM permeability; efflux
Streptogramins	Inhibition of protein synthesis (50S ribosome inhibitors)		Target modification by Erm methylases (mutations in 23S rRNA)	OM permeability; efflux
Sulfonamides	Interference of folic acid synthesis		Mutations or overproduction of dihydropteroate synthases (encoded by *sul1* and *sul2*)	Efflux by RND pumps
Trimethoprim	Interference of folic acid synthesis		Mutations or overproduction of dihydrofolate reductases (encoded by *dhfr* or *dfrA* genes)	OM permeability; efflux by RND pumps
Tetracyclines	Inhibition of protein synthesis (30S ribosome inhibitors by preventing aminoacyl-tRNA association with the ribosome)	Tetracycline-inactivating enzymes: Tet(X) protein; tetracycline destructases	Target ribosomal protection	OM permeability; efflux by MFS and RND pumps

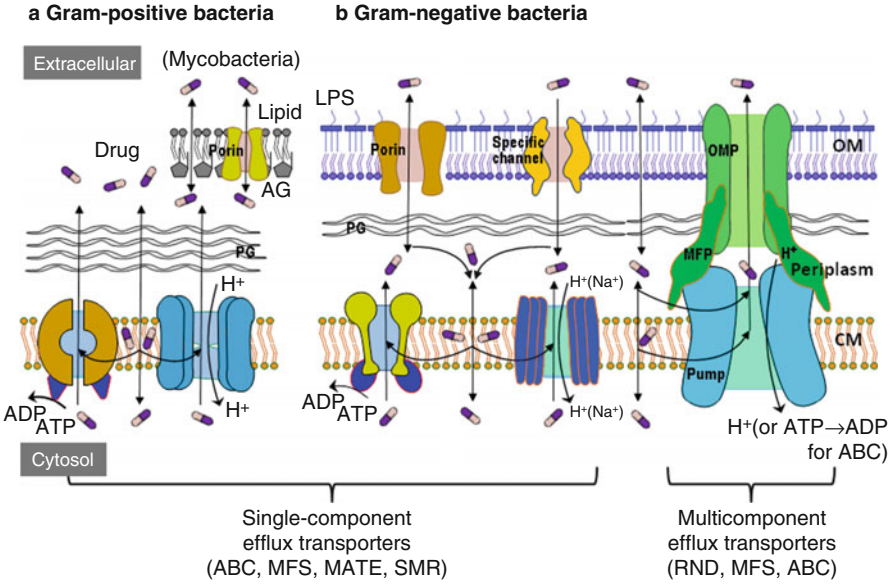

Fig. 6.1 The influx and efflux of antimicrobial drugs across bacterial cell envelope. (**a**) In Gram-positive bacteria, drugs enter the cell via penetration of the peptidoglycan (*PG*) and the cytoplasmic membrane (*CM*) (in mycobacteria, drugs also penetrate an additional outer barrier comprising of porins and mycolyl arabinogalactan [*AG*] that forms a barrier complex with PG). The efflux pumps belonging either ABC transporters or proton-dependent secondary active transporters are single-component exporters that may take the drug from the CM and/or the cytosol. (**b**) In Gram-negative bacteria, the asymmetric lipopolysaccharide (*LPS*)-containing outer membrane (*OM*) functions as a formidable permeability barrier. Drugs enter the periplasm via three possible pathways, porin (e.g., small hydrophilic molecules), specific channel (e.g., carbapenems), or lipid bilayer (e.g., lipophilic molecules). The efflux pumps can be either the single-component pumps or multicomponent pumps that take the drug from the CM, periplasm, or cytosol. A tripartite efflux system typically contains a pump, an OM channel protein (*OMP*), and an accessory membrane fusion protein (*MFP*) (also called periplasmic adaptor protein). A single-component pump may function with protein channels to make an effective efflux process. The available data support that multicomponent RND pumps incorporate drugs from the periplasm or CM and directly extrude the substrate out of the cell. RND pumps are also found in Gram-positive bacteria including mycobacteria (Reproduced from Li [35])

penetration through the cell envelope barrier, such as the cell wall of Gram-positive bacteria or the outer membrane (OM) of Gram-negative bacteria, is essential. Indeed, the differences in cell envelope permeability to drug molecules between Gram-positive and Gram-negative cells characterize differing intrinsic resistances; for example, Gram-positive bacteria are generally more susceptible to antimicrobials (e.g., macrolides, ketolides, oxazolidinones, novobiocin, and rifampicin) than Gram-negative bacteria. A novel class of antibiotics, teixobactin, inhibits peptidoglycan synthesis and displays strong activity against Gram-positive species [38]. However, it lacks activity against *Escherichia coli* and *Pseudomonas aeruginosa* [38], suggesting that this agent cannot sufficiently access its cellular target within Gram-negative bacteria.

The cell wall of Gram-positive bacteria is located outside the cytoplasmic membrane and consists of peptidoglycan and polysaccharide polymers (e.g., teichoic acids, teichuronic acids, and neutral/acidic polysaccharides) [39, 40]. These polymers are linked together via glycosidic, amide, and peptide chemical bonds [40], thus creating a barrier with physiological roles in protection, maintenance of cell shape, and osmotic stability [41]. Though the cell wall is not considered to be a true permeability barrier, it still sieves out large molecules by restricting the entry of molecules larger than 1,200 Da [39]. Since antimicrobials tend to be small, the Gram-positive cell wall is usually permeable to them (with the exception of Gram-positive mycobacteria, as described below). Interestingly, cell wall thickening is a commonly described feature in vancomycin-resistant and/or daptomycin-insusceptible *Staphylococcus aureus* [42], but it remains unclear how this change alone significantly affects drug susceptibility. Mycobacteria, although being Gram-positive organisms, are closer to Gram-negative bacteria in terms of permeability and have an unusual OM [43, 44]. Their OM contains a sparse number of channel-forming porins, but there is an abundance of long-chain fatty acids (mycolic acids) covalently linked to the peptidoglycan-associated polysaccharide arabinogalactan; together these components produce an impermeable cell wall that makes mycobacteria intrinsically resistant to many agents [45, 46]. This decreased permeability suggests that mycobacterial cells have specific uptake pathways to permit the entry of nutrients. Indeed, a major antituberculosis agent, pyrazinamide, takes advantage of a nicotinamide transport system in *Mycobacterium tuberculosis* to enter cells before being converted from a prodrug into its active form [47]. Interestingly, this particular species is unable to pump the drug out due to the lack of an efflux system, while many other pyrazinamide-resistant mycobacteria seemingly possess this machinery [48].

The OM of Gram-negative bacteria comprises of an asymmetric LPS-containing lipid bilayer region with water-filled porins and functions as an effective barrier that prevents the influx of drug molecules [49]. Located in the outer leaflet of the OM, LPS contains more fatty acid chains than phospholipids, and its strongly negatively charged core will bind divalent cations such as Mg^{2+} and Ca^{2+}. Large hydrophobic agents need to traverse the OM lipid bilayer to enter into cells. The loss of LPS integrity, as observed in "rough" phenotype mutants (i.e., with shorter polysaccharide chains), significantly increases OM permeability and produces a drug hypersusceptibility phenotype [50]. Various cationic agents (e.g., polymyxins) can serve as OM permeabilizers that increase drug access to their target and thus sensitize Gram-negative cells to antimicrobials (such as hydrophobic rifamycins and large macrolide molecules) [51]. For instance, polymyxin B enhances the activities of trimethoprim-sulfamethoxazole against multidrug-resistant isolates of *Stenotrophomonas maltophilia* [52], consistent with the role of the OM barrier in resistance. Polymyxins act by disrupting the OM permeability barrier and their resistance is contributable to the LPS modifications which can be caused by chromosomal mutations affecting LPS integrity [53–57] or plasmid-borne *mcr-1* gene [22]. Porins, channel-forming proteins, constitute another major component of the OM and allow the penetration of small hydrophilic molecules, including nutrients and antimicrobial agents [49]. The classical porins of *E. coli*, OmpF and OmpC,

form trimeric pores with certain size limitations. β-Lactam agents are able to cross the OM via these classic porins [58]. Thus, *E. coli* strains are indeed quite susceptible to various β-lactam agents. Consequently, the diminished expression of porins has been widely seen in resistant clinical isolates of several enteric species including *Enterobacter cloacae* and *Klebsiella pneumoniae* [59–63]. Slow porins (another porin type) are represented by monomeric OmpA in *E. coli* and *Acinetobacter baumannii* and OprF in *P. aeruginosa*. In addition to their structural role in maintaining cell envelop stability, these porins can also form larger channels; however, the open fraction of these porins is limited [49, 64, 65]. Indeed, this fact partially explains why *A. baumannii* and *P. aeruginosa* are more intrinsically resistant to many antimicrobials as compared to *Enterobacteriaceae* (to note, efflux also plays a synergistic role) [65]. The OM may also contain substrate-specific protein channels such as OprD of *P. aeruginosa* and CarO of *A. baumannii* that are specific for the diffusion of basic amino acids and peptides [66, 67]. These specific channels facilitate diffusion of carbapenem β-lactams, and both loss in their production and reduced expression have been observed in carbapenem-resistant isolates from patients [68–72].

Drug Efflux by Pumps In addition to the factors affecting drug influx, another major mechanism of drug resistance is through the drug extrusion pathway, which consists of energy-dependent efflux transporters located in the cytoplasmic membrane. Gram-negative bacteria utilize multicomponent drug efflux complexes that can span both the cytoplasmic and outer membranes. The contribution of drug efflux pumps to intrinsic and acquired resistance was recognized relatively late in comparison with other mechanisms of resistance [36, 73]. However, there is an increasing emphasis on their importance to resistance as well as their impact on antimicrobial development and therapy. Drug transporters are categorized into different superfamilies and families by protein sequence features; an example of such a group includes the primary-active transporters belonging to the ATP-binding cassette (ABC) superfamily that utilizes ATP hydrolysis as the energy source. Many other efflux pumps are secondary-active antiporters that are dependent on a proton motive force; examples include the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family [37], and other families such as recently described antimetabolite transporters (the AbgT family) [74] and the proteobacterial antimicrobial compound efflux (PACE) family [75]. There are a large number of known drug exporters which can have an incredibly broad substrate profile or can be drug specific as described below. It should be noted that although many bacterial species possess a large number of drug exporters, only a limited number of pumps (e.g., the predominant RND-type AcrAB-TolC system) can contribute to clinically relevant resistance [76, 77].

(i) *Multidrug efflux pumps*. A feature of many drug efflux pumps is their capacity to accommodate an incredibly wide range of the substrates (Table 6.3), which differs from many other drug-specific enzymatic or target modification mechanisms. Even in Gram-positive bacteria, which have a quite permeable cell envelope (except mycobacteria), multidrug efflux pumps contribute to

basal resistance levels and can facilitate the development of clinically relevant acquired resistance. Several efflux pumps (such as MepA, MedA, NorA, NorB, NorC, and QacA/B) are present in *S. aureus* and may also contribute to resistance to antibiotics and biocides [88, 125–127]. An analysis of more than

Table 6.3 Drug efflux transporters in major pathogenic bacteria

Species	Efflux pump	Transporter family	Substrates	Reference
Enterococcus faecalis	EfrAB	ABC	ACR, CIP, DAU, DOR, DOX, NOR, TPP	[78]
Staphylococcus aureus	Isa(E)	ABC	LIN, PLE, STA	[79]
	MepA	MATE	BIO, EB, FQ, TIG	[80, 81]
	Msr(A)	ABC	ML, TEL	[82, 83]
	NorA	MFS	FQ	[84]
	QacA	MFS	ACR, CHX, CV, EB, QAC	[85]
Streptococcus pneumoniae	MefE	MFS	ML	[86]
	PatAB	ABC	FQ	[87]
Mycobacterium tuberculosis	Mmr	SMR	CAB, CLA, TPP	[88–90]
	Rv1218c	ABC	BAP, BPD, PRI, PYR	[89, 91]
	Tap	MFS	PAS, SPE, TET	[92, 93]
Acinetobacter baumannii	AbeM	MATE	ACR, AG, DAU, DOR, FQ	[94]
	AceI	PACE	ACR, BAC, CHX	[75]
	AdeABC, AdeFGH, AdeIJK	RND	AG, BL, FQ, ML, TET, BIO, BS	[95–98]
	CraA	MFS	CHL	[99]
Campylobacter jejuni	CmeABC, CmeDEF	RND	AG, BL, CHL, FQ, ML, RIF, TET, BS, EB	[100, 101]
Escherichia coli	AcrAB-TolC	RND	BL, CHL, FQ, ML, NOV, RIF, TET, TGC, BS, CV, OS, R6G, SDS	[102–105]
	EmrE	SMR	ACR, EB, QAC	[106, 107]
	MacAB-TolC	ABC	ML	[108]
	MdfA	MFS	CHL, DOR, NOR, TET	[109]
	QepA/ QepA2	MFS	FQ	[110]
	OqxAB	RND	CHL, FQ	[111]
Klebsiella pneumoniae	KpnEF	SMR	BAC, BS, CEF, CHX, ERY, SDS, STR, TET, TRI	[112]
	KpnGH	MFS	CAZ, CEF, STR, TET	[113]
	OqxAB	RND	CHL, FQ	[111]
Neisseria gonorrhoeae	MtrCDE	RND	BL, CAP, EB, ML, TRX	[114, 115]
	NorM	MATE	AG, EB, FQ	[114]

(continued)

Table 6.3 (continued)

Species	Efflux pump	Transporter family	Substrates	Reference
Pseudomonas aeruginosa	MexAB-OprM, MexXY-OprM / OprA, MexCD-OprJ, MexEF-OprN	RND	AG, BL, CHL, FQ, ML, SUL, TET, TGC, TMP, BIO, BS, EB, BS, OS, SDS	[116–119]
Stenotrophomonas maltophilia	FuaABC	ABC	FUA	[120]
	SmeABC, SmeDEF	RND	AG, BL, FQ, ML, TET	[88, 121, 122]
Vibrio spp.	NorM	MATE	AG, EB, FQ	[123]
	VcaM	ABC	CIP, DAU, DOR, NOR, TET	[124]
Various bacteria	TetA	MFS	TET	[73]

ACR acriflavine, *AG* aminoglycosides, *BAC* benzalkonium chloride, *BAP* biaryl-piperazines, *BL* β-lactams, *BIO* biocides, *BPD* bisanilino-pyridines, *BS* bile salts, *CAB* cetyltrimethylammonium bromide, *CAP* cationic antibacterial peptides, *CAZ* ceftazidime, *CEF* cefepime, *CHL* chloramphenicol, *CHX* chlorhexidine, *CIP* ciprofloxacin, *CLA* clofazimine, *CV* crystal violet, *DAU* daunomycin, *DOR* doxorubicin, *DOX* doxycycline, *EB* ethidium bromide, *ERY* erythromycin, *FQ* fluoroquinolones, *FUA* fusidic acid, *LIN* lincosamide, *ML* macrolides, *NOR* norfloxacin, *NOV* novobiocin, *OS* organic solvents, *PAS* *p*-aminosalicylate, *PLE* pleuromutilin, *PRI* pridones, *PYR* pyrroles, *QAC* quaternary ammonium compounds, *R6G* rhodamine 6G, *SDS* sodium dodecyl sulfate, *SPE* spectinomycin, *STA* streptogramin A, *STR* streptomycin, *SUL* sulfonamides, *TEL* telithromycin, *TET* tetracycline(s), *TGC* tigecycline, *TMP* trimethoprim, *TPP* tetraphenyl phosphonium, *TRI* triclosan, *TRX* Triton X-100

100 clinical bloodstream isolates identified increased pump expression, which was found to be correlated with resistance to fluoroquinolones, biocides, and dyes [128]. A newly identified ABC transporter, Isa(E), mediates resistance to lincosamides, pleuromutilins, and streptogramin A in enterococci and staphylococci [79, 129]. Constitutive overexpression of an ABC-type PatAB transporter in *Streptococcus pneumoniae* caused a fourfold increase in MIC values of ciprofloxacin, norfloxacin, and levofloxacin [130]. PatAB overproduction was also confirmed in fluoroquinolone-resistant clinical isolates [131].

Disruption of several efflux pumps makes mycobacterial cells more susceptible to many classes of antimicrobial agents [89–91, 132]. Using macrolide resistance as a model, enhanced efflux pump expression was found to be a general first step in the evolution of mycobacterial drug resistance, i.e., efflux-mediated low-level resistance occurred prior to the development of target mutation-mediated high-level resistance [133]. Multidrug-resistant clinical isolates exhibit hyperexpression of the Rv1218c ABC pump [134]. Efflux also contributes to the development of resistance to three frontline antituberculosis agents: isoniazid, ethambutol, and pyrazinamide [135–137].

In Gram-negative bacteria, the efflux mechanism is very effective in producing high-level resistance. Tripartite RND pumps can directly pump their substrates out of the cell, whereas substrates of single-component

pumps only transport their substrates to the periplasm and may require cooperation with RND pump(s) for their extrusion to the external medium [138–140]. Moreover, RND pumps generally have much broader substrate profiles than drug exporters of other families (Table 6.3) [37]. In particular, many newer antibiotics (such as new-generation cephalosporins, fluoroquinolones, and glycyclines) that have been developed against drug-specific resistance mechanisms are typical substrates of RND pumps [37]. In *P. aeruginosa*, an 8- to 128-fold drop in MIC values for antipseudomonal agents azlocillin, carbenicillin, cefoperazone, ceftriaxone, and ciprofloxacin occurs as a consequence of MexAB-OprM inactivation in wild-type strains [116]. The overproduction of the RND pump(s) (e.g., MexAB-OprM [116] and MexXY [117]) has been widely reported in clinical isolates of *P. aeruginosa* [118, 141, 142].

(ii) *Drug-specific efflux pumps*. Many of these pumps belong to the MFS. The most representative examples include the Tet pumps that provide high-level tetracycline-specific resistance. Tet pumps are often encoded by plasmids and are widely present in both Gram-positive and Gram-negative bacteria [143]. Several chloramphenicol-specific exporters include the CraA pump of *A. baumannii* [99] and the MdfA/Cme and FloR of *E. coli* and other species [88]. In *S. pneumoniae*, the MFS-type Mef pump was shown to cause an 80-fold increase the MIC values of the macrolides azithromycin, clarithromycin, and erythromycin (M-type resistance) [86, 144]. Several ABC-type pumps also mediate drug-specific resistance. The FuaABC pump confers intrinsic resistance to fusaric acid in *S. maltophilia* [120].

6.3.2 Drug Inactivation

Inactivation of antimicrobials occurs through various differing chemical processes which range from hydrolysis to group transfers that include acylation, glycosylation, nucleotidylation, phosphorylation, and ribosylation. Specific examples are provided below.

β-Lactams β-Lactamases represent the most important mechanism of high-level β-lactam resistance in Gram-negative bacteria. These enzymes hydrolyze the four-membered β-lactam ring of penicillins, cephalosporins, and other β-lactams [145]. To date, more than 1,000 naturally occurring β-lactamases have been documented, and this number is certainly expected to rise rapidly since a single amino acid substitution within a β-lactamase can alter its substrate profile and evolve as a new enzyme. Encoded on chromosomes and plasmids, β-lactamases are categorized into four classes (Ambler classes A, B, C, and D) on the basis of their primary amino acid sequences and catalytic mechanisms [146, 147]. These enzymes are further functionally classified into many types/subtypes [145]. Class A, C, and D β-lactamases are serine-dependent enzymes but are evolutionarily distinct. These

enzymes act via a ring-opening nucleophilic attack on active serine sites in the β-lactam ring. Class B β-lactamases are metalloenzymes requiring zinc ions for activity [145].

Class A enzymes are often encoded by plasmids. They not only include many older β-lactamases such as TEM (223 are documented at http://www.lahey.org/studies/ and http://www.laced.uni-stuttgart.de/ as of January 15, 2016) and SHV (193 documented) enzymes (which can have either narrow or broad spectrums toward hydrolyzing β-lactam agents) but also encompass the newer CTM-X enzymes (172). The latter have emerged as the most prevalent extended-spectrum β-lactamases (ESBLs), and have been isolated from hospitalized patients, complicated community patients, as well as from livestock animals and varying environments [15, 148, 149]. Class A ESBLs (e.g., of TEM, SHV, and CTX-M types) hydrolyze aztreonam and oxyimino-cephalosporins but not 7-α-substituted β-lactams and are susceptible to β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) [150]. Most CTM-M producers are multidrug resistant [15]. Class C enzymes include many chromosomally encoded or plasmid-mediated AmpC β-lactamases (>200 are documented) from the *Enterobacteriaceae*, *P. aeruginosa*, and other Gram-negative species. Expression of these enzymes can be promoted by inducers including certain β-lactams, but mutational changes can also lead to their constitutive, high-level production [151]. AmpC enzymes hydrolyze penicillins but are more active on cephalosporins with substrates including cephamycins and oxyimino-cephalosporins. They are inhibited by aztreonam, cloxacilllin, and oxacillin but are less affected by sulbactam and tazobactam [151].

Class D enzymes include OXA β-lactamases (496 are documented in database) that prefer penicillins as substrates and are not significantly inhibited by clavulanic acid. Once thought to be encoded only by plasmids, OXA enzymes can also be encoded by chromosomes. These enzymes can be divided into several subgroups and are becoming increasingly important [152]. Carbapenem-hydrolyzing OXA-type carbapenemases constitute a major resistance problem, as seen in *Acinetobacter* and *Enterobacteriaceae*. Class B β-lactamases require zinc ions for their catalytic activity and thus are subjected to the inhibition by ion chelators such as EDTA. These enzymes are active in hydrolyzing almost all β-lactams including cephamycins, carbapenems, and some class A β-lactamase inhibitors [152, 153]. In this regard, carbapenemases are found in classes A (e.g., IMI, KPC, and GES), B (e.g., IMP, IND, NDM, and VIM), and D (OXA) encoded on either chromosomes or plasmids [153].

Aminoglycosides These agents are mainly inactivated by enzymatic modification, although target modifications and efflux also contribute to their resistance [154–156]. Three types of aminoglycoside-modifying enzymes exist – aminoglycoside *N*-acetyltransferases (AACs), *O*-nucleotidyltransferases (ANTs), and *O*-phosphotransferases (APHs). These enzymes modify different amino and hydroxyl residues, as well as groups on the 2-deoxystreptamine nucleus and sugar moieties. Each of the AACs, ANTs, and APHs has numerous subclasses with different substrate profiles. Many enzymes have several allozymes that may exhibit

varied substrate profiles [155]. AAC(3′) and APH(2″) enzymes have typical substrates of gentamicin, kanamycin and tobramycin, while ANT(2″) enzymes modify gentamicin and tobramycin. Encoded by chromosomes and plasmids, these enzymes are widely distributed in numerous Gram-positive and Gram-negative bacteria including mycobacteria. For instance, *A. baumannii* strains possess about ten aminoglycoside-modifying enzymes (including various AAC, ANT, and APH enzymes) that together have a substrate range for nearly all clinically relevant aminoglycoside agents [16]. Additionally, the association of enzyme-encoding genes with genetic mobile elements (plasmids/transposons/integrons) facilitates horizontal gene transfer among different species. These genes can often be copresent with other resistance determinants – for example, the copresence of *aphA6* and $bla_{OXA\text{-}58}$ in one plasmid and *aacA4*, *ant*, *aphA1*, *armA*, *catB*, *armA*, *mph*(*E*), *msr*(*E*), and *sulI* in other plasmids [157]. Investigation on the inhibition of aminoglycoside-modifying enzymes has been a focus of antimicrobial discovery research [158, 159], but there has been little success toward developing clinically useful inhibitors.

Fluoroquinolones The aminoglycoside acetyltransferase, AAC(6′)-Ib-cr, is a fluoroquinolone-modifying enzyme that not only inactivates amikacin, kanamycin, and tobramycin but also reduces the activity of ciprofloxacin and norfloxacin by *N*-acetylation at the amino nitrogen of the piperazinyl ring substituent [21, 160]. The *aac*(*6′*)*-Ib-cr* gene is widely disseminated in numerous *Enterobacteriaceae* and generally exists as part of a resistance gene cassette (in particular with a quinolone resistance *qnr* gene and/or a $bla_{CTX\text{-}M}$ gene) in MDR plasmids [21].

Lincosamides and macrolides Lincomycin can be modified by nucleotidyltranferases encoded by *lnu*(*A*) and *lnu*(*B*) of staphylococci and enterococci [161]. However, resistance levels conferred by this inactivation mechanism are low. Although likely infrequent in clinical isolates, macrolide inactivation can also occur as a consequence of the hydrolysis of the macrolide lactone ring by an *ere*-encoded esterase [162] or modification by *mph*-mediated macrolide phosphorylation [163]. The *mph* gene can also be present in transferable plasmids with other macrolide resistance genes such as efflux pump genes *msr* or *mef* [164].

Amphenicols Chloramphenicol acetyltransferases (CATs) inactivate chloramphenicol and thiamphenicol. These enzymes are widely distributed in bacteria (encoded in both chromosomes and plasmids) and are grouped via their distinct structures into two defined types, either as classical CATs or as xenobiotic CATs [165]. The *cat* genes have often been a part of resistance gene cassettes in MDR plasmids.

Rifamycins Several mechanisms of enzymatic degradation of rifamycins are currently known. ADP-ribosyltransferases are able to modify rifampicin and contribute to low-level resistance to rifamycin in mycobacteria [166]. Rifamycin-inactivating phosphatases, a new protein family in conferring resistance, have been found in

pathogenic and environmental bacteria [167]. Additionally, a rifamycin-modifying glycosyltransferase was also characterized in actinomycetes [168].

Other Antimicrobials Fosfomycin is subjected to modification by *fosA* or *fosB*-encoding thioltransferases, which cause the adverse opening of the fosfomycin epoxide ring [169]. There are an increasing observations of *fos* genes on transposon-containing plasmids and their copresence with $bla_{CTX\text{-}M}$ genes in *E. coli* and *K. pneumoniae* [170]. The oxidation of tetracyclines, including tigecycline, is mediated by flavin-dependent TetX monooxygenase [143, 171]. Recently, a novel family of tetracycline-inactivating flavoenzymes, tetracycline destructases, was identified through soil functional metagenomic selection, and these enzymes were found to produce high-level tetracycline resistance [172].

6.3.3 Drug Target Alterations, Protection, and Overexpression

Mutations in chromosomal genes that encode antimicrobial targets are responsible for high-level resistance to many antimicrobial drugs, particularly agents that target cell wall, nucleic acid, and protein biosynthesis.

β-Lactams Changes in penicillin-binding proteins (PBPs) are the major mechanism for β-lactam resistance in many Gram-positive bacteria (although this also occurs in Gram-negative bacteria). A typical example includes methicillin-resistant *S. aureus* in which the acquisition and expression of the PBP 2a-encoding *mecA* gene causes high-level β-lactam resistance. The *mecA* gene and another *ccr* gene constitute the major part of staphylococcal cassette chromosome *mec* (SCC*mec*) elements which are currently grouped into 11 types (http://www.staphylococcus.net) [173, 174]. Mosaic PBPs in streptococci have reduced affinity to β-lactams with a resistance reservoir from commensal streptococci [175]. Alterations of PBPs in *Haemophilus*, *Helicobacter*, and *Neisseria* also contribute to clinically relevant β-lactam resistance [176].

Aminoglycosides, Amphenicols, Glycylcyclines, Ketolides, Lincosamides, Macrolides, Oxazolidinones, Pleuromutilins, Streptogramins, and Tetracyclines These agents target the bacterial ribosome, which consists of three rRNAs (16s, 23s, and 5s) and >50 ribosomal proteins [177]. The specific target sites of the ribosome include tRNA delivery (e.g., tetracyclines/tigecycline), tRNA translocation (e.g., aminoglycosides), the peptidyl transferase center (e.g., chloramphenicol), and the ribosome exit tunnel (e.g., macrolides) [177]. Resistance to these ribosome-targeting agents often occurs due to either methylation of the 23S rRNA or mutations in genes encoding the 16S rRNA, 23S rRNA, 30S, and 50S ribosomal proteins, which affect drug binding sites [177]. For example, Erm-methyltransferases mediate adenine-specific methylation in the 23S rRNA within the large ribosomal subunit and produce resistance to macrolides, ketolides, lincosamides, and streptogramin A [177, 178]. Mutations in the 23S rRNA also lead to modification of the

linezolid binding site on the ribosome and thus provide resistance to oxazolidinones [179]. Moreover, mutations in the 16S rRNA gene produce resistance to aminoglycosides [180]. The Cfr methyltransferase modifies the 23S rRNA and mediates resistance to many antimicrobial classes including amphenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A [181]. There is also elongation factor-associated protection of ribosomal drug targets which is involved in resistance to fusidic acid and tetracyclines [177]. Mutations in the ribosomal S10 protein confer reduced susceptibility to tigecycline in both Gram-positive and Gram-negative species [182]. It is important to mention that ribosomal mutations can often lead to cross-resistance to different ribosome-targeting agents when the agents share similar modes of action in inhibiting protein synthesis. For instance, laboratory-generated single and dual mutations within the 23S rRNA conferred different resistance types to (a) macrolides and clindamycin; (b) chloramphenicol, florfenicol, linezolid, and valnemulin; or (c) macrolides only [183].

Fluoroquinolones The target proteins of these agents are chromosomally encoded topoisomerases II (DNA gyrase; encoded by *gyrAB*) and IV (*parCE*), which are heterotetrameric enzymes (i.e., $GyrA_2B_2$ and $ParC_2E_2$, respectively) [184]. Mutations in the *gyrAB* and/or *parCE* genes cause reduced binding of fluoroquinolones to the enzyme-DNA complexes and confer variable levels of resistance to fluoroquinolones resistance [21, 184]. The most frequent mutations occur particularly in the quinolone resistance-determining regions (QRDRs) which correspond to residues 67–106 of Gyr and residues 63–102 of ParC in *E. coli* [184]. The primary targets of different fluoroquinolones may vary against the two topoisomerases. Generally, DNA gyrase in Gram-negative bacteria is often more sensitive to quinolones, while topoisomerase IV is more sensitive in Gram-positive bacteria. The initial mutations occur in more sensitive primary targets. Mutations in both target enzymes often produce high-level resistance. However, certain species (such as *M. tuberculosis*) only possess gyrase and emergence of resistance can be readily selected [184].

The Qnr proteins encoded primarily by plasmids provide protection for fluoroquinolone targets [185]. Represented by the 218-amino acid QnrA, Qnr proteins are part of the pentapeptide repeat family of proteins and are currently grouped into six classes (QnrA, QnrB, QnrC, QnrD, QnrS, and QnrVC) [184, 185]. Plasmid-encoded QnrA1, QnrB11, and QnrS1 are each shown to provide a 32-fold increase for ciprofloxacin and levofloxacin MIC values [184]. Importantly, *qnr*-containing plasmids often carry multiple resistance determinants such as those encoding ESBLs and/or AAC(6′)-Ib-cr [184, 185]. In addition, chromosomally encoded Qnr proteins have also been identified in >90 bacterial species [21], although it was first identified in *S. maltophilia* (in which SmQnr proteins were found to mediate intrinsic resistance to fluoroquinolones) [186]. In this regard, it is noteworthy that no mutations in topoisomerases have been linked to fluoroquinolone resistance in *S. maltophilia*.

Other Antimicrobials Glycopeptide resistance in Gram-positive species, such as vancomycin in enterococci, can largely be attributed to the acquisition of the genes (i.e., *vanA-G*) encoding an altered peptidoglycan precursor terminating in D-Ala-D-

lactate or D-Ala-D-Ser (instead of D-Ala-D-Ala) which diminishes affinity to vancomycin 1,000-fold [187]. Mutations in the *rpoB* gene cause alterations in the RNA polymerase β-subunit, leading to its reduced affinity to rifamycins [188]. Low-level resistance to mupirocin in *S. aureus* occurs via mutations in the mupirocin target, isoleucyl-tRNA synthetase, whereas the acquisition of isoleucyl-tRNA synthetases (that are similar to relevant eukaryotic enzymes) provides high-level mupirocin resistance [189]. Antituberculosis pyrazinamide acts on multiple targets, including the ribosomal protein S1 (RpsA), to inhibit the translation process in *M. tuberculosis* which can be overcome by RpsA overexpression [190].

6.3.4 Interference with Metabolic Pathways

The combination of a sulfonamide and trimethoprim in treating bacterial infections provides a critical example which validates the significance of combination therapy. Sulfonamide agents are competitive inhibitors and alternate substrates (as opposed to the physiological substrate *para*-aminobenzoate) for dihydropteroate synthase (DHPS). Similarly, trimethoprim is an inhibitor of dihydrofolate reductase (DHFR), and together these two classes of agents block two steps within the hydrofolate biosynthesis pathway. Resistance to these agents has been attributed to the production of altered DHPS (encoded by a *sul* gene) and DHFR (encoded by a *dhfr* or *dfr* gene); each of these enzymes is comprised of different variants [191]. Genes encoding for these enzymes are found on both chromosomes and plasmids and often coexist with other resistance genes in resistance gene cassettes or genomic islands containing mobile elements. Isoniazid resistance in mycobacteria has also arisen through changes in metabolic pathways. Two enzymes, NADH-dependent enoyl-acyl carrier protein reductase (encoded by *inhA*) and a β-ketoacyl acyl carrier protein synthase (encoded by *kasA*), participate in the biosynthesis of mycolic acids, and their mutations are involved in isoniazid-resistant *M. tuberculosis* [188].

6.3.5 Loss of Prodrug Activation

Certain antimicrobial agents are prodrugs, in which a conversion to their active forms is essential for their respective activities. Two important antituberculosis drugs, isoniazid and pyrazinamide, require the activation by the *katG*-encoded catalase-peroxidase and the *pncA*-encoded pyrazinamidase, respectively. Mutations in the relevant enzyme-encoding genes are linked to resistance to isoniazid and pyrazinamide in *M. tuberculosis* [192]. Metronidazole is another prodrug that requires its reduction by oxygen-insensitive NADPH nitroreductase (RdxA), NADPH-flavin-oxidoreductase (FrxA), and ferredoxin-like enzymes (FrxB) [193].

Mutations in *rdxA*, *frxA*, and *frxB* confer metronidazole resistance, as observed in *Helicobacter pylori* [193]. The reduction of nitrofuran agents by nitroreductases is also essential for the antibacterial activity of nitrofurans; thus, resistance can be induced by loss-of-function mutations in genes that encode oxygen-insensitive nitroreductases such as *nfsA* and *nfsB* in *E. coli* [194].

6.4 Interplay Between Drug Efflux Pumps and Other Resistance Mechanisms

Bacteria possess multiple resistance mechanisms as described above, and these mechanisms can cooperate with each other to enhance resistance levels and/or profiles [88].

6.4.1 Interplay Between Efflux Pumps

Efflux exporters themselves can interplay with each other to produce additive and even multiplicative impacts on drug resistance levels [138]. In *E. coli*, inactivation of AcrAB alone and together with AcrEF reduces ciprofloxacin MIC values by four and eightfold, respectively (X-Z Li, unpublished data, 2001), suggesting a synergistic effect of two RND efflux pumps on intrinsic resistance to ciprofloxacin. The coexpression of plasmid-encoded AcrAB with either the cloned MFS pump MdfA or MATE pump MdtK (previously known as NorE) confers a higher level of fluoroquinolone resistance than AcrAB expression alone [195]. Two RND systems CmeABC and CmeDEF in *Campylobacter jejuni* play synergistic roles in enhancing intrinsic resistance to bile salts and detergents [100]. In *P. aeruginosa*, co-overexpression of multiple RND pumps MexAB-OprM/MexCD-OprJ, MexAB-OprM/MexEF-OprN, and MexAB-OprM/MexCD-OprJ/MexEF-OprN increases MIC values of antipseudomonal levofloxacin by 16-, 32- and 64-fold, respectively, in comparison with the levofloxacin MICs for strains with wild-type constitutive MexAB-OprM production [138].

In Gram-negative bacteria, typical tripartite component efflux complexes (e.g., RND pumps) directly efflux drug substrates out of the cell. In contrast, single-component Tet efflux pumps can only extrude drug molecules into the periplasm. These molecules then re-diffuse across the cytoplasmic membrane (although in this case, RND pumps can aid the further efflux of these drugs from periplasm out of the cell). Indeed in *P. aeruginosa*, the presence of a functional MexAB-OprM pump can aid singlet pumps TetA and TetC to produce a 128-fold increase in tetracycline MIC values in comparison to only 32- to 64-fold rises without the functional MexAB-OprM pump [138]. Moreover, ethidium bromide (a substrate of many MDR transporters, including AcrAB, EmrE, and MdfA of *E. coli*) is not efficiently extruded

from the periplasm in the absence of a functional AcrAB pump [140]. AcrAB interplays with EmrE and/or MdfA in the efflux of ethidium bromide, which also provides an example of coordinated efflux by multiple transporters [140]. Furthermore, three pumps, RND-type MtrCDE, ABC-type MacAB, and MATE-type NorM in *Neisseria gonorrhoeae* have been shown to contribute to MDR phenotypes of clinical isolates [114].

6.4.2 Interplay Between Efflux Pumps and the Membrane Permeability Barrier

In Gram-negative bacteria, the relationship between the OM barrier and drug efflux pumps together determines the availability of drug molecules to drug targets [37]. Earlier we demonstrated the synergistic interplay between MexAB-OprM and the OM barrier in conferring intrinsic and acquired MDR (see Chap. 14) [196]. Similarly, the widely used, laboratory mutagenesis-generated hypersusceptible *P. aeruginosa* strain Z61 was found to be deficient in LPS structure and MexAB-OprM expression [196–198]. An example in which *E. coli* have also exhibited a synergistic effect of increased influx (due to deficient LPS) and reduced efflux (due to genetically disrupted *acrAB*) on antimicrobial susceptibility is provided in Chap. 9. In particular, the simultaneous deficiency of both the OM barrier and active drug efflux renders the mutant extremely susceptible to large hydrophobic agents such as macrolides. In multidrug-resistant clinical isolates of *Enterobacteriaceae* (e.g., *Enterobacter* and *Klebsiella* species), both reduction/lack of porin expression and RND pump overexpression are frequently observed (reviewed in [37]). These examples compellingly demonstrate that improved drug access by simultaneous disruption of the membrane barrier and increased influence of efflux pumps is a critical means to overcome drug resistance.

6.4.3 Interplay Between Efflux Pumps and the Drug Inactivation Process

Enzymatic inactivation of antimicrobials often produces high-level drug resistance such as β-lactamase-mediated β-lactam resistance, and thus this mechanism may complicate our ability to detect contributions from other resistance pathways. Indeed, inactivation of the AcrAB pump or AmpC β-lactamase in *E. coli* clearly shows differential contributions of these two mechanisms to intrinsic β-lactam resistance; however, disruption of both mechanisms produces higher susceptibility to several β-lactams [199]. Genetic inactivation of the MexAB-OprM pump in a β-lactamase-derepressed *P. aeruginosa* mutant also reduces resistance to third- and

fourth-generation cephalosporins (with MIC values decreased by two- to eightfold) [200]. In *S. maltophilia*, MDR efflux pumps interplay with an aminoglycoside-modifying enzyme, acetyltransferase AAC(6′)-*Iz*, to enhance aminoglycoside resistance [88].

6.4.4 *Interplay Between Efflux Pumps and Drug Target Modification*

Interaction between efflux pump overproduction and mutations in DNA gyrase/topoisomerase IV contributes to high-level fluoroquinolone resistance in clinical isolates of *S. aureus*, *E. coli*, and *P. aeruginosa* [88]. Similarly, simultaneous RND pump overproduction and ribosomal target protein modifications are necessary for high-level macrolide resistance in *Campylobacter* spp. [201]. MtrCDE overproduction in *N. gonorrhoeae* is also required for clinically relevant penicillin resistance in the presence of PBP2 target alteration and PorIB porin mutation [202]. Susceptibility of a PBP *P. aeruginosa* mutant to third- and fourth-generation cephalosporins was enhanced (with two to fourfold reduction of MIC values) when MexAB-OprM was inactivated [200].

6.5 Concluding Remarks

Bacteria possess a range of sophisticated mechanisms to resist the actions of diverse antimicrobial agents. Despite their ancient origins, resistance genes have clearly been enriched under modern antimicrobial use. These genes, present on both chromosomes and plasmids, are widely distributed in the bacterial pathogens concerned in public health as well as in environmental species and together provide the molecular basis and biochemical mechanisms of resistance. Enzymatic degradation of drug molecules and modification of drug targets are generally drug/class specific, while membrane barrier/drug efflux pump-mediated mechanisms often affect multiple structurally unrelated agents. A thorough understanding of these resistance mechanisms is fundamental for the development of new antimicrobials and antimicrobial adjuvants. In particular, our insight on the role of efflux pumps in intrinsic and acquired resistance is critical for therapeutic intervention strategies and the development of drug molecules that can evade/inhibit efflux mechanisms. Finally, the dynamic nature of both chromosomal alterations and the exchange of mobile genetic materials highlights the significance of minimizing antimicrobial selection pressures on the evolution of resistance through prudent antimicrobial use.

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Chapter 7
Antimicrobial Drug Efflux Pumps in *Staphylococcus aureus*

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Abstract The highly adaptive opportunistic human pathogen, *Staphylococcus aureus*, contains a large number of integral membrane transport proteins important in homeostasis and pathogenesis. Included in this group are multidrug efflux pumps which play a role in antimicrobial resistance by reducing the intracellular concentration of drug compounds through active extrusion from the cell. *S. aureus* encodes many such drug efflux proteins, which fall into four out of the six currently recognized drug transporter families. These efflux pumps can confer host resistance against a vast number of clinically relevant antimicrobials, including macrolides, quinolones, streptogramins, and tetracyclines, as well as biocides such as biguanidines, diamidines, and quaternary ammonium compounds. The prevalence of these drug efflux determinants, either chromosomally or plasmid encoded, has been established worldwide, with clinical isolates expressing numerous multidrug transport proteins being continually identified. In addition to the characterized drug transporters, the recent surge in sequencing has also revealed a number of putative drug efflux proteins; some of these may share characteristics of known pumps, but, as seen with the recent addition of the proteobacterial antimicrobial compound efflux family, may represent proteins which are unique.

Keywords *Staphylococcus aureus* • Multidrug resistance • Drug efflux pump • Sav1866 • NorA • NorB • NorC • MgrA • MepA • MepR • QacA • QacB • QacC • QacR

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7.1 Introduction

Identified to be a major cause of both nosocomial- and community-acquired infections, *Staphylococcus aureus* is a highly adaptive opportunistic bacterium that displays a wide spectrum of pathogenicity, largely owed to its ability to acquire mobile genetic elements encoding virulence and resistance determinants [1, 2]. This adaptive power has given rise to the emergence of methicillin-resistant *S. aureus* (MRSA) and continues to contribute to the development of new strains resistant against a number of antimicrobial drugs, including one of the last resort drugs, vancomycin [3, 4]. Resistance genes carried on plasmids, transposons, and other mobile elements can be acquired by *S. aureus* from other Gram-positive bacteria through the process of horizontal gene transfer and genetic recombination, arming it with determinants such as *vanA*, which originated in enterococci, but now contributes to vancomycin resistance in *S. aureus* [5, 6].

History has shown that staphylococci are able to act against each new antimicrobial by employing at least one resistance mechanism. These mechanisms include enzymatic modification or destruction of the drug, alteration of the drug target by mutation, enzymatic inactivation resulting in reduced affinity for the drug, and extruding the drug from the cell via an efflux protein [7, 8]. Of these, drug efflux appears to be one of the most widespread resistance mechanisms. In Gram-positive bacteria, such as *S. aureus* which lack an outer membrane, efflux pumps are vital in limiting the accumulation of toxic compounds within the cell. Found in all living organisms, these proteins fulfill numerous physiological functions including the expulsion and subsequent elimination of endogenous metabolites, bile salts, and host-defense molecules as well as the uptake of essential nutrients [9–12]. Their abundance and the variety of roles they fulfill suggest that drug efflux is a fortuitous event, which has helped to arm bacteria against most, if not eventually all, antimicrobials they come across.

Initially detected on plasmids in staphylococcal strains found to be highly resistant to antimicrobials [13], whole genome sequencing has allowed for the detection of a multitude of putative drug-specific and multidrug transporters. Recent studies analyzing chromosomally encoded multidrug efflux-like proteins from the genomes of three *S. aureus* strains, 8325, COL, and N315, identified 21 open reading frames encoding putative membrane efflux proteins [14]. Although experimental analysis revealed that overexpression of these did not produce resistance to common multidrug efflux substrates, it is still possible that one or more of these can extrude a yet untested compound and that other genes encoding drug efflux-like proteins, not identified by the method employed in the study, exist. To date there are close to 70 whole *S. aureus* genomes sequenced (the National Center for Biotechnology Information database: http://ncbi.nlm.nih.gov), but not all have been analyzed, leaving the possibility that there are a number of genes encoding novel drug efflux pumps which are yet to be assessed.

Although the number of identified multidrug transporters continues to increase, experimental structural analysis of these hydrophobic membrane-bound proteins

such as X-ray crystallography and nuclear magnetic (NMR) spectroscopy is limited by numerous factors and hampered by the experimental challenges which are inherent to such analyses [15, 16]. As a result of these, biochemical analysis such as site-directed mutagenesis, where residues are substituted with other amino acids carrying a different charge or side-chain volume, is carried out in order to assess their importance in function and/or structure. Such analyses, coupled with computational determination of the three-dimensional structure of a protein, have become essential for both functional and structural analysis of membrane transporters.

Falling into six different transport protein superfamilies/families (Fig. 7.1), and based on their energy requirements, drug transport proteins can be termed either as primary or secondary transporters. Primary transporters include the ATP-binding cassette (ABC) superfamily, while secondary transporters include the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the newly discovered proteobacterial antimicrobial compound efflux (PACE) protein family [17–21]. Although some can be specific for a single drug, numerous membrane transporters can extrude a broad range of structurally diverse antimicrobial compounds and as such are designated multidrug efflux pumps. Both specific and multidrug transport proteins have been identified in *S. aureus* and at a protein level are indistinguishable (Table 7.1). Of these, characterized membrane transporters within *S. aureus* include the chromosomally encoded LmrS [35], NorA [38, 39], NorB [42], NorC [43], SdrM [49], MdeA [36, 37], MepA [55], Sav1866 [28], and Tet38 [42], as well as the plasmid-encoded TetA(K) [50], TetA(L) [53], QacA [67], QacB [68], QacC [58], QacJ [61], and QacG transporters [69].

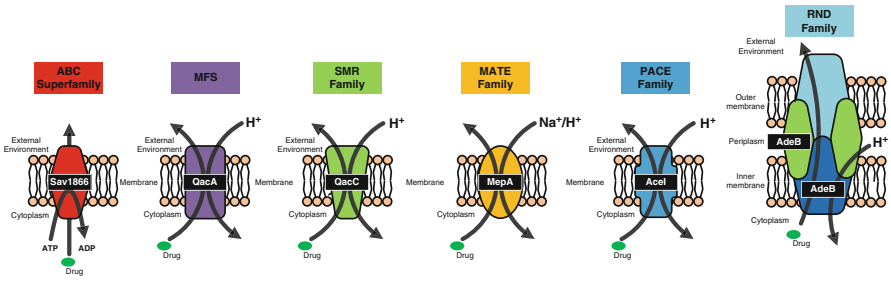

Fig. 7.1 Diagrammatic representation of transporters from the six major drug transporter superfamilies or families. The representative transport systems are shown along with their energy-coupling mechanisms (i.e., ATP hydrolysis for the ABC superfamily export system and the use of electrochemical gradients for the remaining systems). The export systems classified within the ABC, MFS, SMR, MATE, and PACE families typically transport their substrates (drug, *green oval*) across the cytoplasmic membrane. However, RND transporters as well as some MFS and ABC systems assemble with periplasmic- and outer membrane-bound proteins to form tripartite systems (in Gram-negative bacteria) that are able to expel substrates across both the cytoplasmic and outer membranes

Assessment of these transporters has shown that they are capable of transporting a wide range of substrates and in most cases show an overlap in their substrate profiles.

This chapter summarizes the most recent data on the best characterized multidrug transporter that is a representative from within each transport family identified within *S. aureus*. These multidrug efflux pumps are the ABC superfamily Sav1866 protein, the MFS QacA/QacB proteins, the MATE family MepA protein, and the SMR QacC protein. Although RND membrane transporters have been identified in *S. aureus*, including a Mmpl homologue transporter [70] and the SecDF accessory factor to the Sec protein translocation machinery [71], none have been shown to be directly involved in multidrug efflux and as such will not be included in this review. By focusing on a representative of each family/superfamily, the mechanism of transport, substrate profile, structure, and regulation will be discussed.

Table 7.1 Characterized multidrug efflux pumps of *S. aureus*

Family	Efflux pump	Substrate specificity	Reference
ABC	AbcA[a]	CTX, DAM, EB, MET, MOE, NAF, PEN, RHO	[22–24]
	Msr(A)	ERY, OLE, TEL, VIR	[25–27]
	Sav1866	EB, HO, TPP, VIN	[28, 29]
	Vga(A)		
	Vga(A)v	CLI, LIN, PRI, RET, VIR	[30–32]
	Vga(B)	CHL, LIN, PRI	[30]
	Vga(C)	CLI, LIN, PIR, TIA, VIR	[33]
MFS	FexA	CHL, FLO	[34]
	LmrS	CHL, EB, ERY, FLO, FUA, KAN, LIN, LIZ, OXY, SDS, STR, TMP, TPP	[35]
	MdeA	ACR, BAC, CIP, DAU, DEQ, DOR, EB, FUA, HO, MUP, NOR, NOV, R6G, TPP, VIR	[36, 37]
	NorA	ACO, BAC, CET, CHL, CIP, CIX, EB, ENX, FLO, LOM, NAL, NOR, OA, OFX, PA, PER, PPA, PUR, R6G, SPA, TPP	[38–41]
	NorB	CET, CIP, EB, GAR, GMF, MOX, NOR, PRF, SPA, TPP	[42, 43]
	NorC	CIP, GAR, MOX, NOR, PRF, SPA	[43]
	QacA	ACL, ACR, ACY, BAC, CTA, CV, DAP, DAPI, DAZ, DBP, EB, HED, PAD, PPD, PRO, PTD, PY, QR, R6G, SO, STD, TMA-DPH, TPA, TPM, TPP	[44–47]
	QacB	ACY, ACR, BAC, CTA, CV, EB, PRO, PY, R6G, SO, TPA, TPP	[47, 48]
	SdrM	ACR, EB, NOR	[49]
	Tet38[b]	TET	[42]
	TetA(K)	DOX, TET	[50–52]
	TetA(L)[c]	TET	[53, 54]

Table 7.1 (continued)

Family	Efflux pump	Substrate specificity	Reference
MATE	MepA	ACR, BAC, CET, CHX, CIP, CV, DAPI, DEQ, EB, HO, NOR, PTD, PY, RHO, TGC, TPP	[55–57]
SMR	QacC	BAC, CTA, CTP, CV, DEQ, EB, PRO, R6G	[58]
	QacEΔ1	CTA, CTP, CV, DEQ, EB, PRO, R6G	[59, 60]
	QacG	BAC, CTA, EB	[61, 62]
	QacH	BAC, CTA, EB, PRO	[61, 63]
	QacJ	BAC, CTA	[61]

ACL amicarbalide, *ACO* acridine orange, *ACR* acriflavine, *ACY* acridine yellow, *BAC* benzalkonium chloride, *CET* cetrimide, *CHL* chloramphenicol, *CHX* chlorhexidine, *CIP* ciprofloxacin, *CIX* cinoxacin, *CLI* clindamycin, *CTA* cetyltrimethylammonium, *CTP* cetylpyridinium, *CTX* cefotaxime, *CV* crystal violet, *DAM* daptomycin, *DAP* diamidinodiphenylamine, *DAPI* 4′,6-diamidino-2-phenylindole, *DAU* daunorubicin, *DAZ* diminazene, *DBP* dibromopropamidine, *DEQ* dequalinium, *DOR* doxorubicin, *DOX* doxycycline, *EB* ethidium bromide, *ENX* enoxacin, *ERY* erythromycin, *FLO* florfenicol, *FLX* fleroxacin, *FUA* fusidic acid, *GAR* garenoxacin, *GMF* gemifloxacin, *HED* hexamidine, *HO* Hoechst 33342, *KAN* kanamycin, *LIN* lincomycin, *LIZ* linezolid, *LOM* lomefloxacin, *MET* methicillin, *MOE* moenomycin, *MOX* moxifloxacin, *MUP* mupirocin, *NAF* nafcillin, *NAL* nalidixic acid, *NOR* norfloxacin, *NOV* novobiocin, *OA* oxolinic acid, *OFX* ofloxacin, *OLE* oleandomycin, *OXA* oxacillin, *OXY* oxytetracycline, *PA* piromidic acid, *PAD* phenamidine, *PEN* penicillin G, *PER* perfloxacin, *PIR* pirlimycin, *PPA* pipemidic acid, *PPD* propamidine, *PRF* premafloxacin, *PRI* pristinamycin, *PRO* proflavine, *PTD* pentamidine, *PUR* puromycin, *PY* pyronin Y, *QR* quinaldine red, *R6G* rhodamine 6G, *RET* retapamulin, *RHO* rhodamine, *SDS* sodium dodecyl sulfate, *SO* safranin O, *SPA* sparfloxacin, *STD* stilbamidine, *STR* streptomycin, *TEL* telithromycin, *TET* tetracycline, *TGC* tigecycline, *TIA* tiamulin, *TMA-DPH* 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *TMP* trimethoprim, *TPA* tetraphenylarsonium, *TPM* tetraphenylammonium, *TPMP* triphenylmethylphosphonium, *TPP* tetraphenylphosphonium, *VIN* vinblastine, *VIR* virginiamycin

[a]AbcA also exports cytolytic toxins [64]

[b, c]Tet38 also exports certain unsaturated fatty acids [65], whereas as TetA(L) also plays a role in Na^+ homeostasis [66]

7.2 Primary Active Transporters

7.2.1 The ATP-Binding Cassette Superfamily

Consisting of both uptake and efflux transport systems, ABC transporters represent one of the largest superfamilies of proteins known in both prokaryotic and eukaryotic organisms [72, 73]. Proteins within this family have been shown to import or export a wide range of substrates, including, among others, amino acids, sugars, and lipids [74, 75]. This substrate diversity is mirrored by the myriad physiological roles that ABC transporters play in the cell. These include nutrient uptake, elimination of waste products from the cell, and export of cellular components such as cell wall polysaccharides [76, 77]. Found in all species, their importance is exemplified by the number of proteins certain species express. For example, in *Escherichia coli*, almost 5 % of its genomic coding capacity is composed of genes encoding ABC

transporters [78]. Analysis of ABC transporters has also shown some of them to be medically important, including those involved in cystic fibrosis, various eye diseases, liver disease, and multidrug resistance in cancer cells, among others [79].

Analyses of crystal structures of ABC transporters such as BtuCD, an *E. coli* vitamin B_{12} importer [76, 80]; HI1470/1, a metal-chelate transporter from *Haemophilus influenzae* [81]; and the high-resolution structure of the *S. aureus* Sav1866 multidrug transporter [82] show that they follow a basic organization which includes two transmembrane domains (TMDs) which act as the substrate-translocation pathway and the nucleotide-binding domains (NBDs), which bind and hydrolyze ATP [83, 84]. Organized as either heterodimers or homodimers, the NBD and TMD subunits can be encoded separately, or as a single polypeptide, and can be arranged with a carboxyl-terminal NBD and an amino-terminal TMD or vice versa [83]. In contrast to the TMDs, NBDs are homologous throughout the ABC superfamily and contain several motifs which are characteristic of this family. These include the Walker A and B motifs, which are present in many nucleotide-binding proteins, motif C which is specific to the ABC superfamily [74] and the stacking aromatic D, H, and Q loops [77].

Structural studies have revealed that the TMDs, containing the substrate-binding sites, are composed of multiple α-helical transmembrane segments (TMSs) that form the transmembrane channel. The TMSs extend, in many cases, into the cytosol where they fold and form a physical interface with the NBDs [85]. The majority of ABC membrane transporters are predicted to have 6 TMSs per domain and function as homodimers, with each transport complex containing 12 TMSs in total. Although exceptions to this rule exist, most ABC transporters conform to this two-times-six TMS paradigm [86].

Despite representing the largest of the six drug transporter families, the number of ABC drug membrane proteins in *S. aureus* which have been characterized remains low. As mentioned above, the Sav1866 membrane transporter, representing the first staphylococcal drug transporter for which a structure has been determined [82], remains the best characterized of all the currently identified drug membrane transporters and is discussed in more detail below. Msr(A), initially identified on a macrolide resistance plasmid from *Staphylococcus epidermidis*, is a 488-amino acid protein which confers resistance to 14- and 15-membered ring macrolides, type B streptogramins, and telithromycin, among others [87]. Msr(A) shares high homology with Msr(C) and Msr(D), which are characterized by two fused NBDs but lack identifiable TMDs [88]. Substrate efflux is thought to occur from an interaction of its NBDs with TMDs of other chromosomally encoded ABC transporters [25]. It has also been proposed that Msr(A) does not function as a transport protein but confers resistance though a different mechanism. One possibility is that Msr(A) disassociates erythromycin, from the ribosome by inducing a conformational change which would allow the antibiotic to passively diffuse out of the cell [89].

Displaying a low amino acid sequence identity of 35 % to Msr(A), the Vga(A) protein is also plasmid encoded and, along with the variant Vga(A), and the Vga(B) and Vga(C) proteins, mediates resistance against type A streptogramins,

lincosamides, and pleuromutilins [30, 31, 33]. Like Msr(A), the Vga proteins have two NBDs but lack the TMDs, which suggests that these proteins utilize a similar mechanism of transport [13].

Finally, representing the newest addition to the *S. aureus* ABC transporters is the AbcA multidrug resistance protein [22, 64]. Displaying high similarity to ABC multidrug transporters such as the *Lactococcus lactis* LmrA, *E. coli* MsbA, and the *S. aureus* Sav1866 protein, overexpression of this transporter results in resistance against β-lactams, daptomycin, moenomycin, and dyes such as ethidium [64].

7.2.2 The ABC Transporter: Sav1866

The *S. aureus* Sav1866 multidrug transporter was the first ABC membrane transporter structure to be determined at a high resolution of 3.0 Å [28] and is used as a model for homologous human and bacterial ABC multidrug transporters [29, 90]. Although crystallized, little was known about Sav1866 and its role in transport. Functional studies have now revealed that Sav1866 can transport structurally unrelated substrates such as ethidium, tetraphenylphosphonium, and Hoechst 33342 in intact cells as well as from plasma membrane vesicles and proteoliposomes containing purified and functionally reconstituted protein [29].

Following the two-times-six TMS paradigm mentioned above, the solved crystal structure of Sav1866 revealed that this protein consisted of a dimer comprised of two elongated subunits, with each subunit containing an amino-terminal TMD and a carboxyl-terminal NBD [28]. Sav1866 is referred to as a homodimer of half transporters [91], as one TMD is fused to a NBD, which then dimerizes to form a full membrane transporter [28]. These two subunits appear to twist and embrace each other, with the NBD and TMD interacting tightly (Fig. 7.2) [28]. Adopting an outward-facing conformation, the TMD face outward, toward the exterior of the cell, while the NBD subunits are shown to be closely associated and pack around the nucleotide [84]. The two TMDs provide a substrate-translocation pathway, while the two NBDs are the involved in ATP binding and hydrolysis [82].

The exact mechanisms by which multidrug ABC transporters like Sav1866 export their substrates are still unclear, although analysis of crystal structures from members in both inward and outward conformations that are available for this superfamily has presented a possible mechanism for coupling ATP hydrolysis to transport [92]. Occurring in an outward-open conformation, the hydrolysis of ATP results in the TMDs reorienting to produce an inward-open conformation of the protein, which allows substrate and ATP binding to occur. Substrate extrusion then is mediated by the interaction of substrate and TMDs, which initiates conformational changes in both the TMDs and the NBDs, leading to an outward-open conformation. The substrate is then expelled and the transporter is reset for further transport [93, 94]. More structural, biochemical, and biophysical studies are required to elucidate the nature of these conformational changes employed by ABC transporters.

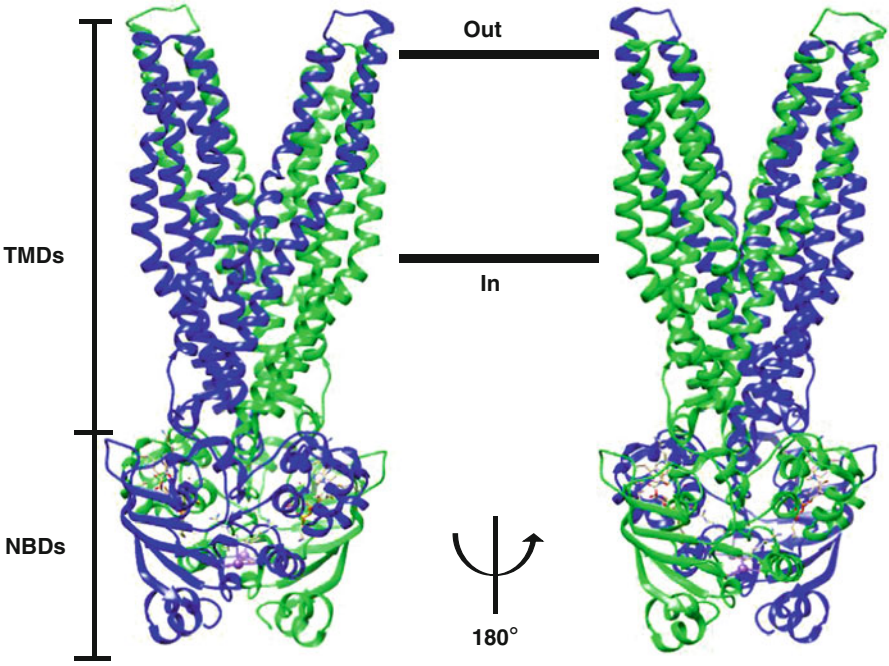

Fig. 7.2 The Sav1866 structure. The Sav1866 structure (PDB code 2HYD) is shown in an outward-facing orientation from the *front* and *back*. The two monomers of the half-transporter are colored *blue* and *green* and bound nucleotide is shown in a *purple* ball and stick representation

7.3 Secondary Active Transporters

7.3.1 The Major Facilitator Superfamily

The MFS is the largest family of secondary transporters, catalyzing the transport of a diverse range of substrates [95, 96]. As found for ABC transporters, proteins belonging to this superfamily are ubiquitously found in the membranes of all living cells [97, 98], making up ~25 % of all known transport membrane proteins in prokaryotes [96]. These proteins can act as uniporters, symporters, or antiporters, harnessing the power in the electrochemical gradient of H^+ or Na^+ ions to transport amino acids, peptides, sugars, vitamins, and drugs, among others [99]. This continually expanding superfamily is currently composed of 82 recognized subfamilies, although most of these have been classified, 17 MFS families are composed of as of yet functionally uncharacterized members and are referred to as unknown major facilitators [98].

Well-defined multidrug transporters belong primarily to three different drug:H^+ antiporter (DHA) subfamilies; DHA1, DHA2, and DHA3. Proteins belonging to the DHA1 and DHA2 subfamilies are known to efflux a wide range of structurally dis-

similar compounds and have been demonstrated to play a major role in bacterial multidrug resistance [100], while members of the DHA3 subfamily, found only in prokaryotes, are shown to efflux antibiotics such as macrolides and tetracyclines [9]. Consisting of between 400 and 600 amino acids, allocation of drug transporters to these subfamilies is primarily based on the number of TMSs. Proteins within the DHA1 and DHA3 subfamily have their amino acids arranged into 12 TMSs, while those belonging to DHA2 are comprised of 14 TMSs [98, 101]. These TMSs are connected by hydrophilic loops, with both the N- and C-termini facing the cytoplasm [102]. Members with 12 TMSs are thought to have evolved from a single 2 TMS hairpin that triplicated, giving rise to a protein with 6 TMSs which through a duplication event resulted in the formation of 12 TMSs. MFS transporters with 14 TMSs are believed to have obtained two centrally localized TMSs as a result of an intragenic duplication event of an adjacent hairpin [98].

Like members of the ABC superfamily, sequence analysis of MFS transporters has identified a number of superfamily- and family-specific sequence motifs [17, 50, 97]. High conservation of these motifs within members implies that they play a role in structure and/or function. Among these motifs, motif C, specific to antiporters, is highly conserved between efflux proteins. Positioned in TMS 5 of all antiporters, it is believed that this motif may influence or specify the direction of substrate transport [103]. Analysis of this motif in the MFS *S. aureus* tetracycline resistance efflux protein TetA(K) revealed that a number of conserved residues within this motif are important for tetracycline transport [50], with glycine residues identified as conferring conformational plasticity required for drug efflux. However, the best characterized motif is motif A, also known as the MFS-specific motif [104]. Recent structural analysis of the MFS transporter YjaR has demonstrated that residues contained in motif A sense and respond to protonation inside the central cavity [104].

Although the structures of 23 different MFS transporters belonging to a variety of MFS subfamilies have been so far elucidated (http://blanco.biomol.uci.edu/mpstruc/), only two of these are of multidrug transporters. These are the *E. coli* EmrD multidrug transporter [105] and, solved more recently, the *E. coli* MdfA drug efflux protein [106]. The MdfA structure (2.0–2.4 Å) is also the first MFS multidrug transporter to be solved in complex with its substrates, chloramphenicol, deoxycholate and *N*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide. The structure shows approximately 30 residues forming the substrate-binding cavity, of which the majority are shown to be hydrophobic. This is in line with the EmrD structure which also possesses an internal cavity of hydrophobic residues [105]. The crystal structures of these are reminiscent of other MFS-solved structures, such as those for the *E. coli* lactose permease (LacY) [107, 108] and glycerol-3-phosphate transporter (GlpT) [109], however, differ in the internal cavity, with LacY and GlpT maintaining a hydrophilic cavity [105].

Despite MFS proteins displaying low sequence similarity, different substrate specificity, and transport coupling mechanisms, MFS protein structures described to date exhibit some generalities [100]. Combining data achieved from the analysis of structures of MFS proteins in different states of the transport process,

i.e., outward-facing, occluded, and inward-facing states, the mechanism for drug translocation has been postulated [105]. According to this model, substrate translocation is assumed to be facilitated by the interconversion of the inward-and an outward-facing alternating conformation of MFS transporters, using a "rocker-like" mechanism to alternately generate a pathway of access to either surface [105, 110, 111].

Representing the largest group of identified multidrug transporters within *S. aureus*, MFS members are also among the best characterized bacterial drug transporters [13]. Included, as shown in Table 7.1, is the NorA multidrug transporter, which was one of the first chromosomally identified and characterized staphylococcal MFS proteins [38, 112]. Conferring resistance to a wide range of chemically diverse compounds such as hydrophilic fluoroquinolones, dyes like ethidium bromide and biocides, this 388-amino acid protein is a 12-TMS member of the DHA1 subfamily [113]. In addition to the NorA protein, *S. aureus* contains MFS-family drug transporters such as the NorB, NorC, LmrS, QacA, SdrM, and MdeA multidrug resistance proteins and Tet38, a tetracycline-specific efflux pump (Table 7.1).

Sharing around 30 % similarity with NorA, the NorB efflux pump confers resistance to a number of compounds, some of which overlap with the NorA substrate profile, while others are NorB specific, such as moxifloxacin and sparfloxacin [42, 114]. Additionally, NorB has been shown to influence bacterial fitness and survival in abscesses alluding to the possibility that this pump may transport other as yet unidentified natural substrates [115]. Predicted to be comprised of 14 TMSs, this protein, along with NorC and Tet38, was identified in a *S. aureus mgrA* knockout mutant [42, 116]. MgrA is a global regulator of a number of proteins including the Nor multidrug efflux pumps and is discussed in detail below. Analysis of this *mgrA* knockout mutant revealed resistance to quinolones, tetracyclines, and antimicrobial compounds, which was not conferred by the NorA multidrug efflux protein. In addition to identifying NorB, this study identified the NorC multidrug efflux pump (61 % similarity with NorB), shown to contribute to quinolone resistance [43] and the 14-TMS Tet38 transporter (26 % similarity to the tetracycline exporter TetA(K)), that exports tetracycline, in addition to certain unsaturated fatty acids [65]. The Tet38 protein has also been shown to contribute to *S. aureus* colonization of mouse skin and aid in the ability of *S. aureus* to invade and survive within epithelial cells [65, 117].

Sharing the predicted 14-TMS topology identified for the above membrane transporters, the *S. aureus* MdeA, SdrM, and LmrS multidrug efflux pumps are all chromosomally encoded and display varying degrees of similarity to other MFS proteins. The MdeA protein, initially identified from the N315 clinical strain [36], when overexpressed confers resistance to quaternary ammonium compounds (QACs) and antibiotics. Sequence analysis shows that it is most closely related (62 % similarity) to the LmrB efflux protein from *Bacillus subtilis* [36]. The SdrM membrane transporter shares 61 % sequence similarity with QacA and has been shown to confer low-level resistance to dyes such as acriflavine and ethidium as well as to the fluoroquinolone, norfloxacin [49]. Finally, representing one of the more recent additions to the *S. aureus* MFS group of multidrug efflux pumps, the

LmrS protein is involved in the increased resistance to chloramphenicol, linezolid, tetraphenylphosphonium, and trimethoprim [35].

7.3.2 *The MFS transporters: QacA and QacB*

The most widely studied multidrug efflux pumps in *S. aureus* are the chromosomally encoded NorA and the plasmid-encoded QacA and QacB membrane transporters [118]. The QacA multidrug efflux pump was the first bacterial multidrug efflux system to be discovered [67] and since has been extensively analyzed [13, 44, 68, 119, 120]. Initially identified in Australian multidrug-resistant strains of *S. aureus*, the *qacA* determinant was found to be carried on the pSK1 large multidrug resistance plasmid [67]. Classified within the DHA2 subfamily of the MFS, QacA consists of 514 amino acids that have been demonstrated to be organized into 14 TMSs (Fig. 7.3) [68]. Assessment of resistance has revealed that QacA confers resistance to a broad range of more than 30 cationic and lipophilic antimicrobials, including monovalent cationic dyes such as ethidium and pyronin Y, QACs such as benzalkonium and cetylpyridinium, and bivalent cations such as chlorhexidine and pentamidine [44–46]. QacA has been identified in clinically relevant *S. aureus* isolates across the world and has been shown to contribute to resistance to disinfectants that are widely used in medical environments and to a number of antiseptics in eye drop formulations [121, 122].

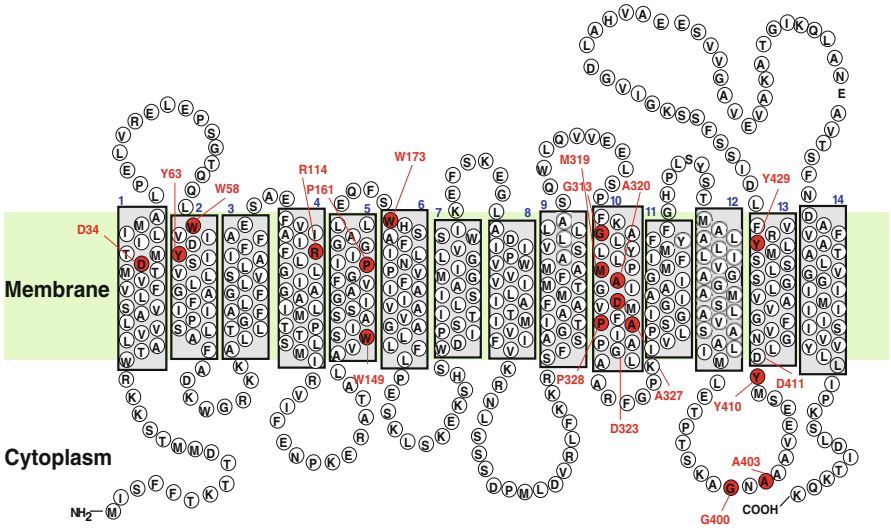

Fig. 7.3 Schematic topological representation of the *S. aureus* QacA multidrug transporter. Topology of the QacA protein is based on hydropathy analysis. The 14 TMSs are indicated in *gray*, with residues found to be functionally important highlighted in *red*

QacA-mediated resistance has been extensively assessed, with results showing that QacA uses a single antiport mechanism for the expulsion of these structurally different compounds [45, 119, 121, 123, 124]. QacA-mediated efflux is dependent on both the $\Delta\psi$ and on the ΔpH components of the proton motive force [46] and is initiated within the inner leaflet of the cytoplasmic membrane, thus preventing the drugs from entering the cytoplasm [46]. Analyses of the kinetic parameters K_m and V_{max} have revealed that QacA-mediated transport of substrates adheres to Michaelis-Menten kinetics, with low K_m values in the micromolar range for a range of QacA substrates indicating high binding affinity [46].

Closely related to the QacA membrane drug transporter and possibly a progenitor of QacA, the QacB plasmid-encoded protein was initially identified in clinical strains isolated in the early 1950s [125]. Differing by only seven base pairs, the *qacB* determinant primarily confers resistance against monovalent organic cations, thus exhibiting a more restricted substrate profile than QacA [46, 47, 125]. This difference in substrate specificity stems from an acidic residue at position 323 (Asp323) in TMS 10, which is an uncharged alanine in QacB, that affords the QacA membrane transporter the capacity to transport a wider range of substrates including bivalent cations, which QacB can only poorly transport [45, 46]. This acidic residue is proposed to play a role in the processive-like transport relay recently identified in MFS proteins for bivalent cations [126]. Not only has the QacA TMS 10 region been shown to be involved in the binding of bivalent cations [46, 120] but additionally Gly377, located in TMS 12, may also facilitate the transport of bivalent cationic substrates. Mutagenesis studies moving an acidic residue from TMS 10 to TMS 12 revealed that QacA double mutants Asp323Cys-Gly377Glu and Asp323Cys-Gly377Asp retained an overall capacity to confer resistance to bivalent cations such as chlorhexidine and dequalinium [123]. Other residues also located in TMS 10 have been found to be functionally important, as Met319 along with Asp323 forms the bivalent substrate-binding site, while Gly313 appears to be important for the extrusion of both bivalent and monovalent substrates [120].

Although residues such as Gly313 may play a role in the efflux of both monovalent and bivalent compounds, competition analyses have demonstrated that QacA possesses distinct binding sites for these compounds [46]. Fluorimetric transport assays measuring the efflux of ethidium in the presence of various nonfluorescent monovalent or bivalents substrates showed that monovalent cations such as benzalkonium competitively inhibited QacA-mediated ethidium efflux, while the addition of bivalent cations such as chlorhexidine resulted in noncompetitive inhibition. These results suggested that monovalent compounds may share the same or have overlapping binding sites, whereas bivalent compounds bind at a distinct site or sites other than those occupied by ethidium, a monovalent cation [46]. This is similar to what has been observed in the regulator of QacA, the QacR multidrug-binding repressor protein, as discussed in more detail below.

The differences between QacA and QacB, and their natural variants, have allowed for the assessment of their evolution and their ability to recognize different subsets of substrates. Initially, beginning with the discovery of the prototypical

QacA protein, carried on the pSK1 plasmid [67] and the QacB protein, carried on pSK23 [125], these two proteins are an excellent example of fortuitous mutations which can extend the substrate profile of membrane transport proteins. As such, although most likely initially being able to expel only one class of antimicrobials, a single mutation, such as exemplified by the change to a charged residue in QacA at residue position 323, is able to render membrane proteins capable of transporting new classes of compounds.

7.3.3 The Multidrug and Toxic Compound Extrusion Family

Exhibiting a membrane topology similar to the MFS, members of the MATE family are ubiquitous to all kingdoms [18]. Well-characterized MATE family multidrug efflux proteins include MepA from *S. aureus* [55, 56] and NorM from *Neisseria gonorrhoeae* (NorM-NG) [127–129] and *Vibrio parahaemolyticus* [130, 131]. Phylogenetic analysis has revealed that the MATE family is composed of 3 large families with 14 small subfamilies [132]. In addition to drug efflux of compounds including cationic dyes such as ethidium, aminoglycosides, and fluoroquinolones [129, 133], MATE family proteins are involved in basic mechanisms which maintain homeostasis, by extruding metabolic waste products and xenobiotics in nature [132, 134]. This is achieved using energy stored in either the Na^+ or H^+ electrochemical gradients.

Membrane transporters of the MATE family have between 400 and 500 amino acids and form 12 TMSs. Although no apparent consensus is observed between MATE proteins, they exhibit around 40 % similarity [134] with multiple sequence alignments revealing slightly conserved regions located in TMS 1 and TMS 7 and within six loops, two extracellular and four cytoplasmic loops. Given this symmetrical repetition of conserved regions, which is distributed between the amino and carboxyl halves of the MATE pumps, it is hypothesized that these transporters evolved from a common ancestral gene that underwent genetic duplication [132], similar to that proposed for MFS proteins.

Although determined to be clinically significant, the transport mechanism of MATE proteins is poorly understood. However, analysis of solved structures of four MATE proteins (http://blanco.biomol.uci.edu/mpstruc/), including structures of the NorM-NG transporter in apo- and substrate-bound forms [129, 131, 135], has revealed a multidrug-binding cavity which is composed largely of negatively charged amino acids, with a limited number of hydrophobic residues. At least three negatively charged amino acids of the NorM-NG transporter were determined to be involved in substrate charge neutralization, as they were essential for precluding electroneutral or negatively charged compounds from being bound and transported, thereby ensuring specificity [129].

Based on the above structural and functional assessment of the NorM-NG transporter, it was suggested that Na^+ triggers multidrug extrusion by inducing conformational changes within the protein. The binding of Na^+ to a drug-bound transporter

promotes the movement of TMS 7 and TMS 8 which causes the drug to disassociate from the binding site. The Na^+ bound, drug-free transporter then switches to an inward-facing conformation, where it can then bind to another drug. It is believed that drug binding and the subsequent movement of TMS 7 and TMS 8 weaken Na^+ binding. As a result of this change, Na^+ is released into the cytoplasm and the transporter takes on an inward-facing conformation, and then the drug-bound protein returns to the outward-facing conformation where the transport cycle is completed [129].

Although Na^+-driven, the mechanism of transport is comparable to the mechanism described for H^+-coupled transporters. In fact, structural analysis and functional assessment of DinF, a H^+-coupled MATE transporter from *Bacillus halodurans*, also identified a membrane-embedded substrate-binding site. It was suggested that drug-mediated DinF transport resulted from the direct competition between H^+ and the drug and occurred through conformational inward- and outward-facing changes [136], which, as discussed above, has also been described for proteins belonging to the MFS.

7.3.4 The MATE Transporter: MepA

The chromosomally encoded MepA protein was the first and is the only MATE family multidrug transporter to be discovered in *S. aureus* [55]. Functional analysis of this protein revealed that when overexpressed, it had a broad substrate profile (Table 7.1) that included both monovalent and bivalent biocides such as ethidium and chlorhexidine, respectively, as well as fluoroquinolone agents such as norfloxacin and ciprofloxacin [55]. The expression of *mepA* is controlled by the transcriptional repressor MepR, a MarR-family member (see below).

Analysis of MepA residues critical for substrate binding and/or translation revealed that like many drug efflux pumps whose substrates are cationic, negatively charged residues play a key role in the substrate-translocation pathway, which in MepA are Glu156 and Asp183, located in TMS 4 and TMS 5, respectively. However, the exact nature of their participation in this process is still to be confirmed [57, 137]. In addition to these, within the substrate-translocation pathway, which *in silico* modeling has revealed to be formed from TMSs 1, 2, 4, 7, 8, and 10 coming together and forming a central cavity, residues Ser81, Ala161, Met291, and Ala302 have been identified as playing a role in substrate interaction. Further examination of this model also showed a residue unique to the MepA protein, Glu295 located in TMS 8, whose side-chain projected into the putative central cavity. Site-directed mutagenesis studies assessing the possible function of the residue revealed that the size and not the charge of the residue played an important role in the function of MepA, as a substitution with residues smaller than Glu resulted in a reduction in function [137]. Other functionally important residues, which are located outside of the putative central cavity and lying within TMSs 7–11 and the intervening cytoplasmic loops, include Lys242, Asn308, Met312, Asn369, Phe375, Met391, Ala392, and Ala397.

7.3.5 The Small Multidrug Resistance Family

Prior to the discovery of the PACE family, the SMR family represented the smallest proteins of the membrane transport system. The SMR family can be separated into three subfamilies: the small multidrug pumps (SMP), paired small multidrug resistance pumps (PSMR), and suppressors of *groEL* mutations (SUG); however, phylogenetic analysis of SMR proteins shows that they are subdivided into two phylogenetic clusters, with only one of these being able to catalyze drug export [138, 139]. As drug transport has not been demonstrated for a number of proteins within the PSMR subfamily, members of this subfamily are spread throughout the two clusters mentioned above. Thus, of the two identified clusters, the largest one is composed of SMR proteins including the multidrug exporters QacC from *S. aureus* and EmrE from *E. coli*, which are grouped with QacE, QacEΔ1, QacF, QacH, and QacJ.

By assessing the diversity and evolution of the SMR family, in search for a common SMR progenitor, it was revealed that this family underwent a high frequency of lateral gene transfer and rapid sequence divergence, giving rise to the current variety of SMR proteins [138]. The emergence of PSMR proteins is thought to have occurred through gene duplication events, explaining the shared relationship that some of these proteins exhibit with both the SMR and SUG subfamilies. Additionally SMR-family proteins display regions of conservation with other larger metabolite and multidrug membrane transporters. Fusions of TMSs of SMR proteins and sequence rearrangements are believed to have contributed to the formation of the bacterial/archaeal transporter family containing proteins with five TMSs, which later led to the formation of the drug/metabolite efflux family [102, 140].

Frequently identified on mobile elements, such as integrons and plasmids, these proteins are typically composed of only 100–150 amino acids and form four TMSs [140]. As found for all other membrane transport families, multiple sequence alignments of SMP-subfamily proteins can reveal a number of signature motifs [138, 139]. These motifs lie in each of the predicted TMSs with the highest residue conservation observed in TMS 1 [139]. Assessment of this TMS revealed that a single negatively charged residue, Glu14, is highly conserved in SMR members, and is essential for drug transport [141–144].

Members of the SMP subfamily confer low-level resistance to a variety of antimicrobial agents, including a number of QACs such as benzalkonium and tetraphenylphosphonium in addition to toxic lipophilic cations such as DNA intercalating agents. They have also demonstrated to have the ability to extrude potentially toxic metabolites such as nicotine intermediates out of the cell [139, 140].

Members of the SMR family are known as proton-coupled transporters and are proposed to function as dimers [139, 145, 146]. Currently the only solved structure of a SMR-family protein is that of the *E. coli* EmrE multidrug transporter, which has been determined at a resolution of 7.0 Å [147] and 3.8 Å [148]. According to the earlier structure, EmrE is an asymmetric homodimer that consists of a bundle of eight TMSs with one substrate molecule that is bound near the center. Such

asymmetry was also seen in the crystal structure of EmrE in complex with tetraphenylphosphonium, which showed the asymmetrical arrangement appeared to stem from an antiparallel topology within the homodimer [148]. This antiparallel orientation proposed for EmrE was surprising, as membrane proteins generally insert in one particular orientation and follow the "positive-inside rule" [149]. However, SMR proteins like EmrE are proposed to form dual-topology dimers with the subunits having an inverted topology. This could be due to the fact that EmrE appears to have a weak charge-bias and as such may not conform to this rule [150]. It is possible through genetic manipulation to force EmrE into one or the other orientation, although this does limit the formation of an active transport protein [150].

In addition to QacC, proteins such as QacEΔ1, QacG, QacH, and QacJ have been identified on plasmids within a number of antimicrobial resistant bacteria, including various *S. aureus* strains isolated from humans [59] and animals [61, 151] and in the food industry [62, 63, 152]. The Qac nomenclature given to these proteins is based on their ability to confer host resistance against QACs [153]. Assessment of resistance has shown that despite conferring resistance to a wide range of QACs and cationic dyes, Qac proteins display their own unique resistance profiles for specific compounds. For example, high levels of resistance to ethidium have been determined for QacH and QacEΔ1, but not for QacG. However, QacG has been shown to confer higher levels of resistance to cetyltrimethylammonium bromide than QacH [139]. Displaying distinct substrate ranges, analysis of human staphylococcal clinical isolates has shown that several *qac* genes coexist in some isolates and appear to act synergistically to remove different compounds from the bacterial cells [154].

7.3.6 The SMR Transporter: QacC

The QacC multidrug transporter was the first SMR-family member identified. It was originally cloned from *S. aureus* strains resistant to disinfectants such as ethidium and benzalkonium chloride [155]. The *qacC* determinant was found to be located on a small 2.4 kb plasmid identified as pSK89, isolated from an Australian hospital [156]. Homology with pSK89 was also identified with the larger pJE1 and pSK41 plasmids, suggesting that these too carried resistance determinants which were similar or identical to the determinant responsible for the resistance attributed to QacC; as such this determinant was referred to as *qacD*. Although initially assumed that the *qacC* gene stemmed from *qacD*, similar to that seen with the QacA/B relationship, it was later shown that that these genes were identical and thus encoded one protein only, QacC [157]. In addition to QacD, the QacC membrane protein has also been referred to as Smr (staphylococcal multidrug resistance) [157] and Ebr (ethidium bromide resistance protein) [158, 159].

Comparative analysis of the QacC amino acid sequence with other drug efflux proteins such as QacA, the *B. subtilis* Bmr, and *E. coli* TetA proteins revealed low sequence identity implying an independent origin for the *qacC* gene [157]. Further analysis revealed that QacC shared high sequence similarity with proteins belonging

to a family of small hydrophobic proteins, which were all encoded by short open reading frames (~330 bp). Of these, QacC was shown to be most closely related to the *E. coli* MvrC protein, now known as EmrE, showing a 42 % sequence identity [157, 160]. In addition to displaying high homology with EmrE, QacC was also shown to contain a number of highly conserved residues identified in other SMR proteins, including Glu13. Mutagenesis of this residue revealed that a substitution of Glu13 with aspartic acid or glutamine resulted in host cell susceptibility to benzalkonium [141]. This highly conserved carboxylic residue, found to be putatively located within the first transmembrane domain of many SMR proteins, has since been extensively analyzed and shown to be essential for drug/proton binding and translocation in SMR-family proteins [139, 161].

Composed of 107 amino acids, topological assessment of QacC, initially investigated by the construction and analysis of a series of *qacC-phoA* and *qacC-lacZ* fusions, supported the proposed four TMS topology seen for SMR proteins (Fig. 7.4) [58]. In addition, the orientation of the N-terminus was shown to be cytoplasmic; however, the location of the C-terminus could not be unequivocally determined. Further analysis, using NMR confirmed that QacC is composed of four TMSs, with residues 6–23 contained in TMS 1, 32–40 TMS 2, 60–71 TMS 3, and 94–101 TMS 4 [162]. As with other members of the SMP subfamily, the QacC functional unit is postulated to be dimeric.

Functional analysis of the QacC multidrug transporter revealed that although possessing a more limited substrate range than QacA, it can confer host resistance

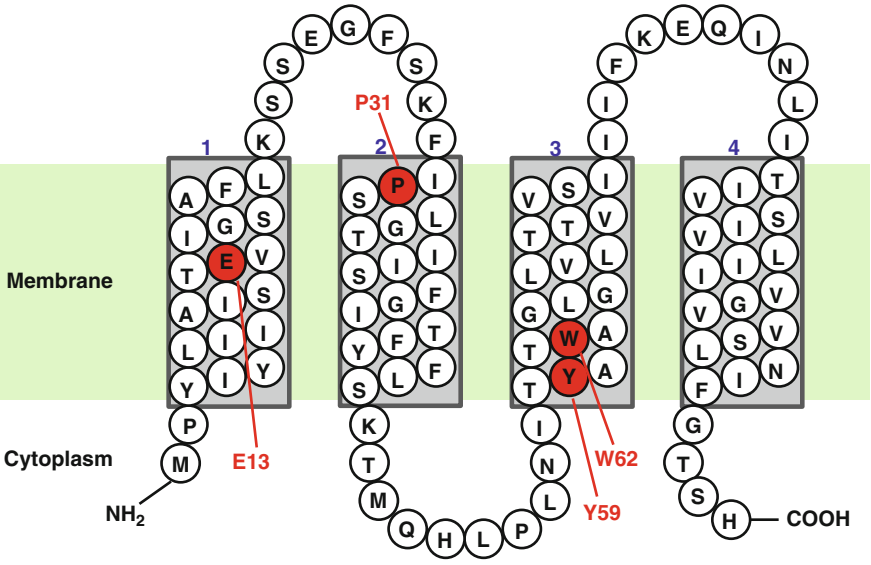

Fig. 7.4 Schematic topological representation of the *S. aureus* QacC multidrug transporter. Secondary structure of the QacC membrane transport protein is based on hydropathy analysis. Residues shaded in *red* have been found to be essential for proper QacC function, as analyzed by site-directed mutagenesis [58, 157]

to a broad spectrum of antimicrobials including QACs such as cetyltrimethylammonium bromide, cetylpyridinium, benzalkonium, and dequalinium, as well as dyes such as crystal violet, ethidium, proflavine, pyronin Y, and rhodamine 6G [58]. In addition to the essential Glu13 residue, site-directed mutagenesis revealed that two other highly conserved residues, Tyr59 and Trp62, were essential for function [58]. Furthermore, the conserved proline at position 31 was found to be essential for full function among members of the SMR family, as substitutions of this residue with either glycine or alanine residues, which carry similar side-chain volumes resulted in a reduction in resistance to both crystal violet and ethidium, suggesting that Pro31 may reside in an region of the protein that is integral in determining QacC substrate specificity [58]. Finally, Glu24, located in a loop region, was shown to be involved in substrate specificity [157], while Cys42, located within TMS 3, was shown to play a role in substrate recognition and could only be replaced with threonine to maintain wild-type function [58].

7.4 Regulation of Staphylococcal Drug Efflux Pumps

The regulatory mechanisms used for the expression of multidrug transporters are complex, intertwined, and not completely understood. Regulation of multidrug efflux pumps can occur at global and specific levels, or not at all, as some pumps appear to not be under the control of regulatory proteins. Regulation is complex, as global regulatory proteins can themselves be modulated by other proteins and/or small regulatory RNAs. Global regulatory proteins are defined by their ability to modulate operons belonging to different metabolic pathways [163, 164], while specific regulators, also known as substrate-responsive regulators as they commonly bind to the substrates of the multidrug efflux pumps, can act as transcriptional activators or repressors for individual pumps [118]. All of these regulatory mechanisms are at play in *S. aureus* providing a means that enable the bacteria to adapt to changing environments, including the presence of antimicrobial agents.

Within *S. aureus*, the global regulator MgrA, also known as Rat or NorR, regulates the expression of a number of genes in the *mgrA* regulon including those encoding virulence factors and multidrug resistance efflux pumps such as NorA, NorB, and NorC [42, 43, 165]. MgrA binding itself is regulated by phosphorylation/dephosphorylation by Stk1 and RsbU, respectively [165]. Recently, the importance of MgrA has been extended, as it was shown that small RNAs, namely, RNAIII, stabilize *mgrA* mRNA by interacting with it, leading to its increased production. RNAIII is integral to the regulation of virulence genes affected by the *agr* quorum-sensing system in *S. aureus* [166]. Possessing a helix-turn-helix motif involved in DNA binding, the MgrA protein is a small transcriptional regulator that functions by directly binding to the promoter region of its target [167, 168]. In *S. aureus*, it can function both as an activator and a repressor, as it can downregulate the expression of NorA and upregulate the expression of NorB [168].

Regulation of efflux pumps can also be carried out at a local level. In addition to the MgrA global regulator, NorA, NorB, and NorC are also regulated by a specific regulator, NorG, which also controls the ATP-dependent membrane protein, AbcA [169]. Among the *S. aureus*-specific regulators, and one of the best characterized, is the QacR multidrug-binding protein that represses the transcription of the *qacA*/*qacB* multidrug transporter genes [170, 171]. QacR functions as a pair of dimers by binding in the absence of QacA substrates to an inverted repeat (IR1) DNA sequence which overlaps the *qacA*/*B* transcriptional initiation sites, resulting in repression of *qacA*/*B* transcription [172]. Assessment of the substrate recognition profile of QacR has revealed that it binds a number of structurally dissimilar cationic lipophilic antiseptics, disinfectants, and cytotoxins including crystal violet, ethidium, and rhodamine [173, 174], all of which are also QacA substrates. Binding of these substrates induces a coil-to-helix conformational change in QacR which renders the protein unable to bind IR1, triggering its release from the *qacA*/*B* promoter region and allowing transcription to occur [172, 175].

Much of what is known pertaining to the structure and function of QacR comes from high-resolution crystal structures of QacR bound to ten structurally diverse compounds [171, 172, 176], including one structure of QacR bound simultaneously to two different drugs [177]. These structures revealed that QacR contains two separate but overlapping drug-binding pockets, which exist in one extended binding site. The orientation of these two pockets, referred to as the "ethidium" and "rhodamine 6G" pockets, shows that the ethidium pocket partially overlaps the rhodamine 6G pocket and lies closer to the proposed drug-binding "portal" entrance than the rhodamine 6G pocket [172]. The volume of the extended binding pocket, when drug-free, is under 400 $Å^3$; however, this expands to 1,100 $Å^3$ when bound to substrate [172], exhibiting immense flexibility. The binding pocket itself is rich in aromatic residues and contains acidic residues which play a role in substrate discrimination by affecting the positioning of the drugs within this pocket [172, 178]. The aromatic residues also play a vital role in the QacR-induction mechanism, whereby drug binding triggers a conformational change of QacR by initiating a coil-to-helix transition of residues Thr89 through to Tyr93, resulting in the elongation of the C-terminus of helix 5 by a turn. This results in the expulsion of Tyr92 and Tyr93 from the interior of the protein leading to the formation of the large multidrug-binding pocket [171, 172, 178]. Upon this conformational change, the relocation of the DNA-binding domain is instigated leading to the QacR protein no longer being able to bind to the IR1-operator site, resulting in derepression of *qacA* [178]. The presence of these distinct pockets provides an explanation as to why QacR is able to bind both charged and neutral compounds as well as two different drugs simultaneously [172]. However, the exact mechanism by which QacR is able to recognize such a wide variety of compounds is still unknown [173]. This is reminiscent of what is seen in the structure and functional analyses of multidrug transporters to date, as it appears that proteins able to bind multiple compounds generally have a pocket where subsets of residues, commonly charged or aromatic in nature, interplay and interact with each individual substrate.

Regulation of the MepA pump by the MepR repressor protein provides the only known example of how a MATE pump is regulated in bacteria. Like the above discussed global regulator MgrA, MepR is a MarR-family repressor that is encoded upstream of *mepA* [55, 56, 179, 180]. MepR binds to a seven-base-pair signature sequence contained within operator regions upstream of both *mepR* and *mepA* where the *mepR* operator contains one repeat and that for *mepA* two, resulting in MepR displaying a higher affinity for the *mepA*-operator site. Mutations in the *mepA*-operator site can impede MepR binding and thus produce an elevated expression of MepA [181]. MepR repression can be relieved by the interaction of MepR with cationic, hydrophobic agents resulting in dissociation of MepR from the DNA. Although the majority of MepR ligands are substrates of MepA [179, 181], some selectivity is seen with bis-indole compounds [182]. MepR-inactivating substitution mutants have been reported among bloodstream isolates with reduced susceptibilities to biocides and fluoroquinolones [183]. Analysis of these variants located these mutations to a linker region between the dimerization and DNA-binding domains rather than in the DNA-binding domain per se; three of these have been crystallized and examined in detail [184]. These structures also show MepR possesses a very open conformation that can accommodate multiple ligands likely to influence the structural and allosteric changes of MepR [185].

Analysis of regulators such as those mentioned above is vital, as they provide valuable insights into the substrate range of the transport proteins that they regulate. Additionally, a detailed understanding of the complex nature of how multidrug exporters are controlled and respond to the changing environment that bacteria inhabit may allow for new strategies to be devised to overcome these survival mechanisms and lead to new treatments.

7.5 Concluding Remarks

The importance of multidrug transporters in antimicrobial resistance has clearly been established. With their wide substrate specificity and ability to produce elevated levels of resistance when overexpressed, they contribute to the high level of resistance exhibited by *S. aureus*. For bacteria such as *S. aureus* lacking an outer membrane, drug efflux pumps may also serve as a first line of defense before other more established mechanisms can be established, allowing for survival in a hostile environment [118]. Given its classification as an opportunistic nosocomial pathogen [186–188], the long-term exposure of *S. aureus* to hospital biocides may also have significantly contributed to its acquisition of numerous membrane drug transport proteins. Such advantageous acquisition can be seen in the prevalence of the *qacA*, *qacB*, and *qacC* determinants, identified in isolates from environments where decontamination with biocides is in use [189, 190], particularly as these genes are commonly carried on mobile genetic elements.

Their prevalence in *S. aureus* and contribution to resistance has made multidrug efflux pumps, such as those discussed in this review, targets for the development of

inhibitors which, when successful, would restore the activity of the antimicrobial agent [133]. Although inhibition studies have led to the development of compounds capable of inhibiting such pumps, for example, inhibition of the Tet efflux pumps [191–193], their development is challenging and complicated by the need to combine them with antimicrobial agents that show similar pharmacokinetic profiles [133]. Thus, the key to the development of inhibitors lies in the understanding of the molecular mechanism of multidrug transporters and highlights the importance of structural and functional analysis as it is through the detailed understanding of their mechanism that effective inhibitors can be designed.

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Chapter 8
Antimicrobial Drug Efflux Pumps in Other Gram-Positive Bacteria

Natalya Baranova and Christopher A. Elkins

Abstract Gram-positive bacteria have a more ancient and primitive membrane structure than their Gram-negative counterparts which generally results in higher levels of intrinsic susceptibility to various lipophilic and amphiphilic antimicrobial drugs. Nonetheless, these bacteria encode similar numbers of efflux pumps in their respective genomes. In this chapter, we provide a historical overview of the identification and current understanding of such systems in Gram-positive genera of practical and industrial significance – including some clinically relevant organisms not covered elsewhere in this book. In general, these systems have been less thoroughly investigated than their Gram-negative counterparts with respect to transporter and substrate identification and their associated regulation. However, some key findings in the progression of the bacterial drug efflux field were first identified in less clinically relevant organisms such as *Bacillus subtilis* and *Lactococcus lactis*. Given this framework, the physiological relevance of efflux has become increasingly significant with concepts involving the innate immune response, metabolites, and bactericidal host-derived resistance and "natural" substrates.

Keywords Gram-positive bacteria • *Bacillus* • *Clostridium* • *Enterococcus* • *Lactococcus* • *Listeria* • *Streptococcus* • Antimicrobial resistance • Multidrug transporters • P-glycoprotein • ABC superfamily • Major facilitator superfamily

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8.1 Introduction

Model Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* encode a number of membrane efflux pumps that are responsible for significant levels of resistance to a variety of noxious compounds. In the *E. coli* genome alone, there are approximately 37 efflux pumps that belong to five different phylogenetic families and represent approximately 9 % of the encoded transporters [1–3]. The main constitutive pump, AcrB, covered in-depth elsewhere in this book, is a member of the resistance-nodulation-cell division (RND) superfamily and is a part of a system of three proteins that spans the inner membrane, periplasm, and outer membrane to coordinate efflux of amphiphiles simultaneously across both membranes into the extracellular milieu. This archetype complex alone is responsible for significant levels of efflux of several classes of antibiotics (β-lactams, macrolides, fluoroquinolones, etc.), dyes, natural and synthetic detergents (including bile acids), organic solvents, and even steroid hormones [4–6]. Furthermore, *E. coli* also encodes five other systems in this phylogenetic family that are poorly expressed except under specialized conditions, usually as a result of upregulation via two-component sensor kinases. Such systems have been extensively characterized in *P. aeruginosa*, a more clinically significant microbe, but are slightly more complex. In fact, several Gram-negative bacteria of various genera have been shown to chromosomally encode AcrB homologs [7].

On the contrary, Gram-positive efflux-based resistance is less well reported in the literature. Nevertheless, genomic analysis suggests that Gram-positive bacteria encode as many putative multidrug transporters as Gram-negative. Thus, the *Enterococcus* genome reveals 34 potential drug efflux-related genes [8]. Likewise, further comparative genomics of 11 Gram-positive bacteria of importance to the health and food industry is generally similar to *E. coli* in genomic prevalence ranging around 10 %, the exception being *Bacillus subtilis* in which 17 % of its transporter cadre is putatively dedicated to drug and toxic compound extrusion [1]. Gram-positive bacteria have a cell envelope that consists of a single phospholipid bilayer, surrounded by a thick layer of peptidoglycan. Consequently, unlike multidrug transporters of Gram-negative bacteria which often assemble into multicomponent complexes designed to span both membranes, multidrug transporters of Gram-positive bacteria have only a single transmembrane component. Since the archetypal RND superfamily transporter, the major clinically relevant superfamily of multidrug transporters in Gram-negative bacteria, consists of three components spanning both inner and outer membrane, it was believed for a long time that there are no RND-type multidrug transporters in Gram-positive bacteria. In 2001, this view was modified when YerP, a transporter of the RND superfamily, was identified in *B. subtilis* [9]. However, the number of multidrug efflux pumps belonging to RND superfamily in Gram-positive bacteria (except mycobacteria) is generally very limited [10]. Four other efflux families or superfamilies found in Gram-negative bacteria are well represented in Gram-positive bacteria: the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the multidrug and

toxic compound extrusion (MATE) family, and the ATP-binding cassette (ABC) superfamily [11]. Considering the vast difference in membrane structure between Gram-negatives and Gram-positives [12, 13], it is important to determine whether such proteins can contribute to similar levels of intrinsic resistance in this, a more ancient division of bacteria.

Identification of multidrug transporters remains quite a challenge even today, in a post-genomic era. Multidrug transporters lack definitive signatures for substrate specificity, so while it is possible to identify putative multidrug transporters by analysis of the genomic DNA sequence, their ability to transport multiple unrelated compounds has to be confirmed experimentally. Thus, putative SMR family proteins PsmrAB were cloned in *E. coli* from metagenomic DNA from a halophilic environment and were found to function as a two-component Na^+/H^+ antiporter, rather than involved in resistance to drugs [14]. Also, often, multidrug transporters are not expressed under physiological conditions, and whereas some of them are activated by their substrates, for many, the activator(s) are still not known. These transporters need to be overexpressed in order to confirm their identity, but for many bacteria, the difficulties in culturing or genetic manipulation and the availability of genetic tools make overexpression or disruption of the gene very difficult. In this chapter, we review the presently characterized multidrug efflux pumps in Gram-positive bacteria.

8.2 *Bacillus subtilis*

B. subtilis, while of little clinical importance, is an excellent model organism, easily cultured and with a lot of genetic tools available. In addition, *B. subtilis* has significant genomic abundance of multidrug transporters relative to other Gram-positives [1]. For these reasons, these *B. subtilis* transporters have been extensively studied. In fact, the phenomenon of bacterial multidrug resistance was first discovered in *B. subtilis* [15]. Authors hypothesized the presence of a mechanism analogous to the mammalian multidrug transporter, P-glycoprotein. Indeed, multidrug-resistant cells were obtained after selection with increasing concentrations of one of the substrates of P-glycoprotein, rhodamine 6G. These cells exhibited resistance to some other known substrates of P-glycoprotein, such as ethidium bromide, chloramphenicol, and puromycin, as well as to tetraphenylphosphonium and cetyltrimethylammonium bromide, which are not transported by P-glycoprotein [15]. The mechanism of resistance was shown to be efflux based and was sensitive to the same inhibitors, reserpine and verapamil, as mammalian P-glycoprotein. These cells were used to clone the first bacterial multidrug transporter, Bmr, whose gene was found to be amplified in resistant cells. Analysis of the Bmr sequence, however, showed little similarity with P-glycoprotein. Indeed, Bmr is a multidrug transporter of the MFS and is very different from an ABC transporter P-glycoprotein. It was shown to use a different energy source – secondary-active transport with the transmembrane pH gradient – whereas P-glycoprotein couples the transport of substrates with

primary-active ATP hydrolysis [16, 17]. Later, a second multidrug transporter of the MFS, Blt, was identified in *B. subtilis* [18].

Blt is 51 % identical to Bmr and transports a similar set of compounds; however, the pattern of their expression is quite different. Bmr is expressed under standard cultivating conditions and is further regulated by BmrR, a member of the family of MerR-like transcriptional activators [19]. BmrR activates the expression of Bmr after binding its substrates. In contrast, Blt expression is normally not detectable. It is regulated by BltR [18], which is related to BmrR, but has a different inducer-binding domain, and its substrates are not yet known. Blt is cotranscribed with a downstream gene encoding spermine-spermidine acetyltransferase, indicating physiological function(s) apart from synthetic drug resistance per se – a theme central to efflux systems in Gram-positive and Gram-negative systems alike [11, 20]. Another layer of regulation was reported later for Bmr and Blt [21]. Their expression was found to be further controlled by a MerR-type regulator Mta. Apo-Mta acted as a repressor of the *bmr* and *blt* gene transcription. Although Mta inducer was not identified in this report, Mta was converted into transcriptional activator by the removal of the C-terminal inducer-binding domain. The authors proposed that this removal mimics the binding of inducer to Mta [21].

Several more multidrug transporters were identified in *B. subtilis*. In 1996, a stunning discovery was made of a first bacterial multidrug transporter of the ABC family, LmrA from *Lactococcus lactis* [22]. LmrA was homologous to both halves of the mammalian P-glycoprotein which is arranged in a 6 + 6 transmembrane motif [23] common to many multidrug transporters of different families [24]. Subsequently, two multidrug transporters of ABC family, BmrA and BmrC/BmrD, which functions as heterodimer, were identified in *B. subtilis*. BmrA was first identified from genome sequencing of *B. subtilis* [25] and demonstrated to transport Hoechst33342 (a fluorescent dye used to stain DNA), doxorubicin, and 7-aminoactinomycin D in highly enriched inverted membrane vesicles from *E. coli* [26]. BmrC/BmrD was likewise shown in the same system to transport Hoechst 33342, doxorubicin, BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, fluorescent compound) and mitoxantrone [27]. BmrC/BmrD expression is regulated, first, by the main transcription phase regulator AbrB, and second, via a dedicated ribosome-mediated transcriptional attenuation mechanism that requires the *bmrB*-encoded leader peptide [28]. Another substrate-specific ABC transporter deserves mentioning here because of its clinical relevance. BceAB is positively regulated by BceRS two-component regulatory system and contributes to intrinsic resistance to bacitracin [29, 30].

Additional work identified Bmr3 [31], a member of the MFS. Bmr3 was shown to transport puromycin, norfloxacin, and tosufloxacin which are also substrates of the Bmr and Blt. Bmr3 expression is growth phase dependent and is drastically reduced as the cells enter late log phase. A spontaneous multidrug-resistant mutant selected by puromycin exhibited an increased stability of *bmr3* transcripts [32]. In addition, MdtP is a multidrug transporter of the MFS that contributes to resistance to actinomycin, fusidic acid, novobiocin, and streptomycin [33]. MdtP expression is induced by its substrate fusidic acid, which binds to repressor MdtR (whose

encoding gene is cotranscribed with *mdtP*) and causes its dissociation from the *mdtP* promoter. YerP is the sole RND-type multidrug transporter characterized in Gram-positive bacteria (except mycobacteria) and has been shown to be involved in resistance to acriflavine, ethidium bromide, and surfactin, a cyclic lipopeptide biosurfactant synthesized by some species of *B. subtilis* [9]. Finally, EbrAB is a paired multidrug transporter which belongs to the SMR family and functions as heterooligomer. Its overexpression in *B. subtilis* confers resistance against acriflavine, ethidium bromide, pyronine Y, and safranin O [34].

8.3 *Clostridium difficile*

C. difficile is a major cause of nosocomial diarrhea. It is also implicated in 95 % of pseudomembranous colitis cases [35]. This species is intrinsically less susceptible to antibiotics, in particular β-lactams, fluoroquinolones, chloramphenicol, and lincosamides, than the other clostridia. Active efflux was long thought to be responsible for this resistance; however, the first description of multidrug transporter from *C. difficile* dates to 2004, when Dridi et al. [36] characterized CdeA, an MATE family transporter. When overexpressed in hypersensitive strain of *E. coli*, CdeA caused resistance to acriflavine, ethidium bromide, ciprofloxacin, and norfloxacin. CdeA was shown to cause energy-dependent efflux of ethidium bromide in *E. coli* cells, and quantitative reverse transcription-PCR assay showed that *cdeA* expression in *C. difficile* was significantly increased by exposure to ethidium bromide, but not to ciprofloxacin. The authors could not test the effect of CdeA inactivation in *C. difficile*, due to inability to genetically transform this species. The same year, Lebel et al. [37] identified four genes in *C. difficile* encoding putative proteins homologous to NorA from *Staphylococcus aureus*, a multidrug transporter of the MFS. Of these sequences, only one, designated *cme*, conferred resistance to ethidium bromide, safranin O, and erythromycin when expressed in *Enterococcus faecalis*. It was not known whether the lack of effect of the other three open reading frames was due to inefficient expression in a different species, to inappropriate substrates, or to inactivity of these open reading frames as multidrug transporters.

8.4 *Listeria monocytogenes*

L. monocytogenes is an important food-borne pathogen that can cause such severe diseases as septicemia, meningitis, stillbirth, and abortion. High-risk groups include immunocompromised patients, neonates, and pregnant women [38]. Infection with *L. monocytogenes* causes significant mortality and morbidity in these groups. *L. monocytogenes* invades host cells and replicates within their cytoplasm [39]. Acquired antimicrobial resistance in *L. monocytogenes* is a very rare event. However, this pathogen is intrinsically resistant to several antimicrobial agents and also to the

bile which has bactericidal properties and to which it is exposed during several stages of its lifecycle in the human gastrointestinal tract. In addition, multidrug transporters play a fascinating role in interaction of *Listeria* with mammalian innate immunity during its infection cycle. For these reasons, information about multidrug transporters in *Listeria* is highly clinically significant.

The first multidrug transporter in *Listeria* spp., MdrL, was identified serendipitously while sequencing genomic region around a gene encoding a putative histone-like protein, *flaR*, in an effort to find genes implicated in its regulation [40]. The authors identified a gene similar to a number of multidrug transporters of the MFS. Disruption of the allele in the wild-type strain of *L. monocytogenes* resulted in a small but reproducible increase in susceptibility to erythromycin, josamycin, clindamycin, and heavy metals and about a tenfold increase in susceptibility to cefotaxime. Functional characterization of MdrL as an efflux pump was confirmed by observing reserpine-dependent inhibition of ethidium bromide efflux, which was virtually eliminated in the MdrL disruption mutant.

Two other multidrug transporters of MFS family present in *L. monocytogenes*, MdrM and MdrT, were identified based on predicted protein sequence similarity [41]. To date, no experimental work addressing directly and conclusively their function as multidrug transporters has been published, but there is a significant indirect supporting evidence. The expression of these genes, as well as of *mdrL*, is induced by common multidrug transporter substrates. MdrL, MdrM, and MdrT were shown to be regulated by repressors LadR, MarR, and BrtA (previously TetR), respectively [42–44]. These repressors are encoded adjacently to the corresponding multidrug transporter genes. The expression of MdrL is induced by rhodamine 6G in the LadR-dependent fashion [43]. The transcription of *mdrM* and *mdrT* is also upregulated in response to rhodamine 6G and tetraphenylphosphonium, although the involvement of the aforementioned repressors in their activation by these compounds has not been demonstrated [42]. Cholic acid, another common multidrug transporter substrate, was shown to bind BrtA and cause its dissociation from the *mdrT* promoter, resulting in the induction of *mdrT* transcription [44]. Moreover, MdrT was shown to transport cholic acid out of the cells [44]. There is significant cross-regulation among these genes. A *ladR* mutant upregulates not only *mdrL* but also *mdrM* [42]. In response to the bile acid and cholic acid, BrtA upregulates not only *mdrT* but *mdrM* as well [44].

The most fascinating function of MdrM and MdrT was described in the Portnoy laboratory [42, 45]. The authors showed that these multidrug transporters control the magnitude of the host cytosolic innate immune response to *L. monocytogenes*. On the entry into the host cytosol, *L. monocytogenes* activates host response that leads to transcription of dozens of genes, including robust expression of interferon beta (IFN-β) [46, 47]. MdrM and MdrT expression was shown to affect the induction of IFN-β in infected macrophages [42, 48, 49]. Disruption of *mdrM* [42] or *mdrT* in the strain with mutated *brtA* [49] decreased IFN-β production, while overexpression of either MdrM or MdrT resulted in increased induction of IFN-β in infected macrophages [42]. The molecule that triggers the cytosolic host response was shown to be the cyclic dinucleotide c-di-AMP [45]. This molecule is produced by many bacteria and is a second messenger that is implicated in a variety of

functions including cell wall metabolism, potassium homeostasis, DNA repair, and control of gene expression [50]. C-di-AMP in *L. monocytogenes* is secreted by MdrM and MdrT [45]. It is sensed by the cytosolic innate immune receptor, STING [51]. Stimulation of this pathway results in the activation of the interferon regulatory factor-3 and nuclear factor-kB transcription factors and, ultimately, to host transcriptional activation of IFN-β [46, 51]. While innate immune system is indispensable for defense against microbial pathogens, paradoxically, the production of IFN-β increases the bacterial burden and lethality of *L. monocytogenes* infection in mouse models [52–54], through mechanisms that are not well understood, but may involve the enhanced susceptibility of lymphocytes to apoptosis in response to a pore-forming toxin and a major virulence factor of *L. monocytogenes*, listeriolysin O [53, 54].

Finally, AnrAB is an ABC-type multidrug transporter that was isolated by screening for nisin-sensitive mutants of *L. monocytogenes* [55]. A mutant strain exhibited enhanced susceptibility to nisin, gallidermin, cefuroxime, cefotaxime, ampicillin, penicillin G, and bacitracin.

8.5 *Lactococcus lactis*

L. lactis is broadly used for food manufacturing. Despite a few case reports of *L. lactis* being an opportunistic pathogen [56], it is a generally regarded as safe organism. However, the "resistance gene reservoir" hypothesis suggests that beneficial and commensal bacterial populations in gastrointestinal tract may play a role in horizontal transfer of antimicrobial resistance to pathogenic microorganisms [57].

Initially, only secondary, proton motive force-driven multidrug transporters were described in bacteria, when, in 1994, Bolhius et al. [58] reported isolation of three mutants of *L. lactis*, selected for resistance to high concentrations of ethidium bromide, daunomycin, or rhodamine 6G. These mutants were found to be cross-resistant to a number of structurally and functionally unrelated drugs, such as quinine, actinomycin D, and gramicidin D. The drug resistance of these strains was due to energy-dependent efflux and was inhibited by reserpine, a multidrug efflux pump inhibitor. Efflux was also inhibited by orthovanadate (an inhibitor of ATP-dependent efflux activity characteristic of ABC transporters) in one of the strains, and in two others, it was partially inhibited both by orthovanadate and by nigericin (an ionophore). This observation suggested that a proton motive force-dependent and ATP-dependent systems were involved in drug efflux. A year later, a lactococcal proton motive force-dependent multidrug efflux pump, LmrP, was characterized in the same laboratory [59]. *LmrP* was cloned in *E. coli* and was shown to belong to the MFS. In *E. coli*, its substrates included ethidium bromide, daunomycin, and tetraphenylphosphonium, which were transported in a proton gradient-dependent manner. Overexpression of *lmrP* in *L. lactis* resulted in elevated resistance to ethidium bromide; however, an *lmrP* deletion mutant was only slightly more susceptible to ethidium bromide than the wild-type strain. The resistance of the *lmrP*-deficient strain to ethidium bromide could be significantly decreased by treating the cells

with orthovanadate. This observation confirmed that an ATP-dependent multidrug transporter was functional in *L. lactis*.

In 1996, LmrA, the first bacterial ATP-dependent multidrug transporter, was characterized in the same laboratory [22]. *LmrA* was homologous to the human *mdr1*, which encoded the P-glycoprotein and, moreover, complemented MDR1 in human lung fibroblast cells [60]. LmrA was targeted to the plasma membrane and conferred typical multidrug resistance in these human cells. Blockers of P-glycoprotein-mediated multidrug resistance also inhibited LmrA-dependent drug resistance. Like P-glycoprotein, LmrA removed drugs from the inner leaflet of the cytoplasmic membrane [61]. The expression of *lmrA* in a hypersensitive *E. coli* strain increased resistance to the very wide variety of drugs, including aminoglycosides, chloramphenicol, β-lactams, lincosamides, macrolides, quinolones, streptogramins, and tetracyclines [62].

LmrA is equivalent to half of the P-glycoprotein and functions as homodimer. Later, however, a functional heterodimeric ABC-type multidrug transporter LmrCD was described in *L. lactis* [63]. LmrC and LmrD were copurified as a heterodimer, and overexpression of both LmrC and LmrD in LmrA-negative strain of *L. lactis* demonstrated ATP-dependent efflux of ethidium bromide, BCECF-acetoxymethyl ester, daunomycin, and Hoechst 33342. As a corollary, the cells did not show drug extrusion when either gene was overexpressed singly. LmrCD is also responsible for bile resistance [64]. The expression of *lmrCD* is controlled by transcriptional repressor LmrR, encoded upstream of the *lmrCD* [65, 66]. LmrR also autoregulates its own expression. LmrR binds the LmrCD substrates: Hoechst 33342, daunomycin, and rhodamine 6G [65, 67]. Drug binding to LmrR relieves the LmrR-dependent repression of the *lmrCD* genes [68]. Interestingly, when four mutant multidrug-resistant strains of *L. lactis* selected by challenging with increasing concentrations of daunomycin, ethidium bromide, rhodamine 6G, or cholate were analyzed, only *lmrCD* multidrug transporter genes were significantly and strongly upregulated in all four strains [69]. These data suggested that LmrCD was a major determinant of multidrug resistance in *L. lactis*. This study, however, did not address the expression of other putative multidrug transporters in mutant strains. Finally, in 2013, CmbT was characterized as an MFS-type multidrug transporter [70]. Overexpression of *cmbT* in *L. lactis* resulted in marginally increased resistance to cholate, ethidium bromide, Hoechst 33342, lincomycin, puromycin, rifampicin, streptomycin, sulbactam, sulfadiazine, and sulfamethoxazole (IC_{50} increased approximately 1.2–3 times). Overexpressed CmbT mediated extrusion of ethidium bromide and Hoechst 33342, and ionophores inhibited the CmbT-mediated transport of Hoechst 33342. Based on the increased level of thiol groups in supernatant of strain overproducing CmbT, the authors hypothesized possible involvement of CmbT in sulfur metabolism [70]. However, this observation was dependent on methionine and cysteine content of the medium and was not further investigated in this report. In addition to LmrP, LmrA, LmrCD, and CmbT, the genome of *L. lactis* contains 36 putative multidrug transporters; however, they are still to be characterized experimentally. Additionally, a multidrug transporter of the ABC superfamily, LmrB, was identified on a plasmid carried by a natural isolate of

L. lactis [71]. LmrB was shown to be an active multidrug transporter capable of the extrusion from the cell of ethidium bromide and Hoechst 3342. Interestingly, two genes encoding polypeptidic bacteriocins LsbA and LsbB are located on the same plasmid as LmrB, in the immediate vicinity of the multidrug transporter gene. LmrB was shown to render the cells immune to both bacitracins, and to mediate their secretion into the medium. In this function, LmrB could be complemented by LmrA but not LmrP [71]. The location of the *lmrB* gene on a plasmid may facilitate transfer of this multidrug transporter from *L. lactis* to pathogenic bacteria and may deserve further investigation.

8.6 *Enterococcus* spp.

The enterococci are commensal bacteria that normally populate the human intestine. Over the last two decades, enterococci were identified as causative agents of nosocomial infections with increasing frequency. Infections caused by enterococci include urinary tract infections, nosocomial bacteremia, intra-abdominal infections, and endocarditis. Most enterococci have intrinsic resistance to various antimicrobial agents. However, increasingly frequent isolation of enterococci with acquired resistance to most commonly used drugs has been observed in recent years. As early as 1997, Lynch et al. [72] hypothesized that intrinsic resistance of enterococci to various antimicrobial agents, in the absence of outer membrane, is due, at least in part, to active efflux system(s). They examined four wild-type strains of *E. faecalis* and a strain of *Enterococcus faecium* and found that all strains showed energy-driven efflux of chloramphenicol, and all but one strain of *E. faecalis* extruded norfloxacin. In contrast, active efflux did not play a role in resistance to β-lactams. In this work, genetic determinants of these efflux pumps were not identified. Four years later, enterococcal genome-scanning identified a potential multidrug transporter EmeA [73] due to its homology to NorA from *S. aureus*, MFS-type multidrug transporter covered in-depth in Chap. 7 in this book. Deletion of this gene in *E. faecalis* resulted in an approximately twofold increase in susceptibility to acriflavine, ethidium bromide, clindamycin, erythromycin, novobiocin, ciprofloxacin, and norfloxacin compared to the wild-type strain. Functional complementation with wild-type plasmid-expressed *emeA* restored the resistance to ethidium bromide and resulted in the resistance to norfloxacin fourfold higher than in the wild-type strain. This resistance was due to energy-dependent efflux. Incubation with reserpine (competitive multidrug transporter blocker), verapamil (a calcium channel blocker), or lansoprazole (a H^+ and K^+-ATPase pump inhibitor) decreased resistance of both wild-type and complemented strains. The resistance of the mutant strain was unaffected by these agents, except for resistance to ethidium bromide which was lowered twofold by reserpine. These data allowed the authors to conclude that EmeA was the main enterococcal pump for these agents.

Later, Lee et al. [74] cloned EfrAB, an ABC multidrug transporter from *E. faecalis*, by using a drug-hypersusceptible mutant of *E. coli* host. When expressed in *E. coli*,

EfrAB conferred resistance to norfloxacin, ciprofloxacin, doxycycline, arbekacin, novobiocin, daunorubicin, doxorubicin, acriflavine, 4′,6-diamidino-2-phenylindole, ethidium bromide, safranin O, and tetraphenylphosphonium. Furthermore, EfrAB demonstrated energy-dependent efflux of acriflavine. This efflux was inhibited by verapamil, reserpine, and sodium orthovanadate (an ATPase inhibitor). Similar to other two-component ABC multidrug transporters, both EfrA and EfrB were required for resistance. The expression of EfrAB is induced by subinhibitory concentrations of chloramphenicol, gentamicin, and streptomycin [75]. In the same laboratory, *E. faecium* multidrug transporter belonging to the MFS, EfmA, was cloned in a similar fashion [76]. *E. coli* harboring EfmA showed energy-dependent efflux of 4′,6-diamidino-2-phenylindole and tetraphenylphosphonium, as well as norfloxacin/H^+ antiport. EfmA was found to be constitutively expressed by *E. faecium*. Overall, 34 putative multidrug transporters in *E. faecalis* have been identified from genome sequencing [8]. However, the majority of them are still experimentally unexplored.

8.7 *Streptococcus* spp.

8.7.1 Streptococcus pneumoniae

S. pneumoniae is the main bacterial cause of community-acquired pneumonia and represents a major disease burden worldwide [77]. Despite the recent introduction of the heptavalent pneumococcal conjugate vaccine, antimicrobial resistance is an increasing problem in this organism due to the spread of multidrug-resistant clones and increases in antimicrobial resistance among nonvaccine serotypes [78].

The initial report of the multidrug transporter in *S. pneumoniae* did not identify the transport protein associated with the phenotype [79]. A few years later, PmrA, a multidrug efflux pump of the MFS family, was identified using *S. pneumoniae* genomic sequence as homologous to *norA* of *S. aureus* [80]. The gene was overexpressed in *S. pneumoniae* and found to confer resistance to norfloxacin, ciprofloxacin, acriflavine, and ethidium bromide [80]. In later reports, a knock-out of *pmrA* [81, 82] did not result in increased susceptibility to drugs, indicating that PmrA is not intrinsically active in *S. pneumoniae*.

By 2006, the overexpression of the ABC superfamily efflux proteins PatA and PatB was found to be responsible for the multidrug-resistant phenotype of a mutant of *S. pneumoniae* selected after exposure to ciprofloxacin [83]. Disruption of *patA* and *patB* resulted in increased sensitivity to acriflavine, ethidium bromide, berberine, erythromycin, oxolinic acid, norfloxacin, ciprofloxacin, and novobiocin [81, 82] thus demonstrating that PatAB is normally expressed by *S. pneumoniae*. Each subunit consists of a nucleotide-binding domain and a membrane spanning domain, and heterodimerization of PatA and PatB is required to form a functional transporter [84]. Expression of *patAB* is induced by subinhibitory concentrations of fluoroquinolones [85, 86]. In clinical fluoroquinolone-resistant isolates of *S. pneumoniae*, whose resistance is ascribable to the overexpression of multidrug transporters, either PmrA [87] or PatA/PatB [88], were found to be responsible for the phenotype.

Similar to other Gram-positive examples covered in this chapter, additional putative multidrug transporters exist in the genome of *S. pneumoniae*, but so far, no phenotype was associated with them [82].

8.7.2 Streptococcus agalactiae

S. agalactiae causes neonatal sepsis, pneumonia, meningitis, as well as infections of the bovine udder. *S. agalactiae* produces α-hemolysin, which is an important virulence factor. It is capable of damaging erythrocytes, lung epithelial cells [89], and brain microvascular endothelial cells [90], which is regarded as an initial step in invasive disease. *cylA* and *cylB* were identified as genes essential for the production of the *S. agalactiae* hemolysin [91] and encode an ABC-type transporter. These genes are part of the 12-gene *cyl* operon, which contains, in addition to *cylA* and *cylB*, 5 genes similar to fatty acid biosynthesis enzymes (*cylD*, *cylG*, *acpC*, *cylZ*, and *cylI*), one similar to an aminomethyltransferase (*cylF*), one carrying the conserved domain of a glycosyltransferase (*cylJ*), a gene predicted to encode an acetyl coenzyme A carboxylase (*cylX*), a putative phosphopantetheinyl transferase (*cylK*), and a putative acyl-coA acyltransferase (*cylE*) [92]. *cylA* and *cylB* deletion mutants resulted in a nonhemolytic phenotype [93]. *cylA* mutant was shown to still harbor intracellular hemolytic activity, which was released by sonication. Since CylAB contained the signature sequence of a multidrug resistance transporter, wild-type and nonhemolytic *cylA* mutant were exposed to known substrates of multidrug transporters. Deletion of *cyl*A resulted in significant increase in susceptibility to daunorubicin, doxorubicin, and rhodamine 6G. Furthermore, the *cylA*-negative strain displayed a markedly reduced capacity to export doxorubicin. Growth in the presence of reserpine resulted in a dose-dependent decrease of extractable hemolytic activity, supporting the hypothesis that hemolysin is transported out of the cell by a multidrug transporter. At the time, the nature of the *S. agalactiae* hemolysin was unknown, and it was believed to be a pore-forming protein toxin. However, Gottschalk et al. [93] raised doubts in the protein nature of hemolysin based on the described work. Multidrug transporters were known to transport small molecules, rather than proteins. Indeed, in 2013, Whidbey et al. [94] showed that an ornithine rhamnolipid pigment known as granadaene [95] is responsible for the hemolytic activity of the bacterium. This is perhaps one of the few cases where the nature of the natural substrate of the multidrug transporters is proven very strongly.

8.7.3 Streptococcus mutans

S. mutans is a major causative agent in human caries and forms biofilm known as dental plaque [96, 97]. Although *S. mutans* strains are generally susceptible to antimicrobial agents [98], prolonged antimicrobial exposure can select antimicrobial resistance [99, 100]. Involvement of multidrug transporters in drug resistance has

been reported [101, 102]. Genome of *S. mutans* UAB159 [103] shows the presence of 71 putative ABC transporters and 10 putative MFS transporters (TransportDB at http://www.membranetransport.org).

Increased susceptibility to methyl viologen (paraquat), benzyl and ethyl viologens, and quaternary ammonium compounds was observed in mutant strains that were deficient in a function of ABC transporter complex, VltAB, which is encoded by an operon (*SMU.905-906*) [104]. The same laboratory also reported another putative ABC transporter complex, SmbFT, which is not present in strain UAB159 [105] and is encoded by genes located in the same locus and provides protection against lantibiotics Smb and haloduracin, but not against other lantibiotics (e.g., nisin) and several peptide antibiotics such as bacitracin, polymyxin B, and vancomycin [106]. A newer study also described two ABC transporter systems, SMU.654-655-656-657 and LctFEG (SMU.1148-1149-1150), which are, respectively, encoded by the genes linked to the two-component regulatory genes *nsrRS* (located downstream of *SMU.654-655-656-657*) and *lcrRS* (located upstream of *lctFEG*). Inactivation of *nsrRS* or *nsrS* (but not *SMU.654-655-656-657*) rendered the mutant strains more susceptible to nisin A (16-fold MIC reduction), while disruption of *lcrRS*, *lcrS,* or *lcrFEG* increased the susceptibility of the mutants to nukacin (eightfold MIC decrease) [101], suggesting involvement of LctFEG transporter in nukacin resistance. In *S. mutans* and *Streptococcus gordonii* (a commensal species), the gene locus *rcrRPQ* encodes an MarR-like transcriptional regulator (RcrR) and an ABC efflux complex (RcrPQ), which are linked to stress tolerance [107–109]. Inactivation of *rcrP* in *S. mutans* or *rcrR* in *S. gordonii* rendered the mutant cells more susceptible to lower pH or oxidative stress agents such as H_2O_2 and methyl viologen [107, 109]. Either overproduction of or deficiency in RcrPQ impaired the biofilm formation in *S. mutans* [107], suggesting an optimal status of this ABC exporters is essential for biofilm formation. In this regard, another exporter, the NrgA ammonium transporter, is also essential for biofilm formation in *S. mutans* [110]. Similar to that observed in *B. subtilis* [29], an ABC transporter named BceAB is encoded by part of the four-gene operon *bceABR*S that also encodes the BceRS two-component regulatory system, and BceABRS contributes in response to bacitracin-induced cell envelope stress [111]. Recently, the *copYAZ* operon (SMU.424-426-427) that encodes CopA ABC copper exporter and CopYZ regulators was demonstrated to play an important role in copper homeostasis, stress tolerance, and biofilm formation [112].

Additionally, a multidrug transporter, MdeA of the MFS predicted with 12 transmembrane domains, conferred resistance to ampicillin, oxacillin, nalidixic acid, ciprofloxacin, kanamycin, tetracycline, acriflavine, and rhodamine 6G (4- to 32-fold increase in MIC values) when expressed on a plasmid in a hypersusceptible *E. coli* host [102]. It is also noted that several regulatory systems such as LytST and ScnRK have been reported to contribute to tolerance to oxidative stress [113, 114]. Whether these regulatory systems are linked to any multidrug transporters remains to be investigated (Table 8.1).

Table 8.1 Summary of characterized drug efflux pumps in Gram-positive bacteria

Species/transporter family	Pump	Substrates[b]	Reference
B. subtilis			
ABC	BceAB	BCT	[29]
ABC	BmrA	AAD, DOR, HO	[25, 26]
ABC	BmrCD	BCECF, BCT, DOR, HO, MTO	[27, 115]
MFS	Blt	ACD, EB, DOR, FQ, R6G, TPP	[18, 21]
MFS	Bmr	ACD, EB, DOR, FQ, R6G, SPD, TPP	[18, 21, 116]
MFS	Bmr3	FQ, PUR	[31, 32]
MFS	LmrB	DOR, FQ, LIN, PUR	[117, 118]
MFS	MdtP	ACT, FUA, NOV, STR	[33]
RND	YerP	ACR, EB, SUR	[9]
SMR	EbrAB	ACR, EB, PY, SO	[34]
C. difficile			
MATE	CdeA	ACR, EB	[36]
MFS	Cme	EB, ERY, SO	[37]
E. faecalis			
ABC	ABC7	DAU, DOR, EB, OFX	[8]
ABC	ABC11	CHX, PTD	[8]
ABC	ABC16	AZI, CLA, ERY	[8]
ABC	ABC23	QD, VIR	[8]
ABC	EfrAB	ACR, CIP, DAP, DAU, DOR, EB, FQ, NOV, NOR, SO, TET, TPP	[74, 75]
ABC	Lsa	CLI, QD	[119]
MFS	EmeA	ACR, CLI, EB, ERY, FQ, NOV	[73]
E. faecium			
ABC	MsrC	ACR, DA, DP, DR, FQ, TC, TPP	[74]
L. lactis			
ABC	LmrA	DAU, DOR, EB, LIN, ML, R6G, TET, VIN, VIT	[22, 61, 62]
ABC	LmrB[a]	EB, HO	[71]
ABC	LmrCD	CHO, DAU, EB, HO, R6G	[63–65, 69]
MFS	CmbT	EB, HO	[70]
MFS	LmrP	DAU, EB, TPP	[59]
MFS	MdtA[a]	LIN, ML, STG, TET	[120]
L. monocytogenes			
ABC	AnrAB	AMP, BCT, CXM, CTX, GAL, NIS, PEN	[55]
MFS	Lde	ACR, BAC, EB, FQ	[121–123]
MFS	MdrL	CTX, EB, ML	[40, 43]
MFS	MdrM		[42]
MFS	MdrT	CHO	[42, 44]

(continued)

Table 8.1 (continued)

Species/transporter family	Pump	Substrates[b]	Reference
S. agalactiae			
ABC	CylAB	DAU, DOR, R6G	[91, 93]
MFS	MefB, MefG	ML	[124]
MFS	MreA	AZI, ERY, SPI	[125]
S. mutans			
ABC	BceAB	BCT	[111]
ABC	CopA	Cu	[112]
ABC	LctFEG	NUK	[101]
ABC	SmbFT	HAL, SMB	[106]
ABC	SMU.654-655-656-657	NIS	[101]
ABC	RcrPQ	H_2O_2 , PQ	[107, 109]
ABC	VltAB	BV, EV, PQ, QAC	[104]
MFS	MdeA	ACR, AMP, CIP, KAN, OXA, NAL, R6G, TET	[102]
S. pneumoniae			
ABC	PatAB	FQ	[81, 83, 85, 126, 127]
MFS	PmrA	FQ	[80]
MFS	MefE	ML	[128]

This table excludes drug efflux pumps identified in staphylococci and mycobacteria, which are described in Chaps. 7 and 21 of this book

[a]The genes encoding these pumps are located on plasmids

[b]*AAD* 7-aminoactinomycin D, *ACD* acridine dyes, *ACR* acriflavine, *ACT* actinomycin, *AG* aminoglycosides, *AMP* ampicillin, *AZI* azithromycin, *BAC* benzalkonium chloride, *BCECF* 2′,7′-*bis*-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, *BCT* bacitracin, *BV* benzyl viologen, *CHX* chlorhexidine, *CHO* cholate, *CIP* ciprofloxacin, *CLA* clarithromycin, *CLI* clindamycin, *CTX* cefotaxime, *CXM* cefuroxime, *DAP* 4′,6-diamidino-2-phenylindole, *DAU* daunorubicin, *DOR* doxorubicin, *EB* ethidium bromide, *ERY* erythromycin, *EV* ethyl viologen, *FQ* fluoroquinolones, *FUA* fusidic acid, *GAL* gallidermin, *HAL* haloduracin, *HO* Hoechst 33342, *KAN* kanamycin, *LIN* lincosamides, *ML* macrolides, *MTO* mitoxantrone, *NAL* nalidixic acid, *NIS* nisin A, *NOR* norfloxacin, *NOV* novobiocin, *NUK* nukacin, *OFX* ofloxacin, *OXA* oxacillin, *PEN* penicillin G, *PQ* paraquat (methyl viologen), *PTD* pentamidine, *PUR* puromycin, *PY* pyronine Y, *QAC* quaternary ammonium compounds, *QD* quinupristin-dalfopristin, *R6G* rhodamine 6G, *SMB* a lantibiotic, *SO* safranin O, *SPD* spermidine, *SPI* spiramycin, *STG* streptogramin, *STR* streptomycin, *SUR* surfactin, *TET* tetracycline, *TPP* tetraphenylphosphonium, *VIN* vinblastine, *VIR* virginiamycin, *VIT* vincristine

8.8 Concluding Remarks

Gram-positive organisms are generally more susceptible to antimicrobial drugs than their Gram-negative counterparts. Typical model archetypes for drug efflux have been more well developed genetically, biochemically, and structurally in Gram-negative organisms. This research bias may be generally attributable to the relative

contributions of tripartite efflux systems to clinically significant drug resistance phenotypes as targets for inhibitory compounds with potential dramatic modulation of drug resistance phenotypes. From a historical perspective, however, it is significant that some of the first bacterial drug efflux systems were identified and characterized in Gram-positive organisms (with little clinical significance) but noteworthy genetic and functional conservation with the major mammalian multidrug transporter, P-glycoprotein. However, counterintuitive to membrane evolution and drug kinetics, Gram-positive organisms encode a similar, if not greater, genomic investment (in the case of *Bacillus subtilis*) to efflux-based transport mechanisms. This observation suggests efflux may be physiologically and functionally more essential and, hence, more intrinsically active in Gram-positive organisms to accommodate their respective environments and survival. Further research into pump regulation and expression-level comparisons may be useful to determine relative functional balance between efflux and cell energetics in context with cell physiology and metabolism. In this regard, Gram-positive organisms described herein may be instructive in identifying physiologically relevant substrates and roles for respective efflux systems in the host-bacterial interface. The increasing availability of genetic tools in these organisms will facilitate genetic manipulation required to conduct such studies and *in vivo* modeling.

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Chapter 9
Antimicrobial Drug Efflux Pumps in *Escherichia coli*

Xian-Zhi Li and Hiroshi Nikaido

Abstract Bacterial active efflux of drugs (encoded by plasmids) was first discovered in *Escherichia coli*. Subsequently, a large number of chromosomally encoded multidrug efflux pumps (represented by AcrAB-TolC system) were identified in this species. Several of these efflux systems have served as prototypical pumps for characterizing substrate specificity, transport mechanisms, regulation, and inhibition of bacterial drug transporters. Efflux pumps are encoded by chromosomes or plasmids and exhibit a variable (broad or narrow) drug substrate profile that can be clinically relevant. Physiological roles of certain pumps have been demonstrated. This chapter provides an updated overview of more than 20 individual efflux systems/pumps of various families in *E. coli* with a focus on their substrate profiles, clinical relevance, and expression-based regulation. A discussion is also made on the interplay between the AcrAB pump and outer membrane permeability barrier on drug susceptibility as well as the AcrB-catalyzed efflux kinetics for β-lactams in intact cells.

Keywords *Escherichia coli* • Antimicrobial resistance • Efflux • RND pumps • Outer membrane • Plasmid • Efflux kinetics • AcrAB • TolC

9.1 Introduction

Escherichia coli is an important commensal bacterial species in the gastrointestinal tract of humans and animals, but some strains possess pathogenicity, causing intestinal and extra-intestinal diseases, including various life-threatening conditions [1]. Initially reported in 1885, *E. coli* is the most commonly studied prokaryotic microorganism, not only for its inherent biology but also as a molecular genetic tool for

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exploiting microbes in general. Indeed, a search in the PubMed database using either "*Escherichia coli*" or "*E. coli*" retrieves >320,000 items (as of February 29, 2016 at http://www.ncbi.nlm.nih.gov/pubmed). Human activities within the past 70 years of the antibiotic era have exacerbated antimicrobial resistance emergence in various microorganisms including *E. coli*. Multidrug-resistant *E. coli* now constitute one of the most prevalent microorganisms with the threat of resistance. A wide range of mechanisms mediate resistance to antimicrobial agents, including the bacterial drug efflux phenomenon, which was first recognized in *E. coli* with the plasmid-encoded tetracycline-specific efflux pumps in the 1970s [2–4]. Subsequently in the early 1990s, chromosomally encoded polyspecific multidrug resistance (MDR) efflux transporters were also identified in *E. coli* with the AcrAB pump [5]. It should be noted, however, that decreased accumulation of tetracycline and acriflavine by *E. coli* mutant cells was observed earlier in the 1960s [6]. To date, *E. coli* is known to utilize a number of drug efflux systems belonging to various families or superfamilies. As a model organism, research on these *E. coli* efflux pumps has advanced our in-depth understanding of biochemistry and genetics of drug efflux pumps, which ranges from their structural and biochemical transport mechanisms to their expression, regulation, and inhibition as well as their clinical significance. Many of these aspects are discussed throughout various chapters of this book. In this chapter, we limit our descriptions to an overview of the *E. coli* drug efflux pumps with an emphasis on their role in clinically relevant resistance.

9.2 Historical Perspectives on the Discovery of Plasmid-Encoded Tetracycline Resistance Pumps and Chromosomal Multidrug Efflux Systems

Transferable MDR with chloramphenicol, streptomycin, and tetracycline in bacteria was initially observed in the 1950s and mediated by plasmids (R-factors). Tetracycline resistance was suspected to be due to reduced permeability of cells to the drug [7] and was also inducible by subinhibitory concentrations of tetracycline [8]. Subsequently, a plasmid-encoded protein (named Tet) was identified and thought to cause reduced uptake in the resistant cells [2]. However, the latter phenomenon was, in fact, attributable to a process of energy-driven active efflux [3]. To date, about 30 different Tet efflux proteins have been reported in bacteria and are known to play a major role in tetracycline resistance.

In the 1960s, Nakamura published a series of studies on the susceptibility of *E. coli* to acriflavine and other basic dyes. Initially, a gene locus termed *acrA* in the chromosome of *E. coli* was involved in controlling MDR to basic dyes (acriflavine, methylene blue, toluidine blue, crystal violet, methyl green, and pyronin B), phenethyl alcohol, and sodium dodecyl sulfate [9, 10]. This phenotype was associated with the reduced acriflavine-binding capacity of *E. coli* [6] and was affected by the content of glucose in the growth medium [11]. This observation was originally attributed to

a structural change in the cytoplasmic membrane of *acrA* mutants [12]. Later, several other genes termed *acrB*, *acrC*, and *acrD* were also found to affect acriflavine resistance [13, 14]. Although the acriflavine hypersusceptibility phenotype in *acrA* mutant strains had been attributed to increased outer membrane (OM) permeability, additional studies were unable to identify compositional changes of the OM [15]. Since *E. coli* is a Gram-negative bacteria, its OM permeability barrier had frequently been used to explain the fact that Gram-negative bacteria are more resistant to many antimicrobial agents that are active against Gram-positive bacteria [16]. However in 1993, Ma et al. [5] reported the cloning of genes at the *acrA* locus (that contained a two-gene operon dubbed as *acrAE* [latter renamed as *acrAB*] encoding both peripheral and transmembrane proteins) and demonstrated an energy-dependent acriflavine efflux process. Both the *acrA* and *acrB* genes affect susceptibility to acriflavine and other structurally unrelated antimicrobial agents. In fact, the original *acrA* mutant obtained in the 1960s carried an IS2 insertion in the *acrA* gene, which thus abolished the function of AcrA [5]. A further study discovered that AcrAB plays a major role in the multiple antibiotic resistance (Mar) phenotype [17], which can be readily selected after exposure of susceptible *E. coli* to low-level inhibitory concentrations of a variety of antimicrobial agents [18–20]. The function of AcrAB in drug resistance was further found to be dependent on an OM protein, TolC [21], which is an exit duct for drugs and proteins [22]. Together, it took nearly three decades to recognize that the initial *acrA* locus encodes a part of the AcrAB-TolC drug efflux system, which plays a critical role in intrinsic and acquired MDR. Meanwhile, homologues of AcrAB-TolC systems were also found in time to contribute to MDR in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* in conjunction with their OM barrier [23–26]. Figure 9.1 shows a historical sketch of the proposed tripartite drug efflux pumps, which eventually led to published models [25, 27, 28]. Overall, the pieces of efflux-mediated MDR puzzle coalesced to elucidate an important mechanism of resistance and also established new important lines of research for the last two decades.

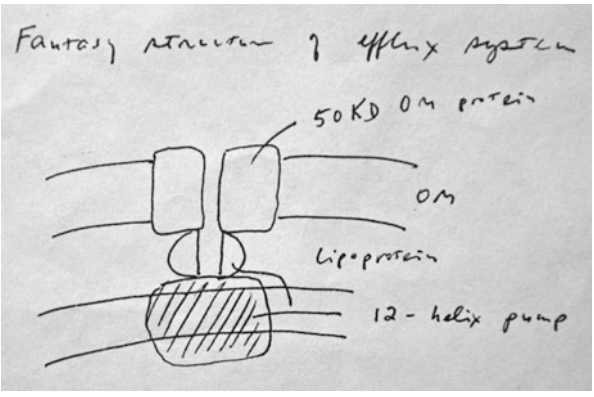

Fig. 9.1 Structural sketch of a proposed multicomponent drug efflux complex. This sketch, drawn in 1993 by H. Nikaido in a correspondence to D. M. Livermore, was based on available genetic and biochemical data supporting the predominant role of tripartite drug efflux pumps in intrinsic and acquired resistance of *E. coli* and *P. aeruginosa*

9.3 Chromosomal and Plasmid-Encoded Drug Efflux Systems and Their Functions in *E. coli*

9.3.1 Genomic and Functional Analysis of Drug Efflux Transporters

Before the availability of the complete *E. coli* genome, several drug exporters were already reported based on sequence comparison and biochemical characterization and included AcrAB [5], AcrEF [29], Bcr [30], Cmr (also called Cml or MdfA) [31, 32], EmrAB [33], EmrD [34], and EmrE [35] (Table 9.1). Furthermore, the first complete genome sequence of *E. coli* became available in 1997 and showed the presence of a large number of functionally identified and putative drug efflux pumps [122]. There are at least three dozen drug exporters of different transporter families [36], i.e., (i) the resistance-nodulation-cell division (RND) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion (MATE) family (part of the multidrug/oligosaccharidyl-lipid/polysaccharide [MOP] export superfamily), (iv) the small multidrug resistance (SMR) family (part of the drug/metabolite transporter [DMT] superfamily), and (v) the ATP-binding cassette (ABC) superfamily. Many of these efflux transporters have been studied via two major approaches in order to characterize their contribution to drug resistance. The first approach measures the susceptibility of pump-deficient mutants to various agents. Sulavik et al. [37] applied this method to assess 16 genes or operons encoding known or putative drug exporters, further confirming that AcrAB-TolC pump has the broadest substrate profile and plays the most important role in drug resistance (Table 9.2). We also used this deletion approach to determine the effect of the status of multiple efflux pumps and lipopolysaccharide (LPS) on drug susceptibility (Table 9.3). More recently, Nichols et al. [113] carried out phenomic profiling that included quantitative susceptibility measurements of an *E. coli* mutant library (i.e., the Keio collection) to >100 antimicrobial agents in an attempt to identify the genes (including efflux pump genes) involved in drug action and resistance. The second approach utilizes the plasmid vector-based expression of putative efflux genes, often in an antimicrobial-susceptible host strain. Nishino and Yamaguchi [36] cloned 37 known and putative efflux genes and revealed that 20 of them confer drug resistance. Since the AcrAB-TolC pump plays a predominate role in MDR and can thus mask the contribution of other efflux pumps, a host strain deficient in AcrAB-TolC function is often required in either the deletion or overexpression approach. Moreover, it should be noted that the findings from plasmid-based overexpression methods cannot differentiate whether the pumps are functioning in wild-type strains. Substrate profiles of the drug efflux pumps vary among pumps and can be either drug specific or multidrug in nature. The large numbers of drug efflux transporters reflect the diverse and sophisticated defense systems of the organism, particularly against the actions of antibacterial toxicants. Nevertheless, the tripartite RND-type AcrAB-TolC efflux system is the predominant efflux pump that produces the broadest defense in this organism.

Table 9.1 Proven drug efflux transporters of *E. coli* and their role in drug resistance and other functions

Family/transporter	MFP	OMP	Regulator	Drug substrates	Other functions	References
RND superfamily						
AcrB	AcrA	TolC	AcrR, MarA, MarR, SoxS, RobA, SdiA, EvgSA, RyeB	ACR, BL, BS, CHL, CV, EB, FA, FQ, ML, OS, RIF, SDS, TGC, TET, TRI	Export of enterobactin, estradiol and progesterone, hydrocortisone, and stress response	[5, 17, 21, 36–40]
AcrD	AcrA	TolC	BaeSR, CpxRA, NlpE	AG, BL	Export of enterobactin, estradiol, and progesterone and biofilm formation	[39–44]
AcrF	AcrE	TolC	AcrS, H-NS	ACR, DOC, EB, FQ, R6G, OS, TGC	Maintenance of cell division	[36, 45–49]
CusA (YbdE)	CusB (YlcD)	CusC (YlcB)	CusRS (YlcA YbcZ)	Ag(I), Cu(I)		[50–53]
MdtBC (YegNO)	MdtA (YegM)	TolC	BaeSR, CpxRA, NlpE	BS, NOV	Enterobactin and free fatty acid export and acid tolerance	[40, 54–57]
MdtF (YhiV)	MdtE (YhiU)	TolC	EvaAS, GadE, GadX, YdeO, H-NS, DsrA	DOC, DOR, EB, ERY, R6G	Export of free fatty acid, estradiol, and progesterone and nitrosative stress response	[36, 39, 57–61]
OqxB (plasmid-borne)	OqxA (plasmid)	TolC		ACR, BAC, CHL, CIP, EB, FLU, NAL, NIT, NOR, OQX, SDS, TMP, TRI		[62–66]
MFS						
Bcr				BCM, TET	Export of L-cysteine and dipeptides	[67, 68]
Dep				DHC		[69]
EmrB	EmrA	TolC	EmrR	CCCP, EB, TLM	Acid tolerance; export of estradiol and progesterone	[33, 39, 56, 70–72]

(continued)

Table 9.1 (continued)

Family/transporter	MFP	OMP	Regulator	Drug substrates	Other functions	References
EmrD				CCCP	Energy stress response, arabinose export, and biofilm formation	[34, 44, 73]
EmrY	EmrK	TolC		DOC, MIT, NAL	Stress response to hydrogen peroxide and UV irritation and biofilm formation	[44, 74–77]
FloR				CHL, FLO, THL,		[78–80]
MdfA/Cmr/CmlA				BAC, CHL, DOR, NOR, SIT, TET	Alkaline pH homeostasis and export of arabinose and isopropyl -*D*-1-thiogalactopyranoside	[32, 72, 73, 81–83]
MdtG (YceE)			MarA, SoxS	FOF		[36, 84]
MdtM				BS, CHL, EB, QAC	Alkaline pH homeostasis	[85–88]
Mef				ML		[89]
QepA and QepA2				FQ		[90]
TetA			TetR	TC		[91]
ABC superfamily						
MacB	MacA	TolC		ML	Export of enterotoxin II and protoporphyrin	[92–95]
McjD		TolC		MJ		[96–98]
MsbA				DAU, EB, VIN	Lipid flippase for export of LPS precursor lipid A and other lipids	[99–104]
YojI		TolC	Lrp	MJ		[105, 106]
YgiA					Alkaline tolerance, completion of cell division	[107, 108]
MATE family						
MdtK (NorE, NorM, YdhE)				ACR, BER, CV, DOC, DOR, ENR, NOR, R6G, TPP	Export of dipeptides	[36, 67, 109–112]

YeeO				BAC, BCM, DOR,FAD, FMN, LVX, PUR, VAN, VER	Export of dipeptides and flavins	[67, 113, 114]
SMR family						
EmrE		OmpW		ACR, CV, EB, PQ, QAC	Betaine and choline export and biofilm formation	[36, 37, 44, 113, 115–117]
MdtJI (YdgFE)				ACR, BAC, CHL, CHO, DOC, ERY, EB, FUA, SDS	Spermidine export	[36, 113, 118]
SugE				CAB, HTA, QAC		[119]
YnfA				CAR		[120]
Other families						
DedA family: YghB and YgjA				ACR, BAC, CAB, CIP, CLX, ERY, EB, NAL, OXA, PIP, PQ	Completion of cell division	[107, 108]
TDT family: TehAB				DEQ, EB, PQ, PRO, TEL		[121]

ACR acriflavine, *AG* aminoglycosides, *BAC* benzalkonium chloride, *BCM* bicyclomycin, *BER* berberine, *BL* β-lactams, *BIO* biocides, *BS* bile salts, *CAB* cetyltrimethylammonium bromide, *CAR* carbenicillin, *CCCP* carbonyl cyanide *m*-chlorophenyl hydrazone, *CHL* chloramphenicol, *CHO* cholate, *CIP* ciprofloxacin, *CLX* cloxacillin, *CV* crystal violet, *DAU* daunomycin, *DEQ* dequalinium, *DHC* 4,5-dihydroxy-2-cyclopenten-1-one, *DOC* deoxycholate, *DOR* doxorubicin, *EB* ethidium bromide, *ENR* enrofloxacin, *ERY* erythromycin, *FA* fatty acids, *FAD* flavin adenine dinucleotide, *FLO* florfenicol, *FLU* flumequine, *FMN* flavin mononucleotide, *FOF* fosfomycin, *FQ* fluoroquinolones, *FUA* fusidic acid, *HTA* hexadecyltrimethyl ammonium, *LVX* levofloxacin, *MIT* mitomycin C, *MJ* microcin J25, *ML* macrolides, *NAL* nalidixic acid, *NIT* nitrofurantoin, *NOR* norfloxacin, *NOV* novobiocin, *OQX* olaquindox, *OS* organic solvents, *OXA* oxacillin, *PIP* piperacillin, *PQ* paraquat, *PRO* proflavine, *PUR* puromycin, *QAC* quaternary ammonium compounds, *R6G* rhodamine 6G, *RIF* rifamycins, *SDS* sodium dodecyl sulfate, *SIT* sitafloxacin, *TEL* tellurite, *TET* tetracycline, *TGC* tigecycline, *THL* thiamphenicol, *TLM* thiolactomycin, *TMP* trimethoprim, *TPP* tetraphenylphosphonium, *TRI* triclosan, *VAN* vancomycin, *VER* verapamil, *VIN* vinblastine

Table 9.2 Impact of the genetic inactivation of *acrAB* locus on antimicrobial susceptibility of *E. coli*

Antimicrobial class/ agents	Wild type (MIC in μg/ml)	Δ*acrAB* (MIC in μg/ml)	MIC ratio of wild type/ Δ*acrAB* (fold)
Ampicillin	12.5	3.12	4
Azlocillin	16[a]	4[a]	4
Carbenicillin	4[a]	1[a]	4
Cloxacillin	256[a]	2[a]	128
Oxacillin	512[b]	4[b]	128
Penicillin G	16[a]	8[a]	2
Piperacillin	4[b]	0.25[b]	8
Nitrocefin	19[c]	1.9[e]	10
Cephalothin	4	4	1
Cefoxitin	4	1	4
Cefoperazone	0.03[a]	0.015[a]	2
Ceftazidime	0.12[a]	0.12[a]	1
Ceftriaxone	0.0015[a]	0.0015[a]	1
Cefepime	0.0075[a]	0.0075[a]	1
Cefpirome	0.015[a]	0.015[a]	1
Imipenem	0.12[a]	0.12[a]	1
Gentamicin	8[b]	8[b]	1
Nalidixic acid	3.13 (16[b])	1.56 (2[b])	2 (8[b])
Ciprofloxacin	0.01	0.0025	4
Levofloxacin	0.063[b]	0.016[b]	4
Norfloxacin	0.004	0.004	1
Trovafloxacin	0.025[c]	0.006[c]	4
Azithromycin	8[c]	0.5[c]	16
Erythromycin	50 (128[b])	1.56 (2[b])	32 (64[b])
Minocycline	>0.5[c]	0.25[c]	>2
Tetracycline	1.25	0.156	8
Tigecycline	0.5[c,d]	0.25[c], 0.125[d]	2–4
Chloramphenicol	6.25	0.78	8
Florfenicol	6.25	0.78	8
Fusidic acid	400	3.125	128
Linezolid	240[b]	15[b]	16
Novobiocin	100	1.56	64
Puromycin	100	1.56	64
Rifampin	5 (16[b])	2.5 (8[b])	2 (2[b])
Streptomycin	1.95	1.95	1
Sulfacetamide	2000	2000	1
Trimethoprim	2[c]	0.25[c]	8
Triclosan	0.5[b]	0.125[b]	4

The MIC data are from Sulavik et al. [37], except: [a][123], [b][124], [c]Table 9.3, [d][45], and [e][45]

Table 9.3 Effect of the lipopolysaccharide (*waaP*) and/or AcrAB efflux pump (*acrAB*) status on antimicrobial susceptibility of *E. coli* K-12 strain AG100 (MIC values in μg/ml)

Antimicrobial	Wild type	Δ*waaP*	Δ*acrAB*	Δ*waaP* Δ*acrAB*
Ampicillin	4	4	1	1
Penicillin G	32	16	8	16
Ciprofloxacin	0.0125	0.025	0.003	0.003
Trovafloxacin	0.025	0.006	0.006	0.003
Azithromycin	8	1	0.5	<0.12
Erythromycin	256	32	8	1
Minocycline	>0.5	>0.5	0.25	0.125
Tetracycline	2	2	0.5	0.25
Tigecycline	0.5	0.5	0.25	0.25
Chloramphenicol	8	4	2	2
Linezolid	1024	256	16	16
Novobiocin	256	4	8	1
Rifampin	8	4	8	1
Trimethoprim	2	0.25	0.25	0.06
Acriflavine	64	32	4	0.5
Crystal violet	16	1	2	0.5
Ethidium bromide	256	256	32	4
Sodium dodecyl sulfate	>50,000	256	32	16

The data are unpublished observations (X.-Z. Li and K. Poole, 2001)

9.3.2 *Efflux Pumps of the RND Superfamily*

E. coli encodes six RND efflux systems (including the MdtABC system that contains a pair of RND pumps, MdtB and MdtC) (Table 9.1). These systems belong to the hydrophobe/amphiphile efflux-1 (HAE-1) family with the exception of CusCBA that is a member of the heavy metal efflux (HME) family [125]. A typical functional RND efflux complex is composed of three components: an RND pump embedded in the cytoplasmic membrane, a periplasmic adaptor protein (also known as membrane fusion protein [MFP]), and an OM channel protein [126]. However, in *E. coli*, the genes coding for four RND pumps (i.e., AcrAB, AcrEF, MdtABC, and MdtEF) each form an operon that encodes only two components, the pump(s) and the MFP. These four systems still require TolC as their OM protein [21, 127, 128]. This genetic organization of two-gene operons is quite different from that of the commonly occurring three-gene operons in *Acinetobacter baumannii* and *P. aeruginosa* [126]. AcrD is encoded by a single gene [41] but is dependent on AcrA and TolC for its functionality [70, 129]. CusCBA is the only RND system in *E. coli* with three components encoded by an operon [50, 51]. Additionally, there is also a plasmid-encoded RND system, OqxAB [62]. Characteristics of each of these RND systems are described below.

AcrAB-TolC This efflux system is the prototype pump of the RND superfamily and plays the most important role in both intrinsic and acquired MDR in *E. coli*. The genes encoding AcrAB and TolC are located in different regions of the chromosome, but their respective expression is coordinated (see Sect. 9.4) [5, 21]. The AcrAB-TolC pump complex represents the typical tripartite efflux machinery containing the pump AcrB, the accessory MFP AcrA, and the OM channel protein TolC (see the Chap. 1 in this book) [126, 130]. A recent study demonstrated that AcrB-AcrA fusion protein can function as a drug exporter [131]. There is an additional component, AcrZ, which is physically associated with AcrB and is also essential for the role of AcrAB-TolC complex in drug resistance [132]. Constitutively expressed in wild-type cells, AcrAB-TolC significantly contributes to intrinsic resistance and exhibits an incredibly broad substrate profile. Inactivation of *acrAB* in wild-type strains results in hypersusceptibilities to not only to clinically relevant β-lactams (including penicillins and cephalosporins), fluoroquinolones, macrolides, tetracyclines, tigecycline, chloramphenicol, and novobiocin but also to basic dyes, disinfectants, detergents, and organic solvents (Tables 9.1 and 9.2; recently reviewed in reference [126]) [5, 133, 134]. Exceptions, not being substrates, are aminoglycoside agents, which are the substrates of another RND pump, AcrD (see next section). Inactivation of *acrAB* can usually decrease MIC values of many antimicrobials by four- to eightfold and in certain cases by 64- to 128-fold (Table 9.2). This also leads to an increased post-antibiotic effect [135]. As discussed later in the Regulation section, the expression of AcrAB-TolC is controlled by multiple regulators involving complex regulatory pathways. Indeed, AcrAB-overproducing mutants carrying mutations in the regulatory genes have widely been reported in clinical isolates and laboratory-selected mutants [38, 134, 136–140]. Lastly, it should be emphasized that in spite of the presence of dozens of drug efflux pumps in *E. coli*, only AcrAB-TolC and a few other systems are likely to be clinically relevant regarding their role in resistance. Nonetheless, other systems with possibly modest role in resistance could still contribute to the survival of bacteria under diverse conditions by elevated expression and thus facilitate the emergence and evolution of resistance.

AcrAD-TolC A single gene, *acrD*, encodes the RND pump AcrD [41] that is dependent on AcrA and TolC for forming a functional complex [42, 70]. The AcrD pump has a much narrower substrate profile than AcrB, and it mostly accommodates hydrophilic, cationic aminoglycosides [41, 43] and anionic β-lactams [36, 141]. *In vitro* reconstitution of AcrD into proteoliposomes has established the efflux of aminoglycosides by AcrD [43]. Substrate specificity of AcrD and AcrB was found to be determined by both the large periplasmic loops of these pumps [129] and dependent on certain critical residues in the access substrate-binding pocket of the pumps [142]. When overproduced from plasmid, AcrD also accommodates the steroid hormones estradiol and progesterone [39].

AcrEF-TolC The AcrEF pump is encoded by an operon *acrEF* (initially known as *envCD* [143]). Deletion of *acrEF* in a wild-type strain did not alter antimicrobial susceptibility [37]. Cloned AcrEF produced a fourfold increase of the MIC values of acriflavine, deoxycholate, ethidium bromide, rhodamine 6G, and tigecycline

against an AcrB-deficient strain [36, 45]. In the absence of AcrB, mutants with overproduced AcrEF were selected *in vitro* due to the insertion of an IS element in the upstream region of *acrEF* and displayed enhanced resistance to fluoroquinolones [46] and organic solvents [47]. Similar IS element-mediated insertional activation of AcrEF production was also selected by fluoroquinolones in a *Salmonella* DT204 strain deficient with AcrB [144]. A regulatory gene, *acrS*, is located upstream of *acrEF* but is transcribed divergently [29]. Deletion of *acrS* in wild-type cells hardly affected antimicrobial susceptibility except to novobiocin [145]. Apart from drug resistance per se, the physiological role of AcrEF may be related to the maintenance of the cell division process [48].

MdtABC-TolC This system contains a pair of RND pumps, MdtBC [54, 55]. A similar organization with heterotrimeric RND pumps is also observed in *P. aeruginosa* (i.e., TriBC) (reviewed in [126]), *Stenotrophomonas maltophilia* (SmeJK) [126], and *Erwinia amylovora* [146]. MdtABC also requires TolC for functionality [56, 70]. Disruption of this system in wild-type strains does not alter antimicrobial susceptibility [37], but its overproduction is involved in resistance to novobiocin and deoxycholate in AcrAB-deficient strains [54, 55]. MdtBC appears to function only as B_2C trimer [147], and site-directed labeling studies suggest that only MdtC binds the substrate [148]. Tannins can induce the expression of MdtABC, which in turn confers resistance to tannins [149]. MdtABC-TolC, together with several other efflux systems, may be involved in the efflux of enterobactin [40] and free fatty acids [57] as well as in acid tolerance [56], and these may likely constitute part of physiological functions of these efflux systems. Simultaneous absence of MdtABC and AcrD also makes *Salmonella* more susceptible to copper and zinc salts [150].

MdtEF-TolC Genetic inactivation of this efflux system has no effect on antimicrobial susceptibility of wild-type cells [37]. When expressed from a plasmid in an AcrAB-deficient strain, MdtEF confers resistance to deoxycholate, doxorubicin, erythromycin, ethidium bromide, and rhodamine 6G with a fourfold increase in their MIC values [36]. Moreover, MdtEF is found to accommodate steroid hormones (estradiol and progesterone) [39] and free fatty acids [57] and also to mediate response to nitrosative stress such as under anaerobic growth conditions [58, 59].

CusCBA This tripartite efflux system encoded by the four-gene operon, *cusCFBA*, confers resistance to Ag(I) and Cu(I) ions [51–53]. Upstream of the *cusCFBA* genes are located the *cusRS* genes which encode a two-component regulatory system and are transcribed in a divergent direction [50, 151]. The additional product from this operon is a periplasmic metallochaperone protein, CusF, which is required for copper and silver resistance and functions to sequester Ag(I) and Cu(I) ions to the Cus efflux pump [152, 153] (thus, this efflux complex is also described as a tetrapartite system [51]). Interestingly, the genetic arrangement of *cusCFBA-cusRS* is identical to that of *silCB-orf-silA-silRS* presented in an MDR/silver resistance mega-plasmid (ca. 180 kb) of *Salmonella* (pMG101; GenBank accession AF067595) that was obtained in the USA in 1973 [154]. Recently, nearly identical clustering of these genes was also found to be present in two MDR mega-plasmids (227 kb and 273 kb),

respectively, from *Salmonella enterica* serovar Heidelberg obtained in the USA (i.e., pSH111_227; GenBank accession JN983042) and from *E. coli* isolated in China in 2011 (pEC5207; GenBank accession KT347600) [155]. Disruption of the chromosomal *cusA*, which codes for the RND pump, renders mutant strains more susceptible to Ag(I) ions (e.g., unable to grow in the presence of 25 μM $AgNO_3$) [52, 53]. Cloned *cusA* gene provides a three- to fourfold increase in MIC values of $AgNO_3$ and $CuSO_4$ in a mutant deficient in both CusA and multi-copper oxidase CueO [156]. Plasmid pEC5207 containing *cusCBA* was experimentally demonstrated to confer an 80-fold increase of silver nitrate MIC value [155]. Crystal structures of CusBA and CusC (the OM channel) suggest the assembly of a CusCBA complex in the form of $CusC_3$-$CusB_6$-$CusA_3$ that spans both the cytoplasmic membrane and OM to extrude Ag(I)/Cu(I) ion substrates out of the cell [157, 158]. A more recent study shows the activation of the CusCBA pump in the periplasm, which involves the metal ion transfer between CusF and apo-CusB and the critical role of metal-bound CusB in the regulation of metal ion movement from CusF to CusA [159].

OqxAB-TolC OqxAB pump is one of a very limited number of RND pumps encoded by plasmids. The conjugative plasmid (named pOLA52) encoding OqxAB was isolated from an olaquindox-resistant strain derived from swine manure of a farm using olaquindox as a feed additive [63]. This plasmid also contains a bla_{TEM} β-lactamase gene and other genes for formation of type 3 fimbriae (*mrkABCDF*, which may enable biofilm formation) [63, 160]. Like several chromosomally encoded RND pumps described above, OqxAB also requires TolC protein for producing resistance [62]. OqxAB pump produced from the original plasmid mediates resistance to olaquindox (16-fold MIC increase) [62]. The susceptibility phenotype generated by the cloned *oqxAB* genes indicates a broad substrate profile of OqxAB pump that ranges from chloramphenicol, quinolones, and trimethoprim to other biocides (Table 9.1) [64]. A recent study further identified nitrofurantoin as another substrate of OqxAB and the requirement of OqxAB in high-level nitrofuran resistance [65].

To date, more than 30 *oqxB* variants have been reported [161]. The clinical importance of OqxAB is supported by evidence for its wide presence not only in *E. coli* but also in other *Enterobacteriaceae* species [66, 162–166] as well as for its copresence with other resistance genes and mobile elements such as integrons [161, 167–171]. In one study conducted in Denmark, *oqxAB*-positive strains were found in nearly all olaquindox-resistant isolates (9/10) that were derived from 556 strains collected from swine farms [66]. A report on the prevalence of quinolone resistance determinants in 1022 *E. coli* isolates of human, animal, and environmental sources in China revealed *oqxAB* as the most frequently found resistance genes, with 20 % of the isolates being *oqxAB* positive (the isolates dated back to as early as 1994) [167]. Another Chinese study reported a ciprofloxacin resistance rate of 51 % for 590 isolates from community-onset patients in 30 hospitals with the occurrence of *oqxAB* in 1.4 % and 6.3 %, respectively, of ciprofloxacin-susceptible and ciprofloxacin-resistant isolates [161]. A study with human isolates from Korea showed the distribution of *oqxAB* genes in *E. coli*, *Enterobacter cloacae*, and

K. pneumoniae with a good correlation for resistance to ciprofloxacin and olaquindox [162]. A multidrug-resistant *E. coli* isolate of chicken origin in China carried a conjugative plasmid containing *oqxAB*, $bla_{CTX\text{-}M\text{-}24}$, $bla_{TEM\text{-}1b}$, and *aac*(6′)-*Ib-cr* genes [172]. Copresence of *oqxAB* and other resistance gene cassettes was also noted in *Salmonella* spp. [163, 173]. Of importance, *oqxAB* genes in *K. pneumoniae* were found to be chromosomally located and widely present in multidrug-resistant isolates (including 100 % presence in *K. pneumoniae* carbapenemase-producing isolates in one study [164]) [162, 165]. Phylogenetic assessment confirmed that *oqxAB* in *K. pneumoniae* was genetically closest to the plasmid-borne counterparts from *E. coli* and *Salmonella*. Chromosomally encoded *oqxAB*-like elements with lower sequence homology were also noted in other Gram-negative species such as *Enterobacter* spp. [174].

9.3.3 Efflux Pumps of MFS

E. coli possesses a large number of MFS transporters. Both chromosomally encoded and plasmid-encoded MFS efflux pumps have been found to be involved in drug resistance (Table 9.1). These efflux systems may be either single-component or multicomponent exporters. The latter often require the TolC OM protein. With the single-component pumps of MFS and other families, the drugs that are pumped out into the periplasm may quickly diffuse back, spontaneously, into the cytosol. Thus, it is important that these pumps can usually produce significant levels of drug resistance only when the drugs in periplasm are exported across the OM into the medium, through collaboration with RND pump complex [175, 176]. Moreover, most of these pumps have a relatively narrow substrate profile with limited clinical relevance; on the other hand, tetracycline-specific TetA pumps constitute one of the major mechanisms of high-level tetracycline resistance.

Emr Pumps These include the EmrAB-TolC [33], EmrD [34], and EmrKY-TolC [74]. Individual inactivation of any of these systems in a wild-type strain does not change antimicrobial susceptibility [37]. However, a modest twofold reduction of MIC values of azithromycin, acriflavine, and proflavine was noted with genetic inactivation of *emrD* in a hypersusceptible *acrAB*/*acrEF*/*emrE* triple deletion mutant (X-Z Li, unpublished, 2001). EmrD is involved in adaptive response to proton uncoupler energy shock [34] and also in the export of pentoses [73]. When overproduced from a plasmid, EmrAB accommodates a variety of cytotoxic substrates (that do not include conventional antibiotics) [33, 71] and mammalian steroid hormones [39] (Table 9.1). Inactivation of EmrKY renders mutants more susceptible to the lethal effects of stress generated by antimicrobials, hydrogen peroxide, or UV irradiation [75], while their overexpression from a plasmid significantly increases resistance to deoxycholate [74]. Interestingly, the ability to form biofilms was compromised in *emrD* or *emrKY* deletion mutants as well as *acrD*, *mdtE*, or *emrE* mutants [44].

Mdt Pumps Under this group are (predicted) MdtD (YegB), MdtG (YceE), MdtH (YceL), MdtL (YidY), and MdtM (YjiO) pumps (Table 9.1). These pumps appear to be single-component exporters, and the genetic inactivation of each of them renders the mutants more susceptible to antimicrobial(s) [85, 113] (also reviewed in [126]). MdtM is the mostly studied pump of these Mdt pumps and can accommodate a variety of substrates including bile salts (see Table 9.1) [85–88]. It also interplays with multicomponent AcrAB-TolC pump to produce a multiplicative effect on bile salt resistance [88]. MdtM also contributes to tolerance to an alkaline pH [87]. Intriguingly, MdtM also serves as an importer for proline-rich antimicrobial peptides such as oncocins to enter bacteria particularly in the absence of the SbmA importer of these peptides [177]. MdtG overexpression was noted in a fluoroquinolone-resistant mutant [84].

MdfA/Cml/Cmr and FloR Pumps One of the well-studied MFS pumps is the single-component MdfA pump, which is also called CmlA or Cmr because of its substrate profile including chloramphenicol (Table 9.1) [32, 81, 82]. Plasmid-borne *mdfA* variants were also recently reported to be copresent with other resistance genes [178, 179]. Although the expression of *mdfA* does not correlate with general fluoroquinolone resistance in clinical *E. coli* isolates [180], correlation between *mdfA* expression and sitafloxacin resistance was noted [180], and an MdfA homologue produced in *Shigella flexneri* was also involved in fluoroquinolone resistance [181]. Yet, overexpression of MdfA in *E. coli* results in hypersusceptibility to spectinomycin [81]. Interestingly, overexpression of *mdfA* could restore the resistance phenotype lost in a mutant deficient in DedA drug exporters [107]. Physiological roles of MdfA are likely related to pH homeostasis, i.e., alkaline pH tolerance [83] as well as pentose export [73]. Recent studies on the export mechanisms of MdfA provide an example on how divalent drug molecules can be transported via two-transport cycles by MdfA, and hence the substrate spectrum can be expanded [182, 183].

FloR is an efflux pump encoded by plasmids initially isolated from multidrug-resistant *Salmonella* spp.; this pump mediates high-level resistance to florfenicol (a veterinary amphenicol agent) [78, 184, 185]. However, *E. coli*, often of animal origin, has also been reported to carry *floR*-containing plasmids that also contain other resistance genes (e.g., *strA*/*strB* for aminoglycoside resistance; bla_{CMY-2} for β-lactam resistance) and mobile genetic elements (e.g., transposons) [78, 79, 185–187]. (Chromosomal *floR* gene was reported in *Bordetella bronchiseptica* of animal origin [188].) A recent study found a *floR* prevalence of 7.4% among multidrug-resistant isolates of Shiga toxin-producing *E. coli* O157:H7 from meat and dairy products [189]. FloR displays 12-transmembrane segments and provides specific resistance to amphenicol drugs (chloramphenicol, florfenicol, and thiamphenicol) [80, 189]. Reduced accumulation of florfenicol was demonstrated in *floR*-positive strains [186]. A study also described the copresence of *floR* and *oqxAB* in the same plasmids of *E. coli* isolates from diseased animals in China [190], likely suggesting a rapid evolution of novel plasmids under antimicrobial selection pressure since the agents to which resistance occurs, florfenicol and olaquindox, are veterinary agents only.

QepA and QepA2 Pumps These are fluoroquinolone-specific resistance pumps that are plasmid encoded and mostly found in *E. coli* of global sources [90, 166, 191–197]. In one study carried out in Japan, two isolates were found to be *qepA* positive among 751 isolates collected from 140 hospitals during the period of 2002–2006 [198]. One study [161] showed the presence of *qepA* in 16 % and 12 %, respectively, of ciprofloxacin-susceptible and ciprofloxacin-resistant isolates in China. Similar to the copresence of multiple resistance genes in many plasmids, *qepA* or *qepA2* often coexists in the same plasmids with transposon elements and other resistance genes including $bla_{CTX\text{-}M}$, *aac*(*6′*)-*Ib-cr*, and/or *qnr* genes [167, 192, 195, 199]. Cloned *qepA2* gene conferred a four- to eightfold increase in ciprofloxacin MIC values and also interplayed with chromosomal fluoroquinolone resistance mechanisms to produce clinically relevant resistance levels [200].

Tet Pumps As the first drug efflux systems discovered in bacteria, Tet pumps have been extensively studied [201, 202]. Heterogeneity of plasmid-borne tetracycline resistance determinants is well established [203]. To date, there are >30 different classes of Tet efflux proteins reported in bacteria, and many of them [i.e., Tet(A), (B), (C), (D), (E), (G), (J), (L), (V), (Y), and (Z)] have been observed in *E. coli*. Importantly, many plasmids containing *tet* genes also carry other resistance genes. Most of the Tet efflux proteins including Tet(A) and Tet(B) belong to the 12-transmembrane segment (TMS) group, while others such as Tet(L) contain 14-TMS. Meanwhile, other tetracycline resistance mechanisms such as ribosome protection [mediated by Tet(M) and Tet(W)] and enzymatic inactivation [mediated by Tet(X)] are also known in *E. coli* (http://faculty.washington.edu/marilynr/; lastly accessed on December 15, 2015) [202]. Tetracyclines are one of the earliest antimicrobial classes that have been widely used in human and veterinary medicine (although their use in humans became limited due to both the widespread tetracycline resistance and the availability of other newer antimicrobials). Current prevalence of tetracycline resistance in *E. coli* is so high that *E. coli* isolates of chicken origin in the USA over the period of 2000–2011 showed tetracycline resistance rates of 40–68 % [204]. Tet efflux pumps provide high-level tetracycline-specific resistance (e.g., tetracycline MIC values differ more than 100-fold for strains with or without *tet*-containing plasmids [205, 206]). Most of them confer resistance to tetracycline but not to minocycline except for Tet(B) [206]. Mutations in *tet* genes (e.g., *tet*(*B*)) can alter tetracycline substrate specificity [207, 208]; for instance, mutated Tet(B) proteins confer a fourfold increase in MIC values of tigecycline [206] which, as a glycylcyline agent, was developed against the organisms containing *tet* genes.

9.3.4 Efflux Pumps of the ABC Superfamily

The MacAB pump of this superfamily has been well studied. It provides resistance to 14- and 15-membered macrolides when overproduced from a plasmid [92]. MacA is an accessory MFP with a hexameric structure and MacB is an ABC protein with

4-TMS and one nucleotide-binding domain [209, 210]. The function of MacAB is also dependent on TolC [92]; MacAB-TolC forms a multicomponent efflux complex similar to the tripartite RND pumps. Inactivation of *macAB* genes does not alter antimicrobial susceptibility (including to macrolides) [37], suggesting limited expression of this pump in wild-type cells and/or masking of its function by the predominant AcrAB-TolC pump. However, with a more sensitive cell-based assay, *macB* inactivation yields enhanced susceptibility to a number of non-macrolide agents such as bacitracin, bicyclomycin, bile salts, cefaclor, ethidium bromide, nitrofurantoin, novobiocin, hydrogen peroxide, sodium dodecyl sulfate, taurocholate, and tunicamycin [113]. Furthermore, from a physiological point of view, MacAB-TolC participates in the secretion of enterotoxin produced by enterotoxigenic *E. coli* [93] as well as the protoporphyrin, a heme precursor [94]. Homologues of MacAB are identified in *Salmonella*, *Neisseria*, and *Stenotrophomonas* (reviewed in reference [126]).

MsbA is another ABC exporter that has been studied extensively regarding its structure and biochemistry [211–214]. With a functional role in the export of LPS precursors [99], MsbA also provides resistance to several cytotoxic agents when overexpressed in *E. coli* [100]. This substrate profile is similar to that of LmrA of *Lactococcus lactis*, a bacterial homologue of mammalian P-glycoprotein [100].

9.3.5 Efflux Pumps of the MATE Family

While reporting the first-identified bacterial MATE pump, NorM of *Vibrio parahaemolyticus*, Morita et al. [109] also showed its homologue in *E. coli*, YdhE. The latter, renamed as MdtK (http://www.tcdb.org), is also known as NorE [110]. MdtK likely possesses 12 TMSs, and when overexpressed from plasmids, it confers resistance to fluoroquinolones and a variety of other structurally unrelated agents including dipeptides (Table 9.1) [36, 38, 67, 110]. Inactivation of *mdtK* in a wild-type strain only slightly increased fluoroquinolone susceptibility [110], but increased susceptibility to multiple agents was observed in a more sensitive cell-based assay [113]. MdtK also synergistically interplays with the AcrAB pump to reduce fluoroquinolone susceptibility levels [110]. Although the clinical significance of MdtK remains unclear, its expression is lower than that of *acrA*, *acrB*, and *tolC* and appears variable in clinical isolates [38]. Expression of *mdtK*, *mdfA*, and *tolC* was induced by ciprofloxacin in clinical fluoroquinolone-resistant isolates [215]. Similar to AcrAB, MdtK is likely also involved in exporting signal molecules for cell-cell communication [216]. In this regard, *ydhE* gene in *Salmonella* is located next to a *Salmonella* pathogenicity island containing a virulence locus for a type III secretion system [217].

YeeO is another MATE efflux protein and its inactivation increases susceptibility to several structurally unrelated agents including levofloxacin (Table 9.1) [113]. Similar to MdtK, overexpressed YeeO also exports dipeptides [67]. A recent study demonstrated the export of flavin mononucleotide and flavin adenine dinucleotide by YeeO [114], consistent with the role of MATE pumps for extruding metabolic wastes and maintaining cellular homeostasis.

9.3.6 *Efflux Pumps of the SMR Family*

The EmrE pump of the SMR family has been extensively studied [218]. EmrE with only 4-TMS is thought to function as a dimer and its substrates mainly include cationic lipophilic biocides. EmrE is one of a limited number of *E. coli* pumps whose inactivation in wild-type cells causes increased antimicrobial susceptibility [37, 113]. As such, disruption of *emrE* results in a four- to eightfold decrease in MIC values of ethidium bromide and methyl viologen [37], while overexpressed EmrE produces a 2- to 16-fold increase of MIC values of biocides (Table 9.1) [36]. EmrE also exports betaine and choline, endogenous osmoprotectants, and thus reduces pH and osmotic tolerance [115]. Although EmrE is usually considered as a single-component efflux pump, a study also suggested the participation of an OM protein, OmpW, in the extrusion of quaternary cationic agents by EmrE [116]. It remains unknown how the quaternary agents can penetrate the narrow channel of OmpW [126].

A dimeric SMR pump may be a heterodimer, often coded by adjacent genes such as *mdtJI* (also called *ydgFE*) [118]. This pump contributes to resistance to deoxycholate and sodium dodecyl sulfate when expressed from a plasmid [36], and its inactivation enhances susceptibility to a number of antimicrobials including chloramphenicol and erythromycin [113]. MdtJI also catalyzes the export of a polyamine and spermidine; their expression can be induced by spermidine [118]. Although *mdtIJ* genes and other disinfectant resistance genes are widely present in *E. coli* [219, 220], their clinical relevance in biocide resistance remains unknown.

SugE, a suppressor of *groEL* mutations, is an SMR efflux pump encoded by both chromosome and plasmids [119, 219]. Cloned *sugE* on a plasmid confers resistance to quaternary ammonium agents [119]. SugE shows a ligand-binding capability similar to EmrE [221]. Expression of *sugE* is increased in mutants deficient in DNA cytosine methyltransferase and can be induced by cytosine DNA methylation [222]. When present on plasmids, *sugE* coexists with other resistance genes including $bla_{CMY\text{-}2}$ β-lactamase gene [185]. Lastly, a recent study also reported the overexpression of an SMR-encoding gene, *ynfA*, in multidrug-resistant isolates from urinary infections. Its deletion rendered the mutant more susceptible to carbenicillin. However, quantitative susceptibility data are not available [120].

9.3.7 *Efflux Pumps of Other Families*

Several pumps of other families are also involved in drug resistance. Inactivation of two genes, *yghB* and *ygjA* encoding putative exporters of the DedA family, produces hypersusceptibility to several agents (Table 9.1) [107]. Tellurite resistance in *E. coli* is linked to a two-gene operon, *tehAB*, which encode TehA exporter and TehB *S*-adenosyl-*L*-methionine-dependent non-nucleic acid methyltransferases [121, 223]. The TehA pump was previously included in the SMR family and is now

grouped into tellurite-resistance/dicarboxylate transporter (TDT) family. Overproduced TehA alone produces resistance to several antiseptics (Table 9.1) [121]. Insertional inactivation of *tehAB* in a wild-type strain leads to no change in antimicrobial susceptibility except, unexpectedly, a fourfold increase of novobiocin MIC [37]. Interestingly, a homologue of TehA in a wild-type *Vibrio cholerae* strain does not mediate resistance to tellurite but provides resistance to chloramphenicol [224].

9.4 Regulation of the *E. coli* Efflux Pump Expression

The presence of a large number of drug efflux pumps and their possibly overlapping functions require a coordinated regulation of the expression of these pumps. In fact, only a small number of efflux pumps are expressed under normal laboratory growth conditions, with the predominant constitutive expression of the AcrAB-TolC system. Many of the *E. coli* efflux systems may only be expressed under certain conditions including the induction by xenobiotic agents [225].

9.4.1 RND Pumps

AcrAB-TolC Expression of this efflux system has been investigated extensively and described in detail in a recent review [126]. Briefly, multiple regulators and pathways are involved in the regulation of the expression of AcrAB-TolC (Fig. 9.2). Although the a*crAB* and *tolC* genes are located in different regions of the chromosome, their expressions are often controlled by common regulators. The local repressor AcrR of the TetR family directly inhibits both *acrAB* expression and its own expression [226]. However, this inhibition is not sufficiently tight and thus still allows constitutive *acrAB* expression. AcrR can also bind ligands such as rhodamine 6G and hence further reduce its inhibition of *acrAB* expression [227–229]. Mutations in AcrR leading to derepression of *acrAB* have been reported in clinical isolates [137]. A recent study suggested the association between *acrR* mutations and high-level resistance to levofloxacin [140]. Additionally, the AcrS repressor of AcrEF pump [145], histone-like nucleoid structuring protein (H-NS) [76], and quorum-sensing receptor SdiA [230, 231] can influence *acrAB* expression (Fig. 9.2).

Furthermore, three global regulators, MarA, SoxS, and Rob, play more important roles in promoting the expression of *acrAB*, *tolC*, and *micF*, which are part of the affected genes under the *marA-soxS-rob* regulon [232]. The *micF* transcript decreases the production of OmpF porin, which provides an entry channel for small hydrophilic molecules [233]. Encoded by the multiple antibiotic resistance (*mar*) locus containing *marRAB* [234, 235], MarA is a transcriptional activator of the AraC family [236, 237] and is involved in the positive regulation of

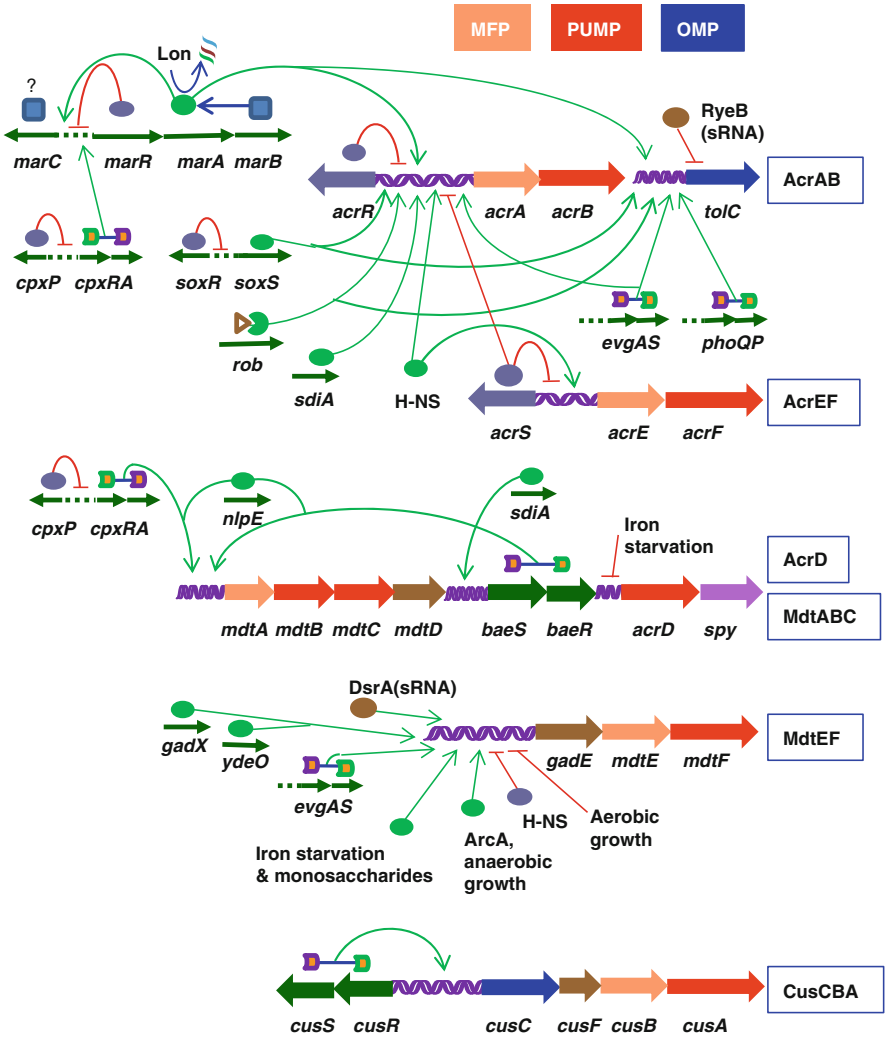

Fig. 9.2 Regulation of the expression of RND efflux pumps in *E. coli*. Six chromosomal RND pump operons are presented (mostly in the *right*) with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (MFP), a pump, or an outer membrane protein (OMP), respectively. Genes encoding the proven or putative regulators are mostly located on the left with their gene transcriptional directions indicated by *arrows*. The *green lines* represent the positive regulation of the efflux gene expression, while the *red lines* denote the repression of relevant gene transcription by repressors. The posttranscriptional regulation via small RNAs and posttranslational via Lon protease are also shown

the expression of *marRAB* and many other genes [238]. MarA also regulates the expression of the small accessory protein AcrZ to AcrB [132, 239]. Importantly, the level of MarA is determined by several proteins: MarR, MarB, and the Lon protease. MarR is the prototypic repressor of the MarR family and negatively controls the expression of *marRAB*, thus playing a critical role in the production level of MarA [240]. MarB also inhibits MarA via a posttranscriptional process [113]. The Lon protease mediates proteolytic degradation of MarA [241]. Additionally, MarA-mediated MDR can be modulated by a number of other chromosomal genes [242]. SoxS and Rob activators [243] also stimulate the expressions of many genes under the *mar* regulon [235]. Overexpression of *marA* and *soxS* was reported in clinical fluoroquinolone-resistant isolates [244]. A recent study identified an Ala12Ser mutation in SoxS that contributed to fluoroquinolone-associated MDR of clinical isolates from dogs [139], further highlighting clinical relevance of SoxS-mediated efflux pump expression. MarA and SoxS are much smaller than Rob, and their expression levels (influenced by MarR and SoxR, respectively) alone can influence the regulation of AcrAB-TolC. In contrast, Rob needs to bind to certain ligands (e.g., dipyridyl [245] or fatty acids and bile salts [246]) to enhance its upregulation of AcrAB. The EvgAS and PhoPQ two-component systems also contribute to the transcriptional regulation of the *acrAB* and *tolC* genes [77, 247–249]. Influence from small RNAs on drug efflux systems has also drawn much attention. For example, the RyeB (SdsR) small RNA inhibits TolC expression via a posttranscriptional pathway [250, 251] with the Hfq small chaperone protein required in this regulation. Hfq mutants with reduced AcrAB production are more susceptible to multiple antibiotics and toxic agents [252]. Repression of *tolC* expression by RyeB occurs by direct base paring, upstream from the ribosome binding site [251]. Lastly, a recent study showed elevated expression of *acrAB-tolC* under simulated microgravity growth condition [253].

Other RND Pumps The production of AcrEF is negatively controlled by a local repressor, AcrS [145], but is upregulated by H-NS [76] (Fig. 9.2). While an *in vitro* selected AcrEF overproducer resulted from an IS2 insertion in the promoter region of *acrEF* [46], another ciprofloxacin-selected triclosan-resistant mutant displayed elevated expression of *acrF* and *marA* with additional fitness cost [254]. The expression of MdtABC and AcrD pumps is regulated by multiple factors including the BaeSR [54, 255] and CpxRA [225] two-component regulatory systems. In fact, such regulation is part of the BaeSR and CpxRA regulons, which are known to respond to extracytoplasmic stress [256–258]. The *baeSR* genes are within a cluster of eight genes including those coding for the MdtBC pump (*mdtABCD-baeSR-acrD-spy*) (Fig. 9.2). BaeSR is essential for envelope stress-induced CRISPR RNA-mediated DNA silencing [259]. While the BaeR overproduction upregulates about 60 genes that are related to two-component signal transduction, chemotactic responses, and flagellar biosynthesis in addition to multidrug transport [260], there are likely upward of 100 genes/operons under the regulation of the Cpx regulon [258, 261]. Indole can induce the production of both AcrD and MdtABC via BaeSR

and CpxRA to yield an MDR phenotype. Yet, the contribution of CpxRA requires the participation of BaeSR (but not vice versa) [225]. The expression of the *nlpE* gene encoding an OM lipoprotein also upregulates, via BaeSR and CpxRA, the AcrD and MdtABC production [262, 263]. Involvement of BaeSR in resistance to the condensed tannins [149] and allyl isothiocyanate (responsible for the pungent smell of mustard) [264] has been reported, likely attributed to the elevated efflux pump expression. Interplay of BaeSR and CpxRA on efflux pump expression has been observed in several bacterial species including *Salmonella* and *Erwinia* [265–267]. Other possible envelope stress-causing agents such as flavonoids and tungstate also induce expression of BaeSR and the MdtABC efflux system [255]. Iron starvation negatively influences the *acrD* expression [268].

The *gadE-mdtEF* operon encodes an acid resistance regulator GadE and the MdtEF system, and its regulation involves multiple factors [59, 269]. The *mdtEF* expression is repressed under the aerobic growth conditions or by H-NS but is enhanced under the anaerobic conditions (via the ArcA global regulator), iron starvation, and by EvgAS and two AraC-type activators, YdeO and GadX [59, 268–271] (Fig. 9.2). Several monosaccharides including *N*-acetylglucosamine and glucose can induce expression of MdtEF, and such induction is dependent on catabolite control involving the cAMP receptor protein [269]. Overexpression of a small (85-nt) noncoding RNA, DsrA, was noted to be linked to the elevated expression of *mdtE* and an MDR phenotype [60]. DsrA acts as an antisilencer of the H-NS-silenced genes [272]. The regulation of CusCBA is positively regulated by the CusSR two-component regulatory system, which exhibits increased activity in response to external copper [151, 273].

9.4.2 Non-RND Efflux Pumps

The control of non-RND drug efflux pump expression is less understood than that of RND pumps, due to at least two reasons: (i) these pumps have been less well studied and (ii) the genes encoding many of these pumps (Table 9.1) are not immediately accompanied by any regulator genes. Likely, the most well-studied non-RND pump regulators of *E. coli* are EmrR of EmrAB pumps and TetR of Tet pumps. The expression of EmrAB is downregulated by EmrR repressor that is encoded by the gene belonging to the same *emrRAB* efflux operon [274]; EmrR binds directly to the *emrAB* promoter [275]. Several chemicals including nalidixic acid can induce *emrAB* expression likely through their interaction with EmrR [274, 276]. Mutations in the *emrR* gene render *E. coli* cells tolerant to elevated sodium levels and lactic acid [277]. EmrR also represses the expression of the *mcb* operon that contributes to microcin B17 production. Interestingly, the production of EmrR (together with that of SoxR and the glutathione synthetic enzyme GshA) is stimulated by polyamines under oxidative stress conditions [278], an observation consistent with the substrate profile of EmrAB (Table 9.1).

The regulation of the *tet* pump gene expression by TetR repressors has been extensively characterized (reviewed in detail in reference [279]). In fact, the knowledge on the regulators of TetR family has helped understand prokaryotic signal transduction mechanisms in general (reviewed in [280]). There are multiple TetR classes, which are often encoded by the genes that are divergently transcribed from the *tet* efflux genes. TetR proteins repress the expression of *tet* efflux genes by binding to the operator of the efflux pump genes. However, the strong affinity of TetR proteins to tetracyclines explains the induction of Tet pump-mediated tetracycline resistance by tetracyclines.

Additionally, EmrKY expression is negatively influenced by H-NS [76]. The expression of several non-RND pumps (including EmrAB, EmrD, EmrE, EmrKY, MdfA, and YdgFE) is relatively stable during the various phases of growth [281]. A study also identified the expression of *mdtG* as part of the *marA-soxS-rob* regulon, in which a truncated SoxR protein resulted in constitutive expression of SoxS in an *in vitro* norfloxacin-selected resistant mutant [84]. The elevated expression of *mdtG* was similar to that of *acrA* and *acrB*, together with the higher expression of TolC protein and reduced expression of *opmF* gene. A marbox was identified in the promoter region of *mdtG* [84]. Multiple factors were recently reported to influence expression of the *mdtJI* operon in *Shigella* with H-NS to repress its expression and the substrates spermidine and bile compounds to increase its expression [282].

9.5 Drug Access in *E. coli*: Competition Between Drug Influx and Efflux

The effectiveness of drug efflux pumps is intimately tied to the strength of the OM permeability barrier in Gram-negative bacteria. Indeed, the drug efflux pumps and the OM interplay to limit the access of antimicrobials to their cellular targets and thus to protect the cell from the action of antimicrobials [27, 283]. The OM barrier of *E. coli* includes the asymmetrically organized lipid bilayer with the inner leaflet of phospholipids and the outer leaflet of LPS [284]. Such a structure prevents the entry of large and/or hydrophobic noxious agents, allowing only a slow influx of drugs. This phenomenon explains the fact that many antibiotics (such as macrolides or lipophilic penicillins) are much less active against Gram-negative bacteria than Gram-positive bacteria [16, 283]. Disruption of the LPS by chemicals or genetic means can significantly increase susceptibility to large and/or hydrophobic agents [15, 285] (see also Table 9.3). The OM also contains the water-filled protein channels (e.g., the major porins, OmpF and OmpC) [284]. The importance of the porin pathway in the entry of small and relatively hydrophilic antimicrobial drugs can be seen by the selection of mutants lacking the larger channel porin OmpF by β-lactams, first reported in *E. coli* in 1981 [286], and is now a major mechanism of resistance in species such as *Klebsiella pneumoniae* (reviewed in [126]). Moreover, additional resistance mechanisms (such as β-lactam hydrolysis by β-lactamases) in the periplasm can further limit access of the drugs to targets [287].

However, newer drugs that are not enzymatically inactivated, such as β-lactamase-stable β-lactams and fluoroquinolones, are often the substrates of the broad-spectrum drug efflux pumps located in the cytoplasmic membrane. Multicomponent pumps such as the major RND system, AcrAB-TolC, have their efflux complex spanning the cytoplasmic and outer membranes. Such pumps take up drug substrates from the periplasm (or cytoplasm through cooperation with single-component pumps in the cytoplasmic membrane) and directly extrude them out of the cell. Because the exported drugs are located extracellularly, not in the periplasm, they cannot easily reenter the cells due to the OM permeability barrier [130]. The competition between the influx and efflux processes ultimately determines the steady-state concentration of drug molecules in the cell and thus antimicrobial susceptibility.

An example for the effect of the influx (e.g., affected by the LPS status) and efflux (determined by *acrAB* status) on antimicrobial susceptibility is shown in Table 9.3, where the inactivation of the *waaP* and/or *acrAB* is achieved genetically. The kinase-encoding *waaP* gene is essential for the addition of phosphate to the LPS inner core region, and its inactivation reduces the OM stability [288]. Although the impact of influx and efflux may vary among different antimicrobials, these two processes generally interplay to affect the drug susceptibility. This knowledge helps us analyze the role of the efflux mechanisms under drug- and species-specific circumstances and thus provides insights for therapeutic intervention of relevant resistance mechanisms.

9.6 Kinetics of AcrAB-TolC Pump

The comparison of antimicrobial susceptibility data generated with strains of different AcrAB status (wild-type level, deficient, and overproduction) has demonstrated the polyspecific substrate profile of AcrAB-TolC system and also provided an indirect measurement of the efficiency of drug efflux process toward different substrates (Tables 9.2 and 9.3). However, the direct determination of substrate transport kinetics of the multicomponent pumps has been very challenging. In early, proteoliposome reconstitution of the purified AcrB or AcrD protein was carried out to assess their efflux activity of several clinically relevant antimicrobial agents [43, 289]. Several other efflux assay methods have also been developed for quantitative, real-time assessment of efflux pump activity in intact cells of *E. coli* [290–294]. One method first requires to de-energize bacterial cells by a proton uncoupler, preload them with a fluorescent probe (e.g., *N*-phenyl-1-naphthylamine and ethidium bromide), followed by monitoring fluorescence after re-energization of the cells through an energy source [290, 291]. Another method is an optimized, semiquantitative assay with the lipophilic Nile red dye that was also developed for further characterization of the AcrAB-TolC pump [292]. This approach found that several tetracyclic antimicrobials (doxorubicin, chlortetracycline, doxycycline, minocycline, and tetracycline) and efflux pump inhibitors (phenylalanyl-arginine β-naphthylamide and 1-naphthyl-methylpiperazine) can compete with the Nile red dye substrate [292].

More recently, a real-time assessment of β-lactam efflux by AcrAB-TolC in *E. coli* intact cells has been achieved through spectrophotometrical monitoring of the β-lactam hydrolysis by a periplasmic β-lactamase, followed by mathematical calculation of various parameters for establishment of kinetic constants [293, 294]. The principle of this assay relies on assessing the periplasmic concentration of β-lactams from their rate of hydrolysis by a β-lactamase in the periplasm and the rate of efflux as the difference between the influx rate and the hydrolysis rate. With this method, a number of conventional cephalosporins and penicillins were tested. The results provided for the first time important information on kinetic constants of the AcrB-catalyzed efflux process for clinically relevant β-lactam antibiotics [293–295]. For example, while nitrocefin efflux indicated a V_{max} of 0.024 nmol/mg/s and a K_m of 5 μM with little sign of cooperativity, the efflux of cephalothin, cefamandole, and cephaloridine displayed lower affinity to the AcrB pump, yet with strong positive cooperativity [293]. In comparison, penicillins were found to possess stronger affinity to AcrB, and the efflux of penicillins also showed strong positive cooperativity [294]. The measured kinetic constants were further validated by the similarity between the experimentally determined MIC values and the theoretically predicated (efflux- and hydrolysis kinetic-based) MIC values [294]. With this efflux transport kinetics assay, certain ligands such as chloramphenicol or organic solvents were revealed to enhance cephalosporin efflux, likely through their effect on AcrB conformational changes needed for substrate extrusion [296]. Additionally, Kinana et al. [297] conducted a quantitative assessment of efflux kinetics of phenylalanyl-arginine β-naphthylamide by AcrB and revealed aminoacyl β-naphthylamides as both substrates and modulators of AcrB. Nitrocefin efflux kinetics can be altered via inhibition of phenylalanyl-arginine β-naphthylamide [297]. Overall, efflux kinetics data not only provide insights on the transport mechanisms of RND pumps but also highlight the clinical implications of these exporters.

9.7 Concluding Remarks

In the past two decades, a number of drug efflux systems of *E. coli* have been identified, with the most-studied AcrAB-TolC pump characterized as the predominant drug exporter. These various drug exporters often serve as the prototypic pumps for bacteria in general. The in-depth understanding gained from these studies has revealed the complexity of these efflux systems and their expression as highlighted in this chapter. The clinical significance of the AcrAB-TolC pump is attributable to both its incredibly broad substrate profile (particularly including most advanced antimicrobial agents) and its contribution to intrinsic and acquired MDR to clinically relevant antibiotics. Resistant isolates overproducing efflux pumps can be readily obtained after antimicrobial exposure. Efflux systems often possess other functions beyond antimicrobial resistance and can, for example, affect stress responses and pathogenesis. This calls for continued efforts to develop antimicrobial agents that can overcome the efflux-mediated resistance and to identify the clinically useful efflux pump inhibitors as antimicrobial adjuvants for combinational therapy.

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Chapter 10
Antimicrobial Drug Efflux Pumps in *Salmonella*

Kunihiko Nishino

Abstract *Salmonella* species are causative organisms of salmonellosis, and the prevalence of multidrug-resistant *Salmonella* has increased dramatically. These multidrug-resistant isolates have been found in both humans and animals and thus pose a major public health concern. Drug resistance in *Salmonella* has been shown to be largely attributable to multiple target gene mutations and to active efflux by pumps. At least ten drug efflux system genes in the genome of this organism have been experimentally identified to date, and some efflux pump genes encoded in plasmids have been also identified. This chapter describes the drug resistance and virulence roles of efflux pumps and their regulation in *Salmonella*.

Keywords *Salmonella* • Antimicrobial resistance • Efflux • RND efflux pumps • Plasmid • Virulence • AcrAB • TolC • RamA • RamR

10.1 Introduction

Salmonella species exist all over the world and are responsible for causing acute gastroenteritis and typhoid/paratyphoid [1]. *Salmonella enterica* serovar Typhimurium is contagious in rodents, including mice, causing a systemic infectious disease, closely resembling human typhoid [2, 3]. In humans, it produces acute gastroenteritis and is a cause of food poisoning. Fluoroquinolones represent the drug of choice for the treatment of a wide range of human infectious diseases, and they were also introduced into veterinary medicine in Europe in the late 1980s through the early 1990s and the USA in 1995. Following their introduction, fluoroquinolone-resistant strains of *Salmonella* started to emerge [4]. Fluoroquinolone resistance in *S. enterica* serovar Typhimurium has been shown to be largely attributable to multiple target gene mutations and to active efflux by

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multidrug transporters [5, 6]. Also, the increasing prevalence of multidrug resistance has been found in *Salmonella* isolates from both humans and animals and thus poses an important public health concern [7, 8].

The genome sequences of *Salmonella* spp. indicate the presence of numerous efflux pump genes that encode transporters of various superfamilies and families [9, 10]. At least ten drug efflux pump genes in the genome of *S. enterica* serovar Typhimurium have been experimentally identified to date [11–15]. Some efflux pump genes encoded on plasmids have been also identified [16–18]. In addition to their roles in drug resistance, it was shown that the efflux pumps contribute to *Salmonella* virulence [13, 15, 19, 20]. Physiological functions of efflux pumps in *Salmonella* have been also reported with roles in metal resistance [21, 22], biofilm formation [23], colonization [11], adhesion, and cell invasion [19]. In this chapter, the roles of *Salmonella* efflux pumps in drug resistance and their physiological functions and regulation are described.

10.2 The AcrAB Efflux Pump in *Salmonella*

S. enterica serovar Typhimurium Tn*phoA* mutants with increased susceptibility to biological and chemical detergents were reported [24], and it was found that one mutant LX1054 had a defect in a multidrug resistance pump AcrB [11]. Nikaido et al. [12] found that the previously reported drug-susceptible *S. enterica* serovar Typhimurium [25] carried a mutation in the *acrAB* operon. The mutant of *acrAB* exhibited increased susceptibility to a wide range of antimicrobial agents including antibiotics, bile salts, dyes, detergents, and disinfectants as shown in Table 10.1 [12, 13]. AcrA and AcrB in *S. enterica* serovar Typhimurium strain LT2 exhibit the amino acid identities of 92 and 95 % with those in *Escherichia coli* [13]. High-level fluoroquinolone resistance in *S. enterica* serovar Typhimurium phage type DT204 has been previously shown to be essentially due to both multiple target gene mutations and active efflux by the AcrAB–TolC efflux system [5, 6]. In other drug-resistant isolates of *Salmonella*, overexpression of *acrB* is also reported [29], and antimicrobial treatment of *Salmonella* results in the increased expression of *acrB* [30, 31]. A post-therapy isolate of *S. enterica* serovar Typhimurium (after treatment with fluoroquinolones and β-lactams) was found to carry a Gly288Asp substitution in AcrB [32]. This residue substitution is located in AcrB drug-binding pocket and significantly affects the structural and dynamic properties of AcrB, resulting in alternated substrate specificity (i.e., reduced susceptibility to fluoroquinolone but increased susceptibility to doxorubicin and minocycline) [32]. Low-level exposure of *S. enterica* serovar Typhimurium to a biocide, either a quaternary ammonium compound, an oxidative compound, or a halogenated tertiary amine compound, in the laboratory selected mutants that were cross-resistant to nalidixic acid, ciprofloxacin, chloramphenicol, tetracycline, and/or triclosan [33]. Among multiple mutations carried by these mutants, derepression of AcrAB–TolC expression was observed [33].

Table 10.1 Substrate profiles of characterized *Salmonella* efflux pumps

Transporter family/efflux pump	Substrates	Reference
RND		
AcrAB	ACR, BAC, CAR, CEF, CHL, CHO, CLX, CTX, CV, DOC, DOR, EB, ERY, FOX, FUA, MB, NAF, NAL, NOR, NOV, PEN, R6G, RIF, SDS, SUL, TET, TPP, TRI, TRX	[11–13, 26]
AcrD	AZT, CAR, DOC, NAF, NOV, OXA, SDS, SUL	[13, 27]
AcrEF	ACR, CHL, CV, DOC, DOR, EB, ERY, NAL, NOR, MB, NOV, R6G, SDS, TET, TPP, TRI	[13, 26]
MdsABC (GesABC)	ACR, BAC, CHL, CLX, CV, EB, MB, NAF, NOV, THL, TPP	[13, 22, 28]
MdtABC	DOC, NOV, SDS	[13]
MFS		
EmrAB	NAL, NOV, R6G, SDS, TRI	[13, 26]
MdfA	CHL, DOR, NOR, TET	[13]
SmvA	ACR, EB, MG, NAL, PQ, PY	[14]
MATE		
MdtK	ACR, DOR, NOR	[13]
ABC		
MacAB	ERY	[13]

ACR acriflavine, *AZT* aztreonam, *BAC* benzalkonium chloride, *CAR* carbenicillin, *CEF* cephalothin, *CHL* chloramphenicol, *CHO* cholate, *CLX* cloxacillin, *CTX* cefotaxime, *CV* crystal violet, *DOC* deoxycholate, *DOR* doxorubicin, *EB* ethidium bromide, *ERY* erythromycin, *FOX* cefoxitin, *FQ* fluoroquinolones, *FUA* fusidic acid, *MB* methylene blue, *MG* malachite green, *NAF* nafcillin, *NAL* nalidixic acid, *NOR* norfloxacin, *NOV* novobiocin, *OQX* olaquindox, *OXA* oxacillin, *PEN* penicillin G, *PQ* paraquat (methyl viologen), *PY* pyronine B, *R6G* rhodamine 6G, *RIF* rifampicin, *SDS* sodium dodecyl sulfate, *SUL* sulbenicillin, *TET* tetracyclines, *THL* thiamphenicol, *TPP* tetraphenylphosphonium, *TRI* triclosan, *TRX* Triton X-100

10.3 The *Salmonella* Drug Efflux Pumps Identified by Genomic Information

Genomic analyses revealed that *Salmonella* strains possess five putative RND efflux systems (http://www.membranetransport.org). Four of them, AcrAB (AcrA, membrane fusion protein; AcrB, RND transporter), AcrD, AcrEF (AcrE, membrane fusion protein; AcrF, RND transporter), and MdtABC (MdtA, membrane fusion protein; MdtB and MdtC, RND transporters), have homologs in *E. coli* with approximately ~90 % amino acid identity (Table 10.1) [13]. MdtB and MdtC are each an RND pump and usually function as one drug efflux system [34]. The last putative RND system is the *Salmonella*-specific MdsABC (MdsA, membrane fusion protein; MdsB, RND transporter; MdsC, outer membrane protein). In addition to the RND pumps, efflux systems belonging to the major facilitator superfamily (MFS) (EmrAB, MdfA, and SmvA), multidrug and toxic compound extrusion (MATE)

family (MdtK), and the ATP-binding cassette (ABC) superfamily (MacAB) transporter families were also experimentally identified (Fig. 10.1) [13, 14, 35].

The genes of *acrAB*, *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, and *macAB* were cloned into the multicopy number plasmid, and their ability to confer drug resistance upon the *Salmonella acrB* mutant was investigated (Table 10.1) [13]. The plasmids carrying efflux operons or genes that confer multidrug resistance phenotypes against various antimicrobial compounds are shown in Table 10.1. It was also reported that the deletion mutant of the *smvA* gene showed increased susceptibility to a range of cytotoxic agents (Table 10.1) [14]. Overproduction of SmvA provided acriflavine resistance in the *Salmonella acrB* mutant (unpublished data). A recent study also showed that *Salmonella* EmrAB and AcrEF pumps may have additive effects with the major efflux system AcrAB in decreased susceptibility to triclosan [26]. Deletion of the *tolC*, *acrB*, or *acrAB* genes resulted in strains with increased susceptibility to various compounds, and the *acrB*, *acrAB*, and *tolC* mutant strains have overlapping substrate susceptibility profiles, which is in agreement with the notion that the encoded proteins interact as a tripartite efflux complex system. The *tolC* mutant was more susceptible to certain compounds including novobiocin, deoxycholate, and sodium dodecyl sulfate than the *acrAB* mutant [13, 36] – suggesting a functional role in other efflux systems. And a strain with nine drug exporters (*acrAB*, *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, and *macAB*) deleted was shown to be more susceptible to novobiocin, deoxycholate, and sodium dodecyl sulfate, compared to the Δ*acrAB* mutant. On the other hand, strains deleted for the *acrD*, *acrEF*, *mdtABC*, *mdsABC*, *emrAB*, *mdfA*, *mdtK*, or *macAB* genes exhibited the same drug susceptibility as the wild-type strain [13]. These two lines of data suggest, similar to *E. coli*, a predominant if not overwhelming role of the AcrAB in the drug resistance phenotype. Furthermore, that other pump expression is minimal and/or their functions are masked by overlapping substrate repertoires with AcrAB. The expression levels of drug transporter genes under laboratory conditions were investigated by streaking out onto X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) LB agar plate strains in which the *E. coli lacZY* genes replaced the chromosomal copy of the drug efflux genes in *Salmonella* [13]. The *tolC-lacZY* and *acrA-lacZY* strains were blue on plates, whereas the *acrD-lacZY*, *emrA-lacZY*, *mdfA-lacZY*, *mdtK-lacZY*, and *macA-lacZY* strains were only faint blue. Thus, the AcrAB–TolC efflux system is expressed in the complex laboratory media, whereas the other efflux systems appear to require additional cues for expression [13].

TolC is required for the function of seven drug efflux systems AcrAB, AcrD, AcrEF, MdsAB, MdtABC, EmrAB, and MacAB in *S. enterica* serovar Typhimurium [27]. Therefore, plasmids carrying the *acrAB*, *acrD*, *acrEF*, *mdsAB*, *mdtABC*, *emrAB*, or *macAB* genes do not confer resistance to the *tolC* mutant, whereas they conferred drug resistance in the *acrB* mutant. Plasmids carrying *mdsABC*, *mdfA*, or *mdtK* provide resistance to the *tolC* mutant, indicating that these three efflux systems function without TolC. The crystal structure of TolC (i.e., ST50) from *Salmonella* Typhi was recently reported, showing the structural basis for TolC role in multidrug efflux pumps across the outer membrane [37]. The *Salmonella*-specific

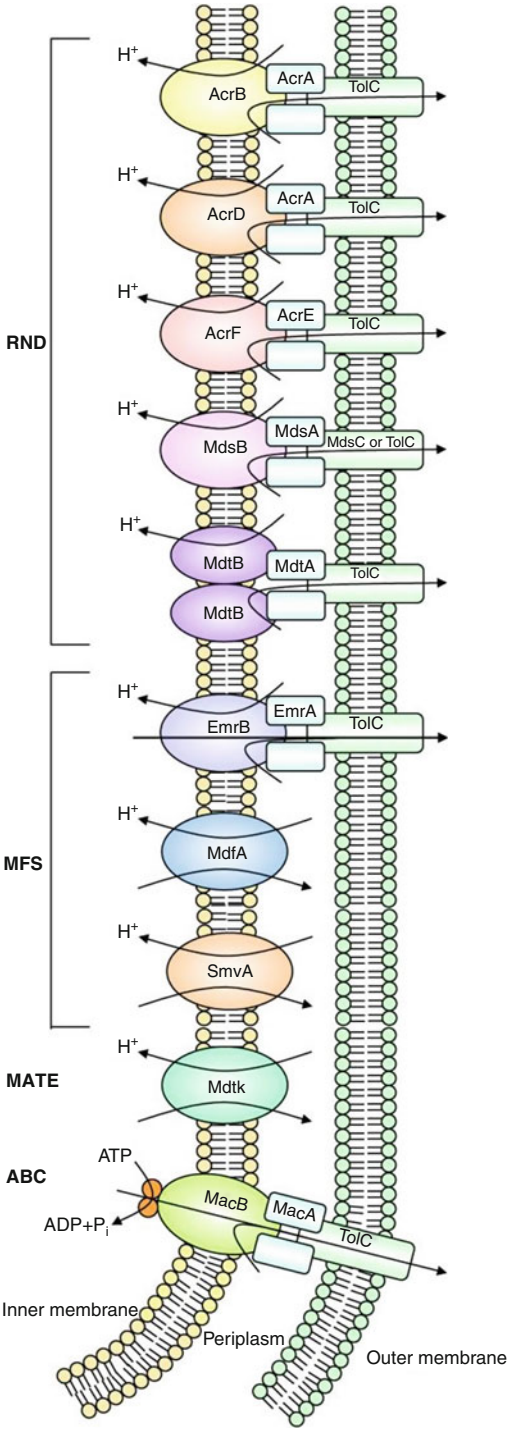

Fig. 10.1 Diagrammatic representation of the structure of multidrug efflux pumps in *Salmonella* and their location on the membranes (Modified from Horiyama et al. [27])

drug efflux system *mdsABC* operon codes for a putative outer membrane protein – MdsC – which is in contrast to the other operons coding for RND-type drug transporter genes. In *E. coli*, most operons coding for RND-type drug transporter homologs lack genes for outer membrane proteins [38] because they rely on TolC as their outer membrane component [39–42]. Overexpression of both the *mdsABC* and *mdsAB* genes produced drug resistance in the Δ*acrB mdsABC* strain. On the other hand, overexpression of *mdsABC*, but not *mdsAB*, resulted in drug resistance to the Δ*acrB tolC mdsABC* strain. These findings indicate that the drug resistance phenotype conferred by the MdsAB system is dependent on the presence of either the MdsC or TolC proteins and that the MdsAB system can function with both TolC and MdsC outer membrane components [13, 27].

Except for the *acrD* gene, all RND efflux system genes also code for a membrane fusion protein in the same operon. The overproduction of AcrD yielded multidrug resistance in the Δ*acrB* mutant against β-lactam antibiotics and other agents (Table 10.1). It was revealed that AcrD requires AcrA and TolC to function (Fig. 10.1) [27, 43]. One possibility for AcrD utilizing AcrA, coded in a different operon, is that AcrD may form a complex with AcrA and TolC when mutations occur in AcrB and compensate for the lost function of AcrAB–TolC multidrug efflux system. Another possibility is that AcrA contributes to different biological functions by forming complexes with two different RND pumps, AcrB and AcrD. Such a functional network of multidrug efflux pumps may contribute to bacterial adaptation to various environmental conditions [43].

10.4 Plasmid-Mediated Fluoroquinolone Efflux Pumps

In addition to the efflux systems encoded in the *Salmonella* genome, plasmid-mediated fluoroquinolone efflux pumps have been identified. The MFS efflux pump QepA was originally identified in *Escherichia coli* clinical isolate [44]. Resistance levels against ciprofloxacin, enrofloxacin, and norfloxacin were significantly elevated in *E. coli* transformants harboring *qepA* under AcrB–TolC-deficient conditions. The intracellular accumulation of norfloxacin was decreased in a *qepA*-expressing *E. coli* transformant [44]. In *Salmonella*, *qepA* was first detected in the clinical isolates obtained in the hospital clinic in Spain [45]. Subsequently, *qepA* was detected in several quinolone-resistant *Salmonella* spp. clinical isolates [46, 47].

Plasmid-encoded multidrug efflux genes *oqxAB* were also identified in *Salmonella* [18, 48–51]. The quinoxaline-di-*N*-oxide olaquindox has been a growth enhancer in pigs. Its antimicrobial activity is due to inhibition of DNA synthesis [52]. The *oqxAB* genes were originally identified from a conjugative plasmid isolated from *E. coli* [53]. OqxA, a membrane fusion protein, and OqxB, an inner membrane protein, are homologous to several RND family efflux systems from different species. Plasmids containing the *oqxAB* genes yielded high resistance to olaquindox in *E. coli*. The *oqxAB*-encoded pump also conferred high resistance to

chloramphenicol [53]. H^+-dependent ethidium efflux abilities of OqxAB were also confirmed in *E. coli* [53]. A derivative of the plasmid encoding OqxAB was readily transferred to enterobacterial pathogens and transconjugants showed reduced susceptibility to chloramphenicol, ciprofloxacin, and olaquindox [54]. OqxAB were found in human clinical isolates on a plasmid in *E. coli* and on the chromosome of *Klebsiella pneumoniae*. IS26-like sequences flanked the plasmid-mediated *oqxAB* genes, suggesting that they had been mobilized as part of a composite transposon [55]. After the first detection of *oqxAB* in *Salmonella* spp. isolated from food [47], the genes were identified in many *Salmonella* isolates which exhibited resistance to fluoroquinolones [48–51, 56, 57].

10.5 Virulence Roles of *Salmonella* Drug Efflux Pumps

Drug efflux systems are evolutionarily ancient and are found throughout the three domains of life [58, 59]. These systems are fundamental to the bacterial physiology and some have roles other than conferring resistance to antimicrobials. Recognizing that the AcrAB–TolC system serves as an important antimicrobial resistance determinant [11, 12], it was also reported that this efflux system is required for *Salmonella* resistance to bile salts [11, 60] which are found exclusively associated with higher vertebrates. It was shown that the *acrB* mutant of *S. enterica* serovar Typhimurium exhibited a reduced capacity to colonize the intestinal tract, and this suggests that AcrAB–TolC efflux system play an important role in mouse intestinal colonization [11]. It was also reported that the deletion of the *macAB* genes attenuated *Salmonella* virulence, and a strain lacking all drug efflux systems was avirulent when mice were inoculated by the oral route [13]. These results indicate that drug efflux genes are required for *Salmonella*'s ability to cause a lethal infection in mice. Utilizing similar approaches, Buckley et al. [19] studied the role of efflux systems on virulence of *S. enterica* serovar Typhimurium using efflux-defective 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. enterica* serovar Typhimurium in chickens. Experiments using BALB/c mice by the oral route with isogenic strains harboring deletions in efflux genes showed that the mutation in *tolC* of *S. enterica* serovar Typhimurium attenuated virulence [13], as reported for an *S. enterica* serovar Enteritidis *tolC* mutant [61]. Inactivation of the MarA or RamA activator (which upregulates AcrAB–TolC expression; see Sect. 10.7) reduced both the invasion and survival ability of *Salmonella choleraesuis* in the host cells and virulence in mice [62].

Salmonella MacAB pump plays a role in the detoxification of reactive oxygen species, compounds that salmonellae are exposed to at various stages of infection [63]. The *macAB* operon is induced upon exposure to hydrogen peroxide and is critical for survival of *S. enterica* serovar Typhimurium in the presence of oxidative stress. Furthermore, *macAB* is required for intracellular replication inside murine macrophages but is not required for survival in reactive oxygen species-deficient

macrophages [63]. Bogomolnaya et al. [63] suggested the presence of a soluble anti-peroxide compound secreted by *Salmonella* cells through a MacAB-dependent mechanism. In *E. coli*, MacAB is involved in the secretion of heat-stable enterotoxin II [64], and MacA binds lipopolysaccharide core specifically with high affinity [65]. Also, it was recently reported that protoporphyrin is exported by MacAB–TolC in *E. coli* [66]. Because high protoporphyrin levels result in production of reactive oxygen species [67], Turlin et al. [66] proposed that MacAB is involved in the efflux of intracellular protoporphyrin which decreases reactive oxygen species formation in the bacterial cytoplasm, providing a possible explanation for the role of MacAB in *Salmonella* pathogenicity.

10.6 Physiological Functions of *Salmonella* Drug Efflux Pumps

There are several reports about the physiological functions of *Salmonella* drug efflux systems. The BaeSR two-component signal transduction system activates the *acrD* and *mdtABC* expression in response to indole, copper, and zinc. BaeSR, AcrD, and MdtABC contribute to copper and zinc resistance in *Salmonella* [21]; andiron and sodium tungstate are inducers of the BaeR regulon suggesting MdtA, AcrD, and AcrB exist for the waste disposal of tungstate from the cell [68]. Additionally, the MdsABC pump (also called GesABC) is required for gold resistance and the *mdsABC* operon is controlled by GolS which is a MerR-like sensor and highly selective for Au ions [22]. In contrast to heavy metal-specific CusCBA RND pump of *E. coli*, MdsABC, accommodates a large number of substrates including many antibiotics (Table 10.1) [28].

Recent studies have showed that defects in efflux activity impair biofilm formation. In *S. enterica* serovar Typhimurium, deletion of any efflux pump or chemical inhibition of the efflux activity results in compromised ability of *Salmonella* to form biofilm [23]. The defect of biofilm formation in efflux mutants 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 [69] (also see Chap. 25).

10.7 Regulation of *Salmonella* Drug Efflux Pumps

The key to understanding how bacteria utilize multidrug efflux pumps lies in the regulation of pump expression. The data currently available show that multidrug efflux pumps are often expressed under precise and elaborate transcriptional control. For example, expression of *macAB* is controlled by the PhoPQ system, the master regulator for the virulence of *Salmonella* (Table 10.2) [13]. A sequence

resembling the PhoP binding box exists in the upstream of the *macAB* operon [78]. DNase I footprinting analysis with the purified PhoP protein showed protection of the region upstream of the *macA* open reading frame [13], indicating that the PhoPQ two-component signal transduction system controls *macAB* directly. Analysis of mRNA levels of drug efflux genes revealed that the expression of *macAB* is induced when the organism infects macrophages [15]. A recent study also showed that hydrogen peroxide induces expression of *macAB* [63], supporting the induction of *macAB* inside macrophages and the existence of additional regulator to control the *macAB* genes responsive to hydrogen peroxide.

Moreover, positive regulation of the multidrug efflux pump *mdtABC* and *acrD* genes by the BaeSR two-component signal transduction system was found (Table 10.2) [21]. In addition to the roles of MdtABC, AcrD, and BaeSR in multidrug resistance, they contribute to copper and zinc resistance in *Salmonella* as described above. Both copper and zinc are essential for organisms but can be toxic at high levels, and microorganisms express diverse resistance mechanisms. The expression of *mdtABC* and *acrD* is induced by copper or zinc, and BaeSR is involved in this induction (Table 10.2). This finding indicates that the MdtABC and AcrD efflux systems have physiological roles in metal homeostasis beyond multidrug resistance [21]. It was also reported that GolS controls MdsABC in response to Au ions [22].

Table 10.2 The known regulators of multidrug efflux pumps in *Salmonella*

Efflux pump	Regulator	Regulator family	Inducible signal	Reference
AcrAB	RamA	AraC	Bile, indole	[70]
	RamR	TetR	Berberine, bile, crystal violet, dequalinium, ethidium bromide, rhodamine 6G	[71, 72]
	AcrR	TetR	Unknown	[73]
	MarA	AraC	Unknown	[74]
	SoxS	AraC	Paraquat	[75]
AcrEF	AcrS	TetR	Unknown	[76]
	H-NS	Histone-like protein	Unknown	[77]
AcrD	BaeSR	Two-component system	Indole, copper, iron, zinc tungstate	[21, 68]
	CpxAR	Two-component system	Indole, copper, zinc	[21]
MdtABC	BaeSR	Two-component system	Indole, copper, zinc, tungstate	[21, 68]
	CpxAR	Two-component system	Indole, copper, zinc	[21]
MdsABC	GolS	MerR	Gold	[22]
MacAB	PhoPQ	Two-component system	Magnesium	[13]

Mutations in *acrR* contribute to overexpression of *acrAB* and increases resistance to multiple drugs in *Salmonella* [73]. The histone-like protein (H-NS) modulates multidrug resistance through repression of the *acrEF* genes [77]. Eaves et al. [74] suggested that *acrB*, *acrF*, and *acrD* are coordinately regulated and that their expression is also influenced by the expression of the transcriptional activators *marA* and *soxS*. Nikaido et al. [75] found that *acrAB* induction in response to methyl viologen is dependent on SoxS. Indole, bile salts, and an *E. coli*-conditioned medium were also able to induce the expression of *acrAB* in *Salmonella*. The *acrAB* induction by these three signal sources is completely dependent on the *Salmonella*-specific regulator RamA, indicating that RamA plays a major role in inducing *acrAB* (Table 10.2) [70]. RamA belongs to the AraC transcriptional activator family, and this gene appears to be specific for *Salmonella* serovars and is absent in many other Gram-negative microorganisms; notable exceptions are *Klebsiella pneumoniae* and *Enterobacter* species [79–81]. The AcrAB induction pathway in *Salmonella* is different from that in *E. coli*. Bile induces AcrAB in both *Salmonella* and *E. coli*. In *E. coli*, the transcriptional factor Rob plays a major role in inducing *acrAB* expression in response to bile [82]. However, bile induction of *acrAB* in *Salmonella* is dependent on RamA, not Rob. Other regulators, including MarA, SoxS, SdiA, and AcrR, are not involved in AcrAB induction by indole and bile [70]. These facts suggest that RamA is the major regulator of *Salmonella acrAB* and may mask the contributions of any other *acrAB* regulators.

Abouzeed et al. [83] demonstrated that the inactivation of the *ramR* gene upstream of *ramA* resulted in an increased expression of *ramA* and the AcrAB efflux pump, indicating that RamR is a local repressor of *ramA*. Inactivation of *marR*, *marA*, *soxR*, and *soxS* did not affect the susceptibilities of the *S. enterica* serovar Typhimurium strain LT2, whereas the disruption of *ramR* resulted in a multidrug resistance phenotype with this strain. In *E. coli*, multiple regulators, including MarA, Rob, SoxS, and SdiA, work together in controlling *acrAB* expression in response to *acrAB* inducers. This may be related to the lack of RamA in *E. coli*. Indeed, overproduction of RamA has induced the drug resistance level of *E. coli* [84, 85]. There may also be different induction mechanisms for *acrAB* via the RamA regulator. Indole was shown to induce *ramA* expression, and such increased expression of *ramA* can induce *acrAB*, whereas bile binds to RamA. This is reminiscent of the binding of bile to the Rob protein involved in regulation of *acrAB* in *E. coli* [82]. It seems that RamA can be converted from a low-activity state to a high-activity state in response to bile. More recently, Baucheron et al. [71] also identified a different induction mechanism of *acrAB* in response to bile whereby the bile-mediated activation of the *acrAB* and *tolC* multidrug efflux genes occurs via transcriptional derepression of the *ramA* activator gene, likely via the RamR repressor protein controlling expression of *ramA*. Indole and bile salts are found in various internal human environments, especially in the intestine [86, 87]. Indole is produced by many enteric bacterial species [87], and bile is often present in high concentrations in the intestinal tract [86]. Therefore, RamA may be required for *Salmonella* to detect environmental signals and for subsequent induction of the AcrAB–TolC system, resulting in excretion of toxic compounds into the surrounding environment

in the above examples, the intestine. A recent study showed heterogeneity in *ramRA* mutations and its differential impact on expression of regulator genes *ramA*, *marA*, *soxS*, and *acrR* and efflux component genes *acrB*, *acrF*, *emrB*, and *tolC*, revealing deletions that affected RamR-binding site exhibiting a major impact on the *ramA* transcript level and the multidrug resistance phenotype [88].

10.8 Structure of Multidrug Efflux Pump Regulator RamR with Multiple Drugs

As described above, RamR and RamA are important regulators for AcrAB–TolC in *Salmonella*. From the structural and biochemical analysis of RamR, a multidrug recognition mechanism of RamR occurs, whereby the DNA-binding activity is controlled by multiple drugs in order to induce *ramA* expression [72]. Yamasaki et al. [72] identified five substrates of the RamR protein, including berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G (Fig. 10.2). Similar approaches in crystallizing the TetR family regulators with multiple drugs have been also reported in QacR [89], TtgR [90], and CmeR [91]. The molecular weight of RamR in solution was calculated to be 36 kDa using gel filtration chromatography, which was conducted during the purification of the RamR protein. Dissolved RamR was found to exist in the dimer form in solution, and the molecular weight of the RamR monomer was 21 kDa [72]. The structure of RamR was initially determined at a resolution of 2.6 Å by multiple wavelength anomalous dispersion using selenomethionine modification. Subsequently, the RamR structure was determined at 2.1 Å by molecular replacement. Approximate overall dimensions of the RamR dimer were $58 \times 47 \times 44$ Å^3. RamR is composed of nine α-helices, and the three-helix bundle structures formed at the N-terminus maintain a helix-turn-helix motif conserved in DNA-binding sites. The structure of the RamR DNA-binding site is similar to that of other TetR family regulators. By the surface plasmon resonance analysis, it was found that five compounds, berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G, bind to the RamR protein. In contrast, tetracycline did not show any indication of binding to RamR. Using a *ramA* reporter plasmid, a ß-galactosidase assay showed the enhanced promoter activity of *ramA* when bacterial cells were treated with berberine, crystal violet, dequalinium, ethidium bromide, or rhodamine 6G. The crystal structures of RamR in complex with berberine, crystal violet, dequalinium, ethidium bromide, and rhodamine 6G were determined at a resolution of 2.4, 2.2, 2.6, 1.6, and 2.5 Å, respectively [72]. The structure reveals that RamR binds two molecules of berberine, ethidium bromide, or rhodamine 6G per dimer. And RamR binds one crystal violet or dequalinium molecule per dimer. It was originally reported that all the ligands bind to QacR with a 1:2 stoichiometry (one ligand per QacR dimer) [89], while either 1:2 or 1:1 stoichiometry has been observed for RamR. Similar observations were reported in TtgR [90]. The orientation of all agents is parallel with the Phe155 of RamR, suggesting that all these drugs bind with RamR through π–π stacking interactions. In contrast

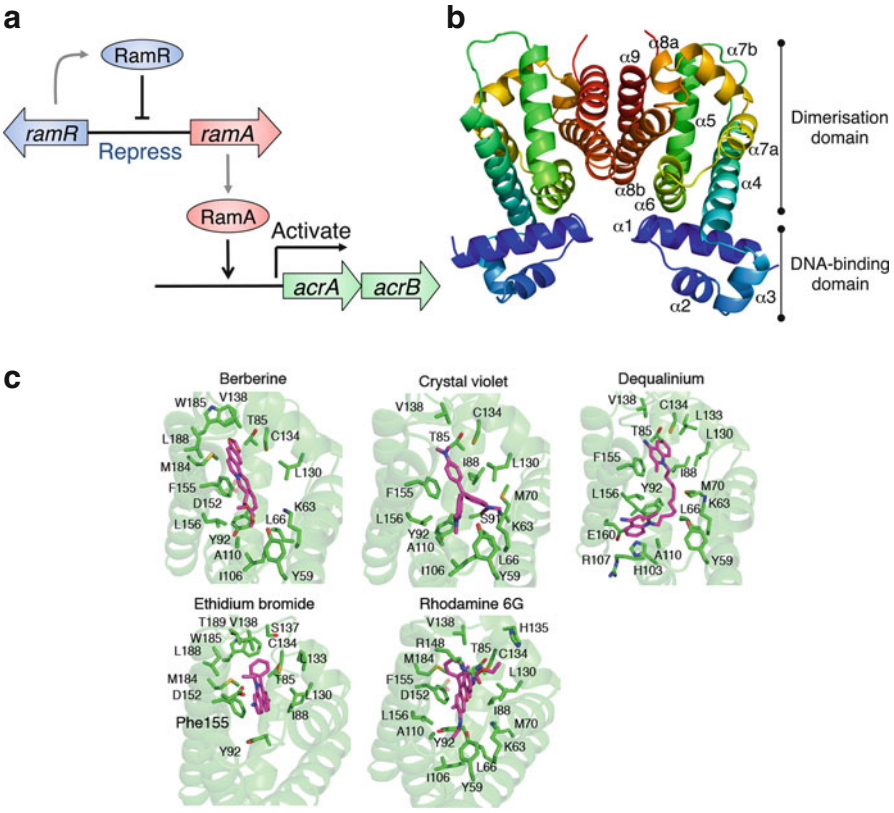

Fig. 10.2 Regulatory cascade and structure of RamR. (**a**) Model for gene regulation by RamR. RamR represses expression of the *ramA* gene, which encodes the activator protein for the *acrAB* efflux pump genes. RamR binds to the intergenic region between the *ramR* and *ramA* genes, and RamA binds to the upstream region of *acrAB*. (**b**) Crystal structure of the RamR dimer. Each monomer is colored as follows: the α-helices are represented in *blue* (*α1*), *marine* (*α2*), *sky blue* (*α3*), *cyan* (*α4*), *green* (*α5*), *limon* (*α6*), *yellow* (*α7a*), *deep olive* (*α7b*), *orange* (*α8a*), *brown* (*α8b*), and *red* (*α9*). (**c**) Multidrug recognition by RamR. Substrate binding site of RamR with a bound molecule berberine, *crystal violet*, dequalinium, ethidium bromide, or rhodamine 6G. Key residues are shown, including residue Phe155, which is involved in π–π stacking interactions with drugs. Carbon atoms of drugs and RamR are shown in *magenta* and *green*, respectively. Nitrogen, oxygen, and sulfur atoms are shown in *blue*, *red*, and *yellow*, respectively (Figure is modified from Yamasaki et al. [72])

to the common interaction of all of these drugs with Phe155, each individual drug was also found to interact with a different set of amino acid residues other than Phe155. The interaction of different sets of amino acid residues with each drug indicates that multiple drugs are recognized by the multisite binding of RamR [72]. Comparison of the liganded structures with an unliganded RamR structure reveals that drug binding triggers an expansion of the distance between the N-termini of the helix-turn-helix motifs in the RamR dimer. This expansion occurred as a result of the binding of all of the drugs examined. By the electrophoretic mobility shift assays

and surface plasmon resonance experiments, RamR substrates interact with their recognition sites to reduce the DNA-binding affinity of RamR, resulting in the induction of *ramA* [72]. Because RamA has also been reported to negatively influence virulence in *S. enterica* serovar Typhimurium by downregulating expression of the *Salmonella* pathogenicity island 1 [92], determining the crystal structure of RamR is the first step in understanding the structural basis for the function of the regulatory proteins that control both drug resistance and virulence in pathogens. This effort extended our knowledge of transcriptional regulation mediated by RamR, a regulator of multidrug resistance in several enterobacterial pathogens.

10.9 Concluding Remarks

Post-genomic research has demonstrated that bacteria possess a large number of drug efflux system genes. As described in this chapter, at least ten drug efflux systems in the genome of *S. enterica* have been experimentally identified to date. Under normal growth conditions, most of drug efflux pumps are thought to be weakly expressed [13]. Increased expression of such efflux systems is possible when mutations occur in their regulatory factors. In fact, various types of mutations in *ramR* and the *ramR–ramA* intergenic region were identified in multidrug-resistant strains of *S*. Typhimurium, other *S. enterica* serovars, and *K. pneumoniae*, which result in increased expression of *ramA* and an increase in efflux-mediated multidrug resistance [83, 93, 94]. Also, it was reported that overexpression of the multidrug efflux operon *acrEF* occurs by insertional activation with IS1 or IS10 elements in *S. enterica* serovar Typhimurium DT204 *acrB* mutants selected with fluoroquinolones [76]. A mutation in *acrR*, the local repressor of *acrAB*, was found for two ciprofloxacin-resistant selected mutants of *S. enterica* serovar Typhimurium [73]. In addition to these mutations, the structural and biochemical analysis showed that toxic compounds bind to RamR resulting in the increased efflux activity of *Salmonella* to protect this organism against the compounds [72].

Association of resistance mechanism with two-component signal transduction systems, which control the expression of drug efflux pumps, has also been identified in *Salmonella*. These findings suggest that the expression of efflux systems is transiently induced through some types of stimulation. In fact, this induction occurs as a result of various environmental stressors, such as low pH, osmotic changes, metals, and oxidative stress. The mechanism by which efflux pumps are expressed in response to the environment suggests that they might be expressed in the growth environments of bacteria such as at infection sites. It is reasonable to assume that efflux systems are induced inside hosts because these contribute not only to drug resistance but also to bacterial virulence. Therefore, it is necessary to identify the regulatory network of multidrug transporters in order to understand their physiological functions. Moreover, determining the physiological substrate of efflux systems is an important area of study, which will contribute to the understanding of the role of drug efflux systems in virulence.

The mechanism by which drug efflux pumps contribute to bacterial virulence has three features. Firstly, the efflux system has the capacity to transport substrates necessary to establish virulence, for example, toxins. Secondly, the efflux system is able to export antibacterial substances present in the host (such as bile acid and antimicrobial peptides) in order to protect the bacteria from the host environment. Thirdly, it can transport factors contributing to bacterial homeostasis or promoting bacterial regulatory functions within the host (such as autoinducers). Currently, several research groups and pharmaceutical companies are conducting research to develop drug efflux pump inhibitors. As efflux systems contribute to multidrug resistance and bacterial virulence, efflux systems are an attractive target for the development of new drugs. If an effective inhibitor is found, it could play a role in the development of new therapies that could conquer bacterial multidrug resistance and virulence.

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Chapter 11
Antimicrobial Drug Efflux Pumps in *Enterobacter* and *Klebsiella*

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Abstract *Enterobacter* and *Klebsiella* spp. are recognized as important opportunistic and multidrug-resistant bacterial pathogens and now classified in the ESKAPE microorganism group. These Gram-negative bacteria exhibit a rapid and efficient adaptation to antimicrobial agents and are responsible for several healthcare-associated infections. The modification of the transporters involved in the drug translocation through membrane barrier represents the first line of bacterial defense. Multidrug resistance is primarily due to modifications of membrane transporters involved in the antimicrobial translocation. This results from the activation of several regulatory pathways such as Mar or Ram that control membrane permeability through the expression of porins and efflux pumps. The overexpression of efflux pumps dramatically reduces the intra-bacterial concentration of various classes of antimicrobials, and the extrusion takes place rapidly when the pumps are active. The *Enterobacter* and *Klebsiella* prevalence in human infections and the major contribution of efflux for controlling the intracellular concentration of antimicrobials highlight the role of the membrane barrier in bacterial strategies facing our antimicrobial arsenal.

Keywords *Enterobacter* • *Klebsiella* • Antimicrobial resistance • Efflux pumps • Outer membrane • Porin • Activator • Repressor • Virulence • AcrAB-TolC • EefABC • OqxAB • AcrR • RamA • RamR • RarA

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11.1 Introduction

Enterobacter and *Klebsiella* are members of common Gram-negative, facultative anaerobic, rod-shaped bacteria belonging to the family *Enterobacteriaceae*. Both genera are very close and it was even suggested twice to reclassify *Enterobacter aerogenes* into the *Klebsiella* genus [1]. Today, *Enterobacter* and *Klebsiella* species represent the prominent enterobacterial members of the ESKAPE pathogen group that encompasses the main multidrug-resistant strains described in the most worrying and complicated infections in hospitals [2, 3]. This ranking is associated with the rapid adaptation of these species face to antimicrobial therapy, including the control of membrane permeability barrier and the expression of several mechanisms of resistance [4]. A recent publication has clearly demonstrated the correlation between the emergence of resistant isolates during the treatment with imipenem of a patient infected with *E. aerogenes* and the modification of membrane permeability associated with increasing resistance toward β-lactam antibiotics [5].

In *Enterobacter* and *Klebsiella* species, involvement of several regulators and genetic cascades has been described in the development or selection of efficient mechanisms that provide a serious resistance against various antimicrobial classes [6]. Importantly, the activity of these global or local regulators has been reported in many clinical isolates in addition to the expression of different genes directly involved in the control of intracellular accumulation of antimicrobial agents. This chapter provides an up-to-date review of efflux pump-mediated antimicrobial resistance in *Enterobacter* and *Klebsiella* including the regulation of pump expression and the methods used for pump characterization.

11.2 Efflux and Resistance in *E. aerogenes* and *K. pneumoniae* Clinical Strains

11.2.1 E. aerogenes *and* K. pneumoniae*: Relevance in Gram-Negative Bacteria Infection and Antimicrobial Resistance*

E. aerogenes has been responsible for a significant European outbreak between 1993 and 2003 and is considered as the paradigm of opportunistic bacteria. Until 2003, *E. aerogenes* was considered as an important emerging multidrug-resistant pathogen, particularly in intensive care units [7, 8]. The situation in the 1990s in Europe pointed to the dispersion of an epidemic clone [9]. The event corresponded to the international spreading of the extended-spectrum β-lactamase (ESBL) TEM-24 ($bla_{\text{TEM-24}}$), located on an epidemic plasmid [7, 9]. The prevalence of *Enterobacter* spp. infections in clinical institutes has also increased greatly with the introduction of extended-spectrum cephalosporins and carbapenems into antimicrobial therapy [10, 11]. *Enterobacter cloacae* is among the most common *Enterobacter* spp. causing only nosocomial infections in the last decade, and a number of studies have been

published on the antimicrobial resistance features of these microorganisms. Interestingly, due to the diffusion of most frequent ESBLs and carbapenemases in this species, *E. cloacae* has now become the third *Enterobacteriaceae* species involved in nosocomial infections after *Escherichia coli* and *K. pneumoniae* [12, 13]. Recent observations suggest that *E. cloacae* harbors clonal lineages of increased epidemic potential that may be associated with resistance spread in Europe [14].

Despite its intrinsic resistance to ampicillin and constant expression of ESBLs, which is associated to other resistance mechanisms and results in multidrug resistance (MDR) phenotype, the prevalence of ESBL-producing *E. aerogenes* has significantly dropped by 18-fold in France from 2002 to 2012 [10, 12]. Its position was displaced in the context of hospital-acquired infections, because of the drastic rise of the *E. coli* pandemic clone O25:H4-ST131 along with *K. pneumoniae* and *E. cloacae*, ESBL- and/or carbapenemase-producing strains [15]. *E. cloacae* is now the most frequently observed clinical species among *Enterobacter* spp. [6]. It can be associated to the dissemination of actual epidemic plasmids bearing most prevalent resistant genes and expressing the widest range of new β-lactamases or carbapenemases.

Resistance of *Enterobacter* spp. to third-generation cephalosporins is most typically caused by overproduction of AmpC β-lactamases, and if strains are also ESBL producers, they become resistant to all members of this class of antibiotics. The prevalence of AmpC and CTX-M producers represented approximately 5 % of the isolates in most recent studies, and these enzymes are most often plasmid mediated [13]. These characteristics, associated with the frequent endogenous intestinal carriage of *E. cloacae*, may result in abnormally high levels in the bowels of hospitalized patients, especially those who have received cephalosporins [13].

K. pneumoniae is, after *E. coli*, the main enterobacterial species involved in a wide range of nosocomial infections [4]. Following epidemiology of the plasmid-mediated resistance to extended-spectrum cephalosporinases, *K. pneumoniae* has been for the last 30 years the nosocomial enterobacterial species with the highest proportion of isolates producing plasmid-mediated β-lactamases, including ESBLs, cephalosporinases, and carbapenemases [16–19]. Contrary to *Enterobacter* spp., *K. pneumoniae* also causes invasive community-acquired infections, including liver abscess, pneumonia, severe bacteremia, and meningitis [20]. The hypervirulent strains causing these infections are exceptionally resistant to antimicrobials, whereas nosocomial strains are commonly multidrug resistant. Interestingly, hypervirulent and multidrug-resistant strains belong to different clonal groups following a recent study that provides genomic definition of clonal groups in *K. pneumoniae* [21].

11.2.2 First Description of Efflux Mechanisms, Prevalence, and Associated Mechanisms of Resistance

In 1998, the involvement of an efflux mechanism in the extrusion of molecules such as chloramphenicol, fluoroquinolones, and tetracycline in *Enterobacter* spp. was first described [22]. This mechanism is highly efficient since about 80–90 % of the norfloxacin content can be extruded by the AcrAB-TolC efflux pump during the first

10–15 min [22]. This process is energy dependent and requires membrane energy (proton motive force) as extensively described (for a review, see [18]). Approximately 40 % of multidrug-resistant clinical strains have an active efflux contributing to resistance [8]. The role of efflux mechanism in *E. aerogenes* resistance has been studied with isolates from an 8-year period, which indicated a noticeable increase in the number of clinical isolates containing an efflux mechanism susceptible to a pump inhibitor in one decade [8].

The EefABC and AcrAB-TolC efflux pump genes of *E. aerogenes* and *K. pneumoniae* have been described (Table 11.1), and their involvement in antimicrobial exportation has been studied [23–26, 36, 37]. Several studies on *E. cloacae* have also reported the presence of efflux pumps belonging to the resistance-nodulation-division (RND) superfamily such as AcrAB-TolC and OqxAB [38–41]. In *E. aerogenes* and *E. cloacae*, the sequence similarities and biological activity are particularly high with regard to AcrAB-TolC [24, 39]. Various chemicals such as chloramphenicol, imipenem, and salicylate are also able to trigger the genetic cascade controlling

Table 11.1 RND-type efflux pumps in *E. aerogenes* and *K. pneumoniae*

Accession number[a]	Efflux component	Protein family	Phenotype	References
EAE_12955 EAE_12950	AcrA AcrB	Periplasmic adaptor RND transporter	Multidrug resistance	[24]
EAE_4740 EAE_4735	AcrE AcrF	Periplasmic adaptor RND transporter	Not determined	
EAE_00485	AcrD	RND transporter	Aminoglycoside resistance	
EAE_06725 EAE_06730 EAE_06735	EefA EefB EefC	Periplasmic adaptor RND transporter Outer membrane TolC	Acid tolerance and virulence	[25, 26]
EAE_19065		Putative RND transporter	Not determined	
EAE_16625 EAE_16630	OqxA OqxB	Periplasmic adaptor RND transporter	Not determined	[27–29]
EAE_23590 EAE_23595 EAE_23600	MdtA MdtB MdtC	Periplasmic adaptor Heterodimeric RND transporter Heterodimeric RND transporter	Not determined	[30, 31]
EAE_09970 EAE_09975 EAE_09980 EAE_09985	CusA CusB CusF CusC	Periplasmic adaptor RND transporter Chaperone Outer membrane TolC	Resistance to metallic cations	[32, 33]

[a]Accession numbers are given accordingly to the deposited genomes of reference strain *E. aerogenes* KCTC2190 [34]. The complete genome for *K. pneumoniae* is also available such as for strain KCTC2242 [35]

the expression of *Enterobacter* AcrAB-TolC pump [42]. The regulation seems to be associated with the internal concentration of chemicals that plays a role in activation of the efflux pump expression cascade [43]. This coordinated control of influx and efflux directly and efficiently governs the intracellular accumulation of antimicrobial agents. Importantly, this internal accumulation of antimicrobial molecules below the threshold corresponding to the minimal inhibitory concentration (MIC) can favor the emergence and the acquisition of additional mechanisms of resistance such as target mutation and production of detoxifying enzymes (e.g., β-lactamases and acetyltransferase) and contribute to the extension of MDR phenotype [23, 44]. The involvement of efflux pumps in resistant isolates has been largely underevaluated due to the scarcity of precise assays to determine the level of efflux activity in Gram-negative isolates (see Sect. 4 below). Only a limited number of studies have reported an evaluation of the efflux pumps in various clinical isolate collections or have tried to follow the evolution of efflux resistance measurement in *Enterobacteriaceae* pathogens during the last decades. Importantly, a recent report has described an increase of the efflux concern in hospital isolates of *E. aerogenes* suggesting that this resistance mechanism can be underestimated or masked by other mechanisms [5, 8]. Another recent study further confirmed AcrAB-TolC as the most important RND pump for its contribution to multidrug resistance in *E. cloacae* [45]. This study also identified additional known or putative RND pumps in *E. cloacae*, which include CusCBA homologues (ECL_01960-01962-01963 and ECL 04893-04891-04888), EefABC, MdtABC, OqxAB, ECL_01759-01758, ECL_02243-02244, ECL_03150-03149, ECL_03767, and ECL_04649-04650. Trans-complementation of *acrB* by EefABC, ECL_01758, or OqxAB largely restored the wild-type drug susceptibility phenotype [45].

In *K. pneumoniae*, β-lactamase production and reduced porin levels have been the major mechanisms described as to be involved in β-lactam resistance until the middle of the 2000s [46]. Involvement of overexpression of efflux pumps in β-lactam resistance in clinical isolates of *K. pneumoniae* was suspected with reduced susceptibility to cefoxitin in isolates without cephalosporinase production and with a low level of resistance to antimicrobial families (e.g., nalidixic acid and chloramphenicol) well-known to be substrates of drug efflux pumps [23, 47–50]. On the basis of this particular resistance phenotype, henceforth confirmed to be related to overexpression of efflux pumps in *K. pneumoniae* [27, 51], 5 % of clinical isolates displaying this phenotype were assessed [52]. However, as this mechanism of resistance is often associated with others in clinical isolates [5], its precise detection is difficult, and subsequently its prevalence is probably underestimated.

The basal efflux present in bacteria is certainly the first mechanism with membrane impermeability that opposes the action of antimicrobials on the bacteria [42]. A subinhibitory intracellular concentration of antimicrobials will then promote the development of more specific mechanisms of resistance such as target mutations for fluoroquinolones or enzymatic response for β-lactams. For example, several mutations are found in genes *gyrA* and *parC* coding quinolone targets, and they are frequently associated to efflux, resulting in high MIC levels. Thus, most of the isolates studied combine efflux to specific resistance mechanisms. Inhibition of efflux

mechanisms alone in such cases never restores susceptibility to the antimicrobial despite a significant diminution of the MICs [53]. At this moment, several efflux pumps have been genetically and functionally characterized in *E. aerogenes* and *K. pneumoniae* (Table 11.1).

11.2.3 Involvement of Efflux Pumps in E. aerogenes *and* K. pneumoniae *Functions Other Than Antimicrobial Resistance*

Padilla and colleagues have described a moderate increase in cefoxitin MIC, concomitantly to resistance to chloramphenicol and ciprofloxacin, in a derivative of *K. pneumoniae* strain 52145 in which the gene *acrR*, encoding the repressor of the efflux system AcrAB had been inactivated. They showed in a murine model of pneumonia that the *K. pneumoniae* AcrAB efflux system is involved in resistance to the host antimicrobial peptides present in the lung, one of the first host barriers of the innate immune system against infections [54]. This finding strongly suggests the participation of efflux systems of *K. pneumoniae* in its virulence, as did the results published by Coudeyras et al. [25]. The latter showed that the potential efflux pump EefABC of *K. pneumoniae* conferred an advantage to this bacterial species to colonize the digestive tract in a murine model [25].

In order to determine the respective role in virulence of the two efflux pumps AcrAB and OqxAB identified in *K. pneumoniae*, Bialek-Davenet et al. tested several isogenic strains, differentially expressing both efflux pumps, in the *Caenorhabditis elegans* model [27]. A strain normally producing AcrAB and overproducing OqxAB, consecutively to a mutation in the gene encoding *oqxR*, was significantly more virulent than its isogenic strain producing AcrAB and deleted for *oqxABR*. Deletion of the *acrB* gene in a strain overproducing OqxAB led to a significant decrease in its virulence. Furthermore, the same effect was observed with a strain deleted for *oqxABR* and overexpressing *acrB* due to a deletion of the *ramR* gene. Therefore, deletion of the *acrB* gene seems to reduce the virulence potential, while overexpression of this gene does not have, by itself, a positive effect on virulence [27]. Recently, it has been demonstrated by De Majumdar et al. [55] that RamA can trigger changes on the bacterial surface that allow *Klebsiella* cells to survive to antimicrobial challenge, to evade from attack by host immune peptides, and to resist to phagocytosis. More precisely, molecular basis of increased survival of RamA-overexpressing *K. pneumoniae*, against host-derived factors, is associated with RamA-driven alterations of the lipid A moiety of *Klebsiella* lipopolysaccharide. This modification is likely to be linked to *Klebsiella*'s ability to resist the host response so that it remains undetected by the immune system [55]. Finally, RamA overproduction protects against macrophage uptake and internalization, thus providing a basis for the greater dissemination of the RamA-overexpressing strain. The relationship between AcrAB-TolC and bacterial virulence has been clearly established in *Salmonella* Typhimurium where inactivation of *acrB* causes a decreased

expression of genes in the pathogenic island that are known to be required for infections [56].

Using the *C. elegans* model, it has been reported that a reduction of *E. aerogenes and K. pneumoniae* virulence is associated with the membrane modifications involved in drug resistance [57, 58]. The combination of outer membrane alterations induces an important decrease in bacterial fitness and adaptation to environmental stresses. Porin and lipopolysaccharide organizations contribute to the physiological state involved in the mid-late/late steps of host colonization. In contrast the overexpression of AcrAB-TolC efflux pump does not modify the *E. aerogenes* virulence in this model [58]. Consistently, inactivation of *acrB* also reduced pathogenicity of *E. cloacae* in an invertebrate model of infection [45].

11.3 Efflux Regulation and Role in Antimicrobial Resistance

Numerous regulatory proteins have been implicated in the development of MDR, and both structural and genetic investigations endeavor to understand and decipher their mechanisms of action [23, 50, 59]. Control of efflux pump expression is carried out on several levels: (i) positive regulation by general or specific transcriptional activators that coordinate the expression of several genes, (ii) negative regulation by repressors of efflux pump components, and (iii) response to chemical or pharmaceutical factors that trigger one or more complex interlinking regulatory cascades.

11.3.1 Positive Regulation by Global Transcriptional Activators

The chromosomal transcriptional regulators of bacterial influx and efflux genes described in *Enterobacteriaceae* belong to one of several regulatory protein families, in particular AraC, MarR, and TetR [60–62]. All possess α-helix-turn-α-helix DNA-binding motifs and the most described is the global activator MarA.

The *mar* locus The *mar* (multiple antibiotic resistance) locus is a continual operon which encodes for the key regulator MarA and controls both porin and efflux pump expression. The *marRAB* operon exhibits a genetic organization preserved among the *Enterobacteriaceae* [63, 64]. It is constitutively repressed by MarR, its specific repressor, and MarB that encodes a small periplasmic protein that reduces the transcription rate of *marA* [65]. The expression (or derepression) of *marRAB* is the consequence either of (i) mutations in the MarR binding sites (observed in *E. coli*), (ii) modification of MarR at the protein level preventing its repressor function, or (iii) the direct action of inductors of the system [66, 67]. However, no clinical *Enterobacter* spp. and *K. pneumoniae* strains with *marR* mutations were characterized among multidrug-resistant isolates (data not published).

MarA is an important regulator implicated in adaptation to the environment and protection against external aggressions, by inducing the direct or indirect action of more than 60 genes [68, 69]. MarA activates the *acr* operon by binding to the intergenic region between *acrR* and *acrA*, lifting the repressive action caused by the AcrR repressor protein. The expression of *marA* triggers a cascade that leads to the MDR phenotype by simultaneous reduction of influx and increase in efflux of antimicrobials. The overall effect of the *mar* regulon is to reduce intracellular and periplasmic concentrations of antimicrobials that enter the bacterial cell through porins and are substrates of active efflux via AcrAB-TolC [70].

The oxidative stress regulon *soxRS* SoxS is the effector of the *soxRS* global superoxide response regulon. SoxS exhibits about 50 % homology with MarA [71]. It is activated by superoxide-generating agents via conversion of SoxR, a divergently transcribed local transcriptional activator, into an active form [72]. In the presence of oxidizing agents (e.g., hydrogen peroxide, nitric oxide, and methyl viologen), SoxR shifts from the reduced to the oxidized state and triggers the transcription of gene *soxS* [71]. SoxS was demonstrated to be involved in activation of the MDR phenotype in *E. cloacae* by upregulating *acrAB* [40]. The marboxes are also target sequences for the binding of SoxS, and the phenotype induced by SoxS is similar to that induced by MarA [73]. SoxS is also able to activate MarA expression and together they activate many of the same genes [74]. Mutations in the SoxR activator have been identified in clinical isolates of *E. coli* and *S. enterica* from patients undergoing quinolone treatment and shown to confer an increased MDR phenotype, but no mutations were found in multidrug-resistant *E. aerogenes*, for instance [75, 76]. With regard to *K. pneumoniae*, a mutant strain selected *in vitro* with ciprofloxacin, with a point mutation in the *soxR* gene (C375G) resulting in an amino acid substitution (Asn125Lys) in the SoxR protein, has been described [51]. This mutant showed an increase in the transcription level of gene *soxS* compared to its parental strain. Complementation with the wild-type *soxR* gene normalized the transcription level of *soxS* and restored the strain's susceptibility to antibiotics. Interestingly, the *soxR* mutant did not overexpress AcrAB, which suggests that in *K. pneumoniae* the *soxRS* operon is involved in the regulation of a distinct efflux pump.

The *rob* regulon Rob is also a member of the AraC family and it regulates genes involved in resistance to antibiotics, organic solvents, and heavy metals [77, 78]. Overexpression of Rob in *E. coli* produces both increased organic solvent tolerance and low-level resistance to multiple antimicrobial agents, due to increased expression of AcrAB [79]. There is considerable overlapping of the genes targeted by Rob and those under the control of MarA and SoxS, but Rob has a limited effect on the level of expression [80]. Rob activates the target genes only after the binding of inducers such as medium-chain fatty acids and bile salts, with its C-terminal end. Its constitutive expression in the cell should saturate target genes with MarA and SoxS and should induce a continuous MDR phenotype.

RamA super-regulator RamA was first described in a multidrug-resistant *K. pneumoniae* mutant [81–83]. It shares 45 % identity with MarA with high conservation of

the two DNA-binding motifs essential to the regulatory function. Thus, it can be expected that RamA and MarA might recognize an overlapping set of operator sequences. RamA in *E. aerogenes* elicited a high-level resistance to diverse antimicrobials (chloramphenicol, fluoroquinolones, tetracycline, tigecycline, trimethoprim, etc.) as result of a decreased expression of OmpF and an active efflux [84]. RamA has been identified in *Salmonella enterica* serovar Paratyphi B and Typhimurium and in *E. aerogenes* and *E. cloacae* [6]. Schneiders and colleagues found that transcription levels of the gene *ramA* were elevated in three out of ten fluoroquinolone-resistant and *acrA*-overexpressing *K. pneumoniae* isolates, whereas no *marA* or *soxS* overproduction was detected [85]. In a recent study, De Majumdar et al. [55] identified 40 genes upregulated and 50 downregulated by RamA (both AcrAB and TolC are upregulated). In a similar study by Källman and colleagues [86], overexpression of *ramA* was detected in nine out of ten clinical *K. pneumoniae* isolates, resistant to chloramphenicol, nalidixic acid, tigecycline, and cefuroxime and displaying increased expression of *acrA* and decreased transcription of *ompK35*, whereas only four of them overexpressed *marA* and no one overexpressed *soxS*.

RamA has also been demonstrated to bind to the *mar* operator in *E. coli* and enhance its transcription, suggesting an interaction between these two systems [87]. This suggests that MarA could regulate the transcription of *ramA*; however, constitutive expression of RamA results in an MDR phenotype even in the absence of the *mar* locus. RamA is a transcriptional activator of the Mar regulon and is also a self-governing activator of the MDR cascade. RamR is the repressor of *ramA* expression, and mutations that impact the RamR dimerization and responsible for a MDR phenotype have been found in multidrug-resistant *E. aerogenes* isolates. In carbapenem-resistant *K. pneumoniae* isolates, mutations, insertions, or deletions associated to frameshift and premature stop codon were identified in the *ramR* gene for three strains [88]. Additional gene (rather than *ramR*) may also be involved in controlling *ramA* expression [89]. RamA plays a role in the oxidative stress response in partnership with *soxRS*, but seems more important than *marA* and *soxS* in the development of MDR in *Salmonella* spp. [90]. At this moment, it is also important to mention that the RamA regulator is described in *Enterobacter*, *Klebsiella*, and *Salmonella* but not reported in *E. coli* in contrast to the Mar regulon [91].

The RarA regulator Besides *acrRAB*, the chromosome of *K. pneumoniae* contains another recently characterized locus, *rarA-oqxABR*, encoding an efflux system together with its regulatory elements [41]. The *oqxAB* operon was originally described on plasmid pOLA52, carried by an *E. coli* strain of porcine origin and shown to confer resistance to olaquindox (a growth enhancer used in pig farming), chloramphenicol, and quinolones [92, 93]. RarA is an AraC-type transcriptional regulator. It is overproduced as OqxAB when the negative regulator OqxR does not work [27, 41].

De Majumdar and colleagues [94] found by using transcriptome analysis that *rarA* overexpression resulted in the differential expression of 66 genes (42 upregulated and 24 downregulated). Under the clusters of orthologous group functional classification, the majority of affected genes belonged to the category of cell

envelope biogenesis and posttranslational modification, along with genes encoding the previously uncharacterized transport proteins (e.g., KPN_03141, *sdaCB*, and *leuE*) and the porin OmpK35. However, genes associated with energy production and conversion and amino acid transport or metabolism (e.g., *nuoA*, *narJ*, and *proWX*) were found to be downregulated.

11.3.2 Other Regulators

H-NS, SdiA, and Fis regulators Another global regulator of outer membrane permeability, H-NS (histone-like nucleoid-structuring protein), regulates the expression of porins and several efflux pumps in *E. coli* and *E. aerogenes* in response to osmotic stress [95, 96]. There is evidence that this H-NS also controls expression of the cation-selective outer membrane protein OmpX [97, 98].

A recent study demonstrated that AcrAB is also positively regulated by SdiA, a protein that regulates cell division genes in a manner dependent upon quorum sensing [99]. Fis is a nucleoid-associated global regulatory protein that modifies *acrAB* transcriptional activity in response to various growth conditions and can also bind to a site within *marO* upstream the marbox. This regulator is proposed to limit the overall level of negative superhelicity and stabilize the local DNA architecture of certain promoters, providing an additional twofold stimulation to MarA-, SoxS-, and Rob-mediated activation of transcription [100].

Repressors of efflux pump genes Many operons that encode efflux pump components contain a physically linked regulatory gene that plays a monospecific role in modulating expression of that pump. *acrR* encodes a TetR-type repressor, which is known to repress both its own and *acrAB* transcription [101]. Mutations in *acrR* have been shown to derepress *acrB*. Such mutations have been found in clinical isolates of *E. coli*, *S.* Typhimurium, *E. aerogenes*, and *K. pneumoniae* [24, 85, 102–104]. These mutations lead to the overexpression of *acrAB* and partially contributed to increased resistance to several unrelated antimicrobials as demonstrated for tigecycline in *K. pneumoniae* [24, 102, 103]. The gene *oqxR* downregulates *rarA* and *oqxAB* expression in *K. pneumoniae* [27].

11.3.3 Chemical Effectors or Inductors

When the *mar* system was first described, salicylate, chloramphenicol, and tetracycline were reported to induce the transcription of the *marRAB* operon [105]. Some inducers have been shown to bind directly to the repressor MarR following entry into the bacterial cell. Indeed, salicylate and (to a lesser extent) tetracycline exhibit strong affinity for MarR, preventing binding to *marO* or shifting the repressor out of the operator site [106]. Similarly deoxycholate interacts with MarR to prevent DNA

binding in *S*. Typhimurium [107]. More generally, the transcriptional activators involved in MDR respond to a variety of chemically unrelated compounds including antibiotics (tetracycline, tigecycline, and chloramphenicol), biocides (triclosan and household disinfectants), the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cyclohexane, salicylate, acetylsalicylate (aspirin), acetaminophen, sodium benzoate, plumbagin, menadione, methyl viologen, dinitrophenol, and more generally phenolic rings [108]. In the same way, a multidrug recognition of RamR has been recently demonstrated, whereby the DNA-binding activity is controlled by multiple drugs [109]. Five substrates were identified including berberine, crystal violet, and ethidium bromide. Removal of such toxic substances and subculture in favorable conditions often results in the reversion of MDR to the sensitive phenotype as repression of global activators is restored. The exact mechanism of induction by each of these compounds is yet to be deciphered, and further investigation of these processes may aid in the understanding of how MDR develops.

11.3.4 Model and Regulation Cascade

The regulation of MDR in *Enterobacter* is quite complex and redundant [42, 91] and contributes to the rapid adaptation of the clinical isolate via the porin and efflux balance [110, 111]. Interestingly, regarding the genetic control of efflux pump expression, activators MarA, RamA, and RarA and repressors MarR, RamR, and AcrR could be intimately associated at a global and local level to conjointly organize the resistance in clinical *Enterobacter* and *Klebsiella* isolates [6]. A tentative model for an integrated schema of regulation is proposed (Fig. 11.1). This model tries to integrate the various mechanisms (e.g., influx and efflux) and respective regulation pathways (global, local, etc.) acting in the control of intracellular accumulation of antimicrobials in *E. aerogenes* and *K. pneumoniae*.

11.4 Drug Efflux Activity

11.4.1 Methods for the Detection and Characterization of Efflux

As mentioned in the previous sections, the tripartite AcrAB-TolC efflux complex of *E. coli* belongs to the RND efflux pump family and is well conserved in other clinically relevant *Enterobacteriaceae* such as *E. aerogenes* and *K. pneumoniae*. Based on its substrate specificity with an exceptional range and its high constitutional expression levels under physiological conditions, it acts as a major contributor to the intrinsic resistance in these species. *E. coli* AcrAB-TolC is also the best

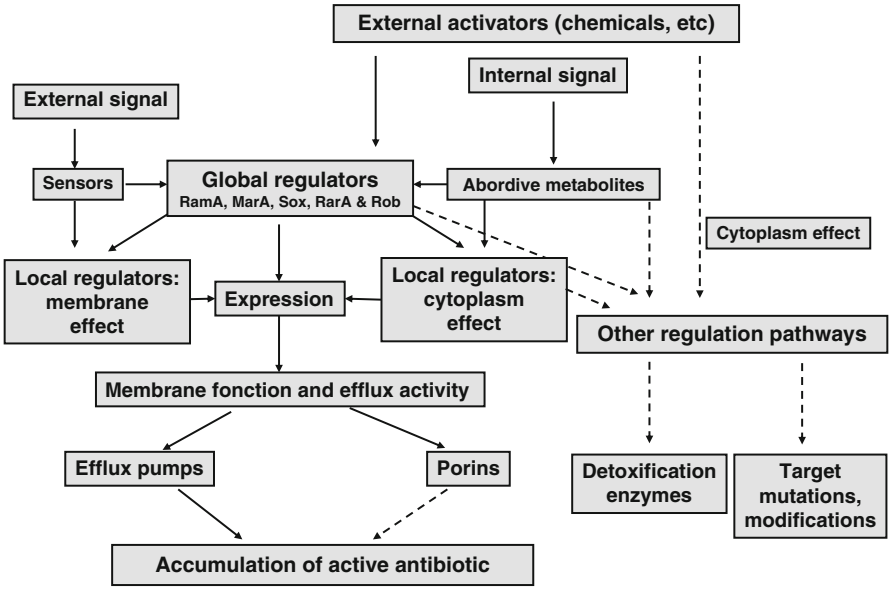

Fig. 11.1 Regulation of intracellular accumulation of antimicrobials in *E. aerogenes* and *K. pneumoniae* in response to external stresses. The *dashed arrows* (*right part*) represent the ways that contribute to control the antimicrobial accumulation in addition to efflux system (Adapted from Davin-Regli and Pagès [6])

structurally characterized RND efflux complex, since the crystal structures of AcrA, AcrB, and TolC have been solved to high resolution [112–114]. While structural models are relatively well advanced, the phenotypic characterization of AcrAB-TolC (as well as other efflux pumps) has mainly relied on comparisons between the MIC values of the wild-type strain and *acrAB-tolC* deletion strains. However, determination of MICs cannot offer real-time information for direct observation of resistance mechanisms in multidrug-resistant clinical isolates or activities of new antimicrobial molecules. In addition, these methods are laborious and time- and material consuming.

The intent of this section is to describe a variety of new methods that allow to (i) characterize intrinsic efflux pumps and their contribution in lowering the intracellular concentration of antimicrobials, (ii) identify efflux-mediated MDR in clinical isolates, and (iii) screen for efflux pump inhibitors (EPIs). These are mostly based on the use of fluorescent probes or intrinsically fluorescent antimicrobials such as fluoroquinolones to monitor their accumulation and efflux in bacterial cells. Bulk measurement techniques use fluorescent spectroscopy, representing the balance between entry and efflux of a given molecule, while single-cell observations use fluorescent microscopy.

Radiolabeled antimicrobial assay The first assay developed to evaluate the efflux activity in *E. aerogenes* and *K. pneumoniae* cells was the measurement of

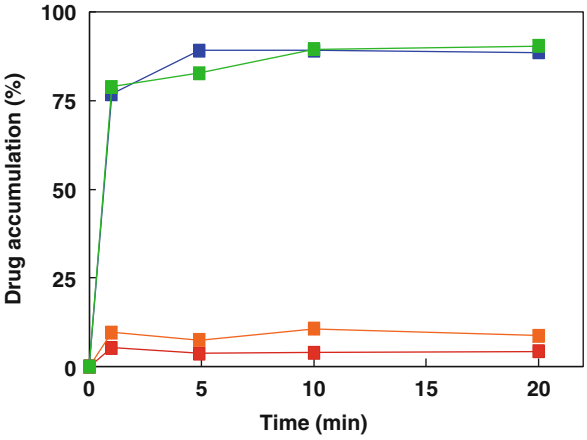

Fig. 11.2 Efflux and intracellular accumulation of norfloxacin in *E. aerogenes* strains. About 80–90 % of norfloxacin is expelled during the first several minutes of incubation in multidrug-resistant strains that overexpress the efflux pump (in *red* and *orange*) compared to a susceptible strain with deleted efflux pump (in *blue*). In the presence of CCCP that collapses the energy-driven force required by efflux pump, the intracellular accumulation is restored in a bacterial multidrug-resistant strain (in *green*)

radiolabeled chloramphenicol and norfloxacin accumulation in resistant strains [22, 115]. The efflux activity was demonstrated in several multidrug-resistant isolates by using CCCP that collapsed the energy component of the efflux and consequently blocked the pump and restored the intracellular drug concentration at a level similar to the normal susceptible reference strains. Interestingly, it was calculated that about 80–90 % of the internal norfloxacin that penetrates during early times of incubation was expelled during the first 10 min of the efflux assay (Fig. 11.2). Regarding *E. aerogenes*, this radiolabeled assay was further used to test several compounds that can block the efflux activity and restore intracellular accumulation of antimicrobials (for reviews see [23, 49]). The radiolabeled assay presents some advantages about precision and sharpness of quantification but also large disadvantages regarding the availability/production of marked molecules, safety protocols, internal controls, and waste treatments.

The ethidium bromide-agar-based method MDR phenotypes of clinical isolates have been shown to be related to the overexpression of efflux pumps. Recently, a simple and instrument-free method that uses agar plates containing increasing concentrations of ethidium bromide (a fluorescent efflux pump substrate) has been developed [116]. This method, called the ethidium bromide-agar cartwheel method, has been validated for screening large collections of clinical strains for the identification of overexpressed efflux pumps that contribute to MDR phenotypes [36, 116–118]. Briefly, it is based on the determination of the minimal concentration of ethidium bromide in agar that produces fluorescence associated to confluent bacteria growing at the surface of the agar defines the maximum extrusion rate of this substrate. Data

showed that the minimal concentrations of ethidium bromide for strains that overexpress efflux pumps are considerably higher than those for reference strains.

The semiautomated "real-time" method using fluorescent probes Conventional methods for testing bacterial susceptibility to antimicrobials are not sensitive enough to detect increased efflux and/or decreased permeability in a given bacterial population. Therefore, it was of great interest to develop fast, easy-to-perform, and reliable methods in order to identify and characterize these mechanisms. Automated methods are advantageous in that they allow quantification of influx and efflux levels on a real-time basis and can be set up for high-throughput analysis of bacterial strains. However, feasibility of this approach requires (i) that the chosen molecule does not affect cell viability or physiological functions at the working concentrations, exhibits properties of good diffusion, is a substrate of efflux pumps, and yields high signal only when inside the cells and (ii) a methodology that detects the variation of this signal when molecules are pumped out of the cells. First, several fluorescent probes, which change their spectroscopic properties upon entering the cells, are particularly suitable for these assays. Second, if the above characteristics are checked, cells can be preloaded with dyes before monitoring efflux in energized cells. This result may be improved by increasing the concentration of dye during the loading step. Nevertheless, these assays are likely to severely underestimate true efflux rates.

Lipophilic membrane-partitioning dyes would therefore be better suited for efflux assays. *N*, *N*, *N*-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate efflux has been examined in *Pseudomonas aeruginosa* but found rather slow [119], while efflux of *N*-phenyl-1-naphthylamine was found much faster [120, 121]. Recent procedures for efflux assays have been optimized by examining the role of the proton motive force inhibitor CCCP and the dye concentration as well as cell growth and treatment conditions. This has been done for the use the two lipophilic compounds Nile red (with log*P* of 6) and 1,2′-dinaphthylamine (log*P* of 3.8) [122, 123], which both give negligible fluorescence in aqueous solutions but become strongly fluorescent in nonpolar environments such as bacterial membranes [124]. Special attention should also be given to the self-quenching fluorescence of the dye with membranes in case of high concentrations. After loading, cells are then energized by the addition of glucose, which produces the proton motive force across the inner membrane and triggers the dye efflux.

Determination of antimicrobial accumulation in single bacteria using UV fluorescence microscopy/spectroscopy methods Membrane-associated mechanisms of bacterial MDR (i.e., membrane impermeability and drug efflux) tend to lower the concentration of antimicrobials at the vicinity of their targets. Therefore, a key point in the understanding of these mechanisms is to determine and compare the intracellular concentration of antimicrobials in bacterial cells accordingly to their phenotypic and genotypic status. Several approaches have been developed in the past by using plate assays [125, 126], radiometry [122, 127, 128], and fluorimetry [129], mainly for measuring the accumulation of quinolones by *Enterobacteriaceae*.

Quinolones are broad-spectrum antimicrobials that act by inhibiting the DNA gyrase and topoisomerase IV enzymes located in the bacterial cytoplasm. The ability of quinolones to permeate the bacterial envelope to reach their intracellular targets is an important factor in determining their activity. In Gram-negative bacteria, quinolones have been shown to translocate across the outer membrane porins with a preference for OmpF [130] and PhoE rather than OmpC. The mechanism of permeation across the inner membrane is less clear, but most likely an energy-independent passive diffusion. Of note, quinolones are also excellent substrates of efflux pumps.

Several groups have used radiolabeled quinolones associated with a removal of samples at timed intervals, filtration, and scintillation counting. To overcome the limitations due to antimicrobial modification and activity, Chapman and Georgopapadakou have developed a fluorimetric method based on the natural fluorescence of the quinolone nucleus present in fluoroquinolones such as the clinically used ciprofloxacin and norfloxacin [129]. It is now accepted that spectrofluorimetry of cell lysates allows fine quantification of the antimicrobial content (in ng/ml) in a given bacterial population, based on a calibration curve [118]. In our team, we recently set up an optimized procedure to monitor fleroxacin accumulation. In particular, after the incubation step in the presence of an antimicrobial, cells are loaded onto a sucrose solution and spun down in order to overcome problems of reproducibility and data interpretation due to nonspecific absorbance of antimicrobial molecules at the cell surface [131]. Moreover, special attention should be given to the spectral properties of the fluorescent molecule (here fleroxacin) in order to detect its fluorescence in bacterial lysates. Results have been obtained with two isogenic strains of *E. aerogenes*: EA289, a multidrug-resistant clinical isolate that lacks outer membrane porins Omp35 and Omp36, overexpresses the AcrAB-TolC efflux pump, and carries mutations in quinolone targets; and EA298, a *tolC*::Km derivative. Experimental conditions were set up with concentrations of fleroxacin approaching saturation without cell killing (i.e., 64 μg/ml, which corresponds to 8 × MIC of fleroxacin for EA298), in the absence or in the presence of CCCP to collapse the energy force that drives efflux. The data showed that steady-state accumulation of fleroxacin was stable after a 10–15 min incubation time for the two strains. Fleroxacin accumulation level showed an increase of about twofold in EA298 as compared to that in EA289 but was similar when EA289 was co-incubated with CCCP, consistently with the inactivation of efflux mechanisms [131].

Although strongly reliable, this method cannot provide details on the antimicrobial concentration at the single-cell level or on the antimicrobial localization. To do this, our group, together with the DISCO beam line of Synchrotron SOLEIL, recently showed an innovative method to investigate the transport kinetics of fluoroquinolone accumulation in single bacteria and estimate the contribution of efflux pumps on this process by using deep UV synchrotron radiations [132]. Deep UV has been used to study new peptide molecules with metal-binding abilities and their activity against multidrug-resistant clinical isolates. A dansyl-derivative compound has been imaged inside single bacteria with a heterogeneous subcellular localization [133].

The fluorescence intensity is clearly related to the intracellular accumulation level of this drug, which is dependent on its extracellular concentration and the incubation time with the cells.

11.4.2 Examples of EPIs Active Against E. aerogenes *and* K. pneumoniae *Efflux Pumps*

In our team, we undertook efforts to identify and characterize series of alkoxy- and alkyl-aminoquinolines as EPIs showing significant activities against laboratory susceptible and clinical multidrug-resistant strains of *E. aerogenes* and *K. pneumoniae*. This novel class of EPIs was primarily discovered from chemical screens on strains of *E. aerogenes* [134, 135]. These compounds have been assayed for their activity against various multidrug-resistant clinical strains overexpressing efflux pumps [134–140]. Active quinoline derivatives have then been evaluated for their ability to restore the activity of various antibiotic families (i.e., fluoroquinolones, phenicols, and tetracyclines). Several lines of evidence confirmed the potential of these EPIs, and several quinoline derivatives are now considered as broad-spectrum EPIs for both *E. aerogenes* and *K. pneumoniae* [136].

The analyses of structure-activity relationships have indicated that the alkyl side chain linked to the heterocyclic moiety of alkyl-aminoquinolines plays a key role in EPI activity. Alkoxy- and thioalkoxy-quinolines that share piperidino ethyl chains restore susceptibility to chloramphenicol, with the degree of susceptibility increase depending upon the type of derivative-less so with oxo-derivatives than with amino substitutions in thioalkyl molecules [135]. In addition, the connecting heteroatom and the position of substituted groups on the ring seem also to be of importance. Additional studies are necessary to define the role of pharmacophoric groups and their reactivity with the affinity pockets reported in AcrB [141–146]. Alkylamino-, alkoxy-, thioalkoxy-, and chloro-quinoline derivatives present several advantages: a strong similarity with quinolone antibiotics, a negligible intrinsic activity, and no side effect on membrane permeability. However, toxicity assays and pharmacodynamics studies are still needed to determine the therapeutic potency of these compounds. Lastly, several other classes of EPIs have been described [147–149] and recently reviewed in [150, 151].

11.5 Concluding Remarks

During the last decades, we observed the emergence of several infectious episodes due to resistant *Enterobacter* and *Klebsiella* strains in hospitals. It is important to mention that the two genera, *Enterobacter* and *Klebsiella*, exhibit a strong conservation of the two global regulatory systems, Ram and Mar. These key systems can act

independently or synergistically to control the transport of antimicrobial agents. The redundancy and cooperativity between these global regulators of membrane permeability can explain the rapid adaptation of clinical isolates during antibiotic treatment. In addition, other systems (Sox, Rob, and RarA) can also contribute to the significant level of resistance mentioned in clinical isolates.

The efflux strategy is identified in resistant bacteria that efficiently pump out antimicrobial molecules that can pass the membrane permeability barrier, and the contribution of this mechanism in the early step of resistance is now recognized [23, 49, 59]. Several international consortia are working on this concern regarding the scarcity of new antimicrobial molecules and the continuous emergence of Gram-negative resistant bacteria. Among them, the Innovative Medicines Initiative-Translocation is involved in the understanding of antibiotic translocation (http://www.translocation.eu). At this moment, a key point is the precise determination of the intra-bacterial concentration of antimicrobials depending on the membrane transport and the correlation between the efflux activity and the bacterial susceptibility [131]. This important contribution of membrane permeability, comprising Influx-Efflux, and the efficient management via global and local regulators can explain the emergence of these *Enterobacteriaceae* in ESKAPE [2, 3]. For instance, our recent assessment of the role of outer membrane proteins and efflux pumps in the susceptibility of *Enterobacteriaceae* to ceftazidime-avibactam suggested a key role of outer membrane permeability in the activity of ceftazidime-avibactam with no or little evidence of any efflux effect on antibacterial activity of the combination [152].

The knowledge and definition of these parameters will be the platform of the development of new molecules or new protocols and combinations that could be used to circumvent the membrane and the efflux barrier that represent the early step of bacterial response face to antimicrobial use. MDR in Gram-negative bacteria, such as *Enterobacteriaceae* and *P. aeruginosa*, is a significant problem for treatment of bacterial infections worldwide. Overexpression of RND-type efflux pumps is a major component in the development of MDR phenotypes in these species. Consequently, efflux pumps are considered as attractive targets for inhibition in combination therapy, i.e., EPI and antimicrobial combinations should increase potency of poorly active antimicrobials and reduce the emergence of MDR in clinics. To date, several EPIs have been characterized [48, 54, 151, 153] on resistant *E. aerogenes* and *K. pneumoniae* multidrug-resistant strains. Some series have been optimized by pharmaco-modulation but none has been tested in clinical programs due to cell toxicity. The correlation between intracellular accumulation, the activity of efflux pumps, and the killing rate of specific antimicrobials is an important challenge for the future.

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Chapter 12
Antimicrobial Resistance and Drug Efflux Pumps in *Vibrio* and *Legionella*

Yuji Morita and Xian-Zhi Li

Abstract The two genera, *Vibrio* and *Legionella*, are associated with aquatic environments and cause severe illnesses such as cholera and legionellosis, respectively. The representative species, *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Legionella pneumophila*, are generally susceptible to a range of antimicrobial agents, but their resistance to antimicrobials can be readily selected after exposure to antimicrobial agents. The genomes of these species contain a large number of genes encoding proven and putative drug efflux transporters (including the prototypical NorM drug exporter identified in *Vibrio* spp.), some of which have been demonstrated to play an important role in intrinsic resistance to structurally unrelated antimicrobials as well as to involve in other functions such as virulence. However, the expressional regulation of these drug efflux pumps and their contribution to acquired antimicrobial resistance remain a key area for future research. This chapter provides an overview of antimicrobial resistance in *Vibrio* and *Legionella* with a focus on current understanding of drug efflux pumps in resistance and other functions.

Keywords *Vibrio cholerae* • *Vibrio parahaemolyticus* • *Legionella pneumophila* • Antimicrobial resistance • Efflux • Outer membrane • RND • MFS • ABC • VexAB • VceCAB • NorM

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12.1 Introduction

The bacterial species in the genera of *Vibrio* and *Legionella* are often present in aquatic environments and can cause severe illness such as cholera or legionellosis (frequently occurring in epidemic outbreaks) in humans [1–3]. The *Vibrio* species are facultatively anaerobic, straight, curved rods that are primarily in marine waters, of which some species are pathogenic for humans [4]. The latter species can be classified into two groups according to the type of diseases they cause: the gastrointestinal infection group (e.g., *Vibrio cholerae*) and the extraintestinal infection group (e.g., *Vibrio vulnificus*) [5]. *V. cholerae* strains (mostly serogroup O1 and O139) produce cholera toxin and are associated with epidemic of cholera, and others are agents of watery and severe disease diarrhea usually milder than typical cholera [2, 4, 5]. *Legionella pneumophila*, the causative, intracellular agent of legionellosis, was initially isolated in 1976 from patients in an outbreak of fatal pneumonia [6, 7]. *L. pneumophila* serogroup 1 that includes the three initially sequenced strains Philadelphia [8], Paris, and Lens [9] is the predominant serogroup responsible for Legionnaires' disease [7].

Antimicrobial therapy constitutes an important part of the management of *Vibrio*- and *Legionella*-causing diseases. However, antimicrobial resistance including multidrug resistance (MDR) has been observed in these two genera, in particular in *Vibrio* spp. [10–12]. Among various mechanisms of resistance, drug efflux pumps are also present in these species. In fact, *V. cholerae* and *Vibrio parahaemolyticus* are two well-studied species with respect to their drug efflux systems. In this chapter, current status of drug resistance and major resistance mechanisms in *Vibrio* and *Legionella* are reviewed with an up-to-date description of drug efflux pumps.

12.2 Antimicrobial Resistance and Major Resistance Mechanisms

Antimicrobial resistance including MDR in *Vibrio* spp. has been a major concern [13]. In fact, rapid resistance development in *V. cholerae* was observed in the 1970s during therapeutic and preventive use of tetracycline [14]. One of the mechanisms for resistance emergence was likely due to the acquisition of transferable resistance plasmids carrying determinants of resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline [15]. Outbreaks of resistant *Vibrio* spp. containing resistance plasmids have been well documented in literature [13, 16–19]. It is now clear that these MDR plasmids often carry resistance gene cassettes and mobile genetic elements such as integrative conjugative elements (also referred as SXT elements) or integrons [18–20]. One report described plasmids containing *dfrA1* (for trimethoprim resistance), *sul2* (for sulfonamide resistance), *strA*/*B* (for streptomycin resistance), and *floR* (for amphenicol exporter) genes reported in one plasmid [18], while another article showed two types of plasmids with one type

containing three resistance regions that included *sul2* region (*floR-tetA-strAB-sul2*), *cmy-2* insertion region (for β-lactam resistance and Tn21-like region (*aad-aac*) (for aminoglycoside resistance), and another type containing *sul2* and *cmy-2* insertion regions, an *arr3-drfA27-aadA16-sul1* resistance gene cassette at the Tn-21 location, and other resistance genes (*aac(3)-IIa*, $bla_{CTX\text{-}M\text{-}2}$, $bla_{TEM\text{-}1}$, *mphA*, and *sul1*) [21].

Chromosomal mutations also mediate drug resistance. Mutations in quinolone resistance-determining region of gyrase-encoding *gyrA* gene or in topoisomerase IV-ending *parC* gene confer quinolone resistance [22, 23]. Repressed expression of the outer membrane protein OmpU is linked to resistance to cationic antimicrobial peptides including polymyxin B and a bactericidal/permeability-increasing peptide [24]. A distinctive class of integron that includes *V. cholerae* repeated sequence-associated, integrase-encoding *intl4* gene has been identified in the *V. cholerae* genome and this helps heterologous gene acquisition [25]. In *V. parahaemolyticus*, resistance to β-lactams occurs by induction of β-lactamase production by β-lactam antibiotics via the action of β-lactams on the two-component regulatory system histidine kinase sensor/response regulator pair VbrK-VbrR. Mutants deficient in *vbrK* or *vbrR* do not produce β-lactamase and are not resistant to β-lactams [26]. This study shows the histidine kinase sensor as a β-lactam receptor, which represents a novel mechanism for bacterial β-lactamase production. Additionally, resistance mechanisms are also suggested to link to virulence process to facilitate an evolution response of invasive *Vibrio* spp. [27].

L. pneumophila is generally susceptible to antimicrobial agents such as macrolides, ketolides, rifamycins, fluoroquinolones, and carbapenems [28–33]. β-Lactams show varied activities against *L. pneumophila* [34]. A new fluoroketolide agent, solithromycin, exhibits a strong *in vitro* activity against *L. pneumophila* with its MIC_{50} and MIC_{90} values to be 8- and 32-fold, respectively, lower than those of the macrolide azithromycin [35]. Omadacycline of the aminomethylcycline class also displays significant *in vitro* activity [36]. Since it is an intracellular pathogen, the antimicrobials of choice for the treatment of *L. pneumophila* infections include agents such as macrolides, rifamycins, and fluoroquinolones that can have adequate intracellular drug concentrations [32, 37]; resistance or reduced drug susceptibility may have significant adverse impact of legionellosis therapy. A major challenge is to interpret antimicrobial susceptibility data because of no standardized testing assay. The existing methods are extracellular susceptibility testing, making the results to be difficult to predict clinical outcomes [7]. Currently, only limited information is available regarding drug resistance in *L. pneumophila*. Fluoroquinolone resistance can be readily obtained by *in vitro* selection in the presence of a fluoroquinolone agent, and this is attributable to target modifications in GyrA and ParC [11]. High-level resistance to clindamycin (with minimal inhibitory concentration [MIC] values of 4–32 μg/ml) has been reported [30]. An unusual aminoglycoside phosphotransferase, APH(9)-*Ia*, mediates resistance to spectinomycin in *L. pneumophila* [38]. A recent study showed the *in vivo* selection of fluoroquinolone resistance during hospitalization after fluoroquinolone therapy [12]. Involvement of the membrane permeability and drug efflux pumps in resistance will be discussed in next section.

12.3 Drug Efflux Pumps in *Vibrio* and *Legionella*

Efflux, or the energy-dependent extrusion from bacterial cells, is recognized as one major mechanism of antimicrobial resistance [39, 40]. Some pumps are drug-/class-specific to only extruding a narrow range of antimicrobials such as a variety of tetracycline efflux pumps [41]. Other pumps are multidrug transporters that are able to export a broad range of antimicrobials, which differ in structures and in mode of action [39, 40]. Bacterial chromosomes encode various drug efflux pumps which fall into at least six families or superfamilies, i.e., the resistance-nodulation-cell division (RND) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the ATP-binding cassette (ABC) superfamily, and the proteobacterial antimicrobial compound efflux (PACE) family [40, 42]. Most drug efflux pumps function as secondary active transporters coupled with the H^+-motive force (and also, rarely, the Na^+-motive force) to antiport drug with ion (H^+ or Na^+), while ABC systems are primary active transporters which hydrolyze ATP to drive drug efflux. In Gram-negative bacteria, drug efflux pumps can be divided in single-component transporters (which act at the cytoplasmic membrane) or multicomponent transporters (which span the entirety of the Gram-negative cell envelop and typically contain a cytoplasmic membrane pump, an outer membrane channel-forming protein, and a periplasmic accessory membrane fusion protein) [39].

12.3.1 V. cholerae

V. cholerae strains (mostly serogroup O1 and O139) which produce cholera toxin and are associated with epidemic of cholera and others are agents of watery and severe disease diarrhea usually milder than typical cholera [4, 5]. Following ingestion, *V. cholerae* colonizes the small intestine via a process that is dependent upon the induction of genes (including transporter genes) which are required for intestinal colonization and disease development [2, 43–46]. Persistence in the intestine is dependent upon *V. cholerae*'s ability to overcome antibacterial barriers intrinsic to gastrointestinal tract, including the presence of high concentrations of toxic small molecules such as bile salts and other detergent-like molecules, antimicrobial products generated by resident flora, and products of the innate immune system [43, 45–47].

Wild-type non-plasmid-containing *V. cholerae* isolates are generally susceptible to a wide variety of antimicrobials, particularly hydrophobic and amphipathic agents (such as macrolides and rifamycins) [48, 49], and this is likely attributable to the presence of phospholipids in the outer leaflet of the outer membrane [50]. The lipopolysaccharide moiety in the outer membrane also has a relatively low negative charge [50]. These characteristics are expected to produce a rapid permeation of large hydrophobic/lipophilic agents [40]. Moreover, the major porins of *V. cholerae*,

OmpU and OmpV, also produce channels which are even larger than the classic trimeric porins of *Escherichia coli* [51]. However, even with a relatively high permeability outer membrane, several drug efflux pumps have been shown to play an important role in drug resistance.

RND Pumps Six RND-encoding loci were annotated in the *V. cholerae* genome (strain El Tor N161962) [44], although eight RND transporters are predicted based on the TransportDB (http://www.membranetransport.org; accessed on November 25, 2015) [52, 53]. Five of the loci map to the larger chromosome I (of 2.96 Mb) and one to the smaller chromosome II (of 1.07 Mb) [44, 47]. These RND efflux systems are arranged each in a probable operon structure and named *vexAB*, *vexCD* (also known as *breAB* for bile response genes [54]), *vexEF*, *vexGH*, *vexIJK*, and *vexLM* [47, 55]. As shown in Fig. 12.1a, each operon includes an RND pump gene (*vexB*, *vexD*, *vexF*, *vexH*, *vexK*, or *vexM*) and at least a gene for the membrane fusion protein gene (*vexA*, *vexC*, *vexE*, *vexG*, *vexI*, *vexJ*, and *vexL*) with the *vexIJK* operon containing a pair of genes (*vexIJ*) for two membrane fusion proteins. However, these operons lack the genes that encode the outer membrane protein components of typical RND tripartite efflux complex. In this regard, the *V. cholerae* genome (chromosome I) contains several outer membrane protein genes (e.g., *VC1565*, *VC1606*, *VC1621*, and *VC2436*) that encode the homologs to the outer membrane efflux channel protein TolC of *E. coli*. Yet, only the VC2436 protein shows the highest similarity (71 %) to *E. coli* TolC, and only its inactivation results in hypersusceptibility to bile salts, erythromycin, novobiocin, and polymyxin B [56], similar or identical to the inactivation the RND pumps [47]. Thus, the VC2436-encoded protein is considered as the outer membrane channel protein which plays a functional role in the Vex RND pump complexes.

Bina et al. [47] showed that *V. cholerae* RND efflux systems are required for antimicrobial resistance, optimal virulence factor production such as cholera toxin and the toxin co-regulated pilus, and colonization of the infant mouse small intestine using *V. cholerae* O1 biovar El Tor N16961 and its derivatives. The RND-null strain displayed significant decreases in the MICs for the bile salts cholate (>160-fold) and deoxycholate (>500-fold), the detergents Triton X-100 (>250-fold) and sodium dodecyl sulfate (>40-fold), and the antibiotics erythromycin (100-fold) and polymyxin B (fourfold) but not for chloramphenicol, carbenicillin, cefotaxime, kanamycin, nalidixic acid, ciprofloxacin, rifampicin, and tetracycline [47]. Among the six RND pumps, VexB, VexD, VexH, and VexK are responsible for *in vitro* antimicrobial resistance and are required for virulence factor production and intestinal colonization [45, 47]. Although these four pumps are redundant for some substrates, they do not have equal activity [45, 47]. VexB and VexD are major contributors to bile acid resistance *in vitro*, while VexH and VexK play minor roles [45]. VexB is the primary RND efflux pump-mediated resistance to the broadest range of antimicrobials including bile acids, detergents, and antibiotics [erythromycin, novobiocin, penicillins, and polymyxin B]) [47, 55]. VexD is limited to bile salts and has overlapping substrate profile with VexB. Contribution from VexH and VexK to resis-

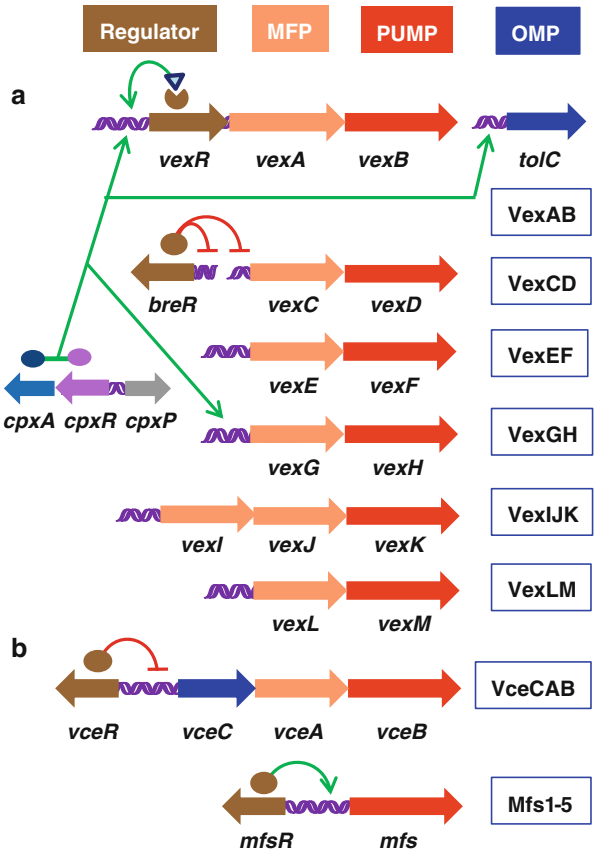

Fig. 12.1 Genetic organization of the known and putative chromosomally encoded RND (**a**) and MFS (**b**) efflux pumps in *V. cholerae* strain El Tor N161962. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (*MFP*), a pump, or an outer membrane protein (*OMP*), respectively. Genes encoding the proven or putative regulators including a two-component regulatory system (CpxRA) are shown on the *left*. The *green lines* represent the positive regulation of the efflux gene expression, while the *red lines* denote the repression of relevant gene transcription by repressors

tance is masked due to redundancy with VexBD (for bile salts) or VexB (for detergents and antibiotics) [45, 47]. VexH possesses a relatively broad specificity (only less broad than VexB) and is involved in resistances to bile salts, Triton X-100, novobiocin, and ampicillin, but not to penicillin and erythromycin [45]. Moreover, VexB is conserved in *Vibrionaceae* (at least in *V. parahaemolyticus*, *Vibrio fischeri*, *Vibrio harveyi*, and *V. vulnificus*) [57] and is also highly similar to MexW and MexI of *Pseudomonas aeruginosa* (50 % and 47 % identity, respectively) among the characterized RND pumps [58, 59]. VexK possesses a limited specificity and contributes to resistance to bile salts and detergents [47]. VexF and VexM of the remaining

two RND pumps do not affect *in vitro* antimicrobial resistance but do negatively affect cholera toxin and the toxin co-regulated pilus production [45].

Rahman et al. [60] cloned each of the six RND operons (Fig. 12.1) from *V. cholerae* non-O1 NCTC4716 in efflux-deficient hypersusceptible *E. coli* mutants. VexAB, VexCD, and VexEF were functionally associated with *Vibrio* TolC in the *E. coli* mutant [45, 60]. Judging from the MIC profiles, VexB and VexD of strain non-O1 NCTC4716 possess similar substrate specificities in comparison with those of strain O1 biovar El Tor N16961 [47, 55, 60]. Still, VexF of strain non-O1 NCTC4716 was shown to mediate broader resistance to antimicrobials than VexB when both were compared in the *E. coli* host, including bile salts, antibiotics (erythromycin and novobiocin), disinfectants (benzalkonium chloride), and others (crystal violet, ethidium bromide, Hoechst 33342, rhodamine 6G, and tetraphenylphosphonium), but not antibiotics (norfloxacin, tetracycline, and streptomycin) in the *E. coli* [60]. Moreover, VexF-mediated efflux requires Na^+ in *E. coli*, indicating that VexF is either a Na^+-activated or Na^+-coupled transporter [60].

The expression of certain RND pumps is under control by regulators. Upstream of the *vexAB* operon is a gene named *vexR* that encodes a TetR family transcriptional regulator [47, 55]. Deletion of *vexR* was found to cause reduced expression of *vexRAB* [46]. Indeed, bile salts within the concentration of the intestinal lumen (0.2–2 %) was revealed to induce the *vexRAB* and *vexCD* (*breAB*) operons [54, 55]. Expression of *vexRAB*, not *vexCD* (*breAB*), was also induced by erythromycin, novobiocin, and sodium dodecyl sulfate, all of which are substrates of the VexAB pump [46, 54]. Such induction of *vexRAB* expression is dependent on cognate VexR transcriptional activator which binds to certain inducers, including deoxycholate (also a substrate of VexAB), indole, and other cellular metabolites [46]. Expression of the *vexCD* efflux operon is repressed by BreR belonging to TetR transcriptional regulator family and the *breR* gene is not located immediately up of the *vexCD* operon and is also transcribed divergently in comparison with the *vexCD* transcription (Fig. 12.1) [54]. Additionally, the two-component regulatory system, CpxAR, a critical system in bacteria stress response [61, 62], also positively participates in regulation of the expression of at least two RND operons (i.e., *vexRAB* and *vexGH*) and the *tolC* gene (Fig. 12.1), thereby enhancing the RND pump-mediated antimicrobials resistance [63, 64]. Yet, the functional status of the VexAB pump was also found to affect the expression of Cpx system, thus revealing the reciprocal effect of these gene expressions [63].

Non-RND Pumps From the genome sequence of *V. cholerae* O1 N16961, 22 non-RND family efflux systems (11 MFS, 6 MATE, 1 SMR, and 4 ABC pumps) are present [65]. Among them, VceCAB and NorM were shown to contribute to antimicrobial resistance in *V. cholerae* cells [66–68]. VceCAB is the earliest-reported tripartite efflux pump from *Vibrio* spp. [66] that shares many characteristic features of the EmrAB-TolC of *E. coli* [69, 70]. This MFS-type efflux system consists of the cytoplasmic membrane transporter (VceB), outer membrane channel protein (VceC), and periplasmic membrane fusion protein (VceA), which are encoded by

the *vceCAB* operon (Fig. 12.1b) [66, 67]. This operon is under the negative control of the product of the divergently transcribed *vceR* repressor gene [67], which codes for a TetR family transcriptional autoregulatory protein [71]. The VceABC-inactivated strain displayed significant decreases in the MICs of bile acids (e.g., deoxycholate [fourfold]) and antimicrobials (e.g., nalidixic acid [eightfold]) and others (e.g., carbonyl cyanide *m*-chlorophenylhydrazone [80-fold], phenylmercuric acetate and pentachlorophenol [both with fourfold]) in *V. cholerae* [66]. Another study assessed five MFS pumps of *V. cholerae* (named Mfs1-5), and the upstream of each of these pump's encoding genes is paired with a divergently transcribed gene that encodes a LysR-type transcriptional activator (named MfsR1-5) [72]. Gene inactivation study demonstrated the involvement of these pumps in resistance to bile salts and tetracycline as well as the positive control of the pump gene expression by LysR-type regulators [72].

NorM of *V. cholerae* is a member of the MATE family transporters [68] and has a high level of sequence similarity to the NorM of *V. parahaemolyticus* which is the first example of MATE proteins [68, 73]. The NorM-null strain displayed significant decreases in the MICs of norfloxacin (16-fold) and ciprofloxacin (tenfold) as well as ethidium bromide (fourfold) in *V. cholerae* [68], indicating that NorM is a major fluoroquinolone intrinsic resistance determinant in *V. cholerae*. Tsuchiya and colleagues characterized all six MATE family pumps (VcmA [identical to NorM], VcmB, VcmD, VcmH, VcmN, and VcrM) and one ABC pump (VcaM) of strain non-O1 N16961 expressed from a plasmid in *E. coli* mutant lacking the major multidrug pump gene *acrB* [65, 74–76]. Their substrates are shown in Table 12.1. All MATE pumps except for VcrM rendered the *E. coli* mutant more resistant to fluoroquinolones [65, 74]. The VcaM expression produced elevated MICs of fluoroquinolones and tetracycline in the tested *E. coli* host [76]. It is noted that the *vceABC* and *norM* were induced in the presence of bile acids at the levels available in the intestinal lumen [54]. Recently, using the proteoliposome reconstituted with the purified protein, NorM of *V. cholerae*, was demonstrated to simultaneously couples to the sodium-motive force and proton motive force [87].

12.3.2 V. parahaemolyticus

V. parahaemolyticus is a slightly halophilic marine bacterium that is found in estuarine, marine, and coastal environments and the leading causal agent of human acute gastroenteritis following the consumption of raw, undercooked, or mishandled marine products [88]. Upon entering the human host, *V. parahaemolyticus* cells pass through the gastric acid barrier of the stomach and colonize the small intestine where bile acids are a key factor to influence bacterial colonization [80]. Drug efflux pumps contribute to antimicrobial resistance and other functions as detailed below.

RND Pumps The genome of clinical *V. parahaemolyticus* RIMD2210633 is relatively large in size (ca. 5.2 Mb with chromosome I of 3.3 Mb and chromosome II of

Table 12.1 Antimicrobial drug efflux pumps in *Vibrio* spp. and *L. pneumophila*

Species/transporter family	Efflux pump (regulator)	Substrates	References
V. cholerae			
RND	VexAB-TolC (VexR, CpxRA)	AMP, DT, ERY, NOV, PMB, SDS	[54, 55, 60]
RND	VexCD-TolC (BreR)	BS, DT, ERY	[47, 54, 55, 60]
RND	VexEF-TolC	BAC, DOC, EB, ERY, NOR, NOV, SDS, TET, TMP	[60]
RND	VexGH (CpxRA)	DT, NOV	[45]
RND	VexIJK	BS, DT	[45, 47]
RND	VexLM		[45, 47]
MFS	EmrD-3	CHL, EB, ERY, LZD, MIN, R6G, RIF, TPP	[77]
MFS	Mfs1-5 (MfsR1-5)	BS, TET	[72]
MFS	VceCAB (VceR)	CCCP, DOC, NAL, PCP, PMA	[66, 67]
MATE	NorM/VcmA	ACR, EB, CIP, DAU, DOR, NOR, KAN, STR	[68, 74]
MATE	VcmB, VcmD, VcmH, VcmN	AG, EB, FQ, HO	[65]
MATE	VcrM	ACR, DAP, EB, HO, R6G, TPP	[75]
ABC	VcaM	CIP, DAP, DAU, DOR, HO, NOR, TET	[76]
V. fluvialis			
MATE	VFD, VFH	CIP, NOR	[78]
V. parahaemolyticus			
RND	VmeAB-VpoC (VP0425)	ACR, BS, CIP, CLX, CV, DOC, EB, ERY, NOR, NOV, OXA, R6G, SDS, TET, TMP, TPP	[57, 79]
RND	VmeCD-VpoC (VP0040-TetR)	BAC, BS, CV, EB, ERY, NOV, R6G, SDS, TPP	[57]
RND	VmeEF-VpoC	BS, EB, NOV, R6G, SDS	[57]
RND	VmeGHI-VpoC	SDS	[57]
RND	VmeJK-VpoC		[57]
RND	VmeLM-VpoC		[57]
RND	VmeNO-VpoM (VPA0366)		[57]
RND	VmePQ		[57]
RND	VmeRS-Vpa0482		[57]
RND	VmeTUV-VpoC (VdeR-TetR)	ACR, BAC, BS, CHX, CLX, EB, OXA, R6G, SDS, TPP	[57, 80]

(continued)

Table 12.1 (continued)

Species/transporter family	Efflux pump (regulator)	Substrates	References
RND	VmeWX (VPA0947-ArsR)		[57]
RND	VmeYZ-VpoC	BS, NOV, SDS	[57]
MATE	NorM	EB, FQ, KAN, STR	[73]
MATE	VmrA	ACR, DAP, EB, TPP	[81]
PACE	VP1155	ACR, BAC, CHX, PRO	[82]
V. vulnificus			
RND	VexAB-TolC	ACR, BS, EB, ERY, NOV, SDS	[83, 84]
RND	VexCD	ACR	[83]
L. pneumophila			
RND	CeaABC	BAC, ERY, NOR, NOV	[85]
RND	HelABC	NOR, NOV, Ni, Zn	[85]
RND	LmxFE-LprN	ERY, NOR, Zn	[85]
RND	Lpl0757-0758	CATB, MB, NOR, R6G, SDS, Ni, Zn	[85]
RND	Lpl2104-2103	CTAB, ERY, NOR,	[85]
MFS	LbtB	LGB	[86]
ABC	LssDB	BAC, ERY, NOR	[85]
ABC	Lpl0278-0279-0280	BAC, EB, ERY, NOR, Ni	[85]
ABC	Lpl0695-0696-0697-0698-0699	BAC, NOR, NOV, R6G, Ni	[85]
ABC	Lpl0880-0881-0882	BAC, ERY	[85]
ABC	Lpl2849-2850-2851-2852	ACR, BAC, CTAB, ERY, NOR, SDS, Ni	[85]

ACR acriflavine, *AG* aminoglycosides, *AMP* ampicillin, *BAC* benzalkonium chloride, *BS* bile salts, *CCCP* carbonyl cyanide *m*-chlorophenylhydrazone, *CHL* chloramphenicol, *CHO* cholate, *CHX* chlorhexidine, *CIP* ciprofloxacin, *CLX* cloxacillin, *CTAB* acetyl trimethylammonium bromide, *CV* crystal violet, *DAP* 4′,6-diamidino-2-phenylindole, *DAU* daunorubicin, *DOC* deoxycholate, *DOR* doxorubicin, *DT* detergents, *EB* ethidium bromide, *ERY* erythromycin, *FQ* fluoroquinolones, *HO* Hoechst 33342, *KAN* kanamycin, *LGB* legiobactin (a siderophore), *LZD* linezolid, *MB* methylene blue, *MIN* minocycline, *NAL* nalidixic acid, *Ni* nickel sulfate, *NOR* norfloxacin, *NOV* novobiocin, *OXA* oxacillin, *PCP* pentachlorophenol, *PMA* phenylmercuric acetate, *PMB* polymyxin B, *PRO* proflavine, *R6G* rhodamine 6G, *RIF* rifampicin, *STR* streptomycin, *SXT* trimethoprim-sulfamethoxazole, *TET* tetracycline, *TMP* trimethoprim, *TPP* tetraphenylphosphonium, *Zn* zinc sulfate

1.9 Mb) [89] and is estimated to contain ca. 560 transporters including 16 putative RND pumps (http://www.membranetransport.org; accessed on November 25, 2015) [52, 53], although the published studies only described 12 RND pump-encoding loci in the same genome [57, 79, 80]. Each of these RND efflux systems is arranged in a probable operon structure (Fig. 12.2). Five of the operons (*vmeAB*, *vmeCD*, *vmeEF*, *vmeGHI*, *vmeJK*, and *vmeLM*) map to the chromosome I (Fig. 12.2a) and seven (*vmeLM*, *vmeNO-vpoM*, *vmePQ*, *vmeRS*, *vmeTUV*, *vmeWX*, and *vmeYZ*) to the chro-

mosome II (Fig. 12.2b) [57]. Each operon includes RND pump genes, at least a membrane fusion protein gene, and an outer membrane protein gene (Fig. 12.2) [57]. The *vmeGHI* and *vmeTUV* operons each include a pair of genes that encode the membrane fusion proteins (*vexGH* and *vexTU*). The TolC homolog of *E. coli*, VpoC, is encoded by a gene that is located at a remote site of the chromosome I (gene *VP0425*) from any RND genes [57]. The expression of TolC was found to be differentially regulated under various culture conditions [90]. Yet, we note an additional gene, *VP1998*, which also encodes a TolC homolog as well as several putative regulator genes (in addition to the reported *vdeR* gene [80]) in the genome (Fig. 12.2). *V. parahaemolyticus* possess twice more RND pumps than *V. cholerae*. Four of the 12 RND pumps of *V. parahaemolyticus* are phylogenetically orthologues of *V. cholerae* RND pumps, i.e., VmeD, VmeK, VmeF, and VmeI to VexB, VexF, VexH, and VexK [57]. VmeAB and VmeCD pumps were mainly involved in antimicrobial resistance because the double knockout mutant showed almost the same antimicrobial susceptibility phenotype as the RND-null strain [57]. VmeB is similar to AcrB of *E. coli* and MexB of *P. aeruginosa* (64 % and 61 % identity, respectively), both of which are major multidrug transporters in these organisms [40]. VmeD seems to be an orthologue of VexB (88 % identity) phylogenetically and functionally [57].

Among the 12 RND pump-encoding operons, four of them are locally linked to a regulatory gene, either located immediately or separately by a few genes from upstream of the RND pump operon (Fig. 12.2). These genes mostly encode the regulators of TetR family [71] which often function as repressors to negatively control expression of RND pumps in Gram-negative bacteria [40]. Experimentally, only the VdeR regulator of TetR family was demonstrated to play a role in downregulating the expression of VmeTUV since mutations of either point mutation or deletion in *vdeR* were seen in VmeV-overproducing deoxycholate-resistant mutants [80]. Similarly, *vmeD* was upregulated in response to deoxycholate, which is one of the constituents of bile acids [57]. A putative TetR family transcriptional regulator gene (*VP0040*) is upstream of the *vmeCD* genes [57]. The protein encoded by *VP0040* is similar to VexR, the activator of the *vexRAB* operon [67 % (81) identity (similarity)] in *V. cholerae* [46].

Matsuo et al. [57, 79, 80] published several studies that demonstrated that *V. parahaemolyticus* RND efflux systems are required for antimicrobial resistance including tolerance to bile salts and pathogenicity in the intestine. The RND-null strain displayed significant decreases in the MICs for the bile salts such as cholate (>64-fold) and deoxycholate (64-fold); the detergent such as sodium dodecyl sulfate (1,024-fold); antibiotics such as cloxacillin (128-fold), erythromycin (16-fold), and novobiocin (32-fold); and disinfectants such as benzalkonium chloride (fourfold) and chlorhexidine (eightfold) [57]. The antimicrobial susceptibility profile of the RND-null strain was almost the same to that of the *vpoC* deletion mutant, indicating that VpoC is an outer membrane component for several RND efflux systems [57].

Non-RND Pumps Non-RND family efflux systems of *V. parahaemolyticus* are not characterized except for two MATE efflux proteins (NorM and VmrA) [73, 81, 91, 92] and the AceI homolog of the PACE family [82]. In fact, NorM of *V. parahaemo-*

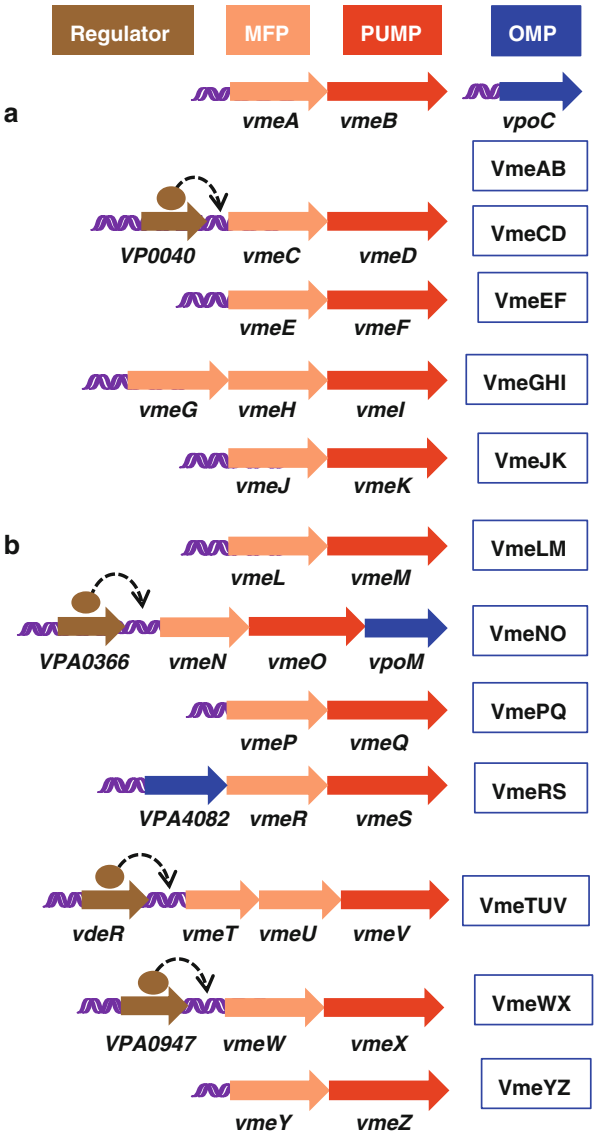

Fig. 12.2 Genetic organization of 12 known and putative RND efflux pumps encoded by chromosome 1 (**a**) and chromosome 2 (**b**) of *V. parahaemolyticus* strain RIMD2210633. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (*MFP*), a pump, or an outer membrane protein (*OMP*), respectively. Genes encoding the putative regulators are shown on the *left*, and yet, the effect of these regulators on the efflux pump expression remains to be determined (denoted by the *dotted lines* with *arrows*)

lyticus is recognized as a prototype of MATE family transporters [73], which are widely distributed in all kingdoms of living organisms [93]. Studies suggested that both NorM and VmrA couple the movement of toxic organic cations out of the cell (against their prevailing concentration gradient) to the energetically favorable movement of sodium ions into cell, along their electrochemical gradient [94]. Among the 24 species tested, the AceI homolog (VP1155) from *V. parahaemolyticus* strain RIMD2210633 was a few pumps that showed to confer, when expressed from a plasmid in a hypersusceptible AcrB-EmrE-MdfA-deficient *E. coli* mutant,

resistance to several biocides including chlorhexidine, benzalkonium chloride, acriflavine, and proflavine (fourfold MIC reduction) [82, 95]. Interestingly, the function of VP1155 and AceI (of *Acinetobacter baumannii*) was not TolC dependent [82]. VP1155-mediated efflux of acriflavine and proflavine in the intact cells of the *E. coli* host was also demonstrated [82]. The PACE exporters may suggest another family of proteins that also contributes to intrinsic drug resistance [42].

12.3.3 Other Vibrio *spp.*

The genomes of several other *Vibrio* spp. also confirm the wide presence of the putative drug efflux pumps such that the marine pathogen *V. vulnificus* (5.2 Mb) has 15–16 putative RND pumps (strains CMCP6 and YJ016) in addition to two TolC homologs (http://www.membranetransport.org) [96]. A study using mutants carrying deletion of one of the three RND systems (which are, respectively, homologous to VexAB, VexCD [both of *V. cholerae*], and AcrAB of *E. coli*) suggested that the VexAB homolog is mainly involved in intrinsic resistance to multiple antimicrobials system [83]. Another earlier study from the same group assessed the effect of the deletion of either *tolCV1* or *tolCV2* on antimicrobial susceptibility. Inactivation of TolCV1 rendered the mutant more susceptible to those agents shown to be substrates of VexAB (Table 12.1) in addition to novobiocin and tetracycline, highly suggesting that VexAB and TolCV1 likely function as a major drug efflux pump in this species. Disruption of TolCV2 had no or little effect on antimicrobial susceptibility [84]. These *Vibri*o TolC proteins can function with MacAB ABC transporter of *E. coli* [97]. An RND pump (containing VV1_1681) is involved in the export of vulnibactin that is required for iron acquisition from the environment in *V. vulnificus* [98]. VV1_1681 is an orthologue to VmeK (VP2472) of *V. parahaemolyticus* (87 % identity). In *Vibrio tasmaniensis*, five genes, *cusCBAF* and *copA*, are predicted to encode an RND efflux system and an ABC transporter for copper efflux that provides copper resistance in order to resist the action from phagocytes, induce cytosis of immune cells, and colonize the host [99].

12.3.4 L. pneumophila

The genome of *L. pneumophila* Philadelphia 1 contains a single circular chromosome of ca. 3.4 Mb in size [8] with genes encoding a relatively small number of putative transporters (only 156 are predicated on the basis of TransportDB at http://www.membranetransport.org; accessed on March 25, 2016). However, based on the phylogenetic analysis, there are still a number of genes encoding transporters of three superfamilies, e.g., 9 RND, 35 MFS, and 35 ABC (in strain Philadelphia) as well as genes encoding membrane fusion proteins and OM channel proteins [8]. Of note, no member of the MATE family was identified [100]. Similarly, the genomes

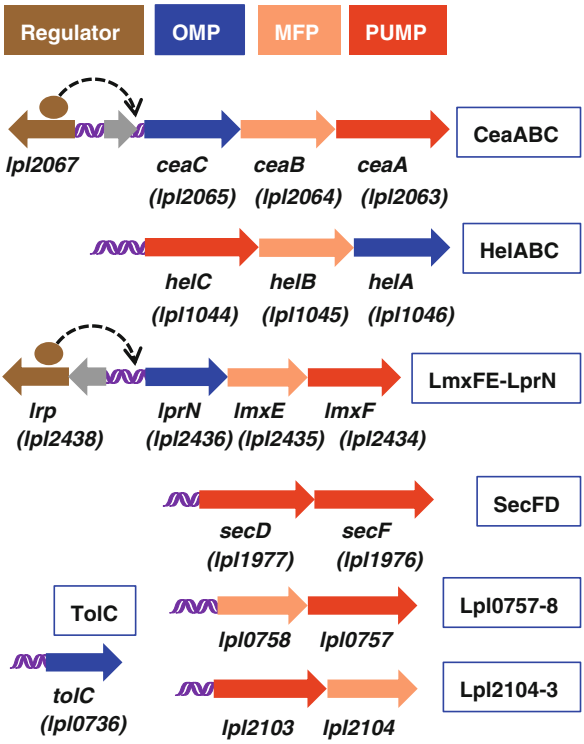

Fig. 12.3 Genetic organization of six known and putative chromosomally encoded RND efflux pumps in *L. pneumophila* strain Lens. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (*MFP*), a pump, or an outer membrane protein (*OMP*), respectively. Genes encoding the putative regulators are shown on the *left*, and yet, the effect of these regulators on the efflux pump expression remains to be determined (denoted by the *dotted lines* with *arrows*)

of strains Paris and Lens [9] contain, respectively, 7 and 11 putative RND systems [85, 100]. The transcriptional organizations of the genes that encode the putative RND pumps from strain Lens are shown in Fig. 12.3 [9]. To estimate the potential role of an efflux mechanism in antimicrobial resistance, there is a need to consider the influx of antimicrobial agents, thus the outer membrane permeability barrier features of *L. pneumophila* [40, 101]. This species possesses a major 28 kDa outer membrane protein that is similar to *E. coli* porins in terms of channel-forming activity and forms cation-selective and voltage-independent gating channel [102]. *L. pneumophila* strains also display high-level *in vitro* susceptibility to macrolides, rifamycins, fluoroquinolones, aminoglycosides, and β-lactams [103, 104]. These data may likely suggest the limited contribution from the outer membrane permeability barrier or drug efflux pumps to intrinsic resistance in *L. pneumophila*. However, mutants with both low-level and high-level resistance phenotypes have been generated *in vitro* [11, 30]. For instance, the presence of erythromycin or ciprofloxacin selected *in vitro* mostly low-level resistance, which is often seen in Gram-negative bacteria as an indicator of possible drug efflux involvement [30]. High-level resistance with an increase of 8- to 512-fold moxifloxacin MIC values was associated with DNA gyrase-based target mutations [11].

To date, there is only a limited characterization regarding the possible drug efflux transporters of *L. pneumophila*. The study of Ferhat [85] assessed the expression of a

large number of the genes that encode 5 RND, 5 MFS, 4 SMR, and 15 ABC transporters by quantitative reverse transcription PCR assays for strain Lens to compare the gene expression between exponential and stationary phase of the growth. Among the RND pump-related genes, expression of *lpl2063* (*ceaA*), *lpl2434* (*lmxF*), and *lpl0736* (*tolC*) was highly increased during the exponential phase of growth, while *lpl1046* (*helA*) expression was elevated in the stationary stage. Ferhat also constructed a number of deletion mutants in order to assess their contribution to antimicrobial susceptibility. Mutants with inactivation of RND-type *lpl2065-2063* (*ceaABC*), *lpl1044-1046* (*helABC*), *lpl2436-lpl2434* (*lmxFE-lprN*), *lpl0757-0758*, *and lpl2103-2104* became more susceptible to a variety of antimicrobial agents including heavy metal salts as specified in Table 12.1, mostly with a moderate twofold MIC reduction. Disruption of several putative ABC transporter genes or operons (such as *lpl1509-1510* [*lssD-lssB*], *lpl 0278-279-280*, *lpl0695-0696-0697-0698-0699*, *lpl0880-0881-0882*, and *lpl2849-2850-2851*) rendered mutants with similar increased susceptibilities to several agents listed in Table 12.1 (generally a twofold MIC reduction) [85]. These data support a modest role of drug efflux pumps in drug resistance.

L. pneumophila has a homolog of 455 amino acids (encoded by *lpg0699* [strain Philadelphia], *lpl0736* [strain Lens] [9] or *LPC2595* [strain Corby]) that is 36 % identical to the TolC channel protein of *E. coli* (475 amino acids), and inactivation of this protein rendered the mutant susceptible to a wide range of antimicrobial agents (e.g., 16-fold erythromycin MIC reduction and two- to eightfold decrease for MIC values of benzalkonium chloride, deoxycholate, ethidium bromide, methylene blue, nickel sulfate, norfloxacin, novobiocin, and rhodamine 6G [8, 85, 100]. This phenotype is highly indicative of the operation of a drug efflux mechanism in *L. pneumophila*. Comparing the modest reduction of the MIC values and the overlapping substrate profiles for various RND or ABC pump mutants described above (Table 12.1), it is likely that TolC functions with multiple multicomponent efflux pumps since the hypersusceptible phenotype of the *tolC* mutant supports that inactivation of TolC function would simultaneously abolish the operation of multiple efflux systems that are functionally dependent on TolC. Consistently, ethidium bromide accumulation assay in intact cells revealed significant accumulation of ethidium bromide in *tolC* mutant cells than the wild-type cells and increased accumulation to the same levels in both cell types after the treatment of the cells by the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone [85, 100].

Moreover, as expected with multifunctional role of TolC protein as a key component of multiple efflux systems in Gram-negative bacteria, *L. pneumophila* TolC also contributes to oxidative stress response caused by hydrogen peroxide or cooling tower biocides and is required for virulence against protozoa and macrophages [85, 100]. It is also involved in secretion of a lipid-containing unidentified surfactant that promotes *Legionella* motility [85, 100, 105]. An MFS exporter with 12 transmembrane segments, LbtB, is a homolog of several efflux proteins (23 % and 21 %, respectively, identical to bicyclomycin resistance protein Bcr and tetracycline efflux pump TetA of *E. coli*) and is involved in secretion a siderophore named legiobactin that helps the intracellular growth of the species [86].

Lastly, it is necessary to emphasize that the intracellular nature of *L. pneumophila* may particularly suggest an important role which a drug efflux pump could play in acquired resistance affecting efficacy of antimicrobial treatment regime. This is because the multiplication of *L pneumophila* within macrophages has limited the choice of antibiotics to those that can penetrate phagocytic cells such as macrolides, rifamycins, and fluoroquinolones [106, 107], which are generally good substrates of typical drug efflux pumps [40].

12.4 Concluding Remarks

The two species, *Vibrio* and *Legionella* discussed in this chapter, are associated with aquatic environments. They both have a relatively high permeable outer membrane and thus are generally susceptible *in vitro* to a wide range of antimicrobials including those typically against Gram-positive bacteria such as macrolides. These species also possess a large number of proven and putative drug efflux transporters including the prototypical MATE pump, NorM, first identified in *V. parahaemolyticus*. Some of these transporters have been demonstrated to mediate intrinsic resistance to multiple antimicrobial agents and are also involved in function beyond drug resistance such as colonization and virulence. However, a major question remains to be answered on whether or how these transporters could contribute to acquired drug resistance, although there is already evidence to support their role in low-level multidrug resistance. It is also important to see whether loss of porins could occur in these species, and this could synergistically interplay with drug efflux systems to raise resistance level. Moreover, there is little information regarding the regulation of the expression of these transporters, particularly *in vivo* conditions. *Vibrio* spp. infect people through digestive tract, where various chemicals such as bile salts can induce the expression of drug efflux pumps. *L. pneumophila* resides intracellularly and contribution from drug efflux pumps may significantly affect the drug accessibility. All of these aspects warrant future research to better understand the role of drug efflux pumps in antimicrobial resistance and beyond.

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Chapter 13
Antimicrobial Resistance and Drug Efflux Pumps in *Acinetobacter*

Bao-Dong Ling, Li Zhang, and Xian-Zhi Li

Abstract Infections associated with *Acinetobacter baumannii* represent a major threat to public health around the globe. This pathogen possesses the most sophisticated mechanisms of resistance in bacteria and has been characterized by its significant intrinsic resistance and propensity to develop acquired multidrug resistance, extensive drug resistance, or pandrug resistance, which all adversely affect antimicrobial therapy. A wealth of evidence indicates that multidrug efflux pumps play an important role in resistance of *Acinetobacter* spp. to a wide range of antimicrobial agents. This mechanism can readily evolve through *in vitro* or *in vivo* exposure of *Acinetobacter* spp. to antimicrobials including clinically used antibiotics and biocides. This chapter provides an overview of current status of antimicrobial resistance and the contribution of drug efflux pumps to clinically relevant resistance, with a focus on the characteristics of efflux pumps of the resistance-nodulation-cell division superfamily.

Keywords *Acinetobacter baumannii* • Antimicrobial resistance • AbaR resistance island • Efflux • RND efflux pumps • Outer membrane • AdeABC • AdeFGH • AdeIJK • AbeM • AbeS • CraA

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13.1 Introduction

Acinetobacter spp., represented by *Acinetobacter baumannii*, are widely present in various environments including soil and water [1]. As one of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) [2], *A. baumannii* has emerged globally as a common nosocomial pathogen, particularly in patients with severe underlying diseases [3]. Meanwhile, non-*A. baumannii* species have also increasingly been showing their clinical importance with respect to both infections and antimicrobial resistance [4–7]. Isolates of *Acinetobacter* spp. display the ability for persistence in various environments and also produce various virulence factors and high-level intrinsic resistance to a wide range of antimicrobial agents [8]. Moreover, this genus can also rapidly develop acquired resistance [8, 9] which provides an advantage for the microbe to survive in antimicrobial pressure environments such as hospitals. Epidemic clones of *A. baumannii* with multidrug resistance (MDR) phenotype have also been frequently reported around the globe [9–12]. Recent studies also showed the high prevalence and outbreaks (including fatal outbreaks) of extensively drug-resistant or pandrug-resistant *A. baumannii* [12–14]. In this chapter, we provide an overview of the resistance features of *A. baumannii*, with an emphasis on the role of drug efflux pumps in both intrinsic and acquired resistance.

13.2 *A. baumannii*: A Global Multidrug-Resistant Pathogen

13.2.1 Emergence and Prevalence of Multidrug-Resistant Isolates

In contrast to many other pathogens, several species of *Acinetobacter* including *A. baumannii* were only designated in the mid-1980s after a long history of taxonomic changes [1, 15]. Routine biochemical identification may only group *A. baumannii* and certain other *Acinetobacter* spp. into the *A. baumannii-Acinetobacter calcoaceticus* complex. For the last three decades, the clinical significance of *A. baumannii* has increasingly become important, as evident with high prevalence and outbreaks of *Acinetobacter* infections [1, 6, 12, 13, 16, 17]. This non-fermentative Gram-negative rod produces various virulence factors and causes pneumonia or bloodstream infections among various infections, in particular in critically ill patients [6, 8].

A. baumannii exhibits significant high-level intrinsic resistance to a wide range of antimicrobial drugs. The Clinical and Laboratory Standards Institute (CLSI) has listed ampicillin, amoxicillin, amoxicillin-clavulanate, aztreonam, ertapenem, chloramphenicol, fosfomycin, and trimethoprim as the agents to which isolates of the *A. baumannii-A. calcoaceticus* complex are intrinsically resistant [18]. *A. baumannii* also features an ability to develop acquired drug resistance. In fact,

there are currently a large number of studies targeting resistance in *Acinetobacter* spp. Among the >5,000 items retrieved under the keywords "*Acinetobacter*" and "resistance," more than 50 % of these items have only become available as of 2010 (retrieved from the PubMed as of February 29, 2016).

Surveillance data from the USA suggest that 63 % of *Acinetobacter* isolates were multidrug resistant, and about 7 % of critically ill patients on mechanical ventilations were infected with *Acinetobacter* spp. [19]. A recent study from China showed 53 % of 120 *A. baumannii* isolates were multidrug resistant [20], consistent with our earlier study for high-prevalent MDR in clinical isolates [17]. Our recent study also indicated that most isolates were resistant to carbapenems [21]. Resistance to carbapenems in *A. baumannii* is particularly a major clinical problem [14, 22–26]. Worrisomely, a number of extensively drug-resistant or pandrug-resistant isolates have also been reported globally [12–14, 27]. Additionally, a recent study showed the association between reduced susceptibility to biocides (chlorhexidine, benzalkonium chloride, and triclosan) and resistance to aminoglycosides, ciprofloxacin, and tetracycline [28]. It is necessary to note that although *A. baumannii* has been isolated less than *P. aeruginosa* from cystic fibrosis patients, *Acinetobacter* spp. including *A. baumannii*, like *P. aeruginosa*, also possess the virulence factor Cif (cystic fibrosis transmembrane conductance regulator inhibitory factor) that may promote airway colonization in lung infections [29]. As a result, it suggests a need to investigate *A. baumannii* prevalence in cystic fibrosis patients.

13.2.2 Molecular and Biochemical Mechanisms of Resistance

Acinetobacter spp. are characterized by their high-level resistance to a wide range of clinically used antimicrobial agents. This trait is attributable to the presence of all major resistance mechanisms of bacteria [30]. In particular, resistant *A. baumannii* isolates frequently feature the presence of chromosomal AbaR resistance islands [31]. For instance, an 86-kb genomic region (designated AbaR1) containing 45 resistance genes was reported a decade ago in the first available genome data of *A. baumannii* [31]. To date, more than two dozen diverse AbaR variants (ca 20–86 kb in size) have been reported globally, and this information provides insight regarding resistance evolution and spread [11, 32, 33]. A recent study identified ten AbaRs including the first type of Aba that also encodes putative drug efflux components [24]. Interestingly, various resistance genes or mobile genetic elements observed in the *A. baumannii* genome are considered to be likely acquired from the species of *Pseudomonas*, *Escherichia*, and *Salmonella* [31].

Drug inactivation *A. baumannii* isolates produce a large number of antimicrobial-inactivating enzymes which are encoded by chromosomes and/or plasmids [30, 31, 34, 35]. These β-lactamases include, for example, either narrow-spectrum TEM enzymes (Ambler class A) or broad-spectrum TEM variants; CTX-M and VEB enzymes (class A); metalloenyzmes IMP, NDM, SIM, and VIM (class B); AmpC-type

ADC enzymes (class C); and a number of OXA enzymes (class D, in particular, OXA-23, OXA-51, OXA-58, and OXA-66) [20, 21, 24, 26, 27, 31, 34, 36]. These enzymes are together able to hydrolyze almost all β-lactams. In particular, class B and class D β-lactamases are involved in hydrolyzing carbapenems, a last resort of antimicrobial agents against several major pathogens [21, 26, 37]. *A. baumannii* also produce aminoglycoside-modifying enzymes including AAC3, AAC6′, AAD, and APH [20, 31, 35, 38] that are often encoded by a part of Aba resistance island-associated gene cassettes containing class 1 integrons [31, 32]. Genes encoding chloramphenicol acetyltransferase (for chloramphenicol resistance), ADP-ribosyltransferase (for rifamycin resistance), and tetracycline modification enzyme (TetX1) were also revealed in the genome of *A. baumannii* [31, 37, 39].

Drug target modification Target alteration and protection-associated resistance mechanisms are responsible for resistance to aminoglycosides (due to 16S rRNA methylation) [40], quinolones (due to DNA gyrase mutations or Qnr-mediated target protection) [35, 41–43], and β-lactams (because of alterations in penicillin-binding proteins) [44–46]. Reduced susceptibility to minocycline and tigecycline occurs via mutations in gene encoding *S*-adenosyl-L-methionine-dependent methyltransferase [47]. Polymyxins act on the outer membrane (OM), and multiple mutations in genes such as *lpx* and *pmrB* affecting lipopolysaccharide structure are known to cause polymyxin resistance [48–50]. (In this regard, a recent study has identified, for the first time, plasmid-mediated resistance to polymyxins in *Enterobacteriaceae*, but not in *Acinetobacter* spp. The plasmid-borne gene named *mcr-1* encodes a phosphoethanolamine transferase enzyme that adds phosphoethanolamine to lipid A [51].)

Outer membrane permeability barrier In addition to these drug-specific inactivation and target modification mechanisms, drug influx and efflux across the cell membrane barrier play a critical role in influencing the susceptibility of *Acinetobacter* spp. to a broad range of antimicrobials. First, similar to that of the well-studied *P. aeruginosa*, the OM of *A. baumannii* also shows very low permeability to cephalosporins (i.e., about 100-fold lower than that of *E. coli* when tested using a liposome reconstitution assay with the isolated OM) [52, 53]. This trait is due to the lack of classic high-permeability trimeric porins (of *Enterobacteriaceae* spp.) in *A. baumannii* [54]. The proteins with porin activity in *Acinetobacter* spp. belong to the minor proteins [52]. As such, it is not surprising to find only minimal changes in the OM protein profiles in isolates with various drug susceptibilities [35, 45]. The monomeric OmpA protein, the major OM protein of *A. baumannii*, was experimentally demonstrated as the principal nonspecific slow porin [53], similar to the slow porins OmpA of *E. coli* and OprF of *P. aeruginosa* [54]. Genetic inactivation of the *A. baumannii ompA* gene resulted in reduced susceptibility to aztreonam and chloramphenicol (eightfold MIC decrease), two agents to which *A. baumannii* is intrinsically resistant [18]; however, there was only a moderate impact (≤twofold) on the MIC values of colistin, imipenem, and tigecycline [55], agents implicated in anti-*A. baumannii* infections [18].

A minor 488-amino acid OM protein named AbuO shows 28 % identity and 48 % similarity to the TolC OM channel protein of *E. coli* [56]; TolC is required for the function of several multicomponent drug efflux systems [57]. Inactivation of AbuO rendered the mutant more susceptible to amikacin, streptomycin, carbenicillin, and ceftriaxone (fourfold MIC reduction) as well as to ethidium bromide, benzalkonium chloride, chlorhexidine, and triclosan [56]. The *abuO* mutant was also more susceptible to deoxycholate and hydrogen peroxide and also showed increased expression in several putative drug efflux pump genes [56].

A small OM protein dubbed CarO (for carbapenem resistance-associated OM protein) has been found to function as an influx channel for carbapenems [58]. In contrast to the findings in an earlier study [58], CarO shows imipenem binding sites (but not for meropenem) [59] and is essential for the influx of *L*-ornithine and basic amino acids [60]. A recent crystallographic study showed CarO as a monomeric eight-stranded β-barrel protein that lacks an open channel [61]. CarO possesses a substantial extracellular domain. Interestingly, an *in vitro* liposome reconstitution study appeared to dispute the penetration of carbapenems via CarO [61]. However, the loss of CarO expression, due to gene disruption by IS*Aba1*, IS*Aba10*, or IS*Aba825* insertion, was found to link to carbapenem resistance phenotypes in clinical isolates [62–65]. Since the expression of carbapenem-hydrolyzing OXA β-lactamases is associated with IS*Aba*, the loss of CarO and the production of an OXA β-lactamase can be copresent [64, 65], likely together contributing to carbapenem resistance. Extensive genetic diversity of *carO* within clinical populations has been observed [66] with three groups of the CarO proteins being proposed recently [67]. Such CarO diversity may serve as a survival strategy for *A. baumannii* under diverse growth conditions [66]. Furthermore, the deficiency in additional OM proteins of 31–36 kDa has also been shown to be associated with carbapenem resistance [68–70].

Drug efflux systems also exert an indispensable role in drug resistance as described in the next section. It should be noted because of the low OM permeability, drug efflux pumps become more effective in generating clinically relevant resistance [71]. For instance, the loss of CarO protein and pump overproduction have been observed in multidrug-resistant isolates [36, 64]. Expressional upregulation of 14 distinct transporter genes and downregulation of *carO* and 31–36 kDa OM protein genes were noted in response to a physiological level of NaCl, which induced tolerance to amikacin and levofloxacin [72].

13.3 Drug Efflux Pumps in *Acinetobacter* spp.

In 2001, Magnet et al. [73] reported the identification of the first multicomponent drug efflux system in *Acinetobacter* spp., the AdeABC pump, which falls into the resistance-nodulation-cell division (RND) superfamily. Subsequently in 2006, completed genome sequence data [31] revealed the presence of a large number of putative drug efflux systems in *A. baumannii* that belong to the RND superfamily, the

major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, and the small multidrug resistance (SMR) family [71]. A newer study also described a novel transporter family in *Acinetobacter* spp., the proteobacterial antimicrobial compound efflux (PACE) family [74]. The analysis of the complete genome sequences of three *A. baumannii* isolates and one *Acinetobacter baylyi* strain suggests that the total number of predicted drug efflux transporters varies between these strains (i.e., 31, 46, 51, and 56 transporters were estimated) [75]. Newly available sequence of *Acinetobacter bereziniae* also shows a large number of putative drug efflux transporters including five RND pumps [76]. To date, numerous studies have investigated the role of *Acinetobacter* multidrug transporters in resistance to clinically relevant antibiotics as well as to biocides, dyes, and detergents [77, 78]. Table 13.1 shows lists of experimentally studied drug efflux pumps of *Acinetobacter* spp.

13.3.1 RND Efflux Pumps

Two decades of *E. coli* and *P. aeruginosa* investigations have revealed the clinical importance of the RND efflux pumps (see Chaps. 9 and 14), which has been further supported by the characterization of RND pumps in *Acinetobacter* spp. The first complete sequence of a multidrug-resistant epidemic clone of *A. baumannii* identified 30 putative genes that encode products associated with the RND efflux systems [31]. A recent study indicated the presence of eight RND pumps in *A. baumannii* [101], while the Transporter Database showed ten RND transporters in *Acinetobacter* sp. ADP1 (http://www.membranetransport.org; accessed as of March 15, 2016). Figure 13.1 shows the genetic organization of known and putative RND efflux systems in *A. baumannii*. As expected with an RND efflux system [71], each of the three characterized RND efflux pump systems, AdeABC, AdeFGH, and AdeIJK, is a tripartite efflux complex containing an RND pump located in the cytoplasmic membrane, a periplasmic adaptor protein/membrane fusion protein, and an OM channel protein (Table 13.1).

AdeABC This efflux system is present in *A. baumannii* [73, 82] and other *Acinetobacter* spp. [99]. However, it is apparently not well expressed in wild-type strains [73, 82]. AdeA, AdeB, and AdeC show identities of approximately 40 %, 50 %, and 40 % to AcrA/MexA, AcrB/MexB, and OprM, respectively, of the RND efflux components of *E. coli* and *P. aeruginosa* [73]. Inactivation of *adeB* in a wild type did not alter the susceptibility to ten classes of clinically used antimicrobial agents [79]. Overproduction of AdeABC makes an important contribution to acquired MDR as evident in clinical isolates of different geographic regions [17, 23, 35, 36, 39, 80, 102–109]. This efflux system accommodates a large number of substrates covering β-lactams, aminoglycosides, macrolides, lincosamides, amphenicols, tetracyclines, and tigecycline (Table 13.2) [79, 110]. Importantly, based on current clinical susceptibility interpretive criteria established by the CLSI

Table 13.1 Antimicrobial drug efflux pumps in *Acinetobacter* spp.

Species/transporter family	Efflux pump (regulator)	Substrates	References
A. baumannii			
RND	AdeABC (AdeSR, BaeSR)	ACR, AG, BAC, BL, CHL, CHX, DOC, EB, ERY, FQ, NAL, PAR, RHO, SDS, TET, TGC, TPP	[73, 79–81]
RND	$AdeAA_2B$ (BaeSR)	TGC	[82]
RND	AdeFGH (AdeL)	CM, EB, ERY, FQ, NAL, SDS, SUL, TET, TGC, TMP	[79, 83]
RND	AdeIJK (AdeN, BaeSR)	ACO, ACR, AZI, BAC, BL, CHL, CLI, CV, DAPI, DOC, EB, ERY, FAR, FQ, FUA, MIN, NAL, PYR, RIF, RHO, SA, SDS, SUL, TET, TMP, TPP, TRI	[17, 79, 80, 84–88]
MFS	AmvA	ACO, ACR, AO, BAC, BS, CHL, DAPI, DEO, EB, ERY, NOR, NOV, PAR, TPP	[89]
MFS	CraA	CHL	[90]
MFS	CmlA	CHL	[31]
MFS	FloR	CHL, FLO	[31]
MFS	TetA(B) (TetR)	TET	[24, 91]
MATE	AbeM	ACR, DAPI, DAU, DOX, FQ, GEN, R6G, TCS	[92]
SMR	AbeS	ACO, ACR, BAC, BS, CHL, CIP, DAPI, DOC, ERY, EB, NOV, RHO,SDS, TPP	[93]
SMR	QacE/QacEΔ1	QAC	[20, 31, 80, 94]
SMR	Smr (A1S_0710)	DOC, SDS	[95]
ABC	MacAB-TolC (BaeSR)	ERY	[87]
PACE	AceI	CHX	[74, 96]
A. baylyi			
RND	AdeIJK	BAC, CHL, CHX, EB, NOR, SDS, TET, TMP	[75]
MFS	CraA	CHL, EB	[75]
Acinetobacter genospecies 3			
RND	AdeDE	AM, CHL, CP, CT, EB, ERY, MP, RF, TET	[17, 97]
RND	AdeXYZ		[17, 98]

(continued)

Table 13.1 (continued)

Species/transporter family	Efflux pump (regulator)	Substrates	References
Acinetobacter genospecies UT13			
RND	AdeABC	AG, BL, CHL, FQ, TET, TGC, TMP	[99, 100]
RND	AdeIJK		[99]
RND	AdeXYZ		[99]

ACO acridine orange, *ACR* acriflavine, *AG* aminoglycosides, *AZI* azithromycin, *BAC* benzalkonium chloride, *BL* β-lactams, *CHL* chloramphenicol, *CHX* chlorhexidine, *CIP* ciprofloxacin, *CLI* clindamycin, *CV* crystal violet, *DAPI* 4′,6-diamidine-2-phenylindole, *DAU* daunomycin, *DOC* deoxycholate, *DOX* doxorubicin, *EB* ethidium bromide, *ERY* erythromycin, *FAR* farnesol, *FLO* florfenicol, *FQ* fluoroquinolones, *FUA* fusidic acid, *GEN* gentamicin, *MIN* minocycline, *NAL* nalidixic acid, *NOR* norfloxacin, *NOV* novobiocin, *PAR* paraquat (methyl viologen), *PYR* pyronin Y, *QAC* quaternary ammonium compounds, *R6G* rhodamine 6G, *RHO* rhodamine 123, *RIF* rifampicin, *SDS* sodium dodecyl sulfate, *SUL* sulfonamides, *TET* tetracycline, *TGC* tigecycline, *TMP* trimethoprim, *TPP* tetraphenylphosphonium, *TRI* triclosan

[18], AdeABC overproduction alone can change the susceptibility category from susceptible ("S") to reduced susceptibility (either intermediate "I" or resistance "R") (see β-lactams and aminoglycosides in Table 13.2).

AdeABC is also involved in resistance to tigecycline, an alternative drug for treating *Acinetobacter* infections, in isolates including epidemic clones, and in extensively drug-resistant isolates [35, 39, 64, 103, 106, 110–115]. Although the susceptibility interpretative criteria remain to be established for tigecycline against *Acinetobacter* spp., the "S," "I," and "R" values for *Enterobacteriaceae* have been defined as ≤2, 4, and ≥8 μg/ml, respectively, by the US Food and Drug Administration. In this regard, tigecycline MIC of ≥8 μg/ml has been observed with multidrug-resistant isolates, where efflux pumps such as AdeABC were investigated [114]. A serial *in vitro* exposure of a clinical isolate to tigecycline resulted in >tenfold tigecycline MIC increase (from 2 to 24 μg/ml) for a mutant showing 50-fold overexpression of *adeB* [112]. A difference of 16-fold tigecycline MIC between isogenic parental and AdeABC-hyperproducing strains was also reported [78].

Fig. 13.1 Genetic organization of the known and putative chromosomally encoded RND efflux pumps in *A. baumannii*. (**a**) Regulation of three characterized RND efflux systems. (**b**) Additional five uncharacterized RND pumps that are obtained based on sequence homology in the genome of *A. baumannii* ACIUCU, an epidemic multidrug-resistant isolate (GenBank accession NC_010611 [101]). The RND pump operons or genes are presented with *arrows* showing their transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (MFP), a pump, or an outer membrane protein (OMP), respectively. Genes encoding the proven or putative regulators are located on the left. The *green lines* represent the positive regulation of the efflux gene expression, while the *red lines* denote the repression of relevant gene transcription by repressors. Certain growth conditions or cell status (such as lipopolysaccharide [LPS] deficiency) also affect pump expression

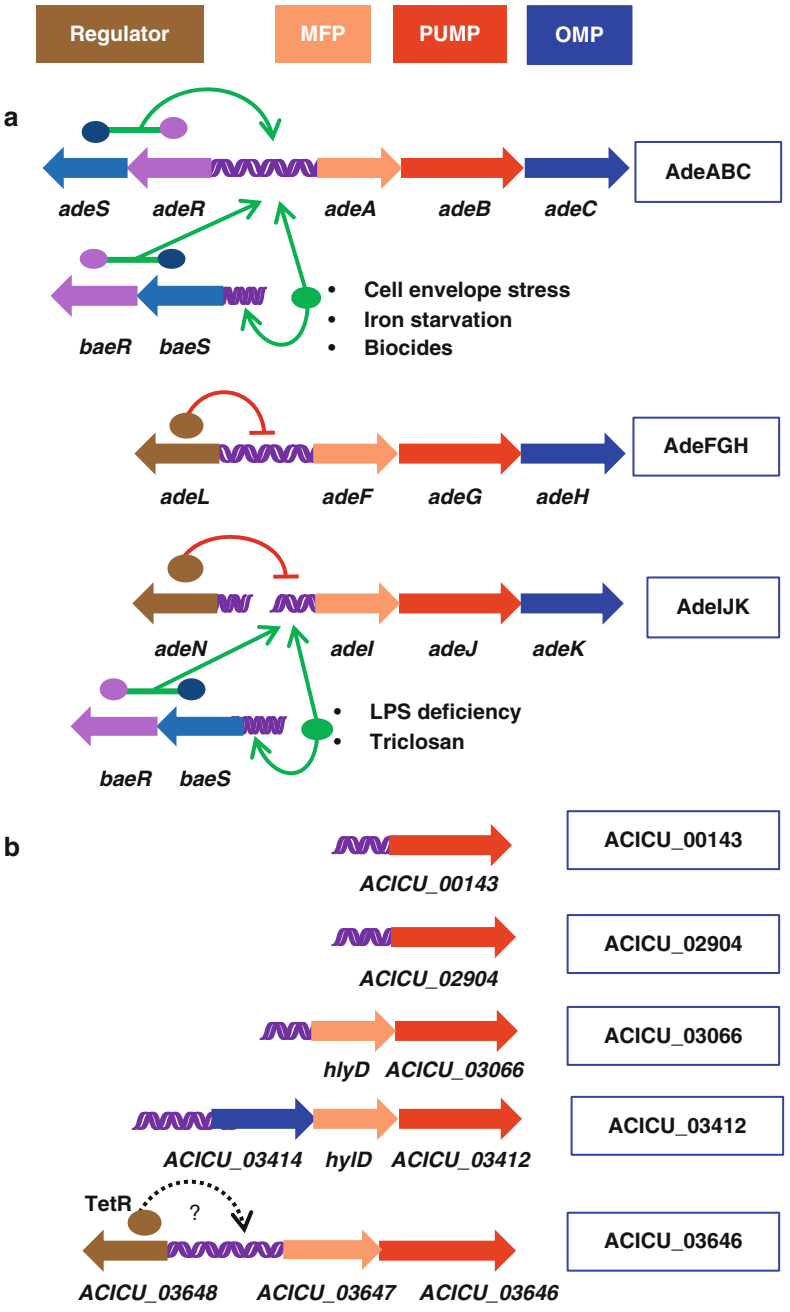
Regulator
MFP
PUMP
OMP
a
adeS
adeR
adeA
adeB
adeC
AdeABC
baeR
baeS
Cell envelope stress
Iron starvation
Biocides
adeL
adeF
adeG
adeH
AdeFGH
adeN
adeI
adeJ
adeK
AdeIJK
baeR
baeS
LPS deficiency
Triclosan
b
ACICU_00143
ACICU_00143
ACICU_02904
ACICU_02904
hlyD
ACICU_03066
ACICU_03066
ACICU_03414
hlyD
ACICU_03412
ACICU_03412
TetR
?
ACICU_03648
ACICU_03647
ACICU_03646
ACICU_03646

Table 13.2 Contribution of the overexpression of three RND efflux pumps to antimicrobial resistance (MIC values in μg/ml)

Antimicrobial	Susceptible breakpoint[a]	Parent strain	Overproduction of		
			AdeABC	AdeFGH	AdeIJK
Ceftazidime	≤8	4	**16**	4	8
Ceftriaxone	≤8	4	4	2	**16**
Cefepime	≤8	1	**16**	0.5	4
Doripenem	≤2	0.25	4	0.25	0.5
Imipenem	≤2	0.125	0.5	0.125	0.125
Meropenem	≤2	0.5	2	0.5	2
Ticarcillin	≤16	8	8	8	32
Amikacin	≤16	2	16	2	2
Gentamicin	≤4	1	**32**	1	1
Netilmicin	≤8	1	**128**	1	1
Tobramycin	≤4	2	**16**	2	2
Ciprofloxacin	≤1	0.125	1	1	0.5
Minocycline	≤4	0.25	0.25	0.25	0.5
Tetracycline	≤4	0.5	4	0.5	2
Tigecycline	Not available	0.125	2	0.25	0.5
Trimethoprim-sulfamethoxazole	≤2/38[b]	16/128[c]	32/128[c]	64/1,024[c]	128/256[c]
Colistin	≤2	0.5	0.5	0.5	0.5

The MIC data are derived from Yoon et al. [79]. The values shown in bold are those with reduced susceptibility based on the CLSI susceptible breakpoints

[a]The susceptible breakpoints are taken from CLSI [18]

[b]This breakpoint is for this combinational agent

[c]These values were obtained from testing two agents (trimethoprim and sulfadoxine) separately [79]

Inactivation of *adeB* in a multidrug-resistant isolate (with tigecycline MIC of 6 μg/ml) resulted in a 32-fold reduction of tigecycline MIC value [110]. Thus, although tigecycline displays activity against isolates possessing tetracycline-specific resistance mechanisms (ribosomal protection and efflux) [116], the AdeABC pump plays a key role in the emergence of tigecycline resistance in *Acinetobacter* spp., similar to the observations in other Gram-negative bacteria [117–119]. Lastly, AdeABC also provides resistance to several biocides (Table 13.1) [80].

AdeFGH This pump complex was first identified in the search for resistance genes from multidrug-resistant mutants deficient in AdeABC and AdeIJK [120]. It was subsequently confirmed to be present in the genome of *A. baumannii* [79]. Isolates overproducing AdeFGH were obtained *in vitro* by exposure to chloramphenicol or norfloxacin [83, 120]. This pump contributes to acquired MDR with a narrower substrate profile than that of AdeABC. Substrates for AdeFGH include chloramphenicol, quinolones, erythromycin, lincosamides, sulfonamides, and trimethoprim [78, 79, 83]. It is noted that either the disruption or overproduction of AdeFGH alone in the presence of AdeABC and AdeIJK (see below) does not alter drug susceptibility

to many classes of agents affected by the hyperexpression of AdeABC or AdeIJK (Table 13.2) [79, 84]. Nevertheless, in the absence of the latter pumps, inactivation of overexpressed *adeFGH* produces a >128-fold reduction of chloramphenicol and clindamycin MICs and a >16-fold reduction of the MIC values of fluoroquinolones, sulfamethoxazole, and trimethoprim [83]. One study conducted in Canada described AdeFGH as the most prevalent overexpressed RND pump in clinical isolates [107]. AdeFGH overproduction was also observed in tigecycline-resistant clinical isolates [115]. Recently, biofilm formation was found to be correlated well with the elevated expression of AdeFGH in multidrug-resistant *A. baumannii*, and higher *adeG* expression was induced by subinhibitory levels of levofloxacin or meropenem [121].

AdeIJK This efflux pump contributes to both intrinsic and acquired resistance in *A. baumannii* [17, 39, 64, 79, 80, 84–86, 115] and *A. baylyi* [75]. Its inactivation in a wild-type strain mostly caused 4- to 16-fold MIC reduction of β-lactams, chloramphenicol, clindamycin, erythromycin, fluoroquinolones, tetracyclines, and tigecycline (Tables 13.1 and 13.2) [78, 79, 85]. There is a good correlation between tigecycline insusceptibility and increased *adeJ* expression in clinical isolates [110]. AdeIJK also contributes to resistance to biocides including chlorhexidine, triclosan, and other disinfectants used in hospitals [79, 80, 122]. Triclosan was found to be able to select AdeIJK-overproducing mutants with MDR phenotypes [122].

The three aforementioned RND pumps show overlapping yet differing substrate profiles and may interplay synergistically in raising resistance levels. The latter was observed for AdeIJK and AdeABC which contribute together to resistance to chloramphenicol, fluoroquinolones, and tetracyclines including tigecycline [78]. Sugawara and Nikaido [123] have characterized the efflux properties of AdeABC and AdeIJK pumps by comparing with those of the *E. coli* AcrAB-TolC efflux system. AdeABC and AdeIJK pumps are both able to pump out β-lactams, and this activity for many β-lactams is masked in *Acinetobacter* due to the endogenous β-lactamases. AdeABC is more effective than AcrAB-TolC in the extrusion of tetracycline, while AdeIJK is remarkably more active in pumping out multiple agents [123]. It should be noted that the overproduction AdeABC and AdeIJK also shown a fitness cost including decreased biofilm formation [79].

Other RND pumps In addition to AdeABC, AdeFGH, and AdeIJK, operons or genes encoding at least five other putative RND pumps have been identified in *A. baumannii* after analyzing the complete genome data of three strains using AdeB as the reference efflux protein (some pumps may be involved in heavy metal resistance) [101, 107]. In this regard, the total number of RND efflux operons in *A. baumannii* is quite comparable to those of several well-studied Gram-negative bacteria (seven from *E. coli*, 12 from *P. aeruginosa*, and eight from *Stenotrophomonas maltophilia*) (see Chaps. 9, 14, and 15). These five new putative efflux genes were confirmed to be mostly present in international clones of *A. baumannii* (Fig. 13.1) [101]. Some of these pumps lack the OM component of the tripartite efflux complex, but the putative OM channel protein AbuO has been identified as described

above [56]. Although their clinical significance in resistance to antimicrobials or heavy metals remains unknown, these genes may likely include the gene that encodes an AdeT pump involved in resistance to aminoglycosides when expressed in a hypersusceptible *E. coli* host (reviewed in [77]). Increased expression of *adeT* was noted in an AbuO mutant [56].

Additionally, an efflux system containing a pair of membrane fusion proteins and an exporter without a linked OM channel protein (i.e., AdeA-AdeA2-AdeB) was identified in *A. baumannii* with an involvement in tigecycline resistance [82]. The presence of a pair of membrane fusion proteins was first observed for TriAB of the RND TriABC pump of *P. aeruginosa* [124]. The RND efflux systems from non-*A. baumannii* species of *Acinetobacter* are included in Table 13.1 [17, 77, 86, 99]. For example, homologues of the AdeIJK pump and its regulator AdeN are present in all 11 examined non-*A. baumannii-Acinetobacter* spp. [86].

13.3.2 MFS and MATE Efflux Pumps

The Transporter Database lists 48 MFS transporters from *A. baylyi* ADP1 (http://www.membranetransport.org; accessed as of October 15, 2015) [75]. However, only a few of these transporters have been experimentally proven to be drug exporters within the literature. The CraA pump, belonging to the drug/H^+ antiporter-1 family with 12 transmembrane segments, is present in all tested isolates and specifically contributes to chloramphenicol resistance; its inactivation produced >128-fold reduction of chloramphenicol MIC [90]. CraA is considered to contribute to intrinsic resistance to chloramphenicol since the efflux pump inhibitor, phenylalanine-arginine β-naphthylamide (PAβN), was able to reduce chloramphenicol MIC by eightfold for a wild-type strain but has a minimal impact on the isogenic *craA*-disrupted strain [90]. The *craA* gene is among the 25 upregulated transporter genes in response to exposure to NaCl induction [72]. An *A. baylyi* mutant with *craA* overexpression was obtained by a single exposure of the parental strain to chloramphenicol. Deletion of *craA* from this mutant and the wild-type strain resulted in eight- and fourfold reduction of chloramphenicol MIC, as well as four- and twofold reduction of ethidium bromide MIC [75], respectively, suggesting that CraA is involved in both intrinsic and acquired resistance. However, *craA* gene status did not alter susceptibility to many other antimicrobial agents [75], supporting that CraA is drug specific with a very narrow substrate spectrum.

Another MFS pump, AmvA, falls into the drug/H^+ antiporter-2 family with 14 transmembrane segments [89]. When expressed in a hypersusceptible *E. coli* host from cloned *amvA* gene, this pump conferred resistance to a broad range of substrates including antibiotics and, in particular, cytotoxic agents (Table 13.1). Deletion of the *amvA* gene further confirmed its involvement in resistance, and there was a higher *amvA* expression in several multidrug-resistant clinical isolates

[89]. However, the latter was not found in another study [64]. Additionally, a recent study using a rapid multiplexed phenotypic method further identified the contribution of two MFS transporter genes ABAYE_0913 and A1S_2795 (and an ABC transporter gene A1S_1535) to resistance to a narrow range of antimicrobials when expressed in *E. coli* host [125].

The well-studied tetracycline-specific MFS-type Tet pumps (such as Tet(A), Tet(B), and Tet39) have also been found in *A. baumannii* [31, 125–128]. These pumps are encoded on the chromosome (including AbaR resistance islands) and plasmids [31, 91, 129, 130]. Plasmid-borne *tet*(*B*)-*tetR* genes were associated with the IS*CR2* mobile element in multidrug-resistant isolates [129], suggesting a possible rapid horizontal resistance spread. Similarly, amphenicol-specific efflux pumps, CmlA and FloR, are also encoded by genes located in *AbaR* resistance islands [31]. A homologue to MFS-type SmvA efflux of *Salmonella* spp. [131] and an RND pump-associated membrane fusion protein were encoded by an AbaR genomic island [24].

The first available genome of *A. baumannii* showed the presence of two putative drug transporters of the MATE family [31]. One of the pumps dubbed AbeM provided resistance to gentamicin and biocides when expressed from cloned gene in *E. coli* (Table 13.1) [92]. Increased expression of *abeM* with moderately increased expression of *adeABC* and *adeIJK* was noted in imipenem-resistant isolates [132], but other studies suggested no correlation between *abeM* expression and resistance phenotype [35, 39, 133, 134]. Newer genome data suggested three additional AbeM homologues – AbeM2, 3, and 4 [135]. One study included *abeM* with other resistance genes (*adeB*, *adeR*, *ampC*, and *ompA*) to assess the genetic linkage of multidrug-resistant endemic clones [105].

13.3.3 SMR Efflux Pumps

The AbeS pump is an efflux pump of the SMR family and shows ca. 50 % of identity in comparison with the prototypical SMR pump, EmrE, of *E. coli* [93]. Inactivation of AbeS in *A. baumannii* led to increased susceptibility to chloramphenicol, ciprofloxacin, erythromycin, novobiocin, and biocides (≥threefold MIC reduction) (Table 13.1). Expression of cloned *abeS* in *E. coli* also demonstrated its role in MDR [93]. Interestingly, the cloned *abeS* gene in *E. coli* conferred only twofold MIC increase for amikacin (the only aminoglycoside agent tested) [93]. Furthermore, there was significantly higher average expression of *abeS* in 5 amikacin-resistant isolates (MIC ≥64 μg/ml) in comparison to amikacin-susceptible isolates (MIC ≤64 μg/ml) [134], and none of other efflux genes tested (*adeB*, *adeJ*, *craA*, *macB*, *emrA*, and *emrB*) exhibited such a relationship, with the exception of the *abeM* expression (which was about twofold higher in amikacin-resistant isolates than the susceptible isolates) [134]. In this regard, an SMR homologue in *P. aeruginosa* was shown to be involved in intrinsic resistance to aminoglycosides [136]. However, the

contribution of AbeS to amikacin resistance, if existed, could be masked by aminoglycoside enzymatic inactivation resistance mechanisms since inactivation of *abeS* did not alter amikacin susceptibility in *A. baumannii* [93]. A recent report compared the substrate specificity of AbeS with the prototypical *E. coli* EmrE pump and identified the residues involved in AbeS substrate recognition; this report highlights the molecular basis of AbeS as a multidrug transporter [137].

QacE (or attenuated QacEΔ1) is an SMR pump encoded by plasmids or chromosomal genomic resistance islands in several Gram-positive and Gram-negative bacteria [138, 139]. The *qacE* and in particular *qacEΔ1* genes are thus generally present with resistance gene cassettes (containing various resistance genes; especially closely linked to the sulfonamide resistance gene *sul*) and mobile genetic elements such as integrons and transposons [139]. QacE contributes to resistance to quaternary ammonium compounds. In *Acinetobacter*, *qacEΔ1* and *qacE* have been found to coexist with other resistance genes and mobile elements [20, 80, 94]. The *A. baumannii* resistance island, AbaR1, carries four copies of *qacE* [31]. In one recent study, *qacE* was highly prevalent (73 % of 112 tested isolates) in multidrug-resistant *A. baumannii* [140]. It is noted that other types of *qac* genes such as those that encode the MFS-type QacA and QacB, as well as the SMR-type QacC, QacG, Qac H, and QacJ, were not present in *A. baumannii* isolates [80, 140].

13.3.4 PACE Efflux Pumps

A new type of multidrug efflux pump, AceI, was found to mediate resistance to chlorhexidine (eightfold MIC increase when AceI was overexpressed in *E. coli*) [96]. The putative pumps of the PACE family are also identified in >20 bacterial species including *E. coli*, *Salmonella enterica* serovar Typhi, *Enterobacter cloacae*, *K. pneumoniae*, *P. aeruginosa*, and *Vibrio parahaemolyticus*. When expressed from a cloned vector in *E. coli*, *aceI* homologous genes from some of these species provided resistance to not only to chlorhexidine but also to acriflavine, benzalkonium chloride, dequalinium, and/or proflavine [74]. Chlorhexidine itself also displays strong induction ability for upregulating *aceI* expression [96]. In this regard, exposure of *Acinetobacter baylyi* to chlorhexidine also induced resistance to chlorhexidine and oxidants [141].

13.4 Regulation of Drug Efflux Pump Expression

13.4.1 RND Pumps

The operons encoding AdeABC, AdeFGH, and AdeIJK pumps are, respectively, linked to regulatory genes as shown in Fig. 13.1.

AdeABC The two-component regulatory system, AdeRS, negatively regulates the expression of AdeABC [81]. Thus, elevated expression of AdeABC has been attributable to mutations in *adeR* and *adeS* in multidrug-resistant or extensively drug-resistant isolates [81, 110, 113, 142–144]. To date, the mutations identified in AdeR include Ile14Val, Asp20Asn, Aln91Val, Pro115Leu, Met197Ile, Val120Ile, Ala136Val, and Ser200Cys, while those in AdeS contain Gly103Asp, Ala130Thr, Thr156Met, Gly186Val, Asn213Asp, Asn268His, and Ile285Leu [81, 110, 113, 145]. The Asp20Asn substitution alone increased the *adeB* expression by sevenfold [145]. Missense mutations in *adeR* have also been reported from *in vivo* selected *adeB*-overexpression multidrug-resistant isolates [23]. The insertion of IS*ABa1* to *adeS* produced a truncated AdeS protein, and this insertional activation correlated with tigecycline resistance in clinical isolates [113]. One study also identified two mutational hot spots, one in AdeS near His149 and another in the DNA-binding domain of AdeR [146]. However, some mutations identified in AdeS may not change the drug susceptibility phenotype as shown by a recent study which also revealed that Gly186Val substitution alone had a significant impact on reduced drug susceptibility (e.g., fourfold tigecycline MIC increase) [147]. In fact, *adeRS* genetic variability was linked to tigecycline resistance [114]. AdeR has been recently demonstrated for its ability to bind to a direct-repeat motif in the intergenic region between *adeABC* and *adeRS* [148]. Expression of *adeRS* is also affected by several factors. Increased temperature (i.e., 42 °C), high osmolarity, and salicylate were all found to downregulate *adeR* expression [149].

Another two-component regulatory system, BaeSR, has been increasingly shown to be involved in the regulation of the cell envelope stress response and efflux pump expression in several Gram-negative bacteria [150–152]. *A. baumannii* also showed the presence of the *baeSR* operon, and its expression was detectable in a wild-type strain and further increased in response to a high osmolarity-caused stress [82]. (However, another study suggested an inhibitory effect from high osmolarity on *adeB* expression [149].) Lipopolysaccharide-deficient *A. baumannii* showed the upregulation of various genes including *baeSR* (ca. tenfold increase) (and efflux pump genes *adeIJK* and *macAB-tolC*) [87]. Both an *in vitro* selected and a clinically derived tigecycline-resistant *A. baumannii* had significantly higher expression of the *adeAA$_2$B* and *baeSR* genes, and inactivation of *baeR* decreased *adeAA$_2$B* expression [82], suggesting that BaeSR positively regulates expression of AdeAB.

adeABC expression is also inducible. The *adeABC* genes are the most highly upregulated genes induced by the cationic biocide, chlorhexidine [96]. However, a recent study showed the inhibition of *adeABC* expression in biofilm cells by chlorhexidine and the quaternary ammonium compound cetrimide [153]. The differential impact on *adeABC* expression between planktonic cells and biofilm cells requires further investigation. Similarly, expression of *adeS* and *adeB* was both increased in certain clones after their exposure to chlorhexidine [28], suggesting a need to further verify the relationship between AdeRS and AdeABC. Additionally, iron limitation can also result in increased expression of *adeABC* and two quorum-sensing genes *luxI* and *luxR* [154]. A high concentration of 500 μg/ml tannic acid

was also able to induce expression of *adeB* and *baeR* [155]. Lastly, salicylate, an agent that often induces low resistance in several bacteria (reviewed in [77]), showed an inhibitory effect on *adeB* expression (but not on *adeG* or *adeJ* level) [149]. An iron-dependent effect of white and blue light on reduced susceptibility to minocycline and tigecycline was reported to be likely as a result of the induction of AdeABC expression [156].

AdeFGH This efflux pump is not likely expressed in wild-type strains [84]. Upstream of the *adeFGH* operon is a gene encoding the AdeL repressor (Fig. 13.1) [83], which belongs to the LysR regulator family involved in regulation of diverse genes in metabolism, quorum sensing, and virulence [157]. Mutations in *adeL* are linked to AdeFGH hyperexpression [83]. Yet, several strains with moderate expression of AdeFGH carried no mutations in *adeL* [146]. Interestingly, a recent study revealed that biofilm formation was significantly induced by subinhibitory levels of levofloxacin with accompanying upregulation of *adeFGH* expression [121].

AdeIJK A gene encoding the AdeN repressor of the TetR family is located ca. 800 kbp upstream of the *adeIJK* operon (Fig. 13.1) [86]. Its inactivation produced fivefold *adeJ* overexpression with resistance to aztreonam, ertapenem, meropenem, minocycline, and tigecycline. AdeIJK overproduction also resulted from AdeN mutations in the region required for dimerization of TetR proteins, suggesting that remotely encoded AdeN represses AdeIJK expression [86]. A triclosan-selected AdeIJK-overproducing mutant carried a 73-bp deletion in *adeN* [122]. Farnesol, a quorum-sensing molecule of *Candida albicans*, was recently found to dysregulate a large number of *A. baumannii* genes including upregulation of *adeIJK* genes and another set of *acrAB*-like genes [88]. This study also revealed the role of efflux pumps in farnesol resistance. Mutants with lipopolysaccharide deficiency have an elevated expression of *adeIJK* [87], suggesting possible involvement of the AdeIJK pump in response to cell envelope stress.

13.4.2 Non-RND Pumps

The genetic organization of Tet efflux pump systems (e.g., *tet*(*A*)-*tetR* or *tetA39*-*tetR39* with the two genes transcribed divergently) in *A. baumannii* and in many other bacteria is highly similar [31, 128]. Thus, the TetR repressor is expected to repress the expression of the Tet efflux pumps. The latter would also be inducible by tetracyclines. Additionally, transcriptional upregulation of *mdfA* was observed among >200 up- or downregulated genes in a multidrug-resistant mutant isolates following exposure to a subinhibitory concentration of tigecycline [158]. One nucleic acid substitution upstream of the *craA* translation initiation codon resulted in more stable *craA* transcripts [75]. The *macAB-tolC* expression was increased in a lipopolysaccharide-deficient mutant [87].

13.5 Prevalence of Efflux Pump-Overproducing Isolates and Effect of Efflux Pump Inhibitors on Drug Resistance

Selection of the efflux pump-overproducing isolates of *Acinetobacter* spp. can be readily obtained *in vitro* under the laboratory conditions. Multiple agents including amikacin, chloramphenicol, chlorhexidine, imipenem, meropenem, levofloxacin, norfloxacin, moxifloxacin, and tetracycline have been reported to select efflux-mediated resistance [75, 79, 83, 121, 134, 155, 159, 160]. Exposure of *A. baumannii* to subinhibitory imipenem or tigecycline has showed global transcriptional response [158, 161]. Similarly, *in vivo* emergence of multidrug-resistant clinical isolates during antimicrobial therapy has also been frequently reported [23, 49, 115, 134]. A recent study assessed the expression of eight efflux pump genes (*adeB*, *adeJ*, *abeM*, *abeS*, *craA*, *macB*, *emrA*-like, and *emrB*-like) in multidrug-resistant clinical isolates and laboratory-selected mutants [134]. Although the expressions of these targeted genes were variable among the tested strains, there was an overall trend suggesting increased expressions of *adeB* and *abeS*. Increased expression of *adeJ* and *macB* appeared to be limited to certain resistant strains [134]. Overexpression of RND pumps was also observed in multidrug-resistant non-*A. baumannii* environmental isolates of *Acinetobacter* spp. [162].

The clinical significance of the RND pumps in MDR in *Acinetobacter* spp. suggests that inhibition of pump efflux activity can provide a possible therapeutic means to combat resistance. Two frequently used efflux pump inhibitors (EPIs) of the RND pumps, PAβN and 1-(1-naphthylmethyl)-piperazine (NMP), have been tested in combination with various antimicrobial agents against *A. baumannii*. At relatively high levels, these two agents exhibit antibacterial activity with their respective MIC values of ≥400 and 200–≥400 μg/ml [104, 160, 163]. The effect of these EPIs on antimicrobial susceptibility may vary from one study to another, and obviously, standard susceptibility testing should be established to better assess the implication of the EPIs. In one study, for these two EPIs, a low concentration of 25 μg/ml showed a very limited activity in sensitizing the cells or reversing the resistance phenotype, but either of the two EPIs at the higher concentration of 100 μg/ml restored drug susceptibility with a MIC decrease by eightfold to certain antimicrobial agents (e.g., ciprofloxacin, levofloxacin, chloramphenicol, clarithromycin, linezolid, rifampicin, and/or tetracycline) [160]. In other studies, PAβN at 10 μg/ml decreased mostly two- to fourfold MIC values of chloramphenicol, clindamycin, and trimethoprim against clinical isolates [164], while PAβN at 20 μg/ml reduced up to 16-fold nalidixic acid MIC but displayed little effect on ciprofloxacin susceptibility [165]. PAβN and NMP, each at 100 μg/ml, restored susceptibility to fluoroquinolone (2- to 16-fold reduction of MICs) and tigecycline (mostly by a twofold MIC decrease) [166]. PAβN at 100 μg/ml also sensitized minocycline activity by reducing ≥fourfold MIC values [167]. Apparently, these EPIs have a stronger effect on resistance reversal with agents that have relatively high MIC values such as chloramphenicol, clarithromycin, clindamycin, linezolid,

rifampicin, and trimethoprim [164, 165, 168]. Intriguingly, one study has suggested a paradoxical effect of NMP at 64 μg/ml on susceptibility to tetracyclines (i.e., increased susceptibility to doxycycline, minocycline, and tetracycline) and tigecycline (reduced susceptibility) [163]. Additionally, a study also tested the effect of omeprazole, phenothiazines (chlorpromazine, prochlorperazine, and promazine), reserpine, and verapamil on sensitizing cells [169] with phenothiazines being the only agents to be able to restore susceptibility to certain antibiotics (≥eightfold MIC decrease) [169]. A recent study tested the effect of PAβN, NMP, omeprazole, reserpine, verapamil, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for their impact on colistin susceptibility of colistin-susceptible and colistin-resistant Gram-negative bacteria including *A. baumannii* [170]. The expression status of any drug efflux pump was not assessed, and only CCCP was shown to mostly exhibit an impact on reversing colistin resistance of colistin-resistant *A. baumannii* (and *S. maltophilia*). However, proton conductors such as CCCP act on disruption of proton motive force across the cytoplasmic membrane and do not act on pump per se [71]. The mechanism of the CCCP's effect on colistin resistance remains unknown. Two serum-associated agents, (E)-4-((4-chlorobenzylidene)amino)benzenesulfonamide and *N*-tert-butyl-2-(1-tert-butyltetrazole-5-yl)sulfanylacetamide, were found to enhance minocycline accumulation and also potentiate activity of several antimicrobials against *A. baumannii* [171].

The use of EPIs can sensitize the activity of new antimicrobial agents. The compound, 3-(phenylsulfonyl)-2-pyrazinecarbonitrile, is an agent developed against resistant nosocomial pathogens. Its MIC against *A. baumannii* is 64 μg/ml but the addition of PAβN can decrease this MIC value by fourfold [172]. Another recently discovered natural antibiotic, kibdelomycin, exhibits a broad-spectrum activity with the MIC_{90} value of 0.125 against *A. baumannii* [173]. This agent appears to be a poor substrate of efflux pumps [173]. Finally, any agents that can permeabilize the OM of *A. baumannii* are expected to counter the action of the efflux pumps in increasing the drug access to their targets. In this regard, several plant extracts (including steroidal alkaloid conessine) appear to be able to sensitize the OM barrier to exert a synergistic effect on restoring activity of various antimicrobials against *A. baumannii* [174–177].

13.6 Concluding Remarks

The presence of the drug efflux pumps is one of the major mechanisms for MDR in *Acinetobacter* spp. and offers an advantage for these organisms to be successful pathogens in the presence of antimicrobial selection pressure. Resistant isolates with fitness advantages for enhanced virulence have been recently reported [178]. Hence, reversing *Acinetobacter* resistance by targeting resistance mechanisms, such as efflux pumps, provides a strong rationale for the development of novel antimicrobials that can either escape the action of efflux pumps or inhibit the efflux process. Certain drug combinations for possible anti-*Acinetobacter* therapy, such as minocycline-polymyxin, were able to increase intracellular drug concentrations and have

good *in vitro* activity [167], warranting further clinical investigations. Two engineered peptides of a phage lysin were shown to be active against multidrug-resistant *A. baumannii* by disrupting the cytoplasmic membrane [179]. The latter mode of action would be expected to somehow thwart drug efflux process. Moreover, the use of various antimicrobial agents such as broad-spectrum aminoglycosides, carbapenems, and fluoroquinolones as well as biocides in hospital settings constitutes an important risk factor for resistance emergence and dissemination, which includes selection of efflux pump-mediated acquired MDR, again highlighting the importance of prudent use of antimicrobial agents.

13.7 Addendum in Proof

A new RND-type pump, AbeD, was functionally characterized in *A. baumannii* [180]. Homologous to AcrD of *E. coli*, AbeD also lacks cognate membrane fusion protein and outer membrane protein. AbeD contributes to resistance to multiple antimicrobial drugs and oxidative agents and is also involved in virulence. Moreover, the AdeRS two-component regulatory system was recently reported to regulate genes involved in multidrug efflux, biofilm formation and virulence in a strain-specific manner [181]. Lastly, a new study has further described the contribution of AdeABC and AdeIJK to fitness and pathogenesis of *A. baumannnii* [182]. Overexpression of these pumps resulted in a significant reduction in fitness when measured by *in vitro* competition assays, while in mice infected intranasally, the mutant with AdeABC overproduction displayed an elevated virulence.

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Chapter 14
Antimicrobial Drug Efflux Pumps in *Pseudomonas aeruginosa*

Xian-Zhi Li and Patrick Plésiat

Abstract *Pseudomonas aeruginosa* is a major opportunistic pathogen that exhibits high-level intrinsic and acquired multiple antimicrobial resistance. In addition to the accumulation of individual drug-specific resistance mechanisms, such resistance phenotypes are attributed to the interplay between the polyspecific multidrug efflux pumps and the low outer membrane permeability, and this reflects evolution of *P. aeruginosa* in exposure to diverse hostile environments. A dozen drug efflux pumps, which belong to the resistance-nodulation-cell division (RND) superfamily, have been characterized in *P. aeruginosa*. Several RND pumps, as represented by MexAB-OprM and MexXY, play important roles in clinically relevant resistance, stress responses, and virulence. Regulation of these pumps is often under the control of local regulators (repressors or activators), global regulators, two-component regulatory systems, and modulators, whose mutations produce elevated antimicrobial resistance in many clinical isolates. This chapter provides an up-to-date overview of antimicrobial drug efflux pumps in *P. aeruginosa* with a focus on their substrates, regulation, inhibition, and clinical significance.

Keywords *Pseudomonas aeruginosa* • Multidrug resistance • Efflux pumps • Outer membrane • Efflux pump inhibitor • RND • MexAB-OprM • MexXY

14.1 Introduction

Pseudomonas aeruginosa is a non-fermentative Gram-negative rod thriving in aquatic environments impacted by human activities. It is a notorious cause of severe healthcare-associated infections in immunocompromised patients, as well as

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pro-inflammatory chronic lung colonization in cystic fibrosis patients [1, 2]. This species is also a pathogen for animals such as dogs, cats, and bovines [3], whose virulence holds on production of multiple cell wall-associated or secreted factors (e.g., alginate, pili, lipopolysaccharide, toxins, and proteases) [4–6] and formation of biofilms [7, 8]. Because of the relatively high-level intrinsic resistance of this species to a wide range of structurally diverse antimicrobials, chemotherapy of *P. aeruginosa* infections relies on a limited number of antipseudomonal antimicrobials [1]. However, clinically significant resistance to these agents is commonly developed by clinical strains via various adaptive or acquired mechanisms [9, 10]. In the USA, it was estimated that 13 % of severe healthcare-associated *P. aeruginosa* infections are due to multidrug-resistant strains [11]. A more recent US study showed non-susceptible rates of up to 25 % toward major antipseudomonal drugs (except colistin) among 1,743 *P. aeruginosa* isolates [12]. In addition to the accumulation of individual drug-specific resistance mechanisms, multidrug resistance (MDR) may be achieved through the synergistic interplay between the low permeability outer membrane (OM) barrier and a number of multidrug efflux pumps belonging to the resistance-nodulation-cell division (RND) superfamily of transporters [13, 14]. Initially discovered in the early 1990s with the predominant role of the MexAB-OprM efflux system in both intrinsic and acquired resistance [15–18], multidrug transporters of *P. aeruginosa* have been further characterized for their roles in drug resistance and other functions [14, 19–21]. This chapter provides an up-to-date overview of efflux pump-mediated drug resistance in *P. aeruginosa* with an emphasis on the substrates, regulation, inhibition, and clinical relevance of these export systems. The roles of MDR efflux pumps beyond drug resistance such as in biofilm formation, stress responses, and pathogenicity of *P. aeruginosa* are described elsewhere (see Chaps. 25, 26, and 27).

14.2 Historical Perspectives on *P. aeruginosa* Chromosomal MDR Efflux Pumps

During the early studies on *P. aeruginosa* in the 1960s, MDR phenotypes characterized by a simultaneous resistance to aminoglycosides, chloramphenicol, penicillins, sulfonamides, and tetracyclines were observed [22, 23]. While at that time resistant *P. aeruginosa* strains were known to produce drug-inactivating enzymes (e.g., β-lactamases and aminoglycoside-modifying enzymes) [23, 24], these drug-specific enzymatic mechanisms offered no satisfactory explanation of resistance to structurally distinct antimicrobials. Because of the barrier function of the OM, most Gram-negative bacteria are less susceptible than Gram-positive bacteria to amphiphilic or bulky drug molecules [25, 26]. Breakthrough studies also specifically demonstrated that *P. aeruginosa* is a species with exceptionally low OM permeability [27–31], which is due to its major porin OprF mainly existing as closed channels [32, 33]. (Of note, *P. aeruginosa* and *Escherichia coli* have similar low permeable asymmetric lipid bilayer domains [34, 35].) Indeed, an antimicrobial-hypersusceptible

P. aeruginosa mutant had OM lipopolysaccharide deficiency with easy drug access [29, 36, 37]. (This mutant was later found to be also deficient in drug efflux activity [16].) Moreover, drug uptake in *P. aeruginosa* may be further reduced in isolates resistant to aminoglycosides (e.g., streptomycin) or carbapenems (e.g., imipenem) by quantitative or qualitative changes in the lipopolysaccharide or porin (OprD) content of the OM [38, 39].

In the 1980s, the use of advanced broad-spectrum β-lactamase-stable β-lactams and fluoroquinolones was accompanied with increased isolation of multidrug-resistant isolates *in vivo* during drug administration [40–44]. These agents were also found to readily select MDR *in vitro* under laboratory conditions. While investigating the biochemical mechanisms of MDR or fluoroquinolone resistance, the OM protein profiles of *P. aeruginosa* isolates were assessed in numerous studies, which showed overproduction of ca. 50 kDa OM proteins that were associated with several gene loci named as *nalB*, *nfxB*, and *nfxC* [45–51]. One of these reports by Masuda and Ohya [51] designated the MDR-associated OM protein as OprM. Importantly, quinolone-resistant isolates also showed reduced uptake of ciprofloxacin [47] and active extrusion of ofloxacin [52]. Regardless of these studies, it became clear that the OM permeability barrier and periplasmic β-lactamase activity [53, 54] cannot fully explain MDR phenotypes (including β-lactam resistance in multidrug-resistant isolates/impermeability-type carbenicillin-resistant isolates) [55], which led to our initiative to investigate intrinsic and acquired MDR of *P. aeruginosa* [16, 17, 56]. In 1993, Poole et al. [15] reported the identification of the *mexAB-oprK* (i.e., *mexAB-oprM*) operon from *P. aeruginosa* which encodes a three-component efflux system involved in MDR. Together, these studies demonstrated a predominant role of drug efflux mechanism in intrinsic and acquired MDR (including β-lactam resistance) and expression of multiple drug efflux pumps in *P. aeruginosa* [15–18]. Subsequently, three MexAB-OprM homologues, MexCD-OprJ [57], MexEF-OprN [58], and MexXY (initially referred to as MexGH or AmrAB) [59–61], were also reported to be involved in *P. aeruginosa* MDR before the availability of the first whole genome annotated sequence for *P. aeruginosa* strain PAO1 [62]. All these Mex pumps belong to the RND superfamily of secondary active transporters [63], which typically require multiple components to form an energy-dependent functional extrusion complex across the entire cytoplasmic (inner) and outer membranes of Gram-negative bacteria [14].

14.3 Antimicrobial Drug Efflux Pumps and Their Clinical Significance in *P. aeruginosa*

P. aeruginosa genome sequences show the presence of a larger number of primary and secondary active transporters (TransportDB at http://www.membranetransport.org; accessed on February 15, 2016) [62, 64]. Both the widely studied strain PAO1 and more virulent strain UCBPP-PA14 contain 17 RND-type transporters. To date, 12 RND efflux pumps have been characterized for their substrate profiles as shown

in Table 14.1. The rest of the RND members include homologues to protein export components such as SecD and SecF, but their role in drug resistance, if any, remains unknown. These RND pumps are generally encoded by operons and are each composed of three components that include a cytoplasmic membrane transporter (e.g., MexB), a cytoplasmic membrane-associated periplasmic adaptor protein (membrane fusion protein) (e.g., MexA), and an OM channel protein (e.g., OprM) (see Chaps. 1 and 5). These multicomponent pumps reflect the complex structures of *P. aeruginosa* cell envelopes and provide the structural and functional basis to directly extrude substrates out of the cell. *In vitro* transport activity of an assembled MexAB-OprM in proteoliposomes was recently demonstrated to show energy-dependent substrate translocation in a system mimicking Gram-negative dual-membrane envelope architecture [129]. Additionally, members of other transporter superfamilies or families have been identified, including the members of the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, and the ATP-binding cassette (ABC) superfamily (Table 14.1). For instance, of five proteins of the SMR family present in *P. aeruginosa*, one pump with the highest identity to the EmrE homologue of *E. coli* was shown to contribute to intrinsic resistance to aminoglycosides and dyes in low ionic strength media [120]. An ABC exporter was recently noted to be regulated by the PhoPQ two-component regulatory system and to contribute to tetracycline resistance [125]. However, the roles of the non-RND pumps in drug resistance remain largely unclear. Hence, we limit the descriptions below to RND efflux pumps.

14.3.1 MexAB-OprM

This efflux system, which has a constitutive though growth phase-dependent expression in wild-type strains, significantly contributes to intrinsic drug resistance of *P. aeruginosa* [130]. Inactivation of any component of MexAB-OprM renders the wild-type strains extremely hypersusceptible with ≥8-fold reduction in the values of the minimal inhibitory concentrations (MICs) for diverse antimicrobial agents (e.g., carbenicillin MIC was reduced by ≥128-fold) [18, 65–67]. MexAB-OprM overproduction contributes to the acquired MDR and is observed in clinical isolates of several mutant types including *mexR* (*nalB*), *nalC*, and *nalD* mutants [18, 51, 131–144]. An investigation of 12 multidrug-resistant MexAB-OprM-overproducing strains showed an equivalent distribution of *mexR*, *nalC*, and *nalD* mutants [136], which was supported by similar findings from independent studies [138, 145]. MexAB-OprM overproducers have also been selected *in vitro* after exposure to β-lactams, quinolones, chloramphenicol, macrolides, tetracyclines, as well as biocides and organic solvents [16, 45, 51, 146–148]. *In vitro* studies on reference strains have shown that any mutations inactivating genes *mexR*, *nalC*, or *nalD* or impairing the activity of their respective products, MexR, ArmR, or NalD (see Sect. 14.4 below), can result in overexpression (≥threefold) of *mexAB-oprM* with

Table 14.1 Non-exhaustive substrate specificity of characterized drug efflux pumps in *P. aeruginosa*

Transporter family/efflux pump	Regulator/modulator	Substrates	References
RND			
MexAB-OprM	MexR, NalC, NalD, ArmR, RocS1/S2-A2, BrlR, MexT	AG, BL, CHIR, CHL, COL[a], CP, CRL, CV, EB, FQ, ID, QL, ML, NOV, OS, PDM, QS, SDS, SUL, TC, TLM, TMP, TRI, TTO	[18, 65–81]
MexXY-OprM, MexXY-OprA	MexZ, ArmZ, AmgRS, ParRS, SuhB	ACR, AG, BPR, EB, FEP, FQ, LBM, ML, TC, TGC	[59–61, 82–91]
MexCD-OprJ	NfxB, EsrC, AlgU, VqsM	AZI, BPR, CHIR, CHL, COL[a], CHX, FEB, FQ, NBTI, NCD, OS, PDM, QAC, QL, TC, TGC, TRI	[57, 67, 74, 76, 82, 92–99]
MexEF-OprN	MexT, MexS, MvaT, AmpR, BrlR, ParRS	CHIR, CHL, DA, FQ, HHQ, TET, TMP, TRI	[58, 67, 76, 100–103]
MexGHI-OpmD	SoxR	ACR, EB, FQ, TET, TPP, QS, Va^{2+}	[104–106]
MexJK-OprM	MexL	ERY, TET	[107, 108]
MexJK-OpmH	MexL	TRI	[107, 108]
MexMN-OprM		CHL, TML	[109]
MexPQ-OpmE		ML, QL, TPP	[109]
MexVW-OprM		ACR, CHL, EB, ERY, FQ, QL, TC	[110]
MuxABC-OpmB		ATM, COL[a], ML, NOV, TET	[111–113]
TriABC-OprM		?	[114]
TriABC-OpmH		TRI	[114]
CzcCBA	CzcRS, CopRS	Cd^{2+}, Zn^{2+}	[115–117]
MATE			
PmpM		ACR, BAC, EB, TPP	[118]
MFS			
Cml[b]		CHL, THL	[119]
TetA[b]	TetR	TC	[119]
SMR			
EmrE		AG, EB	[120]
QacE[b]		QAC	[121]
QacF[b]		QAC	[122]
ABC			
PA1874-1877		CIP[a], GEN[a], TOB[a]	[123]
PA2812 (CcmA)		CIP	[124]

(continued)

Table 14.1 (continued)

Transporter family/efflux pump	Regulator/modulator	Substrates	References
PA4456	PhoPQ	TET	[125]
PvdRT-OpmQ		PMC, SMC	[126–128]

ACR acriflavine, *AG* aminoglycosides, *ATM* aztreonam, *AZI* azithromycin, *BAC* benzalkonium chloride, *BL* β-Lactams (except carbapenems), *BPR* ceftobiprole, *BS* bile salts, *CHIR* CHIR-090 (LpxC inhibitor), *CHL* chloramphenicol, *CHX* chlorhexidine, *CIP* ciprofloxacin, *COL* colistin, *CP* carbapenems (except imipenem), *CRL* cerulenin, *CV* crystal violet, *DA* diamide, *EB* ethidium bromide, *ERY* erythromycin, *FEP* cefepime, *FQ* fluoroquinolones, *GEN* gentamicin, *HHQ* 4-hydroxy-2-heptylquinoline, *ID* indoles, *LBM* LBM415 (peptide deformylase inhibitor), *ML* macrolides, *NBTI* novel bacterial type II topoisomerase inhibitor NBTI5463, *NCD* N-chloramine derivative, *NOV* novobiocin, *OS* organic solvents, *PDM* pacidamycin, *PMC* pyoverdine-metal complexes, *QAC* Quaternary ammonium compounds, *QL* quinolones, *QS* quorum-sensing molecules/inhibitors, *SDS* sodium dodecyl sulfate, *SMC* siderophore-monobactam conjugate, *SUL* sulfonamides, *TC* tetracyclines, *TET* tetracycline, *TGC* tigecycline, *THL* thiamphenicol, *TLM* thiolactomycin, *TMP* trimethoprim, *TOB* tobramycin, *TPP* tetraphenylphosphonium, *TRI* triclosan, *TTO* tea tree oil

[a]Efflux pump contribution to resistance to these agents was observed in biofilms only

[b]These pumps are plasmid borne

concomitant increase in resistance (2- to 16-fold MIC increases) to the pump substrates compared to baseline levels, with *nalC* mutants being in general twofold more susceptible than the *nalB* and *nalD* mutants [68–70, 82, 132, 149–151].

Similar to *E. coli* AcrAB-TolC (see Chap. 9), the MexAB-OprM efflux system shows the broadest substrate profile among the known multidrug efflux pumps of *P. aeruginosa* (Table 14.1) [15–18, 51, 65–67, 71, 72, 120, 152–156]. Those antimicrobial agents that have been confirmed as substrates are comprised of β-lactams (including β-lactamase inhibitors), chloramphenicol, quinolones/fluoroquinolones, macrolides, novobiocin, sulfonamides, trimethoprim, tetracyclines, cerulenin, pacidamycin, and thiolactomycin [16–18, 65, 73, 74, 157]. Moreover, the substrates also extend to nonantibiotics, such as dyes (acridine orange, acriflavine, crystal violet, and ethidium bromide), detergents, triclosan, organic solvents, tea tree oils, and quorum-sensing molecules/inhibitors [66, 72, 73, 75, 120, 148, 155, 158]. MexAB-OprM is also involved in reduced aminoglycoside susceptibility in low ionic strength medium [120]. Intriguingly, antipseudomonal activity of imipenem, a carbapenem β-lactam, appears not a substrate of the MexAB-OprM pump since MexAB-OprM overexpression has no impact on imipenem MIC in an OprD-deficient mutant vs. wild-type OprD strain [159]. (The OprD channel protein functions as a specific pathway for active basic amino acid uptake and also permits rapid penetration of imipenem [39], thus potentially masking the role of an efflux pump.) Nevertheless, other carbapenems such as doripenem, panipenem, and meropenem are substrates for MexAB-OprM [51, 160–162].

Reminiscent of *E. coli* TolC protein, OprM serves as a universal OM efflux protein and functions in multiple efflux systems (Table 14.1) [163–165]. OprM contributes to MDR, not only in conjunction with MexAB [18] but also independent of

MexAB [166]. To date, OprM is known to work with other RND transporters (whose encoding operons often lack a linked gene for an OM component) including MexXY [59–61], MexJK [107], MexMN [109], MexVW [110], and TriABC [114], although other OM proteins can function with some of these transporters such as OpmH [114, 167] and OprA [168] (Table 14.1). Moreover, OprM can functionally replace the role of either OprJ of MexCD-OprJ or OprN of MexEF-OprN without affecting substrate profiles of these systems [163, 169].

Relevant to its clinical significance, MexAB-OprM when overproduced decreases the susceptibility of clinical isolates to antipseudomonal antimicrobials by a two- to eightfold in MIC values in comparison with the baseline levels in the absence of non-efflux resistance mechanisms such as enzymatic drug inactivation and drug target alterations [133, 142, 154, 170]. Based on the clinical susceptibility breakpoints from the Clinical and Laboratory Standards Institute (CLSI) [171], a maximal effect from the elevated MexAB-OprM efflux mechanism (eightfold MIC increase) would change strain categorization for a small number of antipseudomonal drugs such as aztreonam and ticarcillin (from drug susceptible [S] to intermediate [I] or resistant [R]) and meropenem, ciprofloxacin, and levofloxacin (from S to I). Another study showed that MexAB-OprM overproduction (via measuring *mexA* expression) was linked to median MIC values above the clinical resistance breakpoints (from the European Committee on Antimicrobial Susceptibility Testing [EUCAST]) for ciprofloxacin, cefepime, and meropenem [142]. Although further investigations are required to assess the therapeutic impact of MexAB-OprM *in vivo* [172], a recent study demonstrated that isolates with overproduction of either MexAB-OprM, MexCD-OprJ, or MexEF-OprN negatively affected antimicrobial efficacy in a *Galleria mellonella in vivo* infection model [173]. Higher drug dosages or antimicrobial-efflux pump inhibitor combinations are expected to be required in the treatment of infections associated with MexAB-OprM overproducers [173, 174]. Additionally, elevated MexAB-OprM expression also facilitates the emergence of other resistance mechanisms [147, 175]. Simultaneous expression of MexAB-OprM and other Mex pumps (e.g., MexXY or MexEF-OprN) have been reported, and this can produce additive effects in raising drug MIC levels as evident with fluoroquinolones [136, 141, 142, 176–180].

As a key mechanism responsible for high-level intrinsic resistance, the role of MexAB-OprM is also tightly linked to the low OM permeability barrier. Thus, the OM barrier and MexAB-OprM interplay to limit the access of antimicrobials to their cellular targets. The differential MIC values shown in Table 14.2 clearly demonstrate such synergistic interplay between the membrane barrier and the major efflux system in *P. aeruginosa* [181, 182]. Membrane disorganizers, such as chelating agent ethylenediaminetetraacetate (EDTA), potentiate antimicrobial activity of amphiphilic agents (which are expected to cross the OM through the lipidic domains), especially in the absence of MexAB-OprM (Table 14.2) [181]. This is also supported by an observation on the association of the deficiency in both MexAB-OprM and lipopolysaccharide with the hypersusceptible phenotype of strain Z61 [16, 37, 183]. Together, these data support a strategy to reverse antimicrobial resistance through the inhibition of drug efflux pumps and disruption of the OM barrier.

Table 14.2 Contributions of MexAB-OprM and the outer membrane permeability barrier to intrinsic and acquired antimicrobial resistance in *P. aeruginosa* (MICs, μg/ml)

Antimicrobial	Permeabilizer (EDTA[a] at 1 mM)	Wild-type strain (basal MexAB-OprM expression)	MexAB-OprM-deficient mutant[b]	MexAB-OprM-overproducing mutant
Carbenicillin	–	64	2	512
	+	4	0.0125	32
Piperacillin	–	4	0.5	16
Cefoperazone	–	8	0.5	32
	+	1	<0.125	2
Cefotaxime	–	16	1	64
Ceftazidime	–	2	0.5	8
Cefpirome	–	2	0.5	8
Ciprofloxacin	–	0.1	0.05	0.4
	+	0.1	0.025	0.4
Norfloxacin	–	0.5	0.5	2
	+	0.125	0.03	1
Chloramphenicol	–	128	2	512
	+	4	0.5	8
Erythromycin	–	512	64	1,024
	+	128	8	256
Novobiocin	–	512	64	>512
	+	64	2	256
Fosfomycin	–	524	64	1,024
Streptomycin	–	64	16	128
Tetracycline	–	8	1	64
	+	1	0.06	2

The data were from Refs. [181, 182]

[a]EDTA (disodium ethylenediaminetetraacetate at pH 8.0)

[b]Inactivation of MexAB-OprM in wild-type cells abolishes the function of at least two efflux systems, MexAB-OprM and MexXY-OprM

14.3.2 MexXY-OprM/MexXY-OprA

Encoded by a two-gene operon that lacks a gene for an OM protein, the MexXY system utilizes OprM to form a functional efflux pump in most *P. aeruginosa* strains [59, 60]. However, in the phylogenetically distinct isolate, PA7, and related strains, the *mexXY* genes are linked to a downstream gene encoding an OM protein dubbed OprA [168]. MexXY can function with either OprM or OprA in PA7 [168]. MexXY can also operate with another OM protein, OpmB, under still unclear conditions [83]. Inducibly expressed in *P. aeruginosa*, MexXY pump provides intrinsic resistance to aminoglycosides, a class of highly hydrophilic antimicrobial drugs, and to other agents that can, at subinhibitory levels, induce MexXY expression [59, 184]. Intriguingly, all of these inducers target ribosomes and this feature is related to

regulation of MexXY expression (see Sect. 14.4 below) [184, 185]. Inactivation of MeXY in wild-type strains leads to a four- to eightfold reduction in MIC values of aminoglycosides (e.g., amikacin, gentamicin, isepamicin, netilmicin, and tobramycin), erythromycin, and tetracycline [59]. Aminoglycoside resistance in so-called "impermeability-type" clinical isolates is caused by MexXY overproduction [61]. Amino acid residues important for aminoglycoside recognition in MexY have been identified recently [186]. Elevated MexXY expression confers a 2- to 16-fold higher resistance to its pump substrates. When overexpressed from plasmid vectors in *P. aeruginosa* or *E. coli*, MexXY also mediates resistance to fluoroquinolones [59, 60]. Interestingly, induction of MexXY expression by spectinomycin is correlated with an increased susceptibility to polymyxins (up to a fourfold MIC reduction), due to the reduced expression of polymyxin resistance-promoting lipopolysaccharide modification locus [187].

MexXY-overproducing mutants can be easily selected *in vitro* and *in vivo* in the presence of substrate antimicrobial agents including peptide deformylase inhibitors [82, 84–86, 142, 188] [85]. Indeed, MexXY overproducers are highly prevalent in clinical isolates from cystic fibrosis [61, 189–194] and non-cystic fibrosis patients worldwide [137, 140, 178, 179, 195–205]. Abundance of reactive oxygen species in the cystic fibrosis lung environment may offer an explanation for such high rates of resistance [206]. Consistent with this, prolonged exposure of *P. aeruginosa* to hydrogen peroxide was shown to facilitate the emergence of MexXY overproducers *in vitro* [207].

Based on the locations of mutations, MexXY-overproducing mutants can be divided into three types: *agrZ*, *agrW1*, and *agrW2* mutants. With *agrZ* mutants, various mutations occur in gene *mexZ* that encodes a repressor of MexXY [84, 138, 168, 188–191, 193, 197, 200, 205, 208, 209]. With *agrW1* mutants, mutations affect ribosomal proteins such as L1 [61], L25 [210], L21, and L27 [211] or components of the methionyl-tRNA^{fmet} formylation bypass [85]. Actually, lines of evidence suggest that whatever its origin (e.g., mutations, ribosome targeting drugs) impairment of protein synthesis is a stimulus for MexXY expression. For *agrW2* mutants, mutational activation of the two-component regulatory system ParRS results in constitutive expression of MexXY [86, 203]. The presence of these three types of mutants in clinical isolates was confirmed among non-cystic fibrosis isolates that exhibited a moderate, nonenzymatic resistance to aminoglycosides [205]. However, the *agrZ* type predominates over the two others in cystic fibrosis isolates [189–191].

Isolates with overexpression of MexXY (via measuring *mexX* expression) also showed median MIC values higher than the EUCAST resistance breakpoints for amikacin, ciprofloxacin, cefepime, and meropenem [142]. However, only a few studies have assessed the potential role of MexXY in clinical therapeutic outcomes. In a rabbit experimental model of pneumonia treated with intravenous administration of tobramycin, a modest influence from MexXY overexpression on animal survival and post-treatment bacterial loads was observed [212]. Elevated efflux activity due to *mexXY* derepression is likely one of the multiple means *P. aeruginosa* can accumulate gradually to increase its resistance toward potent antimicrobials [205, 210]. As mentioned earlier, simultaneous overexpression of multiple efflux pumps

(e.g., MexAB-OprM, MexXY, and MexEF-OprN) in conjunction with other resistance mechanisms is common in hospital strains [16, 136, 140, 178, 179, 213].

14.3.3 MexCD-OprJ

This efflux system is apparently quiescent in wild-type strains under normal laboratory growth conditions, and thus, chromosomal disruption of the *mexCD-oprJ* operon does not alter antimicrobial susceptibility of wild-type cells [57, 158]. MexCD-OprJ expression is inducible by various membrane-damaging nonantibiotic toxicants, including acriflavine, ethidium bromide, rhodamine 6G, chlorhexidine, and tetraphenylphosphonium, which are also the substrates of MexCD-OprJ [92, 93]. Exposure of *P. aeruginosa* to waste water was found to lead to MexCD-OprJ overexpression [214]. Mutation-mediated overexpression of this operon in *nfxB*-type mutants significantly contributes to resistance to fourth-generation cephalosporins (cefepime and cefpirome), quinolones/fluoroquinolones, chloramphenicol, cerulenin, pacidamycin, tetracycline, and novel inhibitors of lipid A synthesis such as CHIR-90 [57, 67, 74, 76, 94, 215]. Similar to MexAB-OprM, the substrates for MexCD-OprJ also include other cytotoxic compounds, such as acriflavine, ethidium bromide, quaternary ammonium compounds, rhodamine 6G, triclosan, and organic solvents [66, 92, 93, 95]. Based on the variability in drug resistance levels, MexCD-OprJ-overproducing *nfxB* mutants can be grouped into two types [215]. Type A mutants are resistant to erythromycin, ofloxacin, and zwitterionic cephems (cefclidin, cefozopran, cefoselis, and cefpirome), while type B mutants are resistant not only to these aforementioned agents but also to chloramphenicol and tetracycline [215]. Type B mutants are, however, four- to eightfold more susceptible to many conventional penicillins (e.g., carbenicillin), atypical β-lactams (aztreonam and moxalactam), carbapenems (biapenem and imipenem), and aminoglycosides (gentamicin and kanamycin) than the wild-type PAO1 strain [215]. This hypersusceptibility to conventional β-lactams and aminoglycosides [215, 216] is possibly attributable to the downregulation of MexAB-OprM [183, 216], MexXY [94], and the AmpC β-lactamase [217] in the MexCD-OprJ-overproducing mutants, although other mechanism(s) may exist [218].

MexCD-OprJ overproducers are associated with fluoroquinolone resistance, although fluoroquinolone-resistant isolates may also overexpress other efflux pumps (e.g., MexAB-OprM, MexXY, or MexEF-OprN) and/or carry quinolone-target mutations [94, 178, 219]. Indeed, overexpression of MexCD-OprJ, as with that of MexAB-OprM or MexXY, was reported to occur in a large proportion of fluoroquinolone- and/or carbapenem-resistant clinical isolates [220, 221]. (However, this MexCD-OprJ overexpression issue remains controversial and requires further investigations since *nfxB*-type MexCD-OprJ-overexpressing mutants are strongly deficient in fitness. The reverse-transcription-qPCR thresholds used to arbitrarily define *mexCD-oprJ* overexpression may have a strong impact on the mutant rates found in the clinical setting such as non-cystic fibrosis patients.) An *in vitro* study

suggested newer fluoroquinolone agents were in favor of the selection of MexCD-OprJ producers [147]. Supporting this notion, elevated MexCD-OprJ expression is linked to levofloxacin resistance in *P. aeruginosa* isolates from urinary tract infections [222]. A patient treated by two substrates of MexCD-OprJ, ciprofloxacin and cefepime, produced over the treatment period *nfxB* mutants which had a change of bacteria from S to I or R as regards their susceptibility to fluoroquinolones based on the CLSI resistance breakpoints [223]. *nfxB* mutations causing MexCD-OprJ overproduction can be the first-step mutations in addition to further mutations in other resistance determinants as evident by a selection with a novel bacterial type II topoisomerase inhibitors [96, 224]. Here, it is worth mentioning that the genotypic alterations in MexCD-OprJ overproducers may not correlate with the phenotype [213, 225], likely attributed at least partly to global changes in the physiology and metabolism caused by *nfxB* mutations [111, 226]. MexCD-OprJ overexpression produces an increased susceptibility to complement-mediated killing and consequently results in reduced virulence [227]. In this regard, a recent study showed that quaternary compounds were substrates of MexCD-OprJ but were unable to select resistant mutants including MexCD-OprJ-overproducing mutants with these biocides [95]. Nevertheless, the negative resistance selection results warrant further investigation. Another study revealed that *P. aeruginosa* adapted to 2-phenoxyethanol displayed reduced susceptibility to different biocides but increased susceptibility to several antipseudomonal antibiotics including amikacin, tobramycin, ceftazidime, and ciprofloxacin [228]. Another biocide, triclosan, can select MexCD-OprJ overproducers [229, 230]. High-level resistance to this biocide was speculated to play a role in antibiotic resistance in an epidemic isolate [231].

14.3.4 MexEF-OprN

This efflux system is also not well expressed in wild-type cells of *P. aeruginosa*, and thus its inactivation leads to no or little change in antimicrobial susceptibility [58, 183]. MexEF-OprN is highly expressed in *nfxC* mutants to confer an increased resistance to chloramphenicol, quinolones/fluoroquinolones, tetracycline, and trimethoprim [58, 169, 178]. Decrease in susceptibility to carbapenems, a phenotype characteristic of some *nfxC* mutants [58], is partly attributable to the downregulation of OprD expression [232, 233]. *nfxC* mutants are readily selected by chloramphenicol and fluoroquinolones, but not by carbapenems [147, 234, 235]. They have been observed in clinical isolates from cystic fibrosis and other patients [221, 225], but their prevalence varies from one study to another. While many studies apparently suggest low frequencies or even no detection of *nfxC* mutants among clinical isolates [178, 195, 199, 204, 233], recent studies revealed more prevalence of MexEF-OprF overproducers [221, 236]; for example, about 30 % of 62 isolates (mostly obtained from intensive care unit patients and with reduced carbapenem susceptibility) had an increased production of MexEF-OprN (from >4- to 19-fold in *mexF* mRNA transcripts in comparing with a wild-type reference isolate) [236].

MexEF-OprN overproducers can likely also be among the first-step mutants, which may further acquire higher resistance [178]. Unexpectedly, tobramycin-hypersusceptible mutants (eightfold MIC reduction) were associated with insertional inactivation of *mexF* that generated the aberrant hybrid MexF-alkaline phosphatase proteins. These hybrid proteins were interpreted to cause cytoplasmic membrane stress for gain-of-function changes with increased aminoglycoside susceptibility [87].

14.3.5 MexJK-OprM/OpmH

This efflux system is expressed at low levels in wild-type cells [107, 112]. Despite the lack of a gene for OM protein in its encoding operon, MexJK requires an OM channel protein for drug efflux. While OprM is involved in the extrusion by MexJK of ciprofloxacin, erythromycin, and tetracycline, MexJK is dependent on another OM protein, OpmH, for providing resistance to triclosan [107, 167]. Clinical significance of this pump remains unknown. Nevertheless, MexJK overproduction was observed in two MexXY-hyperexpressing cefepime-resistant isolates [197] as well as in a MexXY-/MexVW-overproducing isolate [179].

14.3.6 MexGHI-OpmD

Encoded by a four-gene operon, PA4205-PA4208 genes [62], MexGHI-OpmD is operative in wild-type cells and mediates intrinsic resistance to vanadium [237]. While MexH and MexI are, respectively, the cytoplasmic membrane exporter and the accessory membrane fusion protein, MexG is a protein with unknown function. This system is involved in PQS (pseudomonas quinolone signal) homeostasis and is associated with quorum sensing in *P. aeruginosa* [112]. Its inactivation results in reduced production of several virulence factors, thus linking antimicrobial susceptibility and pathogenicity [104, 237]. Intriguingly, vanadium hypersusceptibility of the *mexGHI-opmD* null mutants is accompanied by increased resistance to netilmicin, tetracycline, and ticarcillin-clavulanic acid [237], likely due to the compensating overexpression of other MDR pumps [183].

14.3.7 Other RND Efflux Pumps

Several additional RND efflux systems, when expressed from vectors, were able to confer resistance in *P. aeruginosa* or *E. coli* host deficient in major RND pumps (Table 14.1): MexMN-OprM for resistance to fluoroquinolones and macrolides [109]; MexPQ-OpmE for resistance to amphenicols [109]; MexVW-OprM for

resistance to chloramphenicol, fluoroquinolones, macrolides, and tetracycline [110]; MuxABC-OpmB for resistance to aztreonam, macrolides, novobiocin, and tetracyclines [112, 113]; and TriABC-OpmH for triclosan resistance [114]. While the MuxABC-OpmB system possesses two RND components, MuxBC [114], TriABC-OpmH requires two periplasmic accessory membrane fusion proteins, TriA and TriB [114], which play different roles in the assembly and function of TriABC pump [238]. MuxABC-OpmB is expressed in wild-type strains, and interestingly, its inactivation results in elevated β-lactamase production with increased β-lactam resistance [112]. MuxABC-OpmB is one of the RND efflux systems that affect the development of colistin-tolerant subpopulations in *P. aeruginosa* biofilms [111]. Co-overexpression of MexVW and MexXY was also reported [179]. Finally, one RND efflux system, CzcCBA (also called CzrCBA), contributes to resistance to cadmium, cobalt, and zinc salts [115, 116]. Phenotypically, CzcCBA-mediated heavy metal resistance is also linked to imipenem resistance as a result of the down-regulated OprD expression and the elevated expression of quorum-sensing autoinducer molecules, due to a shared two-component regulatory system, CzcRS [117, 239]. We have observed one imipenem-insusceptible isolate with overexpressed CzcCBA and reduced OprD production from an intensive care unit [203].

14.4 Regulation of *P. aeruginosa* RND Efflux Pumps

Regulation of *P. aeruginosa* RND efflux pumps has been studied intensively over the last two decades, which shows the complexity of RND pump regulatory network with involvement of various transcriptional regulators and modulators at multiple levels [14]. Changes in natural or host environments of *P. aeruginosa* such as antimicrobial exposures and nutrient, oxidative, and nitrosative stresses can affect the expression of drug efflux pumps and subsequently contribute to phenotypic adaptations such as the development of MDR [20, 240]. The expressional status of one drug efflux pump may also be linked to the production of other pumps through complex regulatory networks [94, 183]. Together, different regulatory pathways interconnect antimicrobial susceptibility, stress responses, pathogenicity, and even biofilm formation.

14.4.1 MexAB-OprM

Although constitutively expressed, the *mexAB-oprM* operon is subject to a complex and finely tuned regulation. Multiple gene products MexR, NalD, ArmR (via NalC), and a two-component regulatory system, RocS1/S2-RocA2, are involved in the regulation of *mexAB-oprM* expression (Fig. 14.1).

MexR, a MarR-family repressor, is encoded by a self-regulated gene (*mexR*/*nalB*) that is divergently transcribed upstream of the *mexAB-oprM* operon [68]. Binding of

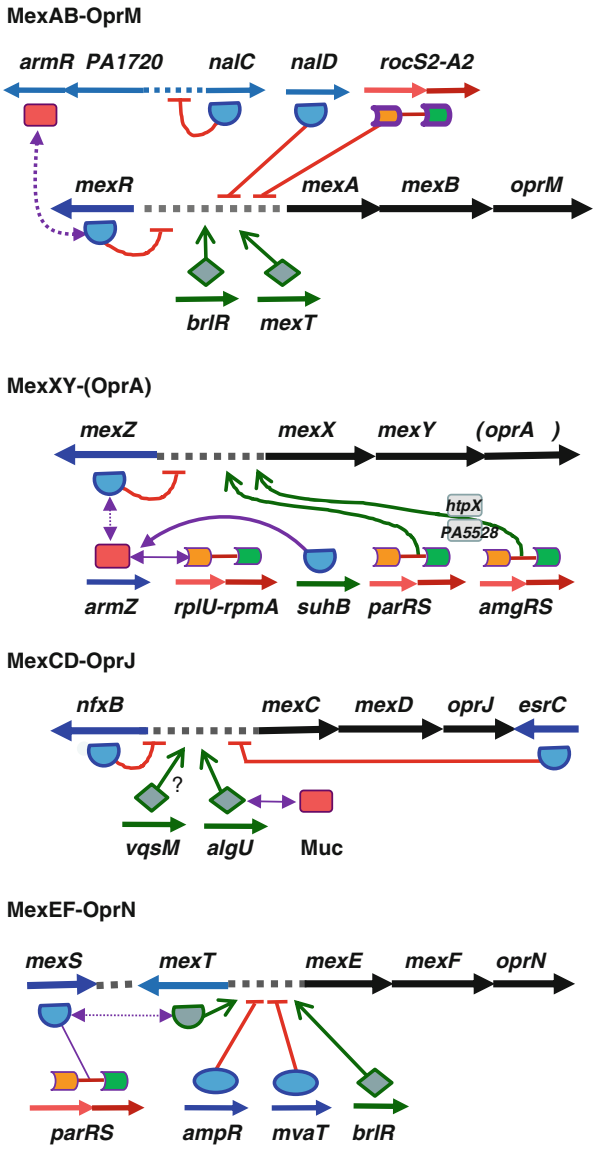

Fig. 14.1 Regulation of the expression of four RND multidrug efflux systems of *P. aeruginosa*. These pumps are controlled by a local regulator (mostly by a repressor [MexR, NfxB, EsrC, or MexZ] or by an activator [MexT]). Positive and negative regulations of the relevant gene transcriptions are, respectively, denoted by red and green lines. Local repressors are controlled by anti-repressor proteins (ArmR and ArmZ) and can also bind to ligands (e.g., antimicrobial agents) or be induced under various conditions (nitrosative, oxidative, or cell envelope stress). Mutational changes can also lead to inactivation of these regulators. See text for details

MexR as a dimer to the intergenic divergent overlapping promoters of *mexR* and *mexAB-oprM* produces a balanced transcription of both *mexR* and *mexAB-oprM*, which provides *P. aeruginosa* with a protective baseline level of wide-spectrum efflux activity [149, 241]. Mutations in *mexR* (*nalB* mutants) are typically associated with MexAB-OprM overexpression [68, 135, 144, 148]. The crystal structure of MexR suggests an effector-induced conformational change for inhibiting DNA binding [242]. Based on *in vitro* data that MexR dimerization through the formation of intermonomer disulfide bonds between two redox-active cysteines prevents MexR from interacting with its cognate DNA binding sites, redox modulation of MexR was proposed to occur *in vivo* under stressful conditions such as the presence of oxidative agents (e.g., hydrogen peroxide) or antibiotics (meropenem and nalidixic acid) [243, 244]. However, several agents including hydrogen peroxide, colistin, and tobramycin apparently do not significantly induce *mexAB-oprM* transcription [245–248].

The expression of *mexAB-oprM* is positively modulated by ArmR, a 53-residue peptide, whose encoding gene is located in a two-gene operon, PA3720-*armR* [69]. By an allosteric polypeptide-protein interaction of high affinity, ArmR function as an anti-repressor to sequester MexR, consequently reducing the MexR repressor activity [249, 250]. Unless mutations inactivate gene *nalC* (*PA3721*) which encodes a TetR-family repressor to strongly downregulate the adjacent *PA3720-armR* operon, basal amounts of ArmR do not affect MexAB-OprM production in wild-type cells [69]. In fact, *nalC* mutants only show modestly elevated expression of *mexAB-oprM*, and disruption of ArmR in these *nalC* mutants reduces MexAB-OprM expression to wild-type levels and compromises MDR [69]. By reversible, non-covalent binding to NalC, various chlorinated phenols including pentachlorophenol at relatively high levels can induce the expression of operons *PA3720-armR* and *mexAB-oprM* [77, 251]. Although pentachlorophenol affects expression of *armR*, MexAB-OprM induction by pentachlorophenol can also be ArmR-independent, yet MexR-dependent [252]. This suggests that *in vivo* generated catabolite effectors may mimic more specific phenolic antimicrobial compounds than pentachlorophenol that *P. aeruginosa* encounters in its natural habitat [252].

NalD, a TetR-family repressor, is another regulator of *mexAB-oprM* that binds to the proximal promoter upstream of the efflux operon [70, 78], resulting in *mexAB-oprM* being expressed essentially from its distal promoter [78]. A recent study demonstrated direct binding of novobiocin to NalD to result in dissociation of NalD from the promoter with subsequent derepression of *mexAB-oprM* expression [253]. The combinational mutations in *mexR*, *nalC*, and *nalD* have been observed in clinical isolates including epidemic strains which are MexAB-OprM overproducers [136, 138, 145, 254].

The *mexAB-oprM* expression is also growth-phase regulated and reaches a maximum level at the onset of the stationary phase, independently of MexR and of LasR, a transcriptional regulator controlling the production of quorum-sensing cell-to-cell signal *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) [130, 255, 256]. *P. aeruginosa* has several intertwined quorum-sensing systems, such as Las, Pqs, and Rhl, that control virulence gene expression [257, 258]. The Rhl

quorum-sensing signal *N*-butanoyl-L-homoserine lactone (C_4-HSL) can induce *mexAB-oprM* expression [152, 259], possibly via its role in the growth-phase-dependent regulation of MexAB-OprM [72, 79] with MexR being not required in this control [260]. This regulation of MexAB-OprM can be canceled by MexT, the activator of *mexEF-oprN* operon in *nfxC* mutants [79]. Additionally, AmpR, a LysR-family global transcriptional regulator implicated in AmpC β-lactamase expression and other genes of the AmpR regulon, was reported to potentially derepress the MexAB-OprM expression by negatively modulating the *mexR* expression [261]. Several 14- and 15-membered macrolides including azithromycin at subinhibitory levels can repress the cell density-dependent expression of MexAB-OprM in a MexR-dependent manner through yet-unidentified mechanism [262].

MexAB-OprM expression in biofilm cells is further regulated by additional mechanisms. MexAB-OprM pump contributes to tolerance to colistin in a metabolically active subpopulation of biofilm cells [263]. The histidine kinase sensors RocS1 and RocS2 act through their cognate response regulator RocA2 to repress *mexAB-oprM* expression in biofilms [80]. BrlR, a biofilm-specific MerR-family regulator, functions as an activator and is required to sustain expression of *mexAB-oprM* (and *mexEF-oprN*) during an early stage of biofilm development through its binding to the promoter regions of the two operons [81, 264]. Intriguingly, BrlR is responsive to the secondary messenger, cyclic dinucleotide c-di-GMP, which is required for BrlR production and function [265]. During the early developmental stage of biofilms, the two-component hybrid histidine kinase SagS is also produced, and it positively affects the production of c-di-GMP and BrlR, which contribute to increased expression of MexAB-OprM and MexEF-OprN and high-level biofilm-specific resistance to antimicrobial agents [266, 267]. Therefore, *mexAB-oprM* expression in biofilms is likely affected by at least two distinct signal transducing systems (i.e., RocS1/RocS2-RocA2 and SagS-BrlR). However, contribution of the MexAB-OprM pump to antimicrobial resistance in *P. aeruginosa* biofilms remains controversial and might depend upon the experimental conditions used or stage of biofilm development [268].

14.4.2 MexXY

The MexXY efflux system is also subject to a multi-level regulation (Fig. 14.1). MexZ is the local transcriptional repressor of the *mexXY* or *mexXY-oprA* operon [59, 168]. Binding of dimerized MexZ to the overlapping promoters of mexXY and *mexZ* allows very low baseline production of MexXY [208, 269, 270]. Unlike many other TetR-family regulators [271], MexZ's DNA binding is not relieved by antimicrobials through a direct ligand-regulator interaction but seemingly via indirect protein-protein sequestration, which is dependent on the anti-repressor of *mexZ*, ArmZ (PA5471) [270, 272, 273]. Induction of *mexXY* expression occurs through an ArmZ-dependent manner [88, 272] in response to the exposure of *P. aeruginosa* to a number of ribosome-targeting antimicrobials (such as aminoglycosides,

chloramphenicol, macrolides, and tetracyclines) [185] or oxidative stress conditions [207]. Expression of *armZ* itself is induced by ribosome-targeting agents through a transcriptional attenuation, ribosome stalling mechanism that involves a short 13-amino acid leader peptide, PA5471.1 [88]. Ribosome stalling at this leader peptide mRNA yields *armZ* transcription to subsequently upregulate *mexXY* expression [88]. In this regard, another protein, SuhB, was found to interact with the ribosome [89]. The *suhB* gene was first identified as an entragenic suppressor of a component of the type II secretion system in *E. coli* [274] and was also revealed to be involved as a regulator of multiple virulence genes implying types III and VI secretion systems and biofilm formation in *P. aeruginosa* [275]. A *suhB* mutant exhibited higher level of *PA5471.1* mRNA with elevated *mexXY* expression, which was consistent with the reduced susceptibility of the *suhB* mutant to aminoglycosides [89]. Additionally, in pan-aminoglycoside-resistant mutants, reduced expression of the *rplU-rpmA* operon is attributable to mutations in the promoter region of the operon, which encodes ribosomal proteins L21 and L27 [211]. This change is also linked to an ArmZ-dependent MexXY overproduction. Hence, the ribosome-perturbing mutations act in a way reminiscent of *mexXY* induction by ribosome-targeting antimicrobials [89, 211]. However, *mexXY* expression still remains inducible to some extent in *mexZ* and *mexZ-armZ* null mutants [185, 273], suggesting the presence of additional contributors in induction of *mexXY*.

Regulation of MexXY is also mediated by the two-component regulatory system ParRS. Either mutations or exposure to subinhibitory levels of polycationic compounds such as polymyxins can activate ParRS [276], which upregulates both *mexXY* and lipopolysaccharide modification operon *arnBCADTEF-ugd* and downregulates the *oprD* expression, yielding an MDR phenotype by activation of three distinct mechanisms (efflux, lipopolysaccharide modification, and OprD reduction) [86, 205]. Analysis of tobramycin-hypersusceptible mutants revealed mutations in more than a dozen genes that included *mexXY*, *oprM*, and the two-component regulatory system genes *amgRS* with *amgRS* mutants showing 8- to 16-fold reduction of tobramycin MIC values [87]. AmgRS was shown to be required for tobramycin induction of several genes, including three genes, *htpX*, *PA5528*, and *yccA* (which encode, respectively, a cytoplasmic membrane-associated protease, a modulator of the FtsH protease, and a protease-associated factor) involved in positively stimulating *mexXY* expression [87, 90, 91]. A recent study showed AmgRS-dependent potentiation of the activity of 4,5-linked aminoglycosides (such as neomycin, paromomycin, and ribostamycin) by rifampicin [277]. The latter targets AmgRS and repressed expression of AmgRS-dependent genes including *htpX*, *yccA*, and *mexXY*. Rifampicin also potentiated the activity of two 4,6-linked aminoglycosides such as amikacin and gentamicin in two clinical isolates [277].

Inactivation of either gene *PA2572* (for a non-canonical response regulator) or *PA2573* (for a probable methyl-accepting chemotaxis protein) strongly increased *mexXY* expression by >10-fold when measuring *mexX* or *mexY* transcripts, and this explains the reduced susceptibility of these mutants to two aminoglycosides, amikacin and tobramycin (10- to 40-fold MIC increase) [278]. However, the detailed cascade affecting *mexXY* expression remains unknown.

14.4.3 MexCD-OprJ

The MexCD-OprJ pump is negatively controlled by NfxB and EsrC repressors, whose encoded genes are located, respectively, upstream and downstream of the *mexCD-oprJ* operon (Fig. 14.1) [57, 97, 279]. NfxB acts as a multimer (dimer of dimers) with C-termini required for multimerization and N-termini in DNA binding [97, 280]. *nfxB* mutations can occur over the entire *nfxB* gene with the deletion-generated frameshifts frequently observed in clinical strains [94, 281]. Inactivation of DNA oxidative repair system also increases frequencies of *nfxB* mutations [282]. Intriguingly, VqsM, an AraC-family master transcriptional regulator involved in the regulation of virulence factors and quorum-sensing compounds, can bind to the promoter of *nfxB* to likely increase *nfxB* expression, although *vqsM* mutants derived from wild-type PAO1 strain show higher resistance to kanamycin and tetracycline (16- and 32-fold MIC increase, respectively) with no changes in susceptibility to ceftazidime, ciprofloxacin, polymyxin B, and tobramycin [98]. Given the low-level expression of *mexCD-oprJ* in wild-type cells, it would be interesting to know whether VqsM influences MexCD-OprJ production in *nfxB* mutants.

Another regulator of MexCD-OprJ, EsrC, is functionally dependent on NfxB for repressing *mexCD-oprJ* expression when cells are under envelope stress [279]. Expression of *mexCD-oprJ* is induced by a number of biocides (e.g., benzalkonium chloride and chlorhexidine), dyes (ethidium bromide), and other membrane-damaging agents (detergents, solvents, polymyxin B, and antimicrobial peptides including human host defense peptide LL-37) [92, 93, 283]. Exposure to chlorhexidine diacetate produces a significant transcriptomic response [284]. These membrane-damaging agents apparently generate membrane lipid derivatives to stimulate the membrane-associated Muc proteins and to eventually activate the stress response sigma factor, AlgU, for upregulating MexCD-OprJ expression. *nfxB* mutation-related *mexCD-oprJ* hyperexpression is also dependent on AlgU [93]. Finally, disruption of the aforementioned gene *PA2572* that codes for a putative response regulator was also found to modestly increase *mexCD-oprJ* activity (a fourfold increase in *mexC* transcripts) [278].

14.4.4 MexEF-OprN

Expression of MexEF-OprN is also controlled by several regulators (Fig. 14.1). MexT, a LysR-family global regulator, controls expression of multiple genes in *nfxC* mutants including *mexEF-oprN*, *oprD*, and genes for virulence factors [232, 285–288]. Inactive and active forms of MexT exist, respectively, in wild-type strains and nfxC mutants. One gene of the MexT regulon, *mexS* (encoding an oxidoreductase of unknown function [285]), because of its alteration in *nfxC* mutants, promotes *mexEF-oprN* expression with concomitant development of MDR [289]. This induction occurs as a result of MexS-MexT interplays through presumed

intracellular accumulation of toxic metabolites recognized by MexT as co-inducers [289]. Indeed, exposure of *P. aeruginosa* to nitrosative stressors such as S-nitrosoglutathione activates *mexEF-oprN* transcription via MexT [290]. Disulfide stress response and the type III secretion system are affected by MexS-MexT interaction [100, 291], thus providing another example for the linked regulation among drug efflux pumps, redox stress response, and virulence factor production. But, MexS-independent *mexEF-oprN* overexpression has also been observed [292]. Similar upregulation of *mexS* and *mexEF-oprN* was noted when *P. aeruginosa* was exposed to human airway epithelial cells releasing unknown efflux-inducing signals [293]. Expression of *mexEF-oprN* was also found to be abolished by the downregulation of MexS through mutations in the ParRS two-component regulatory system [101]. The latter is also involved in the regulation of MexXY, OprD, and lipopolysaccharide modifications [86, 276]. A recent study showed single amino acid substitutions in MexS in a good proportion of clinical *nfxC* mutants, which had an association with moderate effects on drug resistance and virulence factor production, supporting the notion of *in vivo* selection of partially defective *mexS* mutants retaining some degree of pathogenicity [294]. Additionally, the global regulator MvaT influences expression of hundreds of genes including *mexEF-oprN* and others involved in biofilm formation, quorum sensing, and virulence [295–297]. Independent of *mexT* or *mexS*, inactivation of *mvaT* results in MexEF-OprN hyperexpression and marginal OprD reduction (associated with increased susceptibility to imipenem) [102], suggesting the complexity in MexEF-OprN expression. Consistently, despite the observed mutations in *mexS*, *mexT*, and *mvaT* in MexEF-OprN-overproducing clinical isolates [178], a good proportion of *nfxC* mutants do not show any mutations in these genes [294], revealing involvement of additional regulatory mechanisms. In this regard, AmpC β-lactamase regulator AmpR affects expression of >500 genes, and its inactivation increases MexEF-OprN production with an MDR phenotype [261]. The abovementioned BrlR also positively affects MexEF-OprN expression in biofilm cells [81]. The reduced virulence of *nfxC* mutants has been attributed to MexEF-OprN-dependent extrusion of 4-hydroxy-2-heptylquinoline [103] and/or kynurenine [298, 299], to two precursors of quorum-sensing molecule PQS, and to MexT-dependent downregulation of type III secretion system and pyocyanin production [286, 291].

14.4.5 Other RND Pumps

The *mexGHI-ompD* operon is positively regulated by SoxR transcriptional regulator as part of an oxidative stress response to the presence of methyl viologen [105], the phenazine pyocyanin (a heterocyclic, redox-active agent) [106], and oxidative compounds such as 7-hydroxyindole involved in anti-virulence [300]. A human host defense peptide, LL-37, is also able to induce expression of MexGHI-OmpD [283]. Expression of MexJK is negatively regulated by MexL repressor, which is encoded by a gene transcribed divergently from the adjacent *mexJK* operon [107]. CzcCBA

metal exporter is upregulated by at least two two-component regulatory systems, CzcRS (CzrRS) [115] and CopRS [117]. Subinhibitory concentrations of zinc or copper salts can induce expression of *czcCBA*, *czcRS*, and *copRS*. CzcRS and CopRS are also involved in the downregulation of OprD expression with concomitant resistance to carbapenems [116]. CzcR further affects various genes involved in virulence including gene expression of quorum-sensing 3-oxo-C12-HSL and C4-HSL autoinducers [239]. A *mvaT* mutant also shows a decreased expression of the two-component regulator gene (PA2570) located immediately downstream of the *czcABC* efflux operon [296].

14.5 Overcoming *P. aeruginosa* Drug Efflux Activities

The characterization of RND pumps shows the scientific challenge of finding antimicrobial drugs that can bypass the efflux mechanisms (see Chap. 28). Numerous newer antimicrobial agents are substrates of RND pumps, such as ceftobiprole, doripenem, and tigecycline [14, 82, 301, 302]. In fact, recent success in clinical use of a new β-lactam-β-lactamase inhibitor combination product, ceftazidime-avibactam, has faced an unexpected challenge from archived *P. aeruginosa* isolates, i.e., drug efflux and membrane permeability barrier to reduce activity of this product [303]. To combat the efflux impact, rational drug design can be exploited to minimize or to avoid efflux. This approach is becoming increasingly feasible due to the in-depth structural and biochemical understanding of RND efflux pumps [129, 165, 304–309].

The following examples show that despite the multi-specificity and multiplicity of RND transporters in *P. aeruginosa*, novel antimicrobials can be developed to escape efflux mechanism. The activity of a novel parenteral aminopyrazolium cephalosporin, FR264205, is unlikely affected by the expression of MexAB-OprM, MexCD-OprJ, MexEF-OprN, or MexXY [310, 311]. A methylcarbapenem, tomopenem, displays broad-spectrum activity against Gram-positive and Gram-negative pathogens including *P. aeruginosa*, and this is at most minimally impacted by overexpression of Mex pumps [312, 313]. The latter may, however, be partly attributable to the high affinity of tomopenem to the major lethal targets, penicillin-binding proteins 2 and 3 [314]. Antimicrobial polypeptides generally do not appear to be impacted by efflux systems including RND pumps [315]. Polymyxins are often active against multidrug-resistant *P. aeruginosa* despite reports suggesting that MexAB-OprM, MexCD-OprJ, and MuxABC-OpmB pumps contribute to nonspecific adaptive resistance to polymyxins in biofilms [111, 263]. In comparing with several fluoroquinolones such as ciprofloxacin, activity of clinafloxacin is less compromised by Mex pumps [71]. Overall, multiple factors such as efflux pump effect, affinity to the drug targets, and membrane permeation contribute to collectively the antipseudomonal activity of drug molecules.

The role of clinically relevant efflux pumps also highlights a needed strategy to look for agents that can function as efflux pump inhibitors either to restore susceptibility of multidrug-resistant strains or to prevent the emergence of

mutation-driven resistance mechanisms, when combined with conventional antibiotics. Since the discovery of RND pumps, efforts have also been undertaken to identify pump inhibitors and *P. aeruginosa* RND pumps have particularly been a major target (see Chaps. 29 and 30) [240, 316]. Phenylalanine-arginine β-naphthylamide is one of the earliest efflux pump inhibitors identified and is accepted as a typical efflux pump inhibitor of RND pumps [316, 317]. It potentiates *in vitro* activity of a number of antipseudomonal agents against multidrug-resistant strains [316, 318], but its clinical applications have been challenged by various factors including unfavorable pharmacokinetics and toxicity [14]. Compounds of synthetic pyridopyrimidine series have also been investigated for MexAB-OprM-specific inhibition, and these include a potential preclinical candidate, quaternary analogue D13-9001 [319, 320], which potentiates the activity of aztreonam and levofloxacin and reduces *in vitro* invasiveness of *P. aeruginosa* into mammalian cells [319, 321]. Molecular modes of action of these inhibitors including their interaction with RND pumps were reviewed recently [14, 320, 322]. Similar to the effect from genetic inactivation of PvdRT-OpmQ efflux pump [126, 127], reserpine was found to inhibit this exporter to synergize both *in vitro* and *in vivo* activities of a siderophore-monobactam conjugate [128].

Certain existing drug agents have also been assessed for their potential to be used as efflux pump inhibitors such as sertraline and trimethoprim [173]. (However, further studies are required since only wild-type strains, not efflux-upregulated mutants, were affected.) Various natural extracts have been assessed for combinational use with conventional antibiotics against *P. aeruginosa* [240, 323–326]. The compound 3,4-dibromopyrrole-2,5-dione isolated from a *Pseudoalteromonas* spp. was shown to potentiate activity of multiple antimicrobials against Mex pump overproducers [327]. However, more investigations are needed to rule out any non-efflux inhibitory effects of these compounds on cell growth. Transcriptional inhibition of the RND pumps has been shown to reduce efflux-mediated resistance although clinical implications of this approach remain unknown. Andrographolide, isolated from an herb, appears to reduce MexAB-OprM expression via transcriptional inhibition and to increase drug susceptibility [328]. The use of a deoxyribozyme (i.e., DNA molecules with catalytic action in gene replication) against the mRNA of a probable ATP-binding component of an ABC transporter (which is likely PA2812, homologous to CcmA involving in cytochrome c maturation) seems to be able to decrease ciprofloxacin resistance *in vitro* [124]. The antisense phosphorothioate oligodeoxynucleotides which targeted the *oprM* gene and were encapsulated in anionic liposomes were shown to reduce *oprM* expression and to increase antimicrobial susceptibility of multidrug-resistant isolates [329].

14.6 Concluding Remarks

Over the last two decades, huge advances have been achieved in our in-depth understanding of multidrug efflux systems of *P. aeruginosa*. These efflux pumps play a predominant role in clinically relevant MDR, which demonstrates a

remarkable ability of *P. aeruginosa* to develop sophisticated defense mechanisms against a variety of old and new antimicrobial agents. Actually, very few existing drugs appear to escape the multiple and complementary efflux pumps in this microorganism. Efflux phenomenon can not only serve as the initial mechanism of resistance to acquire other means of resistance but also interplay synergistically with them to raise resistance levels. The high percentages of efflux mutants from clinical settings around the globe further highlight the significance of these drug efflux systems as a major *in vivo* mechanism of resistance, which also link resistance selection and cross-resistance between conventional antibiotics and biocides. Minimizing exposure of *P. aeruginosa* to multiple structurally unrelated efflux selecting antimicrobial agents would limit the development of resistance, including multidrug-resistant efflux mutants, thus providing another compelling argument for antimicrobial stewardship in any environment that includes prudent antimicrobial use in both clinical settings and community hygiene practice. Efflux mechanisms can also be taken into consideration in pharmacokinetic-pharmacodynamics of individual antimicrobial agents to guide clinical drug use in minimizing resistance emergence [330]. Evidently, therapeutic approaches to intervene in efflux mechanisms are attractive for antimicrobial research and development, in particular because drug efflux systems also contribute to stress responses and virulence factor production. The increasing structural and biochemical understanding of drug efflux pumps such as drug recognition or binding sites and transport kinetics should facilitate such an effort. However, despite the progress made in the field of drug efflux research to date, challenges continue to be faced in the development of novel antimicrobial agents or efflux pump inhibitors that can be applied to combat infections associated with multidrug-resistant *P. aeruginosa*.

14.7 Addendum in Proof

A lytic bacteriophage of the *Myoviridae* family was recently shown to utilize OprM as a receptor-binding site and consequently to compromise the function of MexAB-OprM and MexXY-OprM efflux systems, leading to restore antimicrobial susceptibility in multidrug-resistant isolates [331]. A new study has reported the transport, via MexGHI-OpmD pump, of 5-methylphenazine-1-carboxylate, an intermediate involved in phenazine biosynthesis in the conversion of phenazine-1-carboxylic acid to pyocyanin [332]. Expression of MexGHI-OmpD is sufficiently induced by 5-methylphenazine-1-carboxylate and this induction is required for biofilm development. Finally, a recent study has revealed that MexR with Arg21Trp mutation displays a mutation-induced allosteric coupling of contact networks that are independent of the wild-type MexR protein in the regulation of MexAB-OprM expression, suggesting a novel mechanism for MarR family derepression that mimics derepression by small-molecule binding to MarR proteins [333].

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Chapter 15
Antimicrobial Drug Efflux Pumps in *Stenotrophomonas maltophilia*

María B. Sánchez, Guillermo García-León, Alvaro Hernández, and José L. Martínez

Abstract *Stenotrophomonas maltophilia* is an emerging opportunistic pathogen with an environmental origin. One of the most cumbersome characteristics of *S. maltophilia* is its natural low susceptibility to different antimicrobial agents that are currently in use in the clinical practice. Because of that, this species is considered as a prototype of intrinsically resistant microorganism. Part of its capability to resist the action of antimicrobials resides in a number of chromosomally encoded efflux pumps. Notably, overexpression of some of these efflux pumps can confer clinically relevant resistance to quinolones. This is likely the reason why, in contrast to other pathogens, no *S. maltophilia*-resistant isolates have been found presenting mutations in the genes encoding bacterial topoisomerases. Along this chapter, we describe different efflux pumps that have been so far reported in *S. maltophilia* as well as the mechanisms that allow their regulation. The clinical relevance these efflux pumps may have for the success of *S. maltophilia* in producing infections in patients is also discussed. Finally, we focus on the function that efflux pumps may have in the adaptation of *S. maltophilia* to nonclinical ecosystems, such as rhizosphere, where antimicrobial selective pressure is likely low.

Keywords *Stenotrophomonas maltophilia* • Antimicrobial resistance • Drug efflux pumps • SmeABC • SmeDEF • SmeIJK • SmeYZ • SmeT

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15.1 Introduction

Stenotrophomonas maltophilia is an opportunistic pathogen with an environmental origin, which is considered to be a prototype of a bacterium intrinsically resistant to antimicrobial agents [1, 2]. Before the wide use of antimicrobial agents, most opportunistic pathogens were species belonging to the commensal microbiota capable of colonizing previously debilitated patients. Whereas commensals are still responsible for a large fraction of nosocomial infections, bacteria with an environmental origin, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, or *S. maltophilia*, also constitute a problem in habitats, such as hospitals hosting immunosuppressed patients [3], patients with a basal disease or needing care after a surgery, and generally speaking, debilitated patients. This situation, in which the host response to infection is too weak to avoid a disease, allows different organisms to colonize a human host that otherwise will not be infected. In the presence of immune system deficiency, the most important line of defense consists of the use of antimicrobials, and, in this context of high antimicrobial load, only antimicrobial-resistant organisms are able to produce an infection, despite their reduced virulence. One of these types of microorganisms is *S. maltophilia*, which is not highly virulent but is an increased cause of infections in hospitals and in people suffering from cystic fibrosis [4]. The reason behind is likely its low susceptibility to antimicrobial agents currently in clinical use. Indeed, one of the risk factors for being infected by *S. maltophilia* is a previous treatment of a patient with antimicrobial drugs.

Reasons for the reduced susceptibility to different antimicrobials are multifaceted. *S. maltophilia*, as other Gram-negative non-fermenting bacteria, presents a reduced membrane permeability, which precludes the fast and easy entrance of antimicrobial agents. In addition, the chromosome of *S. maltophilia* contains several genes coding for proteins that contribute to its resistance to several drugs. Among them, it is important to highlight two β-lactamases (including a metalloenzyme) that are capable of inactivating penicillins, cephalosporins, and/or carbapenems [5, 6]; two aminoglycoside-modifying enzyme genes, *aph*(*3′*)*-IIc* encoding an aminoglycoside phosphotransferase [7] and *aac*(*6′*)*-Iz* coding for an *N*-aminoglycoside acetyltransferase [8, 9], which can inactivate most aminoglycosides; one quinolone-resistantce protein (SmQnr) [10–12]; and several efflux pumps, belonging to different families of transporters [13], which altogether constitute one of the most important mechanisms of antimicrobial resistance in *S. maltophilia* [1].

Since *S. maltophilia* is not a producer of antibiotics used in clinics for treating human infections, such as β-lactams or aminoglycosides, it is intriguing to know the reasons why this bacterial species presents such a wide range of antimicrobial resistance determinants. Two possibilities (not necessarily mutually exclusive another) may explain this situation: (i) given that *S. maltophilia* colonizes the rhizosphere, it can encounter there a dense bacterial population (including antibiotic producers) and needs to present elements for avoiding the inhibitory activity of competitors; (ii) as proposed for different antimicrobial resistance determinants, their function in nature, an environment where the antimicrobial load is not high, is likely not to

provide resistance to the inhibitory action of antimicrobials [14–17]. As described below, this can be the situation of the SmeDEF efflux pump which seems to be involved in the colonization of the roots of the plants [18]. In the current chapter, we discuss the relevance of *S. maltophilia* efflux pumps for the acquisition of resistance to clinically relevant antimicrobial drugs, as well as the role these efflux pumps may have in other bacterial processes.

15.2 Characterization of Efflux Pumps in *S. maltophilia*

The efflux pump of *S. maltophilia*, SmeDEF, was described in 2000 [19]. Later on, in 2002, the efflux pump SmeABC was also described [20], and in 2008 the sequencing of complete genome of *S. maltophilia* K279a strain [13] allowed the identification of genes encoding several multidrug transporters belonging to three different superfamilies of transporters: the chromosome of *S. maltophilia* contains genes coding for eight multidrug resistance (MDR) efflux pumps belonging to resistance-nodulation-cell division (RND) superfamily, three efflux pumps belonging to the major facilitator superfamily (MFS), and two ATP-binding cassette (ABC) superfamily pumps [13]. After their first identification, different studies aimed to characterize them in detail, in particular their role in antimicrobial resistance, but also their involvement in resistance to biocides as well as their role in other cellular processes, including the colonization of plant hosts and the response to stress.

15.2.1 RND Efflux Pumps

The structure of *S. maltophilia* RND efflux pumps is the same as for other members of the superfamily. These efflux pumps form a tripartite complex composed of three proteins: an inner membrane protein, an RND transporter that binds and translocates substrates, an outer membrane protein (OMP), and a membrane fusion protein (MFP), which links the outer and inner membrane proteins in the periplasmic space. In general, the genes encoding the three efflux pump proteins are located in the same operon, with some exceptions in which the operon does not contain the OMP (Fig. 15.1). In other Gram-negative bacteria, when the OMP is not encoded in the same operon, the substrates can be extruded to the periplasmic space and leave the bacteria through an OMP encoded elsewhere on the chromosome, which is recruited to form a tripartite pump. For instance, the *P. aeruginosa* MexXY efflux pump can accommodate OprM [21, 22], whose gene is located in the operon encoding MexAB-OprM [23]. Usually, located upstream and divergently transcribed from the efflux pump operon are gene(s) encoding for a transcriptional regulator protein [24] or a two-component regulatory system [20]. Expression of these efflux pumps is tightly downregulated by such regulators, and high expression levels can be achieved either in the presence of an inducer or upon mutations of the regulators.

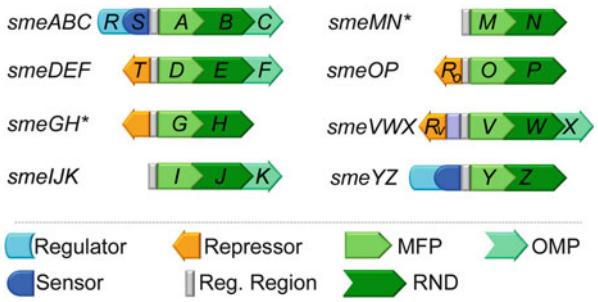

Fig. 15.1 RND operons in *S. maltophilia*. Operons containing RND genes and the genes for their local regulators are represented. The same figure is used for genes codifying for proteins with the same function, as detailed in the figure. RND genes are *smeB*, *smeE*, *smeH*, *smeJ*, *smeN*, *smeP*, *smeW*, and *smeZ*. Adjacent membrane fusion protein (MFP) genes and outer membrane protein (OMP) genes are shown. Genes encoding local regulatory proteins, as well as their DNA regulatory regions (Reg. Region) containing promoters and operator regions, are shown. *Asterisks* (*) indicate operons whose role in antimicrobial resistance has not been proven yet

Six of the eight RND efflux pumps identified in the *S. maltophilia* chromosomal sequence have been functionally characterized, SmeABC, SmeDEF, SmeIJK, SmeOP, SmeVWX, and SmeYZ (Table 15.1), while the putative role of SmeGH and SmeMN in antimicrobial resistance remains unknown. Among them, SmeDEF, SmeIJK, SmeOP, and SmeYZ, as well as the ABC efflux pump MacABCsm (see below), have shown to have a role in intrinsic resistance to different compounds (Table 15.1). The overexpression of SmeABC provides resistance to ß-lactams, aminoglycosides, and quinolones, although only the deletion of the OMP of the system, SmeC, has an effect in the susceptibility to several antimicrobial drugs [20]. This may suggest that the observed effect is not due to the efflux pump itself but of another efflux pump that can make use of SmeC for its activity in extruding antibiotics. SmeDEF has an important role in susceptibility to chloramphenicol, tetracycline, quinolones, and cotrimoxazole, as well as to biocides such as triclosan [28, 35–38]. When overexpressed, the efflux pump SmeVWX is mainly involved in acquired resistance to chloramphenicol and quinolones [28, 29]. The efflux pump SmeOP confers low susceptibility to aminoglycosides, nalidixic acid, doxycycline, and macrolides, as well as to some nonantibiotic compounds, such as carbonyl cyanide *n*-chlorophenylhydrazone (CCCP), crystal violet, sodium dodecyl sulfate, and tetrachlorosalicylanilide. However its overexpression only alters the susceptibility to toxic compounds not used in therapy such as CCCP and tetrachlorosalicylanilide [27]. Other two efflux pumps, SmeIJK and SmeYZ, provide levofloxacin resistance when they are overexpressed [26]. Nevertheless, their substrate profile is different. While efflux pump SmeIJK overexpression affects susceptibility to aminoglycosides, ciprofloxacin, and tetracycline, SmeYZ overproduction only changes aminoglycosides' susceptibility [13].

The overexpression or the inactivation of efflux pumps affects antimicrobial susceptibility in different ways. The fact that the deletion of OMPs, such as SmeC and

Table 15.1 Functionally characterized *S. maltophilia* efflux pumps

Efflux pumps	Superfamily	Regulator (family)	Intrinsic resistance	Antimicrobial resistance	Reference
SmeABC	RND	SmeSR (two-component system)	No	Aminoglycosides, ß-lactams, and quinolones	[20]
SmeDEF	RND	SmeT (TetR)	Yes	Chloramphenicol, quinolones, and tetracyclines	[19, 25]
SmeIJK	RND	Unknown	Yes	Aminoglycosides, ciprofloxacin, and tetracycline	[13, 26]
SmeOP	RND	SmeRo (TetR)	Yes	Aminoglycosides, doxycycline, macrolides, and nalidixic acid	[27]
SmeVWX	RND	SmeRv (LysR)	No	Chloramphenicol and quinolones	[28, 29]
SmeYZ	RND	Smlt2199-2130 (two-component system)	Yes	Aminoglycosides and trimethoprim-sulfamethoxazole	[13, 26, 30]
EmrCABsm	MFS	EmrRsm (MarR)	No	CCCP and nalidixic acid	[31]
SmtcrA	MFS	SmqnrR (DeoR)	No	Tetracycline	[32]
SmrA	ABC	Unknown	Unknown	Doxorubicin, fluoroquinolones, and tetracycline	[33]
MacABCsm	ABC	Unknown	Yes	Aminoglycosides, macrolides, and polymyxins	[34]

TolCsm, alters the susceptibility to antimicrobial agents, together with the absence of OMP-encoding genes in the operons encoding some efflux pumps such as SmeIJK, SmeOP, and SmeYZ, suggests that such OMPs are not efflux pump specific and several pumps could use the same OMP. Actually, the deletion of *smeC*, encoded in the *smeABC* operon, affects susceptibility to several antimicrobials, whereas the deletion of the gene encoding the actual inner membrane efflux pump *smeB* does not have any relevant effect on antimicrobial susceptibility [20]. In a similar way, the *tolCsm* gene encodes an OMP and is located upstream *smeOP* in another operon, *smeRo-pcm-tolC*, which suggests that it might form part of the SmeOP efflux pump [27, 39]. Nevertheless the deletion of *tolCsm* increases the susceptibility to several compounds, without showing any correlation with the phenotype observed in a mutant lacking *smeOP* [39].

It has been recently shown that the use of antibodies targeting efflux pumps might increase *S. maltophilia* susceptibility to antimicrobials [40]. These OMPs that are shared by different efflux pumps might be good targets for such antibodies since their inactivation will potentially inhibit simultaneously the action of different efflux pumps.

15.2.2 MFS Efflux Pumps

The study of efflux pumps belonging to the other families already found in *S. maltophilia*, namely, MFS and ABC, has received less attention than in the case of RND pumps. Four MFS efflux pumps have been so far identified in the genome of this bacterial species [13, 41]. One of these, EmrCABsm, is encoded in an operon of four genes that encodes the three efflux pump components and the transcriptional regulator of the expression of the pump *emrRsm* (MarR type) transcribed in the same direction. The deletion of *emrRsm* causes the overexpression of the efflux pump, which reduces *S. maltophilia* susceptibility to nalidixic acid and CCCP [31]. This indicates that *emrRsm* encodes a repressor of *emrCABsm*. The second MFS-type efflux pump (Smlt0032) has been identified only by bioinformatics analysis, and information on its role in antimicrobial resistance remains to be confirmed [13]. The third MFS-type efflux pump, MfsA, has been associated with methyl viologen resistance. Its regulator, SoxR, is a sensor of superoxide-generating agents [41]. However, whether or not this efflux pump has a role in the resistance of *S. maltophilia* against REDOX compounds remains to be established. Sm*tcrA* is another MFS efflux pump, which is associated to tetracycline resistance. It is localized near another antimicrobial resistance gene, Sm*qnr*. Previous work shows that both genes seem to be regulated by the same regulator SmQnrR [32], although a more recent study indicated that the effect of SmQnrR on Sm*qnr* regulation is strain specific and that this repressor regulates Sm*tcrA* expression in the two *S. maltophilia* strains studied so far [42].

15.2.3 ABC Efflux Pumps

Two ABC efflux pumps, SmrA and MacABCsm, are present in *S. maltophilia* [33, 34]. The activity of the MacABCsm efflux pump is associated with reduced susceptibility to aminoglycosides, macrolides, and polymyxins. Interestingly, a mutant presenting the deletion of the OMP, *macCsm*, has a different phenotype than mutants lacking *macAB*. These data suggest that the MacABCsm efflux pump can use an alternative, still-unidentified, OMP [34], as it was reported for some of the RND-type efflux pumps (see above). The second ABC efflux pump, SmrA, has been studied only in the heterologous host *E. coli*, in which it provides resistance to fluoroquinolones, tetracycline, doxorubicin, and multiple dyes. Whether or not this efflux pump is involved in resistance to these compounds in *S. maltophilia* remains to be established [33].

15.3 Regulation of the Expression of *S. maltophilia* Efflux Pumps

Expression of efflux pumps is in general tightly controlled by both local and general regulators. A possible reason why they are expressed at low levels unless an effector is present is that their expression can entail fitness costs. This cost can be due to the waste of energy required for the synthesis and activity of these macromolecular complexes and also because efflux pumps may extrude molecules that are important for microbial physiology such as quorum-sensing signals or virulence determinants among other compounds [43–45]. As shown in Table 15.1, hypothetical regulators, belonging to different structural families, have been identified for five RND efflux pumps. Most studies are based on the *in vitro* inactivation of the regulator. In two cases [42], the efflux pumps *smeDEF* and *smeVWX*, mutations inactivating their regulators, SmeT (TetR type) and SmeRv (LysR type) that lead to the overexpression of SmeDEF and SmeVWX, respectively [28, 46], were characterized both *in vitro* and *in vivo* [28, 38, 47–52]. Another TetR-type protein (SmeRo) regulates the efflux pump SmeOP [27]. In the case of SmeABC, a two-component system seems to be responsible of its regulation [20], a situation also observed for the efflux pump SmeYZ [13].

The molecular and structural basis of the regulation of *smeDEF* has been studied in detail. Expression of this efflux pump is downregulated by the transcriptional repressor SmeT, which is encoded upstream of *smeDEF*, in its complementary DNA strand [46]. In addition to repressing *smeDEF* transcription, SmeT also downregulates *smeT* transcription [46]. Crystal structure and biochemical analyses have shown that, like other members of the TetR family of transcriptional repressors, SmeT behaves as a dimer. A pair of dimers binds to a pseudopalindromic 28 bp region that overlaps both *smeT* and *smeD* promoters [53]. Binding of SmeT to this operator region precludes the transcription of *smeT* and *smeDEF*, likely by steric hindrance impeding RNA polymerase binding to DNA (Fig. 15.2). It is to be noticed

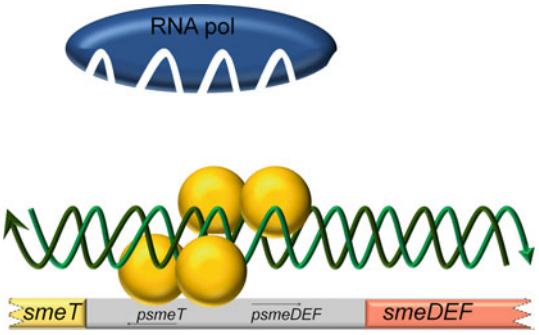

Fig. 15.2 Transcriptional repression of *smeDEF* by SmeT. Representation of a pair of dimers of the transcriptional repressor SmeT (*yellow spheres*) bound to its DNA operator region (*green double helix*). The binding of the protein impedes the *smeDEF* pump transcription since the *smeD* promoter overlaps with the repressor's operator, making impossible the RNA polymerase-DNA initiation complex. A *bar* indicating the order and position of the *smeT* and *smeDEF* genes (*yellow* and *pink*, respectively), as well as their promoters (*psmeT* and *psmeDEF*), is included

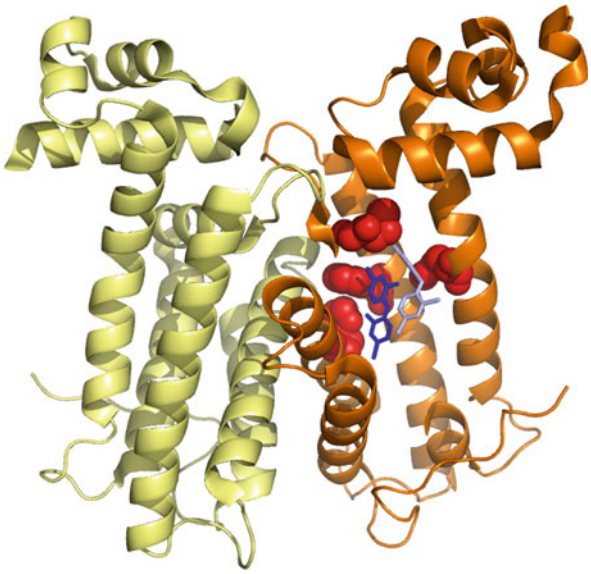

Fig. 15.3 Crystal structure of the SmeT-triclosan complex. Shown is a cartoon representation of a dimer of the transcriptional regulator SmeT (one monomer in *pale yellow* and the second one in *orange*) accommodating two molecules of the biocide triclosan (*blue* and *light blue* sticks). The residues responsible for the key interactions protein-biocide (His-67, Met-113, Phe-133, and His-167) are represented as *red spheres*

that while most studied clinical isolates overexpressing SmeDEF present mutations in *smeT*, one *smeDEF*-overexpressing clinical isolate did not present relevant changes neither in *smeT* nor in its operator sequence [47]. This result strongly suggests that other proteins besides SmeT may participate in the regulation of *smeDEF* expression.

High-level expression of SmeDEF can be achieved by mutating SmeT or by releasing its binding from its operator in the presence of a SmeT effector. It has been shown that, while antibiotics that are good substrates of the efflux pumps are not effectors of SmeT, other compounds such as biocides or flavonoids present in plant exudates can be accommodated by the SmeT binding pocket (Fig. 15.3), releasing the regulator from its operator and, consequently, allowing the induction of SmeDEF expression [18, 37, 54]. The fact that flavonoids, and not antibiotics, are good SmeT effectors suggests that the original function of SmeDEF is not necessarily the extrusion of antibiotics (see below).

15.4 Clinical Relevance of *S. maltophilia* Efflux Pumps

Most works on the substrate range and regulation of *S. maltophilia* efflux pumps have been performed *in vitro*. From these studies, we can state which efflux pumps are involved in intrinsic *S. maltophilia* resistance to antimicrobial agents (Table 15.1) and predict which ones may contribute to the acquired resistance upon their overexpression. Nevertheless there are few detailed epidemiological analyses describing mutants overexpressing these efflux pumps in clinical isolates. These studies are

hampered because, in contrast to the situation in other Gram-negative bacteria in which efflux can be inferred by determining the minimal inhibitory concentrations (MICs) of the antimicrobials of choice in the presence and in the absence of the efflux pump inhibitor phenylalanine-arginine-ß-naphthylamide, this compound does not inhibit the activity of *S. maltophilia* efflux pumps [55]. Despite this situation, functional and molecular assays have shown that overexpression of SmeDEF is common among clinical *S. maltophilia* isolates [47, 48, 56]. In addition, clinical isolates overexpressing SmeVWX have been also found, some of them presenting the same mutation in *smeRv*, which encodes the transcriptional regulator of this efflux pump that has been described for *in vitro* selected *smeVWX* overexpressing mutants [38, 50–52]. These data support the idea that *in vitro* evolution studies may help in predicting mutations appearing *in vivo* during the treatment of patients [57–60]. This could help in improving therapeutic regiments in order to avoid the emergence of antimicrobial resistance, particularly MDR, because selection by a single antimicrobial of mutants that overexpress an efflux pump will render resistance to all the antimicrobials that are substrates of such an efflux pump.

One aspect in which *S. maltophilia* efflux pumps' activity is particularly cumbersome concerns acquired resistance to quinolones. Quinolones are synthetic antimicrobials with a broad spectrum of activity. They were discovered in the 1960s while studying chloroquine for malaria treatment [61]. Afterward, it was proven that a fluoride group added to the central ring of the molecule increases the efficiency of the antimicrobial, creating the fluoroquinolones. Since there are no quinolone producers in nature, it was expected that resistance to this family of drugs would appear just through mutations in the genes encoding for their targets, the bacterial topoisomerases [62]. In fact, mutations in these genes, especially in the quinolone resistance-determining regions (QRDRs), are the main cause of high-level quinolone resistance in all studied microorganisms.

The exception to this rule is *S. maltophilia*. Different works have shown that, in sharp contrast with what happens for other bacterial species, such mutations are not found in quinolone-resistant *S. maltophilia* strains, neither in mutants selected *in vitro* nor in clinical isolates [28, 63–65]. The reason behind this situation is likely that, whereas usually overexpression of efflux pumps confers low-level resistance to quinolones in all bacterial pathogens so far studied, the overexpression of efflux pumps may confer the clinically relevant resistance in the case of *S. maltophilia*. Indeed, it has been shown that the overexpression of SmeDEF correlates with the quinolone resistance of *in vitro* selected mutants [28] and several clinical isolates overexpress this system [38, 47, 52, 56, 66]. Further, even when this efflux pump is inactivated, quinolone-resistant mutants do not present changes in the QRDR of the topoisomerases, but resistance is caused by the overexpression of yet another different efflux pump, SmeVWX [28]. It is important to notice that SmeVWX-overexpressing clinical strains presenting the same mutations in its regulator as the *in vitro* selected mutants have been reported [28, 52].

One intriguing finding is that although SmeDEF overexpression is found more frequently in both the *in vitro* obtained mutants and the clinical isolates, SmeVWX

overexpression leads to higher quinolone MICs in *S. maltophilia* [28, 52]. It is possible that, once a clinically relevant resistance is achieved, the only factor affecting the selection of one mutation over another consists of a fitness cost associated with the acquisition of each of such mutations [59, 60, 67, 68]. In this regard, it has been described that SmeDEF overexpression impairs *S. maltophilia* fitness and virulence [66]. Whether or not the effect of SmeVWX overexpression in *S. maltophilia* is similar (or eventually more drastic) remains to be established.

While efflux pumps seem to be major players in the acquisition of quinolone resistance by *S. maltophilia*, this bacterial species also contains in its genome a gene encoding a quinolone resistance protein SmQnr, which also might contribute to this resistance [11, 12]. Although the *in vitro* studies show that the SmQnr overexpression increases MICs of quinolones [11, 12], the SmQnr overexpression has not been found as the cause of resistance neither in *in vitro* selected quinolone-resistant mutants nor in clinical isolates. A reason for such situation is that in contrast to the overexpression of *S. maltophilia* efflux pumps, the SmQnr overexpression renders only a low-level quinolone resistance [28, 52]. We can then conclude that the overexpression of either SmeDEF or SmeVWX is the only known mechanism of quinolone resistance in *S. maltophilia*, which is present after the *in vitro* selection of quinolone-resistant mutants, and has been described in clinical isolates of this bacterial species [28, 52, 69].

15.5 *S. maltophilia* Efflux Pumps: More Than Antimicrobial Resistance Determinants

Even though the majority of studies related to efflux pumps explore their role in antimicrobial resistance, it is known that functions of MDR pumps are very diverse [14]. It has been shown that, besides antibiotics, efflux pumps can extrude a huge variety of compounds like heavy metals [70], organic solvents [71], dyes [72], detergents [25], bile salts [73], biocides [36], quorum-sensing signals [43–45], or plant-produced compounds [74–77]. However, little is known about functions that *S. maltophilia* efflux pumps have in dealing with the behavior of this microorganism in its natural habitat.

Some recent works begin to address this issue; SmeDEF, for example, is involved in the colonization of plant roots by *S. maltophilia* [28]. Plant-produced flavonoids can bind to SmeT, derepressing *smeDEF* expression. They are bona fide effectors regulating *smeDEF* expression, since they specifically induce this pump and not other *S. maltophilia* efflux pumps. Consistent with these findings, the deletion of *smeE* impairs *S. maltophilia* colonization of plant roots. These results show that one original function of SmeDEF is the colonization of plant roots, and only in clinical settings the quinolone resistance is manifested as a function of this efflux pump, being a good example of exaptation, an evolutionary process in which a

change of function is achieved by an environmental shift, not by a genetic change [17, 78].

Not only has SmeDEF a role in the interaction of *S. maltophilia* with plants, but SmeVWX might also be somehow related. The diffusible signal factor (DSF) is a quorum-sensing molecule of fatty acid nature that was first detected in *Xanthomonas campestris* [79]. This molecule is also present in *S. maltophilia* and is involved in the regulation of the expression of numerous genes directly or indirectly involved in plant growth promotion and biocontrol, including antimicrobial resistance genes such as SmeW [80]. When the gene encoding the DSF synthase, *rpfF*, is deleted, the *smeW* expression is downregulated, indicating that SmeW is an efflux pump that may also be somehow involved in colonization of plants by *S. maltophilia*, although further studies are needed to address this hypothesis. DSF also regulates chaperone biosynthetic genes and therefore the stress resistance [80], establishing an indirect link between SmeVWX efflux pump and stress response.

SmeIJK operon contains two RND-type transporters (SmeJ and SmeK) and is constitutively expressed in the absence of antimicrobial pressure, indicating that antimicrobial resistance is not likely the main function of this pump. A recent study showed a direct relationship among the SmeIJK efflux pump, the cell envelope integrity, and the envelope stress response mediated by the sigma factor RpoE [81]. When *smeIJK* is deleted, *S. maltophilia* is more susceptible to hyposmolarity and to membrane-damaging agents (MDAs) such as sodium dodecyl sulfate or Triton X-100. In addition, the loss of SmeIJK activates the *rpoE* regulon. This regulon can also be activated in the presence of MDAs, and this activation further upregulates SmeIJK expression. In the presence of antimicrobials that are known substrates of SmeIJK efflux pump, *smeIJK* expression is not increased, further confirming the main function of SmeIJK to be a part of the *S. maltophilia* envelope stress responses via RpoE [81]. Another pump with a known function in *S. maltophilia* envelope stress responses is the ABC-type efflux pump, MacABCsm [34]. This pump is also constitutively expressed, and besides envelope stress responses, it also has a role in oxidative tolerance and biofilm formation.

S. maltophilia is an opportunistic pathogen with increasing relevance in nosocomial infections [4]; however, its natural habitats are water and soil, where this microorganism can colonize all parts of plants, including the whole endosphere [82]. As stated previously, little is known about the role of efflux pumps of *S. maltophilia* in the natural environment, but with this natural origin, it is predicted that the majority of efflux pumps should display different functions in these habitats. The interest in knowing the original functions of efflux pumps is increasing, so it is expected that, in the next years, more natural functions for these antimicrobial resistance determinants will be deciphered.

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Chapter 16
Antimicrobial Drug Efflux Pumps in *Burkholderia*

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Abstract The genus *Burkholderia* includes more than 90 species able to colonize different environments and characterized by a versatile metabolism. Some members of the *Burkholderia* genus are opportunistic pathogens, especially for immunocompromised and cystic fibrosis patients. Of note, they show a high level of intrinsic drug resistance, and many genes encoding virulence factors were identified in their genomes. Main contributors to antimicrobial resistance of these bacteria are efflux pump proteins which span the cytoplasmic and outer membranes. These systems are able to recognize and extrude very dissimilar compounds, thus rendering the antimicrobial therapy challenging. A detailed description of the resistance-nodulation-cell division (RND) transporter superfamily, which is the most represented in Gram-negative bacteria such as *Burkholderia* spp., is given. This includes the distribution of RND-encoding genes in the various *Burkholderia* spp. genomes and the list of the principal RND pumps in *B. cenocepacia*, *B. vietnamiensis*, *B. pseudomallei*, *B. mallei* and *B. thailandensis*. The clinical significance of RND efflux transporters in *Burkholderia* spp. and relevant existing efflux pump inhibitors is also discussed.

Keywords *Burkholderia* • *Burkholderia cepacia* complex • Antimicrobial resistance • Efflux pumps • RND

16.1 Introduction

Currently the genus *Burkholderia* consists of more than 90 formally described species and a large number of candidate species [1–4]. *Burkholderia* species are Gram-negative bacteria occurring in very different environments and possessing very

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diverse metabolisms; they can be found in pristine and contaminated soils, in plant rhizospheres and phytosphere, in invertebrate intestinal tracts and in the human respiratory tract [5–7]. The metabolic versatility of these species is partially due to their large genomes that are among the largest bacterial genomes known, with sizes spanning from 7 to 9 Mb [8]. These large genomes consist of two to three different chromosomes and, in some species such as *Burkholderia cenocepacia*, also of plasmids [9]. Most members of the *Burkholderia* genus are pathogens characterized by well-known drug resistance and virulence factors [10, 11]. Among various mechanisms of antimicrobial resistance, drug efflux pumps play an important role in *Burkholderia* spp. In this chapter, we first describe the major features of several species of *Burkholderia* of public health concern and then provide a review of the current status on the role of drug efflux pumps in drug resistance in these species.

16.2 The Genus *Burkholderia*

16.2.1 Burkholderia cepacia *Complex*

Members of the *Burkholderia cepacia* complex (Bcc) bacteria share a high level (>97.5 %) of 16S rRNA gene sequence similarity and moderate (30–60 %) DNA-DNA hybridization values. They are characterized by very different biological features making them both a friend and a foe to humans [12]. Bcc bacteria have large genomes (7.5–8.5 Mb) with a G+C base composition of approximately 67 %, and they are characterized by multiple replicons, providing them with exceptional metabolic capacities [13]. Currently, Bcc has been dissected into more than 20 species, but all available data demonstrated that there is still a large number of unnamed Bcc species. From old and new classification techniques, it is clear that Bcc bacteria constitute a genotypic continuum in which separated entities, called species, have only developed in the last years. New methods are better than the traditional 16S rRNA-based approach, as they analyse a larger part of the genome with a higher resolution in order to precisely discriminate closely related bacteria [13].

Presently, the complex consists of more than 20 genetically closely related species, isolated from human infections, as well as from natural environments [14–16]. Many members of the Bcc are opportunistic pathogens particularly dangerous for immunocompromised individuals and cystic fibrosis patients. Several Bcc species are transmissible from one cystic fibrosis patient to another, thus causing epidemic outbreaks [17–19]. Among Bcc bacteria, *B. cenocepacia* and *B. multivorans* predominate in cystic fibrosis, accounting for 85–97 % of all Bcc infections [14].

B. cenocepacia is one of the most dangerous pathogens in cystic fibrosis, and infection with this organism is associated with reduced survival and a high risk of developing fatal cepacia syndrome [20, 21]. Research on the pathogenicity of Bcc bacteria is focused on *B. cenocepacia* because of the preponderance of epidemic strains and because the first Bcc genome sequenced was that from *B. cenocepacia*

J2315 [9, 22]. The latter was isolated from a cystic fibrosis patient and is a member of the epidemic ET12 lineage, which is responsible for infecting many patients in Canada and the United Kingdom. The *B. cenocepacia* J2315 genome of 8.06 Mb consists of three circular chromosomes plus a plasmid [9]. It contains 14 genomic islands not found in other *Burkholderia* spp. [9]. In the evolution of the ET12 lineage, the exchange of genomic islands was shown as crucial, introducing features necessary for the survival and for the pathogenesis in the cystic fibrosis lung. In particular, J2315 strain has developed increased resistance to many antimicrobials [23], and the genome sequencing showed that it contains drug resistance determinants in genomic islands, underlining the important role of a horizontal transfer [9]. Comparative genomic studies highlighted that gain of functions through horizontal transfer and loss of functions via mutations were necessary for J2315 strain to sustain the growth and persistence in cystic fibrosis infections [9].

Pharmacological treatment of Bcc infections is very difficult due to the high intrinsic and acquired resistance of most strains to a broad range of antimicrobial drugs. Such resistance is due to various mechanisms, including reduced permeability, changes in lipopolysaccharide structure, the presence of numerous multidrug efflux pumps, inducible chromosomal β-lactamases and altered penicillin-binding proteins [24]. Furthermore, Bcc bacteria are able to form biofilms that contribute to increase the survival in the cystic fibrosis lung environment protecting bacteria from antimicrobials [24]. In this scenario, the treatment of Bcc-infected patients should be based on a combination therapy driven by antimicrobial susceptibility tests, with two or three antimicrobial agents that function synergistically.

16.2.2 Burkholderia pseudomallei

B. pseudomallei is a saprophytic intracellular opportunistic pathogen that multiplies within macrophages. It causes melioidosis, a disease characterized by sepsis, pneumonia and abscess formation in almost any organ. It is endemic in tropical and subtropical regions [25–27]. *B. pseudomallei* is a potential bioterrorism agent and should be manipulated in biosafety level 3 (BSL-3) laboratories only. The genome of *B. pseudomallei* has been sequenced and found to comprise two chromosomes of 4.07 Mb and 3.17 Mb, respectively [28]. The larger chromosome contains genes associated with core function such as cell growth and metabolism, while the smaller one carries genes for accessory functions and for adaptation and survival in different environments. Approximately 6 % of the genome is constituted by putative genomic islands, probably derived from horizontal gene transfer, but it is not known if these regions are involved in pathogenesis [29]. Using multilocus sequence typing to study the molecular epidemiology of *B. pseudomallei*, a high level of genetic recombination was hypothesized [30]. From the comparison of *B. pseudomallei* and *B. mallei* (see below), it seems that the latter derived from a single clone of the former through a “genomic down-sizing” [29].

The clinical symptoms of melioidosis are multifarious, ranging from acute sepsis to chronic recurrent infections as well as disease without clinical symptoms. These different aspects are due to a combination of infecting dose, type of infection, host risk factors and still unknown bacterial virulence determinants [27]. If the diagnosis is not rapid, and without appropriate antimicrobial treatment, the mortality rate is ~40 % and can increase to >90 % in subjects with septic shock [26]. The disease affects at-risk patients, like those suffering from cystic fibrosis [28, 29, 31, 32], non-cystic fibrosis bronchiectasis [33] and also diabetes.

Infections caused by *B. pseudomallei* are characterized by particular morbidity and mortality. Therapy is extensive and divided in many phases: parenteral (ceftazidime, amoxicillin-clavulanic acid or meropenem) and oral (trimethoprim-sulfamethoxazole).

The resistance mechanisms documented in *B. pseudomallei* are the modification of the cell envelope constituents to decrease the cell permeability, efflux pump activation and modification or deletion of target sites [34]. Moreover, other factors that contribute to antimicrobial resistance are the biofilm formation like in Bcc strains [35], the intracellular and non-replicative metabolic state [36] and growth under stress conditions [37].

16.2.3 Burkholderia mallei

B. mallei, an obligate mammalian pathogen, is a non-motile, facultative intracellular bacterium known as the etiologic agent of glanders. *B. mallei* infection can be chronic or acute: in the first case, the clinical symptoms are mucopurulent nasal discharge, lung lesions and nodules involving the liver and spleen, while the acute infection results in high fever and emaciation, with ulceration of the nasal septum, accompanied by haemorrhagic discharge [38]. Rarely, *B. mallei* can infect humans, including laboratory workers and those in contact with infected animals. Bacteria enter the body through the eyes, nose, mouth or wounds in the skin. Human symptoms are initial onset of fever, rigors and malaise and rapid onset of pneumonia, bacteraemia, pustules and abscesses, with death coming in 7–10 days without antimicrobial treatment. Ninety-five percent of untreated infections and 50 % of antimicrobial-treated cases are fatal [38]. *B. mallei* is highly infectious in the aerosol form, and only few bacteria are required to establish the infection, thus rendering it a potential biological threat agent. The use of this bacterium is confined to BSL-3 laboratories.

The genome of the *B. mallei* comprises two circular chromosomes (5.8 Mb) and a G+C content of 69 % [39]. The comparison with the closely related species *B. pseudomallei* and *B. thailandensis* reveals a significant similarity, with 99 % identity between the conserved genes in *B. pseudomallei*, even if *B. mallei* contains approximately 1.41 Mb less DNA than *B. pseudomallei* [39]. It is probable that *B. mallei* evolved from a single strain of *B. pseudomallei* after a colonization of an equine-like ancestral host [40].

The evolution was a result of intergenic sequence (IS)-mediated gene loss and genomic recombination [39, 41]. The IS intervention was found in diverse symbionts

and obligate pathogens, suggesting an elaborated genome transition during the initial bacterial evolution after establishing constant association with the host. The structural flexibility is the major feature of *B. mallei* genome in order to adapt to multiple distinct mammalian hosts and to increase the ability to escape the adaptive immune responses.

Many *B. mallei* strains show resistance to a high number of antimicrobial agents; in fact the genome contains at least 33 genes involved in the drug resistance [39]. *B. pseudomallei* is resistant to macrolide and aminoglycoside antibiotics because of the presence of multidrug efflux pumps, while *B. mallei* shows susceptibility to these drugs. In *B. mallei*, the 50 kb region where these genes are located in *B. pseudomallei* genome is absent [39].

16.2.4 Burkholderia thailandensis

B. thailandensis is a soil saprophyte common to tropical and subtropical regions, and it is used, as generally considered non-pathogenic, for antimicrobial and vaccine studies because it can be manipulated in BSL-2 laboratories. It is closely related to *B. pseudomallei*, and only occasionally it is reported to cause human disease in association with traumatic event or reduced immune competence [42].

B. thailandensis and *B. pseudomallei* diverged from a common ancestor about 47 million years ago, and the two species show a high level of 16S rRNA sequence similarity [43]. Their genomes are highly syntenic and approximately 85 % of their genes are conserved, with only four large inversions. It has been demonstrated that the use of live *B. thailandensis* expressing capsular polysaccharide on *B. pseudomallei* induces protective responses [44]. This result revealed the importance of capsular polysaccharides in the stimulation of immune response against *B. pseudomallei* and the efficacy of *B. thailandensis* E555 strain as potential vaccine in protecting against melioidosis [44].

16.3 Drug Efflux Pumps

Efflux pumps are considered among the three principal causes of drug resistance in bacteria, together with drug-modifying enzymes and alterations of the antimicrobial target [45]. Efflux pumps are able to extrude chemically very different compounds (including cationic dyes, detergents, solvents and antimicrobials) out of the cell, thus preventing these compounds from reaching their target [46].

Efflux pumps can be divided into five major families/superfamilies: ATP-binding cassette (ABC) superfamily, the resistance-nodulation-cell division (RND) superfamily [47], the multidrug and toxic compound extrusion (MATE) family [48], the major facilitator superfamily (MFS) [49] and the small multidrug resistance (SMR) family [50]. The ABC superfamily is the only one that uses ATP as the energy source [51], while the others obtain energy through the proton motive force. All the families are

found in bacteria, including pathogens [52]. Here we will focus on RND efflux transporters, as the members belonging to this super family are the principal mediators of multidrug resistance in Gram-negative bacteria, including *Burkholderia* spp. [53].

RND efflux transporters are good examples of the typical translocators of Gram-negatives, being composed of three proteins: an inner membrane protein; a membrane fusion protein, located in the periplasm; and an outer membrane protein [47, 54]. These components span the Gram-negative membranes, thus allowing the translocation of different kind of molecules from the outer leaflet of the inner membrane to the outside of the cell.

While the other families of efflux transporters can be composed of only one unit or form similar three-component complexes, it is thought that RND members cooperate with other transporters to deliver their substrates through their periplasmic and outer membrane proteins [55]. For example, in *Pseudomonas aeruginosa*, the TetA efflux pump has been shown to work in concert with MexAB-OprM and enable the resistance to tetracycline higher in the presence of both efflux pumps [56].

The regulation of the expression of these systems is at the transcriptional level and involves DNA-binding proteins acting as repressors or activators that, in turn, sense the presence of various compounds (including the ones that are translocated by the efflux pump). As an example, the crystal structures of AcrAB-TolC of *Escherichia coli* [57–59] and of its regulator AcrR [60] helped to shed light on its mechanism of extrusion and activation. Moreover, transcriptional regulation can be performed by global regulators belonging to the Mar, Sox and Rob families [61].

Additional information may be gained from studying kinetics. To this aim, both *in vitro* and *in vivo* experiments can be performed. The latter is interesting, but it is difficult to discriminate whether the data collected only come from one transporter or from the whole cellular environment. In this way, many knock-out mutants should be tested to properly assess the contribution of one protein in respect to the other ones. Examples of *in vivo* assays are the efflux of fluorescent dyes [62] and antimicrobial minimal inhibitory concentration (MIC) determination in wild type and mutant strains [63]. As regarding *in vitro* approaches, they allow to obtain kinetic constants of purified proteins [64]. The two assays can be combined due to the difficulties in designing a classical protein assay for efflux transporters. One useful method is the preparation of liposomes.

The *in vitro* approach presents limitations due to the hydrophobic properties of the substrate and its tendency to non-specifically bind to the membrane. Moreover, the proton gradient needed by the RND to perform the translocation is not easy to produce [64]. Different strategies to create the proton gradient and to perform liposome assays have been described by Verchère and collaborators [65]. Their conclusions are that the reconstitution of an efflux pump in a membrane-like environment and the vectorial substrate translocation represent the bottleneck step in developing functional *in vitro* assays, but they remain an excellent tool for the characterization of transport activity at the molecular level.

The importance of RND efflux pumps in different clinical isolates has been well described [66]. In clinical infections due to *E. coli*, about 50 % of the isolates exhibited an efflux pump overproduction [67]. Similarly, a serious increase in the prevalence of efflux-producing *Enterobacter aerogenes* strains was observed in a French

hospital [68]. Moreover, the involvement of active efflux in clinical isolates of *Klebsiella pneumoniae* has been reported to be responsible for the extrusion of 90 % of ciprofloxacin [69]. RND multidrug efflux pumps have been well described also in *P. aeruginosa*, another opportunistic pathogen of the respiratory tract. Examples are the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM transporters [70–72]. A clear involvement of MexAB-OprM and MexXY-OprM in the expulsion of commonly used antimicrobial drugs has been described [73–77].

16.4 RND Efflux Pump Distribution in the Genus *Burkholderia*

The members of the RND superfamily are further classified into nine subfamilies: aryl polyene pigment exporter (APPE), eukaryotic (putative) sterol transporter (EST), hydrophobe/amphiphile efflux-1 (HAE1), hydrophobe/amphiphile efflux-2 (HAE2), hydrophobe/amphiphile efflux-3 (HAE3), heavy metal efflux (HME), putative nodulation factor exporter (NFE), secretion system DF family (SecDF) and hopanoid biosynthesis-associated RND (HpnN) [54, 78]. The names of the subfamilies depend on the substrate that they translocate: heavy metals (HME), multiple compounds (HAE), lipooligosaccharides (NFE) [79] or hopanoids (HpnN) [80]. APPE, HME, HAE1 and NFE are restricted to Gram-negative bacteria; HAE2 are typical for Gram-positives, while EST are found in eukaryotes. HAE3 are distributed among Archaea and Spirochaetes, while representatives of the SecDF family can be found in Gram-negatives, Gram-positives and Archaea [79].

In the *B. cenocepacia* J2315 genome, 16 operons encoding putative RND efflux pumps were described [9, 81], while in *B. pseudomallei* K96243, 10 operons encode RND transporters [82]. Most of these pumps consist of a polypeptide chain of 700–1,300 amino acids, with a characteristic topology of a transmembrane segment (TMS) at the N-terminus, an extracytoplasmic domain, six TMSs, another extracytoplasmic domain and five C-terminal TMSs.

In 2010, Perrin and collaborators analysed the 16 operons of *B. cenocepacia* and confirmed the presence of four highly conserved motifs [83] in all of them [84]. The 12 TMSs and the 2 large loops that are characteristic of RND proteins [79] were found in all of them. The organization of the operons was then studied, revealing three different arrays, based on the *ceoB* (the inner membrane portion) encoding gene position, while a phylogenetic analysis further splits the 16 sequences into 5 clusters [84]. Then, the distribution of the CeoB-like proteins was checked in the entire *Burkholderia* genus, and a variable number of proteins, ranging from 6 (in *B. mallei* strains) to 18 (in *B. cenocepacia* strains), were found. All the sequences identified in the *Burkholderia* genus were assigned to two RND subfamilies: HAE1 (further divided into three groups that likely transport unrelated substrates) and HME (split into two different groups, one for the export of monovalent and one for divalent cations) [84]. While no apparent relationship between bacterial lifestyle (in the environment, in the host or in both), pathogenicity or genome size and RND protein number was detected, a correlation between the number of proteins and

taxonomy could be found. In fact, a similar number of RND proteins are present in strains of the same species and/or related species [84]. As regarding the evolution of RND-encoding genes, they seem to derive from an ancestral *ceoB*-like sequence. In fact, the degree of sequence similarity is very high. Probably the ancestor was able to recognize different substrates, and then, through differentiation and duplication events, the transporters acquired the substrate specificity [84].

These analyses were subsequently deepened by performing a comprehensive comparative analysis of the RND superfamily efflux systems in 26 completely sequenced *Burkholderia* genomes [84, 85]. In this way, a new uncharacterized RND family was discovered, and the distribution of the other subfamilies was evaluated. In particular, at least one copy of the genes belonging to the HAE1 and SecDF families and to HpnN transporters was found in all the genomes analysed, indicating that these proteins are involved in the extrusion of different antimicrobial and/or toxic compounds in different microorganisms [86, 87], thus mediating the resistance.

16.5 RND Efflux Pumps in *B. cenocepacia*

The first evidence of the contribution of efflux transporters to *Burkholderia* spp. drug resistance came in 1989 when Burns and collaborators determined the mechanism of chloramphenicol resistance of a cystic fibrosis clinical isolate [88]. An outer membrane protein homologous to *P. aeruginosa* OprM was found to be responsible for that phenotype [89]. The entire efflux gene cluster was subsequently isolated and characterized and named "*ceo*" for "*cepacia* efflux operon" encoding CeoAB-OpcM [90]. It showed the ability to actively efflux chloramphenicol and salicylate out of the cell, and its involvement in the transport of trimethoprim and ciprofloxacin was assessed [90].

In 2006, a bioinformatic analysis allowed our group to identify 14 putative operons encoding RND efflux transporters in the genome of *B. cenocepacia* J2315 [81]. After the completion of the genome sequencing, two additional transporters were added to the list [9]. By reverse transcription-PCR experiments, *orf3*, *orf9*, *orf11* and *orf13* were shown to be expressed in *B. cenocepacia* J2315, and *orf3* expression was strongly induced in the presence of chloramphenicol [81]. One of the RND-encoding genes (*orf2*) was cloned into an inducible vector and transformed into an *E. coli* strain which lacks the *acrAB* genes. Orf2 was able to confer resistance to streptomycin, tetraphenylphosphonium, ethidium bromide, nalidixic acid, ciprofloxacin, ofloxacin and norfloxacin and was demonstrated to efflux ethidium bromide out of the cell [81].

Subsequently, to better understand the role of efflux pumps in the intrinsic drug resistance of *B. cenocepacia* J2315, we performed gene knock-out experiments. Firstly, we deleted three operons encoding RND-1 (*BCAS0591-BCAS0593*), RND-3 (*BCAL1674-BCAL1676*) and RND-4 (*BCAL2820-BCAL2822*). Then, the MICs of different compounds were determined for the deleted strains and compared to the wild type [91]. Strain D1 with inactivation of RND-1 did not show any increased

susceptibility to tested compounds, while an eightfold reduction in the MIC of nalidixic acid was observed in strain D3 with inactivation of RND-3. As regarding strain D4 with RND-4 disruption, it showed a 4- to 16-fold increase in drug susceptibility to aztreonam, chloramphenicol, ethidium bromide, gentamicin, tobramycin, nalidixic acid, ciprofloxacin, levofloxacin, norfloxacin and sparfloxacin, indicating that RND-4 plays a crucial role in the intrinsic resistance of *B. cenocepacia* [91]. RND-3 and RND-4 were also shown to be involved in *N*-acyl homoserine lactone export, an important trait which contributes to quorum sensing signalling and to the virulence of the bacterium [91].

In another work, the tolerance of *B. cenocepacia* to the disinfectant chlorhexidine was evaluated [92]. To verify whether efflux pumps contribute to this, chlorhexidine MIC was determined for the wild type and various mutant strains, both grown as biofilms or planktonically. The results indicated that RND-3 and RND-9 (*BCAM1945–BCAM1947*) are associated with chlorhexidine tolerance when cells are grown as a biofilm, while inactivation of RND-4 rendered *B. cenocepacia* planktonic cells more susceptible than wild type cells [92]. The double mutant D4-D9 was hypersusceptible, both in sessile and planktonic cultures. All these data suggested the presence of specific chlorhexidine tolerance mechanisms related to the bacterial lifestyle [92].

More features about RND-4 and RND-9 were elucidated by analysing the transcriptome of three mutants: the single mutants D4 and D9 and the double mutant D4-D9 [93]. Major classes of *B. cenocepacia* genes, with differential expression in the deleted strains as compared to the wild type, belonged to flagellum assembly, motility and chemotaxis. In particular, D4 and D4-D9 mutants shared 26 upregulated flagellum-related genes and 13 upregulated chemotaxis-related genes. Instead, the genes that showed a decreased expression profile in D4 and D4-D9 mutants belonged to many different functional classes. Exactly the contrary was true for D9 mutant. Microarray data were confirmed by quantitative reverse transcription-PCR and phenotypic experiments, as well as by phenotype microarrays. Together these results showed a phenotypic and molecular similarity between D4 and D4-D9 strains and suggested that the RND-4 and RND-9 pumps might have a biological role not only restricted to transport but also related to motility and/or chemotaxis [93].

RND-4 was further characterized by comparing the intracellular proteome of the deletion mutant to that of the wild type strain using two-dimensional electrophoresis [94]. The results pointed out 70 differentially expressed proteins, with 13 protein spots upregulated and 35 downregulated. Fifty percent of the 35 downregulated proteins belonged to the functional categories: “amino acids transport and metabolism”, “nucleotides transport and metabolism”, “lipid transport and metabolism”, “translation” and “ribosomal structure and biogenesis”. Conversely, 46 % of the 13 upregulated proteins belonged to the categories: “energy production and conversion”, “posttranslational modification” and “protein turnover, chaperones”. Together these results confirmed a wider role than just in drug resistance for RND-4 [94]. However, the prominent role in drug resistance of RND-4 was further highlighted when, in attempt to identify the cellular target of a new thiopyridine derivative effective against *B. cenocepacia*, a mechanism of resistance was characterized

which relied on RND-4 itself [95]. In addition, RND-9 has been very recently shown to contribute to resistance of *B. cenocepacia* J2315 against a new benzothiadiazole derivative [96].

To finally assess the role of each of the 16 RND efflux transporters of *B. cenocepacia*, we created knock-out mutants for all of them [97]. First of all, we checked if differences could be detected in the MICs of some drugs for the deleted strains in respect to the wild type strains. Strains D3 and D4 were more susceptible to ciprofloxacin, minocycline and tobramycin, while the behaviour of most mutants was identical to the one of the wild type. These data suggest that RND-3 and RND-4 efflux pumps are involved in resistance of planktonic *B. cenocepacia* cells, while the other RND systems do not play a major role. As regarding to the sessile cells, strain D3 showed the highest reductions in the number of cells in the presence of high concentrations of tobramycin and ciprofloxacin, thus indicating that this efflux system is important also for the protection of *B. cenocepacia* when grown as biofilm. RND-8 and RND-9 seem instead to protect sessile cells against tobramycin [97].

In summary, at present only a few RND efflux transporters out of 16 appear to play a role in drug resistance or to be involved in virulence (due to the transport of quorum sensing signal molecules) in *B. cenocepacia*. In particular, (a) RND-3 is involved in the efflux of nalidixic acid, ciprofloxacin, tobramycin and *N*-acyl homoserine lactone in planktonic cells and seems to have a role in the protection of sessile cells against ciprofloxacin, tobramycin and chlorhexidine; (b) RND-4 plays a role in the efflux of aztreonam, ethidium bromide, chloramphenicol, gentamicin, tobramycin, fluoroquinolones, chlorhexidine, a thiopyridine derivative and *N*-acyl homoserine lactone in planktonic cells; (c) RND-8 is important for the efflux of tobramycin in sessile cells; (d) RND-9 is involved in the transport of chlorhexidine and tobramycin in biofilm grown cells; it contributes to the resistance towards a new benzothiadiazole derivative; (e) RND-10 (Ceo) transports chloramphenicol, salicylate, trimethoprim and ciprofloxacin out of the cell.

In *B. cenocepacia*, only two non-RND efflux transporters have been described for their contribution to resistance, BcrA and a homolog to the *E. coli* Fsr, both belonging to the MFS. The former is able to confer resistance to tetracycline and nalidixic acid when overexpressed in *E. coli* [98] and the latter to fosmidomycin [99, 100].

16.6 RND Efflux Pumps in Other *Burkholderia* Species

16.6.1 Burkholderia pseudomallei *and* Burkholderia mallei

Ten operons encoding RND efflux pumps are present in the genome of *B. pseudomallei* [82], but their clinical importance is difficult to study. Only the role of AmrAB-OprA [101], BpeAB-OprB [102, 103] and BpeEF-OprC [104] was elucidated.

AmrAB-OprA is a multidrug efflux system required for both aminoglycoside and macrolide antibiotic extrusion. This efflux pump shows homology to multidrug

efflux systems studied in *E. coli*, *P. aeruginosa* and *Neisseria gonorrhoeae* [101]. *B. pseudomallei* strains susceptible to aminoglycosides and macrolides have single point mutations or deletions in the *amrAB-oprA* operon [105]. The presence of this efflux mechanism in *B. pseudomallei* explains the lack of therapeutic effect observed for aminoglycosides and macrolides. AmrAB-OprA is also able to reduce the activity of newer antimicrobials like cethromycin: indeed the exposure to cethromycin induces the selection of mutants overexpressing the operon and results in high resistance levels [106].

Another *B. pseudomallei* efflux pump is BpeAB-OprB which extrudes macrolides, fluoroquinolones, tetracyclines and chloramphenicol and contributes to the intrinsic resistance. However, except for macrolides, the resistance levels are low. Despite the relationship between BpeAB-OprB and the *P. aeruginosa* MexAB-OprM, they are quite different: the latter is broadly expressed and it is involved in the intrinsic resistance to many compounds [107], while BpeAB-OprB has lower expression levels and it plays a minor role in the resistance. Studies regarding the correlation between RND efflux pump and quorum sensing or virulence traits showed that BpeAB-OprB in *B. pseudomallei* KHW strain is used for the secretion of *N*-acyl homoserine lactones [108] and virulence-associated determinants, such as siderophores [109].

The last described efflux system in *B. pseudomallei* is BpeEF-OprC, the most important pump for antimicrobial resistance. Initially, it was identified as chloramphenicol and trimethoprim transporter. The *bpeEF-oprC* operon is expressed only in *B. pseudomallei* strains carrying mutations in the regulatory region. Its expression confers resistance to chloramphenicol, fluoroquinolones, tetracyclines and trimethoprim, and it is responsible for the spread of trimethoprim resistance in *B. pseudomallei* isolates [110]. In *P. aeruginosa*, there is a related efflux pump, MexEF-OprN, which is characterized by similar substrate efflux profile [111]. The clinical significance of BpeEF-OprC is described below in the section "RND efflux pumps in *Burkholderia* clinical isolates".

B. mallei, as already described above, is generally more susceptible than *B. pseudomallei* to antimicrobial agents. The ATCC 23344 strain is susceptible to aminoglycosides because of a chromosomal deletion which involves the *amrAB-oprA* operon [39]. In *B. mallei*, the genes coding for BpeAB-OprB and BpeEF-OprC efflux pumps are present, but it is not known if the corresponding efflux systems are functional or not.

16.6.2 Burkholderia thailandensis

B. thailandensis becomes multidrug resistant following chloramphenicol exposure due to the overexpression of two RND efflux systems very similar to the already studied BpeAB-OprB and BpeEF-OprC of *B. pseudomallei* [112]. In another work, Biot and colleagues [113] showed that doxycycline resistance was correlated with the overexpression of AmrAB-OprA or BpeEF-OprC efflux pumps. The expression

levels varied depending on the antimicrobial concentration, and this indicated a reversible multidrug resistance phenotype [113]. Moreover, analysis of mutants overexpressing the efflux pumps highlighted that BpeAB-OprB is able to partially substitute the absence of AmrAB-OprA or BpeEF-OprC [113]. Furthermore, another efflux pump of the MFS family, responsive only to urate, xanthine and hypoxanthine and controlled by a multiple antimicrobial resistance regulator MarR-like, has been described [114]. However, its contribution to drug resistance is still unclear.

16.6.3 Burkholderia vietnamiensis

In contrast to many other Bcc species, *B. vietnamiensis* is susceptible to aminoglycosides and to a broad range of other antimicrobials, while it remains highly resistant to other cationic agents [115]. The same study reported the acquisition of aminoglycoside resistance of *B. vietnamiensis* in cystic fibrosis chronic infection or during the *in vitro* exposure to the drugs [115]. This resistance was caused by an efflux pump homologous to the *B. pseudomallei* and *B. thailandensis* AmrAB-OprA. Mutations in *amrR*, the putative efflux pump regulator, influenced the expression of the *B. vietnamiensis* gene *amrB* [116]. Moreover, in *B. vietnamiensis*, the *norM* gene encoding a MATE-type efflux protein was described. The disruption of *norM* alone was shown to be insufficient to reduce high levels of norfloxacin resistance, because of the presence of other efflux systems [117]. Although the physiological role of NorM is yet unclear, it is probably involved in resistance to the cationic peptide polymyxin B, especially under stress conditions [117].

16.7 RND Efflux Pumps in *Burkholderia* Clinical Isolates

Due to the high degree of antimicrobial resistance among *Burkholderia* species, it is very important to evaluate the contribution of efflux transporters, especially in clinical isolates. In the last years, some papers describing this topic were published.

Recently, Tseng and collaborators [118] evaluated the role of efflux pumps in 66 clinical Bcc isolates recovered between 2009 and 2011 in Taiwan. In order to assess the presence of active efflux, resistance patterns were determined by measuring the MICs of antimicrobials in the presence of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the efflux pump expression was evaluated through quantitative reverse transcription-PCR. The results showed that 78.6 % isolates (resistant to ceftazidime, chloramphenicol, levofloxacin, meropenem and trimethoprim-sulfamethoxazole) demonstrated presence of the efflux pump activity. Moreover, RND-3 and RND-9 transcripts were more abundant in all the tested strains compared to a strain without efflux pump activity. DNA sequences of the regulators of these two pumps were also sequenced, together with the promoter,

thus revealing five nucleotide deletions in RND-3 regulator which affected RND-3 efflux pump expression in *B. cenocepacia* clinical isolates causing antimicrobial resistance [118].

In *B. pseudomallei*, mutations in the *amrB* gene have been reported to be responsible for aminoglycoside sensitivity [119]. The whole-genome sequencing revealed non-synonymous mutations in a highly conserved region of *amrB* gene [119]. In another work, susceptibility of three isolates from Thailand was shown to be due to the lack of or greatly reduced expression of AmrAB-OprA, caused by deletions affecting the pump itself [105]. The role of AmrAB-OprA in the high-level cethromycin resistance of *B. pseudomallei* clinical isolates was further assessed by measuring the *amrB* transcript levels, the *amrR* repressor gene and the *amrR-amrA* intergenic region for presence of mutations and deleting the *amrAB-oprA* operon [106].

The clinical significance of BpeEF-OprC was corroborated by sequencing genomes of isolates from patients suffering from melioidosis with increased resistance to chloramphenicol, ofloxacin and trimethoprim-sulfamethoxazole [120]. As an example, a large inversion of 800 kb resulted in a deletion of the last 24 codons of *bpeT*, coding for the transcriptional regulator of the efflux pump. All these data sustained the hypothesis that BpeEF-OprC efflux pump has an important role in antimicrobial resistance of *B. pseudomallei* [34]. In another report by Podnecky and co-workers, the BpeEF-OprC efflux pump has been shown to contribute to trimethoprim resistance in *B. pseudomallei* clinical and environmental isolates from northeast Thailand and northern Australia [110].

The role of efflux transporters in intrinsic drug resistance of clinical isolates was also shown in the case of *B. vietnamiensis* strains [115, 116]. The authors demonstrated that strains that acquired aminoglycoside resistance during infection and after exposure to tobramycin or azithromycin overexpressed AmrAB-OprM and contained missense mutations in its repressor gene *amrR* [116].

16.8 Efflux Pump Inhibitors

A main concern for the treatment of *Burkholderia* infections is the inability to eradicate them with the available drugs. A possible new approach could be a combination of antimicrobial agents with efflux pump inhibitors (EPIs). This could potentially improve the antimicrobial therapy as the phenotype driven by efflux transporters frequently results in multidrug resistance. In this way, an EPI appears useful to block many pumps at a time, thus rendering bacteria more susceptible to drugs. Moreover, EPI administration should reduce the rates of resistance development [121].

Unfortunately, until now no EPIs entered the clinical trials because of toxicity, even if many have been developed [122, 123]. As an example, phenylalanine-arginine-β-naphthylamide (PAβN) exhibited a great activity in *P. aeruginosa* [124, 125], but it is nephrotoxic. Also, inhibitors of the MexAB-OprM efflux system were

developed and tested *in vivo* but subsequently abandoned [126, 127]. Moreover, some generic uncouplers (such as CCCP) are available. However, these compounds, causing the dissipation of the proton motive force and reducing the viability of the cells themselves, are cytotoxic, so this road is not feasible.

Moreover, EPIs, which have been shown to be effective in one microorganism, not always showed their activity in other species. As an example, PAβN seems to be ineffective in the genus *Burkholderia* [91, 102]. Also, not all the compounds extruded by a pump are potentiated by PAβN because it works by competing with antimicrobials for their binding site [124].

The unsuccessful development of EPI could be ascribed to the difficulties in understanding the features and mechanism of action of efflux pumps, in particular of RND. In fact, only a few crystal structures of individual components of these pumps are available, while a comprehensive knowledge of their assembly and mechanism of translocation is missing. In this way, it is very difficult to predict the specificity of the potential inhibitor and to study its pharmacokinetics. This is complicated also by the fact that the EPI has to be administered together with other antimicrobials, and their pharmacokinetics should be tailored. Even if the structure of the inhibitor D13-9001 bound to AcrB has been solved [128], another main problem for the development of Gram-negative EPIs is the uptake inside the cell (which is preferential for small hydrophilic molecules) and the specific binding to the inner membrane portion of RND pumps (usually hydrophobic molecules). The genetic and biochemical studies and the computational methods which could help to overcome these issues have been recently reviewed by Opperman and Nguyen [129].

16.9 Concluding Remarks

This chapter presents the genus *Burkholderia*, which includes more than 90 species. These species show a high level of intrinsic drug resistance and have many virulence factors. One of the contributors to resistance is the presence of efflux pump-encoding genes in their genomes. These pumps are able to recognize and extrude very dissimilar compounds, thus rendering the bacteria particularly difficult to eradicate. We described the distribution of RND pump-encoding genes in the various *Burkholderia* genomes and showed the role of the principal RND pumps in *B. cenocepacia* (especially studied in our laboratory), in *B. pseudomallei*, in *B. mallei*, in *B. thailandensis* and in *B. vietnamiensis* in drug resistance. A brief description of non-RND systems (MFS and MATE) has been added for each species in which they were studied. The clinical significance of RND efflux transporters in *Burkholderia* spp. has been demonstrated, thus confirming the importance of the research in this field and highlighting the need for new therapeutic solutions to be combined with the existing antimicrobial drugs to overcome the resistance problem of these infections.

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Chapter 17
Efflux Pumps in *Neisseria gonorrhoeae*: Contributions to Antimicrobial Resistance and Virulence

William M. Shafer, Edward W. Yu, Corinne Rouquette-Loughlin, Daniel Golparian, Ann E. Jerse, and Magnus Unemo

Abstract This chapter deals with the structure-function relationships, genetics, gene regulation systems, and clinical/biologic significance of efflux pumps expressed by the sexually transmitted human pathogen *Neisseria gonorrhoeae*. The overarching theme emphasized herein is that bacterial efflux pumps contribute not only to the ability of *N. gonorrhoeae* to evade many antibiotics in current or past treatment regimens for gonorrhea but they also help this pathogen to evade antimicrobials that contribute to innate host defense during infection. Accordingly, gonococcal drug efflux pumps should be viewed not only in the context of their capacity to negatively impact antimicrobial therapies but in the larger picture as virulence factors that promote survival of *N. gonorrhoeae* during infection. Based on this

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hypothesis, we posit that strategies that cripple gonococcal efflux pump activities may prove useful in the design of new therapies, which is of special importance in this era when antibiotic-based treatment options for gonorrhea are dwindling due to mechanisms of bacterial resistance that include the action of drug efflux pumps.

Keywords *Neisseria gonorrhoeae* • Antimicrobial resistance • Efflux pumps • Regulation • Structure • Virulence • Vaccine • MtrCDE • MtrF • FarAB • MacAB • NorM • MtrA • MtrR • MpeR

17.1 Introduction

Neisseria gonorrhoeae is a strictly human, Gram-negative pathogen that is typically transmitted by sexual contact and causes the disease termed gonorrhea also known as "the clap," which is a slang term thought to be derived from the name of the district Les Clapiers in Paris, France, that housed prostitutes in the late fourteenth century [1]. Gonorrhea typically presents as urethritis in men and cervicitis in women, but rectal and pharyngeal infections also occur in both sexes; the spectrum of clinical manifestations of gonorrhea has been reviewed recently [1]. If gonorrhea is left undetected, untreated, and inappropriately treated or fails to respond to conventional antibiotic treatment due to resistance (an important contemporary concern), the disease can ascend to the upper genital tract where it can cause severe reproductive complications (especially for women) such as endometritis, pelvic inflammatory disease, penile edema, and epididymitis. These complications can have a significant, devastating impact on general and reproductive health, including infertility, of infected individuals. Moreover, involuntary loss of life due to gonorrhea-associated ectopic pregnancy is not uncommon especially in the developing world. On rare occasions, gonococci can enter the bloodstream causing disseminated gonococcal infection and triggering damage to organs and joints distant from the site of infection. Conjunctivitis, more frequent in newborns (ophthalmia neonatorum) than adults, can result in blindness; transmission to the newborn can occur during vaginal delivery when the mother has cervical gonorrhea. It is also important to note that gonorrhea can increase transmission and host susceptibility to other sexually transmitted infections (STIs) including HIV, which emphasizes the importance of effective antimicrobial treatment regimens for gonorrhea. Thus, to prevent these medical issues and complications, it is imperative that effective and accessible antibiotic treatment and prevention regimens exist.

Historical writings suggest that gonorrhea has afflicted humans for thousands of years [1]. In this respect, a passage from the *Book of Leviticus* of the Old Testament warning women to avoid men with discharges and the writings of the second-century Greek physician Galen that described purulent exudates observed often in men with what is now considered as gonorrhea ("flow of seed") suggest that this is an ancient disease; a more in-depth and recent historical review of gonorrhea can be

found in a recent review by Unemo and Shafer [1]. Presently, gonorrhea is without question a major public health problem as emphasized by the estimated 78 million infections worldwide per year and the emergence of strains resistant to first-line antibiotics [1–3]. The looming public health disaster of gonococcal resistance to current first-line antibiotics, combined with retained resistance to previous first-line antibiotics, and the lack of new, effective antibiotics that can serve as replacement therapies has been recently emphasized [3]. In this context, it is important to emphasize that in the absence of a vaccine, effective antibiotic therapy is crucial for reducing spread of gonorrhea in the community.

As a strict human pathogen, gonococci have over the millennia found ways to resist the multitude of innate and adaptive immune responses that occur during infection (summarized in [4]). Pathogens, like gonococci, encounter numerous host biocides (e.g., antimicrobial peptides, toxic-free fatty acids, bile salts, and progesterone), and their capacity to resist these antimicrobials likely promotes their survival and proliferation during infection. It is now evident that many of these antimicrobials are substrates for gonococcal multidrug transporters (see Sect. 17.7 below). Moreover, strong evidence exists that implicates gonococcal efflux pumps in exporting important antibiotics (e.g., β-lactams and macrolides) that are currently used in the clinic (see Sect. 17.6 [5]). In sum, studies on efflux pumps produced by gonococci provide a unique opportunity to understand the role of bacterial drug efflux pumps in the overall pathogenic mechanisms of bacteria during infection and how they also contribute to resistance to antibiotics used to treat infection. This review concentrates on the genetics, structure-function relationships, gene regulation systems, and overall significance of gonococcal drug efflux pumps.

17.2 Types and Substrate Profiles of Gonococcal Drug Efflux Pumps

In contrast to other bacteria that express a wide number and type of drug efflux pumps (the reader is directed to other chapters in this book for the vast repertoire of efflux pumps produced by bacteria), gonococci produce much fewer drug efflux pumps (Fig. 17.1; summarized in [6]). These pumps belong to the resistance-nodulation-cell division (RND) superfamily (MtrCDE), the major facilitator superfamily (MFS) (FarAB-MtrE), the ATP-binding cassette (ABC) transporter superfamily (MacAB-MtrE), and the multidrug toxic compound extrusion (MATE) family (NorM); see other reviews [1, 6] for a description of the discovery of these efflux pumps. Recently, a fifth gonococcal efflux pump, the MtrF protein first described by Veal and Shafer [7], which belongs to the AbgT family of transporters that typically behave as importers, was designated as an efflux pump by virtue of its ability to export sulfonamides [8]. Finally, some (rare) clinical isolates have been reported to harbor the *mef* gene [9], which encodes a pump that exports macrolides and appears to have been acquired from *Acinetobacter junii*.

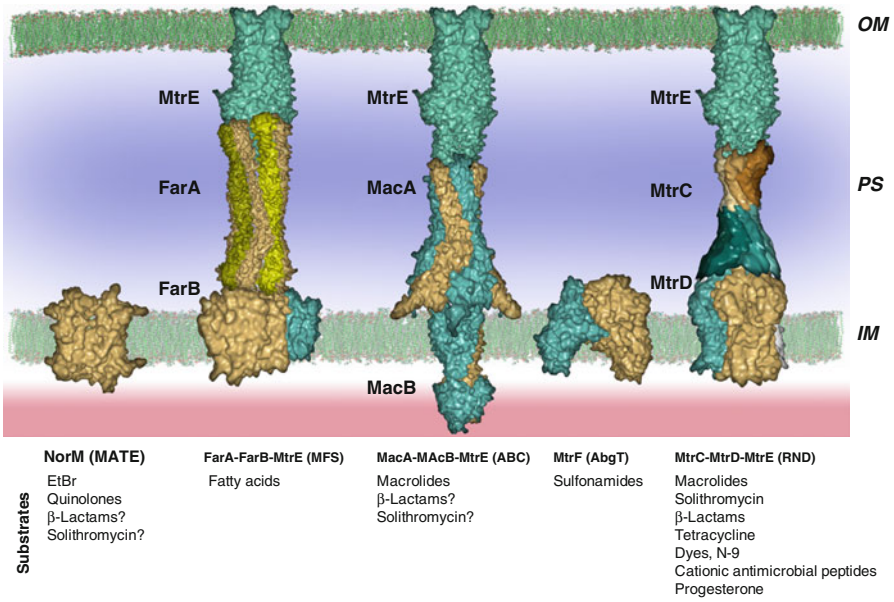

Fig. 17.1 Membrane organization of drug efflux pumps in gonococci. The proposed membrane organization and class of the known drug efflux pumps common to gonococcal strains (NorM, FarAB-MtrE, MacAB-MtrE, MtrF, and MtrCDE) as well as their substrates are depicted. MtrE most likely acts as an outer membrane channel protein for three of the pumps. MtrF possibly also acts as an accessory protein for the MtrCDE efflux pump. The crystallized NorM (PDB code: 5C6P), MtrF (4R1I), MtrD (2DHH), and MtrE (4MT0) from gonococci are shown. For MacA, MacB, FarA, FarB, and MtrC, the crystallized homologues MacA (3FPP), MacB (2HYD), EmrD (2GFP), EmrA (4TKO), and MexA (1VF7) are shown. *OM* outer membrane, *PS* periplasmic space, *IM* inner membrane. The ratio of monomeric unit number for the MtrC, MtrD, and MtrE proteins are 2:1:1, respectively, as defined earlier [25]

With respect to efflux of antibiotics, it is now recognized that the MtrCDE efflux pump can export structurally diverse hydrophobic antibiotics, β-lactams (including penicillin, nafcillin, and extended-spectrum cephalosporins) and tetracycline [5, 10–12], while the NorM pump exports quinolones [13] and the MacAB-MtrE pump exports macrolides [14]. The newly described MtrF pump recognizes sulfonamides and is proposed to be used by gonococci to export antimetabolites that accumulate within the cytosol [8]. The MtrCDE and FarAB-MtrE efflux pumps also recognize host (human and mouse)-derived antimicrobials, including cationic antimicrobial peptides (such as human cathelicidin LL-37 and murine cathelin-related antimicrobial peptide CRAMP), bile salts and progesterone (MtrCDE) [15, 16], and long-chain fatty acids (FarAB-MtrE) [17] that often bathe mucosal surfaces infected by gonococci. A functionally intact MtrCDE pump, but not FarAB-MtrE pump, is required for long-term colonization by gonococci of the lower genital tract of female mice [18], suggesting that its capacity to export host antimicrobials that pass the outer membrane helps this pathogen to establish an infection. Importantly, overproduction of the MtrCDE pump due to *cis*- or *trans*-acting regulatory mutations

(see below) cannot only increase the resistance to antimicrobials but can also increase gonococcal fitness during experimental infection of the lower genital tract of female mice [16, 19]. In total, the cumulative evidence supports the notion that the MtrCDE efflux pump is a virulence factor. Interestingly, this pump also recognizes nonionic detergents such as the spermicide nonoxynol-9, and its use over the past six decades may have inadvertently selected for gonococcal mutants that overproduce this pump [20]. The MacAB-MtrE efflux pump exports macrolides (e.g., azithromycin and erythromycin) [14]. The NorM efflux pump behaves as a Na^+-dependent transporter [21] and exports compounds harboring a quaternary ammonium on an aromatic ring (e.g., acriflavine, berberine, ethidium bromide, and 2-*N*-methylellipticinium) as well as ciprofloxacin and norfloxacin to the periplasmic space [13]. It is unknown if these latter two efflux pumps recognize and export host-derived antimicrobials.

17.3 Structural Features of Gonococcal Drug Efflux Pumps

Structural data on proteins that function as drug transporters or critical components of efflux pumps contribute to basic knowledge that can facilitate or advance studies on new drug development (e.g., efflux pump inhibitors (EPIs)) or alternative therapies and prevention strategies (e.g., vaccine development). With respect to gonococci, critical structural information is now available regarding two proteins (MtrD and MtrE) [22, 23] of the MtrCDE efflux pump as well as the MtrF inner membrane protein; as described above, MtrF appears to be an independent efflux pump that recognizes sulfonamides [8] and, possibly, acts as an accessory protein of the MtrCDE pump [7]. It is important to stress that, while structures have been solved for proteins that are highly similar to MtrD, MtrE, and MtrF, the information for the gonococcal proteins emphasizes that "one structure does not fit all" and that critical differences can exist between homologous proteins. These differences, as well as the potential ramifications of such, are discussed below.

17.3.1 Crystal Structure of the MtrD Inner Membrane Efflux Pump Transporter

The gonococcal MtrD inner membrane transporter protein of strain PID332, which is 11 amino acids shorter at the C-terminus in comparison with FA19 MtrD, was crystallized using vapor diffusion [24]. This membrane protein exists as a trimer linked to a hexamer of the periplasmic adapter MtrC, which is in turn linked to a trimer of the MtrE outer membrane channel protein [25]. At the amino acid sequence and substrate recognition levels, MtrD shares many, but not all, features of other transporters in the RND efflux pump family produced by Gram-negative bacteria.

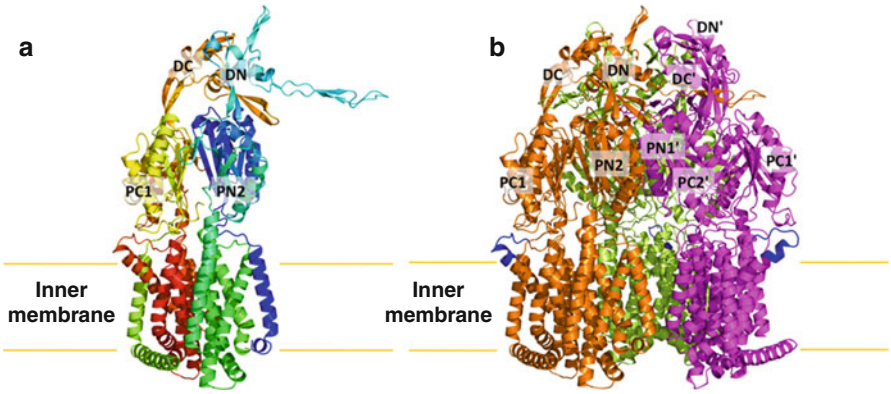

Fig. 17.2 Structure of the MtrD inner membrane transporter. (**a**) Ribbon diagram of a protomer of MtrD viewed in the membrane plane. The molecule is colored using a rainbow gradient from the N-terminus (*blue*) to the C-terminus (*red*). Sub-domains DN, DC, PN2, PC1, and PC2 are labeled. The location of PN1 is behind PN2, PC1, and PC2. (**b**) Ribbon diagram of the MtrD trimer viewed in the membrane plane. Each subunit of MtrD is labeled with a different color. Residues 917–927 (only found in MtrD) forming the upper portion of TM9 and the loop connecting TM9 and TM10 are in blue color (This figure has been reproduced from [22])

The crystal structure of the full-length PID 332 MtrD multidrug efflux pump transporter protein (114 kDa) was determined to a resolution of 3.53 Å (Protein Data Bank [PDB] code: 4MT1) [22]. The final model included 97 % of the amino acids (residues 2–493 and 508–1,040) (Fig. 17.2a). The structure of MtrD is closer to the conformation of the "access" protomer of AcrB of *Escherichia coli* [26]. However, superimposition of these two structures results in a high root-mean-square deviation (RMSD) of 7.6 Å over 1,000 C^{α} atoms, suggesting that there are significant differences between these two transporters. MtrD assembles as a 125-Å-long and 95-Å-wide homotrimer (Fig. 17.2b). Each protomer comprises 12 transmembrane helices (TM1-TM12). Like other RND transporters, the N-terminal (TM1-TM6) and C-terminal (TM7-TM12) halves of MtrD are related by a pseudo-twofold symmetry. A large periplasmic domain is created by two extensive periplasmic loops connecting TM1 with TM2 and TM7 with TM8, respectively. As in AcrB [26–30] and MexB of *Pseudomonas aeruginosa* [31], this periplasmic domain can be divided into six sub-domains: PN1, PN2, PC1, PC2, DN, and DC. Sub-domains PN1, PN2, PC1, and PC2 form the pore domain, with PN1 making up the central pore and stabilizing the trimeric organization. However, sub-domains DN and DC contribute to form the docking domain, presumably interacting with the outer membrane channel MtrE (structural information for MtrE is described below). The trimeric MtrD structure suggests that sub-domains PN2, PC1, and PC2 are located at the outermost core of the periplasmic domain, facing the periplasm. Sub-domains PC1 and PC2 also form an external cleft, and this cleft is open in the MtrD structure (Fig. 17.2). Based on the co-crystal structure of

CusBA [32, 33] of the CusCBA tripartite efflux complex of *E. coli* [34–39], the upper regions of PN2, PC1, PC2, and sub-domains DN and DC should directly interact with the MtrC membrane fusion protein to form a functional adaptor-transporter complex.

Structures of AcrB in complex with a variety of substrates [26, 29, 30] have identified that the periplasmic cleft of the pump forms several mini-binding pockets within the extensive, large periplasmic multidrug binding site. This site is supposed to play a predominant role in the selection of drugs for export. Protein sequence alignment reveals that many of the amino acids forming the large periplasmic binding site of AcrB are conserved with MexB and MtrD, indicating that these three multidrug efflux pumps may have a similar substrate binding profile for drug recognition. These conserved amino acids in MtrD are made up of several charged and polar residues, such as Ser79, Ser134, Arg174, Asp272, Glu669, and Arg714, and aromatic residues, such as phenylalanine at 136, 176, 610, 612, and 623. In addition, a flexible loop is found inside the large periplasmic cleft, which forms the multidrug binding site of the pump. This flexible loop is located deep inside the cleft between sub-domains PC1 and PC2, composed of residues 608–619, and should correspond to the Phe-617 loop [29] in AcrB. The loop is highly conserved among MtrD, AcrB, and MexB. It is expected that this flexible loop is important for drug recognition and extrusion. There is a chance that this loop may shift positions during the course of the extrusion process to facilitate drug export.

Perhaps the most interesting secondary structural feature appears in TM9 of the MtrD pump [22]. In contrast to other known structures of the RND transporters, MtrD contains an extended region that protrudes into the periplasm and contributes part of the periplasmic domain (Fig. 17.2). This region (residues 917–927) comprises an α-helix extending from the upper portion of TM9 and also to the loop connecting TM9 and TM10. Protein sequence alignment suggests that these extra residues are only found in MtrD, but not in other homologous RND proteins. Therefore, this fragment should represent a unique feature of this pump that cannot be found in other RND pumps. TM9 is distinct in that it is not vertically oriented. Instead, it is inclined from the horizontal membrane plane by 54°. The spatial arrangement between the extra elongated helix and loop (upper portion of TM9) and the periplasmic cleft formed between PC1 and PC2 suggests that these extra structural features may help the pump to transport its substrates more effectively from the outer leaflet of the inner membrane to the multidrug binding site at the periplasmic domain.

Drug export by RND multidrug transporters is proton motive force dependent [40]. Based on the crystal structure of MtrD, it is expected that the charged residues Asp405 and Asp406 of TM4 and Lys948 of TM10 are important for forming the proton-relay network of the pump. These residues are supposed to undergo protonation and deprotonation within the transport cycle. The involvement of these charged amino acids in proton translocation was supported by a previous study showing mutations of these residues inhibit proton translocation [41]. In turn, the MtrE outer

membrane channel protein is blocked and unable to dissociate from the MtrCDE tripartite efflux complex [41].

17.3.2 Crystal Structure of the MtrE Outer Membrane Channel Protein

The gonococcal MtrE protein serves as the outer membrane protein channel for the MtrCDE efflux pump, and most likely also for the MacAB and FarAB efflux pumps, that facilitates exit of antimicrobial pump substrates from the bacterial cell (Fig. 17.1; [42]). In this respect, its function in the trimer state resembles that of other outer membrane channel proteins (e.g., OprM of *P. aeruginosa* and TolC of *E. coli*) of RND efflux pumps possessed by Gram-negative bacteria. However, important differences between MtrE vs. OprM/TolC exist, and these were illuminated by structural work [23] and are summarized below.

The gonococcal MtrE protein of strain FA136 was used for crystallization using vapor diffusion. Protein sequence alignment suggests that FA136 MtrE and FA19 MtrE are nearly identical, except for the amino acid residues between 178 and 197. In FA136 MtrE, these residues are NAVRIAVQGRRDFRRRPAPA. The corresponding residues in FA19 MtrE are KLSELRYKAGVISAVALRQQ. As mentioned in the following paragraph, these residues form a loop region between the periplasmic helices H3 and H4 of FA136 MtrE. It was later found that the protein sequence of FA136 MtrE was in error, possibly due to a DNA sequencing error when the sequence of FA136 *mtrE* gene was determined two decades ago. Nonetheless, the crystal structure of the FA136 MtrE outer membrane channel protein (prepared from expression of a synthetic gene based on the FA136 sequence) was determined to a resolution of 3.29 Å (PDB code: 4MT0) [23]. The final model comprises 99 % of the total amino acids (residues 1–445) (Fig. 17.3). The MtrE and OprM channels share 40 % protein sequence identity. However, superimposition of the structure of MtrE with that of OprM [41] results in an RMSD of 18.2 Å over 445 C^{α} atoms, suggesting a highly significant difference in the overall tertiary structures between these two channel proteins. An important insight gained from the MtrE structural data is the recognition of the presence of two, short surface-exposed domains (6 per trimer) that are antigenic and now under investigation as part of the newly rejuvenated vaccine effort (see below); these surface-exposed domains represent residues 93–98 and 300–310 [23].

Like OprM [43], TolC [44], CusC [36, 37], and CmeC [45], MtrE exists as a homotrimer that forms a ~130-Å-long α/β barrel (Fig. 17.3). Each subunit of MtrE contains four β-strands (contributing to the 12-stranded outer membrane β-barrel) and eight α-helices (forming the elongated periplasmic α-barrel): H1 (41–53), H2 (55–78), S1 (85–92), S2 (99–114), H3 (119–182), H4 (188–226), H5 (254–260), H6 (262–284), S3 (288–299), S4 (311–320), H7 (328–395), and H8 (400–435). These four β-strands (S1, S2, S3, and S4) constitute the β-barrel domain and are organized in an antiparallel fashion, spanning the outer membrane. In contrast, the

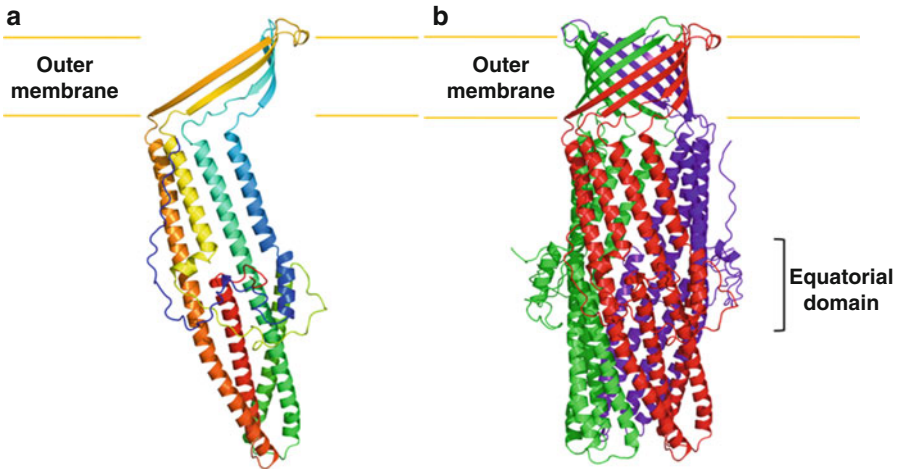

Fig. 17.3 Structure of the MtrE outer membrane channel protein. (**a**) Ribbon diagram of a protomer of MtrE viewed in the membrane plane. The molecule is colored using a rainbow gradient from the N-terminus (*blue*) to the C-terminus (*red*). (**b**) Ribbon diagram of the MtrE trimer viewed in the membrane plane. Each subunit of MtrE is labeled with a different color (This figure has been reproduced from [23])

elongated periplasmic tunnel of MtrE contains six α-helices. Similar to the structure of TolC [44], two long α-helices (H3 and H7) are found to extend across the entire length of the periplasmic α-helical tunnel. The α-helical tunnel of MtrE also includes two pairs of shorter α-helices (H2 and H4) and (H6 and H8). These two pairs of shorter α-helices stack end to end to form pseudo-continuous helices, which contribute coiled-coil interactions with the two long helices. The equatorial domain of MtrE is composed of two α-helices (H1, H5), and the remaining elements at this domain are mostly unstructured. The periplasmic tunnel of MtrE is ~100 Å long with an outermost diameter of ~35 Å at the tip of the tunnel.

To date, all available structures of outer membrane channel proteins, such as TolC [44], OprM [43], CusC [36, 37], and CmeC [45], indicate that the interior surfaces of these channels are highly electronegative. However, MtrE is distinct in that its internal surface does not have extensive positively or negatively charged patches. On the contrary, the charge distribution of the outside surface of MtrE is very similar to other outer membrane channels, in which the outside surfaces of all these channels have no extensive charged patches. In view of the crystal structure of MtrE, the internal surface of the protein forms a continuous channel. This channel is completely open and fully accessible through both the periplasmic end and outer membrane surface, suggesting that the MtrE channel is at its open conformational state. Most of the available structures of outer membrane channels, including TolC, OprM, CusC, and CmeC, are closed at one or both sides [42–45]. The widest section of the channel is located at the surface of the outer membrane, with the internal diameter of ~22 Å. The volume of the continuous channel formed by the internal surface of the MtrE trimer is ~45,000 $Å^3$ [23].

The architecture of the interior of the MtrE channel is quite similar to that of TolC [42, 46]. An aspartate ring is found at the periplasmic entrance of the interior of the MtrE channel. Each protomer of MtrE contributes Asp402 and Asp405 to form two concentric circles of negative charges in the inner cavity of the trimeric MtrE channel [23]. Thus, this interior aspartate ring is composed of six aspartate residues. In TolC, the corresponding aspartate ring creates a selectivity gate for this channel and this ring can be blocked by large cations. The internal diameter of the MtrE aspartate ring is ~12 Å, which creates the narrowest region of the tunnel. It is likely that this aspartate ring is responsible for the selectivity of the channel, similar to the case of TolC [46]. Indeed, it has been demonstrated that the aspartate ring of MtrE can be blocked by the large positively charged hexamminecobalt (III) complex [41]. During the course of substrate import or export, the aspartate ring may still need to dilate more and increase its internal diameter to allow for the passage of substrates through the channel. Although the structure indicates that MtrE is capable of opening this channel by itself, it has been suggested that the dilation and constriction of the aspartate ring may be controlled by the MtrC periplasmic membrane fusion protein [25]. In addition, it has been observed that the MtrE channel is able to allow the large vancomycin molecule to enter the cell but only does so in response to the binding of the membrane fusion protein MtrC adaptor [25], presumably enhancing the degree of dilation of the MtrE channel. It appears that the opening and closing of the MtrE channel may be induced by the change in conformation of the MtrC protein, which propagates the progressive motion of the MtrD multidrug efflux pump within the transport cycle to the MtrE channel. As MtrD is a proton motive force-dependent pump, this may imply that active proton translocation within the MtrD efflux pump provides the energy to open and close the MtrE channel.

As mentioned earlier, it is likely that the sequence of the reported FA136 *mtrE* gene (and the originally predicted MtrE amino acid sequence) was incorrect due to a DNA sequencing error; we thank V. Bavro (University of Birmingham, United Kingdom) for bringing this to our attention. We have started crystallizing the FA19 MtrE channel protein. Hopefully, its structure will be determined and reported in the near future so that a more complete understanding of MtrE can be obtained.

17.3.3 Crystal Structure of MtrF: A Novel Drug Efflux Pump Protein

The MtrF transporter protein was recently implicated as a novel efflux pump protein possessed by gonococci that has the capacity to export antimetabolites and sulfonamide drugs [8]. The capacity of MtrF to export sulfonamide drugs was first realized when recombinant MtrF expressed in *E. coli* reduced levels of bacterial susceptibility to sulfonamides, which was verified by biochemical and genetic

studies using mutant versions of the *mtrF* gene. At the amino acid level, the 522-amino acid MtrF protein belongs to the *p*-aminobenzoyl-glutamate transporter (AbgT) family that typically serve as importers [7]. MtrF shares 38 % identity with *E. coli* AbgT. Interestingly, AbgT has been shown to enable uptake of the folate catabolite *p*-aminobenzoyl-glutamate, but work with MtrF indicates that it can export the folate metabolite paraminobenzoic acid (PABA). Circumstantial genetic evidence also suggests that MtrF can serve as an accessory protein for the MtrCDE efflux system when the latter is overexpressed in strains of gonococci harboring regulatory mutations ([7]; see below).

We have determined the crystal structure of gonococcal MtrF (strain FA19) to a resolution of 3.95 Å (PDB code: 4R1I) (Fig. 17.4) [8]. Crystals of MtrF belong to space group $P6_5$. Two molecules of MtrF, which assemble as a dimer, are found in the asymmetric unit. Superimposition of these two MtrF molecules gives an RMSD of 0.5 Å over 506 C^{α} atoms, indicating that their conformations are nearly identical to each other. The crystal structure of the MtrF dimer reveals a bowl-shaped concave aqueous basin, approximately 75 Å tall, 80 Å wide, and 50 Å thick. The rim of the basin is as large as 45 Å in diameter and penetrates into the inner leaflet of the cytoplasmic membrane by approximately 25 Å. This deep basin probably allows aqueous solution to reach until the midpoint of the membrane bilayer.

Overall, the secondary structure of the MtrF dimer is very similar to that of *Alcanivorax borkumensis* YdaH [47], which also belongs to the AbgT family of transporters. A pairwise superimposition of the MtrF dimer onto YdaH results in overall RMSD of approximately 4 Å. Each molecule of MtrF comprises nine

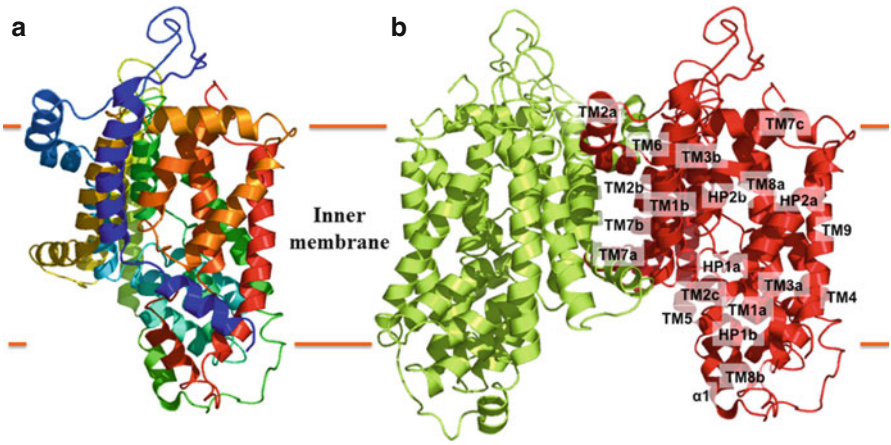

Fig. 17.4 Structure of the MtrF efflux pump. (**a**) Ribbon diagram of a protomer of MtrF viewed in the inner membrane. The molecule is colored using a rainbow gradient from the N-terminus (*blue*) to the C-terminus (*red*). (**b**) Ribbon diagram of a dimer of MtrF viewed in the inner membrane. The right subunit of the dimer is colored *red*, whereas the left subunit is colored *green*. The MtrF dimer forms a bowl-shaped structure with a concave aqueous basin facing the intracellular solution (This figure has been reproduced from [8, 48] (with permission from the publisher))

transmembrane α-helices and two helical hairpins: TM1 (a (12–22) and b (26–47)), TM2 (a (78–92), b (94–112), and c (114–125)), HP1 (a (128–145) and b (147–164)), TM3 (a (168–182) and b (191–205)), TM4 (218–240), TM5 (269–292), TM6 (310–334), TM7 (a (341–353), b (356–374), and c (376–391)), HP2 (a (396–413) and b (417–434)), TM8 (a (438–451) and b (462–471)), and TM9 (480–506). In addition to HP1 and HP2, which are only long enough to span half of the membrane, the transmembrane helices TM1, TM2, TM3, TM7, and TM8 are broken into multiple segments within the membrane. These segmented loops allow the transporter to form an internal cavity within the membrane [8, 48].

Each protomer of MtrF contains a relatively small periplasmic domain. This domain is made up of two long loops formed between TMs 1 and 2 and TMs 5 and 6, respectively. Below the inner leaflet of the membrane, a small cytoplasmic domain links TMs 4 and 5 together. This domain is comprised by a relatively long random loop and helix (α1). The MtrF dimer can also be divided into inner and outer core regions. The inner core, comprising TM1, TM2, TM5, TM6, and TM7, creates a frame-like housing for the inner core and contributes to the dimerization domain. Involved in this dimerization interface are TM1b, TM2a, TM2b, TM6, TM7a, and TM7b, as well as the corresponding segments from the next subunit.

The outer core comprises TM3, TM4, TM8, TM9, HP1, and HP2. Like YdaH [47], the helices and hairpins of the outer core of MtrF form a tunnel spanning approximately from the middle of the inner membrane up to the periplasm. Interestingly, this tunnel is connected to the cytoplasm via an opening in the basin formed by the loop regions of HP1, HP2, TM3, and TM8. Importantly, several conserved residues, including Asp193, Trp420, Pro438, and Asp449, line the wall of the tunnel. It is expected that these residues may play an important role for the function of this transporter. It has also been shown that MtrF is able to export PABA and sulfonamide drugs from the bacterial cell and that the conserved residues Asp193, Trp420, Pro438, and Asp449 are important for this function [8, 48]. Accordingly, these conserved residues are critical for the function of the *N. gonorrhoeae* MtrF efflux pump and strongly support the idea that MtrF act as efflux pumps and participate in exporting the metabolite PABA and sulfonamide antimetabolites from the cell.

17.4 Gonococcal Efflux Pump Genes

Of the five efflux pumps associated with all gonococci strains thus far examined [1, 6, 8], participating proteins of three are encoded by genes organized in an operon (i.e., *mtrCDE*, *farAB*, and *macAB*). The multiple transferable resistance (*mtr*) locus, which includes *mtrF*, was first identified by Pan and Spratt [49] and then further elucidated by Hagman et al. [10, 24], Delahay et al. [42], and Veal and Shafer [7] by conventional cloning and sequencing technologies. The other gonococcal efflux pump systems [13, 14, 17] were identified by mining the FA1090 genome sequence that was made available online by D. Dyer and colleagues in 1998 (http://www.genome.ou.edu).

17.4.1 The mtrCDE *Efflux Pump Operon*

The genetics of the *mtr* system have been extensively studied for the past two decades, and it is the best understood gonococcal efflux pump system to date. Mtr has its origin from a phenotype identified by Maness and Sparling in 1973 [50] when they isolated a spontaneous gonococcal mutant that exhibited increased resistance to multiple, structurally diverse antimicrobial hydrophobic compounds. It was originally thought that the associated, but then unknown, mutation decreased cell envelope permeability by overproducing a 52-kDa outer membrane protein and increasing the degree of peptidoglycan cross-linking [51]. However, subsequent cloning/sequencing experiments in the mid-1990s [10, 49] showed that the Mtr phenotype was due to mutations within the coding region or in a promoter upstream of a gene encoding a transcriptional repressor (MtrR) in the TetR/QacR family [52]. The *mtrR* gene is positioned 250 base pairs (bp) upstream and transcriptionally divergent from the *mtrCDE* operon that encodes the tripartite MtrCDE efflux pump similar to other RND-type pumps (e.g., AcrAB-TolC) of Gram-negative bacteria [10, 24, 42, 49]. As described above, like other RND efflux pumps, the three proteins that form the pump are a cytoplasmic (inner) membrane transporter (MtrD), a membrane fusion protein (also called periplasmic adapter protein; MtrC), and an outer membrane channel protein (MtrE) (Fig. 17.1); as mentioned above, it is most likely that MtrE also serves as the outer membrane channel protein for the FarAB and MacAB efflux pumps (Fig. 17.1; [14, 17] and W. M. Shafer et al. unpublished).

Directly or indirectly, other proteins or outer membrane components also participate in efflux mediated by the MtrCDE pump. In this respect, Veal and Shafer [7] suggested that MtrF could serve as an accessory protein for high levels of antimicrobial resistance mediated by the MtrCDE efflux system. Additionally, energy supplied by the TonB-ExbBD system is needed for inducible antimicrobial resistance mediated by MtrCDE [53] and this induction process requires the participation of a transcriptional activator termed MtrA (see below). Lipooligosaccharide structure is also important in the function of the MtrCDE efflux pump in strains overexpressing *mtrCDE* because a deep rough lipooligosaccharide mutant expressing a core oligosaccharide that was severely truncated was unable to express high-level resistance to substrates of the pump [54].

While the *mtr* locus is highly conserved in gonococci, clinical isolates frequently contain mutations within the *mtrR* structural gene or the associated promoter that increase *mtrCDE* expression and resistance to antimicrobial substrates (see below). The *cis*- or *trans*-acting regulatory mutations are of clinical and biological significance since they appear to be important in the ability of gonococci to resist certain clinically useful antibiotics as well as provide a fitness advantage over wild-type gonococci (see below). Interestingly, a minority of strains, some of which also have *mtrR* mutations, contain small deletions in the *mtrC* or *mtrD* genes that increase gonococcal susceptibility to antimicrobials recognized by the MtrCDE efflux pump [55]. These so-called *env* (*env*elope) mutants were originally identified by the Sparling laboratory in the mid-1970s [56]. The advantage such strains might have

during infection is unclear, but their ability to donate antimicrobial resistance due to *mtrR* mutations is of interest since such genetic exchange may help in the emergence of natural gonococcal variants with decreased susceptibility to efflux pump substrates during infection.

17.4.2 The mtrF *Gene*

The *mtrF* gene was discovered by Veal and Shafer [7] during a study of a gonococcal strain that displayed an *env* phenotype (hypersusceptibility to antimicrobials recognized by the MtrCDE efflux pump) yet contained a wild-type *mtrCDE* operon. Subsequent cloning and sequencing efforts showed that the clinical isolate contained a small deletion in an open reading frame downstream of *mtrR* that would encode a protein within the AbgT family of transporters [8]; the structural aspects of MtrF and AbgT proteins are described above. Interestingly, loss of *mtrF* seemed to have an impact on gonococcal resistance to antimicrobials recognized by the MtrCDE efflux pump only when *mtrCDE* was overexpressed due to *cis*-acting mutations (described below) and the gonococcal strain had a mutant version of the PorB1b porin as opposed to wild-type PorB1b or PorB1a [7]; the latter phenomenon highlights the interplay between membrane components for gonococcal resistance to antimicrobials [57]. In this context, amino acid replacements in PorB1b at position 120 alone (Gly120Lys) or positions 120 and 121 (Gly120Asp/Ala121Asp) are frequently observed in gonococcal strains expressing decreased susceptibility to β-lactam antibiotics [1, 57]. Thus, it was proposed that MtrF serves as an accessory protein used by the MtrCDE efflux pump when gonococci are confronted by high levels of antimicrobials [7]. While this original hypothesis may still be valid, more recent work [8] implicates MtrF as an independent efflux pump that exports sulfonamide antimetabolite drugs. As a member of the AbgT family of proteins that normally import substrates, the illumination of MtrF efflux activity defines a new family of bacterial efflux pumps; continued work is necessary to better understand its roles in gonococcal physiology and metabolism.

17.4.3 The farAB *Efflux Pump Operon*

The *farAB* operon in gonococci was identified by mining the FA1090 genome sequence (http://www.genome.ou.edu) for open reading frames that would encode drug efflux pumps similar to those possessed by other Gram-negative bacteria [17]; the *farAB* gene products are similar to the *emrAB*-encoding efflux pump system of *E. coli*. Expression of *farAB* is regulated by both *cis*- and *trans*-acting systems (see below). As mentioned above, the FarAB efflux pump in gonococci most likely uses MtrE as its outer membrane protein channel (Fig. 17.1) to export long-chain fatty acids such as palmitic and oleic acid. Fecal-derived fatty acids can have potent anti-gonococcal activity under

laboratory conditions [58], and their efflux by the FarAB-MtrE pump may help gonococci survive during rectal infections, which are frequently asymptomatic [1].

17.4.4 The macAB *Efflux Pump Operon*

The *macA* and *macB* genes are organized in an operon, and their predicted products are similar to the MacA and MacB proteins produced by other Gram-negative bacteria [14]. The MacAB efflux pump most likely uses MtrE as its outer membrane protein channel, belongs to the ABC transporter superfamily, and exports macrolides and, at least to some extent, possibly also β-lactams and the fluoroketolide solithromycin (Fig. 17.1; [5]). The promoter that drives expression of *macAB* in strain FA19 has a point mutation in the –10 hexamer sequence that dampens expression of this operon [14]; this mutation has been observed in other gonococcal strains (Reimche et al. unpublished observations). Recent evidence (Kandler et al. manuscript in preparation) suggests that *macAB* expression is enhanced by a response regulator (MisR) of a two-component regulatory system (MisRS).

17.4.5 The norM *Efflux Pump Gene*

The *norM* gene encodes the NorM efflux pump [13], which belongs to the MATE family and exports compounds harboring a quaternary ammonium on an aromatic ring, quinolones, and, at least to some extent, possibly also β-lactams and solithromycin [5]. The *norM* gene is located upstream of open reading frames that have been provisionally annotated (http://www.genome.ou.edu) as encoding the MurB enzyme involved in *N*-acetylmuramic acid biosynthesis and a TetR-like repressor. Additional work is needed, however, to confirm these functions and if the three genes in this region are co-transcribed by a common promoter. Point mutations within the *norM* ribosome binding site and a putative −35 promoter hexamer sequence have been identified, and these likely modulate expression of *norM* and, as a consequence, levels of NorM [13].

17.5 Transcriptional Regulation of Gonococcal Efflux Pump Genes

Discoveries of bacterial efflux pumps were often made prior to the availability of whole genome sequences and were facilitated by the isolation of mutants that expressed decreased susceptibility to antimicrobials [52, 59]. These resistance-conferring mutations frequently mapped to a gene that encoded a DNA-binding protein that would normally dampen expression of a closely linked gene or operon

encoding efflux pump proteins. As an example of this and with respect to gonococci, Pan and Spratt [49] discovered the *mtr* locus in strain CH95 by identifying a mutation in *mtrR*, which encodes a transcriptional repressor (MtrR) of the *mtrCDE* efflux pump-encoding operon (see below). It is now recognized that, in addition to mutations that impact the activity of DNA-binding proteins, *cis*-acting mutations located within promoter sequences can have stronger influences on efflux pump gene expression and levels of bacterial susceptibility to antimicrobials recognized by the cognate efflux pump. Against this background, the complexities of *cis*- and *trans*-acting transcriptional factors that modulate gonococcal efflux pump gene expression and bacterial susceptibility to antimicrobials are described below.

17.5.1 Cis-*Acting Factors that Regulate Efflux Pump Genes*

Cis-acting regulatory mutations can have a profound impact on the expression of gonococcal genes encoding efflux pumps and can be responsible for levels of antimicrobial resistance due to export mechanisms; the most comprehensively studied *cis*-acting regulatory system involves expression of the *mtrCDE*-encoded pump (Fig. 17.5). In this respect, point mutations, deletions, or insertions in the nucleotide sequences between *mtrR* and *mtrCDE* can provide gonococci with higher levels of antimicrobial resistance than mutations within the *mtrR*-coding region. For instance, a single-bp deletion within the 13-bp inverted repeat element localized in the *mtrR* promoter (Fig. 17.5) can significantly enhance transcription of *mtrCDE* [10, 60]. While not all macrolide-resistant clinical gonococcal isolates have this bp deletion [61], it was frequently observed in a panel of strains isolated in Seattle, USA, obtained from men who have sex with men during an outbreak of macrolide-resistant gonorrhea [62]. Some clinical isolates have a dinucleotide insertion within this inverted repeat [63]. In either case, the optimal 17-bp spacing between the −10 and −35 elements is disrupted, and this significantly reduces *mtrR* transcription to nearly undetectable levels. This mechanism, however, cannot be the sole reason why such strains express high-level antimicrobial resistance since their level of *mtrCDE* expression is greater than strains with *mtrR* loss-of-function or null mutations [10]. Instead, because the promoters for *mtrR* and *mtrCDE* transcription overlap and are divergent, it is more likely that the mutations enhance RNA polymerase interactions with the *mtrCDE* promoter.

A limited number of gonococcal strains (e.g., MS11 and WHO L) have a point mutation (C→T) located 120 nucleotides upstream of the *mtrC* translational start codon (mtr_{120}), changing the sequence at this region from TATAAC to TATAAT [19, 64] and thereby generating a consensus −10 element (Fig. 17.5) [65]. This new −10 element acts as a stronger promoter (mtr_{120}) for *mtrCDE* transcription than the wild-type promoter, and its use results in high levels of *mtrCDE* expression and resistance to hydrophobic agents, as well as enhanced *in vivo* fitness [19]; importantly, this new promoter is outside of the control by MtrR, which exerts repressive activity on the wild-type promoter. The identification of this novel promoter that enhanced

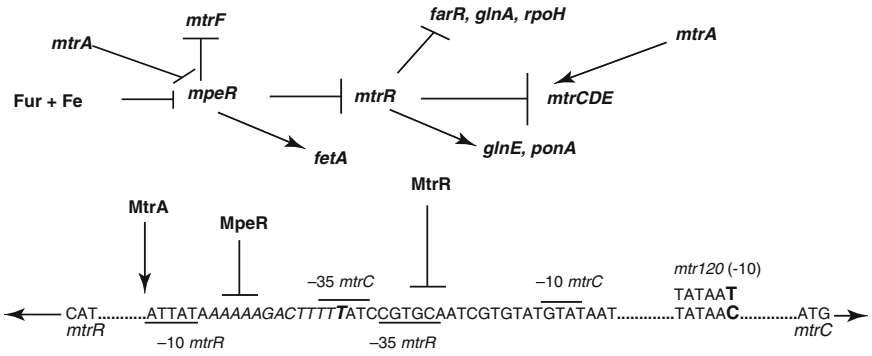

Fig. 17.5 Regulatory features of the *mtr* locus. Shown are the *cis*- and *trans*-acting regulatory elements that control expression of the *mtrCDE* operon as well as "off-target" genes (see text for details). Negative regulation of genes by DNA-binding proteins is shown by the *barred lines*, while transcriptional activation of genes by proteins is shown with *arrows*. Sites of binding by MtrR, MtrA, and MpeR are shown. The main mutations impacting promoters are the single-base pair (bp) deletion in the *mtrR* promoter region, which is designated by the larger font T nucleotide as is the mutation that generates the novel mtr_{120} promoter. The nucleotide sequence shown represents the *mtrCDE* coding strand; the translational start codons for *mtrR* and *mtrC* are shown at the ends of the sequence. The 13-bp inverted sequence within the *mtrR* promoter is shown in italics (This figure has been modified from [1])

transcription of *mtrCDE* independently of MtrR repression allowed Ohneck et al. [66] to study the genome-wide transcriptional influence that might result when the MtrCDE pump is produced at elevated levels. Such a study had not been possible previously since all other mutants that overexpressed *mtrCDE* did so in a background where MtrR would be absent making it difficult to know if any observed transcriptional changes were influenced by this transcriptional regulator. In brief, use of the mtr_{120} promoter (Fig. 17.5) in an otherwise wild-type background resulted in increased expression of 13 genes outside of the *mtr* locus. Included in this set of genes was *ccp*, and its overexpression due to elevated expression of *mtrCDE* was correlated with decreased susceptibility of gonococci to peroxides. Thus, it is important to realize that overexpression of bacterial efflux pumps can have unexpected consequences on gene expression that could influence metabolism and resistance to antimicrobials that are not pump substrates.

Some gonococcal strains, notably those from an outbreak of azithromycin-resistant gonorrhea in Kansas City, USA, in 1999 [67], contain a 153-bp insertion in the *mtrR-mtrCDE* intervening region. These isolates expressed resistance to MtrCDE efflux pump substrates. Interestingly, this (or a closely related) insertion, identified as a Correia element (CE) [68], can be found within this region of the *mtr* locus in many, but not all, strains of *Neisseria meningitidis* (meningococci) [69, 70]. Some serogroup Y meningococcal strains also have a tandemly linked IS*1301* sequence [69]. In meningococci, the presence of the Correia element dampened *mtrCDE* expression as a result of providing a binding site for integration host factor (IHF) and a new site for posttranscriptional processing of the *mtrC* transcript [71];

IHF is also important in regulating the *farAB* efflux pump operon (see below). It is unclear how the Correia element and perhaps more importantly the IHF-binding site and IHF function in gonococci since hydrophobic agent resistance is elevated in strains bearing the Correia element sequence. Nevertheless, the presence (albeit rare) of the Correia element at this site in gonococci suggests that horizontal gene transfer occurred between meningococci and gonococci. If true, this event emphasizes the importance of recombination in generating diversity in clinical strains of both pathogens.

Expression of *norM* and *macAB* genes is also modified by *cis*-acting control elements that were defined by mutations in gonococcal clinical isolates. The presence of point mutations in the −35 hexamer of the *norM* promoter (C to T) or in a putative ribosome binding site (A to G; reference 70 for consensus sequence) can enhance gonococcal resistance to all substrates of the NorM pump [13]. A point mutation in the −10 hexamer of the *macAB* promoter (G to T) has been identified that increases expression of *macAB* and levels of macrolide resistance in gonococci [14]. Together with other mutations that impact antimicrobial susceptibility levels in gonococci, the presence of these *cis*-acting mutations could influence the efficacy of antimicrobial therapy, particularly for strains with susceptibilities near the minimal inhibitory concentration (MIC) breakpoint for the relevant antimicrobials. Like the mtr_{120} mutation for *mtrCDE*, the point mutations found to regulate *norM* and *macAB* alter the sequence of their respective promoter elements to be closer to consensus [65]. Thus, it appears that point mutations within bacterial promoters can upregulate gene expression by enhancing promoter recognition by RNA polymerase outside of the control of transcriptional regulators or, in the case of *norM*, may also increase translation through improved ribosomal recognition of the *norM* transcript.

17.5.2 Trans-*Acting Factors that Regulate Efflux Pump Genes*

A number of DNA-binding proteins directly or indirectly control expression of genes encoding structural proteins of bacterial drug efflux pumps [51, 58], including those of gonococci [12]. Their function proved crucial in early studies dealing with the identification of cognate efflux pump-encoding genes [52]. For gonococci, the DNA-binding proteins that control *mtrCDE*, *mtrF*, and *farAB* expression are especially understood and are the subject of discussion below.

DNA-binding proteins that control *mtrCDE* and *mtrF* gene expression Expression of genes within the *mtr* locus is directly or indirectly controlled negatively or positively by a number of DNA-binding proteins (summarized in Fig. 17.5 and references [1, 6, 12]). Central to this regulation is MtrR, which was first described by Pan and Spratt [49] that acts to repress *mtrCDE* expression by binding as two homodimers to the *mtrCDE* promoter region [72, 73]. Studies on antimicrobial-resistant clinical isolates have shown that such strains often contain mutations that cause radical amino acid replacements in the helix-turn-helix (HTH)

motif (residues 32–53) of MtrR that decrease its binding to target DNA sequences [72] while other mutations map outside of the HTH region that might alter dimer formation or drug interactions. All of these loss-of-function mutations can enhance *mtrCDE* transcription two- to threefold and, depending on the substrate, increase resistance to antimicrobials by four- to tenfold [11, 74]. It is important to emphasize that, because loss-of-function mutations of *mtrR* have been observed in gonococcal clinical isolates, their occurrence is relevant for considering antibiotic treatment regimens used worldwide.

In addition to its prominent regulatory action on the *mtrCDE* promoter, MtrR can directly or indirectly control expression of a number (>65) of other genes at different phases of growth [75]. Cumulative results from genetic and transcriptional profiling studies identified MtrR as a global regulatory protein. Briefly, MtrR has been shown to control genes involved in the generalized stress response (*rpoH*) [75], peptidoglycan biosynthesis (*ponA*) [76], amino acid biosynthesis (*glnA* and *glnE*) [77, 78], polyamine uptake (*potF and potH*) [75], and regulation of a regulator (*farR*) of the *farAB* efflux pump operon [79]; the capacity of MtrR to activate or repress expression of a non-*mtr* related genes is shown in Fig. 17.5. We have termed these genes as being "off-target," since MtrR is acting as a *trans*-regulator to distinguish them from the adjacent *mtrCDE* genes [77].

Expression of *mtrR* is negatively controlled by the product of the *mpeR* gene [80], which in turn is negatively controlled by Fur (ferric uptake regulator) + iron [81] and the MtrA transcriptional activator of *mtrCDE* (Fig. 17.5; [1]). This result suggests that levels of antimicrobial resistance and *in vivo* fitness due to the MtrCDE efflux pump could be influenced by the availability of free iron during infection or inducing agents that work through MtrA (see below). Accordingly, it is important to realize that MIC values obtained for gonococci grown on iron-rich laboratory media may not reflect absolute susceptibility of the test strain during infection and these MICs might accordingly be higher *in vivo*. MpeR was discovered during a search of the FA1090 genome sequence for regulators [81], in addition to MtrR, that might control expression of *mtrF* [82]. The capacity of Fur + iron to repress *mpeR* and the ability of MpeR to repress *mtrR* likely explain why *mtrCDE* is maximally expressed late in growth when levels of free iron would be low [81]. It has been shown that MpeR activates expression of *fetA* [83], which encodes a single-component TonB-dependent receptor that allows the gonococcus to acquire iron from enterobactin-like siderophores produced by enteric bacteria [84]. Taken together, these findings show that MpeR plays important roles in both drug efflux and iron acquisition by gonococci, emphasizing the need to consider regulators of efflux genes in a larger context that includes general concepts of bacterial physiology and pathogenesis.

Gonococci, like other bacteria, can activate transcription of drug efflux pump-encoding genes when confronted with antimicrobial substrates. In this instance, transcriptional activators typically bind "inducers" (pump substrates) and then more effectively interact with DNA sequences that are usually located near the efflux pump-encoding genes. The best example of this phenomenon in gonococci is the action of MtrA [20], a member of the AraC family of transcriptional regulators such

as Rob, which is critical in activating transcription of efflux pump-encoding genes ([85]; see [1, 86] for summary reviews). MtrA was discovered during experiments that evaluated whether growth of the gonococci in sublethal level of substrates recognized by the MtrC-MtrD-MtrE efflux pump could enhance bacterial resistance to such antimicrobials. A panel of strains was tested and some were found to increase their resistance to Triton X-100 by an MtrA-dependent manner when incubated overnight in sublethal levels of Triton X-100 [20]. Interestingly, many gonococcal isolates [20, 87], including strain FA1090 [20], contain an 11-bp deletion within *mtrA* and are unable to transcriptionally activate *mtrCDE* in the presence of an inducer. This MtrA-dependent inducible resistance property requires energy delivered from the TonB-ExbBD system. MtrA has been purified, and its DNA-binding action upstream of the *mtrCDE* promoter has been defined [88]). Consistent with Triton X-100 being an inducer, its presence increased MtrA binding to its target DNA and even outcompeted MtrR in the latter's binding to the *mtrCDE* promoter (Fig. 17.5; [88]). This result provides a mechanism for upregulation of *mtrCDE* expression in the presence of an inducer. MtrA, like MtrR and MpeR, has global regulatory action and can modulate expression of a number of genes outside of the *mtr* locus (W. M. Shafer et al. unpublished) including direct or indirect repression of *mpeR* (Fig. 17.5; [1]).

DNA-binding proteins that regulate the *farAB* operon The *farAB* operon in gonococci is subject to both direct and indirect regulation by DNA-binding proteins. First, it is subject to direct repression by FarR, a member of the MarR family of transcriptional regulators, by its interaction with the *farAB* promoter [79]. This binding is enhanced by the DNA-binding/DNA-bending action of IHF [89]. Second, but indirectly, transcription of *farAB* is controlled by MtrR as this protein binds to the *farR* promoter resulting in dampening of *farR* transcription [79]. Thus, with opposing consequences, MtrR can directly and indirectly regulate levels of two gonococcal efflux pump operon systems, which results in repression of one operon (*mtrCDE*) and activation of another (*farAB*).

17.6 Efflux Pumps as Contributors to Gonococcal Antimicrobial Resistance

From a historical perspective (summarized in [1, 6]), the first line of evidence that an efflux pump could impact levels of gonococcal susceptibility to antimicrobials can be gleaned from the early work of the Sparling laboratory conducted in the 1970s [50, 90]. Their studies on antimicrobial resistance showed that the then undefined *mtr* mutation, now known to be the single-bp deletion in the inverted repeat sequence of the *mtrR* promoter region (Fig. 17.5), could increase gonococcal resistance (two- to eightfold) to relatively hydrophobic antibiotics such as erythromycin, rifampicin, chloramphenicol, and tetracycline, as well as benzylpenicillin. This increased antimicrobial resistance afforded to gonococci by overexpression of the MtrCDE efflux pump was not by itself clinically significant. However, subsequent

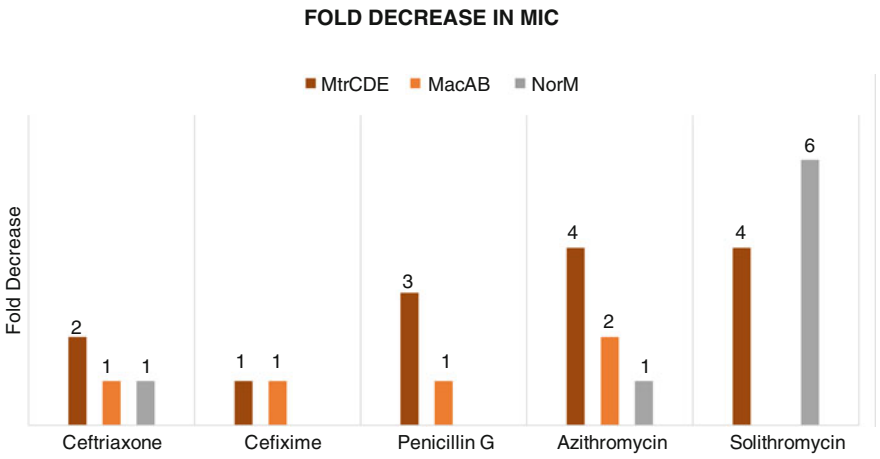

Fig. 17.6 Loss of gonococcal efflux pumps MtrCDE, MacAB-MtrE, and NorM increases bacterial susceptibility to antimicrobials. The impact of loss of individual pumps on gonococcal (strain HO41) susceptibility to selected antimicrobials is shown as fold decrease in MIC relative to parental strain HO41 (as designated by the numeral over each bar). A onefold difference represents a one dilution change in the susceptibility of the mutant compared to parent strain HO41, and the absence of a bar signifies that loss of the respective pump had no impact on the MIC value (The figure was adapted from data reported by Golparian et al. [5])

work by the Sparling group, and later by the Nicholas laboratory, showed that coresident mutations that altered the structure of the β-lactam targets penicillin-binding protein 2 (PBP2) and PBP1 [91, 92], as well as the porin PorB1b [57, 93], which decrease the influx of antimicrobials, would act with the *mtr* mutation to provide full penicillin resistance.

In hindsight, the findings of Sparling and coworkers were remarkable in that we now can better appreciate that, while overexpression of bacterial drug efflux pumps may not provide bacteria with clinical resistance to antimicrobials, it can in conjunction with other mutations be of clinical importance. Work with the *mtr* system in gonococcal clinical isolates such as strain FA6140, which caused the penicillin-resistant (non-β-lactamase) outbreak of gonorrhea in North Carolina, USA, in 1983 [94], and others is an example of such cooperativity of mutations in providing clinically relevant levels of resistance to antimicrobial drugs. Veal et al. [11] showed that loss of the MtrCDE efflux pump rendered this isolate clinically sensitive to penicillin. FA6140 contains the single-bp deletion in the *mtrR* promoter as well as mutations in *ponA* (encoding PBP1), *penA* (encoding PBP2), and *porB1b* (encoding PorB1b). Zarantonelli et al. [63] further showed that *mtrR* promoter mutations that increased expression of *mtrCDE* provided gonococci with low, but clinically relevant, levels of azithromycin resistance and that this could be reversed by genetic inactivation of the *mtrCDE* efflux pump operon. More recently, Golparian et al. [5] showed that loss of the *mtrCDE* efflux pump system in clinical isolates, including the infamous HO41 strain from Japan [95], which caused the first reported case of high-level ceftriaxone-resistant gonorrhea, reversed both penicillin and azithromycin resistance; resistance to extended-spectrum cephalosporins (cefixime and ceftri-

axone) was reduced by up to twofold and solithromycin by fourfold (Fig. 17.6; [5]). In addition to the contributions of the MtrCDE pump, the NorM and MacAB-MtrE efflux pumps can contribute to gonococcal resistance levels to certain antibiotics. Based on results from MIC determination assays that compared wild-type and efflux pump-deficient mutant strains, NorM antimicrobial substrates seem to include quinolones, possibly β-lactams, and solithromycin, while MacAB-MtrE substrates include macrolides and solithromycin (Fig. 17.6; [5, 13, 14]). Taken together, it is now clear that the presence and overexpression of gonococcal efflux pumps can contribute significantly to bacterial resistance to antimicrobial drugs used previously (e.g., penicillin), presently (azithromycin and cephalosporins) or in the future (solithromycin) in gonorrhea treatment regimens. As is discussed below, efforts that target these pumps in drug therapy or vaccines may help in future treatment protocols or prevention strategies.

17.7 Gonococcal Efflux Pumps as Virulence Factors

In the absence of classical antibiotics, does expression of an efflux pump provide an advantage for gonococci (or any bacteria) during infection? This is a critical question because contributions of efflux pumps to bacterial survival prior to appearance of symptoms may influence proliferation and dissemination of the invading pathogen before antibiotic therapy begins. Results from studies on the MtrCDE efflux pump suggest that this is the case since it was required for gonococcal survival during experimental infection of the lower genital tract of female mice [18]. Loss of this efflux pump increases gonococcal susceptibility to intracellular killing by mouse and human polymorphonuclear leukocytes (A. E. Jerse et al. unpublished) and mouse macrophages, but not human ME180 cervical epithelial cells (D' Andrea and Shafer, unpublished). Importantly, differential expression of the *mtrCDE* operon by the transcriptional regulators MtrA and MtrR can modulate fitness levels of gonococci in the murine infection model [16, 19]. Briefly, loss of MtrA in strain FA19 decreased *in vivo* fitness of gonococci by ca. 1,000-fold, while null mutations in *mtrR* or promoter mutations (e.g., single-bp deletion in the *mtrR* promoter or the novel mtr_{120} promoter) increased *in vivo* fitness by ca. 100- and 1,000-fold, respectively. In total, these results with the murine experimental infection model suggest that the MtrCDE efflux pump is important for gonococci to survive *in vivo* when challenged by mediators of innate host defense [16, 19]. This capacity to resist mediators of the innate host defense may be important during human infection because gonococci elicit a strong T_H17 pro-inflammatory response [96], and such a response is often characterized by enhanced production of antimicrobial peptides. The capacity of gonococci to export free long-chained fatty acids (e.g., palmitic and oleic acid) via the FarAB-MtrE efflux pump may help it survive in the rectum where concentrations of such fatty acids are elevated [58, 74]; however, direct experimental evidence is lacking since FarAB-MtrE was not required for *in vivo* survival in the genital tract of female mice, and currently, there is no animal model of rectal infection by gonococci [18].

Based on the above observations, one might ask: why did gonococci not dispense with MtrR since its loss increases antimicrobial resistance and *in vivo* fitness? It is possible that so-called "off-target" genes activated by MtrR (see above) hold the key. Thus, in the mouse infection experiments performed by Warner et al. [16], loss of MtrR afforded a fitness advantage for the first 5 days, but this advantage waned, suggesting that possession of MtrR may be advantageous at later stages of infection. One of these MtrR-activated genes that may be important *in vivo* is *glnE* [75, 78], which is of importance in *Salmonella enterica* serovar Typhimurium growth and fitness *in vivo* [97]. GlnE encodes the enzymatic regulator of glutamine synthetase and can activate or deactivate the enzyme. Since levels of glutamine are low at mucosal surfaces and within phagocytes, the ability to synthesize glutamine *in vivo* may be important for survival and maximal fitness of gonococci. A possible advantage for MtrR-producing gonococci is also suggested by work of Kunz et al. [98], which concentrated on the impact of *gyrA* and *parC* mutations on gonococcal fitness during infection; these mutations confer gonococcal resistance to fluoroquinolones. In relationship to *mtrR*, a spontaneous mutant recovered from mice infected with a strain bearing *gyrA* and *parC* mutations as well as a single-bp deletion in the *mtrR* promoter displayed increased fitness. This mutant had, surprisingly, repaired the deletion such that a wild-type *mtrR* promoter would exist, but the reason why this *in vivo* evolved mutant had a competitive advantage over the parental strain remains unclear [98]. Additional work that uses transcriptional profiling and whole genome sequencing to identify additional mutations that might contribute to this phenotype and construction of mutant strains in this genetic background is needed to pinpoint the mechanism(s) by which this mutant strain has a competitive advantage *in vivo*.

17.8 Targeting Gonococcal Efflux Pumps for Drug or Vaccine Development

Given the importance of the MtrCDE efflux pump in resistance to classical antibiotics and host-derived antimicrobials, would it be beneficial to target this pump for future drug development (e.g., EPIs) or vaccine development? Using EPIs, coadministered with antimicrobials, could allow for the return of previously used and relatively inexpensive antibiotics (e.g., penicillin [5]) and/or render gonococci more susceptible to host-derived antimicrobials, thereby promoting natural clearance by the host. Furthermore, if the EPI has the identical binding site on the efflux pump as the antimicrobial administered, a target modification would be disadvantageous for the bacteria, and this would likely also suppress the resistance development. Below, two recent approaches that target the MtrCDE efflux pump are described.

Efforts to develop safe and effective EPIs have to date failed largely due to toxicity issues [99]. It is important to emphasize, however, that these EPIs targeted inner membrane transporters (e.g., MtrD-like proteins) and cross-recognition by eukaryotic importers/exporters may explain the observed toxicity. A proposed alternative

strategy [100] would be to target the membrane fusion/adapter component (MtrC-like proteins) of the RND pumps as their structures do not have eukaryotic homologues. Indeed, one of our laboratories (E. W. Yu) is developing peptides that bind MtrC and prevent assembly of the Mtr pump *in vitro*. Follow-up studies are in progress to determine if they can sensitize gonococci to antimicrobial drugs such as β-lactams and macrolides.

After decades of relative futility in developing a vaccine to prevent gonorrhea, renewed efforts are now underway by several international groups to develop a gonorrhea vaccine [101]. To a large extent, these efforts have been driven by the emerging problem of antimicrobial resistance and the fear of untreatable infections in the future. One of us (A. E. Jerse) is targeting MtrE as it has two short immunogenic peptide sequences exposed on the surface (see Sect. 17.3.2 above; [23]). The value of targeting MtrE is that it is highly conserved among gonococci, stably produced, and most likely used by at least three efflux pumps (MtrCDE, FarAB-MtrE, and MacAB-MtrE) to export antimicrobials (Fig. 17.1). In preliminary experiments, female mice immunized with recombinant MtrE lacking the first two N-terminal amino acids in conjunction with CpG adjuvant were found to clear gonococci faster than mice immunized with MtrE + cholera toxin adjuvant or CpG adjuvant alone. An important consideration herein is that the CpG adjuvant would drive a T_H1 response thought to be important in generating protective immunity against gonococci [96, 101]. Although these studies are in their infancy, the initial results give hope that MtrE could be part of a future vaccine to at least protect at-risk populations from gonorrhea. It should also be noted that anti-MtrE antibodies might also sensitize gonococci to antimicrobials (e.g., β-lactams and macrolides) recognized by the MtrCDE efflux pump system, which would in theory counteract efflux-dependent resistance mechanisms.

17.9 Concluding Remarks

As has been recently emphasized [1, 3], the public health problem of multidrug-resistant gonococcal strains is becoming more severe, and strains expressing clinical resistance to extended-spectrum cephalosporins would significantly impact therapeutic options in controlling gonorrhea worldwide. In the absence of new antimicrobials that recognize novel targets, this problem will only worsen, and new efforts in the drug development field are required. Based on the considerable progress made in the past 20 years on gonococcal efflux pumps with respect to their identification, structure, function, gene regulation, and important contributions to antimicrobial resistance and virulence, which have been the major topics covered herein, we posit that targeting efflux pumps for drug/vaccine development has merit and should be part of the current anti-infective pipeline to combat gonorrhea. Finally, given the importance of efflux pumps in antimicrobial resistance in gonococci, these basic research efforts over the past decades have helped to contribute to

a better understanding of the genetics, gene regulation, structure, and biologic functions of bacterial efflux pumps in general.

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Chapter 18
Efflux Pumps in *Campylobacter*: Key Players for Antimicrobial Resistance and Environmental Adaption

Lei Dai, Zhangqi Shen, Edward W. Yu, and Qijing Zhang

Abstract *Campylobacter jejuni*, a Gram-negative organism, is a major enteric pathogen causing gastroenteritis in humans. Antimicrobial-resistant *Campylobacter* is increasingly prevalent, posing a serious threat to public health. *Campylobacter* species have developed multiple mechanisms for antimicrobial resistance, including active extrusion of antimicrobial agents using efflux transporters. Some of these transporters have been well characterized, revealing their important functions in conferring resistance to antibiotics used for clinical therapy, organic arsenic compounds used for animal production, and antimicrobials naturally present in animal host. Among the characterized efflux transporters in *Campylobacter*, the CmeABC pump of the resistance-nodulation-cell division superfamily serves as the primary efflux system and is essential for *Campylobacter* colonization in the intestinal tract owing to its key role in bile resistance. Several transcriptional regulators, such as CmeR, CosR, and ArsR, are found to modulate the expression of these efflux transporters in response to the presence of inducers, which are normally the substrates of the corresponding efflux systems. In this chapter, we discuss the current knowledge on antimicrobial transporters in *Campylobacter*, with an emphasis on structure, function, and regulation. Additionally, we describe inhibition of antimicrobial efflux as a potential approach to combating antimicrobial-resistant *Campylobacter* spp.

Keywords *Campylobacter jejuni* • Antimicrobial resistance • Efflux pumps • Bile resistance • RND • CmeABC • CmeR • CosR • ArsR

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18.1 Introduction

Thermophilic *Campylobacter* species, particularly *Campylobacter jejuni*, have been recognized as a major cause of acute bacterial gastroenteritis in humans since the late 1970s [1]. As an enteric organism and a foodborne pathogen, *Campylobacter* is carried in the intestinal tracts of a wide range of domestic animals and wildlife and is transmitted to humans via contaminated food and milk [2, 3]. *C. jejuni* is one of the world's most prevalent enteric bacterial pathogens and thus poses a large public health burden [4–6]. In addition to causing enteritis, *C. jejuni* is also associated with other extraintestinal conditions, such as Guillain-Barré syndrome and irritable bowel syndromes. With more than 800,000 annual cases of campylobacteriosis in the USA and 400–500 million worldwide [1, 7], development of antimicrobial resistance in *Campylobacter* is a serious issue. Clinical treatment of campylobacteriosis requires the use of fluoroquinolone or macrolide antimicrobials, but antimicrobial-resistant *Campylobacter* is increasingly prevalent. The U.S. Centers for Disease Control and Prevention (CDC) reported that almost 25% of *Campylobacter* were resistant to fluoroquinolones in the USA [8], and the resistance rates are much higher (up to 100%) in some other countries [5, 9–19]. Development and transmission of antimicrobial-resistant *Campylobacter* is complicated by the fact that *Campylobacter* is a zoonotic pathogen and is exposed to antimicrobial agents used in both animal production and human medicine. Due to the rising antimicrobial resistance trend, the CDC has recently identified drug-resistant *Campylobacter* as a serious antimicrobial resistance threat in the USA [8].

Similar to other bacterial organisms, *Campylobacter* has acquired multiple mechanisms for antimicrobial resistance [20, 21], including synthesis of drug-inactivating enzymes, alteration or protection of drug targets, reduced permeability to drugs, and active extrusion of drugs via efflux transporters. Antimicrobial efflux transporters are significant players in drug resistance, and *C. jejuni* possesses multiple drug efflux transporters, which contribute significantly to intrinsic and acquired resistance to multiple antimicrobials [22–26]. In addition, the efflux pumps have important physiological functions, facilitating adaptation of *Campylobacter* to various environments and conditions [27–31]. In this chapter, we summarize the function and regulation of these efflux systems in *Campylobacter*, with an emphasis on their roles in antibiotic resistance, arsenic resistance, and oxidative stress response. Additionally, the potential of inhibiting efflux as an anti-*Campylobacter* approach is also discussed.

18.2 The RND-Type Efflux Systems: Function, Regulation, and Structure

There are two multidrug efflux systems of the resistance-nodulation-cell division (RND) superfamily in *C. jejuni*: CmeABC and CmeDEF [22, 32, 33]. Each of these two systems consists of three components including an outer membrane protein

(CmeC/CmeD), an inner membrane transporter (CmeB/CmeF), and a periplasmic fusion protein (CmeA/CmeE). CmeABC is the predominant efflux pump in *Campylobacter* [22, 23, 33]. The genes encoding CmeA, CmeB, and CmeC are organized in a single operon and are widely distributed in both *C. jejuni* and *Campylobacter coli*. CmeABC contributes to resistance to structurally diverse antimicrobials by reducing drug accumulation within bacterial cells, and its inactivation significantly increases the susceptibility of *Campylobacter* to multiple classes of antimicrobials [22–24, 34, 35].

Notably, the CmeABC efflux pump is a significant player in the resistance to fluoroquinolone antimicrobials [22–25], which are clinically important for the treatment of campylobacteriosis. CmeABC reduces the accumulation of fluoroquinolones in *Campylobacter* cells and functions synergistically with the *gyrA* mutations in conferring and maintaining high-level fluoroquinolone resistance in clinical isolates [24, 25]. Inactivation of CmeABC led to at least 24-fold reduction in the minimal inhibitory concentration (MIC) values of ciprofloxacin for various fluoroquinolone-resistant isolates harboring resistance-conferring *gyrA* mutations. This finding indicates that the function of CmeABC is essential for achieving clinically relevant resistance to fluoroquinolones. Additionally, CmeABC plays an important role in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure because many of the spontaneous *gyrA* mutants are not able to survive the killing effect of ciprofloxacin in the absence of CmeABC [36]. Inactivation of cmeB greatly reduced the frequency of emergence of fluoroquinolone-resistant mutants compared to the wild type background, while overexpression of *cmeABC* increased the frequency of emergence of these mutants that are highly resistant to ciprofloxacin [36]. Together, these findings establish the key role of CmeABC in fluoroquinolone resistance and the emergence of fluoroquinolone-resistant mutants under antimicrobial selection.

CmeABC is also important in mediating resistance to macrolides, which represent another drug class of choice for clinical therapy of campylobacteriosis. Insertional mutagenesis of *cmeABC* led to a significant reduction of erythromycin MIC in *Campylobacter* [22, 23, 34, 37, 38]. In the macrolide-resistant mutants that lack known target mutations, inactivation of CmeABC completely reversed to susceptibility [34, 38], while in those mutants that harbor resistance-associated mutation in L4, L22, or 23S rRNA, CmeABC functions synergistically with the target mutations in conferring the acquired resistance to macrolides such as erythromycin and tylosin [34, 37, 38]. In addition, temporal overexpression of *cmeABC* preceded the occurrence of resistance-conferring mutations in 23S rRNA during the development of macrolide resistance, suggesting that the increased expression of this efflux system facilitates the development of high-level macrolide resistance [39].

In addition to conferring resistance to antimicrobial drugs, CmeABC is also a key player in the resistance to bile salts and is essential for *Campylobacter* colonization in the intestinal tract [27, 40]. The CmeB or CmeC mutant grows normally in conventional media, but shows a severe growth defect in bile-containing media. Using a natural host (chicken) of *Campylobacter* as a model system, it was demonstrated that the CmeB or CmeC mutant failed to colonize the inoculated birds, while

complementation of the mutants with a wild type *cmeABC* allele *in trans* fully restored the growth of the CmeB mutant in bile-containing media and its ability to colonize chicken cecum [27]. Additionally, bile compounds induce the expression of *cmeABC* [28], suggesting that *cmeABC* is overexpressed in animal intestinal tracts. Since *C. jejuni* reside in the host intestinal tract, where bile compounds are normally present in high concentrations, bile resistance conferred by CmeABC may have been evolutionally selected and maintained. These findings provide compelling evidence that bile resistance is a natural function of CmeABC.

CmeDEF is another RND-type efflux pump encoded by a three-gene operon (*Cj1031-1032-1033* in *C. jejuni* NCTC 11168 genome), but its contribution to antimicrobial resistance is only modest [33]. Thus, CmeDEF does not play a significant role in antimicrobial resistance as determined under conventional culture conditions. Interestingly, deletion of either *cmeABC* or *cmeDEF* did not affect the viability of C. *jejuni*, but double inactivation of both appeared to be lethal to certain strains (such as NCTC 11168) and reduced the growth of others (e.g., strain 81–176, a frequently studied virulent strain originally isolated from an outbreak of *C. jejuni* diarrhea) in Mueller-Hinton broth [33], suggesting complementary role of these two efflux systems and that their interplay is involved in maintaining cell viability in *Campylobacter*. However, the exact function of CmeDEF and its regulatory mechanisms are still unknown.

The *cmeABC* operon is subject to regulation by two transcriptional factors: CmeR and CosR. The *cmeR* gene is located immediately upstream of *cmeABC* (Fig. 18.1) and encodes a 210-amino-acid protein belonging to the TetR family of transcriptional regulators. CmeR binds to a specific site in the promoter of cmeABC and inhibits the expression of the *cmeABC* operon [41]. Mutations either in the binding site or in the coding sequence of CmeR can reduce CmeR binding to DNA, resulting in overexpression of *cmeABC* [42]. CmeR is a pleiotropic regulator and modulates the expression of additional genes in *C. jejuni* [29]. Particularly, CmeR also directly controls the expression of Cj0561c (a periplasmic fusion protein) and Cj0035c (a major facilitator superfamily [MFS] transporter). CmeR has a dimeric two-domain structure with a DNA-binding motif and a large flexible ligand-binding pocket [43, 44]. Interaction of ligands with the pocket induces a structural change in the DNA-binding motif, preventing CmeR binding to the target DNA. Known inducers of CmeR are bile salts and salicylate, both of which are substrates of CmeABC and increase expression of the efflux operon [28, 45]. As bile is normally present in the intestinal tract, CmeR is expected to assume an induced state during *in vivo* infection, which is indicated by overexpression of *cmeABC* in the intestinal tract [46]. Importantly, the CmeR regulon plays an important role during *in vivo* infection as inactivation of *cmeR* or its regulated transporters (CmeABC, Cj0561c, etc.) severely affected the fitness of *C. jejuni* in the intestinal tract [29, 47].

Recently, it was found that *cmeABC* is also regulated by CosR, a key regulator for oxidative stress response in *Campylobacter* [31]. CosR binds to a site that is 17 bp upstream of the CmeR binding site in the promoter region of *cmeABC* and represses the expression of this efflux operon [31]. CosR is predicted to be a response regulator of a two-component regulatory system, but it is an orphan

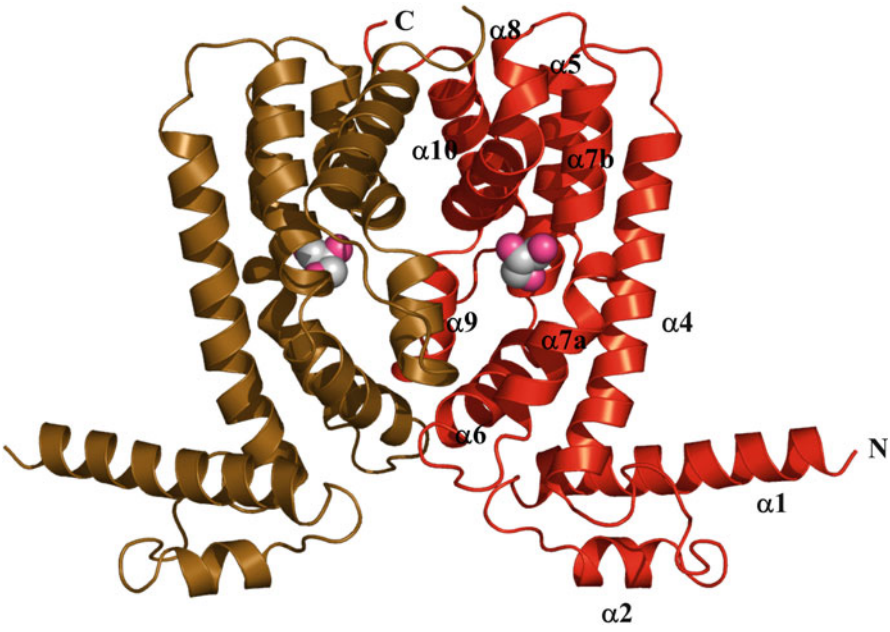

Fig. 18.1 Structure of the *C. jejuni* CmeR transcriptional regulator. Ribbon diagram of the CmeR homodimer bound with glycerol (in balls). The CmeR molecules are colored *red* and *brown*

response regulator and lacks a cognate sensor kinase. The consensus phosphate-accepting aspartate residue in the canonical phosphorylation site of CosR is replaced by asparagine (Asn51) [48], and a recent study indicated that CosR was not phosphorylated [49]. Thus, how the function of CosR is modulated is unknown. CosR appears to be essential for *C. jejuni* since an attempt to construct the deletion of the coding gene was not successful. Inhibition of CosR by an antisense peptide nucleic acid (PNA) led to overexpression of *cmeABC* as determined by both transcriptional fusion and immunoblotting [31]. In addition to inhibiting *cmeABC*, CosR also modulates (both activates and inhibits) the expression of multiple genes involved in oxidative stress response, such as *ahpC* (encoding alkyl hydroperoxide reductase) and *sodB* (encoding superoxide dismutase) [48]. These findings suggest that oxidative stress response and the antibiotic efflux system are interactive in *C. jejuni*, and CosR plays a bridging role in the interaction.

The crystal structure of CmeR was determined at a resolution of 2.2 Å [43]. The structure revealed a dimeric two-domain molecule with an entirely helical architecture similar to members of the TetR family of transcriptional regulators (Fig. 18.1). Each subunit of CmeR contains nine α-helices. CmeR is unique in that it is the only regulator in the TetR family that lacks the N-terminal helix-turn-helix (HTH) DNA-binding motif, in which the recognition helix α3 is replaced by a random coil [43, 50]. In addition, it possesses a large center-to-center distance (54 Å) between the two N-termini of the dimer, making it incompatible to bind *B*-DNA. The larger C-terminal domain is composed of helices α4-α10, forming a very large hydropho-

bic tunnel for substrate binding. This hydrophobic tunnel is ~20 Å long with a total volume of ~1,000 Å^3, which is distinctly larger than the binding pockets of many other TetR-family regulators. A fortuitous glycerol molecule was also found to bind in the binding tunnel of each monomer [43]. Residues Phe99, Phe103, Phe137, Ser138, Tyr139, Val163, Cys166, Thr167, and Lys170 are responsible for forming this glycerol-binding site. Based on the structural information, the crystal structure of CmeR should represent the induced form of this regulator.

As the volume of the hydrophobic ligand-binding tunnel of CmeR is large enough to accommodate a few of the ligand molecules, it is suspected that CmeR might be able to bind more than one drug molecule at a time or possibly accommodate a significantly larger ligand that spans across the entire binding tunnel. It is likely that this tunnel contains multiple binding sites for different ligands. The tunnel is rich in aromatic residues and contains four positively charged amino acids (three histidines and one lysine). Thus, CmeR may utilize these positively charged residues to recognize negatively charged ligands, like bile acids. Indeed, the crystal structures of CmeR in complexes with taurocholate and cholate revealed that CmeR utilizes these cationic residues to recognize bile acids [44]. These two ligands bind distinctly in the binding tunnel (Fig. 18.2). Taurocholate spans the bile acid-binding site adjacent to and without overlapping with the glycerol-binding site. The anionic aminoethanesulfonate group of taurocholate is neutralized by a charge-dipole interaction. Unlike taurocholate, cholate binds in an antiparallel orientation but occupies the same bile acid-binding site. Its anionic pentanoate moiety contributes a water-mediated hydrogen bond with a cationic residue to neutralize the formal negative charge. These structures underscore the promiscuity of the multifaceted binding pocket of CmeR. The binding of these bile acids is extensive. Residues Leu65, Ile68, Cys69, His72, Phe103, Ala108, Phe111, Gly112, Ile115, Trp129, Gln134, Phe137, YTyr139, Val163, Cys166, Lys170, Pro172, His174, His175, Leu176, and Leu179 are involved in the binding [44].

To begin to understand the structural basis of CmeABC function, the crystal structure of the CmeC outer membrane channel protein was recently determined to a resolution of 2.4 Å [51]. CmeC assembles as a trimer of 492 residues per protomer. The CmeC trimer is cannon shaped (Fig. 18.3), forming a 130 Å-long tunnel to export antimicrobial agents, such as fluoroquinolones and macrolides. Each monomer contributes four β-strands and six α-helices to form the β-barrel transmembrane and α-helical periplasmic domains. In addition, the CmeC trimer contains an equatorial domain, which constitutes 12 short α-helices (four from each protomer), enringing the midsection of the α-helical periplasmic domain. The N-terminal end of CmeC forms an elongated loop. This loop extends from the membrane surface and leads down to the equatorial domain in the periplasm. The first N-terminal residue of CmeC is a cysteine. The structure suggests that this residue is covalently linked to the lipid elements of the outer membrane. The corresponding cysteine residue in the CusC outer membrane channel protein of the RND-type copper/silver efflux system CusCBA of *Escherichia coli* has been shown to play an important role in the protein-membrane interaction and is critical for the insertion of the channel protein into the outer membrane [52]. Thus, it is expected that this N-terminal cysteine residue would also be crucial for the function of the CmeC channel.

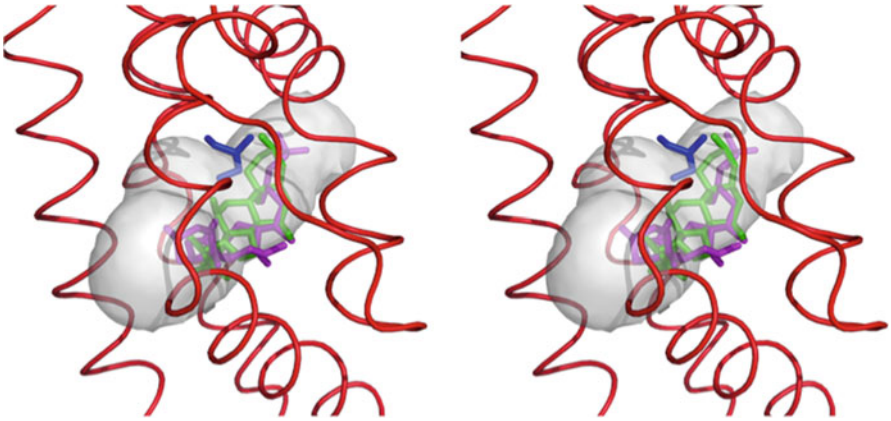

Fig. 18.2 Stereo view of the bile acid and glycerol-binding sites of CmeR. This is a composite figure showing the locations of the bound ligands in the ligand-binding tunnel of the left subunit of the CmeR dimer. The ligands shown in stick models are taurocholate *(magenta)*, cholate (*green*), and glycerol (*blue*). The hydrophobic binding tunnel is colored *gray*. The surrounding secondary structural elements, based on the structure of the CmeR-glycerol complex, are shown as *red* ribbons

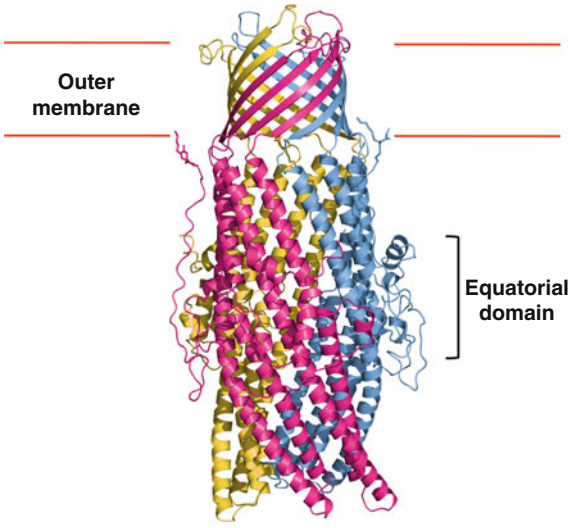

Fig. 18.3 Structure of the *C. jejuni* CmeC outer membrane channel. Ribbon diagram of the CmeC trimer viewed in the membrane plane. Each subunit of CmeC is labeled with a different color. The CmeC protomer is acylated (in sticks) through the first N-terminal cysteine residue to anchor onto the outer membrane

The structure of CmeC also indicates that this channel is in its closed form [51]. The interior of the CmeC outer membrane β-barrel is partially occluded. Residues 96–108 appear to form a flexible loop between strands S3 and S4. This loop may be responsible for the opening and closing of the top end of the β-barrel. The periplasmic end of the α-barrel of CmeC is also partially occluded. The α-helices at this end,

inner H7/H8 and outer H3/H4, are densely packed through coiled-coil interactions. During antimicrobial extrusion, the CmeC channel may need to sequentially dilate to allow passage of the substrates.

The outermost surface of the periplasmic domain of the CmeC trimer forms three intra-protomer and three inter-protomer grooves [51]. These CmeC grooves are likely to provide interaction sites for the CmeA membrane fusion protein. The α-helical coiled-coil domain of CmeA is likely to fit into these grooves to function. In turn, this CmeA-CmeC interaction could control the opening and closing of the CmeC outer membrane channel during drug extrusion.

18.3 Arsenic Efflux Transporters: Role in *Campylobacter* Adaptation

Arsenic exists in nature and organic arsenic compounds have been used for growth promotion and control of diseases in poultry production worldwide. As a foodborne pathogen prevalent in poultry, *Campylobacter* has the ability to resist the toxicity of arsenic compounds. Indeed, *Campylobacter* isolates from conventional poultry showed significantly higher arsenic resistance than those isolates from antimicrobial-free poultry products [53]. Recently, several arsenic detoxification systems have been identified in *C. jejuni* [54–57]. Among them include a four-gene arsenic resistance operon (*ars*) and a single ArsB transporter gene, which are all of chromosomal location. The arsenic resistance and arsenic-sensing operon encode a membrane permease (ArsP), a transcriptional repressor (ArsR), an arsenate reductase (ArsC), and an arsenite efflux protein (Acr3) [54]. The ArsB transporter gene is distantly located from the operon on the chromosome of *C. jejuni* [55]. Functionally, ArsB and Acr3 confer resistance to inorganic arsenic, while ArsP confers resistance to organoarsenicals in *Campylobacter* [54–57]. All three are efflux transporters.

ArsB in *C. jejuni* consists of 428 amino acids and contains 11 predicted transmembrane helices. It shows modest homology (32–33 % identity) to ArsB homologs in *Acidithiobacillus caldus* [26], *E. coli* [58–60], *Shewanella* sp. ANA-3 [61], and *Staphylococcus aureus* [62]. Inactivation of the *arsB* gene resulted in eight- and fourfold reduction in the MICs of arsenite and arsenate, respectively, but had no effect on the MIC of roxarsone, an organoarsenical. In addition, *arsB* is not associated with resistance to other examined heavy metals and antimicrobial agents, including antimonate, copper sulfate, mercury bichloride, azithromycin, erythromycin, ciprofloxacin, clindamycin, ethidium bromide, florfenicol, telithromycin, and tetracycline [55]. Overexpression of *arsB* showed 16-fold increase in the MIC of arsenite compared to the wild type strain, further indicating the role of ArsB in arsenic resistance. The *arsB* gene in *Campylobacter* is inducible by both arsenite and arsenate, but the exact regulatory mechanism is unknown [55].

Acr3 in *C. jejuni* consists of 347 amino acids and contains ten predicted transmembrane helices. The presence of the *acr3*-containing operon is significantly

associated with elevated resistance to arsenite and arsenate in *Campylobacter*. Furthermore, inactivation of *acr3* led to eight and fourfold reductions in the MICs of arsenite and arsenate, respectively, but mutation of *acr3* did not affect the susceptibility to the different classes of antibiotics tested, including erythromycin, tilmicosin, ciprofloxacin, enrofloxacin, oxytetracycline, ceftiofur, and polymyxin B [54].

arsP (*cje1730* in *C. jejuni* RM1221 genome) is the first gene in the four-gene *ars* operon and encodes a transmembrane permease (315 amino acids). ArsP is predicated to have eight putative transmembrane helices, which are quite different from those of ArsB and Acr3. Additionally, ArsP shares little sequence homology to ArsB or Acr3, which function as transporters for As(III) [63–65]. Based on amino-acid sequences, ArsP homologs were found in at least 27 bacterial and archaeal genera. The amino-acid lengths of the ArsP homologs range from 294 to 365, and all of them contain similar transmembrane helices with large hydrophilic loops between the fourth and fifth transmembrane helices, ranging from 37 to 109 amino acids in length. Multiple sequence alignment indicated that ArsP homologs are highly conserved in the transmembrane regions, but quite diverse in the central loop region. Using WebLogo, a highly conserved signature motif "TPFCSCSTIP" located in the second transmembrane domain was identified among the ArsP homologs [56].

The presence of an intact *arsP* gene is significantly associated with elevated resistance to roxarsone in *C. jejuni*, but not to arsenite or arsenate as revealed by the study with inactivation and expression of *arsP*. In addition, *arsP* also confers resistance to other two organoarsenic compounds, arsanilic acid and nitarsone, but has no effect on resistance to other examined antibiotics, including azithromycin, erythromycin, clindamycin ciprofloxacin, nalidixic acid, florfenicol, gentamicin, telithromycin, and tetracycline [56]. Recently, Chen et al. [57] extended the study on ArsP, revealing that reduced trivalent roxarsone, not the pentavalent roxarsone, is the true substrate of ArsP. Moreover, ArsP confers resistance to methylarsenite (MAs(III)) and the reduced forms of phenylarsenite [PhAs(III)], nitarsone (4-nitrophenyl) arsenite [Nit(III)], and *p*-aminophenyl arsenite [pASA(III)], but not to arsenite As(III) or pentavalent organoarsenicals [57]. Thus, ArsP functions as an efflux transporter for trivalent organoarsenicals.

The expression of the four-gene *ars* operon is regulated by ArsR, which is a transcriptional regulator encoded by the second gene in the operon [54]. In-frame deletion of *arsR* drastically increased the expression of the other three genes in the operon including *arsP*, *arsC*, and *acr3*, indicating that ArsR functions as a repressor. ArsR binds directly to an 18-bp inverted repeat in the promoter region of the *ars* operon [54]. In *Campylobacter*, the expression of the *ars* operon is inducible by arsenite and arsenate, and there is a conserved metal-binding motif (ELCVCDL) in the ArsR protein. Therefore, it is likely that arsenic compounds induce the expression of the *ars* operon by inhibiting the interaction of ArsR with the promoter. Interestingly, electrophoretic mobility shift assay demonstrated that arsenite, but not arsenate, inhibited the binding of ArsR to the promoter DNA, providing an explanation for arsenite-induced expression of the operon and suggesting that arsenate, which is reduced by ArsC to arsenite in bacterial cells, indirectly induces the expression of the *ars* operon in *Campylobacter* [54].

At present Acr3, ArsB, and ArsP are the known efflux transporters conferring resistance to arsenic compounds in *C. jejuni*. Some strains carry all three transporters, while others only harbor ArsB [54, 55, 66]. Both Acr3 and ArsB extrude arsenite, but it appears Acr3 has a dominant function. For example, inactivation of *arsB* in strain CB5-28, which harbors both *arsB* and the 4-gene *ars* operon, did not affect the MICs of arsenite and arsenate, suggesting the function of *arsB* in CB5-28 is masked by the fully functional *ars* operon. Possession of multiple arsenic resistance mechanisms provides *Campylobacter* with flexibility in adaptation to various environments.

18.4 MFS Transporter CmeG: Function in Antimicrobial Resistance and Oxidative Stress Response

In *Campylobacter*, there are four putative MFS transporters: Cj0035c, Cj1257c, CmeG (Cj1375), and Cj1687. At present, CmeG is the only functionally characterized MFS transporter in *Campylobacter* [30]. The *cmeG* and *cmeH* (*cj1376*) genes are tandemly positioned on the chromosome of *C. jejuni* NCTC 11168 and were confirmed to be co-transcribed by quantitative reverse transcription PCR. CmeG shares modest amino-acid sequence homology to some MFS efflux transporters involved in multidrug resistance in other bacteria species such as *Bacillus subtilis* Bmr (27 %) [67] and *S. aureus* NorA (27 %) [68]. Structural prediction of CmeG indicated that it possesses 12 transmembrane domains [30], which is a common feature of MFS efflux transporters. Jeon et al. [30] discovered that inactivation of *cmeG* rendered *C. jejuni* more susceptible to ciprofloxacin, erythromycin, gentamicin, rifampicin, tetracycline, ethidium bromide, and cholic acid. Complementation with a wild type *cmeG* restored the susceptibility to near wild type level. These results indicate that CmeG plays a role in intrinsic resistance to antimicrobials in *Campylobacter*. A drug uptake assay revealed that the CmeG mutant accumulated more ciprofloxacin and ethidium bromide than the wild type strain, suggesting that CmeG is a multidrug efflux transporter. Interestingly, CmeG is also a factor in the resistance to oxidative stress as the CmeG mutant was more susceptible to hydrogen peroxide than the wild type strain, while complementation of the mutant with a plasmid-borne *cmeG* restored the resistance to near wild type level [30]. Thus, CmeG plays a role in oxidative stress response in *Campylobacter*. How CmeG contributes to oxidative stress resistance remains unclear and awaits further studies.

While *cmeG* is conserved in various *C. jejuni* strains, the downstream gene *cmeH* is absent from some isolates such as *C. jejuni* 81–176 and 81116. The *cmeH* gene encodes a periplasmic protein, which has been confirmed to be a trilactone esterase and related to iron acquisition in *Campylobacter* [69]. *cmeG* and *cmeH* are co-transcribed, suggesting that the two genes form an operon and might be functionally linked [30]. Another study by Palyada et al. [70] found that *cmeG* was upregulated by the inactivation of the ferric uptake regulator (Fur), which suggests Fur might repress the expression of this operon, either directly or indirectly. Together, these

results reveal that CmeG may have uncharacterized physiological functions in *Campylobacter*, which remain to be examined.

18.5 Combating Antimicrobial-Resistant *Campylobacter*: Targeting Efflux

Inhibition of efflux is a promising approach to the control of antimicrobial-resistant pathogens. There are two possible strategies for inhibiting bacterial efflux pumps: blocking function by using efflux pump inhibitors (EPIs) or inhibiting expression. EPIs can directly interact with efflux transporters and inhibit the extrusion of substrates, enhancing accumulation of antimicrobials within bacterial cells [71, 72]. The most extensively studied EPIs for bacterial efflux pumps are the family of peptidomimetics, which have broad-spectrum activities potentiating different classes of antimicrobial agents [73, 74]. Phenylalanine-arginine β-naphthylamide (PAβN) was the first EPI identified in this family and was first examined in *P. aeruginosa* [75]. Owing to its key role in antimicrobial resistance and in the colonization of the intestinal tract, inhibiting CmeABC is a novel and ideal target for the control of antimicrobial-resistant *Campylobacter*. Potentially, inhibition of CmeABC may sensitize *Campylobacter* not only to antimicrobials but also to bile compounds, which normally exist in animal intestinal tracts. Several studies have attempted to inhibit CmeABC and efflux in *Campylobacter* by using PAβN [35, 76–79]. The general findings are that PAβN has variable potentiating effects on antimicrobials. It showed good potentiating activities for macrolides (8- to >64-fold reduction in MIC), but had limited or no effect on the MICs of fluoroquinolones in *Campylobacter* [78, 80]. This EPI also showed good potentiating activities for other antimicrobials including rifampicin, novobiocin, fusidic acid, and bile salts. Other evaluated EPIs such as 1-(1-naphthylmethyl) piperazine are much less effective than PAβN in potentiating antimicrobials [80]. Other drawbacks of these EPIs are their lack of specificity, inhibiting efflux in both commensals and pathogens, and toxicity to eukaryotic cells.

Recently, peptide nucleic acids (PNAs) have been explored to specifically inhibit *cmeABC* expression. Chemically, PNAs are artificial DNA-mimic polymers, with a backbone made of repeating *N*-(2-aminoethyl)-glycine units connected by peptide bonds and with nucleic acid bases linked to the backbone [81]. PNAs lack phosphate groups in the backbone and thus are not negatively charged, giving high affinity to nucleic acids due to the absence of electrostatic repulsion. In addition, PNAs are highly resistant to proteases and nucleases and are stable in acidic pH, providing an ideal approach to various antisense applications for inhibiting gene expression *in vivo* [82]. The feasibility of using PNAs to specifically inhibit the expression of *cmeABC* and sensitize *Campylobacter* to antimicrobial drugs was demonstrated in culture media [83, 84]. Various PNAs were designed to bind to the translational initiation regions of *cmeABC*, covering the ribosome-binding site and the start codon of the *cmeABC* genes. The antisense PNAs significantly reduced the expres-

sion of CmeA and CmeB, but did not affect the expression of CmeC as determined by immunoblotting. CmeA- and CmeB-specific PNAs increased the susceptibility of *C. jejuni* to ciprofloxacin and erythromycin in a dose-dependent manner. Further work optimized the PNAs that produce the strongest inhibition on CmeABC [85]. These findings proofed the concept that antisense PNAs against CmeABC can be exploited to combat antimicrobial-resistant *Campylobacter*. However, PNAs are expensive to synthesize at present, and bacterial cell permeability to PNAs varies greatly. Thus, PNAs are still considered far away from practical applications. Additionally, the efficacy of PNAs in inhibiting CmeABC in animal models remains to be examined in future studies. With the advance of technology, the cost for PNA production is likely to drop, and PNAs may be improved to enhance cell permeability. In addition, PNAs may be combined with EPIs to increase their efficiency of potentiating antimicrobial drugs against *Campylobacter*.

18.6 Concluding Remarks

From the information discussed above, it is apparent that antimicrobial efflux transporters in *Campylobacter* play a significant role not only in antimicrobial resistance but also in the adaptation to the environments within and outside a host. These efflux systems are regulated by transcriptional factors and can be induced by their corresponding substrates. Some of these transporters are multidrug efflux pumps (e.g., CmeABC), while others are substrate specific (e.g., arsenic efflux proteins). CmeABC is the best characterized efflux system in *Campylobacter*, and its natural substrates are bile compounds present in animal intestinal tracts. Thus, CmeABC is essential for *Campylobacter* colonization in an animal host. Despite the significant advance in understanding the function and regulation of CmeABC, its structural basis for antimicrobial extrusion and if it has additional physiological functions remain to be determined. CmeG is the only functionally characterized MSF transporter in *Campylobacter*, but the physiological role of CmeG remains unclear. Additionally, evidence is accumulating that antimicrobial efflux systems are intertwined with oxidative stress response in *Campylobacter* as demonstrated by the contribution of CmeG to oxidative stress resistance and the regulation of *cmeABC* by CosR, an oxidative stress regulator. The nature of the interaction and how the interaction facilitates the adaptation of *Campylobacter* remain to be characterized. Finally, inhibition of antimicrobial efflux represents a promising approach for controlling antimicrobial-resistant *Campylobacter* as shown by the potentiating effect of anti-CmeABC PNAs and some EPIs on antimicrobials against *C. jejuni*. Additional efforts should be directed toward optimizing the approach to combat antimicrobial resistance in *Campylobacter*.

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Chapter 19
Antimicrobial Resistance and Drug Efflux Pumps in *Helicobacter*

Jennifer Li and Xian-Zhi Li

Abstract *Helicobacter* spp. play important etiological roles in the pathogenesis of gastroenteric diseases such as in the case of *Helicobacter pylori*. Despite wild-type strains of *H. pylori* being generally susceptible to multiple antimicrobial agents, increasing prevalence of antimicrobial resistance in this species constitutes a key risk factor that affects the effective therapy of *H. pylori* infections. Resistance to anti-*H. pylori* agents is mainly mediated by multiple drug-specific mechanisms. However, drug efflux systems, represented by the Hef pumps of the resistance-nodulation-cell division superfamily, are implicated in both intrinsic and acquired multidrug resistance as well as in bile salt/nitrosative stress response and gastric colonization of these pathogens. This chapter provides an overview of antimicrobial resistance and mechanisms in *Helicobacter* with an emphasis on drug efflux systems.

Keywords *Helicobacter pylori* • Antimicrobial resistance • Efflux • RND pumps • Outer membrane • Stress response • Amoxicillin • Clarithromycin • Metronidazole • Bile salts • HefABC • HefDEF • HefGHI

19.1 Introduction

Helicobacter spp. are Gram-negative bacteria belonging to the *Epsilonproteobacteria* class. The representative species, *Helicobacter pylori*, is believed to infect at least 50 % of the world's human population [1]. Although most individuals with *H. pylori* infection do not experience any clinical complications, these infections are often

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implicated in the development of chronic gastritis, peptic and duodenal ulcers, as well as gastric cancers [1, 2]. Indeed, *H. pylori* has been identified as a carcinogen [3]. *H. pylori*, along with additional non-*H. pylori Helicobacter* species, can be divided into three groups (i.e., gastric, enterohepatic, and unsheathed flagella) based on 16S rRNA sequence similarity [4]. Although many of these species have primary animal hosts, some are also known to be associated with gastroenteric and/or hepatic diseases in humans and include, for example, *Helicobacter hepaticus*, *Helicobacter bilis*, and *Helicobacter cinaedi* [4–8]. Furthermore, the clinical significance of *Helicobacter* spp. in the development of gastrointestinal diseases has been supported by microbiome data generated for gut microbiota [9, 10]. *Helicobacter* species can persist and cause chronic inflammation in human gut, thus contributing to the pathogenesis of various gastroenteric diseases [7, 8, 10, 11]. Antimicrobial therapy is needed for the eradication of these bacterial infections; however, the antimicrobials available for the treatment of these infections are quite limited, and a combination therapy is required to achieve optimal clinical effectiveness. Moreover, increasing prevalence of antimicrobial resistance has been observed in *H. pylori* against agents used in *H. pylori* treatment emerges as a crucial issue when tackling *H. pylori* infections [12]. Drug efflux pumps are one of many mechanisms increasingly recognized to play an important role in the emerging resistance in *H. pylori* and other species. Drug efflux pumps of various known transporter families are inherently encoded in *Helicobacter* genomes. In this chapter, we examine the current status of antimicrobial resistance in this genus with an emphasis on drug efflux pumps.

19.2 Antimicrobial Susceptibility, Therapeutic Options, and Resistance Prevalence

Antimicrobial susceptibility studies of *Helicobacter* spp. have been mostly limited to *H. pylori*, which displays significant *in vitro* susceptibility to a number of antimicrobial agents including β-lactams, macrolides, fluoroquinolones, nitroimidazoles, nitrofurans, and tetracyclines [1, 13, 14]. Wild-type strains of *H. pylori* generally have greater susceptibility to antimicrobial agents compared to *Escherichia coli* and *Pseudomonas aeruginosa*, with certain exceptions such as polymyxins, glycopeptides, nalidixic acid, sulfonamides, trimethoprim, and streptogramins (Table 19.1) [13, 17]. The lowered pH within the gastrointestinal tract, the habitat of *H. pylori*, has a negative impact on antimicrobial activity of β-lactams, macrolides, tetracycline, and fluoroquinolones, with values of minimal inhibitory concentrations (MICs) decreased by 4- to 130-fold with pH changes from 7.2 to 5.5 [13]. Susceptibility data for non-*H. pylori Helicobacter* species are very limited, though several isolates of *H. hepaticus* show significant intrinsic resistance to amoxicillin with MIC values of 8–64 μg/ml (cf. with values of ≤0.5 μg/ml for *H. pylori*) [28].

Despite the high *in vitro* susceptibility of *H. pylori* to numerous agents (Table 19.1), *in vivo* therapy of *H. pylori* infections may not correlate well with expectations based on *in vitro* data [13]. The harsh environment within the stomach

Table 19.1 Antimicrobial susceptibility of wild-type strains of *H. pylori*

Antimicrobial	MIC (μg/ml)	Antimicrobial	MIC (μg/ml)
Amoxicillin	0.008[a], 0.06[b]	Gentamicin	1[a]
Ampicillin	0.015[a], 0.06[c]	Tobramycin	0.25–0.5[i]
Mezlocillin	1[b]	Metronidazole	2[e]
Penicillin	0.03[d], 0.12[c]	Furazolidone	0.06–0.25[i]
Piperacillin	0.125[a]	Nitrofurantoin	1[e]
Aztreonam	4[a]	Linezolid	8[a]
Cefaclor	0.5[a]	Novobiocin	0.1[c], 2[a]
Cephalexin	2[d]	Rifampin	0.25[j]
Cefuroxime	0.5[b]	Rifabutin	0.008[j], <0.015[k]
Cefotaxime	0.02[c], 0.125[a]	Tetracycline	0.03[c], 0.125[a], 0.19[l]
Ceftazidime	0.5[a]	Doxycycline	0.19[l]
Ceftriaxone	0.125a, 0.5b	Minocycline	0.19[l]
Nalidixic acid	32[a]	Tigecycline	0.015[d]
Ciprofloxacin	0.12[c], 0.25[a]	Polymyxin B	5[c]
Gemifloxacin	≤0.006[d]	Polymyxin E	8[c]
Levofloxacin	0.25–0.5[e]	Streptogramin A	4[h]
Moxifloxacin	≤0.25[d]	Streptogramin B	8[h]
Nemonofloxacin	≤0.12[d]	Amixicile	0.5[m]
Clarithromycin	0.008[a], 0.03[g]	Bismuth subcitrate	16[e]
Erythromycin	0.06[e], 0.25[a], 0.5[c]	Ethidium bromide	8[a]
Chloramphenicol	0.5[c], 4[a]	Glutaraldehyde	1–10[n]
Clindamycin	1[a], 32[h]		

The data are derived from: [a][15], [b][16], [c][17], [d][18], [e][13], [f][19], [g][20],[h][21], [i][22], [j][23], [k][24], [l][25],[m][26], and [n][27]

poses a challenge for drug selection among orally administered antimicrobial agents. Indeed, therapy has been limited to certain individual agents of various classes which include amoxicillin, clarithromycin, furazolidone, fluoroquinolones, metronidazole, rifabutin, and tetracycline [13, 29]. Therapeutic regimens require combination therapy or sequential therapy with the abovementioned antimicrobials [13, 29–31]. The recommended first-line therapy for the treatment of *H. pylori* infections consists of a standard triple-drug therapy with any two of three antibiotics (amoxicillin, clarithromycin, and metronidazole) and either a proton pump inhibitor (e.g., esomeprazole) or ranitidine bismuth citrate for a duration of 7–14 days [13]. Proton pump inhibitors and bismuth salts possess anti-*H. pylori* activity at high concentration levels [13]. A second-line therapy consists of a quadruple regimen of tetracycline, metronidazole, a bismuth salt, and a proton pump inhibitor [13, 29–31]. Third-line treatment regimens and other rescue therapies are based on the antimicrobial susceptibility profile of the specific strain in question and may include fluoroquinolones, tetracyclines, rifabutin, and furazolidone [32–35].

Antimicrobial resistance is increasingly being recognized as a risk factor affecting treatment efficacy against *Helicobacter* infections [31, 36–39]. A review from 20 years ago has documented a variable but overall high prevalence of 10–70 %

metronidazole resistance [1]. Antimicrobial treatment failure has been linked to increased prevalence of resistant isolates [40]. In a recent study that tested around 340 isolates (including those from patients with up to three treatment failures), the MIC values of amoxicillin varied from <0.015 to 4 μg/ml, with higher prevalence of amoxicillin resistance in isolates from the treatment failures [41]. The rates of resistance to clarithromycin, a major agent for first-line therapies, have increased from 9 % to 18 % in 1998–2008 in Europe and from 7 % to 28 % in 2000–2006 in Japan (reviewed in reference [42]). A recent report has described the rates of resistance to clarithromycin (18 %), levofloxacin (14 %), and metronidazole (35 %) in Europe, and the increased prevalence of resistance was attributable to the increased use of fluoroquinolones and macrolides in clinic [43]. Similarly, a study conducted in China showed increased rates of resistance to clarithromycin (9 % in 2000 to 21 % in 2009) and levofloxacin (10 % in 2000 to 33 % in 2009) with stable rates of about 40–50 % for resistance to metronidazole within a 10-year period [44]. Yet resistance to amoxicillin, furazolidone, or tetracycline was not detectable and/or rarely occurred [44]. A surveillance of nearly 18,000 isolates that were sampled in China between 2009 and 2012 revealed resistance rates of ca. 21 % for clarithromycin and levofloxacin and 94 % for metronidazole with only 0.1 % for amoxicillin, furazolidone, and gentamicin [45]. Resistance to rifabutin remains generally low with the rates of 1.4 % in Germany and 0.24 % in Japan [24, 46]. A German study identified simultaneous resistance to three or four agents in 15 % of isolates contributing to unsuccessful antimicrobial treatment [47]. Furthermore, a Canadian study has also suggested a general increase in resistance to clarithromycin, ciprofloxacin, levofloxacin, and metronidazole beginning from the early 2000s. Together, these data also suggest variable prevalence of resistance in different regions and countries [31]. Newer agents such as finafloxacin and linezolid have been tested for their activity against *H. pylori*, but their implications for therapy require clinical trials [48, 49]. Lastly, heteroresistance, a circumstance in which subpopulations of isogenic strains develop varying antimicrobial susceptibilities [50], was also observed in isolates from the same patients (even before antimicrobial treatment) [51, 52]. Resistance identification can be hindered by heteroresistance with an undesired consequence of selecting more resistant isolates via antimicrobial therapy [50, 51, 53].

It is also noteworthy that the combinatory use of antimicrobials for treating *H. pylori* infections can have an adverse long-term *in vivo* impact on resistance development and persistence in the gut microbiota. For instance, a short-term clarithromycin-metronidazole combination regimen dramatically reduced the diversity of gut microbiota and resulted in a 1,000-fold increase in the *ermB* gene (encoding the macrolide target-modifying RNA methylase), which then persisted in the gut microbiota for at least 4 years [54]. This observation is consistent with an earlier study showing the persistence of *ermB*-mediated resistant enterococci for 1–3 years following an anti-*H. pylori* treatment regimen [55]. Additionally, by modifing lipid A and biofilm formation, *H. pylori* can adapt *in vivo* to resist the antimicrobial activity of calprotein, which is a component of the host innate immune system and is present during the inflammatory response [56].

19.3 Mechanisms of Antimicrobial Resistance

H. pylori displays intrinsic resistance to multiple-unrelated antimicrobials including glycopeptides and polymyxins (Table 19.1) [13], suggesting that access to drug targets likely contributes to resistance manifestation. Acquired resistance can be further developed. One early study from 1990 showed the *in vitro* selection of resistant mutants by antimicrobials at the levels of 4× or 8× MIC, with spontaneous resistance frequencies in the range of 10^{-8}–10^{-6} for ciprofloxacin, erythromycin, metronidazole, and tobramycin [22]. Another study in 2001 reported the frequencies of the *in vitro* spontaneous mutants resistant to clarithromycin, ciprofloxacin, metronidazole, and rifampin being 3×10^{-9} to 7×10^{-8}, while no mutants were recovered for amoxicillin [57]. Development of increasing resistance in *H. pylori* has prompted the investigation of resistance mechanisms. Table 19.2 lists the identified mechanisms of resistance to the major antimicrobials used in the treatment of *H. pylori* infection. Although antimicrobial target changes are a major form of resistance for *H. pylori*, the role of drug efflux systems should not be underestimated.

19.3.1 Amoxicillin Resistance

Resistance to amoxicillin in *H. pylori* appears to occur less frequently [36, 44, 57]. This phenomenon is attributable to mutations in the genes encoding penicillin-binding proteins (PBPs) [41]. *H. pylori* possess three to four major PBPs [58]. Amoxicillin-resistant mutants show significant reduction in the affinity of PBP1 to amoxicillin [59]. Furthermore, amino acid substitutions were observed in PBP1 of resistant isolates [41]. Although mutations in the *pbp2* gene were also noted with those in the *pbp1*, they did not affect amoxicillin resistance [85]. In addition to mutations in PBP1, other unidentified mechanism(s) are likely also needed for high-level amoxicillin resistance [85]. A cysteine-rich protein named HcpA (encoded by *HP0211*) was earlier suggested to not only be a PBP but also a β-lactamase of *H. pylori* that slowly hydrolyzes penicillin derivatives [86]. However, more recent studies only demonstrated HcpA as a bacterial virulence factor triggering the release of a concerted set of cytokines [87, 88]. No further studies support HcpA as a typical β-lactamase. Indeed, typical β-lactamase activity is not detectable in *H. pylori* [89], although it is well known that PBPs generally may have certain β-lactamase activity. This is consistent with the observation that the *H. pylori* genome does not contain genes encoding typical β-lactamases, whose production constitutes the predominant mechanism of β-lactam resistance in Gram-negative bacteria. However, given that many β-lactamase genes are located on plasmids and that *H. pylori* has a strong natural transformation capability, it is not surprising to see the report of a high-level amoxicillin-resistant isolate (≥256 μg/ml amoxicillin) carrying the bla_{TEM} gene [60]; it remains unclear whether this gene was plasmid borne or chromosome encoded.

Table 19.2 Mechanisms of resistance to antimicrobials used for the treatment of *H. pylori* infection

Drug (class)	Mode of action	Resistance	References
Amoxicillin (β-lactams)	Inhibition of cell wall synthesis by targeting penicillin-binding proteins	Mutations in PBP1 with reduced affinity to amoxicillin; reduced porin production; drug efflux	[16, 58–61]
Clarithromycin (macrolides)	Inhibition of protein synthesis by binding to 23S rRNA	Mutations in genes *rrn*, *infB*, and *rpl22* encoding 23S rRNA, translation intuition factor IF-2, and ribosome protein L22; RND efflux pumps	[15, 17, 20, 57, 62–65]
Furazolidone (nitrofurans)	Inhibition of DNA synthesis by cross-linking to DNA	Mutations in nitroreductase genes *porCDAB* and *oorDABC* (*nfsA* and *nfsB* in *E. coli*)	[66–69]
Levofloxacin (fluoroquinolones)	Inhibition of DNA synthesis by targeting DNA gyrase	Mutations in DNA gyrase genes (*gyrA* and *gyrB*)	[32, 57, 70, 71]
Metronidazole (nitroimidazoles)	Production of superoxide radicals and interaction with DNA	Decreased prodrug reduction due to the mutations in *rdxA*, *frxA*, and *frxB*; reduction of superoxide radicals (due to the mutations in ferric uptake regulator); efflux pump overexpression	[72–75]
Rifabutin (rifamycins)	Inhibition of RNA synthesis by targeting the DNA-dependent RNA polymerase	Mutations in *rpoB* gene	[23, 24, 57, 76, 77]
Tetracycline (tetracyclines)	Inhibition of protein synthesis by preventing aminoacyl-tRNA association to ribosome	Mutations in 16S rRNA *rrnA*/*B* genes; drug efflux pumps	[25, 78–84]

Interestingly, two studies have revealed that amoxicillin-resistant/multidrug-resistant mutants accumulate less penicillin, chloramphenicol, and/or tetracycline than susceptible strains [59, 89]. Yet, the accumulation of penicillin and tetracycline by resistant strains was not affected by the ionophore proton conductor, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) [59, 89]). Thus, a reduced accumulation of drugs was explained by investigators as due to reduced uptake and not active efflux. However, the involvement of drug efflux systems needs to be carefully assessed before a solid conclusion is made regarding additional mechanisms of amoxicillin resistance. The outer membrane permeability barrier alone cannot sufficiently explain

drug accumulation differences in the steady state (e.g., within 30–60 min of accumulation). These drug molecules are expected to cross the outer membrane barrier in less than a second [90]. Based on various drug accumulation assays, the steady state of drug levels in intact cells should generally be reached within 30 min [59, 89, 90]. Indeed, the contribution from porin alterations and efflux pumps to amoxicillin resistance in *Helicobacter* spp. has been observed [16, 28] as discussed in the next section. This finding explains a phenotypic relationship between high β-lactam resistance and low- to moderate-level multidrug resistance [89].

19.3.2 Clarithromycin Resistance

Macrolides inhibit bacterial protein synthesis by targeting 23S rRNA. Mutations in the 23S rRNA genes reduce the binding of macrolides to the 23S rRNA [62–64, 91] and are the major mechanism of resistance to macrolides (and particularly clarithromycin for *H. pylori*) [42]. Major mutations include A2142G and A2143G transitions and an A2142C transversion [62, 92] with additional mutations in the 23S rRNA genes reported in the literature [19, 36]. Mutations within other genes, including those in *infB* (encoding translation initiation factor IF-2) and *rpl22* (ribosomal protein L22), were also found to cooperate with 23S rRNA gene mutations in raising resistance level [20]. Lastly, macrolides are often the substrates of multidrug resistance or macrolide-specific efflux pumps in various bacteria [93, 94], and indeed efflux pumps also mediate resistance to clarithromycin, as described in the next section.

19.3.3 Metronidazole Resistance

As a nitroimidazole agent, metronidazole requires reduction by oxygen-insensitive NADPH nitroreductase (RdxA), NADPH-flavin oxidoreductase (FrxA), and ferredoxin-like enzymes (FrxB) to be activated from its prodrug form. Thus, mutations in relevant encoding genes (*rdxA*, *frxA*, and *frxB*) are responsible for metronidazole resistance [66, 85, 91, 95, 96]. Annotated mutations in *rdxA* consist of frame shift mutations, missense mutations, deletions, and insertions [36, 72]. The *rdxA* mutations are also better correlated to clinically relevant resistance (metronidazole MIC >8 μg/ml) than those in *frxA* [73]. Mutations in *frxA* alone may not be sufficient in generating metronidazole resistance [53, 96]. In addition to confirming the role of *rdxA* and *frxA* mutations, a recent study also identified, via whole genome sequencing and natural transformation approaches, mutations in another gene, *rpsU* [97]. The *rpsU* gene encodes ribosomal protein S21; mutations *rpsU* alone do not produce sufficient resistance levels but instead cooperate with *rdxA* mutations to achieve high resistance [97]. Inhibition of superoxide dismutase production in strains with mutations in the ferric uptake regulator is also known to be involved in metronidazole resistance [74]. The contribution of efflux mechanism to metronidazole

resistance is discussed in the next section. Interestingly, the loss of metronidazole resistance occurs under low oxygen conditions (that mimic *in vivo* microaerophilic situation) or in the presence of chloramphenicol [98], suggesting multiple factors affecting metronidazole susceptibility.

19.3.4 Fluoroquinolone Resistance

Fluoroquinolones act on DNA gyrase (A_2B_2 complex) and topoisomerase IV. However, *H. pylori* strains lack the gene encoding topoisomerase IV [42]. Thus, resistance occurs mainly as a result of mutations in the quinolone resistance determining region of the gyrase A gene (e.g., Asn87Lys; Asn87Tyr; Asp91Gly, Asp91Asn, or Asp91Tyr) [42, 70, 91]. Mutations in the gyrase B gene were have also been noted [70]. Furthermore, although fluoroquinolone resistance frequently occurs as a result of efflux pump overproduction in Gram-negative bacteria [94], no efflux pumps affecting quinolone susceptibility have been identified in *H. pylori* to date.

19.3.5 Furazolidone Resistance

The broad-spectrum furazolidone inhibits DNA biosynthesis by crossing-linking to DNA molecules [67]. Mechanisms for furazolidone resistance in *Helicobacter* spp. are not well understood. However, resistance to nitrofurans in *E. coli* has primarily been linked to mutations in genes encoding nitroreductases such as *nfsA* and *nfsB* [99]. A recent study further demonstrated involvement of these mutations in furazolidone-resistant *E. coli* [68]. Pyruvate/flavodoxin oxidoreductase (PorCDAB) and 2-oxoglutarate oxidoreductase (OorDABC) act as nitrofuran nitroreductases in *H. pylori* [66], and mutations in the *porD* and *oorD* genes have been noted in all furazolidone-resistant (>2 μg/ml furazolidone) clinical isolates of *H. pylori* that were obtained from patients previously treated with metronidazole [69].

19.3.6 Rifabutin Resistance

Rifabutin acts on the β-subunit of the DNA-dependent RNA polymerase encoded by the *rpoB* gene. Amino acid substitutions resulting from point mutations in *rpoB* confer high-level resistance to rifampicin and rifabutin (with >128-fold MIC increases) [23, 24]. These resistance levels are dependent on the amino acid substitutions with four distinct regions identified in *rpoB* [76]. Similar to many other species, rifamycin resistance in *H. pylori* occurs more frequently than resistance to other agents [57]. The history of rifamycin use has been linked to the emergence of rifabutin-resistant isolates including those from cases with treatment failure [24, 46, 77].

19.3.7 Tetracycline Resistance

Tetracyclines inhibit protein synthesis by binding to the 30S subunit of the ribosome and preventing association between aminoacyl-tRNAs with the ribosome [78, 79]. Mutations in the 16S rRNA *rrnA*/*rrnB* genes reduce drug binding to the ribosome [100] and yield high-level tetracycline resistance (>40-fold MIC increases for tetracycline, doxycycline, and minocycline [25, 80, 81]). However, other types of tetracycline-resistant isolates were found to lack any mutations in the 16S rRNA genes and instead rely on the altered uptake or efflux [82, 83]. One study has shown proton motive force-dependent efflux of tetracycline in clinical isolates without identifying specific pump(s) [84]. The requirement for multiple mutations in the development of tetracycline resistance may explain its low prevalence in clinical resistance [101, 102]. The involvement of efflux pumps (HP1165) in tetracycline resistance [83] will be discussed in the next section.

19.3.8 Molecular Methods for Resistance Detection

Molecular methods have been developed to detect resistance caused by the specific gene mutations, and have been facilitated by advances in technology such as whole genome sequencing [92]. Commercially available molecular methods for detection of antimicrobial resistance in *H. pylori* also exist as reviewed in the reference [42]. The GenoType HelicoDR test is able to identify point mutations in the *rrn* and *gyrA* genes that are linked to clarithromycin and levofloxacin resistance, respectively [103], but this application has limited in its sensitivity and specificity, apparently making it infeasible for clinical applications [104]. Overall, genetic molecular methods are only applied to known resistance mechanisms for certain genes, as mutations can also be independent of the resistance phenotype [13]. Molecular approaches for examining genetic mutations alone will not be sufficient in characterizing resistance attributable to efflux mechanisms, particularly because multiple regulatory genes can impact efflux gene expression and ultimately the resistance phenotype.

19.4 Outer Membrane Permeability Barrier and Drug Efflux Systems

In Gram-negative bacteria, the outer membrane permeability barrier and drug exporters affect the influx and efflux of antimicrobial agents, respectively, and thus play a role in determining the susceptibility phenotype [94]. A large number of outer membrane and efflux proteins are encoded in the *Helicobacter* genomes [105–107], although the sizes of several known *Helicobacter* genomes are relatively small (only about 1.7–2.0 Mbp) [105, 108–110]. For example, at least 32 outer membrane proteins

[111] and 27 proven or putative drug transporters [112] have been identified in *H. pylori*. These transporters belong to one of the following superfamilies or families [94]: (i) resistance-nodulation-cell division (RND) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion (MATE) family, (iv) the small multidrug resistance (SMR) family, and (v) the ATP-binding cassette (ABC) superfamily (Table 19.3).

19.4.1 Outer Membrane Permeability Barrier

The outer membrane consists of a lipopolysaccharide-containing lipid bilayer with water-filled porins and serves as an effective barrier in limiting the influx of antimicrobial molecules [117]. Small hydrophilic agents, such as amoxicillin, cross the outer membrane via the porin channels, while large or hydrophobic agents require penetration of the outer membrane lipid bilayer [94]. Many outer membrane proteins of *H. pylori* have been studied for their role in infection pathogenesis [107, 118, 119], with five proteins (HopA to E) investigated for their channel-forming activity [120, 121]. The HopA to HopD porins form similar pores with relatively small channel size [120], while the less abundant HopE protein forms a larger nonspecific channel *in vitro* [121]. The presence of these porins explains the high susceptibility of *H. pylori* to small hydrophilic antimicrobials such as amoxicillin, which is expected to enter the periplasm through the porin channels. Indeed, mutations in HopB and HopC proteins render cells less susceptible to β-lactams (two- to eightfold reductions in the MIC values) and cooperate with PBP1 mutations to raise levels of β-lactam resistance (16- to 64-fold amoxicillin MIC reductions) [16]. Alterations in outer membrane protein profiles were observed in high-level amoxicillin-resistant isolates [89]. The outer membrane permeability of *H. pylori* to the small hydrophobic agent, 1-*N*-phenylnaphthylamine, was found to be higher than that of *E. coli* [17], an observation consistent with the low MIC values of many hydrophobic agents (Table 19.1). An increased susceptibility to metronidazole occurred in the presence of aspirin, which enhanced intracellular concentrations of tetracycline, but no significant changes in the transcriptional expression of the genes encoding the HopA, HopB, HopC, HopD, and HopE porins and HefABC efflux system were observed [122]. Hypersusceptibility to several hydrophobic agents (e.g., erythromycin, novobiocin, and rifampicin) was reported for mutants carrying null mutations in the *ostA* (also called *imp*) and/or *msbA* genes [27], which encode an organic solvent tolerance outer membrane protein and a lipopolysaccharide lipid precursor exporter, respectively – both of which are involved in the biogenesis of lipopolysaccharide [107, 123].

19.4.2 RND Pumps

Multiple putative RND pumps have been identified, based on protein homology, in several *Helicobacter* spp. (as presented in Table 19.3). The total numbers are fewer than those found in *E. coli* (which contains six RND pumps) or *P. aeruginosa* (>12 RND

Table 19.3 Confirmed and putative drug efflux transporters in *Helicobacter* spp.

Species/ transporter family	Transporter	Membrane fusion protein	Outer membrane protein	Affected drug susceptibility and functions	References
H. pylori 26695					
RND	HefC (HP0607)	HefB (HP0606)	HefA (HP0605)	Amoxicillin, aztreonam, cefotaxime, ceftriaxone, ceragenins, clindamycin, deoxycholate, erythromycin, ethidium bromide, novobiocin, penicillin, piperacillin, and tetracycline; stress response to bile salts	[15, 17, 61, 105, 112]
RND	HefF (CznA; HP0969)	HefE (CznB; HP0970)	HefD (CznC; HP0971)	Metronidazole, cadmium, nickel, and zinc; urease activity modulation; gastric colonization	[17, 105, 112, 113]
RND	HefI (CzcA; HP1329)	HefH (CzcB; HP1328)	HefG (CrdB; HP1327)	Copper; potentially in nitrosative response	[17, 105, 112–114]
RND	HP1487	HP1488	HP1489	Ethidium bromide	[112]
MATE	HP1184			Ethidium bromide	[112]
MATE	HP0759				[105]
MFS	HP1165			Tetracyclines	[83]
MFS	HP1181				[105, 115]
ABC	CadA (HP0791)			Cadmium, zinc	[114, 116]
ABC	CopA (HP1072)			Copper	[114]
ABC	CopA2 (HP1503)			Metal	[114]
ABC	MsbA (HP1082)			Erythromycin, ethidium bromide, glutaraldehyde, novobiocin, and rifampin; lipopolysaccharide flippase	[27, 105]
H. hepaticus ATCC51449					
RND	HH0174	HP0175			[28, 108]

(continued)

Table 19.3 (continued)

Species/ transporter family	Transporter	Membrane fusion protein	Outer membrane protein	Affected drug susceptibility and functions	References
RND	HH0222 (HefC)	HH0223 (HefB)	HH0224 (HefA)	Amoxicillin, cholic acid, deoxycholic acid, ethidium bromide, ofloxacin, and rifampin; stress response to bile salts	[28, 108]
RND	HH0625 (HefF)	HN0624 (HefE)	HH0623 (HefD)		[28, 108]
RND	HH1859				[108]
MFS	HH1614				[108]
MATE	HH0031				[108]
MATE	HH0167				[108]
SMR	HH0508-0509				[108]
SMR	HH1451-1452				[108]
ABC	HH1857-1858				[108]
H. cinaedi PAGU611					
RND	HCN_0595	HCN_0594	HCN_0593		[106, 110]
RND	HCN_1563	HCN_1564			[106, 110]
MATE	HCN_0708				[106, 110]
MATE	HCN_0807				[106, 110]
MFS	HCN_0741				[106, 110]
SMR	HCN_1599-1600				[106, 110]
SMR	HCN_2016-2017				[106, 110]
ABC	HCN_0962	HCN_0964	HCN_0965		[106, 110]

pumps) [94]; these differences are likely due to the relatively small genome sizes of *Helicobacter* spp. The four RND systems of *H. pylori* are each encoded by a putative three-gene operon [17], which produces the typical three components of RND tripartite efflux complexes; these components include an efflux transporter located in the cytoplasmic membrane, an accessory membrane fusion protein, and an outer membrane channel protein [94]. For two non-*H. pylori* species, putative RND pumps are instead each encoded by a two-gene operon [106] and likely requires an outer membrane channel protein encoded elsewhere in the genome for proper functioning (Table 19.3). Interestingly, unlike in *E. coli* or *P. aeruginosa* [94], no regulatory genes have been identified adjacent to the structural genes of these RND systems. One exception is HefGHI (also known as CrdB-CzcB-CzcA), where the encoded HP1326 (CrdA) is required for induction of HefGHI by copper [114]. CrdA expression is further controlled by a two-component regulatory system CrdRS (HP1364-1365) [124]. A recent study has demonstrated the importance of CrdRS in

nitrosative response of *H. pylori* and its influence on the transcriptional expression of about 100 genes (including the upregulation of *crdA*) [125]. Overall, regulation of *Helicobacter* RND pump expression remains a mystery.

Phylogenetic analysis of the RND pumps of *H. pylori* suggest that HefC is closer to the RND pumps involved in drug efflux while HefF and HefC are related to RND pumps involved in the extrusion of divalent cations [17]. Further studies have been conducted to analyze their expression and functional roles [17, 113]. Despite their expression in wild-type cells, an early study used a genetic inactivation approach to suggest only a minimal role of HefABC, HefDEF, and HefGHI in the antimicrobial resistance in *H. pylori* [17]. Indeed, pretreatment of *H. pylori* cells with CCCP did not result in increased accumulation of either chloramphenicol or tetracycline (on the contrary, reduced drug accumulation was observed), arguing against involvement of a proton motive force-dependent drug efflux pump in intrinsic resistance to chloramphenicol or tetracycline [17].

Two other studies indicate a strong contribution of the HefABC efflux system to intrinsic and acquired multidrug resistance [15, 126]. The expression of *hefABC* in one study was generally the strongest among the four RND systems in clarithromycin-resistant (≥1.0 μg/ml clarithromycin) isolates [65]. Inactivation of the HefC pump gene in a wild-type strain rendered the mutant hypersusceptible to β-lactams (aztreonam, cefotaxime, ceftriaxone, penicillin, and piperacillin but not amoxicillin), clindamycin, erythromycin, ethidium bromide, novobiocin, and tetracycline with four- to 330-fold MIC reduction (Table 19.4) [15]. These antimicrobials are known substrates for RND pumps. Furthermore, CCCP treatment of wild-type cells increased the accumulation of ethidium bromide [15]. Chloramphenicol accumulation was increased slightly in CCCP-treated resistant cells [89]. However, susceptibility to quinolones was not affected by genetic disruption of the *hefA* or *hefC* gene [15, 126]. In another study, disruption of *hefA* (but not *hefD*, *hefG*, or *HP1489*) made the cells more susceptible to deoxycholate and novobiocin and the simultaneous inactivation of *hefA* and *hefD* sensitized cells to metronidazole [112]. Together, these results support that the HefABC pump plays an important role in the intrinsic drug resistance of *H. pylori*. This conclusion is further supported by the involvement of HefABC (not HefDEF or HefGHI) in resistance to bile salts and their derivatives, ceragenins [127]. Elevated *hefA* expression was noted in multidrug-resistant chloramphenicol-selected mutants [126] as well as in multidrug-resistant isolates from another study [128].

The contribution of HefC pumps to amoxicillin resistance was noted in certain resistant isolates, and the combination of 1-(1-naphthylmethyl)-piperazine (NMP; an RND pump inhibitor) at 100 μg/ml reduced the amoxicillin MIC by 16-fold in HefC overproducers [61]. In this case, given the overall hydrophilic nature of amoxicillin, one would expect that the efflux process alone may have a limited role in amoxicillin resistance, but this process may still be possible if the influx of amoxicillin is also affected by reduced porin expression (as already reported in the reference [16]; see above in the outer membrane permeability barrier section). Thus, it would be ideal to assess the porins for the isolates of this study [61]. Mutations in multiple genes were analyzed in this study [61], and surprisingly mutations in HefC

Table 19.4 Effect of the *hefABC* inactivation on antimicrobial susceptibility of *H. pylori*

Antimicrobial	Parental strain (MIC in μg/ml)	Δ*hefABC* (MIC in μg/ml)	MIC ratio of parental strain to Δ*hefABC* (fold)
Cefotaxime	0.125	0.015	8
Ceftriaxone	0.125	0.008	16
Penicillin	0.002	0.00006	330
Piperacillin	0.125	0.0008	16
Clarithromycin	0.008	0.002	4
Erythromycin	0.25	0.015	16
Chloramphenicol	4	2	2
Clindamycin	1	0.125	8
Novobiocin	2	0.03	6
Tetracycline	0.125	0.015	8
Ethidium bromide	8	0.5	16
Cefotaxime	Not reported	Not reported	32[a]
Clarithromycin	Not reported	Not reported	8[a]
Chloramphenicol	Not reported	Not reported	16[a]
Gentamicin	Not reported	Not reported	8[a]

Data are from Kutschke and de Jonge [15] (where the tested mutant had **Δ***hefC*) except otherwise noted

[a]Data are from Liu et al. [126] (where the tested mutant had **Δ***hefA*)

(Asp131Glu and Leu378Phe) were identified in several resistant strains; these mutations appeared to yield a gain of function. In another paper, the inclusion of RND pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) reduced clarithromycin MIC values by four to eightfolds (from 4–32 to 1–8 μg/ml) for 15 clinical clarithromycin-resistant isolates [65] Lastly, HefABC overproduction was revealed to be the first step in the development of acquired resistance [72] to metronidazole. All of these results jointly suggest that HefABC plays a major role in acquired multidrug resistance.

Although the regulation of HefABC expression remains unknown, this efflux system is clearly inducible. The exposure of five clinical isolates to metronidazole at 8 or 16 μg/ml revealed a concentration-dependent increase in *hefA* expression, even though *hefA* was already constitutively expressed in these isolates [129]. The presence of cholesterol also induces *hefABC* expression and thus contributes to resistance to bile salts [127]. It was also found that the expression of all four RND systems was elevated in biofilm cells in comparison with that of planktonic cells [130].

Another RND pump of *H. pylori*, CznABC (i.e., HefDEF), is a metal efflux pump involved in cadmium, nickel, and zinc resistance. Only minimal growth occurred for pump-deficient mutants in the presence of cadmium (10 μM), nickel (1.2 mM), and zinc (0.8 mM) [113]. Furthermore, CznABC is also critical for gastric colonization and modulation of urease activity [113], providing a possible physiological role of RND pumps in *H. pylori*. (Urease activity plays an essential role in acid tolerance of *H. pylori* [131].) The third RND pump, HefGHI, confers resistance

to copper, and its inactivation renders cells more susceptible to copper – with only minimal growth in the presence of 0.1 mM copper [114]. Based on the CrdRS system's involvement in regulation of *crdA* (located immediately upstream of the *hefGHI* genes in the same transcriptional direction) [125], CrdRS could also influence *hefGHI* expression, and thus HefGHI may possibly contribute to nitrosative stress response. Indeed, the role of RND pumps in nitrosative stress response has been demonstrated in *E. coli*, *Klebsiella pneumoniae*, and *P. aeruginosa* [132–134]. Additionally, in contrast to the HefC pump, both HefF and HefH are not involved in cholesterol-dependent resistance to bile salts [127]. Inactivation of either the HefF or HefI pump did not alter the drug susceptibility of cells (with 20 tested agents) [15].

Several RND systems exist in *H. hepaticus* (Table 19.3). Intriguingly, *H. hepaticus* strains appear to be much less susceptible to amoxicillin than *H. pylori*, and they do not have PBP alterations nor produce β-lactamases [28]. Inactivation of *hefA* rendered a mutant strain hypersusceptible to amoxicillin (256-fold MIC reduction but not to another tested β-lactam tested, cefotaxime), rifampicin (ninefold MIC reduction), ofloxacin (fourfold MIC reduction), ethidium bromide (>fourfold MIC reduction), and bile salts (2.5- to 10-fold MIC decreases) [28]. Thus, HefABC likely contributes to intrinsic resistance in *H. hepaticus* to multiple agents including amoxicillin. Moreover, the expression of *hefA* is inducible by bile salts (but not by amoxicillin) [28]. This fact suggests that HefABC may be involved in the survival of *H. hepaticus* in the gastrointestinal tract where it would be exposed to high bile salt concentrations, similar to the role of the *E. coli* AcrAB-TolC system and the *Campylobacter jejuni* CmeABC system [135, 136].

19.4.3 Non-RND Pumps

This group includes ABC, MFS, MATE, and SMR pumps that remain to be characterized (Table 19.3) [105, 112, 115]. Inactivation of the ABC-type MsbA transporter rendered the mutant strain more susceptible to several agents including erythromycin and glutaraldehyde. The impact of CCCP treatment on the accumulation of ethidium bromide supported an efflux process contributed by MsbA [27]. This pump also cooperates synergistically with another lipopolysaccharide biogenesis protein OstA to enhance hydrophobic drug resistance [27]. However, since MsbA is a lipopolysaccharide flippase [107], mutants with MsbA deficiency (and/or with OstA defect) have a reduced lipopolysaccharide production [27]. A few ABC transporters such as CadA and CopA are involved in heavy metal resistance [114, 116].

The HP1165 protein is a homolog of the TetA(P) efflux pump of *Clostridium perfringens* belonging to the MFS family [83]. Its gene is constitutively expressed in any growth phase of a wild-type tetracycline-susceptible strain and its inactivation renders the mutant strain more susceptible to tetracycline (tenfold MIC reduction). While the overproduction of HP1165 is has been linked to tetracycline

resistance following tetracycline exposure, its absence abolishes the ability of tetracycline to induce tetracycline resistance [83]. Additionally, the HP1181 protein is another putative MFS exporter, a homolog of the NorA pump of *Staphylococcus aureus*, but its functional properties remain to be characterized [115].

19.4.4 Effect of Efflux Pump Inhibitors and Methodological Considerations

As described above, many studies have employed efflux pump inhibitors in characterizing drug efflux contribution to resistance in *Helicobacter*. PAβN and NMP are known inhibitors of RND pumps [94]. We have been unable to find data on the activities of these two inhibitors alone against *Helicobacter* spp. (such as MIC values), but these values can be informative in assessing the effect of these agents themselves on *Helicobacter* [94]. In one study [65], PAβN was used at 10, 20, 40, 60, and 120 μg/ml, and it appeared that this agent alone at levels of up to 40 μg/ml did not affect the growth of a particular *H. pylori* strain [65]. Thus, the observations with effect of 40 μg/ml PAβN on the reduction of the MIC values of clarithromycin [65] and metronidazole [72] are interpreted as the involvement of an efflux mechanism. Similarly, NMP can be used at 100 μg/ml without detectable adverse impact on *Helicobacter*, and thus the effect of NMP on HefC is also considered to be related to efflux inhibition [61]. In this regard, it is worth mentioning that the inclusion of a plant extract (baicalin, berberine, emodin, or schizandrin) enhanced antibacterial activity of amoxicillin and tetracycline [128].

Multiple studies have used the proton conductor CCCP, which abolishes proton motive force across the cytoplasmic membrane and therefore is not an efflux pump inhibitor per se. In two independent studies, CCCP at 40 and 100 μM reduced (instead of increased) the accumulation of chloramphenicol and tetracycline [17, 122], suggesting an impact on uptake processes [17]. However, two other studies showed an increase in accumulation of ethidium bromide or chloramphenicol in the presence of 10 or 100 μM CCCP [27, 89]. No impact of 40 or 100 μM CCCP on penicillin and tetracycline resistance was also reported [61, 89]. CCCP at 100 μM produced more effects in drug-CCCP combination susceptibility testing on chloramphenicol-selected multidrug-resistant isolates than the parental strains for multiple drugs [137]. There are also studies that used CCCP at a high level of 200 μM, which increased accumulation of ethidium bromide and tetracycline [84, 138]. Given the apparently inconsistent results on the effect of CCCP on drug accumulation in *H. pylori*, additional investigations are needed to carefully reassess the use of CCCP including its appropriate concentrations.

H. pylori infection also requires the treatment with the proton pump inhibitor acid-inhibitory drugs as part of a drug combination regimen. Proton pump inhibitors themselves exhibit anti-*H. pylori* activities at the levels which are not achievable *in vivo* [13]. Interestingly, studies have examined the effect of proton pump inhibitors on the multidrug resistance phenotype of either bacterial isolates or

mammalian tumor cells [139–141]. Specific to *H. pylori*, esomeprazole, lansoprazole, omeprazole, pantoprazole, and rabeprazole (each at 10 μg/ml) were found to exhibit certain reduction of MICs of amoxicillin and metronidazole (particularly with pantoprazole and rabeprazole) [137]. Yet, clinical relevance of this observation remains unknown.

19.5 Concluding Remarks

The treatment of *Helicobacter* infections is adversely affected by the increasing emergence of acquired resistance in *H. pylori*. Even though drug-specific mechanisms are predominantly responsible for the clinically relevant resistance phenotypes that compromise *H. pylori* infection therapy effectiveness, the contribution of drug efflux systems (particularly the RND-type pumps) to intrinsic and acquired multidrug resistance in *Helicobacter* spp. is being recognized. Furthermore, as already observed in other bacteria of the same class as *Helicobacter* spp., these efflux systems likely also function beyond drug resistance and are involved in its pathogenesis. However, the data regarding the role of the HefABC pump and its substrate profile vary within the literature. These discrepancies may be due to methodological challenges in conducting antimicrobial susceptibility testing with *H. pylori* as well as the high susceptibility of the wild-type *H. pylori* to many agents *in vitro*. A better understanding of *Helicobacter* drug efflux pumps should be pursued to characterize better strategies for therapeutic interventions. Agents that inhibit the efflux pumps could serve as antimicrobial adjuvants to improve the activities of the existing anti-*Helicobacter* drugs. Furthermore, an open-ended question remains on how expression of these efflux systems is regulated. Physiological roles of the *Helicobacter* drug efflux systems are also likely linked to stress response or colonization [113, 125, 127]. The current knowledge clearly warrants further investigations of *Helicobacter* drug efflux pumps and, in particular, their regulation and functional roles.

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Chapter 20
Antimicrobial Resistance and Drug Efflux Pumps in *Bacteroides*

Julio Aires

Abstract *Bacteroides* spp. constitute an important part of the commensal intestinal microbiota, but some species such as *Bacteroides fragilis* are associated with human infections. There is an increasing occurrence of acquired antimicrobial resistance including multidrug resistance in *Bacteroides* spp., which, together with the limited availability of anti-anaerobe antimicrobials, raises a concern for effective therapy of *Bacteroides* infections. This chapter provides a current overview on antimicrobial susceptibility and resistance mechanisms of *Bacteroides* with detailed descriptions of the known drug efflux pumps, which contribute to both intrinsic and acquired resistance.

Keywords *Bacteroides* • Antimicrobial resistance • Multidrug resistance • Efflux • RND pumps

20.1 Introduction

In terms of bacterial classification and taxonomy, the genus *Bacteroides* is composed of >40 species. It includes the *Bacteroides fragilis* group comprising the most frequent clinically isolated species from human biological samples [1]. Bacteria of this genus are anaerobic, bile-resistant, non-spore forming, Gram-negative rods. They are part of the indigenous microbiota of the human and animal gastrointestinal tracts but can be found in other locations such as the mouth, the upper respiratory tract, and urogenital tract. The *Bacteroides* spp. of the *fragilis* group are the predominant microorganisms isolated by culture methods from feces. Metagenomic sequencing has confirmed that *Bacteroides* is a predominant genus of the gastrointestinal tract microbiota [2].

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Although *Bacteroides* are commensal microorganisms that play an important role in human health [2–4], some species are associated with human mixed infections such as intra-abdominal, obstetric-gynecologic, postoperative wound, complicated skin, and soft tissue infections. They are also causative agents of bacteremia [5]. Among the *B. fragilis* group, *B. fragilis* and *Bacteroides thetaiotaomicron* are the most frequently isolated species from clinical samples. *B. fragilis* may account for 40–78 % of the *Bacteroides* isolates recovered from intra-abdominal as well as other infections [1, 6]; *B. thetaiotaomicron* may account for 10–23 % of the isolates [1, 6]. However, while *B. fragilis* is the predominant species isolated from clinical samples, it is not the case in feces where other intestinal *Bacteroides* species are more frequently isolated [7].

Bacteroides spp. have been considered routinely susceptible to a number of broad-spectrum anti-anaerobic molecules. However, surveys following the long-term resistance trends of *Bacteroides* have reported an overall increase in resistance to classical and more modern antimicrobial agents [6, 8–12]. Although the numbers are still low, multidrug-resistant strains have been reported worldwide [13–18]. Moreover, a new multidrug-resistant species of *Bacteroides* was recently identified [19].

Among the different mechanisms for antimicrobial resistance, efflux transporters have been documented in *Bacteroides* spp. This chapter summarizes antimicrobial susceptibility and resistance mechanisms and subsequently provides an up-to-date description of efflux transporters among species of the *Bacteroides* and particularly the *B. fragilis* group. For more information about *Bacteroides* spp. and their commensal role, and their involvement in human disease or information about their physiology, metabolism, and clinical characteristics, several recent reviews are available [1, 5, 7].

20.2 Antimicrobial Susceptibility

Antimicrobial susceptibility of *Bacteroides* spp. has been monitored through national and regional surveys in different countries [6, 8–12, 18]. Even though there are geographic and institutional variations, resistance rates are dependent on the particular species and can therefore vary widely. For instance, *B. fragilis* is frequently more susceptible to many antimicrobial agents in comparison with other species of the *B. fragilis* group such as *Bacteroides vulgatus* and *B. thetaiotaomicron* [1, 5].

Briefly, based on the most recently published surveys [1, 6, 8–10], lower antimicrobial resistance rates of *B. fragilis* group were observed for: (i) carbapenems such as imipenem and meropenem (resistance rates are, respectively, from 0–1.2 % to 1–7.5 %), (ii) chloramphenicol (none reported), (iii) tigecycline (0–8 %), and (iv) metronidazole (0–1 %). Differences in β-lactam/β-lactamase inhibitor activity were observed when the susceptibility and resistance breakpoints from the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used to evaluate *Bacteroides* resistance

rates. For example, piperacillin-tazobactam and amoxicillin/clavulanic acid show low antimicrobial resistance rates. All these antimicrobial agents are considered clinically relevant for the *B. fragilis* group infection therapy. However, anaerobic species identification and antimicrobial susceptibility testing are important in this regard [20]. Moreover, there are increasing reports of antimicrobial resistance including carbapenem and multidrug resistance in *Bacteroides* [16–18, 21–26], which is expected to affect the effective therapy regimens.

20.3 Antimicrobial Resistance Determinants

Currently, *B. fragilis* group species are considered today to be one of the most antimicrobial resistant among human pathogenic anaerobes. Indeed, as part of the commensal microbiota in the intestine, *Bacteroides* spp. exhibit high-level intrinsic resistance to bile salts [27] and inflammation-associated antimicrobial peptides [4] which are associated with *Bacteroides* fitness [4].

β-Lactamase production is the most common mechanism of resistance to β-lactam agents in the *B. fragilis* group. Cephalosporinase genes, *cepA* from *B. fragilis* [28], *cfxA* from *Bacteroides vulgatus* [29], and *cblA* from *Bacteroides uniformis* [30], have been identified. These enzymes are inhibited by the most commonly used β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam). For a minority of *B. fragilis* strains, carbapenem resistance is associated with a chromosomally encoded carbapenemase gene (*cfiA*) that may be "silent" or overexpressed [31]. There is evidence that resistance of *Bacteroides* spp. to β-lactams can be conferred by alteration of penicillin-binding proteins [32, 33]. Also, changes in outer membrane permeability barrier and particularly porin proteins seem to contribute to β-lactam resistance [34, 35]. However, the relative contributions of permeability changes, production of inactivating enzymes, and target modification to antimicrobial resistance needs to be clarified.

Resistance to macrolides (e.g., erythromycin), lincosamides (e.g., clindamycin), and streptogramins (e.g., pristinamycin and virginiamycin) is attributed to the macrolide-lincosamide-streptogramin B (MLS_B)-type *erm* genes encoding methylases that methylate the ribosome target and thus generate resistance by target modification [36]. Fluoroquinolone resistance has been primarily attributed to mutations in the DNA gyrase and topoisomerase genes and particularly in the quinolone resistance-determining region of the *gyrA* gene [37]. *Bacteroides* mechanism of chloramphenicol resistance has been associated with inactivation of the antibiotic by acetyltransferase [38]. The most common type of *Bacteroides* tetracycline resistance mechanisms is mediated by chromosomal genes encoding ribosomal protection proteins, such as TetQ [36]. Some strains were shown to harbor the *tetX* gene encoding flavin-dependent monooxygenase that inactivates tetracyclines in the presence of oxygen [39, 40].

Metronidazole, an important agent with anti-anaerobic activity is one of the mainstay drugs for the treatment of anaerobic infections. Its resistance is generally

attributed to the presence of *nim* genes that encode 5-nitroimidazole reductase enzymes which convert metronidazole to a nontoxic amine derivative [41]. Expression of a new *nim* gene (dubbed *nimJ*) was found in two multidrug-resistant clinical isolates, but the *nimJ* gene alone was considered to be unable to confer high-level resistance to metronidazole [21]. Of note, a recent study failed to confirm the protection of *B. fragilis* by Nim proteins from metronidazole [42]. Description of *nim*-negative strains resistant to metronidazole led to propose alternative resistance mechanisms [7]. Indeed, because of metronidazole mode of action, modification of bacterial metabolic or DNA repair activity may participate in resistance.

Bacteroides spp. possess a wide range of mobile genetic elements. These include plasmids, conjugative/mobilizable transposons, and bacteriophages [5]. Plasmids are common and can be found in 20–50 % of the *Bacteroides*; conjugative transposons are considered ubiquitous; they can be found in over 80 % of the *Bacteroides* spp. [5]. Many of these mobile genetic elements carry genes conferring resistance to the different classes of antimicrobials [1, 5, 7].

Metronidazole resistance *nim* genes have been identified on transferable plasmids [43, 44]. Transferable plasmid-linked chloramphenicol acetyltransferase conferring high-level resistance was documented for a clinical isolate of *B. uniformis* [45]. *Bacteroides* chromosomal genes encoding cephalosporinases can be transferred among species of the *B. fragilis* group. MLS_B-type *erm* genes are transferrable within and between *Bacteroides* spp. via conjugative plasmids or chromosomally located self-transmissible conjugative elements [1, 5, 7]. The ribosomal protection protein TetQ can be transferred by conjugative transposition among *Bacteroides* spp. but also between *Bacteroides* spp. and other bacteria [1, 5, 7]. Additionally, antimicrobial resistance expression has been correlated with activating insertion sequence (IS) elements located upstream of the *cfiA* gene [23], macrolide resistance genes [46], and metronidazole resistance *nim* genes [23]. Classically, anaerobes are naturally resistant to aminoglycosides as uptake of these antibiotics is likely dependent on oxygen- or nitrate-dependent electron transport chain which is lacking [47]. However, current recognition of multiple mechanisms including involvement of drug efflux mechanisms in aminoglycoside resistance requires more investigations of the intrinsic aminoglycoside resistance in *Bacteroides*. Moreover, a transposon carrying a streptomycin resistance gene (*addS*) was reported in *B. fragilis* [48].

Bacteroides antimicrobial resistance levels are linked to the distribution of the genetic determinants that are suspected or proven to be responsible for the resistance phenotypes. Based on the data of different surveys [12, 49–51], the most frequent resistance genes identified among resistant clinical isolates of *Bacteroides* spp. are *cepA*, *cfxA*, and *cfiA* for resistance to β-lactams, *ermF* (encoding 23S rRNA methyltransferase) for resistance to MLS_B group antibiotics, *nim* genes for metronidazole resistance, and *tet*Q for tetracycline resistance. Other less frequent resistance genes may also be found such as *tetX* for resistance to tetracyclines and glycylcyclines [39, 40], *mrs*(*SA*) for streptogramin resistance [52], or *bexA* for fluoroquinolone resistance [12, 49, 53].

To date, low numbers of multidrug-resistant strains have been reported worldwide for *Bacteroides* [13–18, 24]. Analysis of a multidrug-resistant clinical iso-

late of *B. fragilis* by whole genome sequencing revealed the presence of *nimF*, *cfiA*, and *erm* genes, respectively, related to metronidazole, carbapenem, and clindamycin resistance [24]. With the same approach, a metronidazole- and carbapenem-resistant *B. thetaiotaomicron* isolate was shown to contain *cat*, *ermF*, *nim*, *tetQ*, *tetX*, β-lactamase genes, and several efflux genes [25]. The multidrug resistance phenotype is attributed to several mechanisms including efflux transporters (see below).

20.4 Drug Efflux Pumps

Efflux systems in aerobic Gram-negative bacteria have been extensively studied [54] in contrast to anaerobes. With regard to *Bacteroides*, most of the available data concerns *B. fragilis* more than any other *Bacteroides* spp. The documented efflux pumps of the resistance-nodulation-cell division (RND) superfamily have been shown to transport β-lactams, fluoroquinolones, or metronidazole [15, 55–59]. These systems also transport other substrates such as ethidium bromide, triclosan, sodium dodecyl sulfate [57], bile salts [27], or quorum-sensing homoserine lactone autoinducers [60]. An exporter of the multidrug and toxic compound extrusion (MATE) family in *B. thetaiotaomicron* has been associated with norfloxacin, ciprofloxacin, and ethidium bromide efflux [53]. While numerous studies have been conducted to characterize the drug efflux systems of the RND superfamily (see below), macrolide and tetracycline transporters of the ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS) have also been reported [38, 52, 61] with some being found in conjugative transposons [62]. However, even though such efflux encoding genes were found, their substrate specificity has not yet been demonstrated.

20.4.1 RND Drug Efflux Pumps

Bioinformatic analysis of transporter proteins indicated that *B. fragilis* NCTC 9343 and YCH46 genomes has up to 18 putative RND-type proteins representing over 14 % of its total secondary transporters (TransportDB at http://www.membranetransport.org. Accessed on March 15, 2016). In *B. thetaiotaomicron* VPI 5482 and 19 RND-type putative efflux pumps (14 % of the total secondary transporters) are putatively identified but have not yet been characterized. Some of these RND pump homologs were also confirmed in the genome of a multidrug-resistant clinical isolate of *B. thetaiotaomicron* [25].

On the basis of homology with the *mexAB-oprM* efflux system genes of *Pseudomonas aeruginosa* [63, 64], Ueda et al. identified 16 chromosomal RND-type efflux pump genes, named *bmeABC1* to *bmeABC16* (for *B. fragilis* multidrug efflux) [65]. In terms of genetic organization, each operon encodes all genes for the

tripartite efflux components corresponding to the pump (*bmeB*), the membrane fusion protein (*bmeA*), and the outer membrane channel (*bmeC*). The arrangements of the different genes may vary within an operon. Two unusual features have been identified: the *bmeC10* outer membrane component gene may be fused with the *bmeB10* pump gene and two functional pump genes (*bmeB11* and *bmeB11*') are transcribed separately in *bme11*.

Functional characterization of the BmeABC RND efflux pumps in *B. fragilis* showed that 15 transcripts out of the 16 operons were detectable [57]. At least seven BmeB efflux pumps are considered functional in transporting antimicrobials and have overlapping broad substrate profiles, and four of them are involved in intrinsic resistance [65]. Deletion of the *bmeB3* gene resulted in the increased susceptibility of the mutant strain to β-lactams, fluoroquinolones, ethidium bromide, sodium dodecyl sulfate, and triclosan [66]. Expression of *bmeB3* pump in a hypersusceptible strain of *Escherichia coli* resulted in moderately higher minimal inhibitory concentrations (MICs) of several antimicrobial agents in this host [65]. The *bmeB5* gene was shown to be overexpressed in a metronidazole-resistant laboratory mutant of *B. fragilis*. Inactivation of BmeABC5 yielded a fourfold reduction in the metronidazole MIC and also increased susceptibility to other agents [65]. Single and multiple deletions of selected *bmeB* genes caused changes in MICs, which could be reduced by efflux pump inhibitors [57, 58, 65]. Interestingly, the deletion of more than two *bmeB* genes resulted in increased expression of other genes with corresponding MIC increase [57].

20.4.2 MATE Drug Efflux Pumps

In *B. fragilis* NCTC 9343 and YCH46 and *B. thetaiotaomicron* VPI 5482, 13 MATE-type putative efflux pumps (representing ca.10 % of the total transporters) were identified (TransportDB at http://www.membranetransport.org). To date, only one MATE-type efflux system, BexA, has been characterized in *B. thetaiotaomicron* [53]. This MATE pump is involved in the transport of norfloxacin, ciprofloxacin, and ethidium bromide.

20.4.3 Other Efflux Pumps

Based on a DNA microarray profiling of bacterial genes conferring resistance to macrolides, Cossone et al. identified in *B. fragilis* an ABC-type efflux gene homolog of the *msr*(*SA*) gene of *Staphylococcus aureus* [52]. Also, an MFS-type efflux pump homologous to the MefA transporter from *Streptococcus pyogenes* has been found on conjugative transposons in *Bacteroides* spp. [61]. More recently, three putative efflux pump genes coding for a MefA homolog, an ABC- and RND-type transporters were found in a conjugative transposon isolated from a multidrug-resistant clinical isolate of

B. fragilis [62]. A Mef homolog was also observed in *B. thetaiotaomicron* [25]. However, their contribution to and relevance in antimicrobial resistance remain unknown.

20.4.4 Regulation of Drug Efflux Pumps

Pumbwe and coworkers identified a putative TetR-family regulator gene (*bmeR5*) located upstream of the *bmeABC5* operon of a metronidazole-resistant *B. fragilis* laboratory mutant [58]. Experimental evidence demonstrated that BmeR5 is a local repressor of *bmeABC5* transcription and that mutations in the regulatory sequence intergenic region recognized by BmeR5 can lead to a depression and resistance to multiple antimicrobials. A multidrug-resistant clinical isolate of *B. fragilis* with increased *bmeABC5* expression was reported to show a point mutation in this specific region [58]. The same group reported that bile salts affected the transcription levels of 13 out of the 16 *bmeB* efflux pump genes of *B. fragilis* [27]: *bmeB5*, *bmeB6*, *bmeB15*, and *bmeB16* were overexpressed, and reduced expression was observed for *bmeB1* and *bmeB14*. Homoserine lactones were also revealed to modulate expression of *bmeB* efflux genes (*bmeB3*, *bmeB6*, *bmeB7*, and *bmeB10*) [60].

Since *B. fragilis* possesses a large number of RND efflux pumps and because of the documented emergence of isolates with high-level multidrug resistance phenotype, Pumbwe et al. [67] searched for the existence of putative *marA*-like global regulators. The authors showed that two putative AraC-type MarA homologs were induced by benzene and benzene-derived active compounds and suggested their role in a MarA-like system [59]. Like for other microorganisms [54], these data suggest that *Bacteroides* may turn on certain genes such as efflux pumps to extrude toxic compounds in addition to antimicrobial agents.

20.4.5 Efflux and Multidrug Resistance

Spontaneous resistant mutants relating to enhanced efflux pump activity have been reported in *Bacteroides* [55, 68]. The respective potentials of various antimicrobial agents to select for multidrug-resistant mutants of a wild-type *B. fragilis* strain and a quadruple RND efflux pump deletion have been investigated *in vitro* [66]. Out of 21 molecules tested, ampicillin, cefoxitin, doripenem, imipenem, levofloxacin, metronidazole, and sodium dodecyl sulfate selected mutants overexpressing one or more efflux pumps.

In *B. fragilis* clinical strains, the relationship between *bmeB* efflux pump overexpression and resistance to clinically relevant fluoroquinolones and β-lactams was investigated. The data suggested that low- to intermediate-level resistance to fluoroquinolone and high-level β-lactam resistance were correlated to *bmeB* efflux pump expression [56]. Such studies also confirmed a wide presence of resistance efflux gene overexpression in a number of clinical isolates [49, 56].

There have been reports of *B. fragilis* multidrug-resistant clinical isolates [13–18, 21, 24]. Although not all strains were genetically characterized, the multidrug resistance phenotype was potentially attributable to both chromosomal and plasmid-encoded resistance determinants. For one isolate, the RND-type efflux pump genes *bmeB9* and *bmeB15* were shown to be significantly overexpressed, and the addition of efflux pump inhibitors significantly increased susceptibility of the isolate to several structurally unrelated antimicrobials [15]. Thus, drug efflux likely contributes to resistance in multidrug-resistant isolates of *B. fragilis*. *Bacteroides* resistance to antimicrobial agents can potentially arise upon antimicrobial exposure, and particularly high-level resistance is obtained when efflux mechanisms are present in association with other mechanisms [37].

20.5 Concluding Remarks

Antimicrobial susceptibility surveillance reports highlight the increasing level of *Bacteroides* resistance to antimicrobial drugs commonly used for the treatment of anaerobic infections. In addition, some multidrug-resistant *B. fragilis* strains have been reported worldwide. The exposure of *Bacteroides* spp. to antimicrobial agents during infections or surgery prophylaxis therapies may indubitably select for multidrug-resistant strains. In this process, overexpression of efflux pumps participates to the development of the high level of antimicrobial resistance in *Bacteroides* spp. when associated in combination with other endogenous and/or exogenous resistance mechanisms. Among the putative transporters belonging to the different efflux transporter families, only several of them have been genetically and functionally characterized in *Bacteroides* spp. In terms of physiological functions, it is hypothesized that efflux systems may be relevant to *Bacteroides* adaptation for surviving in the gut and that this may be their primary function.

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Chapter 21
Efflux Pumps in Mycobacteria: Antimicrobial Resistance, Physiological Functions, and Role in Pathogenicity

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Abstract The emergence of multidrug and extensively drug-resistant tuberculosis represents a major threat to the control of the disease. Antimicrobial drug resistance in *Mycobacterium tuberculosis* is not merely a consequence of the occurrence of gene mutations in the drug targets but a balance between the acquisition of mutations and drug efflux. The low permeability of the mycobacterial cell wall acts synergistically with active drug efflux pumps, and this combined mechanism may particularly constitute the first step for the development of drug resistance. Besides drug efflux, efflux pumps also have physiological functions in the bacteria, and their expression is subjected to tight regulation in response to multiple environmental and physiological signals. Understanding the mechanisms underlying drug efflux, efflux pump regulation and their contribution for pathogenicity not only enables the development of more rapid and accurate tools for the guidance of antituberculosis therapy but also provides knowledge for the development of new therapeutic strategies.

Keywords Mycobacteria • Tuberculosis • Multidrug resistance • Efflux • Efflux pump inhibitor • Fitness • Pathogenicity

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21.1 Introduction

Drug efflux is generally described as an intrinsic characteristic involved in antimicrobial resistance. In mycobacteria, intrinsic drug resistance is usually attributed to the low permeability of the cell wall, in particular due to its complex barrier structure that acts synergistically with the activity of efflux pumps present in the membrane [1–3]. Therefore, the intracellular concentration that an antimicrobial agent can reach inside the mycobacterial cell is a balance between the influx and efflux of the drug [2, 4–6]. Hydrophobic compounds, such as rifampicin, macrolides, and quinolones, can cross the hydrophobic bilayer through passive diffusion, whereas hydrophilic compounds, such as isoniazid, aminoglycosides, and ethambutol, use porin-like or outer membrane protein channels [3, 7, 8]. In response to the presence of these and other toxic compounds, bacteria can downregulate their expression, but, apparently, in mycobacteria, porins are less abundant and their organization through the thick, waxy, and multilayer cell wall as well as their role in transport is still matter for discussion [8, 9]. OmpA of *Mycobacterium tuberculosis* and the porin MspA of *Mycobacterium smegmatis* were shown to be integral outer membrane proteins which are accessible at the cell surface. These proteins play important roles in lipid and metal transport and are also correlated with survival after infection and pathogenicity, but their relevance in antimicrobial resistance in mycobacteria is still to be demonstrated [10–12]. Other outer membrane proteins have been identified in *M. tuberculosis*, e.g., Rv1698 and Rv1973, representing a new class of channel proteins whose role in transport is still unexplored [13, 14], and Rv3903c (CpnT) associated with toxin transport [15].

Conversely, efflux pumps are now broadly recognized as playing an important role in induced drug resistance in mycobacteria since they can expel a wide range of structurally dissimilar compounds, being associated with multidrug resistance [2, 3, 5, 6, 16–19]. As seen in other bacteria, phenotypic drug resistance in mycobacteria is a balance between the constitutive and/or induced expression and activity of efflux systems alongside with the acquisition of mutations in drug targets [20–23]. Antimicrobial agents can act as inducers of the expression of efflux pumps in a concerted chain of events that increase bacterial drug tolerance during treatment [2, 24–28]. Overall, efflux pumps are cytoplasmic transmembrane proteins that are involved in the extrusion of toxic compounds from within cells into the external environment using cellular energy, ATP, or proton motive force. Although they are mostly known due to their role in the efflux of antimicrobials, they are also involved in several physiological processes such as cell-to-cell communication, bacterial virulence, cellular homeostasis, detoxification of intracellular metabolites and intracellular signal trafficking [3, 6, 19, 29, 30]. In this chapter, we describe the role of efflux pumps in mycobacterial drug resistance and its relationship with pathogenicity and fitness.

21.2 Structure and Assembly of Mycobacterial Drug Transporters

As detailed in previous chapters, bacterial efflux systems can be grouped into five superfamilies or families, according to their structure, energy source, and phylogenetic relationships: (i) the ATP-binding cassette (ABC) superfamily, (ii) the major facilitator superfamily (MFS), (iii) the multidrug and toxic compound extrusion (MATE) family, (iv) the resistance-nodulation-cell division (RND) superfamily, and (v) the small multidrug resistance (SMR) family [18, 31, 32]. These transporters can be classified as primary active transporters (ABC pumps), which are energized by the hydrolysis of ATP, or secondary active transporters (MFS, MATE, RND, and SMR pumps), which act based on the electrochemical gradient generated by protons that are transported and distributed to the surface of the cell, or proton motive force [6, 31].

The study of efflux systems in mycobacteria is mainly based on the characterization of transporter proteins through comparative genomic sequence homology analysis [2, 5, 33, 34], genetic manipulation, and protein expression using *M. smegmatis*, which is a non-pathogenic, fast-growing species, and is easier to be manipulated than *M. tuberculosis*, because of a successful strategy developed in the late 1990s [16, 17, 35–37]. Following this strategy, various transporters from different families have been cloned, expressed, and characterized, many of which were revealed to be involved in drug transport in *M. tuberculosis* and other mycobacterial species [5, 19, 38]. So far it has been possible to recognize and assign, *in silico*, 267 transporters in the *M. tuberculosis* H37Rv genome, of which, 129 correspond to ABC transporters, 30 MFS, 14 RND, and 1 SMR [39]. However, only a small number of these have been cloned with homologous or heterologous expression. The most well-characterized efflux transporters described to date in *M. tuberculosis* are summarized in Table 21.1.

The ABC transporters exist in large number in the *M. tuberculosis* genome (2.5 % of the genome content). These transporters are involved in the uptake of nutrients and secretion of peptides, lipids, ions, and noxious agents (some of which are antibiotics), through the complex mycobacterium cell wall [3, 33, 56]. Only several ABC transporters have been shown so far to be involved in multiple drug transport in *M. tuberculosis*: namely, DrrAB [41], Rv2686c-Rv2687c-Rv2688c [50], Rv1456c-Rv1457c-Rv1458c [46], and Rv1217c-1218c [43, 44] (that are each encoded by an operon) (Table 21.1).

The second most represented family of transporters in the *M. tuberculosis* genome is the MFS, one of the most disseminated superfamily in prokaryotes, transporting many different substrates from sugars and peptides to nucleosides and amino acids, as well as drugs and toxins. The MFS transporters typically have 400–600 amino acids assembled in a 12 or 14 α-helical transmembrane protein, interconnected by two hydrophilic loops having a NH_2 terminal and a COOH terminal spanning toward the cytoplasm [34, 56, 74].

Table 21.1 Known and putative efflux transporters described in *M. tuberculosis*

Pump	Family	Description	Substrates	Reference
Rv0194	ABC	Drug transport transmembrane ATP-binding protein	AMP, CHL, NOV, STR, TET, VAN	[40]
DrrAB (Rv2936-2937)	ABC	Daunorubicin transport ATP-binding proteins	ETB, RIF, STR, TET	[41, 42]
Rv1218c-Rv1217c	ABC	Probable tetronasin transport ATP-binding protein	BL, BP, BSP, INH, NOV, PA, PD, PR, RIF	[43–45]
Rv1456c-Rv1457c-Rv1458c	ABC	Antibiotic-transport integral membrane ABC transporter	INH; RIF; STR; ETB	[46]
BacA (Rv1819c)	ABC	Drug transport transmembrane ATP-binding protein	AP, INH	[47, 48]
Rv1747	ABC	Conserved transmembrane ATP-binding protein	EB, INH	[33, 49]
Rv2686c-Rv2687c-Rv2688c	ABC	Antibiotic-transport ATP-binding protein	FQ	[50]
PstB (Rv0933)	ABC	Phosphate transport ATP-binding protein	EB, CIP, INH, RIF	[51, 52]
Mmr (Rv3065)	SMR	Integral membrane efflux protein	ACR, EB, ERY, PY, SO, TTP	[53, 54]
Rv1634	MFS	Possible drug efflux membrane protein	FQ, SKI	[34, 55]
Rv0849	MFS	Probable conserved integral membrane transport protein	BL, INH, RIF	[56]
EmrB (Rv0783c)	MFS	Multidrug resistance integral membrane efflux protein	RIF	[34, 42]
EfpA (Rv2846c)	MFS	Integral membrane efflux protein	ACR, EB, FQ, INH	[57]
P55 (Rv1410c)	MFS	Aminoglycosides/tetracyclines transport integral membrane protein	INH; RIF; TET; AGs	[17, 58]
Rv1258c	MFS	Conserved membrane transport protein	EB, ERY, INH, OFX, RIF, SPE, TET	[16, 59, 60]
Stp (Rv2333c)	MFS	Conserved membrane transport protein	SPE, TET	[61]
Rv1877	MFS	Conserved membrane protein	ERY, KAN, TET	[34]
JefA (Rv2459)	MFS	Conserved integral membrane transport protein	EB, INH	[34, 62]
MmpL3 (Rv0206c)	RND	Probable conserved transmembrane transport protein	AU, BM212, IU, SQ109	[63–65]
MmpL4 (Rv0450c)	RND	Probable conserved transmembrane transport protein	CMB, MB, RIF	[66, 67]

MmpL5 (Rv0676c)	RND	Probable conserved transmembrane transport protein	AZ, BDQ, CFZ, TET	[68, 69]
MmpL7 (Rv2942)	RND	Probable conserved transmembrane transport protein	INH	[70, 71]
MmpL8 (Rv3823c)	RND	Probable conserved transmembrane transport protein	SQ109	[63]
MmpL10 (Rv1183)	RND	Probable conserved transmembrane transport protein	SQ109	[63]
MmpL11 (Rv0202c)	RND	Probable conserved transmembrane transport protein		[70]
IniBAC (Rv0341-2-3)	Membrane protein	Isoniazid-inducible proteins	EB, INH	[72, 73]

ACR acriflavine, *AG* aminoglycosides, *AMP* ampicillin, *AP* antimicrobial peptides, *AU* adamantyl ureas, *AZ* azoles, *BDQ* bedaquiline, *BL* β-lactams, *BM212* a 1,5-diarylpyrrole derivative, *BP* biarylpiperazines, *BSP* bisanilinopyrimidines, *CFZ* clofazimine, *CHL* chloramphenicol, *CIP* ciprofloxacin, *CMB* carboxymycobactins, *EB* ethidium bromide, *ERY* erythromycin, *ETB* ethambutol, *FQ* fluoroquinolones, *IA* indoleamides, *INH* isoniazid, *KAN* kanamycin, *MB* mycobactins, *NOV* novobiocin, *OFX* ofloxacin, *PA* pyrazolones, *PD* pyridines, *PR* pyrroles, *PY* pyronin Y, *RIF* rifampicin, *SKI* SKI-356313, an imidazoline, *SO* safranin O, *SPE* spectinomycin, *SQ109* an ethylenediamine analogue of ethambutol, *STR* streptomycin, *TET* tetracycline, *TPP* tetraphenylphosphonium, *VAN* vancomycin

The three most well-studied efflux transporters from the MFS in mycobacteria include LfrA, Tap, and P55. The LfrA efflux pump of *M. smegmatis* was the first efflux pump described in mycobacteria and is associated with resistance to fluoroquinolones [35], ethidium bromide, and acriflavine [75, 76]. No homolog of LfrA is found in *M. tuberculosis* genome. The Tap efflux pump of *Mycobacterium fortuitum* [16] shares 71 % homology with the Rv1258c (Tap-like) efflux pump of *M. tuberculosis* and is associated with reduced susceptibility to tetracyclines, rifampicin, and clofazimine, along with other toxic compounds [17, 58, 77]. Recently, it was clearly demonstrated by Lee et al. [78] that the limited antituberculosis activity of spectinomycin was related to its efflux by the Rv1258c efflux pump which was upregulated in multidrug-resistant strains. By structural modifications of the antibiotic, they have been able to evade efflux and the antibiotic became highly effective against *M. tuberculosis* [78], highlighting the one (of certainly many) hidden connection between efflux and drug resistance in mycobacteria [79]. The P55 efflux pump of *Mycobacterium bovis* and *M. tuberculosis* confers a significant increased resistance to aminoglycosides, clofazimine, rifampicin, tetracyclines, and other toxic compounds [5, 58, 77]. Recently, P55 was found to be associated with cholesterol transport, carbon metabolism, and oxidative stress, with importance for mycobacterial survival and pathogenesis [80].

The MATE family of transporters is found in all living organisms, and they transport a large variety of substrates using the electrochemical gradient of protons and/or ions H^+ or Na^+. Consisting of 12 transmembrane domains divided into two amino regions and two carboxyl regions that are connected by 11 loops, they typically transport aromatic and cationic compounds [81] and DNA-damaging chemicals [82]. So far no MATE transporter is officially assigned to *M. tuberculosis* genome. However, sequence analysis of mycobacterial transporters detected a single-open reading frame for an MATE transporter [83]. This MATE transporter was characterized in *M. smegmatis* and was named as Mmp (mycobacterial MATE protein) which is encoded by the *MSMEG_2631* gene. Mycobacterial MATE protein shares similarities with both NorM and DinF proteins and is involved in resistance to multiple drugs, including aminoglycosides, phleomycin, sulfonamides, and cetylpyridinium chloride [83]. The *MSMEG_2631* gene shares 74 % homology with the *dinF* gene (*Rv2836c*) of *M. tuberculosis*.

The non-fermentative strict aerobe *M. tuberculosis* has a relatively reduced number of ABC and MFS efflux systems per genome megabase, less than any other organisms studied in a genomic comparative analysis of solute transport systems of 18 prokaryotes [84]. *M. tuberculosis* seems to be more dependent on the secondary active transporters as compared to fermentative organisms that much depend on the substrate level phosphorylation to generate ATP [6, 19]. In line with this rationale, the representation of the RND superfamily of transporters in the *M. tuberculosis* genome is comparatively larger than those found in other prokaryotes [84]. The RND transporters are basically drug/proton antiporters to transport a wide variety of substrates. Structurally they form a tripartite structure composed by an integral membrane efflux transporter, an outer membrane channel, which connects the inner side to the outside of the cell wall allowing the bacterium to transport substances through both cell membranes, and a periplasmic protein adapter [23, 85, 86].

Proteins of the MmpL (mycobacterial membrane protein, large) family are exclusively mycobacterial RND efflux pumps [70]. *M. tuberculosis* genome encloses 13 genes that encode for MmpLs that mainly export lipids, in particular mycobacterial mycolic acids, essential for mycobacterial survival and pathogenesis [70, 87, 88]. Their role in lipid transport and their direct relation with metabolism, viability, and virulence during infection make them an important target for new antimycobacterial drugs [88, 89]. Sequence similarities suggested that MmpLs might be analogues of AcrB pump of *Escherichia coli* [3, 90] and that MmpS proteins might be analogues of the membrane fusion protein AcrA [3]. However, the absence of related outer membrane factors or periplasmic proteins in *M. tuberculosis* genome suggests that MmpL proteins function as single component pumps in contrast with the conventional RND efflux systems in Gram-negative bacteria [90]. Four out of 13 genes encoding MmpL proteins are associated with *mmpS* genes, namely, *mmpL1*, *mmpL2*, *mmpL4*, and *mmpL5* [70]. MmpS4/MmpL4 and MmpS5/MmpL5 are similar transport systems that are required for biosynthesis and export of siderophores by *M. tuberculosis* [66]. The connection between MmpLs and drug resistance is controversial. Domenech et al. [70] did not found any significant change in intrinsic drug resistance of *M. tuberculosis* when they selectively inactivated 12 *M. tuberculosis* MmpLs, most probably because of the redundancy and promiscuity of these transporters that replace each other to guarantee cell survival under antimicrobial pressure [27, 53]. In this respect, it was shown that the MmpL7 protein extruded isoniazid in *M. smegmatis* and overexpression of *mmpL7* in *M. tuberculosis* resulted in low-level isoniazid resistance [27, 71]. MmpL7 was the first member of the MmpL family to be studied and was shown to be necessary for the export of phthiocerol dimycocerosate (PDIM) across the cell membrane [91]. Furthermore, the overexpression of the MmpS5-MmpL5 efflux transporter was associated with resistance of *M. tuberculosis* to azoles [68], and more recently, the relation between acquired resistance to bedaquiline and efflux by MmpS5-MmpL5 was demonstrated [92].

The SMR family gathers the smallest prokaryotic transporters [93]. In *M. tuberculosis*, only one protein of this family, the Mmr, has been molecularly and physiologically characterized (Table 21.1). It was demonstrated that Mmr expression decreases susceptibility of *M. smegmatis* and *M. tuberculosis* to acriflavine, ethidium bromide, quaternary ammonium compounds, and other cationic dyes and moderately decreases the susceptibility to isoniazid, erythromycin, and fluoroquinolones [53, 54]. Homologs of Mmr are also present in other species such as *Mycobacterium avium* (i.e., MAV_3949) and *Mycobacterium leprae* (ML1756).

Additional to the efflux pumps referred above, others have been identified in other mycobacterial species, with demonstrable importance for drug resistance. In particular, in *M. avium* and *Mycobacterium intracellulare*, nowadays the second most frequent mycobacterial agents that cause infection in humans [94, 95], efflux activity has been also correlated with acquired resistance. The development of resistance to macrolides, the most effective antimicrobials against *M. avium* and *M. intracellulare* infections, is thought to occur due to the induction of efflux pumps [21]. The most prevalent efflux transporters in *M. avium* and *M. intracellulare* are

the ABC and MFS transporters. The genome of *M. avium* subsp. *hominissuis* strain 104 (representative of human and porcine strains) encodes for 166 ABC and 32 MFS putative transporters, while *M. intracellulare* ATCC13950^T encodes for 156 ABC and 28 MFS transporters [39]. The efflux pumps MAV_1695 and MAV_3306, belonging to the ABC superfamily of transporters, and MAV_1406, a MFS transporter, have been clearly associated to acquired resistance to macrolides [26]. Besides these, other efflux pumps present in *M. avium* complex genome may also be involved in antimicrobial resistance and has to be further explored.

The genome of *Mycobacterium abscessus* encodes efflux pumps of the main families of drug transporters; however, the knowledge of their substrate spectrum, in relation with the intrinsic drug resistance and role in the evolution of resistance to antimicrobial agents used in clinical practice, is still unknown [96–98]. These pumps may, like in other microbes, play a role in the process which results in a drug resistance phenotype. In the few studies that characterized efflux pump systems in *M. abscessus*, the authors identified the presence of the well-characterized *tap* gene encoding a mycobacterial pump in three of nine clinical isolates of *M. abscessus* [99] indicating that there could be a similarity regarding the genes present in the genomes of different species, although their regulation and expression may be quite different. A summary of the *in silico* homologies between putative efflux pumps encoded in some mycobacterial species with clinical relevance and those encoded in *M. tuberculosis* genome is presented in Tables 21.2, 21.3, and 21.4. All mycobacterial species show in their genomes a significant number of genes encoding efflux pumps, from *M. leprae* to the rapid-growing mycobacteria, but their phenotypic expression, correlation with metabolism, survival, pathogenicity, substrate specificity, and drug resistance are still largely unknown and will certainly be subject of intensive studies in the future with a special emphasis on the molecular mechanism for substrate recognition by membrane transporters and their associated regulatory proteins.

21.3 Induction and Genetic Regulation of Efflux Pumps in Mycobacteria

M. tuberculosis genome encodes more than 100 transcriptional regulators, including 13 sigma factors, 30 two-component regulators, and 14 protein kinases [100, 101]. There is now a considerable amount of evidences that drug resistance in *M. tuberculosis* can also be attributed to the constitutive or inducible expression of several drug efflux systems, besides the well-described mutations in drug targets genes, but the transcriptional interaction between the RNA polymerase and the promoters of the efflux pump genes or operons in the *M. tuberculosis* genome has not been much explored [2, 3, 24]. Only recently was demonstrated the association between resistance to isoniazid in *M. tuberculosis* and the loss of the sigma factor SigI [102] and was also shown that SigA contributes to a multidrug resistance phenotype in *M. smegmatis* [103]. As in other bacterial species, it is expected that the levels of resistance will be dependent on the expression levels of *M. tuberculosis* transcriptional regulators. Currently, only a few transcriptional regulators are known to contribute to drug resistance in mycobacteria as described below.

Table 21.2 ABC transporters of *M. tuberculosis* and their putative homologs in clinically relevant nontuberculous mycobacteria

Species	ABC transporters											
	BacA	DrrA	DrrB	Rv1218c	Rv1217c	Rv1456c	Rv1457c	Rv1458c	Rv1747	Rv2686c	Rv2687c	Rv2688c
M. leprae TN	ML2084	ML2352	ML2351	–	–	ML0587	ML0589	ML0590	–	–	–	–
M. intracellulare ATCC13950	OCU_27150	OCU_30840	–	OCU_12660	OCU_12650	OCU_31670	OCU_31660	OCU_31650	OCU_28290	–	–	–
M. avium 104	MAV_2896	MAV_3250	–	MAV_1362	MAV_1361	MAV_3323	MAV_3322	MAV_3322	MAV_3010	–	–	–
M. kansasii ATCC12478	MKAN_00310	MKAN_24010	MKAN_24010	MKAN_06960	MKAN_06970	MKAN_27075	MKAN_27085	MKAN_27085	MKAN_28900	MKAN_25445	MKAN_25440	MKAN_25435
M. marinum ATCC BAA-535/M	MMAR_2696	MMAR_1771	MMAR_1710	MMAR_4220	MMAR_4221	MMAR_2261	MMAR_2262	MMAR_2263	MMAR_2612	MMAR_2028	MMAR_2027	MMAR_2026
M. ulcerans Agy99	MUL_3059	MUL_2014	MUL_2013	–	MUL_4522	MUL_1857	MUL_1858	MUL_1859	MUL_3149	–	MUL_3325	–
M. haemophilum ATCC29548	B586_11855	B586_03525	B586_03525	B586_07700	B586_07695	B586_13305	B586_13295	B586_13290	B586_12185	B586_14450	B586_14455	B586_14460
M. abscessus ATCC19977	MAB_3399	–	–	MAB_1359	–	MAB_2754	MAB_2753	MAB_2752	MAB_3698	MAB_1860	MAB_1859	MAB_1858
M. chelonae ATCC35752	GR01_16330	–	–	GR01_06230	–	GR01_12940	GR01_12920	GR01_12920	GR01_18005	GR01_09110	GR01_09105	GR01_09105
M. smegmatis mc^2155	MSMEG_3655 MSMEG_4380	–	–	–	–	MSMEG_3117	MSMEG_3118	MSMEG_3119	MSMEG_1642	MSMEG_1504	MSMEG_1503	MSMEG_1502

M. smegmatis is shown for comparison since it is used as an experimental model organism. Homologies were retrieved from the National Center for Biotechnology Information, USA (http://www.ncbi.nlm.nih.gov/pubmed; last accessed on October 24, 2015)

Table 21.3 MFS transporters of *M. tuberculosis* and their putative homologs in clinically relevant nontuberculous mycobacteria

Species	MFS transporters							
	EfpA	EmrB	P55	Rv0849	Rv1258c	Rv1634	Rv1877	Rv2459
M. leprae TN	ML1562	–	ML0556	–	–	–	–	–
M. intracellulare ATCC13950	OCU_35340	OCU_06270	OCU_32070	–	–	OCU_29730	OCU_26480	–
M. avium 104	MAV_3702	MAV_0730	MAV_3369	–	MAV_1406	MAV_3140	MAV_2832	–
M. kansasii ATCC12478	MKAN_24765	MKAN_11120	MKAN_26735	MKAN_10170	MKAN_06750	MKAN_28065	MKAN_00680	MKAN_22120
M. marinum ATCC BAA-535/M	MMAR_1887	MMAR_4905	MMAR_2219	MMAR_4767	MMAR_4182	MMAR_2438	MMAR_3970	MMAR_3806
M. ulcerans Agy99	MUL_2115	MUL_0501	MUL_1806	MUL_0338	–	MUL_1617	MUL_3826	MUL_3729
M. haemophilum ATCC29548	B586_15055	B586_05245	B586_13475	–	B586_07880	B586_12605	–	–
M. abscessus ATCC19977	MAB_3142	–	MAB_2807	MAB_0450	MAB_1409	MAB_2310	MAB_0970	–
M. chelonae ATCC35752	GR01_15045	GR01_14005	GR01_13185	GR01_05845	GR01_06475	GR01_11055	GR01_04305	–
M. smegmatis mc^2155	MSMEG_2619	–	MSMEG_3069	–	–	MSMEG_3815	MSMEG_3563	–

See footnote to Table 21.2

Table 21.4 RND efflux transporters of *M. tuberculosis* and their putative homologs in clinically relevant nontuberculous mycobacteria

Species	RND transporters						
	MmpL3	MmpL4	MmpL5	MmpL7	MmpL8	MmpL10	MmpL11
M. leprae TN	ML2620	ML2378	–	ML0137	–	ML1231	ML2617
M. intracellulare ATCC13950	OCU_48870	OCU_30830	OCU_24920	–	OCU_18350	OCU_18350	OCU_48920
M. avium 104	MAV_4968	MAV_3249	MAV_2510	–	MAV_1761	MAV_1761	MAV_4973
M. kansasii ATCC12478	MKAN_16495	MKAN_18145	MKAN_18165	MKAN_23970	–	MKAN_16000	MKAN_16475
M. marinum ATCCBAA-535/M	MMAR_0446	MMAR_0771	MMAR_1095	MMAR_1764	–	MMAR_2342	MMAR_0442
M. ulcerans Agy99	MUL_1096	MUL_4859	MUL_0757	MUL_2007	–	–	MUL_1092
M. haemophilum ATCC29548	B586_01320	B586_19785	B586_19760	B586_03320	–	B586_07475	B586_01340
M. abscessus ATCC19977	MAB_4508	MAB_4116c	MAB_4382c	–	MAB_0937c	MAB_0937c	MAB_4529
M. chelonae ATCC35752	GR01_22035	GR01_11010	GR01_11010	–	GR01_04160	GR01_04160	GR01_22135
M. smegmatis mc^2155	MSMEG_0250	MSMEG_0382	MSMEG_0225	–	MSMEG_4741	MSMEG_0410	MSMEG_0241
			MSMEG_3496				

See footnote to Table 21.2

The most studied transcriptional regulator is WhiB7, which is multidrug-inducible. WhiB7 acts as a transcriptional activator by binding to SigA, a primary sigma factor [103]. Under stress conditions, transcription of *whiB7* is initiated leading to its binding to an AT-rich region located 3 bp upstream the −35 region interacting with SigA. This binding leads to an enhanced transcription activity [104]. Disruption of *whiB7* increases susceptibility to ribosome-targeting dissimilar antibiotics (aminoglycosides, lincosamides, macrolides, and tetracyclines), whereas its overexpression promotes drug resistance [105]. In addition to antibiotics, *whiB7* is also induced by exposure to fatty acids and general stress conditions. Burian and colleagues [104] identified 86 *whiB7* activators, including fluoroquinolones, DNA intercalators, the glutamine analogue acivicin, and the antiseptic dequalinium. Conversely, inhibitors of cell wall biosynthesis do not induce *whiB7* transcription. Activation of *whiB7* was also found to be upregulated within macrophages and in a mouse model [106]. Gene expression profiling analyses demonstrate that *whiB7* transcription determines drug resistance by activating expression of a regulon that includes genes involved in ribosomal protection and antimicrobial efflux. Microarray expression profiles showed that *whiB7* represented a primary regulatory gene whose expression was followed by transcription of other genes that includes two efflux pumps, Rv1258c and Rv1473. Furthermore, it was shown that mutations in the 5′untranslated region of *whiB7* enhanced Rv1258c expression, conferring resistance to streptomycin [105, 107]. WhiB7 is autoregulatory and its regulon includes *eis* (*Rv2416c*), *Rv1258c* (*tap*-like), and *ermMT* (*Rv1988*), among others [104]. The protein Eis is involved in modulation of the immune response and intracellular mycobacterial survival [108]; ErmMT confers macrolide resistance by modification of the 23S rRNA [109] and modulates host immune response [110]; and the Rv1258c efflux pump is required for drug resistance, survival in stationary phase [111] and macrophage-induced tolerance to drugs and intracellular survival [25, 112]. Inhibition of the *whiB7* regulon could be an attractive approach for the development of new drugs for tuberculosis treatment since they will affect several pathways essential for growth, virulence, and drug resistance. Interestingly, the already discussed spectinomycin, an antibiotic with reduced antimycobacterial activity, revealed enhanced activity against a *M. tuberculosis whiB7* mutant strain [104].

Another well-studied transcriptional regulator is LfrR, a putative TetR family transcriptional repressor that regulates the expression of the LfrA efflux pump of *M. smegmatis* [75, 76, 113]. LfrR is located upstream *lfrA* gene and both are co-transcribed by a common promoter [113]. LfrR directly binds to the *lfrRA* promoter region repressing the transcription of the *lfrRA* operon. Overproduction of efflux pumps is deleterious for the cells showing the requirement for regulatory systems to modulate their expression. In this respect, LfrR acts as modulator of the expression of LfrA in order to maintain its balance and the physiological needs for adaptation to environmental changes including antimicrobial resistance. LfrA efflux pump is tightly regulated by LfrR and its repression and induction is switched at cellular toxic concentration [113]. Deletion of *lfrR* increases expression of *lfrA* resulting in high levels of resistance to several drugs [76]. Acriflavine, ethidium bromide, and rhodamine 123 are strong inducers of the transcription of *lfrA*. This induction is due

to the direct binding of the substrates to LfrR, causing the dissociation of the repressor-operator complex [114] and increased levels of expression of the LfrA efflux pump. Fluoroquinolones are also known as inducers of *lfrA* expression although at low levels [75, 76, 113].

Also well characterized is RaaS, a regulator of antimicrobial-assisted survival that plays an important role in mycobacterial survival in prolonged stationary phase of growth and during murine infections [115]. RaaS is encoded by gene *Rv1219c*, which is the first gene of the *Rv1219c-Rv1218c-Rv1217c* operon. RaaS shares homology with members of the TetR family of transcriptional repressors [116, 117]. Rv1219c regulates its own expression and controls the expression of the ABC-type Rv1218c-Rv1217c efflux transporter. Its expression is influenced by metabolites produced during active growth [115, 116]. The binding of RaaS to DNA is regulated by the oleoyl coenzyme A that in turn regulates the expression of RaaS regulon and RaaS-mediated persistence [116]. In stressful conditions, free RaaS binds to its DNA recognition site repressing the transcription of itself and its regulon, a process that is potentiated by drug treatment. Conversely, as free acyl-CoA accumulates, RaaS is released from its binding site leading to the overexpression of the RaaS regulon and impaired long-term cell survival [116]. The RaaS regulon is composed by a cluster of genes located immediately downstream of Rv1219c (i.e., *Rv1215c* and *Rv1216c*, encoding conserved proteins of unknown function, and Rv1217c-Rv1218c) and *drrC* that encodes part of the daunorubicin ABC transporter DrrABC and is required for the transport of PDIM to the cell surface [87, 116]. Rv1219c interacts with rhodamine 6G, ethidium bromide, and safranin O and a variety of toxic aromatic compounds such as bisquinolinium cyclophanes, phosphoramidites, porphyrins, and pyridazines [117].

An *E. coli* MarRAB-like regulon (for multiple antibiotic resistance) was characterized in *M. tuberculosis*, showing that the expression of the efflux system genes *mmpS5-mmpL5*, *mmpS4-mmpL4*, and *mmpS2-mmpL2* is regulated by the MarR-like transcriptional regulator Rv0678. Although the role of these RND systems in efflux of drugs is not well understood, it is possible to infer that similarly to Mar system in *E. coli*, different ligands can act as inducers or repressors of expression of these systems [118–121]. MarR has been shown to function as a local repressor of *marRAB* operon in *E. coli* [122], and the inactivation of *marR* results in increased expression of MarA, which acts at several target genes in the cell leading to reduced drug accumulation by efflux and other mechanisms [123]. It has been suggested that loss of one MarR-like protein in *M. tuberculosis*, the Rv2887, leads to increased drug efflux activity and methylase expression ultimately increasing resistance to an imidazopyridine agent [121].

Less studied but of importance is BlaI, encoded by the *Rv1846c* gene, a transcriptional regulator belonging to the winged helix superfamily of regulators [124], a subfamily within the large group of helix-turn-helix proteins [125]. BlaI is autoregulatory and repression of its regulon occurs through its binding to the inverted repeat sequence located in its promoter region. BlaI of *M. tuberculosis* shows strong similarity with BlaI and MecI of *Staphylococcus aureus*: *Rv1846c* forms an operon with *Rv1845c* corresponding to *blaI-blaR* [124]. The *blaI* regulon is composed of

the following genes: *blaR* (*Rv1845c*), *blaI* (*Rv1846c*), *Rv1847*, *blaC* (encodes the unique β-lactamase in *M. tuberculosis*), *sigC* (encodes an extracytoplasmic function subfamily sigma factor), *Rv1456c* (ABC transporter; Table 21.1), *Rv3921c* (conserved transmembrane protein), and *Rv1303-atpBEFHAGDC-Rv1312* (ATP synthase). Altogether this suggests that Rv1846c possibly controls the expression of a set of genes involved in regulation, drug transport and detoxification, membrane components, and ATP synthase [124]. β-Lactams are direct inducers of Rv1846c regulator since treatment with amoxicillin leads to detachment of Rv1846c from its promoter causing derepression of its regulon and upregulation of ATP synthase transcription. This indicates that BlaI is involved in cell response to different stressful stimuli [124].

Noteworthy is also the TetR transcriptional regulator Rv3066 that controls the expression of the *M. tuberculosis* efflux transporter Mmr [53, 126]. Rv3066 is encoded by the *Rv3066* gene that is located immediately downstream of *mmr* and encodes a 202-amino acid protein. Rv3066 represses the transcription of *mmr* through its direct binding to the inverted repeated sequences located in the promoter. Expression of Mmr is induced by the release of the Rv3066 regulator from its cognate DNA in the promoter region. Acriflavine, proflavine, pyronin Y, safranin O, and thioridazine are inducers of the *Rv3066* expression. The binding of these ligands to the ligand-binding pocket of Rv3066 leads to its release from the promoter and expression of Mmr is initiated [126].

As in other bacteria, the existence of a complex global transcriptional modulation network for drug resistance in mycobacteria seems to be a reality that needs to be further studied and expanded to different mycobacterial species. Indeed, Bowman and Ghosh [127] demonstrated the existence of a master regulatory network upstream of WhiB7 in *M. smegmatis*. This regulatory network is composed by an anti-sigma factor (MSMEG_6129) and an anti-anti sigma factor (MSMEG_6127) under the control of an upstream signaling pathway that involves PknB (MSMEG_5437), a serine/threonine-protein kinase. This regulatory network influences several cellular responses, such as multidrug resistance and response to oxidative stress, by controlling the expression of a large regulon comprising efflux transporters, catalases, WhiB7, and other transcriptional factors. Although no homolog of either MSMEG_6129 or MSMEG_6127 is found in *M. tuberculosis* genome, one cannot exclude the existence of a functional homolog not yet identified in *M. tuberculosis* by considering the similarities in intrinsic drug resistance between both species [127].

21.4 Mycobacterial Efflux Pump Inhibitors

The activity of efflux pumps is dependent on the proton motive force and the availability of ATP within the cell. The decrease of intrinsic resistance of *M. smegmatis* to isoniazid by the use of an efflux inhibitor was first demonstrated by Choudhuri and colleagues [128]. In that study, the accumulation of isoniazid increased upon

the addition of protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) suggesting that a proton motive force-dependent efflux system was involved in the efflux of isoniazid. The proton motive force is established through the development of the transmembrane proton gradient which occurs due to the movement of electrons through the electron transport chain resulting in the establishment of the membrane potential. The proton gradient generated through oxidative phosphorylation leads to ATP synthesis via ATP synthase which is responsible for the conversion of the electrochemical potential energy generated by the proton motive force into chemical energy in the form of ATP [129]. Thus, energy metabolism and ATP production through the proton motive force, which is established by the electron transport chain, significantly contribute to drug susceptibility in mycobacteria. The inhibition of efflux activity by the depletion of energy required for transport results in the accumulation of antimycobacterial drugs inside the cells [6, 19].

To date, several compounds have been appointed as potential efflux inhibitors of mycobacterial efflux systems and all share a common trait: they interfere, directly or indirectly, with bacterial energy production necessary to maintaining the functional pumps [6]. The canonical group of molecules that fulfill the previous description of an efflux inhibitor are the protonophores, which include CCCP, dinitrophenol, and valinomycin, which inhibit efflux pump activity by collapsing the transmembrane electrochemical potential [130, 131]. Many studies have extensively shown that these compounds inhibit the activity of secondary transporters in bacteria and, being also ionophores that act as chemical inhibitors of oxidative phosphorylation, they also inhibit the activity of ATP synthase, with a direct effect on bacterial ABC transporters [56, 132]. CCCP reduced the level of resistance to isoniazid, ethambutol, ethionamide, erythromycin, and acriflavine in *M. tuberculosis* overexpressing the efflux pump Mmr [24, 53], interfered with the activity of the P55 efflux pump, reducing the resistance to a large spectrum of compounds [5, 58, 77], and with the activity of MmpS5-MmpL5 and MmpL3 efflux pumps in *M. tuberculosis* [63, 68]. The limitation of these inhibitors is their toxicity since they affect also the energy production of the eukaryotic cells since they share the same targets as the bacterial respiratory chain, precluding their clinical usage in any circumstance [129, 130].

Conversely, ion channel blockers like the phenothiazines thioridazine and chlorpromazine and their derivative flupenthixol, the butyrophenone haloperidol and the phenylalkylamine verapamil, have been used in clinical practice as neuroleptics, antihypertensives, or antiarrhythmic for many years, even with some associated toxicity [19, 131, 133]. Phenothiazines are divided in three groups: the aminoalkyl compounds (e.g., chlorpromazine), piperidine compounds (e.g., thioridazine), and piperazine compounds (e.g., fluphenazine). This class of drugs is known to inhibit the transport of calcium by preventing its binding to Ca^{2+} dependent enzymes in eukaryotic cells and also in bacteria including mycobacteria [134, 135]. Thioridazine exhibited significant activity against multidrug-resistant tuberculosis in a murine model and was successfully employed to treat extensively drug-resistant tuberculosis on the basis of compassionate reasons [136, 137]. The phenothiazines possess a three-ring structure, in which a sulfur atom and a nitrogen atom are linked to two

benzene rings; a substitution of the nitrogen with a carbon atom in the central ring produces a thioxanthene. Among these compounds, flupenthixol is a high-potency thioxanthene with well-established antipsychotic properties [138].

Verapamil, a phenylalkylamine, is an active synthetic analogue to a derivative (papaverine) from the opium poppy which contains three important alkaloids: morphine, codeine, and papaverine [139]. Verapamil acts by blocking calcium channels and is extensively used for the treatment of various disorders such as angina, hypertension, and cardiac arrhythmia [140]. Verapamil is also an inhibitor of P-glycoprotein in mammalian cells [141]. In prokaryotes, verapamil inhibits ATP-dependent multidrug transporters and indirectly proton motive force-dependent pumps, by interfering with the generation of the proton motive force, and has a significant inhibitory effect on mycobacterial efflux pump activity to decrease resistance (and in certain cases to restore susceptibility) to isoniazid, macrolides, ofloxacin, rifampicin, streptomycin, bedaquiline, clofazimine, and ethidium bromide in *M. tuberculosis* and *M. avium* [21, 142–145]. Adams and colleagues [25] showed that macrophage-induced tolerance to rifampicin in a *Mycobacterium marinum*-infected zebrafish larval model can be inhibited with verapamil. This result was later supported by the finding that the addition of verapamil to standard tuberculosis chemotherapy in a mouse infection model significantly decreased the lung bacillary load [144, 146, 147].

The inhibitory activity of these ion channel blockers is attributed to their effect on the mitochondrial electron transport chain. In eukaryotic cells, the neuroleptics haloperidol and chlorpromazine inhibit the mitochondrial NADH: ubiquinone oxidoreductase (complex I) [129, 148]. Evidence for the involvement of the electron transport chain on the mode of action of neuroleptic compounds in *M. tuberculosis* comes from the studies performed with the phenothiazine class of drugs. The phenothiazines are known to inhibit one of the key enzymes of the mycobacterial respiratory chain, the type II NADH: menaquinone oxidoreductase (NDH-2) [149, 150]. *M. tuberculosis* possesses two NADH dehydrogenases: the NDH-1, encoded by the *nuo* operon, which is nonessential in *M. tuberculosis* [151], and the non-proton-translocating NDH-2, encoded by the *ndh-2* gene, which is essential for *M. tuberculosis* survival [152]. NDH-1 is an equivalent of mitochondrial complex I, while NDH-2 is a single subunit enzyme with NADH/quinol oxidoreductase activity [129, 153]. Rao et al. [151] established a link between NDH-2 and proton motive force through the demonstration that the inhibition of NDH-2 by thioridazine dissipates the membrane potential. Thioridazine and chlorpromazine act as partial uncouplers of oxidative phosphorylation in mitochondria [154]. A recent study showed that besides their inhibitory effect on NDH-2, these agents also act as uncouplers of oxidative phosphorylation in *S. aureus* [155].

Finally, plant-derived efflux pump inhibitors, such as the plant alkaloid reserpine from the roots of *Rauwolfia vomitoria* Afz., the *trans-trans* isomer of 1-piperoyl-piperine from the *Piperaceae* family piperine, berberine, from the *Berberidaceae* family, and also other compounds from plant extracts, have been tested as potential new antimycobacterial agents and new efflux inhibitors for mycobacteria [156, 157]. Reserpine has reversed high-level isoniazid resistance in *M. smegmatis* due to overexpression of the *M. tuberculosis mmpL7* gene [71, 128]

and also in *M. tuberculosis* [2, 158]. Piperine is also a known inhibitor of the human P-glycoprotein and cytochrome P450-mediated pathways that mediates an effective inhibition of transport of metabolites and noxious compounds from eukaryotic and prokaryotic cells [159, 160]. Once again the limitations found for their clinical usage are the toxicity associated to their usage at the required concentrations to be effective as mycobacterial inhibitors [156, 157].

The potential use of these efflux pump inhibitors in combination with antimicrobial drugs can constitute an important alternative as adjuvants of the antituberculosis conventional therapeutic regimen and possess the advantage of being cost-effective and time-saving. However, their mechanism of action is not completely elucidated, and the clinical use of these efflux inhibitors at the concentrations needed to reproduce *in vivo* the *in vitro* inhibitory effects is not advised due to the associated toxicity and severe side effects. The hypothesis raised so far for the *ex vivo* and *in vivo* synergistic effects with antituberculosis therapy is grounded on their concentration to high levels at the macrophage phagolysosome where *M. tuberculosis* resides after infection and their ability to enhance the macrophage-killing activity [19, 25, 161, 162]. In this regard, several efflux pump genes of *M. tuberculosis* are macrophage-induced and are correlated with intracellular survival to deal with antimicrobial selection environments [25, 163]. The observed killing enhancement has been postulated to be due to the inhibition of the macrophage calcium transporters leading to the phagolysosome acidification and activation of hydrolases required for the subsequent killing of intracellular *M. tuberculosis* [25, 164, 165]. The delivery of antituberculosis drugs in conjunction with efflux pump inhibitors that act as ion channel blockers, to the phagosome where the mycobacteria reside, may provide a novel and effective therapeutic approach that obviates the serious side effects resulting from the current chemotherapeutic applications (Fig. 21.1) [19, 25, 162, 166].

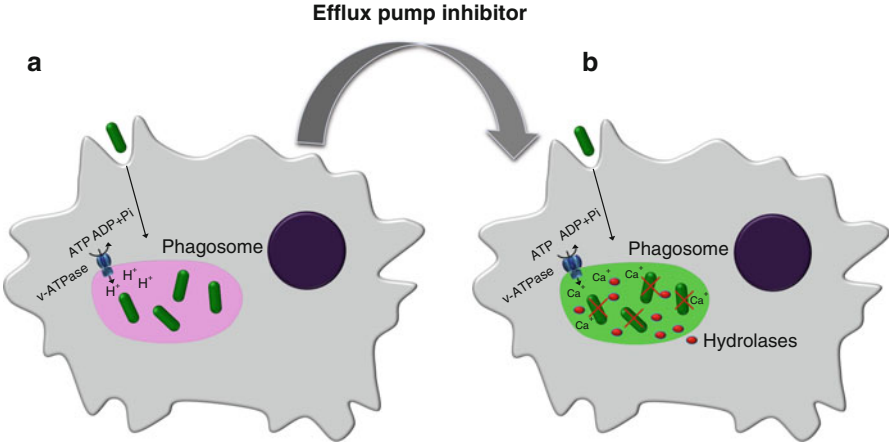

Fig. 21.1 Hypothetical model for the mode of action of efflux pump inhibitors on human macrophages. (**a**) *M. tuberculosis* phagocytosis and beginning of dormancy on the phagosome. (**b**) The addition of an efflux inhibitor leads to an increased transcription of the v-ATPases which increases intracellular calcium concentrations resulting in phagosome acidification. The phagosomal acid synergizes with several components of the host immune response, such as hydrolases leading to bacterial growth restriction

Currently, there are clinical trials ongoing to evaluate the pharmacokinetics of verapamil when co-administered in combination with rifampicin, assessing its tolerability, safety, and potential for shorten the treatment duration in patients with tuberculosis and without basic cardiac disease [165]. These clinical trials were grounded in the recent studies that showed that, in mouse models, verapamil improved the activity of the classical antituberculosis drug regimen [146] reducing the time of treatment completion from 6 to 4 months, decreasing the risk of relapse [147]. Furthermore, *in vitro* and *in vivo* experiments showed that verapamil potentiates the antimicrobial activity of bedaquiline and reduces the dose required for cure reducing the risk of QT prolongation associated with bedaquiline use [144, 165, 167]. Besides verapamil, other compounds such as timcodar and SILA-421, both previously described as mammalian efflux pump inhibitors, have been evaluated as adjuvant in tuberculosis therapy in preclinical studies [168, 169]. Studies to rationally design derivatives of these efflux inhibitors with improved inhibitory activity and reduced toxicity are needed [145, 170].

21.5 Role of Mycobacterial Efflux Pumps in Fitness and Pathogenicity

Besides the different susceptibilities to several substrates, mycobacteria have changed their physiological behavior, such as growth inside and outside of macrophages [25, 171] and pathogenicity [70], when their pattern of efflux activity is altered. These findings show that efflux systems are involved in fundamental cellular physiological processes and that drug extrusion could be a casual nonspecific side-effect [31]. Thus, physiological regulatory systems may determine the levels of drug resistance as well as the presence of a specific substrate of efflux may reveal a change in mycobacterial physiological behavior. Knowledge of the efflux mechanisms in mycobacteria has become intriguing and needful, since the inhibition of efflux can contribute to increase the antimicrobial treatment efficacy and also act in the direct suppression of a physiological activity of bacteria [2, 19].

A conspicuous physiological change related with *M. tuberculosis* pathogenicity is mediated by the ABC transporter DrrC and MmpL7. The doxorubicin resistance operon, *drrABC*, encoding DrrABC proteins, and the gene *mmpL7* encoding MmpL7 transporter are both located in a cluster of 13 genes involved in the biosynthesis of PDIMs and their active transport from the cytoplasm to cell wall surface. PDIMs play a role in cell wall permeability of *M. tuberculosis* [87] and also are virulence factors of *M. tuberculosis*, in particular during the early step of infection when the bacilli encounter their host macrophages [172]. Signature-tagged transposon mutagenesis showed that transposon insertions in the *drr* operon or *mmpL7* lead to a strong growth defect of *M. tuberculosis* in the lungs of intravenously infected mice [173]. Attenuation of the mutants is due to the lack of PDIM in the cell surface and two different efflux pumps are needed for this transport [87, 173]. Camacho and colleagues [173] proposed that MmpL7 and DrrABC transporters interact for efficient translocation of PDIM based on a similar combination involved in

polyketide export in *Streptomyces coelicolor* [174]. In addition to the transport of PDIM, DrrC also exports lipids to the outside and MmpL7 appears to transport a related but structurally distinct phenolic glycolipid expressed only in a subset of highly virulent *M. tuberculosis* strains [70].

Another ABC transporter with an important role in *M. tuberculosis* virulence is BacA. Mice infected with a BacA-inactivated H37Rv strain survived longer once compared with those infected with the wild-type H37Rv strain [47]. BacA seems to play an important role in the outcome of *M. tuberculosis* chronic infections determining whether an infection progresses to active disease or remains latent in a given individual [175]. Furthermore, ABC transporters mediate both influx and efflux activity of molecules as previously described, but their role as importers has been marginalized. Apparently, *M. tuberculosis* is less dependent on external molecules for infection and survival [33], but similar to other prokaryotes, *M. tuberculosis* also has transport mechanisms to internalize molecules and ions, such as the well-studied phosphate-specific transporter (Pst). Pst is responsible for the much needed uptake of inorganic phosphate by mycobacterial cells [176] and is composed of four distinct subunits encoded by the *pstS*, *pstA*, *pstC*, and *pstB* genes. The PstB subunit provides energy for transport through ATP hydrolysis [177]. Unlike other prokaryotic ABC proteins, PstB from *M. tuberculosis* is resistant to known ATPase inhibitors and is thermostable, an uncommon feature in mesophilic bacteria [178]. The importance of PstB as exporter and importer of phosphate was also demonstrated in *M. smegmatis*, where the inactivation of *pstB* resulted in the loss of phosphate uptake and a concomitant hypersensitivity to fluoroquinolones [179]. Interestingly, in *M. tuberculosis* genome, three putative *pst* operons have been identified, but *pstB* gene is present in only one of those operons [51, 100]. Considering that phosphate concentration is determinant for mycobacterial survival inside the vacuoles of the host phagocytic cells, the high-affinity phosphate-specific transporters such as Pst have been considered virulence factors of *M. tuberculosis* [180].

Other efflux pump with impact on virulence of mycobacteria is the P55 efflux transporter. In *M. tuberculosis* and *M. bovis*, the gene encoding the P55 efflux pump belongs to the same transcriptional unit of the gene *lprG* (*Rv1411c*), which encodes an antigenic lipoprotein LprG [181]. The knockout of the *lprG-p55* operon causes strong attenuation in *M. tuberculosis* and *M. bovis* both *ex vivo* and *in vivo* [77, 182]. Likewise, the *lprG* and *p55* genes are conserved across several pathogenic and non-pathogenic mycobacterial species, including *M. avium* complex, *M. leprae*, and *M. smegmatis*, and different studies revealed complementary transport functions: virulence, cell wall maintenance, efflux of noxious agents, and detoxification. In *M. smegmatis*, inactivation of the *lprG-p55* operon promoted changes in colony morphology suggesting alterations in cell wall composition. In *M. avium*, both proteins are required for the transport of noxious compounds across the bacterial cell wall [183, 184]. P55 is also involved in detoxification systems which are linked to respiratory processes and maintenance of the redox balance within the cell. *M. bovis* BCG with the inactivated *p55* gene presented an increased susceptibility to the strong reducing agent DL-dithiothreitol and to diamide, a thiol-oxidizing agent, altering the susceptibility of *M. bovis* BCG to superoxide and to agents that disrupt the systems needed to maintain the thiol redox balance in the bacterial cytoplasm [58].

The ability of *M. tuberculosis* to survive and replicate within macrophages is central to the pathogenesis of tuberculosis and has been inferred that efflux pumps have an important relation with intracellular growth [185]. Several efflux pumps have been reported to be transcriptionally induced in macrophages, namely, Rv0194, Rv2686c-2688c, Rv1258c, and Rv3065 [163]. When Rv1258c is inactivated by transposon insertion, the mutants become compromised for intracellular growth and develop hypersusceptibility to rifampicin, suggesting that this efflux pump is required for both intracellular growth and rifampicin tolerance [25]. Furthermore, it was demonstrated that the disruption of Mb1288c (Rv1258c homolog) in *M. bovis* BCG triggered a general stress response associated with a general repression of genes involved in cell wall biosynthesis. This extensive change in gene expression patterns occurred during stationary phase, with no changes observed during exponential growth, suggesting that the accumulation of substances at toxic concentration levels occurs during stationary phase [186].

Considering that overexpression of drug efflux pumps has direct influence on the physiological behavior of the bacteria, an important question is if this stress-response mechanism influences the fitness of mycobacteria, as seen in other bacteria [2]. The fitness of a strain is a complex characteristic that can be defined as the bacterium's ability to infect a susceptible host, persist and proliferate, and be transmitted to another host [187]. In mycobacteria, studies on evaluation of relative fitness associated to efflux pump overexpression are scarce because the main interest has been the contribution of efflux pumps for clinical resistance of mycobacteria [22, 27], and methods to compare *in vitro* and *in vivo* fitness of mycobacteria are expensive and cumbersome. Recently, new methods based on measurement the metabolic activity to evaluate fitness have been purposed [188, 189]. Of relevance is the usual absence of research and information on the relative fitness cost-associated efflux activity [190]. For the recently developed drug for tuberculosis treatment, bedaquiline, the biological cost of their resistant mutants has been determined. *M. tuberculosis* isolates obtained *in vitro*, in mice, and in patients with increased minimal inhibitory concentrations of bedaquiline presented mutations in the *Rv0678* gene, which causes overexpression of the MmpS5-MmpL5 efflux system. Surprisingly, no biological cost was found comparing the *in vitro* growth rate and *in vivo* virulence between the *Rv0678* mutants in relation to the parental H37Rv strain [92].

Nowadays, with the introduction of massive transcriptomic and whole genome sequencing methods, the analysis of the behavior of mycobacterial clinical isolates in the presence and absence of different substrates, environments, and genetic backgrounds will certainly help to reveal the participation of efflux pumps in their fitness and survival, and their physiological role in virulence and pathogenicity can be further elucidated. The indication that the overexpression of efflux systems can present an advantage to bacterial fitness without imposing a biological cost highlights the need of further studies on the introduction of efflux inhibitors as adjuvants of antimycobacterial therapy [191–193].

21.6 Concluding Remarks

Standard first-line antituberculosis therapy involves isoniazid, rifampicin, ethambutol, and pyrazinamide, and strains that become resistant to at least rifampicin and isoniazid are termed multidrug-resistant. Once additional resistance is acquired to fluoroquinolones and injectable aminoglycosides (second-line drugs), these strains are termed extensively drug-resistant. This process is grounded on the fact that the antituberculosis drugs and the dosages currently in clinical use are toxic and poorly tolerated, with common severe adverse events, combined with the prolonged duration of the treatment to achieve a cure. Both events promote patient noncompliance and treatment failure [194]. Although it is now evident that *M. tuberculosis* drug resistance is predominantly conferred by the accumulation of mutations in genes coding for drug targets and/or drug-activating enzymes, the mechanisms by which clinical resistance emerges are not fully understood. Changes in efflux pump gene expression and regulation also have a significant impact on the development and final level of drug resistance [20, 23, 26, 27, 195].

Understanding the dynamics underlying bacterial antimicrobial-induced transport systems, such as the induction, regulation, activation, and overexpression of efflux pumps, will provide insights on minimizing this chain of events and fostering new chemotherapeutic approaches that would shorten the treatment, ultimately rendering these organisms susceptible to many of the currently accessible antimicrobials, and allowing the recovery of certain compounds that are already out of the clinical usage rather than waiting for the uncertain, costly, and timeless pipeline of new drug development [196]. The search for novel compounds acting as efflux pump inhibitors, to be used in combination with the current armamentarium of antimicrobial agents that are effective against susceptible and drug-resistant tuberculosis (and other difficult to treat mycobacterial infections), is an obvious avenue to explore in the present and should become a major goal of antituberculosis drug discovery programs in the future.

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Chapter 22
Antimicrobial Drug Efflux Genes and Pumps in Bacteria of Animal and Environmental Origin

Lateef Adewoye, Edward Topp, and Xian-Zhi Li

Abstract Antimicrobial drug efflux genes are widely distributed in nature and are important components of the environmental resistome. As with any resistance genetic determinants, drug efflux genes also reflect the dynamic evolution and complex dissemination of antimicrobial resistance across various environments, in particular under antimicrobial selective pressure. A number of efflux genes have been identified and characterized in both pathogenic and non-pathogenic bacteria from animals and other environments including soil, plant and aquatic sources. Drug efflux pumps play important roles in drug resistance as well as other functions including pathogenesis. This chapter describes the distribution of drug efflux genes and functional roles of drug efflux pumps in these bacteria.

Keywords Antimicrobial resistance • Resistome • Resistance genes • Efflux pumps • Efflux genes • Animal • Plant • Soil • Aquatic • Veterinary pathogens

22.1 Introduction

Antimicrobial resistance is a public health issue of epidemiological complexity that is evidently linked to the use of antimicrobial agents in different environments, including human and veterinary medicine, agri-food sector, plant protection, and consumer products [1–4]. The One Health approach, which addresses issues at human-animal-environment interface, has been increasingly recognized as an important strategy to tackle antimicrobial resistance [3, 5]. Indeed, resistant bacteria

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and their resistance genes can disseminate across different environments. Resistance genes acquired by human pathogens typically have their environmental origin, and resistant isolates from different sectors share the same genetic basis and mechanisms of resistance [1, 6].

Drug efflux serves as one of the major mechanisms of antimicrobial resistance and provides a remarkable ability for bacteria to survive and evolve in diverse hostile environments that may contain antimicrobial substances [7, 8]. Thus, not only has drug efflux been demonstrated in bacteria of human sources but it has also been seen in bacteria of animal, plant, and environmental origins. Drug efflux genes constitute an important part of the resistome, i.e., the complete repertoire of resistance genes [9]. These genes typically encode transporters belonging to one of the five major superfamilies/families: ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the multidrug and toxic compound extrusion (MATE) family, and the small multidrug resistance (SMR) family (see Chaps. 1, 2, 3, 4, and 5). Using specific examples, this chapter first briefly describes antimicrobial resistance genes with an emphasis on drug efflux determinants in various environments and then examines the role of drug efflux pumps in resistance and other functions in individual bacteria of animal and environmental origin. Assessment of drug efflux genes in diverse bacteria offers further support on functional importance and therapeutic implications of drug efflux pumps. It should be noted that numerous bacteria (such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Burkholderia* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Vibrio* spp.), which have been predominantly studied as human pathogens, are excluded from this chapter since they are considered elsewhere in this book.

22.2 Antimicrobial Resistance Genes in Bacteria of Animal and Environmental Origin

Investigations (including surveillance) on antimicrobial resistance have to date mainly concerned bacterial pathogens of human source. However, there have been increasingly numerous studies targeting resistance of bacteria from animal and environmental reservoirs including the identification and characterization of resistance genes and mechanisms [2, 10–14]. These studies are revealing the ubiquity of antimicrobial resistance in bacteria, with the general conclusion that "resistance genes are everywhere" [1]. Accumulating evidence has shed light on the epidemiology and ecology of resistance genes and resistant bacteria. Figure 22.1 shows the complex pathways of the epidemiology of antimicrobial resistance. Antimicrobial-resistant bacteria and their resistance genes (including drug efflux genes) exist or can emerge in various environmental settings and can be further disseminated via numerous pathways that interconnect humans and animals through the aquatic and terrestrial environments. In this regard, advances in molecular methodologies such as high-throughput genomic sequencing and metagenomic tools have revolutionized

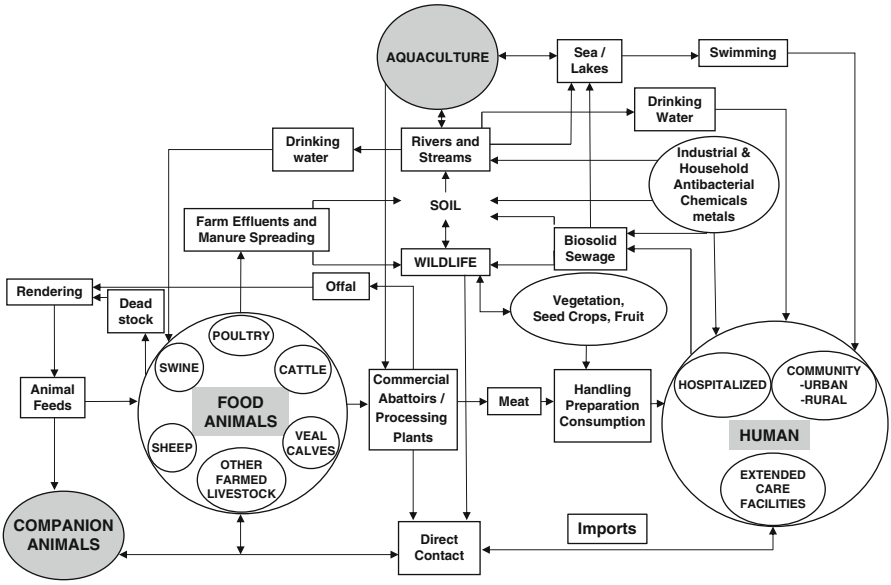

Fig. 22.1 Epidemiology of antimicrobial resistance – possible pathways for the emergence and dissemination of antimicrobial-resistant bacteria and their resistance genes (such as drug efflux genes) from humans, animals, and the environment including soil, plant, and aquatic sources (Figure courtesy of Dr. Rebecca Irwin, the Public Health Agency of Canada)

our approach to studying resistance genes, for example, for large-scale analyses of environmental examples [15–17]. Thus, over the last decade, large amounts of data have become available for in-depth understanding of antimicrobial resistance among bacteria of human, animal, and environmental sources.

Resistance genes in diverse geographical conditions occur naturally or via anthropogenic origin through human or agricultural waste streams. While antimicrobial use is a major risk factor for resistance development in any environmental setting, studies have clearly demonstrated the ancient origin of resistance, revealing resistance as a naturally occurring phenomenon widespread in the environment that had not previously been exposed to the selective pressure of antimicrobial use [13, 18–20]. The detection of resistance genes in remote environments where selection pressure due to antimicrobial use was not a factor [19, 20] reflects additional roles for resistance genes beyond resistance. Resistance genes found in environmental reservoirs are predicted to have diverse physiological functions in bacteria other than conferring resistance to antimicrobials at clinically relevant concentrations [6, 21]. For example, other natural functions of resistance genes can include a role in signaling and amplification of virulence [22]. However, because many antimicrobial substances are produced by soil microorganisms, it can be assumed that these chemicals confer a selective advantage by inhibiting growth of competing organisms in complex microbial ecosystems [9]. There is now a widespread concern that anthropogenic impacts on the environment, such as exposure to antimicrobial

pharmaceuticals in agricultural effluents, wastewater from municipal or hospital, sewage treatment plants, and other exposure routes, have exacerbated the abundance and the persistence of resistance genes and resistant organisms in the environment [2, 11, 14, 15, 23–28]. Loading of antimicrobials, biocides, and other co-selecting chemicals into the environment are likely promoting the acquired resistance in microbes through genetic mutations, recombination, or horizontal gene transfer.

Resistant organisms and resistance genes are prevalent in nature and have been detected or isolated from diverse environments, including human and animal gut microbiota, fecal sources, livestock farms, sewage treatment plants, soils, vegetables, surface waters, ground waters, and oceans [4, 13, 17, 24, 28–38]. The human normal gut microflora is rich in resistance genes that can act as reservoirs of resistance [39]. Specific antimicrobial use practice in humans and animals has been demonstrated to impact the human gut resistome [29]. For instance, antimicrobial exposures in different countries/regions were found to be positively correlated to the median resistance potential of gut microflora samples [29]. Among the resistance gene detected, a significant portion is attributable to drug-specific or multidrug efflux pumps encoded by genes including *bcrA* and *macAB* (for ABC pumps), *acrAB*, *mexB* and *mexF* (for RND pumps), *norM* (MATE pump), and *tet*(*A*), *tet*(*B*), *tet*(*G*), *tet*(*L*), and *tet40* (for MFS pumps) [29]. An *in silico* analysis of resistance genes within the human gut microflora of individuals of diverse geographies and age groups reveals that resistance against tetracycline was the most common, followed by bacitracin [40]. Within this context, the efflux process catalyzed by Tet pumps and the BcrA pump is a major mechanism of resistance to tetracycline and bacitracin, respectively [41, 42].

Recent studies have also focused on the role of the environmental reservoir vis-à-vis the clinically relevant resistance problems in humans [14, 33]. Genes encoding resistance to ampicillin, nitrofuran, sulfadimethoxine, and tetracycline have been detected in diverse marine environments [43]. Decades of antimicrobial use in food animals have created substantial selection pressure for emergence of resistance to a broad range of antimicrobial drugs used in veterinary medicine including livestock production [44]. Resistome diversity was recently also reported in cattle and the environment with identification of more than 300 resistance genes including numerous drug efflux genes [17]. Soils had the most diverse complement of resistance genes, but cattle feces had relatively more abundant genes. When cattle were in the feedlot under antimicrobial selective pressure, resistance gene diversity was reduced. This decrease occurred primarily through the loss of resistance to antimicrobial drugs that were not administered (e.g., amphenicols), but genes responsible for resistance to macrolides and tetracyclines, which were administered to the cattle in this study, were prevalent during the study period, including the exit from the feedlot and transport/slaughter stages [17]. This study demonstrated again that antimicrobial selection pressure can impact the resistome or enrich particular resistance genes. Another recent study reported antimicrobial resistance genes in the sediment samples from fish farms located in Baltic Sea, Finland [45]. Fish farm resistomes were enriched in transposon- and integron-associated genes and in genes encoding

resistance to antimicrobials that had been used at the farms (tetracyclines, sulfonamides, trimethoprim, and florfenicol). Efflux pump genes were among the major resistance genes and included tetracycline efflux genes *tet*(*A*), *tet*(*E*), *tet*(*G*), and *tet*(*H*), amphenicol efflux genes *cml* and *floR*, and multidrug RND pump efflux genes [45]. Indeed, drug residues such as sulfonamides and tetracyclines are frequently detected at significant levels in livestock manures and wastewater runoff from farms where relevant resistant organisms and resistance genes are also prevalent, clearly indicating an association between drug exposures and prevalence of resistance [24, 46–50]. The gut microbiota of honeybees was found to carry eight tetracycline resistance genes including *tet*(*B*), *tet*(*C*), *tet*(*D*), *tet*(*H*), *tet*(*L*), and *tet*(*Y*) efflux pump genes. This observation is attributable to accumulation of resistance genes as the consequence of long-term exposure to tetracycline antibiotics [51]. Roles of tetracycline efflux pumps go beyond tetracycline resistance by their involvement in bacterial stress response and thus improving the bacterial ecological fitness [52]. Overall, among various resistance genes detected in the environment, drug resistance efflux genes have been one group of highly prevalent genes in general, and tetracycline efflux genes have likely been evolved and enriched in particular [15, 24, 28, 53–56].

22.3 Antimicrobial Drug Efflux Genes and Pumps in Bacteria of Animal Source

22.3.1 Gram-Positive Bacteria

***Staphylococcus* spp.** This species is an important facultative opportunistic pathogen responsible for infections in humans and animals. Chapter 8 of this book focuses on the drug efflux pumps in *Staphylococcus aureus*. Drug efflux genes such as *qacG* and *qacJ* have been detected in several animal-related clones of methicillin-resistant *S. aureus* including CC5, CC22, and CC398 [57]. These genes encode drug efflux pumps that contribute to biocide resistance. In another earlier study, high-level prevalence of plasmid-borne biocide resistance efflux genes *qacA*, *qacB*, *qacG*, *qacJ*, and *smr* was found in staphylococci of cattle and goat origin [58]. Interestingly, plasmids carrying *qacG* or *qacH* were also reported from staphylococci from food industry [59, 60] where disinfecting biocides are expected to be routinely used. Transfer of a *qacJ*-containing plasmid among equine staphylococci was also reported [61]. A large number of macrolide- and lincosamide-resistant *S. aureus* isolates from bovine mastitis possessed efflux genes *mrsA* and *mrsB* which were co-present with other macrolide resistance genes [62]. The *msrA* gene was prevalent in *Staphylococcus epidermidis* isolates from bovine milk [63]. Several coagulase-negative staphylococcal isolates, such as *S. epidermidis* and *Staphylococcus xylosus*, have also been isolated from animal-derived ready-to-eat food and shown to harbor genes encoding tetracycline efflux pump genes *tet*(*K*) and *tet*(*L*) and macrolide efflux genes *msr*(*A*/*B*) [64].

Streptococcus suis This organism has long been known as an important zoonotic pathogen responsible for severe infections in swine and humans. Multidrug resistance phenotype has been described among *S. suis* isolates from pigs. In one study of 227 isolates, high levels of resistance to a wide variety of antimicrobial agents, including aminoglycosides, fluoroquinolones macrolides, third-generation cephalosporins, and tetracyclines, were demonstrated with *mef*(*A*), *mef*(*E*), and *tet*(*L*) efflux genes among the identified resistance determinants [65]. Resistance to fluoroquinolones, which are used to treat *S. suis* infections, has previously been shown to be mediated by the SatAB efflux pump, an ABC transporter [66]. SatAB is negatively regulated by a MarR-family regulator, SatR, whose encoding gene is located upstream of the *satAB* efflux genes. *satRAB* genes are co-transcribed and likely form an operon. SatRAB homologs have been identified in several *Streptococcus* species [67]. More recent studies have linked high-level fluoroquinolone resistance to partly to mutations in the DNA gyrase genes and partly to overexpression of an efflux-like protein, a non-SatAB ABC transporter (encoded by gene *SS2069*), which is significantly upregulated in resistant strains [68].

Clostridium perfringens This anaerobic pathogen is responsible for a range of infections in humans and animals, particularly in poultry. It carries two tetracycline resistance genes *tetA*(*P*) and *tetB*(*P*) which encode a tetracycline efflux pump and a ribosomal protection mechanism, respectively [69]. The TetA(P) protein belongs to a typical MFS transporter with 12 transmembrane helices [70]. Acquired resistance to bacitracin is also mediated by the chromosomally encoded ABC transporter BcrAB, which is regulated by BcrR containing a xenobiotic response element [71]. The efflux pump inhibitor thioridazine at a half MIC level was found to reduce bacitracin MIC by eightfold [71]. Another ABC transporter protein (NP_562422) from *C. perfringens* appears to be able to reduce susceptibility to fluoroquinolones and ethidium bromide (Table 22.1) [77].

***Enterococcus* spp.** The BcrAB transporter, encoded by the *bcrABDR* locus, has been reported earlier to confer bacitracin resistance in *Enterococcus* spp. [128], a commensal species in humans and animals as well as a major opportunistic pathogen in humans. A recent study revealed a multidrug resistance plasmid that harbors *bcrABDR* in *Enterococcus faecium* and *Enterococcus faecalis* of both human and swine origin, with much higher prevalence in swine isolates than human counterparts (47 % vs. 14 %) [129]. Enterococci from fecal samples of wild marine species were also found to frequently carry drug efflux genes such as macrolide efflux gene *msrC* and tetracycline efflux gene *tet*(*L*) [130].

22.3.2 Gram-Negative Bacteria

Actinobacillus pleuropneumoniae This species is the major etiological agent of porcine pleuropneumonia. Its genome size is relatively small (e.g., ca. 2.2 Mb for one isolate [GenBank accession CP000687]), but several putative drug efflux pumps

Table 22.1 Drug efflux pumps in bacteria from animals and environmental sources

Source/bacterial species	Transporter family	Pump/homolog	Regulator (family)	Substrates	Reference
Animals					
Aggregatibacter actinomycetemcomitans	ABC	MacAB-TdeA		LTX	[72, 73]
Brucella melitensis	MATE	NorMI		ACR, BB, FQ, GEM, TPP	[74]
Brucella suis	RND	BepDEC	BepR (TetR)	AMI, AMP, BL, CAB, CV, DOC, EB, ERY, NOR, SDS, TET	[75, 76]
	RND	BepFG		CV, DOC, EB	[75]
Clostridium perfringens	MFS	TetA(P)		TET	[69, 70]
	ABC	BcrAB	BcrR	BCI	[71]
	ABC	NP_562422		CIP, EB, NOR	[77]
Pasteurella multocida	?	PM0527/TolC		ACO, CAZ, CV, EB, ERY, LIN, NOV, RIF, SDS, TMP	[78]
	?	PM1980/TolC		CAZ, RIF, VAN	[78]
Streptococcus suis	ABC	SatAB	SatR (MarR)	CIP, NOR	[66, 67]
Soil					
Bhargavaea cecembensis	MFS	Tet45		TET	[56]
Chromohalobacter spp.	RND			BL, CHL, EB, OS, TET	[79]
Flavobacterium johnsoniae	RND	FmeA1B1C1		CHL, ERY, FLO, LZD	[80]
Pseudomonas fluorescens	RND	EmhABC	EmhR (TetR)	AMP, CHL, CV, DPG, EB, FA, NAL, PAH, TET	[81–84]

(continued)

Table 22.1 (continued)

Source/bacterial species	Transporter family	Pump/homolog	Regulator (family)	Substrates	Reference
Pseudomonas putida	RND	SrpABC	SrpSR	OS	[85]
	RND	TtgABC/ArpABC/ MepABC	TtgR/ArpR/MepR (TetR)	AMP, OS, TET	[85–87]
	RND	TtgDEF	TtgT (IclR), SepR (IclR)	OS	[88–90]
	RND	TtgGHI/SrpABC	TtgV (IclR)-TtgW/SrpS (IclR)-SrpR	OS	[90–93]
	ABC	PP26669-2667	AgmR	CHL	[94]
Pseudomonas stutzeri	RND	TbtABM		CHL, NAL, OS, SUL, TRB	[95]
Plant-associated					
Agrobacterium tumefaciens	RND	AmeA	AmeR(TetR)	CB, DOC, NOV, SDS	[96]
	RND	IfeAB	IfeR (TetR)	COU	[97]
Bradyrhizobium japonicum	RND	BdeAB	RegSR	AG	[98, 99]
	RND	Bll7019-7020-7021	Bll7023-7024 (TetR)	FLA	[100]
	RND	FreABC	FrrA (TetR)	FLA	[100, 101]
	RND	RagCD	RagAB		[102]
Erwinia amylovora	RND	AcrAB	AcrR (TetR)	BER, CV, EB, MB, PH, SDS	[103]
		AcrD	BaeSR, CpxAR	BS, CLO, FUA, LUT, NOV	[104, 105]
		MdtABC	BaeSR	Ag, BS, FLA, FUA, JOS, NOV	[106]
		MdtUVW		CLO, FLA, FUA, NOV	[106]
	MATE	NorM		AMP, BER, EB, CV, FQ, KAN, MB, PHL	[107]

Erwinia chrysanthemi	RND	AcrAB-TolC		BER, CAR, CV, LOA, NOR, NOV, SDS, TET	[108, 109]
	MFS	EmrAB-TolC		CAR, CHL, NAR, OLA, TET	[108, 109]
Pseudomonas syringae	RND	MexAB-OprM	PmeR (TetR)	ACR, AG, ACO, BAC, BER, CHL, CV, DAR, EB, ERY, FQ, FLA, FUA, NAL, NIT, R6G, TET, TMP, TPP	[110, 111]
	RND	MexEF-OprN	AefR (TetR)	CHL, CTX, SPC	[112]
	RND	PseABC	GacS/GacA	ACR, ERY, TET	[113]
	ABC	PseEF			[114]
Ralstonia eutropha	RND	AcrA		ISO	[115]
Ralstonia solanacearum	RND	AcrAB	AcrR (TetR)	ACR, AMP, BER, EB	[116]
	MATE	DinF		ACR, AMP, BER, EB, TPP	[116]
Sinorhizobium meliloti	RND	NolFG	NodD (LysR)		[117, 118]
	RND	SmeAB-TolC	SmeR (TetR)	ACR, BER, CHL, ERY, NAR, RIF, SDS, TET	[117]
	RND	SmeCD-TolC		CHL, ERY, NAL	[117]
	RND	SmeEF-TolC		CHL, ERY, TET,	[117]
	MFS	EmrAB	EmrR (TetR)		[119]
	MFS	Tep1		CHL	[120]
Xanthomonas albilineans	MFS	AlbF		ALB	[121]
Xylella fastidiosa	?	?-TolC		BER, GES, RHE	[122]

(continued)

Table 22.1 (continued)

Source/bacterial species	Transporter family	Pump/homolog	Regulator (family)	Substrates	Reference
Aquatic source					
Achromobacter xylosoxidans	RND	AxyABC	AxyR (LysR)	AZT, CHO, CIP, FQ, NAL,	[123, 124]
	RND	AxyXY-OprZ	AxyZ (TetR)	AMI, CB, ERY, FQ, GEM, TOB, TET	[125]
	ABC	MacAB			[123]
Aeromonas hydrophila	RND	AheABC	AheR (TetR)	BAC, BL, ERY, FUA, LIN, PRI, TET, TMP, TRB	[126]
Aeromonas molluscorum	SMR	SugE		CHL, CV, EB, TET, TRB	[127]

ACO acridine orange, *ACR* acriflavine, *ALB* albicidin, *Ag* silver nitrate, *AMI* amikacin, *AMP* ampicillin, *AZT* aztreonam, *BAC* benzalkonium chloride, *BCI* bacitracin, *BER* berberine, *BL* β-lactams, *BS* bile salts, *CAB* cetyltrimethylammonium bromide, *CAR* carbenicillin, *CAZ* ceftazidime, *CB* carbapenems, *CHL* chloramphenicol, *CHO* cholate, *CIP* ciprofloxacin, *CLO* clotrimazole, *COU* coumestrol, *CP* cephalosporins, *CTX* cefotaxime, *CV* crystal violet, *DAR* daunorubicin, *DOC* deoxycholate, *DPG* diacetylphloroglucinol, *EB* ethidium bromide, *ERY* erythromycin, *FA* fatty acids, *FLA* flavonoids, *FLO* florfenicol, *FQ* fluoroquinolones, *FUA* fusidic acid, *GEN* gentamicin, *GES* genistein, *JOS* josamycin, *KAN* kanamycin, *IOS* isobutanol, *LIN* lincosamides, *LOA* linoleic acid, *LTX* leukotoxin, *LUT* luteolin, *LZD* linezolid, *MB* methylene blue, *NAL* nalidixic acid, *NAR* naringenin, *NIT* nitrofurantoin, *NOR* norfloxacin, *NOV* novobiocin, *OLA* oleic acid, *OS* organic solvents, *PAH* polycyclic aromatic hydrocarbons, *PHL* phloretin, *PRI* pristinamycin, *R6G* rhodamine 6G, *RHE* rhein, *RIF* rifampicin, *SDS* sodium dodecyl sulfate, *SPC* spectinomycin, *SUL* sulfonamides, *TET* tetracycline, *TMP* trimethoprim, *TOB* tobramycin, *TPP* tetraphenylphosphonium, *TRB* tributyltin, *VAN* vancomycin

including an arsenic efflux transporter are carried in the genome. An efflux pump involvement in enrofloxacin resistance was observed, but the identity of the transporter(s) is still unknown [131]. Plasmid-borne tetracycline resistance genes (including *tet*(*B*), *tet*(*H*), and *tet*(*L*) efflux genes) were highly prevalent in *A. pleuropneumoniae* [132].

***Bartonella* spp.** These species exist in mammalian host reservoirs and are linked to several human illnesses that comprise Carrion's disease, cat-scratch disease, and trench fever [133]. While *Bartonella* spp. are susceptible to most antibiotic drugs, alteration of drug targets has been identified as the major mechanism of resistance to fluoroquinolones, macrolides, and rifamycins [133, 134]. RND drug efflux pumps and OM efflux components including a TolC homolog are present in *Bartonella* spp. [135–137]); however, functional characterization of these efflux systems remains to be carried out.

Bordetella bronchiseptica This species is a common zoonotic bacterial pathogen responsible for respiratory tract infections in livestock and companion animals. It also occasionally causes pneumonia in humans, particularly in immunocompromised individuals. Bacterial resistance to β-lactams has been reported but no link has been made to efflux mechanism. Resistance to florfenicol and chloramphenicol in porcine isolates of *B. bronchiseptica* has been shown to be mediated by a plasmid-borne *cmlB1* gene, which encodes a putative CmlB1 protein member of a yet uncharacterized chloramphenicol exporter [138]. In this particular study, a chromosomally encoded *floR* gene was also linked to florfenicol resistance in *B. bronchiseptica*. Recent whole genome analysis of Australian respiratory pig isolates of *B. bronchiseptica* found elevated MIC levels for macrolides (64 μg/ml for erythromycin) and reported the detection of several efflux proteins belonging to the ABC, MATE, RND, and MFS pumps [139].

***Brucella* spp.** Six recognized species (*Brucella abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*) are facultative intracellular coccobacilli with distinct host-pathogen association and are the causative agents of zoonotic brucellosis which can be transmitted to humans [140]. The *Brucella* spp. have similar genomes with two circular chromosomes that encode a number of putative drug efflux transporters [141–143]. *Brucella* spp. contain trimeric porins that display similar permeability to *E. coli* porin homologs [144, 145]. Two RND efflux systems, BepDE and BepFG of *B. suis*, interact synergistically with BepDE contributing to resistance to ampicillin, ciprofloxain, norfloxacin, doxycycline (a major agent for the treatment of brucellosis), and tetracycline as well as other biocides [75]. These RND pumps likely also require an outer membrane protein of the tripartite efflux complex such as BepC, a TolC homolog, which has been demonstrated to mediate multidrug resistance (and virulence) [76]. Expression of BepDE is negatively controlled by the BepR repressor and can be induced by deoxycholate [75]. The efflux pump inhibitor phenylalanine-arginine β-naphthylamide can sensitize *B. suis* to erythromycin and moxifloxacin [146, 147], supporting the involvement of efflux pumps in

drug resistance. Genomes of *B. abortus* and *B. suis* also show the presence of genes encoding a putative BicA macrolide efflux pump [143]. NorMI and NorMII are two MATE-type pumps identified in *B. melitensis* with MorMI being functionally confirmed to yield multidrug resistance phenotype (including fluoroquinolone resistance) when expressed in a drug-hypersusceptible *E. coli* strain [74]. However, their role in resistance to fluoroquinolones and rifampicin in clinical isolates was not demonstrated [148].

Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni These species are well-known members of the *Pasteurellaceae* family. They have a broad host range and are major causative organisms for respiratory diseases in food animals. Although they are generally susceptible to a wide range of antimicrobial agents, these pathogens are becoming increasingly resistant, with some multidrug resistant [149–153]. Interestingly, the genome sizes of these species are relatively small (e.g., ca. 2–3 Mb; see GenBank accession CP006957, NZ_CP008918 and NC_010519) in comparison with those of *E. coli* (4–5 Mb) or *P. aeruginosa* (6–7 Mb). This may explain the fewer numbers of transporters encoded by their genomes. For instance, *P. multocida* only encodes 57 ABC, 5 DMT, 11 MFS (including bicyclomycin/multidrug resistance-conferring Bcr/CflA pump), 3 MOP, and 3 RND pumps (one AcrB, SecD, and SecF each) (http://www.membranetransport.org). However, for resistance emergence, mobile genetic elements such as plasmids likely become clinically important. Indeed, in these species, plasmids carrying *tet*(*H*) or *tet*(*L*) efflux genes were detected and found to be responsible for high-level resistance to tetracyclines [154–156], which belongs to one of the most frequently used antimicrobial classes in livestock. In fact, diverse tetracycline resistance genes (including a variety of tetracycline-specific efflux genes) are among the most prevalent resistance genes found in a wide range of bacteria of human, animal, and environmental sources [156, 157].

The gene *msr*(*E*) confers resistance to macrolides (including newer agents such as gamithromycin and tildipirosin in cattle isolates of *Mannheimia* and *Pasteurella* spp. [158–160]. Even with a smaller genome size (ca. 2.3 Mb), *P. multocida* also encodes chromosomally encoded putative transporters including an AcrB homolog (PM1132) [161]. Two outer membrane proteins, TolC homologs (i.e., PM0527 and PM1980) of the tripartite efflux complex (such as OprM and OprJ of *P. aeruginosa* (see Chap. 14)), were identified in *P. multocida*, and their inactivation rendered the mutants hypersusceptible to a wide range of antimicrobial agents, suggesting involvement of multidrug transporters in intrinsic resistance in *P. multocida* [78]. Consistent with this, the involvement of efflux mechanism in fluoroquinolone resistance in *P. multocida* was also described [162]. Exposure of *P. multocida* to subinhibitory levels of various antimicrobials (amoxicillin, chlortetracycline, enrofloxacin, florfenicol, novobiocin, rifampicin, tilmicosin, trimethoprim, and brodimoprim) has been shown to affect the transcriptional responses of *P. multocida* [149, 163, 164]. However, specific drug efflux-related genes were not reported.

22.3.3 Plasmid-Borne Efflux Genes in Animal Isolates

While drug efflux pumps are frequently encoded by genes carried in the chromosome, it is important to mention that several plasmid-encoded clinically relevant drug efflux genes have been first discovered in animal bacteria. For instance, the RND-type OqxAB was first detected in a plasmid from a swine isolate [165]. OqxAB mediates resistance to a range of antimicrobials including olaquindox (a growth promotant in animal feed) and fluoroquinolones (see Chap. 23). OqxAB-encoding plasmids have become increasingly prevalent in *E. coli* of animal origin [166]. A transferable plasmid-encoding FloR pump for florfenicol resistance was also first found in a fish pathogen, *Pasteurella piscicida* [167]. A novel small *floR* gene-carrying plasmid was recently reported from isolates of the swine pathogen *Haemophilus parasuis* isolates that were resistant to florfenicol (8- to 32-fold MIC increase) [168]. The *floR* gene is now well known to be often present on multidrug resistance plasmids as part of resistance gene cassette (see Chap. 23). Novel plasmids carrying an ABC transporter gene, *vga*(*E*), were reported quite recently from swine staphylococci [169]. Vga(E) confers resistance to pleuromutilins, lincosamides, and streptogramin A, which are mostly veterinary antimicrobial drugs. Additionally, the plasmid-encoded ABC transporter OptrA, which mediates resistance to amphenicols and oxazolidinones, is five- to tenfold more prevalent in food animal enterococci than human counterparts [170, 171].

22.4 Antimicrobial Drug Efflux Pumps in Bacteria of Environmental Sources

22.4.1 Soil Bacteria

Soil contains a great diversity of bacteria [172], which are rich in a variety of resistance genes [9, 37, 53]. Here a discussion is limited to several soil bacterial species where antimicrobial efflux pumps are well characterized. Additional plant-associated soil bacteria will be considered in the next subsection.

***Pseudomonas* spp.** *Pseudomonas putida*, a saprotrophic soil organism, has been extensively exploited for its bioremediation and biocontrol capabilities. Before the availability of *P. putida* genome sequence in 2002 [173], four RND-type transporters, SrpABC [85], TtgABC [86, 174], TtgDEF [88], and TtgGHI [91] (the *ttgGHI* operon is present on a self-transmissible plasmid [175]) had been identified based on the sequence homology to Mex pumps of *P. aeruginosa*. These pumps were characterized for their role in organic solvent tolerance (e.g., to toluene) and antimicrobial resistance. Inactivation of the *ttgB* gene rendered the mutant more susceptible to ampicillin, chloramphenicol, and tetracycline [86]. Indeed, TtgABC plays a major role in the intrinsic antimicrobial resistance [91, 176–178] as well as in stress

response and biofilm formation [179]. However, inactivation of TtgABC is evident with enhanced tolerance to phenol in growing bacteria [180]. Negatively regulated by repressor TtgR [176], expression of TtgABC is also inducible by its antimicrobial substrates such as chloramphenicol and tetracycline [178]. The crystal structure of TtgR suggests unique ligand-binding properties of TtgR with two distinct and overlapping ligand-binding sites which form complex with antimicrobials including plant compounds [181]. Molecular mechanism of TtgR binding to antimicrobials has been recently described with an important role of the C-terminal Ser77 for overall protein structure [182].

TtgGHI is also involved in resistance and its expression is regulated by TtgVW (SrpSR) [92, 183]. This expression can be activated by indole, which interacts with TtgV (an Icl-family repressor) [184, 185]. TtgV can bind to the target DNA operators and also to a wide range of structurally distinct effectors which serve as inducers [186, 187]. The crystal structure of TtgV shows a functional tetrameric protein in complex with its target DNA operator containing two continuous recognition sites [188]. Amino acid residues important for intramolecular signal transmission of TtgV are known [189]. SrpR (TtgW) functions as an anti-repressor that binds to SrpS (TtgV) to allow its release from the target DNA site [93]. TtgDEF expression occurs in response to aromatic hydrocarbons [88] and is subject to regulation by TtgT (local repressor of TtgGHI, TtgV, and SepR [89, 90]. The role of TtgDEF in solvent tolerance can be masked by TtgGHI. An ABC transporter (PP2669-2668-2667) and the adjacent AgmR regulator (PP2665) are both required for full resistance to chloramphenicol [94].

A study by Molina-Santiago et al. [190] carried out systemic assessment of the transcriptional response of *P. putida* to subinhibitory concentrations of antimicrobial agents including ampicillin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, rifampicin, and tetracycline. This study showed the expressional changes of a large number of genes including many small regulatory RNAs and genes encoding efflux pumps, transporters, and transcriptional regulators. Except for the increased expression of *ttgB* in the presence of kanamycin, reduced expression of *ttgB* and/or *ttgC* was consistently noted in response to ciprofloxacin, gentamicin, rifampicin, spectinomycin, or tetracycline. Expression of *norA* and *norM* occurred in the presence of ciprofloxacin and gentamicin, respectively [190]. Interestingly, exposure of *P. putida* to various pharmaceuticals results in physiological response that includes involvement of the energy-dependent efflux pumps [191]. Vanillin, an aroma flavor compound, also induces significant proteomic response including upregulation of RND pumps [192].

Pseudomonas fluorescens is another species found in soil and plants. One RND-type system, EmhABC, from *P. fluorescens* can accommodate a range of substrates including nontoxic, highly hydrophobic polycyclic aromatic hydrocarbons and antimicrobials [81]. Mutational analysis of EmhB revealed that the central cavity and periplasmic domains play an important role in the efflux function [82]. Inactivation of any component of EmhABC increases extracellular levels of the polyketide metabolite 2,4-diacetylphloroglucinol [83]. EmhABC is also involved in tolerance to naphthalene by increasing naphthalene metabolism, not by the efflux mechanism

[193, 194]. Negatively regulated by EmhR repressor [83], the expression of EmhABC is influenced by various physicochemical factors including growth temperature, pH, and Mg^{2+} levels [195]. High-level benzalkonium chloride resistance in *P. fluorescens* is also attributable to efflux [196]. *Pseudomonas stutzeri* contains TbtABM pump which contributes to resistance to tributyltin and certain other antimicrobials (Table 22.1) [95].

Other species *Flavobacterium johnsoniae* is a soil and aquatic gliding bacterium. A chloramphenicol-inducible RND-type FmeA1B1C1 is involved in resistance to chloramphenicol, florfenicol, erythromycin, and linezolid [80]. Similarly, putative RND pumps are reported in *Elizabethkingia anophelis*, a dominant species in the gut ecosystem of the malaria vector mosquito *Anopheles gambiae* [197]. Tet45 tetracycline efflux pump was also found in a soil strain of Gram-positive *Bhargavaea cecembensis* isolated from a soil located adjacent to a waste storage on a chicken farm [56]. Using a functional metagenomic approach, a novel gene cloned from Alaskan soil was identified to encode an MFS-type efflux pump named PexA, which mediates reduced susceptibility to chloramphenicol and florfenicol (four- to eight-fold MIC increase when the cloned *pexA* gene was expressed in *E. coli*) [198].

Acinetobacter oleivorans is an environmental organism capable of adhering to and growing on diesel oil [199]. Its genome has revealed the presence of genes/operons that encode more than a dozen RND-type transporters including homologs (e.g., AOLE_RS03650-03655-03660) of *Acinetobacter baumannii* AdeABC/AdeIJK pumps and efflux pump transcriptional regulators [200, 201]. However, experimental assessment of these putative transporters remains to be investigated. Additionally, TetH efflux pump that improves bacterial fitness has been described in *A. oleivorans* [52].

22.4.2 Plant-Associated Bacteria

Agrobacterium tumefaciens This soil bacterium is a well-studied plant pathogen, notably for its ability to transmit and insert DNA into plant genomes. The genome of *A. tumefaciens* C58 (ca. 5.7 Mb) contains two chromosomes and two plasmids [202] and carries a large number of putative transporters (218 ABC, 27 DMT, 47 MFS, 9 MOP, and 11 RND pumps (http://www.membranetransport.org). Two RND-type efflux systems, AmeABC and IfeAB, are negatively controlled by the TetR-family repressors [96, 97], AmeR and IfeR, respectively. Inactivation of AmeABC system does not alter antimicrobial susceptibility, but mutations of AmeR produce increased resistance to carbenicillin, novobiocin, and detergents (Table 22.1) [96], suggesting that AmeABC is not involved in intrinsic resistance and can contribute to acquired resistance. Expression of IfeAB is inducible by the alfalfa root isoflavonoids formononetin and medicarpin and is proposed to contribute to ecological benefit for *A. tumefaciens* [97]. Indole added exogenously was found to upregulate expression of biofilm formation genes, stress response genes (including

marR and *soxR*), efflux genes *emrA* and *norM*, and an RND pump gene (*Atu2551*) and subsequently to result in enhanced biofilm formation and antimicrobial tolerance [203]. Efflux genes involved in arsenic or copper resistance have also been characterized in *Agrobacterium* spp. and other soil bacteria [204–206].

***Bradyrhizobium* spp.** The legume root-nodulating *Bradyrhizobium* spp. include *Bradyrhizobium japonicum* that forms nitrogen-fixing root nodules in association with soybeans. With a large genome size of ca. 9 Mb, *B. japonicum* carries 24 putative RND transporters [98]. The BdeAB RND system is mostly close to MDR-related MexCD of *P. aeruginosa* and AcrAB of *E. coli*. Inactivation of BdeAB led to a marked increase in aminoglycoside susceptibility. The expression of BdeAB is regulated by a two-component regulatory system, RegSR, genetic inactivation of which produces aminoglycoside hypersusceptibility [98, 99]. Nevertheless, the physiological function of BdeAB is likely involved in the formation of an effective symbiosis with the legume plant host, given that the symbiotic nitrogen-fixation activity of either BdeAB or RegSR mutants is strongly reduced [98]. However, BdeAB does not affect the sensitivity to flavonoids (such as genistein) that are nodulation effectors released from the seeds and roots of legumes to induce the nodulation of plants and does not affect the bacterial ability to nodulate [98]. Intriguingly, two flavonoid-responsive RND systems, FreCBA and Bll7019-7020-7021, have been identified in *B. japonicum*, similar to other flavonoid-inducible RND systems identified in other plant bacteria (e.g., AcrAB of *Erwinia amylovora*, IfeAB of *A. tumefaciens*, and MexAB-OprM of *Pseudomonas syringae*) [100, 101, 110]. Deletion of these efflux genes results in defective nodulation performance and nitrogen-fixing capability [100], thus further supporting the role of RND pumps in establishing symbiotic nitrogen fixation.

***Erwinia* spp.** These species belong to a genus of *Enterobacteriaceae* which include mostly plant pathogens. To date, four RND-type drug efflux systems, AcrAB-TolC, AcrD, MdtABC, and MdtUVW, have been functionally characterized in *Erwinia amylovora*. The AcrAB pump is inducible by phytoalexins and is required for the pathogenesis of *E. amylovora* [103]. As expected, the function of AcrAB is dependent on TolC [207]. Interestingly, AcrAB-TolC pumps of both *E. amylovora* and *E. coli* are functionally redundant with respect to conferring resistance and pathogenicity [208]. The AcrD pump mediates resistance to amphiphilic compounds including the antimycotic clotrimazole and the flavonoid luteolin *in E. amylovora*, but not to aminoglycosides in *E. coli*. Expression of *acrD* is regulated by BaeSR and CpxAR two-component regulatory systems [104] and is also induced by deoxycholate, naringenin, tetracycline, and zinc [105]. Both MdtABC and MdtUVW carry a pair of RND transporters. While both pumps mediate resistance to flavonoids, fusidic acid, and novobiocin, MdtABC also confers resistance to bile salts, josamycin, and silver nitrate, and MdtUVW produces resistance to clotrimazole. Expression of MdtABC is subject to regulation by BaeSR [106]. Additional global regulators of the AraC family, Rob, SoxS, PliA, and OpiA, have been characterized for their role in stress response including efflux gene expression [209], consistent with multilevel regulation of RND transporters in Gram-negative bacteria [7]. Another pump of

E. amylovora, NorM, contributes resistance to berberine, ethidium bromide, and norfloxacin as well as to antimicrobial compound produced by plant-associated *Pantoea agglomerans*, likely suggesting its involvement in the competition with other epiphytic microbes [107]. Expression of *norM* is induced at a lower temperature (18 °C) [210].

Erwinia chrysanthemi also possesses multidrug transporters. Four homologs of AcrAB and EmrAB pumps (two for AcrAB and two for EmrAB) were shown to contribute to bacterial survival in the presence of diverse antibiotics, cytotoxic agents, and plant extracts. Individual inactivation of these pumps generally rendered the mutants less virulent [108]. As with other *Enterobacteriaceae* spp., an outer membrane channel protein such as TolC is expected to be required for function of these pumps. Indeed, TolC protein of *E. chrysanthemi* was noted earlier to be involved in resistance to novobiocin and plant-derived chemicals including berberine, coumaric acid, esculetin, genistein, plumbagin, and rhein (4- to 64-fold MIC reduction) as well as being essential for phytopathogenesis [211]. Expression of several efflux pump component genes including *acrA* and *emrA* is induced by salicylate and phenolic acids [109].

Pseudomonas syringae This plant pathogen can infect a variety of species, and its pathogenesis relies on its ability to deliver virulence effector proteins into host cells by type II secretion system [212]. *P. syringae* pv. tomato has a genome of 6.5 Mb in size [213], which encodes a large repertoire of transporters including 12 putative RND pumps (http://www.membranetransport.org). Inactivation of a tripartite RND efflux system, PseABC, in *P. syringae* pv. syringae resulted in reduction in syringopeptin and syringomycin secretion without impairing antimicrobial susceptibility [113]. This pump is under the control of GacSA two-component regulatory system. The expression of the operon in a drug-hypersusceptible *E. coli* host produced phenotypic resistance to acriflavine, erythromycin, and tetracycline [113]. In *P. syringae* pv. phaseolicola, RND-type MexAB-OprM pump was characterized [111]. Disruption of this pump rendered the mutant less virulent to plant and highly susceptible to a broad range of antimicrobial agents including clinically relevant antibiotics, dyes, disinfectants, and plant extracts (Table 22.1). This pump is negatively controlled by a local repressor, PmeR, and can be induced by flavonoids [110]. In *P. syringae* pv. tabaci, MexEF-OprN mediates resistance to cefotaxime, chloramphenicol, and spectinomycin and is negatively regulated by AefR, but independent of the MexT regulator, unlike in *P. aeruginosa* [112]. An ABC-type efflux system in *P. syringae* pv. syringae, PseEF, is homologous to the macrolide-specific MacAB system of *E. coli* [114]. Its inactivation did not alter antimicrobial susceptibility profile but significantly reduced the secretion of syringomycin and syringopeptin by >70 %. The mutant was also much less virulent in immature cherry fruits [114].

***Ralstonia* spp.** *Ralstonia solanacearum* causes bacterial wilt in host plants. With a genome size of 5.8 Mb, *R. solanacearum* GM1000 also possesses a large number of putative transporters including 16 RND and 4 MATE pumps (http://www.membranetransport.org). Inactivation of either RND-type AcrA or MATE-type DinF protein rendered *R. solanacearum* with reduced ability to grow in the presence of

antibiotics, phytoalexins, and/or detergents as well as less virulent on the tomato plant. As expected, substrate profile for RND-type AcrAB is much broader than MATE-type DinF. Expression of these proteins (AcrAB or DinF) individually in a hypersusceptible *E. coli* host confers resistance to ampicillin, acriflavine, and ethidium bromide [116]. *Ralstonia eutropha* AcrA homolog of *E. coli* was also found to contribute to isobutanol tolerance [115].

***Rhizobium* spp.** This species belongs to the plant symbionts that form nitrogen-fixing nodules in association with legumes. In *Rhizobium etli*, a MFS-type multidrug transporter, RmrAB, was identified [214]. Expression of RmrAB is induced by several flavonoids including naringenin, genistein, chrysin, and quercetagetin and contributed to bacterial survival in the presence of kanamycin [214]. RmrAB and another MFS transporter, SalAB, were characterized from *Rhizobium leguminosarum* [215]. Both RmrAB and SalAB are inducible by salicylate. SalAB is also positively controlled by a local regulator SalR, which is a product encoded by the gene divergently transcribed from the *salAB* operon. Inactivation of either *rmrA* or *salA* did not change the drug susceptibility to nalidixic acid and tetracycline, the only two antibiotics tested [215]. The antimicrobial resistance feature of *Rhizobium* spp. can benefit agricultural legumes by increasing the chances of growth, multiplication, and persistence of rhizobial species in the soil [216].

Sinorhizobium meliloti This species is also a nitrogen-fixing Gram-negative soil bacterium that forms a symbiotic relationship with legumes. *S. meliloti* 1021 has a 6.7-Mb genome which encodes 12 putative RND pumps and 45 MFS transporters (http://www.membranetransport.org). Three RND pumps, SmeAB, SmeCD, and SmeEF, were functionally studied [117]. Deletion of *smeAB* rendered the mutant strain more susceptible to antibiotics, dyes, detergents, and plant extracts, and further deletion of either *smeCD* or *smeEF* resulted in further increase in antimicrobial susceptibility, revealing the role of these pumps in drug resistance (Table 22.1). SmeAB inactivation also impaired the ability to compete with the wild-type strain for legume nodulation [117]. An MFS-type EmrAB pump is regulated by flavonoids (e.g., apigenin and luteolin) by interacting with the local repressor EmrR [119]. Either mutation in *emrA* or *emrR* did not affect the symbiotic phenotype of the mutants [117, 119]. Another MFS transporter, Tep1, shows a homology to chloramphenicol-specific Cml pump [217] and indeed contributes to chloramphenicol resistance (as well as the nodulation) [120].

Xylella fastidiosa This plant pathogen possesses a chromosomal genome of 2.7 Mb that encodes a large number of transporters including eight putative RND transporter potentially implicated in multidrug/cation efflux and protein export [218]. *X. fastidiosa* also contains TolC protein which is expected to function with transporters. Disruption of TolC rendered the mutant hypersusceptible (10- to 1,000-fold MIC reduction) to the phytochemicals berberine, genistein, and rhein and resulted in a total loss of pathogenicity on grape [122].

22.4.3 Bacteria from Aquatic Environment

Achromobacter xylosoxidans This species, also known as *Alcaligenes xylosoxidans*, is a waterborne non-fermentative Gram-negative bacterium that causes numerous healthcare-associated infections. It is increasingly isolated from cystic fibrosis patients where it contributes to symptoms of pulmonary exacerbation. *A. xylosoxidans* exhibits high-level intrinsic resistance to aminoglycosides, cephalosporins, monobactams, penicillins, fluoroquinolones, fosfomycin, nitrofurans, sulfonamides, and tetracyclines, with relatively good susceptibility to carbapenems [219]. In one study, MIC_{50} values of aminoglycosides, third- and fourth-generation cephalosporins and tetracyclines (except minocycline) for *Achromobacter* spp. were ≥4 g/ml [220], suggesting significant multidrug-resistant phenotype, similar to that observed with other non-fermentative bacilli such as *Acinetobacter baumannii*, *P. aeruginosa*, and *S. maltophilia* (see Chaps.13, 14, and 15). The genome of *A. xylosoxidans* reveals the presence of a number of putative drug efflux pumps (including nine RND, three MATE, three MFS, and one SMR pumps in addition to one ABC-type macrolide-specific MacAB pump [123, 124, 219]. A transposon carrying class 1 integron with acquired resistance genes and a complete mercury resistance *mer* operon, which resembles the *Salmonella* genomic island 1, was also described in an *A. xylosoxidans* isolate [219].

Two RND transporters, AxyABM and AxyXY-OprZ, mediate multidrug resistance in *A. xylosoxidans* [124, 125]. Disruption of AxyB resulted in up to 20-fold reduction of the MIC values for several third-generation cephalosporins with no changes in susceptibility to the aminoglycosides amikacin and tobramycin [124]. However, AxyXY-OprZ functions as an aminoglycoside/multidrug pump since AxyY inactivation significantly increase the susceptibility of a wild-type strain to aminoglycosides (16- to 128-fold MIC decreases for amikacin, gentamicin, netilmicin, and tobramycin), doripenem, erythromycin, and tetracycline (all with 4-fold MIC reduction). AxyY inactivation also reduced the aminoglycoside susceptibility of an aminoglycoside-resistant mutant to its wild-type level (20- to 192-fold MIC changes) [125]. Importantly, AxyXY-OprZ is correlated to high-level aminoglycoside resistance in various other *Achromobacter* spp. including *Achromobacter aerofaciens*, *A. denitrifican*, *A. dolens*, *A. insolitus*, *A. insuavis*, and *A. ruhlandii*, some of which are also isolated from cystic fibrosis patients [221].

***Aeromonas* spp.** These species are ubiquitously present in freshwater and brackish water and can cause infections in humans and animals. Antimicrobial susceptibility profiles suggest the contribution of drug efflux pumps to resistance in *Aeromonas* spp. [222]. The genome of *Aeromonas hydrophila* shows an abundance of transporters comparable to those of pseudomonads and vibrios, including ten RND transporters [223]. The AheABC RND system is negatively controlled by the AheR repressor and mediates intrinsic resistance to 13 antimicrobials (Table 22.1) [126]. Chlorhexidine resistance in an *A. hydrophila* isolate was speculated to be attributable to an efflux mechanism [224].

Aeromonas salmonicida, a major fish pathogen responsible for diseases such as furunculosis, frequently displays multidrug resistance phenotype. The genome of *A. salmonicida* also contains putative RND (AcrAB, MexF, and MexW homologs), MFS pumps (MdtH and EmrD), MATE (NorM), SMR (EmrE), and ABC (MacAB homologs) pumps [225, 226]. Several drug efflux pumps (including AcrAB-TolC homologs) together with putative pump transcriptional repressors were also identified [226]. This finding is consistent with earlier observation that the efflux pump inhibitor phenylalanine-arginine β-naphthylamide at the relatively high levels of 64–254 μg/ml sensitized *A. salmonicida* fish isolates to two quinolone agents, flumequine and oxolinic acid (mostly 4- to 16-fold MIC reduction) [227]. Another study showed little involvement of drug efflux mechanism in quinolone resistance in six *Aeromonas* species including *A. salmonicida* from humans, eels, and water when the same efflux pump inhibitor was used at the levels of 30 and 100 μg/ml [228].

Aeromonas molluscorum possesses an SMR pump, SugE, which is induced by elevated concentrations of tributyltin, a biocidal hydrophobic derivative of tin that is a component of marine antifouling paint. Expression of *A. molluscorum* SugE in *E. coli* provides resistance to ethidium bromide, crystal violet, chloramphenicol, tetracycline, and tributyltin [127]. Exposure of *A. molluscorum* to tributyltin was found to affect transcription of many genes including moderate upregulation of acriflavine resistance gene and the *tolC* outer membrane efflux protein gene [229].

22.5 Concluding Remarks

The One Health concept stipulates that protection of human health is inexorably linked to that of animals and the environment. A large body of evidence now indicates that genes conferring resistance to human pathogens are recruited from bacteria whose primary reservoirs are nonhuman. Many of these varied genes encode efflux pumps, the primary functions of which in many instances would not be resistance to clinically relevant concentrations of antimicrobial agents. The efflux pumps may have varied role in animal pathogenicity, pathogenic or beneficial interactions with plants, or stress responses. Nevertheless, efflux pumps are clearly a widespread and worrisome means by which bacteria subvert the efficacy of antimicrobial agents of great value in human medicine. Evidence that terrestrial and aquatic environments exposed to urban or agricultural waste streams are enriched in bacteria that express efflux pumps is of concern. The co-selection of these genes by biocides and other chemical agents widely used in commerce is a further concern and should prompt the implementation of measures that mitigate emissions into the environment. Likewise, the use of antimicrobials in food animal production is a strong selection pressure, and should be managed through better stewardship and the deployment of suitable alternatives. Finally, the development of “omics” technologies will lead to insights into the ecology of genes encoding efflux pumps. Identification of hotspots for their proliferation, association with mobile genetic

elements, distribution within real-world bacterial populations, and elucidation of key routes of exposure and transmission will be foundational for targeting intervention strategies to the benefit of public health.

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Chapter 23
Role of Plasmid-Encoded Drug Efflux Pumps in Antimicrobial Resistance

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Abstract Plasmids, as extrachromosomal genetic mobile elements, have been widely documented to mediate high-level bacterial resistance to all major clinically relevant antibiotics and antiseptic agents. The first drug efflux pump discovered in bacteria is plasmid encoded. Naturally occurring drug resistance plasmids are diverse and belong to different incompatibility groups. Multidrug resistance determinants often coexist on the same plasmids with strong linkages to mobile elements such as integrons or transposons. Thus, plasmids play a critical role in the evolution of resistance and in the dissemination of resistant bacteria, which poses a major challenge to antimicrobial therapy. This chapter provides an up-to-date overview of the plasmid-mediated genetic and biochemical mechanisms of antimicrobial resistance with an emphasis on plasmid-encoded drug efflux pumps in major pathogens.

Keywords Antimicrobial resistance • Antiseptic • Multidrug resistance • Plasmid • Integron • Transposon • Efflux pumps

23.1 Introduction

The term plasmid was proposed in 1952 by Joshua Lederberg to be referred to as "a generic term for any extrachromosomal hereditary determinant" [1]. Being extrachromosomal genetic elements, plasmids replicate independently and occur commonly in bacteria. To date, numerous plasmids have been characterized in detail, which include in-depth understanding of the complete nucleotide sequences, gene products, and their functions. Such functions include plasmid transfer elements, metabolic/catabolic degradation enzymes, virulence determinants, and, frequently, antimicrobial resistance genes. The role of plasmids (initially known as "R factor")

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in drug resistance was recognized soon after the beginning of the antibiotic era [2, 3]. In fact, plasmid-mediated resistance is often of high level and thus threatens effective antimicrobial therapy [4, 5]. Plasmids not only possess independent replicons but may also contain other mobile genetic elements (e.g., insertion sequences, integrons, and transposons) [6] and, hence, provide an important means for both vertical and horizontal gene transfer that assist the widespread of resistance within or across bacterial species or genus of different geographical regions. Plasmids make a major contribution to resistance especially in organisms such as the ESKAPE pathogens (i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species); epidemic resistance plasmids have been found globally [7]. Moreover, resistance plasmids that simultaneously carry multiple resistance determinants have been observed frequently [8, 9]. These plasmids can also encode virulence factors and enhance bacterial biofilm formation or colonization in the host, thus contributing greatly to the pathogenicity [10, 11]. It is necessary to mention that there is a very large amount of studies on plasmid-mediated resistance, a topic that has been regularly reviewed over past decades [5, 12–20]. This chapter provides an up-to-date description of plasmids' contribution to current antimicrobial resistance crisis with an emphasis on the role of plasmid-encoded drug efflux pumps.

23.2 Overview of Plasmid-Mediated Antimicrobial Resistance

The significance of plasmid contribution to antimicrobial resistance cannot be overstated since many plasmids from different incompatibility groups, either conjugative or nonconjugative, have been found to confer resistance to all major classes of antimicrobial agents in both Gram-positive and Gram-negative bacteria [5, 15, 19]. These resistance plasmids are widely present in various environments. Plasmid-encoded products exhibit different biochemical mechanisms of resistance, including drug inactivation, modification of drug targets, and drug efflux (Table 23.1).

23.2.1 Genetic Characteristics of Resistance Plasmids

Like any plasmids, resistance plasmids carry replication elements for their independent maintenance. They vary in size (small to mega-plasmids), incompatibility groups, and host ranges [49, 50]. For example, a small plasmid of *Staphylococcus aureus* is only about 2.8 kb and carries a drug transporter gene [51], while mega-plasmids are >100 kb carrying multiple resistance determinants and mobile genetic elements [31]. Some of them have a broad host range and can replicate in different species. They may carry genes for plasmid transfer and become conjugative [49]. The plasmids may encode a single or multiple drug resistance determinants, their

Table 23.1 Examples of plasmid-mediated resistance to major antimicrobial drugs

Antimicrobial resistance	Plasmid (GenBank accession)	Origin of bacterial species	Mechanisms	Reference
β-Lactams	pRSF1030 (RSF1030OR)	*Salmonella* Panama	TEM-1 narrow-spectrum β-lactamase	[21]
	pR997	*Proteus mirabilis*	SHV-1 narrow-spectrum β-lactamase	[22]
	pMG211	*E. coli*	PSE-1 β-lactamase	[23]
	pRGN238 (J02967)	*E. coli*	OXA-1 β-lactamase	[24]
	pMVP-3 (X92506)	*E. coli*	CTX-M-1 extended-spectrum β-lactamase	[25, 26]
	pMVP-2 (X91840)	*K. pneumoniae*	CMY-2 AmpC β-lactamase	[27]
	pAK9373 (D50438)	*S. marcescens*	IMP-1 metallo β-lactamase	[28]
	pKpANDM-1 (FN396876)	*K. pneumoniae*	NDM-1 metallo β-lactamase	[29]
Aminoglycosides	pCTX-M-3 (AF550415)	*C. freundii*	ArmA aminoglycoside methylase	[30]
	pNDM-CIT (JX182975)	*C. freundii*	AadA2 aminoglycoside acetyltransferase	[31]
	pRH-1238 (KR091911)	*Salmonella* Corvallis	AAC(6′)/Aad aminoglycoside acetyltransferases, and AphA6; StrA/B aminoglycoside phosphotransferases	[32]
Amphenicols	pC194 (NC_002013)	*S. aureus*	Cat chloramphenicol acetyltransferase	[33]
	pSCFS1 (AJ249217)	*S. aureus*	Cfr methylase	[34–36]
Fluoroquinolones	pMG252 (AY072035)	*K. pneumoniae*	QnrA DNA gyrase protection protein	[37]
	(DQ303918)	*E. coli*	AAC(6′)-Ib-cr	[38]
	pHPA (AB263754)	*E. coli*	QepA efflux pump	[38]
Fosfomycin	p1E1C (JF411006)	*E. coli*	FosA3 thioltransferase	[39]
Glycopeptides (vancomycin)	pLW1043 (AF017171)	*S. aureus*	VanA	[40]
Macrolides-lincosamides-streptogramins	pE194 (NC_005908)	*S. aureus*	Erm methylase	[41]
Oxazolidinones-amphenicols	pE349 (KP399637)	*E. faecalis*	OptrA efflux pump	[42]

(continued)

Table 23.1 (continued)

Antimicrobial resistance	Plasmid (GenBank accession)	Origin of bacterial species	Mechanisms	Reference
Pleuromutilins-lincosamides-streptogramins	pSA-7 (NG_041699)	*Staphylococcus cohnii*	Vga(E) efflux pump	[43]
	pV7037 (NG_041616)	*S. aureus*	Lsa(E) efflux pump	[44]
Polymyxins	pHNSHP45 (KP347127)	*E. coli*	Mcr-1 phosphoethanolamine transferase	[45]
Rifamycins	In53 (AF205943)	*E. coli*	Arr-2 ADP-ribosyltransferases	[46]
	pTNN01 (AJ277027)	*K. pneumoniae*	Arr-3 ADP-ribosyltransferases	[47]
Sulfonamides/ trimethoprim	pRH-1238 (KR091911)	*Salmonella* Corvallis	SulI /SulII dihydropteroate synthase; Dfra7 dihydrofolate reductase	[32]
Tetracyclines	pOZ100 (L12241)	*Neisseria gonorrhoeae*	Tet(M) ribosomal protein	[48]
	pR100 (NC_002134)	*S. flexneri*	TetA(A) efflux pump	[8]

regulatory genes, and other mobile genetic elements such as insertion sequences, integrons, and transposons [5, 28, 29, 49, 52]. These multiple resistance-associated elements form gene cassettes that occur widely in resistance plasmids. An early reported plasmid, R100, of *Shigella flexneri*, is about 94 kb and contains the *tetA* gene (for tetracycline resistance) in the transposon Tn10, *cat* for chloramphenicol resistance in Tn9, and *aadA1* for aminoglycoside resistance, *sul1* for sulfonamide resistance, and *mer* operon genes for mercury resistance in Tn21 [8]. In another example, the 140-kb plasmid of *Klebsiella pneumoniae* that encodes a gene for the NDM-1 β-lactamase also contains mobile elements (insertion sequences IS26 and class 1 integron) and many other resistance genes including *arr-2* (for rifamycin resistance), *ereC* (erythromycin resistance), *aadA1* (aminoglycoside resistance), *cmlA* (chloramphenicol resistance), *qacEΔ*1 (defective antiseptic resistance), and another gene for an efflux pump [53]. A recent study of a 187-kb plasmid of *Salmonella enterica* Corvallis demonstrated the presence of 15 resistance gene determinants comprising of $bla_{\text{NDM-1}}$, $bla_{\text{CMY-16}}$, *fosA3*, *sulI*, *sulII*, *strA*/*B*, *aac*(*6′*)-*Ib*, *aadA5*, *aphA6*, *tetA*(A), *mphA*, *floR*, *dfrA7*, and *merA* genes that provide resistance to β-lactams (including carbapenems), aminoglycosides, amphenicols, fosfomycin, macrolides, sulfonamides, trimethoprim, and tetracyclines [32]. Of note, this plasmid is considered to originate from Asia and to have been transferred to Germany through a migratory wild bird [32]. Moreover, resistance plasmids may also carry virulence genes and play a critical role in pathogenicity, as shown in the example of a multidrug resistance (MDR) plasmid of *Salmonella* [54]. Current identification

and characterization of plasmids, facilitated by the whole genome sequencing availability together with the bioinformatics analysis, continue to provide insights on the diversity of resistance plasmids derived from various environments [55, 56].

23.2.2 Genetic and Biochemical Mechanisms of Plasmid-Mediated Resistance

β-Lactams Resistance to β-lactams in Gram-negative bacteria is mostly caused by β-lactamases [57], which are encoded by chromosomes or plasmids (Table 23.1). There are more types and numbers of plasmid-encoded β-lactamases than those of chromosomal β-lactamases. Most Ambler class A β-lactamases are produced by plasmids and contain many TEM, SHV, and CTX-M enzymes [57, 58], most of which are extended-spectrum β-lactamases (ESBLs) that hydrolyze aztreonam and oxyimino-cephalosporins and can be inhibited by β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) [57, 59]. Many CTX-M-producing plasmids are MDR plasmids [60, 61]. Class A enzymes also include *K. pneumoniae* carbapenemases (KPCs) that pose a major threat to antimicrobial therapy [62], and these enzymes are also frequently encoded by plasmids [63, 64]. Numerous class B metallo β-lactamases are encoded by plasmids. Perhaps the most noticeable example in recent years is the global spread of NDM-1-encoding plasmids that also contain multiple drug resistance determinants [29, 65]. One major substrate class for these metalloenzymes is carbapenems [29]. A strain harboring both a NDM-1 plasmid and a KPC plasmid was also recently noted [66]. While often encoded by chromosomes, class C AmpC enzymes are also frequently encoded by plasmids, such as the CMY enzymes that are widely distributed in isolates of humans and animals and can particularly hydrolyze cephamycins and oxyimino-cephalosporins [67–69]. Class D OXA β-lactamases are mostly encoded by plasmids and can be divided into several subgroups [70], including carbapenem-hydrolyzing OXA-type carbapenemases [62, 70, 71].

Aminoglycosides Three types of aminoglycoside-modifying enzymes exist and are aminoglycoside *N*-acetyltransferases, *O*-nucleotidyltranferases, and *O*-phosphotransferases, which play a major role in aminoglycoside resistance. Many of them are encoded by plasmids in both Gram-positive and Gram-negative bacteria [72]. Copresence of aminoglycoside resistance genes with other resistance determinants are frequently observed on the same plasmids [8, 32, 44, 73]. Plasmid-encoded methyltransferases such as ArmA, RmtA, and RmtB alter the aminoglycoside binding site of 16S rRNA of the 30S ribosomal subunit and are increasingly seen as a key mechanism of aminoglycoside resistance [49, 58, 74, 75].

Amphenicols Resistance to chloramphenicol and thiamphenicol is mainly mediated by drug-specific chloramphenicol acetyltransferases through drug inactivation. This mechanism can be inducible due to the mRNA structural changes in the 5′-untranslated region of the *cat* gene in the presence of an antimicrobial to enhance

the translation of the *cat* mRNA [33]. These enzymes are often encoded by MDR plasmids containing resistance gene cassettes [76, 77]. Plasmids also mediate non-enzymatic resistance mechanism through amphenicol-specific drug exporters named CmlA or FloR [78, 79] or multidrug exporters such as OptrA [42] (see details in the section on drug efflux pumps). A third mechanism is related to chloramphenicol-florfenicol resistance (Cfr) protein that is encoded by plasmids [34] and is able to methylate 23S rRNA, resulting in reduced binding of amphenicols, lincosamides, oxazolidinines, pleuromutilins, and streptogramins to their ribosome targets [35, 36]. The first described *cfr*-bearing plasmid was an MDR transposon plasmid that also contained *erm*(*33*) for inducible resistance to macrolide-lincosamide-streptogramin B and a streptomycin resistance gene [80]. A novel *cfr*-containing plasmid was found to carry *blaZ* β-lactamase gene, *msr*(*A*) efflux gene, and heavy metal resistance genes [81]. Another plasmid-borne chloramphenicol-florfenicol resistance gene termed *fexA* was also reported with an unknown mechanism of resistance [82]. *cfr*- and *fexA*-carrying resistance plasmids are widely present in staphylococci [83]. A *cfr*-carrying plasmid from *Enterococcus faecalis* was also described [84]. More recently, the *fexA* or *fexB* combination with plasmid-borne *optrA* transporter gene was observed in enterococci of human and food animal sources [42, 85].

Fluoroquinolones While enzymatic inactivation of quinolones by an aminoglycoside-fluoroquinolone acetyltransferase is due to the AAC(6′)-Ib-cr-encoding plasmids, *qnr*-containing plasmids provide quinolone resistance via a target protection mechanism [20, 37, 86–88]. Plasmids carrying *AAC*(*6′*)*-Ib-cr* or *qnr* genes are widely disseminated in numerous *Enterobacteriaceae* and often exist as part of a resistance gene cassette to cause MDR [20, 37, 55, 58]. Additionally, contribution of the QepA and QepA2 transporters to fluoroquinolone resistance will be described in the next major section.

Fosfomycin This agent is increasingly being recognized for its role in therapy against multidrug-resistant pathogens [18, 89]. However, plasmid-borne *fos* genes have been well documented to be responsible for fosfomycin resistance in both Gram-positive and Gram-negative bacteria [90, 91]. The *fos*-encoded thioltransferases cause enzymatic inactivation of fosfomycin [90]. *fos* genes are divided into various groups such as *fosA*, *fosB*, and *fosC*. Of importance, *fos* genes are also frequently observed in MDR plasmids [60, 92]. *fosA3*-containing plasmids of *E. coli* from China and the USA also carry CTX-M-65 β-lactamase gene and *rmtB* methylase gene as well as insertion sequences [39, 91]. Another conjugative plasmid derived from extensively drug-resistant *Enterobacter cloacae* carries *fosA3*, NDM-1 β-lactamase gene, and *armA* aminoglycoside resistance gene [93].

Glycopeptides Transferable resistance to vancomycin and teicoplanin is well recognized [40, 94–98]. Several vancomycin resistance determinants such as *vanA* are associated with mobile genetic elements (e.g., typically Tn*1546*), which also exert an important role in the evolution of vancomycin resistance. For example, plasmid pLW1043 of *S. aureus* encoded six copies of the IS256 transposase, vancomycin

resistance-associated *vanRSHAXYZ* genes, and other resistance genes *dfrA* (for trimethoprim resistance), *qacC* (antiseptic resistance), *aacA-aphD* (aminoglycoside resistance), and *blaZ* (β-lactam resistance) [40]. Van resistance determinants (such as *vanA*, *vanB*, and *vanC*) cause replacement of the terminal D-alanine of the cell wall peptidoglycan precursors with D-lactate or D-serine and consequently result in reduced binding of drugs to the peptidoglycan precursors [99]. In enterococci, in response to a pheromone peptide, pheromone-responsive plasmids help acquisition of resistance genes [100], although pheromone produced by commensal enterococci can also result in killing of multidrug-resistant enterococci [101].

Lincosamides and Macrolides Plasmid-mediated transferable resistance to these classes of agents is well known [52, 102–104]. One example is the plasmid-mediated inducible resistance to three structurally unrelated classes of antimicrobials, macrolides, lincosamides, and streptogramin B [41, 105]. These plasmids contain genes encoding erythromycin ribosome methylases (Erm) that lead to the posttranscriptional modification of the 23S rRNA by the adenine-*N6* methyltransferase. The binding site in the 50S ribosomal subunit for erythromycin overlaps the site of other macrolides, lincosamides, and streptogramin B, resulting in cross-resistance to three antimicrobial classes [106]. The expression of *erm* genes can also often be inducible by an antibiotic such as erythromycin due to a deregulation of posttranscriptional attenuation [107]. In addition to Erm ribosomal methylases, several other macrolide resistance proteins are also encoded by plasmids such as Ere esterase or Mph phosphotransferase (that cause macrolide inactivation) and Mef, Mel, and Msr exporters [31, 52, 104, 108]. The different resistance determinants play synergistic roles in raising macrolide resistance level [52]. A newly reported plasmid-containing methylase-encoding *erm*(*T*) conferred a ≥128-fold increase of the MIC values of azithromycin, erythromycin, clindamycin, and lincomycin [109]. The role of efflux pumps Mef, Mel, and Msr exporters in macrolide resistance [31, 108] will be described below in the section on drug efflux pumps.

Oxazolidinones, Pleuromutilins, and Streptogramins The abovementioned *cfr*-encoding plasmids also mediate resistance to oxazolidinones due to the modification of 23S rRNA and the overlapping mode of action of these agents with that of amphenicols [36, 81, 110]. A new plasmid-borne gene dubbed *optrA* which encodes an exporter is involved in resistance to oxazolidinones and amphenicols (see Drug Efflux Pumps section below) [42, 85]. Either Lsa(E)- or Vga(E)-containing plasmids mediate resistance to pleuromutilins, lincosamides, and streptogramins [43, 44].

Polyketides The plasmid-borne *mupA* gene mediates high-level resistance to mupirocin (≥120-fold MIC increase) in *S. aureus*, in contrast to chromosomal mutation-related low-level resistance (2- to 32-fold MIC increase) [111]. The *mupA* gene encodes a modified isoleucyl tRNA synthetase. Diversity of *mupA*-containing plasmids has been noted, and these plasmids often contain mobile genetic elements and can be conjugative [111, 112].

Polymyxins Until recently [45], resistance to polymyxins had been only known to be caused by chromosomal mutations that affect structure of lipopolysaccharide, the primary target of polymyxins [113]. For instance, the changes from chromosomally encoded PhoPQ and PmrAB systems can modify the lipopolysaccharide-related outer membrane barrier and subsequently mediate resistance to polymyxins [114–116]. The newly discovered colistin resistance plasmid named pHNSHP45 was isolated from *E. coli* of pig origin and was conjugatively transferred to and maintained in *K. pneumoniae* and *P. aeruginosa* [45]. It produced, respectively, 8- to16-fold and four- to eightfold increases in the MIC values of colistin (mostly to 8 μg/ml) and polymyxin B (to 4 μg/ml) for various transconjugants. This plasmid contains a gene dubbed *mcr-1* that encodes a phosphoethanolamine transferase for modification of lipopolysaccharide structure via the addition of phosphoethanolamine to lipid A [45]. The *mcr-1* gene was found to be in *E. coli* isolates, respectively, derived from 15 %, 21 %, and 1 % of raw meat, animal and human inpatient samples [45].

Rifamycins Plasmids encoding ADP-ribosyltransferases [46, 47] or efflux pumps [117] have been reported to confer rifamycin resistance via drug inactivation or extrusion. The *arr* genes are often located in a resistance gene cassette containing integron [46, 47].

Sulfonamides and Trimethoprim Resistance to these anti-folate agents is also frequently attributable to plasmids carrying *sul* genes or *dfrA* genes, which encode, respectively, dihydropteroate synthase and dihydrofolate reductase to provide an alternate folate metabolic pathway [118]. These resistance genes often exist as part of mobile drug resistance gene cassettes [119]. For instance, *sul* is one of the many resistance genes encoded by the mega-plasmid that produces NDM-1 metallo β-lactamase described earlier [29]. *sul* genes are among those frequently identified resistance genes in various environments [120]. The vancomycin resistance plasmid pLW1043 described earlier in this chapter also carries *dhfr* gene for trimethoprim resistance [40].

Tetracyclines Resistance to tetracyclines is often mediated by plasmids in both Gram-positive and Gram-negative species with involvement of active efflux systems (see next section below) and ribosomal protection [121]. The latter includes *tet*(*M*), *tet*(*O*), and *tet*(*Q*) determinants encoding proteins that reduce tetracycline binding to its target [48, 122].

Biocides and Disinfectants These agents such as benzalkonium chloride and chlorhexidine are frequently used in hospital infection control or in preserving food products. Exposure to biocides may also select resistance to clinically used antimicrobial agents [123]. Resistance to these agents is attributed to multiple mechanisms including efflux pumps [124, 125]. Plasmid-mediated resistance to biocides constitutes a major mechanism and include efflux pumps such as Qac pumps to be discussed in the next section.

23.3 Plasmid-Encoded Drug Efflux Pumps

The first drug efflux pump discovered in bacteria, i.e., tetracycline-specific Tet efflux pump, is plasmid encoded [126–128]. The discovery of this mechanism of energy-dependent active extrusion of drugs from bacterial cells was a milestone in resistance studies and expanded our understanding of biochemical mechanisms of resistance. To date, bacteria are known to contain a large number of plasmid-encoded drug efflux pumps that belong to several transporter families and contribute to clinically relevant antimicrobial resistance [125, 129].

23.3.1 Major Facilitator Superfamily

Most known plasmid-borne drug efflux pumps are members of the major facilitator superfamily (MFS), whose characteristics are described in Chap. 2 and have been also reviewed elsewhere [130–132].

Tet Efflux Pumps These pumps are widely found in both Gram-positive and Gram-negative bacteria and include more than two dozen members such as Tet(A) to Tet(E), Tet(G), Tet(H), Tet(J), Tet(K), Tet(V), Tet(Y), Tet(Z), Tet(30), Tet(31), Tet(33), Tet(35), Tet(38), Tet(39) to Tet(43), TetAB(46), and Tet(47) (http://faculty.washington.edu/marilynr/tetweb1.pdf. Accessed on March 20, 2016). A number of plasmid-borne *tet* genes have been revealed to encode Tet efflux pumps that mediate high-level resistance to tetracyclines [129, 133]. The different Tet proteins may vary in their substrate specificities. Many Tet pumps such as Tet(A) confer resistance to chlortetracycline, oxytetracycline, and tetracycline but not the lipophilic minocycline. However, the latter is subject to the extrusion by Tet(B) pump [122]. Glycylcyclines were developed to counter the effect of Tet efflux pumps (and ribosomal protection) and are not the substrates of these pumps [134]. *tet*-containing plasmids often carry other resistance genes such as *sul*, *floR*, and *strA*/*strB* [135] and may also have *tetR* gene (typically seen in various transposons such as Tn10) [135–138] that encode a repressor (the prototype of the TetR repressor family [139]) to inhibit the expression of *tet* efflux gene. Tetracycline binds TetR and thus induces expression of the efflux pump [137].

FloR Pump This pump confers resistance to amphenicols [34, 140]. The gene *floR* was first found in a transferable R plasmid in florfenicol-resistant fish pathogen *Pasteurella piscicida* [141] and subsequently was also located in MDR plasmids isolated from a number of animal-derived bacteria including *Salmonella* [78], *E. coli* [60, 138, 140, 142], *Actinobacillus pleuropneumoniae* [143], *Aeromonas salmonicida* [144], *Haemophilus parasuis* [145], and *Mannheimia haemolytica* [146].

Mef Pumps These pumps include Mef(A), Mef(B), and Mef(I), are encoded by conjugative genetic elements including plasmids, and provide inducible macrolide resistance in streptococci [77, 147, 148] or *E. coli* [119]. The *mef* genes are generally part of the MDR integron/transposon-containing gene cassettes [77, 119]. A *mef* gene and an ABC exporter-encoding gene *mel* was also found to form an operon on mobile genetic elements to produce dual efflux pumps Mef and Mel that are inducible by erythromycin [147, 149].

QacA and QacB Pumps Resistance to antiseptics such as monovalent quaternary ammonium compounds (e.g., benzalkonium chloride) and divalent cations (e.g., chlorhexidine) is frequently mediated by MDR plasmids containing *qac* efflux pump genes such as *qacA* and *qacB* [150–153]. *qac* plasmids are widely distributed in methicillin-resistant *S. aureus* [154–156]. In fact, a description of the *qacB*-containing plasmid dates back to 1951. It was the earliest known *S. aureus* plasmid encoding a drug efflux pump [157]. *qacA* and *qacB* genes differ only by six to nine bases; yet, their proteins produce different phenotypes with QacA displaying higher activity in the efflux of divalent cations [158, 159]. The initially discovered *qacA* gene was located on a 28-kb plasmid (called pSK1) [160] which provided resistance to multiple biocides including quaternary ammonium compounds, chlorhexidine, and the intercalating dyes acriflavine and ethidium bromide [153]. A *qacB* plasmid of *S. aureus* also contains *aacA-aphD* aminoglycoside-modifying enzyme genes, *fosB* fosfomycin resistance gene, cadmium resistance protein gene, and transposase gene [161]. Several variants of plasmid-encoded QacB have been described with one variant being able to confer staphylococcal resistance to fluoroquinolones (fourfold increase of norfloxacin and ciprofloxacin MICs, but no change in levofloxacin MIC values) [159]. It is important to note that a repressor gene dubbed *qacR* is often located upstream of either *qacA* or *qacB* gene and transcribed divergently. QacR negatively controls the expression of *qacA* or *qacB* by binding to the DNA upstream of *qacA* or *qacB*. Certain lipophilic cations can bind to QacR and thus derepress or induce the *qacA* or *qacB* expression [152].

Qep Pumps These pumps including QepA and QepA2 are encoded by MDR plasmids that are mostly of *E. coli* origin and confer fluoroquinolone-specific resistance [20, 162, 163]. Cloned *qepA* provides resistance to ten fluoroquinolone agents of various generations (2- to 16-fold MIC increase) in a hypersusceptible *E. coli* host with virtually no impact on susceptibility to non-fluoroquinolone agents including ampicillin, erythromycin, kanamycin, acriflavine, benzalkonium, crystal violet, deoxycholate, ethidium bromide, rhodamine 6G, and sodium dodecyl sulfate [162]. QepA pump plays a synergistic role with the chromosomal fluoroquinolone resistance mechanism to raise the resistance level [164]. The *qepA* or *qepA2* gene often coexists in the same plasmids with transposon elements and other resistance genes including $bla_{CTX\text{-}M}$, *aac(6′)-Ib-cr*, and/or *qnr* genes [165, 166].

23.3.2 Resistance-Nodulation-Cell Division Superfamily

The drug efflux systems of the resistance-nodulation-cell division (RND) superfamily are generally chromosomally encoded and are predominately found in Gram-negative bacteria, and their importance in MDR is examined in various chapters of this book. Since the RND pump systems are typically a tripartite efflux complex requiring three gene products, the discovery of RND pump genes on plasmids was quite surprising. However, there is an increasing occurrence of plasmid-encoded multicomponent efflux systems including RND pumps.

OqxAB In 1999, a 52-kb plasmid named pOLA52 was obtained from a swine *E. coli* isolate that was resistant to olaquindox, an animal feed additive [167]. This plasmid encoded two gene products (dubbed OqxAB) exhibiting high homology to AcrAB efflux proteins (that are, respectively, a periplasmic adaptor protein and a pump). The plasmid also has another open-reading-frame downstream of *oqxAB* with a divergent transcriptional direction that encodes a repressor (OqxR) [168, 169]. Although lacking an OM protein gene from the plasmid, OqxAB function requires chromosomally encoded TolC protein, which is also an indispensable component of most chromosomal RND systems of *E. coli* (see Chap. 9). Like most chromosomal RND pumps, OqxAB also displays a broad substrate specificity that includes chloramphenicol, fluoroquinolones, nalidixic acid, trimethoprim, olaquindox, benzalkonium chloride, and sodium dodecyl sulfate (cloned *oqxAB* genes increase the MIC values of these agents by 8- to128-fold) [167, 170]. pOLA52 also carries virulence genes such as those for type IV secretion system. Similar to pOLA52 MDR transferable plasmid, newly identified *oqxAB* plasmids carry other resistance genes such as a CTX-M gene or *floR* gene [61, 171]. These plasmids are mostly observed in *E. coli* [61, 172–175] but also in other *Enterobacteriaceae* such as *Salmonella* spp. and *K. pneumoniae* [171, 176–178]. The *oqxAB-oqxR*-containing chromosome of *K. pneumoniae* has been considered as a possible source of plasmid-borne *oqxAB* [179]. Still, additional Gram-negative bacteria such as *Enterobacter aerogenes*, *E. cloacae*, and *Serratia marcescens* were also recently found to contain *oqxAB-oqxR* genes, but the *oqxAB* expression in these bacteria was minimal and appeared not to contribute to quinolone resistance [169]. A newly available study further revealed that plasmid-mediated OqxAB is also involved in resistance to nitrofurantoin and facilitates to high-level nitrofurantoin resistance [180].

SilCBA/CusCBA A 180-kb mega-plasmid, pMG101, was isolated from a multidrug-resistant *Salmonella* derived in 1973 from a severe burn patient in the USA [181, 182]. The plasmid conferred resistance to silver salts (8- to 16-fold silver nitrate MIC increase), ampicillin, chloramphenicol, streptomycin, and tetracycline [181]. Being an MDR plasmid, pMG101 produces three gene products

SilCBA with high homology to an RND system comprised of the antiporter SilA, membrane fusion protein, and outer membrane channel protein SilC. It also encodes an ABC transporter SilP and periplasmic metal-binding protein SilE as well as a two-component regulatory system SilRS [182]. SilABC and SilP likely play a synergistic role in the extrusion of silver salts. pMG101 is the earliest known RND pump encoded by an MDR plasmid. However, the complete nucleotide sequence of this plasmid is not available. Using gene-specific primers, amplification of *silCBA*, *silE*, *silP*, and *silRS* was obtained from several plasmids of silver-resistant *E. cloacae* isolates of human and veterinary origin [183]. The same group also reported the detection of *silE* in methicillin-resistant *S. aureus* and other staphylococci with yet undetermined location of *silE* [184]. These studies warrant the need to investigate the role of *sil* genes in silver resistance, in particular because silver-derived agents are being actively pursued as novel antimicrobials in combating drug resistance.

Recently, a study conducted in China has characterized a 273-kb conjugative IncH1 MDR mega-plasmid named pEC5207 that was isolated in 2011 from an *E. coli* strain of swine origin [185]. The sequence of this plasmid showed the presence of a cluster of genes (*silP-copG-cusA-silB-cusC-cusR-cusS-silE*) [185] that had an identical arrangement in comparison with a region containing *silP-orf-silA-silB-orf-silC-silR-silS-silE* of plasmid isolated in 1973 from *Salmonella* spp. in the USA [182]. This gene cluster is also present in another MDR 227-kb mega-plasmid pSH111_227 of *Salmonella* origin reported in the USA in 2011 (GenBank accession JN983042) [185]. Specifically, these genes encode RND-type efflux system CusC-SilB-CusA, a two-component regulatory system CusRS, ABC transporter SilP, and silver-binding protein SilE [185]. It is important to note that this plasmid-encoded CusA-SilB-CusF-CusC-CusR-CusS is organizationally identical to the chromosomally encoded CusCFBA-CusRS systems of *E. coli* (see Chap. 9 on *E. coli* efflux pumps). In addition, pEC5207 also contain genes encoding homologs to CopABCE (involved in copper resistance), TerZABCDEF (tellurium resistance), EmrE (SMR-type pump for antiseptic resistance), and CMY-2 β-lactamase (β-lactam resistance), as well as the genes for H-NS regulator and RamA activator. *E. coli* transformants with pEC5207 were demonstrated to confer resistance to silver (80-fold increase of $AgNO_3$ MIC) and copper (1.5-fold increase of $CuSO_4$ MIC) [185]. All these findings with plasmids pMG101, pEC5207 and pSH111_227, and *E. coli* genome suggest the importance of CusCBA-CusRS/SilCBA-SilRS for persistency of *E. coli* or *Salmonella* spp. in diverse environments.

Other RND Systems Plasmid DNAs from uncultured bacteria in wastewater treatment plant contain genes with homologs of chromosomal RND pump genes of *E. coli* and *P. aeruginosa* (especially *mexEF-oprN*) [186]. Several large plasmids have also been found to contain RND pump genes. Two conjugative plasmids of ca. 63 and 67 kb were isolated in 1993 as mercury resistance plasmids due to plasmid-borne *mer* resistance genes. However, these plasmids also encode gene products showing homology to MexEF-OprN components of *P. aeruginosa* [187–189]. *E. coli* carrying one of these two plasmids displayed no altered drug susceptibility to chloramphenicol, nalidixic acid, and trimethoprim. An IncHI1 plasmid from an extremely drug-resistant *Citrobacter freundii* isolated from a patient returning from

India [31] contained genes encoding carbapenemase NDM-1, ArmA 16S rRNA transferase, and an RND system (homologous to AcrR-MexAB-CusC which correspond, respectively, to an efflux expression repressor, a membrane fusion protein, an efflux transporter, and an outer membrane channel protein) [190].

23.3.3 Small Multidrug Resistance Family

The most studied chromosomally encoded efflux pump of this family, EmrE of *E. coli*, mediates resistance to antiseptics (disinfectants or biocides). Similarly, plasmid-encoded small multidrug resistance (SMR) exporters such as Smr, QacC, QacD, QacF, QacH, and QacJ and also involved in biocide resistance. They are mostly found in staphylococci [152, 155, 191–194] but also in Gram-negative bacteria [195]. Smr-type *qac* genes were also found in enterococci and *Listeria monocytogenes* [152]. A plasmid-encoded QacZ from *E. faecalis* confers resistance to quaternary ammonium compounds [196]. A small 2.7-kb Smr pump-encoding plasmid of *S. aureus* confers resistance to quaternary ammonium compounds [51]. An MDR plasmid derived from uncultured bacteria contains an *smr* gene for QacF efflux pump, OXA-2 β-lactamase gene, *aadA4* spectinomycin/streptomycin resistance gene, and *sul1* sulfonamide resistance gene and can be transferred to *E. coli* [197]. A recent review examined the phylogenetic relation of *qac* genes that encode either SMR family pumps or MFS-type pumps with SMR genes grouping into four clusters [152].

23.3.4 ATP-Binding Cassette Superfamily

The transporters of the ATP-binding cassette (ABC) superfamily are widely distributed in bacteria. The abovementioned plasmid pMG101 contains ABC silver exporter gene [182]. Another 48-kb MDR plasmid, pRSB101, from an activated sludge of a wastewater treatment plant contains 20-kb resistance region located in a Tn402-like transposon. This plasmid encodes an ABC-binding protein, an ABC transporter, and a periplasmic membrane fusion protein that may possibly form an efflux complex [198]. In addition, it also encodes *sul1* for sulfonamide resistance, *dhfr1* gene for trimethoprim resistance, *aadA2* for spectinomycin/streptomycin resistance, a $bla_{TLA\text{-}2}$ β-lactamase gene, *mph*(*A*) for macrolide resistance (including *mph*[*R*] regulatory protein gene), and *tet*(*A*) for tetracycline resistance (including *tetR* repressor gene) [198]. In a recent study from the USA that investigated $bla_{CTX\text{-}M}$-containing IncF plasmids of *E. coli*, several MDR plasmids (155- to 172-kb) were shown to contain genes for ABC transporter(s) and ABC transporter ATB-binding protein(s), which were considered as putative virulence factors [199]. To date, multiple plasmid-encoded ABC transporters have been reported and include Lsa(E), Msr(A), Mel, and Vga(E) (Table 23.2). Either Msr(A) or Mel can be coproduced with Mph2 from a macrolide resistance cluster of the same MDR plasmid from *S. aureus* [208] or Gram-negative bacteria (*E. coli*, *C. freundii*, *Providencia stuartii*,

Table 23.2 Examples of major plasmid-encoded drug efflux pumps

Transporter family/pump	Plasmid (GenBank accession)	Bacterial species	Plasmid-borne resistance phenotype (resistance gene)	Reference
MFS				
FloR	R plasmid (NG_034640)	*P. piscicida*	Florfenicol (*floR*), sulfonamides (*dhfrIX*)	[141]
QacA	pSK1 (NC_014369)	*S. aureus*	Antiseptic (*qacR qacA*), aminoglycoside (*aacA-aphD*)	[160]
QacB	pTZ2162 (NC_010419)	*S. aureus*	Antiseptic (*qacR qacB*), aminoglycoside (*aacA-aphD*), fosfomycin (*fosD*), arsenate (*arsCBR*), cadmium (*cadD*)	[161]
QepA	pHPA (AB263754)	*E. coli*	Fluoroquinolones (*qepA*) β-lactams (bla_{TEM-1}), aminoglycosides (*rmtB*)	[38]
TetA(A)	pRH-1238 (KR091911)	*Salmonella* Corvallis	β-Lactams (including carbapenems) (bla_{NDM-1}, bla_{CMY-16}), aminoglycosides (*aac(6′)-Ib*, *aadA5*, *aphA6*, *strA/B*), amphenicols (*floR*), fosfomycin (*fosA3*), macrolides (*mphA*), sulfonamides (*sulI*, *sulII*), tetracyclines (*tetA*(*A*)), trimethoprim (*dfrA7*), mercury (*merA*)	[32]
TetA(B)	pHCM1 (AL513383)	*Salmonella* Typhi	Tetracyclines (*tetR-tetA*(*B*)), chloramphenicol (*cat*), β-lactams (*bla*), sulfonamides (*sulII*), streptomycin (*strA/B*)	[200]
RND				
MexAB-CusC	pNDM-CIT (JX182975)	*C. freundii*	β-Lactams (bla_{MBL}, bla_{NDM-1}), aminoglycoside (*aadA2*, *armA*), chloramphenicol (*cat*), macrolides (*mel*, *mph2*), sulfonamides (*sulI*), trimethoprim (*dfrA12*), antiseptics (*qacEΔ1*), tellurium (*terABCDEFWY*)	[31]
MexCD-OprJ	pB4 (AJ431260)	Uncultured bacterium	β-Lactams ($blas_{NPS-1}$), spectinomycin and streptomycin (*strA/B*), chromate (*chrBAC*)	[201, 202]

Table 23.2 (continued)

Transporter family/pump	Plasmid (GenBank accession)	Bacterial species	Plasmid-borne resistance phenotype (resistance gene)	Reference
OqxAB	pOLA52 (EU370913)	*E. coli*	β-Lactams (bla_{TEM}), carbadox, nitrofurantoin, and olaquindox (*oqxAB*), sulfonamides (*sulI*)	[167, 180, 203–205]
SilCBA	pMG101 (AF067954)	*Salmonella* Typhimurium	Ampicillin, chloramphenicol, streptomycin, tetracycline, silver	[181, 182, 206]
SilCBA/ CusCBA	pEC5207 (KT347600)	*E. coli*	β-Lactams (bla_{CMY-2}), aminoglycosides (*aacA7*), sulfonamides (*sulI*), silver (*silP-cusA-silB-cusC-silE-cusRS*), copper (*copABCE*), antiseptic (*emrE*), tellurium (*terABCDEFWXYZ*)	[185]
SMR				
QacF	pB8 (AJ863570)	Uncultured bacterium	Quaternary ammonium compounds (*qacF*), ethidium bromide (*qacEΔ1*), β-lactams (bla_{OXA-2}), aminoglycosides (*aadA4*), sulfonamides (*sul1*)	[197]
QacZ	pTEF1 (AE016833)	*E. faecalis*	Quaternary ammonium compounds (*qacZ*), aminoglycosides (*aac-6′*)	[196, 207]
Smr	pSM52 (NC_025022)	*S. aureus*	Quaternary ammonium compounds (*smr*)	[51]
ABC				
ABC efflux complex	pRSB101 (AJ698325)	Uncultured bacterium	β-Lactams (bla_{TLA-2}), aminoglycosides (*aadA2*), macrolides (*mph*(*A*), *mph*(*R*)), sulfonamides (*sul1*), trimethoprim (*dhfr1*), tetracyclines (*tet*(*A*), *tetR*)	[198]
Lsa(E)	pV7037 (NG_041616)	*S. aureus*	Pleuromutilins, lincosamides and streptogramins (*lsa*(*E*)), aminoglycosides (*aacA-aphD*, *aadE*), lincosamides (*lnu*(*B*)), macrolides (*ermB*)	[44]

(continued)

Table 23.2 (continued)

Transporter family/pump	Plasmid (GenBank accession)	Bacterial species	Plasmid-borne resistance phenotype (resistance gene)	Reference
Mel	pRSB105 (DQ839391)	Uncultured bacterium	β-Lactams ($bla_{OXA\text{-}10}$), macrolides (*mel*, *mph*), sulfonamides (*sulI*), trimethoprim (*dfrB2*), antiseptics (*qacEΔ1*)	[108]
Msr(A)	pMS97 (AB092817)	*S. aureus*	Macrolides (*msr*(*A*), *mph*)	[208]
OptrA	pE349 (KP399637)	*E. faecalis*	Florfenicol, linezolid, and tedizolid (*optrA*)	[42]
Vga(E)	pSA-7 (NG_041699)	*S. cohnii*	Pleuromutilins, lincosamides, and streptogramins (*vga*(*E*))	[43]

Salmonella Paratyphi B, and *A. baumannii*) [31, 108]. Resistance to three structurally unrelated classes of pleuromutilins, lincosamides, and streptogramins is related to Lsa(E) or Vga(E) ABC transporters [43, 44].

A recent new study described a plasmid (named pE349) of oxazolidinone-resistant *E. faecalis* of human origin [42]. This plasmid, 36 kb in size, is conjugative and encodes an ABC transporter dubbed OptrA that is almost identical to a known putative ABC transporter of *E. faecalis* and *E. faecium*. OptrA shows good phylogenetic clustering with several staphylococcal ABC transporters such as Lsa(E) and Vga(A) that are involved in resistance to lincosamides, pleuromutilins, and streptogramins. Although pE349 also contains the *fexA* gene that confers resistance to amphenicols, the cloned *optrA* gene alone produces resistance to linezolid (four- to eightfold MIC increase), tedizolid (fourfold IMC increase), and florfenicol (16-fold MIC increase) in *E. faecalis* and *S. aureus* [42]. According to current clinical resistance breakpoints for linezolid and chloramphenicol from the Clinical and Laboratory Standards Institute [209], the *optrA*-containing plasmid sufficiently changes the interpretive category from susceptible to resistant for enterococci [42]. Of concern, a further survey of 885 enterococci revealed that *optrA* is five- to tenfold more frequently present in *E. faecalis* and *E. faecium* of food animal origin (20 % and ca. 6 %, respectively) than those of human sources (ca. 4 % and 0.6 %, respectively) [42]. An expanded survey of human hospital-derived 1,159 enterococci in China for the *optrA* gene showed prevalence of *optrA* in 34 (2.9 %) tested isolates that had variable molecular typing characteristics [85].

23.4 Concluding Remarks

Plasmid-mediated high-level resistance to all major clinically relevant antibiotics and antiseptic agents has been well documented, with the first bacterial drug efflux pump being plasmid encoded. More importantly, multiple drug resistance

determinants often coexist on the same plasmids with the presence of various mobile elements (insertion sequences, integrons, and/or transposons). Drug resistance plasmids are not limited to encoding only the single-component drug efflux pumps such as the diverse tetracycline-specific Tet pumps. They are increasingly found to carry genes encoding multicomponent drug efflux systems such as RND transporters involved in MDR. Since plasmids play a critical role in the evolution of resistance and in the dissemination of resistant bacteria, strategies to inhibit plasmid transfer could have potential implications in public health. Indeed, a recent study showed the feasibility to minimize resistance spread by blocking bacterial conjugation via the use of synthetic 2-alkynoic fatty acids [210]. Moreover, the enrichment of plasmid-containing bacteria in the presence of antimicrobial selective pressure again supports the significance of prudent antimicrobial use in any setting. Meanwhile, due to the widespread prevalence of plasmid-mediated resistance to antiseptics in hospitals, appropriate infection control measures including optimized disinfectant or biocide use should be taken into consideration in order to reduce the prevalence and spread of resistant pathogens. Lastly, several plasmid-encoded drug efflux pumps such as FloR, Lsa(E), OqxAB, OptrA, and Vga(E) were first identified in isolates of food animal origin, highlighting the important role of antimicrobial stewardship in veterinary medicine.

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Chapter 24
Influence of Regulatory RNAs on Antimicrobial Resistance and Efflux Mechanisms

Xu Jia, Bao-Dong Ling, and Xian-Zhi Li

Abstract Regulatory RNA molecules in bacteria have increasingly been shown to play an important role in influencing gene expression, particularly during the response to intracellular and environmental signals or stress conditions (including exposure to antimicrobial agents). These RNAs include the noncoding small RNA (sRNA) molecules and structured noncoding domains termed riboswitches. sRNA molecules can often have pleiotropic effect by targeting multiple mRNAs, and their activities are frequently dependent on the RNA chaperone Hfq protein. While sRNA molecules play their regulatory role through two major mechanisms, base pairing with RNAs and binding to effector proteins, riboswitches control transcription or translation by selectively binding to metabolites including antibiotics. This chapter provides an overview of regulatory RNA characteristics with a focus on their role in influencing antimicrobial resistance including the expression of drug efflux pumps. Effects of other RNA structural change-related mechanisms, such as ribosome stalling on antimicrobial resistance, are also described.

Keywords Antimicrobial resistance • Efflux pump • Regulatory RNA • Small RNA • sRNA • Riboswitch • Ribosome stalling • Hfq

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24.1 Introduction

Bacteria possess remarkable abilities to adapt to various environments including the development of antimicrobial resistance [1]. The latter can be adaptive or mutational [2, 3] and is caused by one or several of the major biochemical mechanisms such as the prevention of the access of antimicrobials to their cellular targets by reduced influx and increased efflux, drug inactivation, and target alterations [3–6]. Mutations or acquisition of genetic materials related to the action of antimicrobials provides the molecular basis of antimicrobial resistance. Moreover, various regulatory pathways also play an important role in influencing antimicrobial resistance [7]. In this regard, numerous proteins are well known to exert their regulatory functions within a biological system and thus participate in the regulation of gene expression. For instance, regulatory changes can lead to upregulation of antimicrobial-inactivating enzymes (e.g., β-lactamases) [8] and multidrug efflux pumps [4]. However, even in bacteria, gene expression regulatory networks/cascades are far more complex than we previously expected. The increasing studies on regulatory RNAs, including noncoding small RNA (sRNA) molecules and riboswitches, have provided such an example in showing the intricate regulation of the gene expression at multiple levels of transcription, RNA processing, and translation [9–11]. Consequently, regulatory RNAs affect a wide range of cell functions, which include bacterial stress response, virulence, and drug resistance [12–15]. Additionally, structural changes of mRNAs also significantly influence transcriptional and translational gene expression [16, 17]. This chapter provides an overview of regulatory RNAs and structural mRNA changes as well as our current understanding of their influences on gene expression and cellular functions that affect antimicrobial resistance, in particular drug efflux pumps in bacteria.

24.2 Regulatory RNA Molecules

There are a plethora of regulatory RNAs; two major groups include sRNA and the riboswitches, which are described below. Interestingly, noncoding sRNA molecules and riboswitches can also function together in controlling gene expression [18] as evident by the discovery of a riboswitch-containing sRNA in *Enterococcus faecalis* [11] and a riboswitch-regulated sRNA in *Listeria monocytogenes* [19]. However, in this chapter we exclude the discussion on other regulatory RNAs including clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems which are also involved in gene editing and regulation and serve as a defense mechanism in bacteria (the CRISPR-Cas systems also provide a revolutionary technical approach to alter any organism's DNA in a relatively easy manner) [20–26]. It should be noted that the role of CRISPR-Cas in antimicrobial resistance has been uncovered, such as in enhancing the stability of cell envelope and promoting resistance to polymyxins [27]. Meanwhile, multidrug-resistant enterococci were found to lack CRISPR-Cas, possibly due to inadvertent selection, by antimicrobial use, of resistant strains with compromised genome defense [28].

24.2.1 sRNA Molecules

sRNA molecules are referred to as regulatory, noncoding RNA transcripts, usually ~50–550 nucleotides in length, including *cis*- or *trans*-acting antisense RNAs [29, 30]. These sRNAs are encoded by both chromosomes and plasmids and can be produced as primary transcripts or via processing. While sRNAs are mostly derived from the 5′ regions to act via base pairing [31–33], there is an increasing recognition of sRNAs from the 3′ regions of mRNA [34, 35]. The sRNA molecules possess multiple functions, especially as ubiquitous regulators of gene expression, and are known to affect numerous physiological responses, in many cases, stress responses [32, 33, 36]. There is an advantage for the sRNA-based regulation mechanism since it provides a fast response to environmental signals by the fine-tuning of gene expression [37]. sRNAs function via two major mechanisms, i.e., base pairing with RNAs (including mRNAs) and binding to proteins to impact their activity (Figs. 24.1 and 24.2) [9, 29, 30, 32, 33, 35, 36]. Of particular note, the RNA chaperone protein Hfq is an RNA-binding protein that is often essential in promoting pairing between the sRNA molecules and their target mRNAs and subsequently influences translation and turnover rates of specific RNA transcripts [32, 35, 38].

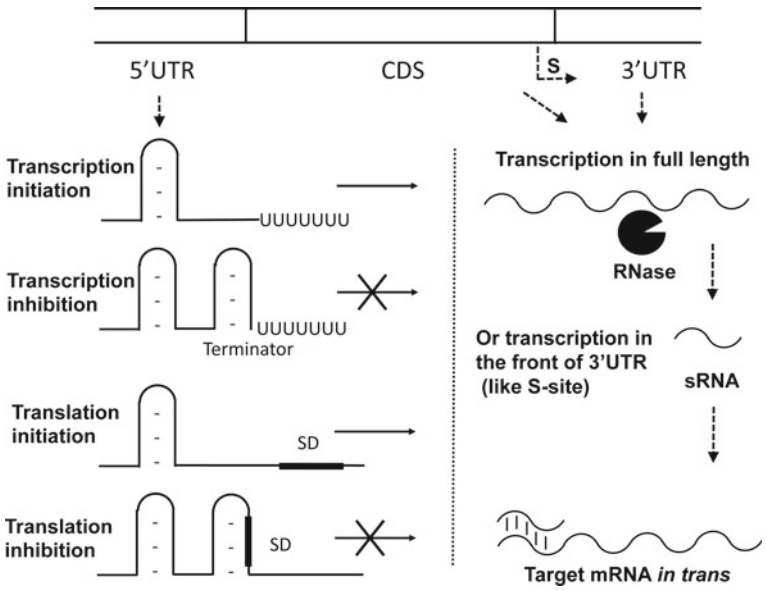

Fig. 24.1 Roles of sRNAs from the 5′ and 3′ regions of bacterial mRNAs in the regulation of gene expression. Bacterial sRNAs repress or activate their gene expression based on the configuration of the corresponding 5′ untranslated regions (5′UTR) (shown on the *left* side). They control transcription termination or translation initiation of the coding DNA sequence (CDS) in response to the change of the microenvironment, through formation of the stem-loop structure of the terminator or as a sequester of the ribosome-binding site (SD). In contrast, sRNAs from the 3′ region (3′ UTR) can be either transcribed from an mRNA-internal promoter (S site) or processed from its parental mRNA with full length (shown on the *right* side). These sRNAs regulate multiple *trans*-encoded mRNAs through short base pairing

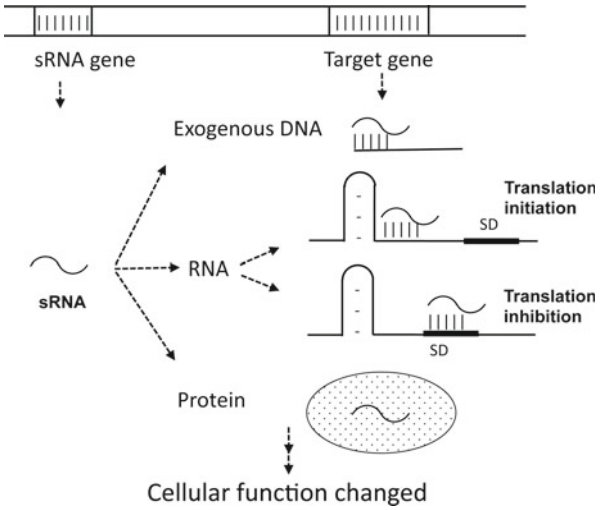

Fig. 24.2 Model for common mechanisms of sRNA-mediated gene regulation. sRNAs control exogenous gene stability, influence protein activity or regulate target mRNA fate, and consequently modulate various aspects of bacterial physiology, virulence, and behavior, including antimicrobial resistance

Functional sRNA transcripts were first discovered in bacteria in the 1980s [9]. A plasmid-specific 108-nucleotide sRNA was reported in 1981 to be untranslatable and to function as an inhibitor to block ColE1 plasmid replication [39, 40]. In 1984, the expressional downregulation of the major outer membrane (OM) porin OmpF of *Escherichia coli* by sRNA (termed as mRNA-interfering complementary RNA [micRNA]) was described [41]. This sRNA is generated from a gene (termed *micF*) that is located upstream of another gene encoding the major OM porin OmpC and is complementary to the 5′ end region of the *ompF* RNA [41]. Initially identified as a non-translated 174-nucleotide RNA [41], the primary sRNA transcript of the *micF* gene was instead found to be smaller as a 93-nucleotide MicF sRNA [42]. The MicF sRNA post-transcriptionally affects the efficient expression of OmpF. *micF* gene expression is now known to be controlled by numerous environmental and internal stress factors including oxidative stress and antibiotics such as cationic antimicrobial peptides [43, 44]. The discovery of the MicF sRNA represents the first example of a chromosomally encoded RNA regulator. Given the effect of porin production on the access of antimicrobials to drug targets in Gram-negative bacteria, the MicF sRNA is also the earliest example of sRNA effects on antimicrobial resistance.

Currently, there is a growing list for the identification and characterization of sRNAs from bacteria; these sRNAs play critical roles in many biological functions [9, 20, 36, 45, 46]. An early study, reported in 2003, summarized 55 sRNA genes in *E. coli* [47] that, as expected, include MicF and SdsR (RyeB) sRNAs currently known to implicate in antimicrobial resistance as described later. Previous studies had also showed the involvement of various sRNAs as regulators in primary and secondary metabolism in *Pseudomonas aeruginosa* [48]. A more recent report has described a genome-wide identification of sRNAs that include 44 known and >500 novel intergenic sRNAs [49]. A study targeting *Acinetobacter baumannii* showed the identification of 31 putative sRNAs, some of which were involved in stress response [50]. Sixty putative sRNAs (including three riboswitches) were also identified in *Stenotrophomonas maltophilia* [51].

As for Gram-positive bacteria, sRNAs of *Staphylococcus aureus* were demonstrated to participate in biological processes related to metabolism, stress response, and virulence [52, 53]. sRNAs related to *S. aureus* genomic and pathogenicity islands were found to be involved in the virulence regulation [54]. A database of 575 staphylococcal sRNAs has recently been made available (http://srd.genouest.org; accessed as of March 25, 2016) [55]. A recent review has discussed sRNAs of low-GC Gram-positive bacteria (such as *Bacillus subtilis*, *S. aureus*, and *Streptococcus pyogenes*); some of the known sRNAs are described to target RNAs that are related to transporters or virulence factors [56]. Additionally, more than 200 sRNAs were found in mycobacteria with certain sRNAs involved in gene expression under environmental stresses [45, 57, 58].

24.2.2 Riboswitches

Riboswitches, also known as RNA switches, are a class of RNA sensors that were first described in 2002 in bacteria in sensing small intracellular vitamin derivatives [59–61]. Over the last decade, remarkable advances have been made toward the in-depth understanding of structural, genetic, and biochemical aspects of riboswitches, which are known to be present in bacteria, archaea, and eukaryotes [62]. A total of 17 riboswitches had been determined as of 2013 [63]. We expect only a continuous dissemination of knowledge regarding the mechanisms behind the riboswitches [64, 65].

Riboswitches include two parts, an aptamer region and an expression platform. For bacteria, these regulatory elements are mainly present in the 5′ untranslated region of mRNA. Despite being composed of only four chemically similar nucleotides, RNAs can base pair with themselves and also interact with other molecules to form complex secondary and tertiary structures [66, 67]. A riboswitch requires its aptamer region to have a local structural flexibility or the ability to transition from one conformation to another in response to environmental small ligand molecules, which leads to the regulation of the downstream gene expression [62, 63]. Riboswitches control gene expression by binding small molecules without the need for protein factors [63]. This mechanism can quickly and correctly allow bacteria in response to the environmental metabolites. Antibiotics are common secondary metabolites of microorganisms for their defense against competitors [68]. It is thus reasonable to predict that antibiotics could serve as a group of potential ligands of the riboswitches and subsequently influence gene expression [69, 70].

24.3 Effect of Antimicrobial Exposures on Expression of sRNAs

The remarkable advances in molecular biology over last two decades have facilitated studies on gene expressions, such as genome-wide transcriptional profiles in bacteria following their exposure to antimicrobial agents [71–75]. Antimicrobial

exposure can affect the expression of a wide range of genes including resistance genes. In recent years, there have been an increasing number of studies that have described the sRNA production in bacteria treated by antimicrobial agents with possible impact on antimicrobial resistance [75–77]. For instance, challenging *Salmonella enterica* serovar Typhimurium with a subinhibitory level of tigecycline or tetracycline resulted in elevated expression of four sRNAs known to be conserved in several bacterial species. One of the sRNAs, sYJ20 (also known as SroA), acts *in-trans* to influence antimicrobial susceptibility [76]. The upregulation of sYJ20 was also seen in cells treated by ampicillin [76], suggesting that this sRNA may be involved in response to a broad range of stresses. More than 400 potential sRNAs were identified in two multidrug-resistant strains of *S. aureus* (with different levels of vancomycin resistance) following their exposure to one of the four antimicrobials tested (ceftobiprole, linezolid, tigecycline, and vancomycin at the half level of the minimal inhibitory concentrations), revealing that a subset of sRNAs contribute to the transcriptional response to specific drug exposures [77]. Recently, a study showed unique transcriptional response profiles (including >150 sRNAs) in multidrug-resistant *Pseudomonas putida* following exposure to a wide range of antimicrobials including ampicillin, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, kanamycin, spectinomycin, and tetracycline, which have different modes of action, again supporting the role of sRNAs in fine-tuning resistance gene expression [75].

24.4 Influence of Regulatory RNAs on Antimicrobial Resistance Including Drug Efflux Pump-Mediated Resistance

Although regulatory proteins such as local or global regulators and two-component regulatory proteins have demonstrated influence on resistance gene expression [4], regulatory RNAs also participate in affecting gene expression including those involved in bacterial stress responses and drug resistance [78]. Indeed, regulatory RNAs can regulate the stability or maintenance of DNA, RNA, and proteins and consequently influence gene expression [32]. Below we describe several pathways by which expression of antimicrobial resistance genes is affected by regulatory RNAs (Table 24.1).

24.4.1 sRNAs

One major mechanistic characteristic for sRNA function is the ability of the sRNA to base pair with the targeted mRNA molecules, which can either increase or decrease the stability and translation of the targeted mRNAs (depending on the circumstances) [96, 97]. This base pairing event often occurs through imperfect

Table 24.1 Influence of sRNA molecules on antimicrobial resistance

Species	sRNA	Target mRNA	Susceptibility or resistance phenotype	Reference
E. coli	DsrA	MdtEF	Multidrug resistance	[79]
	MicA and GcvB	PhoP	Unknown	[80, 81]
	MicC	OmpC	Multidrug resistance	[82]
	MicF	OmpF	Multidrug resistance	[41, 83–85]
	MgrR	EptB	Polymyxin susceptibility	[86]
	RalA	RalR	Fosfomycin resistance	[87]
	SdsR (RyeB)	TolC	Multidrug resistance	[88, 89]
	SdsR (RyeB)	MutS	Unknown	[90]
N. gonorrhoeae	NrrF	MtrF	Multidrug resistance	[91]
S. enterica	SdsR (RyeB)	OmpD	β-Lactam resistance	[92, 93]
	sYJ20	?	Multidrug/tigecycline resistance	[76]
S. aureus	RsaA	MgrA	Unknown	[94]
	SprX	SpoVG	Vancomycin and oxacillin resistance	[95]
	sRNA10	MecA	β-Lactam resistance	[77]

pairing with the ribosome-binding site (the Shine-Dalgarno [SD] sequence) of the targeted mRNAs and consequently leads to the inhibition of effective translation and the degradation of mRNAs [97]. There are numerous examples which demonstrate the role of base pairing RNA in influencing antimicrobial resistance genes.

Membrane permeability The above mentioned sRNA MicF in *E. coli* acts as a *trans*-encoded antisense RNA that negatively regulates the production of OmpF through its binding to OmpF mRNA [41, 43]. The ribosomal binding sites and the start codon of *ompF* transcript base pair with MicF sRNA in an RNA-RNA duplex [98]. Furthermore, MicF can target a diverse number of mRNAs including that of the lipid A-modifying enzyme, LpxR [83]. (LpxR is involved in lipid A deacylation and can thus affect the integrity of lipopolysaccharide [99].) Since OmpF is the major diffusion channel for many small hydrophilic antimicrobial agents such as β-lactams [100], the diminished level or lack of OmpF is well known to contribute to antimicrobial resistance in both laboratory-generated and clinical isolates of *E. coli* [84, 85, 101]. In fact, the MicF-based mechanism constitutes a part of the overall multidrug resistance mechanisms attributable to the decreased influx and increased efflux of drugs. Several global regulators (e.g., MarA, Rob, and SoxS) positively control the expression of the *micF* gene and the predominant drug efflux pump *acrAB* genes (reviewed in [4]). Additionally, the expression of another porin, OmpC, is also affected by an sRNA, the MicC sRNA, which is Hfq associated and inhibits ribosomal binding to the *ompC* mRNA leader [82].

To date, numerous sRNAs are known to be involved in the regulation of the OM composition in response to environmental changes [102–104]. OmpA is a major OM protein which has a structural role and also functions as a slow porin [105,

106]. The sRNA, MicA (initially known as SraD), base pairs with the ribosomal binding region of the *ompA* transcript to inhibit translational initiation and enhance *ompA* mRNA degradation [107–109]. OmrA (also known as RygA) and OmrB (RygB) sRNAs of *E. coli* negatively control production of several OM proteins [110]. MicC sRNA can silence the OmpD translation by endonucleolytic mRNA destabilization [111]. The SdsR sRNA downregulates OmpD production in *Salmonella* via Hfq-dependent base pairing [92]. The reduction of OmpD expression is observed in isolates resistant to ceftriaxone [93] and multiple drugs [112]. OmpD is also one of the genes necessary for the efficient efflux of methyl viologen [113]. The major *E. coli* lipoprotein Lpp resides in the OM and is the most abundant protein in the cell [114, 115]. MicL sRNA specifically targets Lpp mRNA, preventing its translation [115]. Moreover, MicA, RybB, and MicL allow the transcriptional factor δ^E to downregulate the synthesis of all abundant OM proteins in response to stresses [107, 108, 115–120]. RybB also plays a role in the inhibitory effect of the green tea polyphenol epigallocatechin gallate on the biofilm matrix curli fibers via δ^E-dependent cell envelop stress response to reduce biofilm antimicrobial resistance [121].

In addition to porins of the OM, lipopolysaccharide serves as a major barrier for antimicrobials to cross the outer membrane of Gram-negative bacteria [105]. The PhoPQ two-component regulatory system is pleiotropic and often responds to cell envelope stress, for example, its involvement in lipopolysaccharide modifications that affect antimicrobial susceptibility [122]. The expression of *phoP* is also subjected to the negative regulation by multiple sRNAs, including MicA and GcvB, independently via base pairing between the sRNAs and *phoP* mRNA [80, 123]. In fact, GcvB sRNA is pleiotropic and controls expression of multiple target mRNAs [81]. Interestingly, the Hfq-dependent sRNA MgrR of *E. coli* is regulated by PhoPQ system, and this sRNA negatively influences the translation of two mRNAs, which include *eptB* for a lipopolysaccharide-modifying enzyme and *ygdQ* for a hypothetical protein [86]. Deletion of *mgrR* renders the mutant more resistant to polymyxin B [86], which targets lipopolysaccharide. In *Salmonella*, a PhoP-activated sRNA, PinT, affects the expression of invasion-associated effectors and virulence genes required for intracellular survival of the microbe [124]. Overall, these data link sRNA to virulence and/or antimicrobial resistance.

Drug efflux pumps sRNA involvement in the regulation of drug efflux pump expression has also been demonstrated in literature. Nishino et al. [79] showed that the expression of the MdtEF drug efflux pump of the resistance-nodulation-cell division (RND) superfamily is positively influenced by DsrA sRNA, which is 85-nucleotide in length and represses the translation of the global regulator H-NS through its base pairing with H-NS mRNA [125, 126]. The H-NS regulator is one of the complex components involved in the regulation of multiple drug efflux operons including *acrEF*, *emrKY*, and *mdtEF* [127]. Another sRNA, RyeB, produced during stationary phase, represses the expression of TolC, an OM channel component of many tripartite drug efflux pump systems including AcrAB-TolC in *E. coli* [88]. RybB overexpression was shown to reduce resistance to novobiocin and crystal violet [89].

MtrCDE is the major RND-type drug efflux system in *Neisseria gonorrhoeae* (reviewed in [4]), and its regulation also involves a *trans*-acting sRNA, NrrF, which responds to iron availability and acts as a pleiotropic regulator including inhibition of *mtrF* expression [91]. In *A. baumannii*, an sRNA named AbsR25 was recently suggested to negatively influence the expression of the A1S_1331 transporter gene [50]. Putative base pairing between AbsR25 and AIS_1331 mRNA was identified [50].

The RNA chaperone Hfq interacts with sRNAs and mRNA [38]. Deletion of Hfq in *S. maltophilia* resulted in altered production of sRNAs including the accumulation of several RNAs [51]. Hfq-inactivated mutants showed an overall higher resistance to multiple antimicrobials (≥4-fold MIC increase for chloramphenicol, ciprofloxacin, tetracycline, tigecycline, and trimethoprim-sulfamethoxazole) with slightly increased susceptibility to amikacin, colistin, tobramycin, and vancomycin (two- to threefold MIC reduction) [51]. This susceptibility phenotype may possibly suggest the effect of Hfq on gene expression related to cell membranes and drug efflux pumps.

S. aureus expresses a plethora of sRNAs, most of which have unknown biological functions [52, 53, 55]. The RsaA sRNA exerts translational inhibition on the MgrA global regulator [128] via an imperfect base pairing of RsaA with the ribosome-binding site of *mgrA* transcript and a loop-loop interaction within the coding region of the *mgrA* mRNA; this interaction subsequently promotes bacterial persistency but reduces virulence [94]. Since MgrA is implicated in the posttranslational modification of several drug efflux pumps such as NorA and NorB [129, 130], it remains to be seen whether RsaA sRNA can impact these efflux pumps.

Resistance to various antimicrobials Recently, the sRNA SprX was shown to function as a base pairing sRNA in influencing resistance to glycopeptides (such as vancomycin) and β-lactams (e.g., oxacillin) [95]. The *yabJ-spoVG* operon of *S. aureus* encodes YabJ with unknown function and the site-specific DNA-binding protein SpoVG (stage V sporulation protein G) [131]. SprX negatively regulates SpoVG expression through direct antisense pairings at the *spoVG* ribosomal binding site of *yabJ-spoVG* mRNA [95], which is also the target of the abovementioned pleiotropic RsaA sRNA regulator [94]. In another study investigating antimicrobial exposures and sRNA production, the expression of several sRNAs was inhibited by two cell wall-targeting antibiotics, ceftobiprole and vancomycin [77]. One sRNA dubbed sRNA1 is antisense to the *gyrA* gene that encodes the target of quinolone antimicrobials, and another sRNA dubbed sRNA10 is antisense to the penicillin-binding protein 2a-encoding gene *mecA*, suggesting that these sRNAs may facilitate the adaption of *S. aureus* to the presence of antimicrobials [77].

RalR-RalA, encoded by a cryptic prophage in *E. coli*, constitutes a toxin/antitoxin system. RalR functions as a nonspecific DNase, and RalA is an Hfq-dependent antitoxin sRNA with 16 nucleotides that can base pair with the RalR mRNA [87]. Genetic inactivation of *ralR* and *ralRA* renders mutants more susceptible to the peptidoglycan synthesis inhibitor fosfomycin (which inhibits phosphoenolpyruvate transferase), suggesting that RalR-RalA plays a role in fosfomycin resistance [87].

As mentioned earlier, production of several RNAs was elevated in *Salmonella* following exposure to antimicrobials [76]. Deletion of the gene encoding sYJ20 sRNA reduced the survival of the cells in the presence of tigecycline, indicating the role of this sRNA in intrinsic antimicrobial resistance [76].

MutS plays an important role in DNA mismatch repair [132]. An RpoS-dependent sRNA SdsR targets *mutS* mRNA to repress the mismatch repair activity of MutS, and this mechanism contributes the increased mutagenesis frequencies in the presence of subinhibitory concentrations of β-lactam antibiotics (which induce SdsR expression), suggesting a possible role for sRNAs in the emergence of mutational resistance [90]. sRNAs produced by prophage in *E. coli* were reported to contribute to bacterial response to osmotic, oxidative, and acid response including resistance to ampicillin and nalidixic acid [133], and one of the sRNAs named DicF was found to control metabolism and cell division in *E. coli* [134].

24.4.2 Influence of Riboswitches on Antimicrobial Resistance

Aminoglycoside resistance A decade after riboswitch discovery, Jia et al. [135–137] reported an aminoglycoside-sensing RNA in the leader RNA of mRNAs encoding aminoglycoside acetyl transferase (AAC) and aminoglycoside adenyl transferase (AAD), two drug-modifying enzymes conferring high-level aminoglycoside resistance (Fig. 24.3). The 5′ leader RNA shows a typical structure which masks the ribosome-binding site (SD2) of the mRNAs for these enzymes in the absence of aminoglycosides [135, 136, 138, 139]. In the presence of aminoglycosides, these antibiotics bind to the leader RNA and induce a change in its structure such that exposing of the ribosome-binding site becomes beneficial for ribosomal binding and translation of the resistance genes [135, 136, 138, 139]. This instance represents the first description of a riboswitch in antimicrobial resistance. In fact, a sequence in the 5′ leader RNA for the genes encoding acetyl or adenyl transferases is highly conserved in a wide range of microorganisms [135]. The aminoglycoside-binding riboswitch is speculated to help save energy and thus benefit the bacteria in surviving during antimicrobial selection. This example suggests that antibiotic-specific sRNA interference of the 5′ untranslated regions of resistance genes could play an important role in controlling resistance gene expression.

Fluoride resistance Fluorine is one of the abundant elements in the earth's crust and can serve as the ligand of riboswitches [140]. The fluoride-responsive riboswitches, present in bacterial and archaeal species (including oral disease-associated *Streptococcus mutans*), are selectively triggered by fluorine anions (but not by chlorine anions) to activate gene expression of fluoride transporters and fluoride-inhibiting enzymes [140]. These fluoride riboswitches contain a conservative domain termed the *crcB* motif, which is located upstream of genes encoding of diverse functions (including CrcB, enolase, *E. coli*-derived chloride ion channel protein EriC, major facilitator superfamily transporters, MutS, and Na^+/H^+ antiporters). (Overproduction of plasmid-borne *crcB* in *E. coli* was found to confer resistance to camphor and chromosome condensation [141].) An *E. coli* mutant

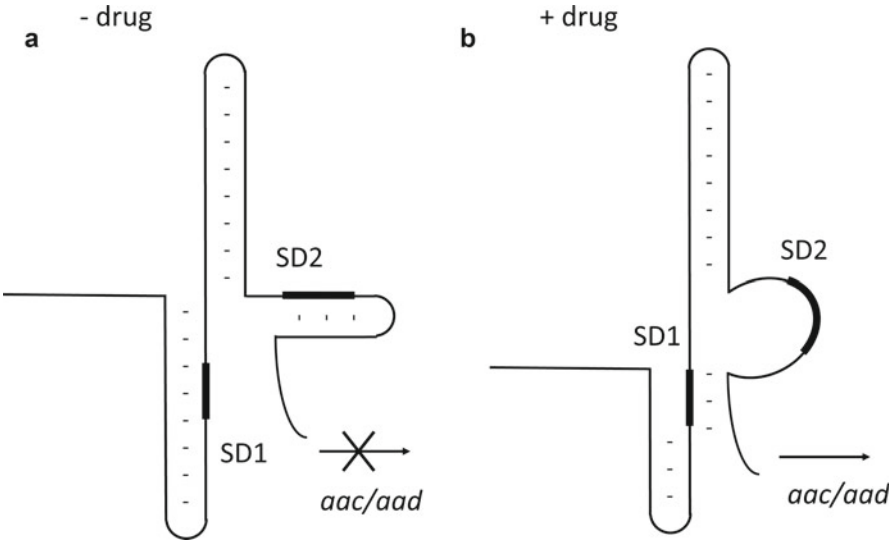

Fig. 24.3 Drug induction of *aad*/*aad* via a mechanism of regulatory riboswitch. Schematic representation of the model for the induction of aminoglycoside resistance. Aminoglycoside binding to the 5′ leader RNA induces a change in the leader RNA structure such that the anti-SD2 sequence base pairs with SD1 consequently unmasking SD2 for ribosomal binding and translation of the resistance gene. In the absence of drugs, the ribosome-binding site SD2 of *aac*/*aad* is sequestered in the mRNA secondary structure (**a**). Therefore, it is inaccessible to initiating ribosomes and *aac*/*aad* is not expressed. When cells are exposed to low concentrations of inducing aminoglycoside antibiotics, the drug bound to leader RNAs engaged in the translation of *aac*/*aad* (**b**). The drugs destabilize the ground-level mRNA secondary structure and shift the equilibrium to the induced conformation. SD2 becomes accessible, and *aac*/*aad* can then be translated by the ribosomes, which is the translation attenuation riboswitch that regulate protein synthesis

carrying *crcB* inactivation showed increased susceptibility to fluoride with a fluoride MIC of ca. 1 mM in comparison with the MIC value of 200 mM for the wild-type strain [140]. Subsequently, fluoride riboswitch-controlled antiporters were shown to be a subclass of bacterial chloride channel anion-transporting proteins which function as F^-/H^+ antiporters and protect bacteria from fluoride toxicity [142]. Moreover, in eukaryotes, resistance to fluoride toxicity is also attributable to fluoride export proteins [143].

24.5 Influence of Other RNA Structural Changes on Antimicrobial Resistance and Efflux Gene Expression

Ribosome stalling causes one of the most dramatic leader RNA structure changes, which results in translational or transcriptional attenuation of downstream gene expression in both bacteria and eukaryotes [144–146]. With this mechanism, the ribosome checks the structure of the polypeptide it is assembling, in response to

certain nascent peptide "stalling" sequences and, often, to specific cellular cues (e.g., antibiotics), which together forms the stable stalled ribosome complex [144, 146–148]. The first description of ribosome stalling dates back to the early 1980s when it was found that inducible macrolide resistance gene expression can be activated by stalling of the ribosome at the leader peptide encoded [149, 150]. In regard to the involvement of antibiotics, ribosome stalling can be grouped into either antibiotic-independent or antibiotic-dependent ribosome stalling [148]. For example, both SecM-mediated ribosome stalling and expression of the tryptophanase *tnaCAB* operon by ribosome stalling in *E. coli* are antibiotic independent. SecM controls the expression of the SecA ATPase that is involved in the protein translocation in *E. coli* via a ribosome stalling mechanism (SecM-encoding gene is located upstream of *secA*) [151–153]. The *tna* operon includes a leader peptide gene, whose product acts in *cis* via ribosome stalling to regulate the *tna* operon [145, 154, 155]. These two examples have emphatically revealed an amazing ability of RNA structures to monitor microenvironmental changes.

The macrolide-induced case [149, 150] provides an example for antibiotic-dependent ribosome stalling-based translational attenuation such as expression of the macrolide-inducible resistance genes, e.g., *ermC*. The *ermC* gene expression is activated by ribosome stalling at the leader peptide encoded by *ermCL* (Fig. 24.4). The stalling occurs in the presence of an inducing antibiotic (e.g., erythromycin) that binds in the nascent peptide exit tunnel [11]. The induction of

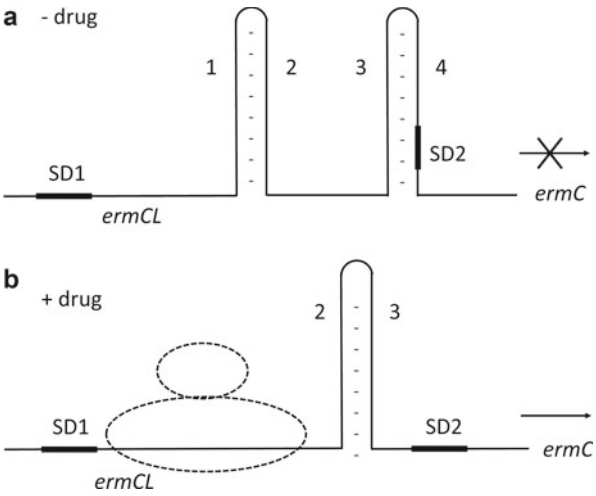

Fig. 24.4 Drug induction of methyltransferase gene *ermC* via a mechanism of translational attenuation. A segment of mRNA spanning the regulatory *ermC* leader peptide (*ermCL*), the intergenic region, and the SD2 of *ermC* are shown in an uninduced (**a**) and induced (**b**) conformation. In the absence of drug, *ermCL* is translated, while *ermC* is not because its ribosome-binding site SD2 (shown in *bold*) is sequestered in mRNA secondary structure. The mRNA segments involved in the conformational switch are marked by (*1–2*) and (*3–4*). During induction, an erythromycin-bound ribosome stalls at *ermCL* leading to a change in the mRNA conformation allowing translation of *ermC*. The mRNA segments involved in the conformational switch are marked by (*2–3*)

ermC expression by ribosome stalling is critically dependent on the ErmCL peptide sequences [11]. In the absence of erythromycin, *ermCL* is translated, while *ermC* is not because its ribosome-binding site is sequestered in the mRNA secondary structure [156]. When erythromycin is available, an erythromycin-bound ribosome stalls at *ermCL* leading to a change in the mRNA conformation that allowings the translation of *ermC* [156]. Expression of another macrolide resistance gene, *ermB*, is also similarly regulated via the macrolide-dependent ribosome stalling. The structure of the erythromycin-dependent ErmBL leader peptide-stalled ribosome complex has become available, providing structural understating of ribosome stalling regulatory process [157].

In *P. aeruginosa*, the RND-type MexXY multidrug/aminoglycoside efflux system undergoes regulation by the MexZ repressor and is inducible by ribosome-targeting antimicrobials including aminoglycosides and macrolides [158, 159]. Dimerized MexZ binds to a 20-bp palindromic sequence of the promoter of *mexXY* to only allow very low-level MexXY expression [4, 160–162]. However, MexZ expression is dependent on the antirepressor ArmZ encoded by *PA5471* (*armZ*) [163], whose own expression is controlled by a transcriptional attenuation mechanism. Drug inducibility of ArmZ requires the participation of the 367-bp *PA5472-PA5471* intergenic region which can be translated to a short 13-amino acid leader peptide, PA5471.1 [164]. In the absence of a drug, the transcribed *PA5471.1* sequence is predicted to form a stem-loop structure with adjacent regions of the leader mRNA ahead of PA5471; this structural form causes transcription termination prior to the PA5471 coding region (Fig. 24.5) [164]. When a ribosome-perturbing antibiotic is present, the PA5471.1 sequence would preclude the formation of these secondary mRNA structures and thus prevent the formation of a transcriptional terminator, permitting the transcription into the PA5471 coding region [164]. However, this structural model does not provide explanation for certain observations such as that elimination of PA5471.1 translation via an M1T (AUG→CUG) mutation also increases PA5471 expression [164] and that PA5471 is substantially upregulated in cells after exposure to oxidative stress caused by hydrogen peroxide [165] or peracetic acid [166, 167], but not by antibiotics. Recently, a novel ribosome-associated protein named SuhB was shown to modulate ribosome stalling activity toward MexXY expression [168]. Deletion of *suhB* resulted in the elevated expression of MexXY and ArmZ and reduced susceptibility to aminoglycosides [168]. SuhB was shown earlier to be a regulator of virulence genes including downregulation of several sRNAs [169].

Lastly, various other examples have also suggested the possible involvement of ribosome stalling in the regulation of antimicrobial resistance gene expression. For example, leader peptide sequences encoded by gene upstream of relevant resistance genes have been identified such as the *armA* gene for 16S rRNA methylase (aminoglycoside resistance) [170]; *cat* for chloramphenicol acetyltransferase [171]; *cfr* and *cml* for chloramphenicol efflux pumps [172]; *ermA*, *ermC*, and *ermD* for macrolide methylases [150, 173, 174]; *lasB*/*mefE*/*msrA* for multidrug or macrolide efflux pumps [175–177]; *tet*(*L*) for tetracycline efflux pump [178]; and *tet*(*M*) for ribosomal protection-based tetracycline resistance [179].

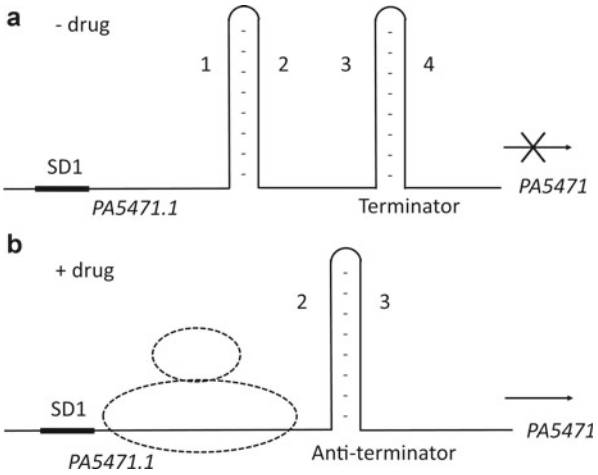

Fig. 24.5 Drug induction of efflux pump antirepressor ArmZ via a mechanism of transcriptional attenuation. Transcription of *armZ* (*PA5471*) of *P. aeruginosa* from an upstream promoter also results in the transcription of an open reading frame of *PA5471.1*, which encodes a 13-residue leader peptide. (**a**) In the absence of a drug, ribosomes bind to the SD1 site of *PA5471.1* and translation proceeds. This event permits the *PA5471.1* mRNA to form a stem-loop structure with a downstream sequence (*1–2*). In the presence of (*1–2*) stem-loop formation, an additional stem-loop is also created (*3–4*) downstream, acting as a transcriptional attenuator located just before the PA5471-coding sequences. Under drug-free growth conditions, transcription is terminated prior to the PA5471-coding region. (**b**) When a ribosome-perturbing antibiotic is present, ribosome stalling within the *PA5471.1* sequence during translation makes 1 unavailable for stem-loop formation with 2, leading to alternate mRNA folding and a stem-loop (*2–3*). The latter loop constitutes an anti-terminator structure to prevent the formation of the transcriptional terminator (*3–4*), and the downstream *PA5471* is transcribed

24.6 Concluding Remarks

This chapter provides examples regarding the contribution of regulatory RNAs and mRNA structural changes to antimicrobial resistance. It should be noted that investigation of the relationship between RNA-mediated regulation and antimicrobial resistance is a relatively new area of research in comparison with the available large amount of studies on regulatory RNAs. Therefore, more studies are warranted for better understanding of the involvement of regulatory RNAs on the development of antimicrobial resistance. Moreover, as a naturally evolved mechanism, RNA-mediated regulation of gene expression provides an efficient means toward the complex gene expression process. In this regard, targeting regulatory RNAs is already regarded as a possible important strategy for new antimicrobial research and development [24, 180]. For example, artificial antisense sRNAs, ligand analogs of riboswitches, and CRISPR system cleaving nucleotides have been utilized for potential candidates of novel antimicrobial agents [24, 181].

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Part III
Role of Drug Efflux Pumps Beyond Antimicrobial Resistance: Natural Functions

Chapter 25
The Relationship Between Bacterial Multidrug Efflux Pumps and Biofilm Formation

Alexander Fahmy, Ananth Srinivasan, and Mark A. Webber

Abstract Multidrug efflux pumps are present in all bacterial species and are known to be important in determining antimicrobial resistance. They are also important contributors to the pathogenicity of many species. Recently a link between multidrug efflux and the ability to form a biofilm has been identified in various species. Biofilm formation is thought to play a role in the majority of infections and bacteria within a biofilm are often highly antimicrobial resistant. Whilst the genes required for biofilm formation vary between species, those which are involved in production of the components of the extracellular matrix which is a hallmark feature of biofilms are crucial. In this chapter, the evidence that links multidrug resistance efflux pumps with a role in biofilm formation is reviewed, and the possible utility of efflux inhibitors as anti-biofilm compounds is discussed.

Keywords Antimicrobial resistance • Multidrug resistance • Biofilm formation • Multidrug efflux pump • Efflux inhibitor

25.1 Introduction

Biofilms are three dimensional surface-adhered bacterial communities embedded in an extracellular polymeric matrix. Growth as a biofilm has been suggested to be the state in which the vast majority of bacteria are found and biofilms are involved in the majority of infections at some stage [1, 2]. The ubiquitous presence of biofilms extends from nature to domestic, industrial and clinical situations. A wide range of human infections are caused by biofilms, for example, *Pseudomonas aeruginosa*

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lung infections in cystic fibrosis, periodontal disease caused by biofilm plaque and medical device-associated infections caused by biofilms of various species [3–6]. Biofilms are problematic in these settings as they are refractory to most antimicrobial treatments employed against them. Biofilms contain a high proportion of metabolically inactive persister cells, and these, along with the biofilm matrix, are thought to be responsible for this antimicrobial tolerance which can be up to 1,000 times greater than their planktonic counterparts [2, 7]. In addition to being highly resistant to antimicrobials, biofilms also facilitate persistence in the clinical domestic and industrial environment. Bacteria such as *Salmonella enterica* serovars are tolerant to bactericidal cleaning agents when in biofilm form and have an increased likelihood of contamination of food and water resulting ultimately in human colonisation and disease [8].

Numerous genes have been shown to be important in the formation of a biofilm, and these are involved in initial adhesion events, production and export of extracellular matrix components, eventual dissemination of cells from a biofilm and communication between cells within a biofilm. In addition to those genes with a biofilm-specific role, many others demonstrate differential expression when cells are grown in a biofilm compared to planktonic cell growth. Bacterial multidrug resistance (MDR) efflux systems have been the focus of much study as a result of their ability to export antimicrobial agents, although they often have additional roles in the cell. There are five main superfamilies/families of MDR efflux systems; members of each can all export a wide range of compounds such as waste metabolites, dyes, toxins and antimicrobials [9]. The most clinically significant efflux systems in Gram-negative bacteria are the efflux pumps of the resistance-nodulation-cell division (RND) superfamily, such as AcrAB-TolC of *S. enterica* and MexAB-OprM of *P. aeruginosa* [10, 11]. Whilst interest has been focused on the antimicrobial resistance properties of MDR efflux pumps, there are other phenotypes which have been associated with these systems; many bacteria lose pathogenicity upon disruption of one or more MDR systems, and the RND family has been linked to events required for cell division.

A growing body of evidence also suggests that MDR efflux systems are involved in biofilm formation. Developing novel anti-biofilm strategies requires a deeper understanding of factors crucial in biofilm formation and maintenance. Therefore, this chapter outlines the experimental evidence to date regarding links between MDR efflux and biofilm formation and the proposed mechanisms that link the two.

25.2 Evidence for a Link Between Drug Efflux Pumps and Biofilm Formation

A link between efflux pumps and biofilm formation has been suggested in multiple studies in the last decade; these links have come from studies where mutants lacking efflux genes have shown a defect in biofilm formation, studies where efflux inhibitors have reduced biofilm formation and studies where expression of efflux pumps has increased in a biofilm. Table 25.1 contains a summary of these studies and a brief description of the evidence from each.

Table 25.1 Summary of studies which have reported a link between MDR efflux pumps and biofilm expression

Report year	Species	Description	Evidence[a]	Reference
1999	*P. aeruginosa*	Inhibition of MexAB-OprM resulted in increased accumulation of autoinducer *N*-3-oxododecanoyl-L-homoserine lactone and reduced biofilm formation	1	[12]
2001	*P. aeruginosa*	No increase in MexAB-OprM or MexAB-OprJ expression during biofilm formation but pump activity was spatially variable and pumps were most active in the substratum of a biofilm	1	[13]
2003	*P. aeruginosa*	Mutants overexpressing MexEF-OprN had a reduction in ability to form a biofilm	1	[14]
2005	*P. aeruginosa*	Within a biofilm the MexCD-OprJ pump is expressed and provides resistance to azithromycin	2	[15]
2007	*E. coli*	Investigated RapA mutants, and a change in biofilm matrix architecture was linked to down-regulation of MDR pump YhcQ	1, 2	[16]
2008	*E. coli*	Investigated aggregation of enteroaggregative wild type vs. TolC mutants, and TolC mutants had a decreased aggregative ability	1	[17]
2008	*E. coli* and *K. pneumoniae*	Various efflux inhibitors repressed biofilm formation alone and in combination. Transporter genes were highly upregulated during biofilm growth. Proposed efflux required for waste disposal within a biofilm	2	[18]
2008	*P. aeruginosa*	A novel efflux pump PA1874-1877 is expressed more in biofilms than planktonic growth and confers resistance to aminoglycosides and ciprofloxacin	2	[19]
2008	*P. aeruginosa*	CCCP inhibits biofilms		[20]
2008	*L. monocytogenes*	Mutation that disrupted a putative ABC transporter resulted in increased biofilm formation	1, 2	[21]
2009	*E. coli*	High-levels of TetA(C) and other SMR pumps were induced in response to osmotic stress correlated with increased production of colanic acid and thereby biofilm formation	1, 2	[22]
2010	*P. aeruginosa*	PAβN in conjunction with an iron chelator works synergistically to reduce biofilm formation	1	[23]
2011	*E. coli*	Deletion of multiple genes encoding different efflux pumps in resulted in a biofilm deficit	1	[24]

(continued)

Table 25.1 (continued)

Report year	Species	Description	Evidence[a]	Reference
2011	*E. faecalis* and *S. aureus*	Naturally occurring efflux inhibitors reduce biofilm formation including caffeoylquinic acid from *Artemisia absinthium* which decreases biofilms when combined with sub-inhibitory concentrations of ethidium bromide and moxifloxacin	1	[25]
2012	*C. violaceum and E. coli*	Trifluoromethyl ketones reduces quorum sensing via inhibition of efflux of quorum sensing signalling molecules – resulted in an anti-biofilm effect		[26]
2012	*Salmonella* Typhimurium	Inactivation of any of the MDR efflux pumps resulted in reduced biofilm formation	1	[27]
2013	*E. coli*	Mutants lacking TolC accumulate toxic metabolites and this results in de-repression of global stress response genes (MarA, SoxS and Rob and BaeRS and CpxARP)	1, 2	[28]
2014	*Salmonella* Typhi	Mutants lacking TolC had compromised biofilm formation	1	[29]
2015	*A. baumannii*	Association of biofilm formation and AdeFGH pump overexpression	1, 2	[30]
2015	*E. coli*	AcrAB and MdtAB pumps contribute to biofilm maintenance	1	[31]

[a]Evidence 1 indicates studies which have linked efflux pumps to biofilm forming ability in both Gram-negative and Gram-positive bacteria, and evidence 2 denotes studies that have suggested a biological role for efflux in forming a biofilm based on gene expression changes

25.2.1 Evidence from Gene Inactivation Studies

As an appreciation of the importance of MDR efflux pumps in the biology of various species has become widespread, there has been an increase in the numbers of these systems which have been characterised [32, 33]. Bacterial genomes all contain multiple MDR efflux pumps belonging to different families and, as molecular genetic toolkits have improved and been adapted for use in various species, a large number of mutant strains lacking components of MDR efflux systems have been created. A significant number of these mutants have been examined in terms of their ability to form a biofilm which has revealed major differences in biofilm formation capacity in various cases.

Several studies (including one from our group) have investigated the impact of multiple efflux pump deletions on biofilm formation. The first study by Matsumura et al. [24] analysed a panel of 22 mutant strains of *Escherichia coli* (created in a K-12 strain) and found decreased formation of biofilms *in vitro* for all the mutants tested with 6 mutants (representing members of the of the major facilitator

superfamily [MFS], the small multidrug resistance [SMR] and RND families) showing extremely reduced biofilm formation. More recently, MDR efflux pumps such as AcrAB and MdtAB were shown to contribute to biofilm maintenance in *E. coli* [31]. Another study which looked at multiple efflux deletion mutants also found a significant biofilm defect for all the efflux mutants tested (nine, again *in vitro*), this time in *Salmonella enterica* serovar Typhimurium [27]. This second study also included a mutant lacking TolC which acts as the outer membrane exit channel for substrates exported by the MDR efflux pumps for *Enterobacteriaceae* and as such is crucial to the effective function of all these systems. Consistent with the hypothesis that loss of TolC should impact all other efflux systems, the TolC mutant demonstrated the greatest efflux deficit in this study. Further evidence that TolC has a key role to play in biofilm formation has come from another two studies which have showed that mutants of *E. coli* or *Salmonella enterica* serovar Typhi lacking TolC demonstrated decreased aggregative capacity or ability to form a biofilm, respectively [17, 29]. Recent studies also assessed the relationship between the expression status of RND pumps in *Acinetobacter baumannii* and biofilm formation [30, 34]. An association between biofilm formation by *A. baumannii* clinical isolates and overproduction of AdeFGH pump was observed [30]. However, overexpression of each of the three RND pumps, AdeABC, AdeFGH and AdeIJK, resulted in ca. 60–80 % decrease in biofilm formation [34], although an *adeB* deletion mutant also displayed a defect in biofilm formation [34], suggesting that optimal biofilm formation is affected by the expression of drug efflux pumps in *A. baumannii*.

Whilst mutants lacking in biofilm capacity have mainly been identified in Gram-negative bacteria, there is also evidence for a link between efflux and biofilm formation in Gram-positive species, although in this case a *Listeria* mutant lacking an ATP-binding cassette (ABC) transporter demonstrated increased biofilm formation, and this was interpreted as being a result of altered regulation of specific biofilm formation genes [21, 35].

25.2.2 Evidence from Studies of Efflux Inhibitors

Even though studies have examined mutant strains lacking efflux components, a wide variety of compounds have been studied due to a suspected ability to inhibit efflux pumps. These include a chemically diverse group of molecules which are thought to act as efflux inhibitors by either acting as competitive substrates, by inhibiting energy generation for efflux or by inhibiting pump gene expression or assembly. Due to the varied nature of the mode of action of these agents, we refer to them as ‘efflux inhibitors’ rather than ‘efflux pump inhibitors’ as some do not directly act on the pump component of MDR efflux systems (e.g. compounds that dissipate the proton motive force will result in loss of efflux from systems which use this for energy although this is an indirect effect). As with efflux mutants, a number of studies have found impacts on biofilm formation which follow exposure to an efflux inhibitor (Table 25.2).

Table 25.2 Summary of efflux inhibitors that have been shown to effectively reduce biofilm formation

Species	Effective efflux inhibitor	Reference
C. violaceum	Trifluoromethyl ketones	[26]
E. coli	NMP, PAβN, thioridazine, trifluoromethyl ketones	[18, 26]
K. pneumoniae	NMP, PAβN, thioridazine	[18]
S. Typhimurium	CCCP, chlorpromazine, PAβN	[36]
P. aeruginosa	CCCP, PAβN	[23, 36]
P. putida	NMP, PAβN, thioridazine	[18]
E. faecalis	4′,5′-O-dicaffeoylquinic acid	[25]
S. aureus	CCCP, chlorpromazine, 4′,5′-O-dicaffeoylquinic acid, thioridazine	[18, 25]

One of the earliest studies by Kvist et al. [18] showed that three different efflux inhibitors, thioridazine, 1-(1-naphthylmethyl) piperazine (NMP) and phenylalanine-arginine β-naphthylamide (PAβN), were able to significantly reduce biofilm formed by *E. coli* isolates and laboratory strains, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas putida*. Whilst there was some variation in the susceptibility to inhibitors in this study, all species did demonstrate reduced biofilm formation when exposed to one or more of the inhibitors [18]. Our group also found a range of different inhibitors, i.e. carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), PAβN, and chlorpromazine, were also effective against biofilms formed by *E. coli*, *S.* Typhimurium, and *S. aureus*, although as with the Kvist study, not all efflux inhibitors were equally effective against all species [36]. Anti-biofilm activity against *S. aureus* and *Enterococcus faecalis* has also been shown for the plant extract 4′,5′-O-dicaffeoylquinic acid produced by the *Artemisia absinthium* which was also revealed to be an effective inhibitor of MFS efflux pumps [25]. The secretion of an efflux inhibitor by a plant that has anti-biofilm activity suggests a self-defence function for this compound in its natural niche.

In nature, quorum sensing is often important in biofilm formation where some species will excrete autoinducer molecules that act as signals to promote expression of biofilm-specific genes when a population density is appropriate. Two recent studies have shown that efflux inhibitors can block export of quorum sensing molecules which, in turn results in reduced biofilm formation – this impact has been seen with *Chromobacterium violaceum* (an opportunistic pathogen [37]), *E. coli* and *P. aeruginosa* rely on the export of autoinducers by proton motive force-driven pumps including MexEF-OprN of *P. aeruginosa* [12, 26]. As a major pathogen and generally a strong biofilm forming organism in laboratory conditions, and with a significant biofilm-associated pathogenicity, *P. aeruginosa* has been studied by various groups as a target for efflux inhibition. Two studies have documented biofilm repression as a result of efflux inactivation by either PAβN, or, the proton motive force uncoupler, CCCP [20, 23]. One key point from these studies with efflux inhibitors is that those which have proved to effectively reduce biofilm formation suggest that the lack of biofilm results from loss of efflux function rather than an artefact caused by loss of the proteins themselves which may impact on properties of the membrane etc.

25.2.3 Evidence from Biofilm Physiology Studies

The final types of study which have implicated a link between efflux pumps and biofilm formation are those which have examined expression of genes within a biofilm and compared these values to planktonic growth. A number of these studies have suggested a functional link for efflux pumps within a biofilm based on increases in expression when in a biofilm. Changes to gene expression and protein production during biofilm formation have been best studied in *P. aeruginosa* [38]. Various efflux systems have been found to be upregulated in *P. aeruginosa* biofilms including MexCD-OprJ (correlated with azithromycin tolerance) [15] and PA1874-1877 (a novel system correlated with tolerance to aminoglycosides and ciprofloxacin) [19]. As well as studies showing increased expression of efflux systems in *Pseudomonas* biofilms, there is evidence of spatial variation of expression within a biofilm with another study showing that the MexAB-OprM system was more active in some parts of a biofilm than others [13]. Evidence for a contribution of efflux pumps to the physiology of cells within biofilms is not limited to *Pseudomonas*. Kvist et al. [18] found that 20 efflux pumps and transporters of *E. coli* were upregulated during biofilm growth, and May et al. [22] noted that the TetA(C) pump was upregulated in biofilms and involved in export of colonic acid, a capsular component important in matrix formation and thereby biofilm formation.

Upregulation of MDR efflux pumps within a biofilm is suggestive of a functional role although there is no clear pattern as to which type of pump is produced and no clear correlation with spatial or temporal expression within a biofilm. It is likely that there will be major differences between species in this respect.

25.3 How May Efflux Contribute to Life Within a Biofilm?

25.3.1 Do Efflux Pumps Export Components of the Biofilm Matrix or Signal Molecules?

One logical explanation for the dependency of biofilming on bacterial efflux would be that efflux pumps export crucial components required for biofilm formation (Fig. 25.1). Whilst there is some evidence for export of matrix components [22] and also transport of quorum sensing molecules [12, 26], this does not appear to be a universal phenomenon. We specifically aimed to address this issue with *Salmonella* efflux mutants and experiments where *Salmonella acrB* and *tolC* mutants were co-incubated with wild type strains resulted in biofilms in which no appreciable numbers of mutants were present (although equally distributed in planktonic cultures). We also found no ability of media conditioned by growth of a wild type to restore biofilm formation of efflux mutants.

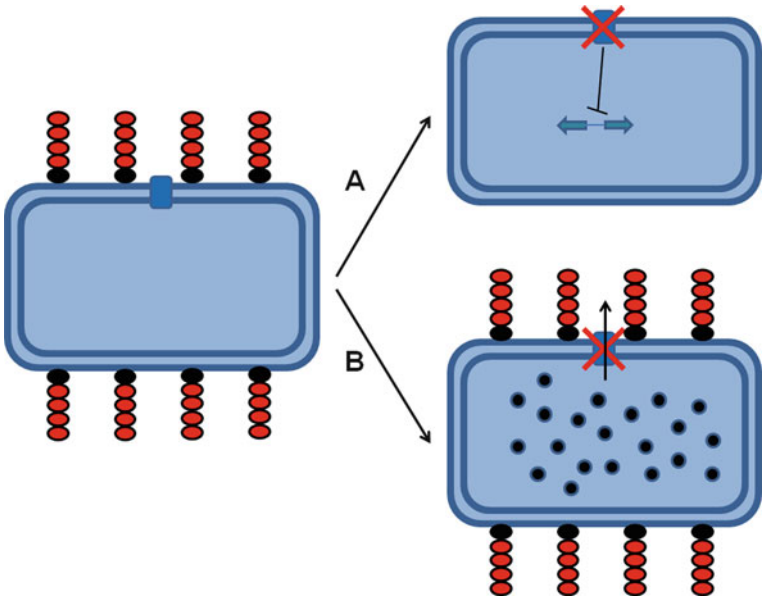

Fig. 25.1 Possible mechanisms linking efflux and biofilm formation. (**a**) Loss of efflux results in repression of curli production (indicated by *black* and *red circles*). (**b**) Loss of efflux prevents export of a metabolic waste product that over accumulates to toxic levels

25.3.2 Are Efflux Pumps Required for Life at High Density?

Another possible way that MDR efflux pumps could contribute to biofilm formation is by allowing high-density life if they act to export waste metabolites and help prevent toxic accumulations of molecules produced as by-products of intracellular reactions. Actively metabolising cells within a biofilm may require efficient means of export of waste, and, given the broad spectrum of substrates of the MDR efflux pumps, it has been suggested they may play a role in detoxifying the host cell from waste products. The studies outlined above which show that efflux pumps are upregulated within a biofilm are suggestive for a physiological role although none directly address waste export [13, 15, 18, 19, 22]. An investigation into a TolC mutant of *E. coli* did specifically examine metabolite accumulation and did find that mutation of TolC resulted in de-repression of stress response pathways including those that control biofilm and efflux expression [28]. As TolC is the common exit duct for many transporters in *E. coli*, it may be that this phenomenon may not be reflected when individual, inner membrane transporters are inactivated.

25.3.3 Do MDR Efflux Pumps Directly Influence Aggregation?

As membrane-bound systems, it is possible that the loss of efflux pumps might influence properties of the cell envelope or that these proteins may have a direct role in adhesion to surfaces. If efflux pumps were involved in adhesion, then loss of

these systems may result in impaired biofilm formation and explain some of the results above. There is however limited evidence for this; an *E. coli* mutant of TolC was found to show decreased aggregative ability although the mutant was created in an enteroaggregative *E. coli* strain [17] which may not be representative of the genus as a whole – we performed the similar experiment in *Salmonella* and found no aggregation defect in a TolC mutant [27].

25.3.4 Linked Regulation of Efflux and Biofilm Genes?

If there are common regulatory networks that controlled both biofilm and efflux genes, then perturbation of efflux might result in altered expression of biofilm genes and an altered biofilm phenotype (Fig. 25.1). As both expression of MDR efflux pumps and growth as a biofilm are protective against antimicrobial stress, it may be expected that they be upregulated together. However, it may be that it is sensible for these pathways to be regulated in an inverse manner: when MDR efflux pumps are required to be upregulated, it infers that the environment may contain antimicrobials or other toxic compounds, and therefore biofilm formation may be counterproductive.

There are various examples of altered regulation of biofilm-specific genes in response to changes in efflux activity; in *Listeria monocytogenes* biofilm matrix components are negatively regulated by an ABC transporter [21, 35], and the MgrA regulator of *S. aureus* has been shown to control both biofilm formation and efflux pump activity demonstrating a regulatory relationship between the two [39]. We have also recently found that in *S.* Typhimurium mutants lacking *acrB* and *tolC* which do not biofilm effectively also have a decreased production of curli [27, 36], a protein that is a major component of the extracellular matrix in many Gram-negative biofilms [40]. This was correlated with repression of *csgD* in the knockout mutants, which is a major curli transcriptional regulator [27, 36].

25.4 Efflux Inhibition as an Anti-biofilm Strategy?

One exciting opportunity which results from a link between efflux and biofilm formation is the potential for efflux inhibitors to be used as anti-biofilm agents. A number of studies have investigated this to some degree as described above (Table 25.2) [12, 18, 20, 23, 25, 26, 36].

For an efflux inhibitor to be effective, it should fulfil a number of criteria, which include demonstration of anti-biofilm activity at concentrations below the minimal inhibitory concentration for the inhibitor (to confirm specific activity rather than a generalised growth defect). This has been seen with inhibitors of different classes against both *E. coli* and *Salmonella* [18, 36].

Potential inhibitors may also demonstrate synergy with antimicrobials which would allow prevention or inhibition of biofilm formation or maturation but may also increase the capacity for conventional antimicrobials to kill cells within a

biofilm; this has been shown for tetracycline and various species [18]. Synergy between pump inhibitors may also be useful. In *E. coli* it was found that a combination of thioridazine and PAβN further increased the biofilm inhibitory effect compared to use of each efflux pump inhibitor alone. A likely explanation is that when the drugs were used together, a wider range of efflux pumps were suppressed, producing a better response [18].

Whilst efflux inhibitors have been developed with a view to clinical application in conjunction with antibiotics, no combination has been licenced as yet – partly due to toxicity or metabolism issues with the inhibitors which have been developed so far. Application against a biofilm may not require systemic application if, for instance, a catheter is being targeted or a prosthetic implant protected – a release into the local vicinity may avoid some of the problems associated with systemic application and represent a novel therapeutic window for efflux inhibitors as anti-biofilm agents.

25.5 Concluding Remarks

It has been accepted for some time that MDR efflux pumps have multiple biological roles relevant to infection including the export of antimicrobial molecules and a regulatory link with specific virulence factors. The identification of another key biological role, a contribution to biofilm formation, confirms the importance of MDR pumps to the biology of the cell. The mechanisms by which efflux and biofilm formations are linked remain to be elucidated in detail, and there are conflicting opinions in the literature to date. It is not clear whether pumps are key players in initial attachment and biofilm formation or are needed for biofilm maturation and life at high density. Some studies have shown increased expression of efflux pumps in biofilms compared to planktonic cells but others have not, in some species initial attachment of efflux mutants is normal but biofilms do not progress beyond micro colonies. Clearly the role of efflux within a biofilm requires greater study – careful examination of efflux activity at different time stages of biofilm development, in different species and in different conditions will help elucidate the biological role of MDR efflux in biofilms. Some early theories have been proposed as to how efflux and biofilm are linked; growth as a biofilm is a high-density lifestyle, and it seems intuitive that waste export may be a problem which efflux pumps could solve. However, in some of the data to date, the biofilm deficit of efflux mutants is apparent relatively early in a biofilm lifecycle where stress from an inability to export waste may not be likely. A link has been observed between production of matrix components and loss of efflux function; it is well established that some matrix components are required for biofilm formation, and loss of matrix production genes will often hamper biofilm formation greatly. A link has been shown between the efflux pumps of *Salmonella* and curli production; however, this link is not fully understood and the regulatory mechanisms underpinning the two remain to be elucidated in detail.

The potential for efflux to be inhibited has been proposed for some time in terms of potentiation of antimicrobial agents, and whilst no inhibitor has reached market to date, a range of efflux inhibitor molecules are effective *in vitro*. The discovery of an additional and infection relevant phenotype linked to efflux pumps now suggests that an effective pump inhibitor could increase drug susceptibility, reduce pathogenicity and prevent biofilm formation; all highly desirable in the treatment of bacterial infection. The potential for efflux inhibition to be of practical use in preventing contamination of surfaces is exciting, but it remains to be seen how effective it may be in practice.

The mechanisms by which efflux pumps contribute to biofilm formation remain to be determined in detail, and this is an area of research in its infancy but with great potential application as anti-biofilm strategies could prevent infection or contamination in multiple settings. Better models of biofilm formation which represent *in vivo* outcomes of biofilm formation are needed, and efflux inhibitors will need to be examined in more representative models before translation into clinical practice, but there is a need for anti-biofilm therapies and this remains a promising area of research.

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Chapter 26
Antimicrobial Drug Efflux Systems as Components of Bacterial Stress Responses

Keith Poole and Michael Fruci

Abstract Chromosomally-encoded broadly specific so-called multidrug efflux systems are widely distributed in bacteria where they are increasingly appreciated as important determinants of antimicrobial resistance. Nonetheless, it is evident that antimicrobial efflux is not the intended function for these efflux systems, many of which are specifically recruited in response to various cell-perturbing environmental stresses. As such, they likely function as components of protective stress responses in these bacteria. The prospect of non-antimicrobial stresses driving efflux gene expression and efflux-mediated antimicrobial resistance has important implications for resistance development in bacteria. A better understanding of the details of their stress regulation and their specific roles in bacterial stress responses may inform efforts to target them therapeutically.

Keywords Antimicrobial resistance • Efflux • RND pumps • Envelope • Stress response • Oxidative • Nitrosative • Bile salts • Heavy metal • pH

26.1 Introduction

Efflux as a mechanism of antimicrobial resistance has been known for more than 30 years, with a tetracycline efflux mechanism first reported in *Escherichia coli* in the early 1980s [1, 2]. As with numerous later examples [3–6], this was a drug-specific efflux mechanism that was encoded by genes resident on a

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mobile genetic element. These systems were and are, thus, typically acquired by horizontal gene transfer and are generally held to function solely in the export of and resistance to antimicrobials and, so, selectable by antimicrobials. In contrast, the more recently described multidrug efflux systems that accommodate a range of structurally diverse antimicrobials are typically chromosomally-encoded and broadly conserved in bacteria [7–10], an indication that they are not drug-selected and not, therefore, intended primarily as determinants of antimicrobial resistance [11–13]. This was, in fact, initially suggested soon after these systems were identified in the early 1990s [14] and has since been supported by the observed multiplicity of chromosomal multidrug efflux systems in individual bacteria, the often overlapping drug selectivity of these systems and their independent regulation by regulatory proteins that do not typically respond to the antimicrobials that are the substrates for these efflux systems [7, 9, 15–18]. Indeed, a plethora of data now point to many of these systems being stress inducible, responding to agents of oxidative and nitrosative stress, membrane perturbation, nutrient limitation, and the adverse consequences of antimicrobial exposure itself (Table 26.1). As such, a number of multidrug efflux systems appear to function as components of bacterial stress responses [107–110], with presumed but as yet undefined roles in ameliorating the damage resultant from stress-mediated perturbations of the bacterial cell. There are five major families (or superfamilies) of drug efflux systems – the resistance-nodulation-cell division (RND) superfamily, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family – although stress-linked drug efflux systems are predominantly of the RND superfamily (Table 26.1). Significantly, RND-type multidrug efflux systems are also major determinants of clinically-relevant antimicrobial resistance in disease-causing bacteria [15, 16, 111–114]. Stress-linked drug efflux systems occur primarily in Gram-negative bacteria, although putative stress-regulated drug efflux systems are reported in Gram-positive bacteria as well as the mycobacteria (Table 26.1).

26.2 Oxidative Stress-Linked Drug Efflux Systems

Organisms that grow aerobically are routinely exposed to oxidative stress in the form of reactive oxygen species (ROS) (e.g., peroxide, superoxide [SO]) that are the unavoidable by-products of aerobic respiration. ROS damage a variety of cellular macromolecules and, thus, elicit adaptive oxidative stress responses in bacteria that facilitate survival in the presence of this stressor [115]. Expression of a number of multidrug efflux systems and/or their regulatory proteins is impacted by agents of oxidative stress (Table 26.1); these efflux systems possibly play some role in ameliorating the adverse effects of oxidative stress.

Table 26.1 Stress-linked antimicrobial efflux systems

Stress	Efflux system	Efflux family	Stress-responsive regulator	Organism	References
Oxidative					
	AcrAB-TolC	RND	SoxRS	*E. coli*	[19]
			SoxRS	*S. enterica*	[20]
			SoxRS[a], OxyR[b]	*K. pneumoniae*	[21]
			SoxRS	*E. cloacae*	[22]
	MexXY-OprM	RND	ArmZ	*P. aeruginosa*	[23, 24]
	MexAB-OprM	RND	MexR	*P. aeruginosa*	[25, 26]
	CmeABC[c]	RND	CosR[d]	*C. jejuni*	[27, 28]
	MdsABC	RND	GolS	*S.* Typhimurium	[29, 30]
	NorA	MFS	MgrA	*S. auerus*	[31]
	NorB	MFS	MgrA, SarZ	*S. auerus*	[31, 32]
	Tet38	MFS	MgrA, SarZ	*S. auerus*	[31, 32]
	MacAB-TolC	ABC	?[e]	*S.* Typhimurium	[33]
	MacABCsm	ABC	?	*S. maltophilia*	[34]
	Persisters/AcrAB-TolC	RND	SoxRS/SOS	*E. coli*	[35]
	EfpA[f]	MFS	?	*M. tuberculosis*	[36]
	AbuO[g]	?	SoxR	*A. baumannii*	[37]
	P55[h]	MFS	?	*M. bovis*	[38]
Nitrosative					
	MexEF-OprN[i]	RND	MexT	*P. aeruginosa*	[39]
	MdtEF(-TolC)[j]	RND	ArcBA	*E. coli*	[40, 41]
	AcrAB-TolC	RND	OxyR[b]	*K. pneumoniae*	[42]

(continued)

Table 26.1 (continued)

Stress	Efflux system	Efflux family	Stress-responsive regulator	Organism	References
Envelope[k, l]					
Membrane-damaging aberrant polypeptides	MexXY-OprM	RND	AmgRS	*P. aeruginosa*	[43]
Solvents, cationic antimicrobial peptides, membrane-active biocides	MexCD-OprJ	RND	AlgU	*P. aeruginosa*	[44, 45]
Non-ionic detegent, membrane-active biocides	SmeIJK	RND	σ^E	*S. maltophilia*	[46]
?[m]	MacABCsm[n]	ABC	?	*S. maltophilia*	[34]
Na-tungstate, plant flavanoids	MdtABC, AcrD	RND	BaeSR	*E. coli*	[47, 48]
Na-tungstate	MdtABC, AcrD	RND	BaeSR	*S.* Typhimurium	[49]
Na-tungstate, plant tannins	MdtABC, MdtUVW	RND	BaeSR	*E. amylovora*	[50]
Sucrose (high osmolarity)	AdeAB	RND	BaeSR	*A. baumannii*	[51]
Misfolded envelope proteins	MdtABC, AcrD	RND	CpxRA	*E. coli*	[48]
?[o]	AcrB, AcrD	RND	CpxRA	*K. pneumoniae*	[52]
?[o]	KpnEF	SMR	CpxRA	*K. pneumoniae*	[53]
?[o]	VexAB, VexGH	RND	CpxRA[p]	*V. cholerae*	[54, 55]
Cell envelope-/wall-active antimicrobials	VraDE	ABC	BraRS	*S. aureus*	[56, 57]
Bile	AcrAB-TolC[q]	RND	Rob	*E. coli*	[58]
	AcrAB-TolC	RND	RamA	*S.* Typhimurium	[59, 60]
	AcrAB	RND	?	*V. cholerae*	[61]
	VexAB	RND	?	*V. cholerae*	[62]
	VexCD (aka BreAB)	RND	BreR	*V. cholerae*	[62, 63]
	CmeABC	RND	CmeR	*C. jejuni*	[64]
	Bme[r]	RND	?	*B. fragilis*	[65]
	AcrAB-TolC[s]	RND	OxyR	*K. pneumoniae*	[42]
	AcrAB-TolC	RND	?	*Y. enterocolitica*	[66]
	MdtM[t]	MFS	?	*E. coli*	[67]

Chlorhexidine	AdeAB	RND	?	*A. baumannii*	[68]
	Bme[u]	RND	?	*B. fragilis*	[69]
	RND-4	RND	?	*B. cenocepacia*	[70]
	RND-8	RND	?	*B. cenocepacia*	[71]
Quaternary ammonium compounds	NorA	MFS	?	*S. aureus*	[72]
Antibacterial fatty acids	AcrAB	RND	Rob	*E. coli*	[58]
	Tet38	MFS	?	*S. aureus*	[73]
Cationic antimcrobial peptides	MexXY	RND	ParRS	*P. aeruginosa*	[74]
	MacAB-TolC, MexB, AdeIJK	RND	BaeSR[v]	*A. baumannii*	[75]
	VraDE	ABC	Aps	*S. aureus*	[76]
Detergents (non-ionic)	MtrCDE	RND	MtrA	*N. gonorrhoeae*	[77]
Tea tree oil[w]	MmpL	RND	?	*S. aureus*	[78]
Thiorizadine[x]	Mmr/Emr	MFS	?	*M. tuberculosis*	[79]
Organic solvents	TtgGHI	RND	TtgV	*P. putida*	[80–82]
pH (akaline)					
	MdfA[y]	MFS	?	*E. coli*	[83]
	MdtM[z]	MFS	?	*E. coli*	[67]
Nutrient limitation/growth impairment					
	Stringent response-driven production of NorA/MepA	MFS, MATE	?	*S. aureus*	[84]
Host					
	Rv1258c/Tap[aa]	MFS	?[c]	*Mycobacterium* spp	[85]
	MdtABC, MdtUVW[bb]	RND	BaeSR	*E. amylovora*	[50]
	MexAB-OprM[cc]	RND	?	*P. aeruginosa*	[86]
	MexEF-OprN[dd]	RND	?	*P. aeruginosa*	[87]
	MexXY(-OprM)[dd]	RND	?	*P. aeruginosa*	[87]

(continued)

Table 26.1 (continued)

Stress	Efflux system	Efflux family	Stress-responsive regulator	Organism	References
Drug[l]					
Isoniazid	Rv1258C/Tap	MFS	?	*M. tuberculosis*	[88–90]
Isoniazid	EfpA	MFS	?	*M. tuberculosis*	[36, 88]
Isoniazid	Mmr	SMR	?	*M. tuberculosis*	[88, 91]
Isoniazid	P55	MFS	?	*M. tuberculosis*	[88]
Isoniazid	Rv2459/JefA	MFS	?	*M. tuberculosis*	[88]
Rifampin	Rv1258c (Tap)	MFS	?	*M. tuberculosis*	[90]
Multiple drugs[ee]	P55	MFS	?	*M. bovis*, *M. tuberculosis*	[38, 92]
Multiple drugs[ff]	Rv1258c (Tap)	MFS	?	*M. tuberculosis*	[93]
Multiple drugs[gg]	Rv1473	ABC	?	*M. tuberculosis*	[93]
Aminoglycosides/chloramphenicol	EfrAB	ABC	?	*E. faecium, E. faecalis*	[94]
Ciprofloxacin	MdtABC	RND	BaeSR	*S.* Typhimurium	[95]
Monochloramine[hh]	MdtB	RND	?	*E. coli*	[96]
Fluoroquinolones/mitomycin C[ii]	PatAB	ABC	?	*S. pneumoniae*	[97]
Ribosome-targetting antimicrobials	MexXY	RND	MexR, ArmZ	*P. aeruginosa*	[24, 98, 99]
Azithromycin	MexCD-OprJ	RND	?	*P. aeruginosa*	[100]
Chloramphenicol	MexEF-OprN	RND	MexT	*P. aeruginosa*	[39]
Formaldehyde	MexEF-OprN	RND	?	*P. putida*	[101]
Heavy metals (*Zn*)	MdtABC, AcrD	RND		*E. coli*	[102]
	MdtABC, AcrD	RND	BaeSR	*S.* Typhimurium	[49]

[a]SoxRS-dependent oxidative stress-induction of *acrAB* was not assessed. SoxS-dependent upregulation of *acrAB* yielding a multidrug resistance phenotype was observed in an *in vitro*-selected fluoroquinolone-resistant mutant harbouring a *soxR* mutation that resulted in increased *soxS* expression

[b]An *oxyR* null mutant showed increases susceptibility to oxidative and nitrosative stress as well as various antibiotics concomitant with reduced expression of *acrB*

[c]*cmeABC* is peroxide-inducible, although the impact of peroxide on antimicrobial resistance in *C. jejuni* has not been assessed

[d]CosR negatively regulates expression of *cmeABC* and several oxidative stress response genes, and a *cosR* knockdown strain shows increased resistance to oxidative stress, suggesting that CosR is an oxidative stress response regulatory protein that is responsible for the oxidative stress inducibility of *cmeABC*
[e]Regulatory protein/system responsible has not been identified
[f]Isonizid exposure triggers a signature stress response in *M. tuberculosis* that includes oxidative stress response and efflux (*efpA*) genes [36]
[g]AbuO is a TolC homologue whose inactivation increases susceptibility to multiple antibiotics and disinfectants as well as to peroxide, suggestive of a role in protection against oxidative stress as well as antimicrobial efflux and resistance
[h]A P55 mutant of *M. bovis* showed enhanced susceptibility to the SO-generating agent menadione, suggesting a protective role against oxidative stress, although oxidative stress inducibility of this efflux system was not assessed
[i]A *mexF* deletion mutant showed increased susceptibility to the disulphide stress elicitor, diamide, and *mexEF-oprN* expression was induced by exposure to diamide, consistent with this efflux system playing a role in disulphide stress resistance and, so, maintaining cellular redox balance
[j]MdtEF expels cytotoxic indole nitrosative compounds that accumulate during anaerobic respiration of nitrate in *E. coli*, thus protecting the organism from nitrosative stress
[k]Where known, the membrane-perturbing envelope stressor(s) that activate the indicated regulators and/or upregulate the indicated efflux systems are identified
[l]*CAP*, cationic antimicrobial peptides; *CHX*, chlorhexidine; *QACs*, quaternary ammonium compounds; *TX-100*, triton X-100; *INH*, isoniazid; *RIF*, rifampicin; *AGs*, aminoglycosides; *CAM*, chloramphenicol; *CIP*, ciprofloxacin; *FQs*, fluoroquinolones; *AZI*, azithromycin
[m]*mexCD-oprJ* induced by naturally-occurring cationic antimicrobial peptide LL-37
[n]Deletion of *macABCsm* rendered cells more sensitive to membrane-damaging detergents (SDS, TX-100) and polymyxin B, suggestive of a link to envelope stress. The impact of these (or any membrane stressor) on *macABCsm* expression was, however, not assessed
[o]While it is assumed that the CpxRA TCS of these organisms responds to the same envelope-perturbing signals as *E. coli* CpxRA this has not been confirmed
[p]CpxRA of *V. cholerae* does not respond to the classical envelope stress signals seen in *E. coli* but seems, instead, to respond to low iron and aberrant disulphide bond formation
[q]*acrAB* is also bile salt-inducible in enterohemorrhagic *E. coli* O157:H7 [103]
[r]Several *bme* RND type efflux gene were upregulated by bile, concomitant with increased multidrug resistance, though no specific link between any efflux system and bile or multidrug resistance was examined
[s]Deletion of o*xyR* rendered *K. pneumoniae* sensitive to bile and hyperosmotic conditions, consistent with this regulator playing a role in an envelope stress response. The *oxyR* mutant also rendered the organism multidrug-susceptible, concomitant with a decrease in *acrB* expression
[t]Purified MdtM bound bile salts and promoted bile export in inverted vesicles, and mutational loss of the *mdtM* rendered *E. coli* more sensitive to bile, indications that this multidrug efflux protein contributes to bile resistance. No evidence has been presented, however, that bile/membrane stress regulates *mdtM* expression
[u]A variety of benzene-containing so-called ‘active compounds’, including chlorhexidine, were shown to promote multidrug resistance and upregulate a variety of *bme* RND-type efflux genes, though no link between any efflux system and resistance was demonstrated

(continued)

Table 26.1 (continued)

[v]A transciptomic study of colistin-exposed *A. baumannii* showed increased *baeSR* expression, suggestive of membrane/envelope damage, concomitant with an increase in *macAB-tolC*, *mexB* and *adeIJK* expression

[w]Tea tree oil is membrane permeabilizing in Gram-positive and Gram-negative bacteria [104]

[x]Thiorizadine, an anti-tuberculosis adjuvant agent, induces the envelope stress-responsive SigB regulon and the *emrE*/Rv3065 multidrug efflux gene. Whether the efflux gene was part of the SigB regulon was not assessed

[y]An *mdfA* deletion strain is sensitive to even mildly alkaline conditions and the cloned gene confers extreme alkaline pH resistance. Induction by alkaline pH was not examined

[z]An *mdtM* deletion strain is sensitive to alkaline pH, although there is no indication that it is induced in response to alkaline pH

[aa]Mycobacterial persisters are selected in zebra fish larvae and in cultured macrophages, dependent on multidrug efflux systems. Rifampin-resistant persisters require Rv1258c, which is known to be induced upon macrophage infection [93, 105]. The *in vivo* inducing/selecting signal for RV1258c in macrophages is unknown

[bb]The genes encoding these efflux systems were induced during *E. amylovora* infection of apple rootstock

[cc]The *mexAB-oprM* genes were upregulated during *P. aeruginosa* growth in tobacco plants

[dd]The genes encoding the efflux systems were induced during cultivation on primary human airway epithelial cells

[ee]P55 gene is induced by chlorpromazine, novobocin, triclosan, clofazimine and thiorizadine

[ff]Rv1258c is induced by the ribosome-targetting agents tetracycline, streptomycin and erythromycin

[gg]Rv1473 is induced by the ribosome-targetting agents tetracycline, streptomycin and erythromycin. Its homologue of *M. smegmatis* is induced during oxygen starvation-induced stationary phase along with various stress response genes [106]

[hh]Monochloramine, a disinfectant used in drinking water, induces a variety of stress response genes in *E. coli*, including those related to oxidative stress, DNA repair and cell wall repair. The disinfectant also induces the *yegN* (now called *mdtB*) gene of the *mdtABCD* multidrug efflux operon, suggesting that this efflux locus might be stress-responsive

[ii]Fluoroquinolones and mitomycin C are DNA-damaging agents, suggesting that DNA damage may be the trigger for *patAB* induction and that PatAB plays a role in a DNA damage stress response

26.2.1 Redox-Cycling Agent-Responsive acrAB

The *acrAB* genes of the AcrAB-TolC multidrug efflux system of *Escherichia coli* is inducible by the SO-generating [116] redox-cycling agent paraquat [117], dependent on the SoxS component of the SoxRS two-component regulatory system (TCS) [116, 118]. While this was originally interpreted as SoxRS being an SO stress-responsive TCS (and AcrAB-TolC being a component, therefore, of an oxidative stress response system), SoxRS is now known to respond directly to redox-cycling agents [19]. As univalent oxidants, these agents can, however, directly oxidize SoxR (to activate the SoxRS regulon) although their major toxic activities may well have to do with depletion of cellular NADPH and, so, interference with NADPH-requiring biosynthetic processes (i.e., SoxRS may be responding to a form of metabolic stress) [19]. SoxS-dependent paraquat induction of *acrAB* has also been reported in *Salmonella enterica* serovar Typhimurium [20, 119] and *Enterobacter cloacae* [22], in the latter promoting a multidrug-resistant phenotype. Not surprisingly, mutations leading to constitutive *soxS* expression and elevated *acrAB* expression and antimicrobial resistance have been described in laboratory and/or clinical isolates of *E. coli* [120–124] and *S.* Typhimurium [125], often as a result of mutations in *soxR* [122, 123]. *acrAB* expression and multidrug resistance owing to constitutive *soxS* expression has also been reported in *S. enterica* serovar Enteritidis [125], *S. enterica* serovar Virchow [126], and *Klebsiella pneumoniae* [21], an indication that AcrAB-TolC functions widely as a redox-cycling agent-responsive efflux system in the *Enterobacteriacae*.

An oxidative stress (i.e., hydrogen peroxide)-responsive regulator, OxyR [127], whose loss results in increased susceptibility to peroxide in *K. pneumoniae* [42, 128], has been linked to multidrug efflux (*acrB*) gene expression in this organism – *acrB* expression is reduced in an *oxyR* deletion mutant [42]. Thus, peroxide-generated oxidative stress may promote OxyR-mediated expression of a multidrug efflux system in *K. pneumoniae*. Methyl viogen has also been shown to promote the formation of persister cells in *E. coli*, dependent on the AcrAB-TolC efflux system. Preincubation of *E. coli* with this redox-cycling agent dramatically increased the number of persisters surviving challenge with a fluoroquinolone though not in a mutant lacking AcrAB. Presumably, methyl viogen-promoted production of AcrAB(-TolC) limits fluoroquinolone accumulation in *E. coli*, yielding a larger fraction of fluoroquinolone-tolerant persisters [35].

26.2.2 Oxidative Stress-Responsive Regulators and Efflux Loci

Like the SoxR regulator of the *acrAB* multidrug efflux operon in *E. coli* [19], the MexR regulator of the *mexAB-oprM* multidrug efflux operon in *Pseudomonas aeruginosa* [25] and the global regulators MgrA [31] and SarZ [32] that control expression of the *norA* (MgrA), *norB*, and *tet38* (MgrA and SarZ) antimicrobial efflux

genes in *Staphylococcus aureus* are sensitive to oxidation, with oxidation promoting derepression of their target genes. Oxidative stress might, thus, be expected to enhance efflux gene expression and, so, promote antimicrobial resistance in these organisms, although this has yet to be tested. Oxidative stress (i.e., peroxide) induction of drug efflux genes has, however, been demonstrated in the case of the *cmeABC* and *mexXY* multidrug efflux operons of *Campylobacter jejuni* [27] and *P. aeruginosa* [23], respectively, as well as the macrolide-specific *macAB* efflux system in *S*. Typhimurium [33]. The MacAB efflux system was also shown to be essential for survival in the presence of peroxide – a deletion mutant was unable to grow *in vitro* in the presence of exogenous peroxide and could only survive in ROS-deficient but not wild-type macrophages [33]. A recent study suggests that the oxidative stress regulation of *cmeABC* in *C. jejuni* may be mediated by CosR, an essential regulatory protein in this organism whose repression with antisense technology yields an increase in expression of oxidative stress response genes [129] and *cmeABC* [28]. CosR binding to the promoter regions of oxidative stress response genes [28, 129] and *cmeABC* [28] has been confirmed, an indication that CosR is a direct negative regulator of these genes. Peroxide induction of *mexXY* is mediated by the *armZ* gene product [23] that is also induced by this ROS [130] and functions as an antirepressor of the *mexXY* repressor MexZ [24]. Significantly, *in vitro* exposure of *P. aeruginosa* to peroxide has been shown to enhance the frequency with which MexXY-dependent aminoglycoside-resistant mutants could be recovered [23], a prime example of environmental (i.e., oxidative) stress driving efflux-mediated antimicrobial resistance.

Similarly, serial peroxide exposure of *Burkholderia vietnamiensis* promotes a 16-fold increase in stable resistance to the aminoglycoside tobramycin that is associated with reduced aminoglycoside accumulation [131], suggestive of involvement of an efflux mechanism. Still, although a *mexY* homologue, *amrB*, has been identified in this organism and its overexpression is linked to aminoglycoside resistance [131], peroxide-selected aminoglycoside-resistant strains of *B. vietnamiensis* do not overexpress *amrB* [131]. Thus, while peroxide-mediated oxidative stress promotes aminoglycoside resistance in *B. vietnamiensis*, it does not appear to involve this efflux mechanism.

26.2.3 Other Stress Response-Related Efflux Systems

The SmeYZ drug efflux system implicated in aminoglycoside [132–134] and trimethoprim/sulfamethoxazole [132] resistance in *Stenotrophomonas maltophilia* has also been linked to oxidative stress – a *smeYZ* null mutant showed increased susceptibility to peroxide and the SO-generating agent menadione [132]. Still, oxidative stress induction of the efflux genes and the impact of this on antimicrobial resistance were not assessed. Similarly, a mutant derivative of *Acinetobacter baumannii* lacking the *tolC*-like gene, *abuO*, was sensitive to peroxide and multiple antimicrobials and showed increased accumulation of a model efflux substrate, ethidium

bromide [37], an indication that it functions as part of a multidrug efflux system that also plays a role in resistance to oxidative stress. What the other components of this efflux system are and whether the system is oxidative stress-responsive is unknown. The MacABC efflux system of *S. maltophilia* contributes to intrinsic resistance to aminoglycosides, macrolides, and polymyxins, and its elimination correlates with modest growth defects in the presence of menadione and peroxide [34], suggesting a link to oxidative stress. Again, however, there is no indication that this multidrug efflux system is responsive to oxidative stress.

A mutant of *Mycobacterium bovis* lacking the P55 multidrug transporter [38, 135] showed enhanced susceptibility to menadione [38], suggesting a protective role for this efflux system against oxidative stress, but, again, there is no evidence that it is responsive to SO or any other ROS. Finally, exposure of *Mycobacterium tuberculosis* to isoniazid triggers a signature stress response that includes expression of oxidative stress response genes [136], as well as the fluoroquinolone resistance-associated [136] efflux gene, *efpA* [36]. While this suggests that one effect of isoniazid on *M. tuberculosis* is oxidative damage, whether this is the inducing signal for *efpA* is unknown.

26.3 Nitrosative Stress-Linked Drug Efflux Systems

Nitric oxide (NO), a product of bacterial denitrification and mammalian immune cells, is toxic for bacterial cells and produces a variety of reactive derivatives (so-called reactive nitrogen species) that are also bacterial cell-damaging [137]. These nitrosative stress agents typically elicit protective responses in bacteria, and in some cases these impact antimicrobial resistance. The *mexEF-oprN* efflux operon of *P. aeruginosa* is, for example, inducible by the NO-generating agents *S*-nitrosoglutathione (GNSO) and diethylaminetriamine NONOate (DETA) [39], dependent on the MexT regulator shown earlier to be required for *mexEF-oprN* expression and multidrug resistance in *mexS* mutants [138]. Moreover, several of the MexT targets identified in an array study of MexT-dependent genes (i.e., the MexT regulon) [139] were also shown to be induced in response to nitrosative stress [39], suggesting that MexT controls the expression of a regulon with some function in a nitrosative stress response. MexT and MexEF-OporN have also been linked to what has been coined "disulfide stress," responding to and protecting *P. aeruginosa* from stress-induced redox imbalances in the cell [140].

The *mdtABCD* efflux operon that encodes an RND family system (MdtABC) and a predicted MFS family exporter (MdtD) in *S.* Typhimurium is also induced by NO [141]. This NO inducibility is dependent on the BaeSR TCS [49] that has previously been shown to regulate *mdtABCD* expression in response to certain membrane-damaging stressors [142]. An *mdtD* deletion strain showed increased NO susceptibility, an indication that this MFS exporter plays a protective role against nitrosative stress [141]. Interestingly, *mdtD* also protected *S.* Typhimurium against peroxide, even though this ROS did not induce *mdtABCD*, and its expression enhanced

survival of this organism in the presence of ciprofloxacin and ampicillin [141]. The antimicrobial protective effect of MdtD (renamed IceT) was shown to result from its ability to export iron citrate from the cell and in so doing slows metabolism and growth, with slow growth ultimately promoting drug tolerance [141]. The products of the *mdtEF* multidrug efflux operon [143] of *E. coli* are also linked to nitrosative stress – it protects the organism from indole nitrosative derivatives, a class of toxic nitrosative stress by-products formed when *E. coli* respires nitrate under anaerobic conditions [40]. *mdtEF* is induced by anaerobiosis [40], mediated by the ArcA regulator of the ArcBA TCS [41], where it also promotes resistance to antimicrobials (e.g., erythromycin) [40].

26.4 Envelope Stress-Linked Drug Efflux Systems

By far the most common stress-inducible drug efflux systems are those responsive to membrane/cell wall damage (Table 26.1). Particularly prevalent in Gram-negative bacteria and generally of the RND family, these systems respond to a myriad of membrane-perturbing stressors, including misfolded and aberrant polypeptides, bile, biocides, antibiotics, fatty acids, detergents, and organic solvents. In several cases, the envelope stress induction of drug efflux systems is mediated by well-characterized envelope stress-responsive regulators.

26.4.1 Drug Efflux Systems Regulated by Envelope Stress-Responsive Regulators

A particularly strong indication of drug efflux systems as components of envelope stress responses comes from observations of their regulation by known envelope stress-responsive TCSs and sigma factors. Presumably these efflux systems play some role in ameliorating membrane damage or facilitating adaptation to membrane stress.

BaeSR This is an envelope stress-responsive TCS present in a variety of Gram-negative bacteria (e.g., *E. coli* [47], *S.* Typhimurium [49], *Erwinia amylovora* [50], and *A. baumannii* [51]) where it responds to membrane perturbation caused by compounds such as Na tungstate [47, 49, 51], plant flavonoids [47], tannins [50], and high osmolarity [51]. In *E. coli*, BaeSR promotes resistance to novobiocin [144, 145] and β-lactams [146] via BaeR-dependent upregulation of the MdtABCD [144] and AcrD [146] multidrug efflux systems. In *S.* Typhimurium, the BaeSR system is also linked to resistance to a variety of antimicrobials and, as in *E. coli* resistance to novobiocin and β-lactams, is dependent on the BaeSR-regulated *mdtABCD* and *acrD* efflux genes [49]. The *mdtABC* efflux operon of *E. amylovora* is also positively regulated by BaeSR, in response to Na tungstate and

plant tannins that also induce a second multidrug efflux operon, *mdtUVW* [50], although a link between BaeSR and antimicrobial resistance has not been established in this organism. Finally, the BaeSR TCS of *A. baumannii* promotes resistance to the glycylcycline, tigecycline, through its positive regulation of the efflux pump genes *adeAB* [51].

CpxRA The CpxRA TCS of *E. coli* and several other Gram-negative enteric bacteria responds to conditions predicted to generate misfolded envelope proteins that adversely impact the cell envelope [147]. Activation of the Cpx response promotes resistance to aminoglycosides, several β-lactams, and novobiocin in *E. coli*, and this is, in part, dependent upon Cpx-mediated upregulation of genes encoding the MdtABCD and AcrD systems [48]. In *Vibrio cholerae*, mutational activation of CpxA promotes resistance to ampicillin, and this appears to be mediated by the RND-type VexGH and VexAB systems, both of which contribute to ampicillin resistance and whose expression is positively influenced by CpxRA [54]. CpxRA of *K. pneumoniae* contributes to intrinsic resistance to β-lactams, chloramphenicol, and the biocide, chlorhexidine, with loss of *cpxRA* compromising resistance to these agents [52]. Resistance may, in part, be dependent upon the multidrug efflux systems AcrB, AcrD, and KpnEF, whose genes are positively regulated by CpxRA [52, 53].

AmgRS The AmgRS TCS of *P. aeruginosa* is activated by aminoglycoside exposure and contributes to intrinsic aminoglycoside resistance in this organism [148, 149]. Although AmgRS shares significant sequence homology to the *E. coli* osmoregulatory OmpR-EnvZ TCS, microarray analysis has revealed that AmgRS regulates genes more reminiscent of the *E. coli* CpxRA envelope stress response regulon [149]. AmgRS appears to respond to and protect *P. aeruginosa* from the membrane-perturbing effects of aminoglycoside-generated aberrant polypeptides [43, 148, 149] and has recently been shown to drive expression of the aminoglycoside resistance-promoting [150] *mexXY* multidrug efflux operon in response to aminoglycoside exposure [43]. MexXY recruitment in response to aminoglycosides is dependent on AmgRS-regulated protease/protease cofactors [43], an indication that this efflux system may play a role in the turnover of aminoglycoside-generated membrane-damaging aberrant polypeptides.

BraRS Initially identified in three separate studies, BraRS [56] (also known as NsaRS [151] or BceRS [57]) of *S. aureus* is induced by a variety of cell wall/envelope active antimicrobials [56, 151, 152] but confers resistance to only a limited number of its inducing agents (e.g., bacitracin and lantibiotics such as nicin) [56, 57]. Resistance to these agents is mediated by the BraRS-regulated ABC transporter, VraDE [56, 57, 153], whose expression has been shown to be promoted by bacitracin [56, 57] and nisin [56]. At present, however, it is unknown whether VraDE functions as an exporter or importer in promoting resistance to bacitracin and nisin. A *braS* point mutation has been reported in a nisin-resistant *S. aureus* strain, and introduction of this mutation into a wild-type strain promoted resistance

to this agent [154] an indication that mutational activation of this TCS can promote antimicrobial resistance. *vraDE* is also induced by membrane-active human [76] and mammalian [155] cationic antimicrobial peptides (CAPs) of innate immunity, mediated, in part, by the CAP-responsive Aps regulatory system [76] (also known as GraRS [156]).

ParRS A CAP-responsive TCS that plays a role in adaptive resistance to cell envelope-perturbing antimicrobial peptides, ParRS [157] promotes resistance to aminoglycosides, β-lactams, and fluoroquinolones [74], as a result of ParRS-mediated CAP induction of the *mexXY* multidrug efflux operon [74, 158]. Drug-resistant clinical isolates of *P. aeruginosa* expressing *mexXY* and harboring mutations in *parR* or *parS* have been described [74, 158], with the mutations confirmed as contributing to *mexXY* expression and antibiotic resistance [158], an indication that activation of this TCS can promote efflux-mediated antimicrobial resistance.

RpoE RpoE (also known as σ^E) responds to a variety of membrane-altering stresses [159] but appears to respond ultimately to misfolded outer membrane proteins [160]. *P. aeruginosa* harbors a functionally equivalent homologue of the *E. coli* RpoE, known as AlgU [161], which was first described as a regulator of alginate biosynthesis in *P. aeruginosa*; AlgU has been shown to mediate the induction of the *mexCD-oprJ* multidrug efflux operon in response to a variety of membrane-damaging agents [44]. The recent demonstration that a mutant hyperexpressing MexCD-OprJ shows increased levels of extracellular fatty acids [162] suggests that fatty acids may be an intended substrate for this efflux system. Possibly, MexCD-OprJ-mediated export of membrane fatty acids is a necessary part of a membrane remodeling process that renders membranes tolerant of envelope stress. The *smeIJK* multidrug efflux operon of *S. maltophilia* is also positively regulated by RpoE in response to a number of membrane-damaging agents [46]. Moreover, loss of SmeIJK efflux system increases the susceptibility of *S. maltophilia* to membrane-damaging agents and activates RpoE [46], an indication that it plays a primary role in protecting cells from envelope stress.

26.4.2 Bile-Inducible Drug Efflux Systems

Bile salts are lipid-solubilizing detergent-like molecules that can perturb bacterial membranes and elicit protective changes to bacterial cell envelopes [163]. Key among these changes, at least in enteric bacteria that are routinely exposed to bile salts in the gut, is expression of bile-exporting efflux mechanisms (e.g., [164]). Significantly, these tend to be multidrug efflux systems that accommodate a range of antimicrobials, including clinically relevant agents. Bile-inducible multidrug efflux systems have been reported in *E. coli* [58] including enterohemorrhagic *E. coli* [103], *S.* Typhimurium [20, 59, 60], *V. cholerae* [61–63], *C. jejuni* [64],

K. pneumoniae [42], *Yersinia enterocolitica* [66], and *Bacteroides fragilis* [65] (Table 26.1). While a primary function of these efflux systems is protection against bile acids [59, 62, 165, 166], their expression can influence antimicrobial resistance [65]. Several bile-responsive regulators of multidrug efflux systems have been identified including Rob (in *E. coli* [58]), RamA (in *S.* Typhimurium [20, 60]), CmeR (in *C. jejuni* [64]), and BreR (in *V. cholerae* [63]). A *cpxRA* knockout mutant of *K. pneumoniae* showed increased susceptibility to bile concomitant with increased susceptibility to several β-lactams and chloramphenicol and reduced expression of the *acrB* and *acrD* efflux genes [52]. This suggests that this envelope stress response TCS may regulate multidrug efflux in response to membrane perturbation although it is unknown at this point if *cpxRA* or the *acr* genes are bile-responsive. Similarly, loss of the OxyR regulator typically associated with bacterial oxidative stress responses renders *K. pneumoniae* sensitive to bile [42, 128], in parallel with a decrease in expression of the *acrB* [42] and *kpnEF* [53] multidrug efflux genes although, again, it is not clear if OxyR or the efflux genes are bile responsive. Finally, the MFS family MdtM multidrug efflux system [167, 168], previously known as YjiO [169], exports bile and contributes to bile resistance in *E. coli* [170] but again has not yet been shown to be bile-membrane stress-regulated.

26.4.3 Membrane-Active Biocide-Inducible Drug Efflux Systems

Chlorhexidine is a membrane-perturbing antiseptic that is active against both Gram-positive and Gram-negative bacteria [171]. It induces the expression of several RND family multidrug efflux systems including MexCD-OprJ in *P. aeruginosa* [44], AdeABC in *A. baumannii* [68], and the RND-4 pump of *Burkholderia cenocepacia* [70] whose link to multidrug resistance has been recently confirmed [71, 172]. These efflux systems also contribute to chlorhexidine resistance in these organisms [44, 70, 173]. An *adeAB* homologue of *B. cenocepacia*, RND-8, is also strongly chlorhexidine inducible [70] although a contribution to multidrug resistance for this presumed efflux system has yet to be demonstrated. Chlorhexidine has also been shown to promote a multidrug resistance phenotype in *B. fragilis*, concomitant with increased expression of a number of RND-type *bme* efflux genes [69], although no evidence was presented that the efflux genes were responsible for resistance. Related membrane-damaging [171] biguanide antiseptics, alexidine and poly(hexamethylenebiguanide)hydrochloride, also induce *mexCD-oprJ* [44] as does cetrimide [44], a membrane-active quaternary ammonium compound antiseptic [171].

Cetrimide and another membrane-active quaternary ammonium compound, benzalkonium chloride [171], are also efflux system inducing – they upregulate the fluoroquinolone resistance-promoting *norA* efflux gene in *S. aureus* [72]. Quaternary ammonium compound antiseptics, including benzalkonium chloride, also induced expression of the *smeIJK* multidrug efflux operon in *S. maltophilia* [46]. Finally, the

membrane-active [174] and fatty acid synthesis inhibitor, triclosan, has been shown to induce expression of the *S. maltophilia smeDEF* multidrug efflux operon and promote antimicrobial tolerance [175]. Still, triclosan was shown to be a direct effector of the SmeT regulator of *smeDEF* expression [175], an indication that SmeT/SmeDEF was not responding to envelope stress per se.

26.4.4 Fatty Acid-Inducible Efflux Systems

Antibacterial fatty acids are membrane-damaging lipidic species [176, 177] that have been shown to positively impact expression of drug efflux genes in *S. aureus* [73, 178] and *E. coli* [58]. In *S. aureus*, these fatty acids induce the *tet38* [73, 178] and *norB* [73] determinants of tetracycline and fluoroquinolone resistance [179], respectively, as well as the *mdeA* multidrug efflux gene [178, 180] and the putative drug efflux gene *mmpL* [178], which has recently been linked to resistance to the oxadiazoles, a new class of membrane-active antimicrobials [181]. Antibacterial fatty acids are common in abscesses where *S. aureus* is found [182], and expression of both *tet38* and *norB* is increased in an experimental skin abscess model of *S. aureus* [183], suggesting that these efflux systems contribute to *S. aureus* survival within abscesses. Indeed, NorB has been shown to contribute to fitness/survival in experimental abscesses [183], and Tet38 has been shown to export antibacterial fatty acids and to protect the organism from their lethal action [73]. As such, *in vivo* selection of these drug efflux systems may impact antimicrobial resistance. These fatty acids also induce *acrAB* expression and promote a multidrug resistance phenotype in *E. coli* [58].

26.4.5 CAP-Inducible Efflux Systems

CAPs include therapeutic agents such as the polymyxin antibiotics and components of innate immunity [184]. Several of these, including the polymyxins, are membrane-damaging [185–188] and capable of inducing expression of drug efflux genes in bacteria. The *mexXY* multidrug efflux operon of *P. aeruginosa*, for example, is inducible by polymyxin B, colistin (polymyxin E), and indolicidin, a bovine neutrophil CAP, mediated by the ParRS TCS [74]. Various CAPs, including the human host defense peptide LL-37 [45] as well as polymyxin B and melittin [44], also induce expression of the *mexCD-oprJ* efflux operon of the same organism, concomitant with an increase in fluoroquinolone and aminoglycoside resistance [45]. Still, a link between MexCD-OprJ and resistance was not examined, and while this efflux system is a known determinant of fluoroquinolone resistance, it is not known to promote aminoglycoside resistance [189]. A transciptomic study of colistin-exposed *A. baumannii* showed increased *baeSR* expression, suggestive of membrane/envelope damage, in parallel with an increase in expression of the *macAB-tolC*,

mexB, and *adeIJK* drug efflux genes (one or more of which may be regulated by BaeSR) [75]. The bacitracin resistance-promoting *vraDE* genes of *V. cholerae* are also induced by membrane-active human [76] and mammalian [155] CAPs, mediated, in part, by the CAP-responsive Aps regulatory system [76] (a.k.a. GraRS [156]).

26.4.6 *Efflux Systems Inducible by Other Membrane-Active Compounds*

Triton X-100, a membrane-solubilizing nonionic detergent [190], induces expression of the *mtrCDE* multidrug efflux genes [191, 192] in *Neisseria gonorrhoeae* [77] and the *smeIJK* multidrug efflux operon in *S. maltophilia* [46]. Tea tree oil also possesses membrane-perturbing activity [104] and has recently been shown to induce the aforementioned oxadiazole resistance-promoting *S. aureus mmp*L efflux gene [78]. A new antituberculosis agent, thiorizadine, induces the SigB envelope stress regulon in *M. tuberculosis*, an indication that it is membrane damaging, together with the *emrE*/*mmr*/Rv3065 multidrug efflux gene [79]. It is, however, unclear whether *emrE* is regulated by SigB and, thus, envelope stress. Organic solvents (aromatic hydrocarbons), toxic agents that disrupt biological, including bacterial, membranes [193, 194], have also been shown to induce expression of the plasmid-borne [195] *ttgGHI* multidrug efflux operon [80, 81] that promotes solvent tolerance and modest antibiotic resistance in *Pseudomonas putida* [80]. Solvent inducibility of the efflux operon is mediated by the repressor, TtgV [82], which binds a variety of inducing solvents [81], consistent with TtgV/TTgGHI responding specifically to these solvents. As such, it is not clear that membrane perturbation per se is an inducing signal or that the efflux system is envelope stress-responsive. Consistent with MexCD-OprJ being a component of an AlgU/RpoE-regulated envelope stress response in *P. aeruginosa*, the efflux operon was induced by a wide range of membrane-perturbing agents in addition to membrane-damaging biocides and CAPS, including a detergent (sodium dodecyl sulfate) and several solvents (hexane, xylene, and ethanol) [44].

26.5 pH-Linked Drug Efflux Systems

Two *E. coli* multidrug efflux systems, MdfA [196] and MdtM [197], contribute to alkalotolerance, their loss in mutant strains rendering the organism more sensitive to even mildly alkaline pH [67, 83]. Expression of *mdfA* from a plasmid also enhanced alkalotolerance, enabling *E. coli* to grow at an external pH of 10 [83]. While this suggests that these efflux systems may be recruited by environmental alkaline stress in order to help maintain stable internal pH, this has not been studied.

26.6 Stringent Response-Linked Drug Efflux Systems

A classic example of nutritional stress is amino acid deprivation, which activates what is known as the stringent response [198]. Associated with increased production of the alarmones guanosine 5′-(tri)diphosphate, 3′-diphosphate [(p)ppGpp] [196], the stringent response is characterized by reduced expression of genes typically associated with growth and increased expression of survival genes that economize the use of scarce nutrients [197]. Activated by a variety of nutritional stresses [depletion of Fe, phosphate (Pi), carbon source or fatty acids] [197], the stringent response-mediated increase in ppGpp has a myriad of effects on bacterial cell physiology and, perhaps not surprisingly, impacts antimicrobial susceptibility [199]. Activation of the stringent response via amino acid starvation promotes increased expression of the fluoroquinolone resistance efflux genes *norA* and *mepA* and a corresponding increase in ciprofloxacin resistance in *S. aureus* [84], although the nature of the link between the stringent response and efflux gene expression or whether the efflux genes were responsible for the ciprofloxacin resistance was not assessed.

26.7 Host-Linked Drug Efflux Systems

Some drug efflux systems are inducible *in vivo* upon infection of a host, in response to some host signals/stressors. The *mdtABC* and *mdtUVW* efflux operons of the plant pathogen *E. amylovora*, for example, are induced *in planta*, apparently in response to antimicrobial plant tannins [50]. Similarly, the *mexAB-oprM* efflux operon of *P. aeruginosa* was induced during growth in tobacco plants [86]. The inducing signal(s) was not assessed in this study although subsequent studies by other authors showed that the efflux genes were induced by chlorinated phenols [200, 201], suggesting that plant phenolic compounds may well be natural inducers and efflux substrates. The *mexXY* and *mexEF-oprN* multidrug efflux operons have been shown to be induced during cultivation of *P. aeruginosa* on primary human airway epithelial cells [87], an indication that some epithelial cell constituent drives efflux gene expression in this organism, though this was not defined. Finally, *M. tuberculosis* infection of macrophages has been shown to promote tolerance to several antituberculosis drugs, including rifampicin and isoniazid, in this organism, as a result of upregulation of efflux mechanisms of resistance by the macrophage environment [85, 202]. The macrophage-specific effector(s) that is promoting efflux gene expression in *M. tuberculosis* has yet to be undefined. In most instances, too, the efflux mechanisms responsible for drug tolerance are not known although macrophage-inducible tolerance to rifampicin has been attributed to the Rv1258c (Tap) drug efflux system [85].

26.8 Antimicrobial Stress-Linked Drug Efflux Systems

Antimicrobial induction of resistance mechanisms, including efflux, is well known [108, 109], and while in some instances it is clear what antimicrobial-generated stress the resistance mechanisms are responding to (e.g., cell wall-/envelope-targeting agents; see Table 26.1), in many cases it is not. Isoniazid is a common antituberculosis drug that has been known to induce drug efflux mechanisms in *M. tuberculosis* and related mycobacteria for some time. Initially, this was evidenced by isoniazid treatment promoting isoniazid resistance that was reversible by efflux inhibitors such as reserpine [203, 204]. More recently, isoniazid has been shown to induce a variety of drug efflux systems in various strains although it is unclear whether their induction by isoniazid has any impact on resistance to this agent (or any other antimicrobial). Isoniazid-inducible drug efflux genes include the fluoroquinolone resistance determinant [136], *efpA* [36, 88, 205]; the low-level aminoglycoside/tetracycline resistance gene [206, 207], Rv1258c (*tap*) [88–90, 205]; the erythromycin resistance-promoting [208] Rv3065 (*mmr*) [88, 89, 91, 205]; the isoniazid resistance-promoting [209, 210] *mmpL7* [88] and Rv2459 (*jefA*) [88, 205]; and the gene for the multidrug transporter, P55 (Rv1410c) [88, 90, 205]. Isoniazid is a prodrug activated by the KatG catalase-peroxidase in *M.* tuberculosis [211], and while it is defined as a cell wall-targeting agent that inhibits mycolic acid synthesis [211], its activation also yields a number of free radicals (e.g., hydroxyl [212] and nitric oxide [213]) that likely also have deleterious effects on the cell. As such, it is unclear what isoniazid-promoted stress(es) is responsible for efflux gene induction in this organism.

A variety of other antimicrobials also induce drug efflux gene expression in *M. tuberculosis*. RNA polymerase-targeting rifampicin drives expression of the Rv1258c (Tap) [90, 205, 214], P55 (Rv1410c) [90, 205], EfpA [205], and JefA [205] drug efflux systems although, again, it is unclear what the inducing stress is. The iosniazid-inducible Rv1258c (*tap*) is also induced by the ribosome-targeting agents tetracycline, streptomycin, and erythromycin [93], as is the predicted macrolide exporter [215] Rv1473 [106]. Finally, *p55* expression is induced by a variety of agents with differing cellular targets including chlorpromazine (respiration), novobiocin (gyrase), PA-824 (protein and cell wall lipid synthesis) [216], triclosan (fatty acid synthesis), clofazimine (membrane potential), and thioridazine (respiration) [92] making it difficult to ascertain exactly what stress(es) this drug efflux system is responding to.

Antimicrobial induction of drug efflux systems is also seen in some Gram-positive organisms, including enterococci [94] and *Streptococcus pneumoniae* [97, 217]. Aminoglycoside and chloramphenicol induction of the *efrAB* multidrug efflux [218] genes has been seen in a few multidrug-resistant isolates of *Enterococcus faecium* and *Enterococcus faecalis* [94], while induction of the fluoroquinolone [97, 219–222] and tetracycline [223] resistance-promoting *patAB*

efflux genes is induced by fluoroquinolones [97, 217, 224] and the DNA-damaging anticancer agents mitomycin C [97] and distamycin [217]. It has been suggested, therefore, that DNA damage may be a primary inducing signal for this efflux system.

A variety of Gram-negative drug efflux systems are also inducible by antimicrobials, including the *mdtABCD* drug efflux operons of *E. coli* and *S.* Typhimurium. In *E. coli*, *mdtB* (previously *yegN* [144]) is inducible by monochloramine, a disinfectant used in drinking water and shown to activate a variety of stress response genes including those related to oxidative stress, and DNA and cell wall repair [96]. Which of these is linked to efflux gene expression is unknown. The *S.* Typhimurium *mdtA* gene is induced by ciprofloxacin, dependent on the BaeSR TCS that is implicated in envelope and nitrosative stress control of *mdtABCD* expression in this organism [95]. What "stress" is being generated (and, so, responded to) in the case of ciprofloxacin is unclear. A DNA microarray study has revealed that the envelope stress-inducible *mexCD-oprJ* efflux operon of *P. aeruginosa* was also upregulated by azithromycin [100] although the nature of the inducing stress is again unknown. As well, chloramphenicol has been shown to induce expression of the MexEF-OprN multidrug efflux system, dependent on the MexT regulator required for nitrosative stress induction of this efflux operon [39]. The observation that the related compound, florfenicol, which lacks a nitro group, is unable to induce *mexEF-oprN* expression [39] suggested that chloramphenicol resembled a nitrated nitrosative stress product that is an intended signal for MexT and a substrate for MexEF-OprN, further support for this efflux system functioning as part of a nitrosative stress response. Intriguingly, the ROS-inducible *mexXY* operon is also induced by antimicrobials that target the ribosome (e.g., aminoglycosides, chloramphenicol, erythromycin, and tetracycline) [98] in response to ribosome disruption [225]. This is mediated by the ArmZ antirepressor [24] that regulates ROS induction of *mexXY* expression and is also induced by ribosome-perturbing drugs [98]. In reconciling *mexXY* induction by both oxidative stress and ribosome disruption, one possibility is that oxidative stress also disrupts ribosome function or in some way perturbs translation (i.e., ribosome perturbation is the inducing "stress" in both cases). Alternatively, since ribosome disruption via mutation or antibiotic exposure leads to the production of aberrant polypeptides that are prone to oxidative modification in *E. coli* [226], it may be that oxidatively modified/damaged proteins (generated by oxidative stress or drug-mediated ribosome perturbation) are the trigger for *armZ* and *mexXY* induction, with MexXY-OprM possibly playing some role in ridding the cells of these aberrant polypeptides. Finally, the lipid-, protein- and DNA-reactive cytotoxin/biocide, formaldehyde, has been shown to induce the *mexEF-oprN* efflux operon in *P. putida*, with MexEF-OprN contributing to formaldehyde resistance [101]. Although a contribution to antibiotic resistance was not assessed, its homologue in *P. aeruginosa* promotes resistance to several antimicrobials, including fluoroquinolones [227].

26.9 Heavy Metal-Linked Drug Efflux Systems

The *mdtABC* and *acrD* drug efflux genes of both *E. coli* [102] and *S.* Typhimurium [49] are induced by zinc, mediated in *S.* Typhimurium by the BaeSR TCS [49]. It is unclear what the inducing signal is although it is noteworthy that MdtABC and AcrD also contribute to zinc resistance in *S.* Typhimurium [49]. Two recent studies also showed the contribution of CopYAZ copper efflux system and MntE manganese efflux pump, respectively, to oxidative stress response in streptococci [228, 229]. A gold resistance-related efflux system RND-type GesABC efflux system of *S.* Typhimurium [29] (also known as MdsABC [230]) is also involved in oxidative stress response [30].

26.10 Concluding Remarks

A substantial number of bacterial drug efflux systems are stress-inducible, particularly members of the RND family that are prevalent in Gram-negative bacteria (Table 26.1). This speaks to these having as their primary intended function protection against the adverse consequences of environmental stress. Nonetheless, in many cases their roles as components of bacterial stress responses and the identities of the inducing signaling/effector molecules remain a mystery. Significantly, many of the stresses that promote drug efflux gene expression can be found in the host (e.g., ROS-mediated oxidative [231, 232] and reactive nitrogen species-mediated nitrosative [233, 234] stress associated with immune cells; envelope stress associated with exposure to bile, antibacterial fatty acids [235], and CAPs of innate immunity [236]). The prospect of host-associated stresses influencing drug efflux gene expression in bacteria, independent of antimicrobial exposure, has profound implications for resistance development in and antimicrobial chemotherapy of disease-causing bacteria. While there is some evidence of stress-induced efflux expression impacting antimicrobial resistance [22, 65, 175], in most instances of efflux-mediated resistance in clinical isolates efflux gene expression is driven by mutation [108, 237]. Still, as components of stress responses, it is possible and, perhaps, likely that host-associated stresses contribute some pressure for their selection. Indeed, host-selected (so-called adaptive) mutations impacting efflux-mediated antimicrobial resistance in bacteria have been described, particularly in bacteria causing persistent infections. In the case of *P. aeruginosa* infections of the cystic fibrosis lung, for example, the most commonly mutated gene in cystic fibrosis lung isolates is the *mexZ* gene encoding a repressor of the *mexXY* multidrug efflux operon that contributes to aminoglycoside resistance [238, 239]. The implication is that the host environment is selecting for *mexZ* mutants, with efflux gene expression enhancing growth and survival in the cystic fibrosis lung but concomitantly promoting

aminoglycoside resistance. Perhaps not surprisingly, MexXY is the primary determinant of aminoglycoside resistance in cystic fibrosis strains of *P. aeruginosa* [240]. *P. aeruginosa* lung infection is characterized by dysregulated pulmonary inflammation leading to release of ROS and, ultimately, chronic lung oxidative stress [241, 242]. In light of the ROS/oxidative stress inducibility of the *mexXY* operon *in vitro*, it seems reasonable to conclude that the inflamed cystic fibrosis lung is the primary driving force for the selection of MexXY-expressing aminoglycoside-resistant *P. aeruginosa*. Consistent with the cystic fibrosis lung providing a selective pressure for MexXY recruitment, cystic fibrosis sputum has been shown to induce *mexXY* expression [243].

There are other examples of *in vivo* selection/promotion of multidrug efflux gene expression in *P. aeruginosa*. Exposure of *P. aeruginosa* to human lung epithelial cells stimulates expression of the *mexEF-oprM* multidrug efflux operon [87], an indication that this resistance determinant may also be selected *in vivo* during lung infection. Similarly, *mexCD-oprJ*- and *mexEF-oprN*-expressing multidrug-resistant *P. aeruginosa* are enriched in the lungs of experimentally infected rats in the absence of antimicrobial exposure [238], further support for the lung environment promoting efflux-mediated antimicrobial resistance. Host recruitment of resistance is also seen in the mycobacteria, whose persistence in macrophages and in a zebra fish infection model depends on efflux pump(s) implicated in antibiotic efflux and resistance [85]. Inducible by the macrophage intercellular environment, these pumps presumably play a role in intracellular survival while also contributing to the demonstrated multidrug resistance of the persisting organisms [85].

Much remains to be done to elucidate the roles of drug efflux systems as components of bacterial stress responses and, thus, provide a more complete reckoning of these protective responses. This may, in turn, shed light on the often characteristically broad specificity of these efflux systems and their apparently unintended ability to accommodate a broad range of clinically-relevant agents and in so doing inform ongoing strategies aimed at countering their contributions to resistance.

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Chapter 27
Involvement of Antimicrobial Drug Efflux Systems in Bacterial Fitness and Virulence

Natalya Baranova

Abstract Multidrug efflux pumps play an important role in antimicrobial resistance and also serve other functions that are related to bacterial cell communication, stress responses, fitness, and virulence. Although it is challenging to define the natural functions of drug efflux pumps, accumulating evidence has revealed both direct and indirect involvement of multidrug efflux systems in these cellular processes. There is also an intertwined regulation of drug efflux and other cellular systems implying various shared regulators. These features explain diverse effects of multidrug efflux pump status on bacterial functions, including interactions between bacterial species and their hosts. Drug efflux pump contribution to improved fitness and increased virulence of pathogens is supported by numerous examples. This chapter describes the current understanding of the roles of drug efflux pumps (in particular those of Gram-negative bacteria) in bacterial pathogenicity, which further underscores the clinical significance of drug efflux phenomena.

Keywords Multidrug efflux pumps • Biological burden • Biofilm • Fitness • Colonization • Pathogenesis • Quorum sensing • Virulence • RND efflux pumps • AcrAB-TolC

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27.1 Introduction

Multidrug efflux pumps, in particular, those of the resistance-nodulation-cell division (RND) superfamily, play an important role in antimicrobial resistance in bacteria. Accumulating evidence has also revealed both direct and indirect involvement of these efflux pumps in other functions that go beyond drug resistance [1–3]. Because of this, the ability of drug efflux pumps to extrude antimicrobial agents has been merely regarded as "incidental" to other functions [2, 4]. Indeed, multidrug efflux systems are ubiquitously distributed in bacteria [5], and their natural substrates have been demonstrated to include bacterial metabolites, whose accumulation inside bacterial cells can be deleterious [6–8]. Studies have also established the substrate profiles of drug efflux pumps that include non-antibiotic substances such as bile salts, phenolic agents, and cell communication molecules, which are encountered by microbes in their natural habitats [9–11]. Furthermore, multidrug efflux systems are now known to interconnect numerous microbial cellular processes such as drug resistance, cell communication, stress response, fitness, colonization, and virulence [3]. This phenomenon is consistent with the fact that intertwined regulation involving various regulators and pathways is often required for coordinated differential expressions of drug efflux and other functional systems. For example, regulators that control directly or indirectly the expression of multidrug transporters also influence quorum sensing, biofilm formation, stress responses, and virulence factor production [12]. This chapter provides an up-to-date overview of the relationship between the status of drug efflux pumps and features related to bacterial biological burden, cell communication, fitness, colonization, and virulence. Since bacterial pathogenicity is not only species specific, it also depends on the host and environmental niches. Thus, discussions are also made on several relevant cell processes such as resistance to bile salts and antimicrobial peptides as well as responses to nitrosative and oxidative stress. Overall, numerous findings support the contribution of multidrug efflux pumps to improved fitness and increased virulence of pathogens via a variety of direct and indirect mechanisms, thus highlighting the clinical significance of hijacking efflux mechanisms as an important therapeutic intervention strategy.

27.2 Bacterial Metabolic Burden and Fitness Associated with Antimicrobial Resistance and Multidrug Efflux Pumps

Bacterial fitness as expressed by growth rate and competitive ability *in vitro* and *in vivo* in the absence of antibiotics is widely supposed to be compromised by expression of multidrug transporters. The association between multidrug resistance and reduced fitness has fueled the hope that a reduction in the use of antibiotics would lead to a reduction in frequency in resistant bacteria. However, the experience shows that multidrug resistance, once acquired, is often eliminated so slowly

that it is unlikely to be of practical importance. Mathematical modeling shows that the fitness cost of drug resistance has to be quite high in order to result in elimination of resistant bacteria in a reasonable amount of time [13], which is often not the case. Thus, when the loss of plasmid-encoded tetracycline resistance from *Escherichia coli* was measured under antibiotic-free conditions, the observed dynamics was best described by a model that included a high-mutation frequency (~10^{-4} mutations per generation) and a 0.7 % reduction in the growth rate of antibiotic-resistant bacteria [13]. With these parameters, replacement of 99.9 % of the population by susceptible bacteria would take approximately 1.5 years. To reach the same replacement level in 5 weeks, the reduction in the growth rate of the resistant bacteria would need to be ~6 %, about nine times higher, which is not realistic for an induced resistance gene on a low-copy number plasmid [14], and presumably even less so for a chromosome-based resistance which is tightly regulated, as is often the case with multidrug pump-encoding genes [15]. Indeed, it has been experimentally shown that introduction of the plasmid that carries the gene encoding β-lactamase resulted in reduced growth rate in *Salmonella enterica* serovar Typhimurium. However, introduction of the regulator of the β-lactamase gene, which made β-lactamase expression inducible rather than constitutive, eliminated these fitness costs [16].

Even in the case of mutations leading to overexpression of multidrug resistance genes, the experimental data differs as to the presence of universal metabolic burden in multidrug-resistant bacteria. Some works support the notion of a fitness cost associated with overexpression of multidrug efflux genes. Thus, two *Pseudomonas aeruginosa* mutants overexpressing MexAB-OprM and MexCD-OprJ multidrug pumps of the RND superfamily were found to be impaired in their survival in water and on a dry surface, when compared to the wild-type strain [17]. In addition, these mutants exhibited impaired virulence in *Caenorhabditis elegans* model system. However, the caveat to this observation is that these mutants overexpressed the multidrug resistance genes as a consequence of mutations in the genes encoding their respective regulatory proteins, *mexR* and *nfxB*. Thus, the expression of other non-related genes could have been affected. Another example of decreased fitness associated with the expression of multidrug efflux pumps comes from *Stenotrophomonas maltophilia*. The strain overexpressing multidrug efflux pump SmeDEF was displaced by the wild-type strain in coculture experiments [18].

On the other hand, other studies failed to detect any metabolic burden that constitutive expression of multidrug efflux pumps might create. Thus, several *Neisseria gonorrhoeae* mutants overexpressing MtrCDE efflux pump to various degrees were identical to a wild-type strain in their growth and survival in *in vitro* competition assays [19].

At the very least, the fitness cost is habitat dependent. Thus, deleterious effect of the constitutive MexEF-OprN pump overproduction in *P. aeruginosa* was possible to detect only under anaerobic conditions [20]. In aerobiosis, overexpression of this pump does not produce a metabolic burden in competition tests. In this case, it appears that *P. aeruginosa* compensates the fitness cost by adjusting its metabolism. MexEF-OprN is a proton antiporter, and its overexpression should lead to an increase in influx of protons. To prevent acidification of the cytoplasm, the mutant

increases oxygen consumption [20]. Increased oxygen consumption may lead to the drop of environmental oxygen in the culture. This situation is rectified by turning on nitrate respiratory chain genes, which are normally not expressed under aerobic conditions, and increasing nitrate consumption and nitric oxide production. As a result of this chain of events, there is no metabolic burden to *P. aeruginosa* overexpressing MexEF-OprN pump under aerobic conditions. Under anaerobic conditions, excess protons are not consumed in the process of respiring nitrate, unlike in oxygen respiration. Cytoplasmic pH of the mutant strain was found to drop to values close to 6.0, presumably contributing to the deleterious impact of MexEF-OprN overexpression observed under these conditions [20]. Another example is *S. maltophilia* strain with mutations in multidrug efflux pump genes *smeIJK*, which displayed a compromised growth in Mueller-Hinton medium, but not in Luria-Bertani medium [21]. It was determined that the defect was due to the low osmolarity of the Mueller-Hinton medium and to the presence of casein hydrolysate as one of its components.

27.3 Effect of Multidrug Efflux Pumps on Virulence

Studies of bacterial fitness in their ecological niches provide necessarily considerably more complicated and difficult to interpret data than those done under controlled conditions. Free-living bacteria are exposed to a chemically and physically complex environment and engage in constant competition with other bacteria. Pathogens are additionally a target of the whole arsenal of antimicrobial devices that their hosts possess as a result of evolution. Nevertheless, it is the fitness of the bacteria in their ecological niche that is, ultimately, the only relevant characteristic. The data on the role of multidrug efflux pumps in fitness *in vivo* and virulence of pathogenic bacteria is somewhat conflicted, but overall the inclination is to give multidrug efflux pumps credit in increasing bacterial virulence and survival in the host or to emphasize the need for precise regulation, when both overexpression and deletion of a pump prove detrimental. In many studies, virulence was investigated in conjunction with observing the involvement of the pump in a specific characteristic of the cell, such as resistance to bile, antimicrobial peptides, etc. While there is necessarily a logical leap in connecting *in vitro* and *in vivo* results, such data will nevertheless be discussed in corresponding sections of this chapter. Here, we will review several studies that concentrated heavily on *in vivo* experiments.

27.3.1 Positive Role of Multidrug Efflux Pumps in Virulence and Survival

Multiple observations suggest that multidrug efflux pumps are necessary for maximum virulence of bacterial pathogens. Thus, Buckley et al. [22] determined virulence of *S. enterica* serovar Typhimurium strains with deletions of the genes

involved in multidrug efflux. The mutants that lacked *acrB* were significantly less able to adhere, invade, and survive in mouse monocyte macrophages. In the human embryonic intestine cells, disruption of *acrB* had no effect on adherence, but bacteria were unable to invade or survive intracellularly. Disruption of *tolC* abolished the ability to adhere, invade, and survive in both cell types. A competitive index assay was used to determine the ability of mutants to colonize and persist in the avian gastrointestinal tract in competition with the parental strain. Mutants with disrupted *tolC* colonized and persisted in the avian digestive tract poorly, while mutants with disrupted *acrB* were able to colonize, but did not persist gastrointestinally.

The genetic background of *Salmonella* strains seems to play an important role in the outcome of these experiments. Indeed, in another work, in which three multidrug-resistant strains of *S. enterica* serovar Typhimurium isolated from cattle, as opposed to the laboratory strain used in the previous work, were used, the results were somewhat different. *acrB* mutants demonstrated a reduced adhesion ability but no change in invasion when human adenocarcinoma cell line HT-29 was used [23]. *tolC* mutants, similar to the previous report, demonstrated lower adhesion and lower invasion rates [23]. Moreover, the authors showed that the low invasion of these *tolC* mutants results from a downregulation of expression of the type III secretion system 1. This system is encoded on the *Salmonella* pathogenicity island 1 and is involved in bacterial entry into cells of the intestinal epithelium. It allows the translocation of a large set of effector proteins from the bacterial cytoplasm into the cytosol of the host cell. These effector proteins induce local cytoskeleton rearrangements leading to membrane ruffling, micropinocytosis, and finally internalization of *Salmonella* [24].

It is interesting that *acrB* and *tolC* mutants have different effects on virulence. In another study, *tolC* mutants of multidrug-resistant *S. enterica* serovar Typhimurium phage type DT104 and DT204 were 64- to 256-fold more susceptible to bile salts than *acrB* mutants, and in contrast to *acrB* mutants, the *tolC* mutants were unable to colonize the cecum, spleen, and liver after 1 week of infection in a day-old chicken model [25]. This may be the result of TolC involvement in other processes, but it can also reflect the global effect of *acrB* or *tolC* deletion on expression levels of other genes. Indeed, the Piddock group later further elaborated on the role of AcrAB-TolC pump in *Salmonella* virulence. Using transcriptomic comparison, they showed that the disruption of *acrA*, *acrB*, or *tolC* resulted in altered expression of multiple genes involved in pathogenesis [26]. Besides confirming the downregulation of *Salmonella* pathogenicity island-1-encoded type III secretion system genes, the affected genes included chemotaxis and motility genes, as well as the genes involved in anaerobic metabolism. In addition, the authors, prompted by somewhat different results reported by Virlogeux-Payant et al. [23], confirmed the altered gene expression in two other *S. enterica* serovar Typhimurium backgrounds. The mechanism of altering gene expression for *acrAB-tolC* mutants is not known. The profound effect of the deletion of *acrA*, *acrB*, or *tolC* on expression of multiple genes underscores the difficulties in the interpretation of data on the involvement of multidrug efflux pumps into bacterial pathogenicity.

N. gonorrhoeae mutants overexpressing MtrCDE pump were shown to have an advantage over wild-type parent strain in competitive infections in mouse [19]. This advantage paralleled *in vitro* resistance to CRAMP-38, a murine antimicrobial peptide, as well as the degree of MtrCDE overexpression.

The disruption of *acrA* or of *tolC* component of the pump AcrAB-TolC in two clinical isolates of *Enterobacter cloacae* was shown to result in a decrease in virulence in a mouse model of systemic infection [27]. However, in this work, various drug resistance cassettes were used to produce gene knockouts, which might have skewed the observed results.

Legionella pneumophila is a pathogenic organism which survives and replicates as an intracellular parasite within free-living amoeba and initiates pneumonia in humans after inhalation of contaminated aerosols. In *L. pneumophila*, the deletion of *tolC* resulted not only in an increase in susceptibility to various drugs but also in a significant attenuation of virulence toward amoebae and macrophages [28]. However, the role of *tolC* was not further dissected in this work, and it is known to be involved in both expression and function of multiple systems.

The effect of six RND efflux pumps on virulence was studied in *Vibrio cholera* [29]. A strain lacking VexB, VexD, and VexK multidrug efflux pumps was attenuated in the infant mouse model, but the production of virulence factors was reported unaffected. In contrast, a strain with all six RND pumps deleted produced significantly less cholera toxin and fewer toxin-coregulated pili than the wild-type strain and was unable to colonize the infant mouse.

27.3.2 Negative Effect of Multidrug Efflux Pump Overproduction on Virulence

Among the observations of the negative impact of multidrug efflux pumps on virulence is the study in which overexpression of MexEF-OprN multidrug efflux pump due to a constitutive activation of the transcriptional activator MexT impaired virulence of *P. aeruginosa* in a *C. elegans* model [30]. The authors ascribed this effect to the activity of the efflux pump and not of MexT, despite the fact that the latter is involved in the regulation of type III and type VI secretion systems and early surface attachment independently of MexEF-OprN [31]. Overexpression of MexEF-OprN resulted in reduced expression of several quorum-sensing-regulated genes. This phenotype was traced back to a delay in the production of quorum-sensing signaling compound due to the extrusion of its precursor through the efflux pump [30]. However, these results may rather highlight the importance of fine-tuning the expression of multidrug efflux pumps. Indeed, in case of *mexAB-oprM* pump genes, it has been shown that both the deletion of the operon and overexpression of the pump genes result in the reduced capacity to invade or transmigrate across canine kidney epithelial cell monolayer and to kill mice [32]. In fact, *mexAB-oprM* deletion mutant exhibited much more dramatic deficiencies than the mutant overexpressing this operon [32].

27.3.3 Plant Pathogens

The interaction between plants and their pathogens and epiphytic bacteria through chemical means is intense. From the point of view of intellectual curiosity, it is regrettable that the volume of research on drug transporters of plant pathogens and epiphytes is quite low. A few published studies examine the role of multidrug efflux pumps in virulence of plant pathogens.

Plants respond to microbial attack with sophisticated defenses that include the production of antimicrobial peptides and phytoalexins, the latter including mostly terpenoids, glycosteroids, and alkaloids. Epiphytic bacteria produce a variety of antibiotics with which they antagonize phytopathogenic bacteria. Both factors are important in fitness and virulence of the plant pathogens. Several works demonstrated that efflux pumps are essential for plant-bacterium interactions. Efflux pump IfeAB of *Agrobacterium tumefaciens* was shown to be important for alfalfa root colonization in a competition experiment between wild-type strain and *ifeAB* mutant [33]. Expression from the operon promoter was induced by alfalfa isoflavonoids formononetin, medicarpin, and coumestrol. Accumulation of coumestrol in mutant cells was abnormal, consistent with coumestrol being exported by the pump. Mutants of *Rhizobium etli* deficient in multidrug efflux pump genes *rmrA* and *rmrB* had enhanced susceptibility to phytoalexins, flavonoids, and salicylate [34]. *rmrA* was inducible by bean-root-released flavonoids; mutants of *rmrA* formed 40 % less nodules in beans. A *Pseudomonas syringae* strain with mutations in the RND pump PseABC exhibited reduced virulence for immature cherry fruit compared to parental strain [35]. *Erwinia amylovora* requires AcrAB for successful colonization and pathogenesis in apple rootstock [36]. An *acrB*-deficient mutant was susceptible to the apple phytoalexins phloretin, naringenin, quercetin, and (+)-catechin, and the expression of *acrAB* was upregulated by the first two compounds. *Ralstonia solanacearum* mutants in multidrug efflux pumps AcrAB and DinF exhibited reduced ability to grow in the presence of phytoalexins and were significantly less virulent on the tomato plant [37].

27.4 Resistance to Bile Acids

Enteric bacteria found in the gastrointestinal tract need to be able to survive antimicrobial effects of up to 20 mM concentrations of bile salts and their free acids [38, 39]. Bile salts are amphipathic, water-soluble, steroidal surfactants, synthesized in the liver from cholesterol and secreted into the bile. They aid emulsification and enzymatic digestion of dietary lipids in the small intestine [40]. In humans, bile salts are secreted by hepatocytes in a form conjugated to glycine or taurine [40]. After a series of events in which intestinal microflora plays a prominent role, they are transformed to unconjugated biliary bile acids consisting primarily of cholate, chenodeoxycholate, and deoxycholate, present in approximately equal

amounts [41]. While over the physiological pH range conjugated bile salts are fully ionized and require the OmpF porin in order to traverse the outer membrane of Gram-negative bacteria [39], the unconjugated bile salts are weakly acidic molecules and in their uncharged, protonated state can diffuse through both bacterial membranes and accumulate in cytoplasm. Bile salts exert a cytotoxic effect on bacteria by way of disruption of cell membrane integrity, promotion of RNA secondary structure formation, DNA damage, denaturation of cellular proteins, and oxidative stress [42]. While Gram-positive bacteria are particularly sensitive, bile acids can also kill Gram-negative bacteria [42].

Multidrug efflux pumps are well known now to participate in providing resistance to these compounds. Bile sensitivity and reduced capacity to colonize the intestinal tract of mice was reported to be associated with *S. enterica* serovar Typhimurium gene *acrB* as early as 1996 [43]. In 1997, it was shown that AcrA of RND-type AcrAB-TolC multidrug efflux pump and, to a lesser extent, EmrB of the EmrAB-TolC of the major facilitator superfamily (MFS) were involved in active removal of chenodeoxycholate from *E. coli* cells [39]. The role of the individual components of transporters was not addressed in this work, but since then, bile salts and acids have been shown to be substrates for multiple multidrug efflux pumps in various bacteria. Moreover, bile salts were shown to induce transcription of multidrug pump genes in a number of cases. Thus, in *E. coli*, bile salts, primarily unconjugated (deoxycholate and chenodeoxycholate), bind to Rob transcription regulator to induce the transcription of *acrAB* [44]. Other pumps that have been shown to play a role in bile resistance in *E. coli* include a single component MFS pump MdtM. MdtM was shown to be involved in resistance to cholate and deoxycholate [45]. Bile salts did not upregulate *mdtM* transcription, but "housekeeping" levels of MdtM were sufficient to contribute effectively to the resistance [45].

In *S. enterica* serovar Typhimurium, the transcription of the genes encoding the AcrAB-TolC multidrug efflux pump was also found to be upregulated by bile. Two mechanisms, not necessarily mutually exclusive, were proposed to be responsible for *acrAB-tolC* activation. Transcriptional activator RamA regulates the expression of *acrAB-tolC* genes in *Salmonella. ramA* transcription itself is normally repressed by the product of the divergently transcribed *ramR* gene. In one work, the authors found that bile prevented the association of RamR with the *ramA* promoter, thereby upregulating the transcription of *ramA* [46]. In another article, the authors did not see increased expression of *ramA* in response to bile salts, but observed the binding of cholic acid to RamA and proposed that this leads to altered conformation and activity of RamA [47]. It seems that significantly lower concentrations of bile salts were used in the second work. It is possible that both mechanisms are involved, and the concentration of bile salts in the second work was insufficient to induce the transcription of *ramA*. Additionally, the altered activity of RamA bound to cholic acid was not demonstrated. Overexpression of *ramA* from the plasmid was found to induce the transcription of *acrA*, *acrB*, and *tolC* in yet another work [48], so at least when greatly overexpressed, RamA does not need an inducer to activate these genes; however, it is not known how RamA behaves when it is present at physiological levels.

In *Vibrio parahaemolyticus*, which is an enteric Gram-negative bacterium that can cause acute gastroenteritis in humans, RND-type multidrug efflux pumps VmeAB, VmeCD [49], and VmeTUV [50] are involved in resistance to bile acids (cholate and deoxycholate).

In *Campylobacter jejuni*, the leading bacterial cause of food-borne enteritis in humans, inactivation of multidrug efflux pump CmeABC drastically decreases resistance to various bile salts [51]. It was also found that the *cmeABC* mutants failed to colonize chickens, and the minimal infective dose for the *cmeABC* mutant was at least 2.6×10^4-fold higher than that of the wild-type strain. Moreover, bile salts drastically elevated the expression of the *cmeABC* operon via binding to a transcriptional repressor CmeR and reducing its affinity to the *cmeABC* promoter [52, 53]. CmeR is encoded by a gene located immediately upstream of the *cmeABC* operon, and its loss changes the expression of a number of genes [54]. Loss of function mutation of *cmeR* severely reduced the ability of *C. jejuni* to colonize chickens [54]. This is not the result expected as a consequence of derepression of the *cmeABC* operon, but CmeR is a pleiotropic regulator, and other genes whose expression level change in response to the deletion of *cmeR* might be responsible for the observed phenotype.

In *Klebsiella pneumoniae*, the efflux pump KpnEF of the small multidrug resistance (SMR) family has been shown to be involved in resistance to bile [55]. The ability of the Δ*kpnEF* mutant to grow in the presence of bile was compromised, as well as its stress response, as discussed later in this chapter. *Yersinia enterocolitica* multidrug efflux pump genes *acrAB* were found to be induced by bile salts [56].

Listeria monocytogenes is a Gram-positive intracellular pathogen causing significant mortality and morbidity, especially among neonates, elderly, and pregnant women [57]. It is food-borne and encounters bile in the gut during initial infection, in the liver where it replicates robustly, and in the gallbladder, from which it can return to the intestine and thence to the environment. *L. monocytogenes* uses its multidrug efflux pump MdrT to protect itself against cholic acid [58]. Cholic acid disrupts binding of the transcriptional repressor BrtA to the *mdrT* promoter, thereby inducing transcription of *mdrT* [58]. The deletion of *mdrT* significantly sensitized the cells to cholic acid. The authors also determined that in a mouse intravenous infection model, bacterial abundance in the liver four days postinfection was tenfold attenuated for Δ*mdrT* cells and 100-fold attenuated for *ΔmdrTmdrM* cells, while the abundance of the wild-type and *ΔmdrM* cells was similar. It suggests that while dispensable in wild-type cells, *mdrM* becomes essential for virulence in the tested situation in *ΔmdrT* background.

27.5 Response to Nitrosative Stress

The gut is mostly an anaerobic environment. Consequently, facultative anaerobic pathogens such as *E. coli* have to switch to the anaerobic physiology when in the gut environment. Since oxygen is limited, the cell must utilize alternative terminal

electron acceptors, with nitrate being the preferable electron acceptor [59]. The generation of reactive nitrogen species during this physiological process results in nitrosylation and subsequent condensation of indole molecules and, ultimately, in significant accumulation of nitrosyl indole derivatives (e.g., indole red) in anaerobically grown *E. coli* when the bacterium respires nitrate [60]. These compounds are toxic to the cell. It has been shown that an RND efflux pump MdtEF actively removes the nitrosyl-damaged cellular components out of the cell [61]. *mdtEF* expression is activated under anaerobic conditions by two global transcription regulatory factors that mediate the transition from aerobic to anaerobic lifestyle in *E. coli*, anaerobic respiration control (ArcA) and, to a lesser extent, fumarate nitrate reduction (FNR) [61]. Deletion of *mdtEF* resulted in a significantly slower growth and increased sensitivity to indole red under anaerobic conditions.

P. aeruginosa is an opportunistic human pathogen that infects the lungs, urinary tract, bloodstream, and surgical wounds [62]. Individuals with cystic fibrosis are particularly susceptible to chronic lung infections caused by *P. aeruginosa* [63]. *P. aeruginosa* can adapt to existence in various niches and is able to grow either aerobically or anaerobically in the presence of alternative terminal electron acceptors, such as nitrate, nitrite, etc. In the cystic fibrosis lung, *P. aeruginosa* grows as a biofilm in stagnant mucus, which was found to be an anaerobic environment rich in nitrate and nitrite [64]. Thus, *P. aeruginosa* likely needs to respire nitrate at the site of infection. *mexEF-oprN* multidrug transporter operon of *P. aeruginosa* has been shown to respond to nitrosative stress [65]. The operon's transcription was induced by S-nitrosoglutathione (GSNO) or diethylamine triamine NONOate (DETA). Both of these compounds generate NO and produce nitrosative stress. The inducibility of *mexEF-oprN* by nitrosative stress was dependent on MexT, a LysR-family-positive regulator of transcription. The deletion of the *mexEF-oprN* operon, however, did not result in changes in the resistance to GSNO. NO nitrosylates multiple substrates in the cell, one or more of which, rather than GSNO or NO itself, may be substrates of MexEF-OprN pump. Interestingly, chloramphenicol, which is a nitroaromatic compound, was capable of rapidly inducing *mexEF-oprN* expression and was also a substrate of MexEF-OprN pump. A derivative resembling chloramphenicol but lacking the nitro-moiety failed to induce *mexEF-oprN* expression. This suggests that chloramphenicol resembles a nitrosative stress product that is deleterious to the cell and serves as a natural substrate for MexEF-OprN pump. A possible link between MexEF-OprN and anaerobic lifestyle was not addressed in this work. Indeed, the link between anaerobic lifestyle, nitrosative stress, and multidrug efflux pumps may not be as straightforward as in *E. coli*. Thus, a *mexEF-oprN*-overexpressing strain was found to have impaired fitness compared to the wild-type strain under anaerobic conditions [20]. MexEF-OprN exchanges external protons for their substrates; thus, constitutive activity of MexEF-OprN may lead to the acidification of the cytoplasm. This, indeed, is the case under anaerobic conditions [20]. When oxygen is available, it is used to consume excess of protons resulting from MexEF-OprN activity. The *mexEF-oprN*-overexpressing mutant consumes oxygen at a much higher rate than wild type, creating local microaerobic conditions, which, in turn, leads to the untimely activation of the nitrate respiratory chain in the

presence of oxygen. This data, however, does not rule out the possible role in detoxification of the cells respiring nitrate, and the fitness levels of the mutant strain overexpressing *mexEF-oprN* under anaerobic conditions may be compromised due to excessive overproduction of the pump.

The authors mention that airway epithelium cells are known to secrete NO in response to lipopolysaccharide exposure or inflammatory signals [65]. It is true that NO is produced in host tissues by inducible nitric oxide synthase (iNOS), whose expression is upregulated following inflammatory stimulus, and NO exhibits antimicrobial activity, reducing adherence of *P. aeruginosa* cells [66]. It is worth noting however that the expression of iNOS and production of NO in the respiratory epithelium of cystic fibrosis patients is markedly reduced, despite chronic severe inflammation [67, 68].

K. pneumoniae, similar to *E. coli*, can respire nitrate under anaerobic conditions [69] and, thus, can potentially encounter nitrosative stress [70]. In this bacterium, the deletion of the SMR-type multidrug efflux pump-encoding genes *kpnEF* leads to the three-times slower growth in the presence of an NO donor, sodium nitroprusside, and up to 20-times reduced growth rates in the presence of acidified nitrite [55]. These results imply that KpnEF expression is involved in nitrosative stress tolerance in *K. pneumoniae*. The deletion mutant also exhibited decreased survival under hyperosmotic conditions and at the elevated (42 °C) temperatures. These results are complicated, however, by the fact that the deletion of *kpnEF* alters the expression of other genes. In this work, the authors used RT-PCR to show that the expression of capsular synthesis genes was changed; the deletion of *kpnEF* may also result in the change in expression of other genes that have not been tested.

27.6 Response to Oxidative Stress

While oxygen provides substantial advantages in energy yield by respiration, it can become life-threatening due to reactive oxygen species (ROS) production by various mechanisms. The most common ROS include superoxide ($O2^{-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO). ROS are detrimental to living organisms since they react with proteins, DNA, lipids, and other biomolecules [71]. Threat from damaging ROS is ever present for both free-living and commensal or pathogenic microorganisms. ROS can be generated both endogenously and exogenously. In aerobic cells, ROS are formed in the process of consecutive addition of electrons to oxygen. H_2O_2 may be generated by various bacteria to inhibit their competitors [72]. Plant [73] and animal [74] hosts generate various ROS as a defense mechanism against microbial pathogens. Interestingly, ROS can be produced by certain bacterial pathogens as virulence factors against their host [75].

There is limited data on the involvement of multidrug transporters in bacterial resistance to ROS. Multidrug efflux pumps are known to be regulated by a global transcriptional system SoxRS in enteric bacteria [76, 77] or directly by SoxR in non-enterics [78]. SoxR-mediated transcription is activated via oxidation of the $[2Fe\text{-}2S]^{+}$ cluster in

the presence of various redox-cycling agents [79]. For a long time, it was generally accepted that redox-cycling agents acted indirectly via superoxide formation, which oxidized SoxR. However, recently it has been shown that redox-cycling agents activate SoxR directly and cause cellular toxicity independently of the production of ROS [80]. This is consistent with the fact that many genes of the SoxRS regulon in *E. coli* have no apparent function in superoxide detoxification, but rather function in export or modification of redox-cycling drugs. Interestingly, in *P. aeruginosa* SoxR is activated by a phenazine pyocyanin, which is a redox-cycling compound, but is also known to be a quorum-sensing signal [81]. Considering the apparent involvement of multidrug efflux pumps in the release of phenazines, the SoxR response may ensure proper shuttling of these compounds [78].

In *E. coli*, the NorM pump of the multidrug and toxic compound extrusion (MATE) family was found to reduce the level of intracellular ROS and protect the cell from oxidative stress via unknown mechanism [82]. Likewise, in *Salmonella*, macrolide-specific efflux pump of the ATP-binding cassette (ABC) superfamily, MacAB, is induced upon exposure to hydrogen peroxide and is critical for the survival of *Salmonella* in the presence of peroxide [83]. Furthermore, MacAB was required for intracellular replication inside wild-type macrophages, but not inside ROS-deficient macrophages. *macAB* mutants also had reduced survival in the intestine of a mouse. In *P. aeruginosa*, expression of MexXY multidrug efflux pump was induced by peroxide via ArmZ (the anti-repressor against MexZ repressor of MexXY pump); however, the MexXY efflux system did not contribute to peroxide resistance [84].

27.7 Combating Iron Deficiency

Another line of defense against bacterial pathogens is the withholding of nutrients to prevent bacterial outgrowth. This process is termed “nutritional immunity” with the most significant form being the sequestration of nutrient iron. The vast majority of vertebrate iron is intracellular, sequestered within the iron storage protein ferritin or complexed with porphyrin ring of heme as a cofactor of hemoglobin or myoglobin [85]. Extracellular iron is insoluble and therefore difficult to access for invading pathogens or tightly bound to the serum protein transferrin. Thus, all bacterial pathogens encounter a period of iron starvation upon entering their host. Most pathogens circumvent iron withholding through high-affinity iron uptake mechanisms that compete against host-mediated sequestration. One of such strategies involves secretion of siderophores; low molecular weight iron-binding molecules which scavenge iron from the host and later in complex with iron are imported back into the bacterial cell. There is a limited evidence of the role of multidrug transporters in the export of siderophores in some bacteria.

As far ago as 1993, genes encoding multidrug efflux system *mexAB-oprK* (i.e., *mexAB-oprM*) in *P. aeruginosa* were identified while trying to complement a mutant strain deficient in the production of siderophore ferripyoverdine receptor and incapable

of growth in an iron-deficient medium [86, 87]. The expression of the efflux operon appeared induced under iron-limiting conditions. The operon was also reported to be co-regulated with other genes of the ferripyoverdine uptake system [87].

Export of the siderophore enterobactin across the cytoplasmic membrane in *E. coli* was first described to be accomplished by a dedicated MFS transporter EntS [88], but it was noted that mutants in *entS* still secrete some enterobactin, suggesting involvement of some other mechanism. Later, enterobactin was shown to be exported through the outer membrane by TolC [89], prompting the analysis of the possibility of involvement in enterobactin export of RND multidrug efflux pumps that engage TolC as an outer membrane component. It was found that multiple deletions in *acrB*, *acrD*, and *mdtABC* resulted in a significant decrease in enterobactin export; however, single deletions of these multidrug efflux systems did not affect the ability of *E. coli* to excrete enterobactin [90].

27.8 Resistance to Host-Derived Antimicrobial Peptides

Host defense peptides are encoded in the host genome and derive from large precursors through one or more proteolytic activation steps [91]. They are known as direct antimicrobial agents as well as innate immune modulators. These peptides are mainly secreted at those anatomical sites that are routinely exposed to environmental challenges, such as skin and mucosal epithelia [92]. Additionally, they are found in the body fluids or stored in the cytoplasmic granules of professional phagocytes [93]. The antimicrobial activity of host defense peptides is the consequence of their ability to interact with and insert into biomembranes [94]. Besides bacteria, they can target fungi, yeast, viruses, and cancer cells [92]. Microbial pathogens utilize a number of resistance mechanisms to subvert the action of host defense peptides [95]. In a few instances, multidrug efflux pumps were shown to be involved in microbial resistance to antimicrobial peptides.

Thus, several mutants of *N. gonorrhoeae* overexpressing MtrCDE multidrug efflux pump exhibited markedly increased resistance to human cathelicidin LL-37, and the level of the resistance corresponded to the degree of MtrCDE overexpression [19]. These mutants also showed similarly increased resistance to CRAMP-38, the murine homolog of the human cathelicidin LL-37, and outcompeted the wild-type strain *in vivo* in a murine infection model [19]. The loss of MtrCDE resulted in increased susceptibility of *N. gonorrhoeae* to cathelicidin LL-37, as well as porcine protegrin-1 and horseshoe crab-derived tachyplesin-1 [96].

Likewise, *Neisseria meningitidis*, which is intrinsically highly resistant to human antimicrobial peptides, utilizes MrtCDE pump as one of the mechanisms of defense against these peptides. Mutants in the *mtrCDE* operon are about 15 times more susceptible to polymyxin B and about 10 times to LL-37 and protegrin-1 [97]. Multidrug efflux system RosA/RosB of *Yersinia pestis* provides bacterium with resistance to polymyxin B [98]. It was also active against cecropin P1 (from pig) and melittin (from bee venom).

AcrAB efflux pump in *K. pneumonia* has been shown to provide the organism with resistance not only to antibiotics but also to host-derived antimicrobial peptides, present among the array of antimicrobial compounds in the mucus. AcrB knockout was more susceptible both to antimicrobial properties of human bronchoalveolar lavage fluid and to such antimicrobial peptides as human neutrophil peptide-1, β-defensin 1 and β-defensin 2 [99]. *acrB* knockout also exhibited a reduced capacity to cause pneumonia in a murine model, compared to the wild-type strain [99].

27.9 Control of Host Immune Response

MdrM and MdrT of *L. monocytogenes* have a fascinating role in controlling the magnitude of the host cytosolic innate immune response to the bacteria [100, 101]. On entry into the host cytosol, *L. monocytogenes* activates a host response that leads to transcription of dozens of genes, including robust expression of interferon β (IFN-β) [102, 103]. MdrM and MdrT expression was shown to affect the induction of IFN-β in infected macrophages [100, 104, 105]. The disruption of *mdrM* [100] or *mdrT* in the strain with mutated *brtA* [104] decreased IFN-β production, while overexpression of either MdrM or MdrT resulted in increased induction of IFN-β in infected macrophages [100]. The molecule that triggers the cytosolic host response was shown to be the cyclic dinucleotide c-di-AMP [101]. This molecule is produced by many bacteria and is a second messenger that is implicated in a variety of functions including cell wall metabolism, potassium homeostasis, DNA repair, and control of gene expression [106]. c-di-AMP in *L. monocytogenes* is secreted by MdrM and MdrT [101]. It is sensed by the cytosolic innate immune receptor, STING [107]. Stimulation of this pathway results in the activation of the interferon regulatory factor-3 and nuclear factor-kB transcription factors and, ultimately, to host transcriptional activation of IFN-β [102, 107]. While the innate immune system is indispensable for defense against microbial pathogens, paradoxically, the production of IFN-β increases the bacterial burden and lethality of *L. monocytogenes* infection in mouse models [108–110]. The mechanisms of this effect are not well understood, but may involve the enhanced susceptibility of lymphocytes to apoptosis in response to a pore-forming toxin and a major virulence factor of *L. monocytogenes*, listeriolysin O [109, 110].

27.10 Export of Virulence Factors

Streptococcus agalactiae, an invasive pathogen, produces β-hemolysin, which is an important virulence factor. It is capable of damaging erythrocytes, lung epithelial cells [111], and brain microvascular endothelial cells [112], which is regarded as an initial step in invasive disease. The genes *cylA* and *cylB* encoding an ABC-type

multidrug efflux pump were identified as genes essential for the production of the *S. agalactiae* hemolysin [113]. The hemolysin is now known to be an ornithine rhamnolipid pigment [114] described previously as granadaene [115]. Growth in the presence of reserpine resulted in a dose-dependent decrease of extractable hemolytic activity, supporting the hypothesis that hemolysin is transported out of the cell by a multidrug efflux pump.

27.11 Effect of Multidrug Efflux Pumps on Cell-to-Cell Communication and Quorum Sensing

Quorum sensing is a density-dependent mechanism by which bacteria coordinate expression of specific target genes in response to a critical concentration of signaling molecules. Effectively, quorum-sensing systems allow bacteria to behave somewhat in the manner of a multicellular organism when their population reaches a threshold level. Quorum sensing controls complex activities such as changes in secondary metabolism, bioluminescence, protein secretion, root nodulation, motility, virulence factor production, plasmid transfer, and biofilm maturation [116]. Quorum sensing plays a critical role in both pathogenic and symbiotic bacteria-host interactions. In pathogens, virulence factors are released and coordinated attack on the host made only when bacterial population reaches certain density and can overwhelm host responses. For symbiotic bacteria, quorum sensing allows synchronization of important cellular responses, for example, bioluminescence and root nodulation, with the host [116].

Five main categories of cell-to-cell signaling systems have been described [117, 118]. Two of these systems, which use *N*-acyl homoserine lactones (AI-1, for autoinducer 1) and AI-3, an aromatic aminated compound whose structure is presently unknown [119], are found in Gram-negative bacteria. Gram-positive cells use an autoinducer polypeptide system. The fourth system, using various furanones collectively referred to as AI-2 as signals, is found in both Gram-positive and Gram-negative cells. *P. aeruginosa* also produces a 4-quinolone molecule called pseudomonas quinolone signal (PQS) [118]. Putative biosynthetic genes homologous to the *P. aeruginosa* genes of PQS system were discovered in *Burkholderia pseudomallei* and *Burkholderia thailandensis* [117], and the production of various 4-quinolones has been detected in *Burkholderia* species and in *Pseudomonas putida* [120].

Quorum sensing is probably best studied in *P. aeruginosa*. Three molecules, 3-oxo-C12-homoserine lactone (3-oxo-C12-HSL), C4-homoserine lactone (C4-HSL), and PQS, are involved in *P. aeruginosa* quorum sensing [7]. Multidrug efflux pumps are intimately involved in the functioning of all three systems, though the details of their function may presently be unclear.

nfxC-type mutants of *P. aeruginosa* overexpress the MexEF-OprN efflux system. It has been shown that variations in the transcriptional activator gene *mexT* can lead to MexEF-OprN overexpression [121]. However, *nfxC* should be considered a

phenotype, since not all of these mutants have been molecularly characterized, and overexpression of MexEF-OprN pump can result from mutations which are not necessarily linked to *mexT*. Köhler et al. [7] reported that production of extracellular virulence factors, such as pyocyanin, elastase, and rhamnolipids, was decreased in *nfxC*-type mutants. The latter were also deficient in swarming motility (a multicellular phenomenon involving the coordinated and rapid movement of a bacterial population across a semisolid surface) [122]. This observation was in agreement with decreased production of rhamnolipids by *nfxC* mutants, since swarming was shown to depend on the properties of rhamnolipids as biosurfactants [123]. The production of these virulence factors depends on *rhl* cell-to-cell signaling system. Indeed, it was found that both the expression of the *rhlI* gene, which encodes for C4-HSL synthase, and the amount of C4-HSL autoinducer in culture supernatants were compromised in *nfxC* mutants. The interpretation of results of this study was hindered by the lack of molecular characterization of *nfxC* mutants, but it was shown that the reduction in the expression of *rhlI* and in the concentration of C4-HSL in supernatant required functional *mexEF* genes [7]. Short-chain autoinducers like C4-HSL diffuse apparently freely across the membrane [124]. It was, therefore, unlikely that MexEF-OprN pump would be involved in an active export of this signaling molecule [7]. Indeed, it was later shown that MexEF-OprN exported the precursor of PQS, 4-hydroxy-2-heptylquinolone, out of the cell, therefore diminishing its availability to intracellular enzyme PqsH which synthesizes PQS [125]. PQS, in turn, regulates *rhl* quorum-sensing system [126]; thus, decreased production of PQS leads to a decrease in expression of *rhlI* and in the production of C4-HSL.

Another multidrug efflux pump, MexAB-OprM, has also been shown to be involved in quorum sensing in *P. aeruginosa. nalB* mutants which overexpress the MexAB-OprM pump, similar to mutants overproducing MexEF-OprN, produced reduced levels of extracellular virulence factors, namely, pyocyanin, elastase, and casein protease [127]. In this case, it was shown that the effect is achieved via regulation of the *las* quorum-sensing system. This system does not directly regulate the expression of pyocyanin; however, it regulates the expression of *rhl* quorum-sensing genes, which, in turn, are responsible for the activation of production of pyocyanin by *nalB* mutants. MexAB-OprM overexpressing mutant produces considerably less 3-oxo-H12-HSL, which is a signal for *las* system. Compromised *las* system activation was proposed to result in diminished expression of *rhl* quorum-sensing genes and in decreased production of virulence factors. It has been proposed that MexAB-OprM efflux pump participates in the export of 3-oxo-H12-HSL, reducing its intracellular concentration and availability to activate LasR [127].

It appears that expressing multidrug efflux pumps would be detrimental to the virulence of *P. aeruginosa*. Nevertheless, MexAB-OprN is expressed constitutively in wild-type *P. aeruginosa*, though not to the same degree as in *nalB* mutants. Minagawa et al. [128] have shown that MexAB-OprM multidrug efflux pump ensures selectivity of the response to *P. aeruginosa*'s own quorum-sensing factor, 3-oxo-C12-HSL. Different species of bacteria use a variety of *N*-acyl-homoserine lactones with different acyl side-chain lengths. LasR, the transcriptional regulator of

the *las* quorum-sensing system, can bind and be activated by *P. aeruginosa* quorum-sensing signal, 3-oxo-C12-HSL, but in a strain with deleted *mexB* gene, it also binds a spectrum of 3-oxo-acyl-HSLs with chains with 8–14 carbons. In fact, in a *mexB* deletion strain, 3-oxo-Cn-HSLs with $n=9$, 10, and 11 are better activators of the transcription of *lasR* target gene, *lasB*, than 3-oxo-C12-HSL. As a result, *P. aeruginosa* mutant with *mexB* deletion can detect 3-oxo-C10-HSL quorum-sensing signal of *Vibrio anguillarum* and start producing pyocyanin in response [128]. In a strain with intact *mexB*, although transcription of *lasB* in response to 3-oxo-C12-HSL is somewhat dampened, it is, however, capable of discriminating between 3-oxo-C12-HSL and other homoserine lactones and is more responsive to the former [128].

The MexGHI-OpmD efflux pump appears to play a role in *P. aeruginosa* quorum sensing that is more difficult to interpret, due either to the complexity of metabolic and regulatory pathways or to insufficient data. The deletion of *mexI* or *opmD* genes resulted in the inhibition of the PQS biosynthetic genes, *phnA* and *pqsA*, and inability to produce PQS [129]. As a consequence, *mexI* and *opmD* deletion mutants failed to produce a number of virulence factors and demonstrated attenuated virulence in rat and plant infection models. These mutants were also impaired in growth. The authors suggested that mutants in *mexGHI-opmD* pump have a growth defect because they accumulate a toxic PQS precursor, anthranilate [129]. Anthranilate is produced by anthranilate synthase PhnAB and further modified by PqsA during the initial steps in PQS biosynthesis. In support of this hypothesis, introduction of *phnA* mutation, which would abolish anthranilate production, into Δ*mexI* strain restores its growth, while introduction of the mutation in *pqsA*, which would accumulate anthranilate, makes Δ*mexI* strain nearly unviable [129]. It is, however, not clear why a wild-type strain with intact MexGHI-OpmD would produce more PQS than Δ*mexI* strain. According to this hypothesis, MexGHI-OpmD pump would export anthranilate out of the cell and therefore make it unavailable for further intracellular steps in PQS biosynthesis.

The quorum-sensing systems of *B. pseudomallei* are known to produce up to six different types of acyl-homoserine lactones, the composition of which differs somewhat from strain to strain [130]. The secretion of all of them was abolished in the null mutant in *bpeAB-oprB* multidrug efflux pump [130]. Only one of the six acyl-homoserine lactones, *N*-octanoyl-homoserine lactone, was still synthesized intracellularly by this mutant. The defect in the synthesis of other acyl-homoserine lactones was suggested to result from the deficiency in the secretion of quorum-sensing compounds [130]. The lack of quorum-sensing signaling abolished the expression of autoinducer synthase BpsI and might also negatively regulate the expression of other relevant biosynthetic genes. BpeAB-OprB function is necessary for optimal production of quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and for biofilm formation. *bpeAB* mutant also exhibited significantly attenuated cell invasion and cytotoxicity toward human lung epithelial and human macrophage cells [131]. These results are however dependent on the genetic background of the *B. pseudomallei* cells. Despite dramatic results obtained for KHW strain, BpeAB-OprB pump in the strain 1026b did not play any role either in the export of acyl-homoserine lactones or in virulence factor production [132].

Multidrug transporters appear to be involved in the export of a quorum-sensing signals or signals of unknown nature in *E. coli*. It was shown that cells with deletion of genes coding for AcrAB or NorE multidrug efflux pumps grow to a higher cell density in stationary phase [133]. Overproduction of either pump caused cells to reach lower density. Conditioned medium from pump mutant cells and conditioned medium from cells overexpressing *acrAB* were, correspondingly, less and more repressive to cellular growth than medium conditioned by growth of wild-type cells. Also, expression of the *rpoS* gene encoding the stationary phase sigma factor is induced earlier in cells overexpressing *acrAB* and later in *acrAB* mutant cells. These results are consistent with AcrAB-TolC and NorE efflux pumps being involved in exporting a quorum-sensing signal out of the *E. coli* cell. Indeed, entry into stationary phase is typically controlled by quorum sensing [134], although the quorum-sensing signal or system responsible for stationary phase entry remains unidentified in *E. coli*. The authors have shown that the deletion of LuxS, which synthesizes the quorum-sensing signal AI-2 in *E. coli*, does not affect these results; therefore, some other molecule(s) must be involved.

27.12 Effect of Multidrug Efflux Pumps on Biofilm Formation

Biofilm is a structured adherent microbial community encased in extracellular matrix. Many bacteria in natural, industrial, and clinical settings predominantly live in biofilms. Biofilms constitute tremendous clinical challenge, being extremely resistant to antibiotics, host immune response, disinfectants, and some physical treatments. The process of biofilm formation is highly complex and involves multiple genes. This process is known to be regulated by cyclic diguanosine-5′-monophosphate, small RNAs, and quorum sensing. A separate chapter of this book is devoted to biofilm (see Chap. 25); here, we will discuss it briefly.

Multidrug efflux pumps appear to play an important role in biofilm formation. Matsumura et al. [135] studied biofilm formation in 22 *E. coli* mutants that were missing various multidrug efflux pumps. They found that all of the mutants showed compromised biofilm formation. The highest inhibition of biofilm formation was observed in mutants lacking *emrD*, *emrE*, *emrK*, *acrD*, *acrE*, and *mdtE* genes. Similarly, ten *Salmonella* strains with mutations in multidrug efflux systems were compromised in their ability to form biofilm [136]. Efflux inhibitors phenylalanine-arginine β-naphthylamide, carbonyl cyanide *m*-chlorophenylhydrazone, and chlorpromazine also repressed biofilm formation in *S. enterica*. Efflux inhibitors were also shown to be effective in preventing biofilm formation in *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus* [137], as well as in *P. putida* [138]. Focused research on inhibition of biofilm production in *acrB* and *tolC* mutants of *S. enterica* showed that both strains exhibited transcriptional repression of the structural genes responsible for biosynthesis of a proteinaceous filament named curli, which is a major component

of the biofilm extracellular matrix [137]. Interestingly, it was demonstrated that the defect in biofilm formation does not result from the inability of *acrB* and *tolC* mutants to export a biofilm-promoting compound [137].

27.13 Concluding Remarks

Antimicrobial resistance is a key phenotype of bacterial pathogens that adversely affects the effectiveness of chemotherapy. How the resistance feature influences interaction between bacterial species and their hosts such as humans remains a topic of intense significance. In this chapter, specific examples have been provided to support the involvement of multidrug efflux pumps in bacterial pathogenicity, because of impacts of multidrug exporters on bacterial responses to diverse hostile environments (antimicrobial substances, nitrosative and oxidative conditions), fitness, colonization, and virulence production. Obviously, this relationship well reflects a general observation that the overall effects from drug resistance on bacterial pathogenicity are dependent on four major factors: the specific bacterial species, resistance and virulence mechanisms, the ecological niche, and the host [139]. However, it is evident that baseline or inducible expression of multidrug efflux pumps is often essential for bacterial virulence, suggesting that an optimized efflux pump expression has likely been evolved for bacterial survival and persistency. Although overproduction of certain drug efflux pumps comes with biological burden and fitness cost, the roles of multidrug efflux pumps in bacterial pathogenesis cannot be underestimated. This is consistent with isolation of a large number of multidrug efflux pump-overproducing strains (e.g., *P. aeruginosa*) from clinical samples. Hence, the contribution of drug efflux systems to bacterial fitness and virulence provides another strong argument for targeting efflux process for the discovery and development of antimicrobial agents or antimicrobial adjuvants.

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Part IV
Antimicrobial Development and Efflux Pump Inhibitors

Chapter 28
Impact of Antimicrobial Drug Efflux Pumps on Antimicrobial Discovery and Development

Xian-Zhi Li

Abstract Antimicrobial efflux pumps reduce intracellular drug levels and play important roles in intrinsic and acquired drug resistance in bacteria. Their impact on antimicrobial discovery and development is multifaceted. Bacterial strains deficient in drug efflux can be used in cell-based assays (including high-throughput screening) to facilitate identification of antibacterial candidates. Drug efflux pump components and their expressional regulatory pathways are potential targets for antimicrobial discovery; these targets can be exploited for the development of drug efflux pump inhibitors to be administered as antimicrobial adjuvants in antimicrobial combination therapy. Although the scientific boom within the current "omics" era has increasingly identified numerous novel drug targets, many newly developed antimicrobials are mainly against Gram-positive bacteria, and their activities are hindered by promiscuous multidrug efflux pumps and the membrane permeability barrier of Gram-negative pathogens. Bypassing this efflux action and improving drug penetration provide important strategies for rational drug design which require careful consideration of both antimicrobial physicochemical properties and the features of drug efflux pumps/membrane barrier. This chapter describes the various impacts of drug efflux pumps on antimicrobial discovery and development.

Keywords Antimicrobial resistance • Antimicrobial discovery • Development • Novel antimicrobial • Mode of action • Efflux pumps • Outer membrane • Permeability • Physicochemical properties

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28.1 Introduction

Antibiotics or antimicrobials are "wonder drugs" [1, 2] vital for human medicine. However, the global crisis in increased antimicrobial resistance within microorganisms has become a major threat to effective antimicrobial therapy of infectious diseases [3–5]. Within the last decade, multidrug resistance (MDR) including extensive drug/pandrug resistance has increasingly been reported in numerous clinical isolates from hospitals and communities worldwide [5, 6]. In particular, increased resistance in Gram-negative bacilli and mycobacteria has been a growing trend [5, 7]. Recent reports of novel plasmid-mediated colistin resistance in *Enterobacteriaceae* [8, 9] further highlight the resistance threat against one of our last-resort antimicrobials. Meanwhile, treatment of resistant infections has been challenged for decades by the limited availability and lack of suitable antimicrobial drugs [10, 11]. For instance, the past four decades in antimicrobial research have not uncovered any novel classes of antimicrobials targeted for clinical use against Gram-negative bacteria [11–14]. This paucity or lack of success in antimicrobial discovery and development constitutes a major challenge in tackling antimicrobial resistance [5, 15, 16].

Among the various mechanisms of resistance, antimicrobial drug efflux pumps play a critical role in both intrinsic and acquired resistance for a variety of bacteria [17, 18]. This mechanism of active extrusion of antimicrobial agents is ubiquitously present in these organisms. Although their role in resistance may vary from one species to the next, drug efflux pumps have shown to provide clinically relevant resistance in Gram-negative bacteria, including multidrug-resistant bacilli such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [18] (see Chaps. 13 and 14). Drug exporters are either drug/class specific or exhibit an incredibly broad substrate profile that covers a wide range of antimicrobials. They also exert functions that go beyond drug resistance, such as virulence [18]. Thus, drug efflux phenomenon must be considered for effective antimicrobial discovery [14, 19–22]. This chapter briefly describes the implications of drug efflux pumps in the research and development of antimicrobial agents.

28.2 Bacterial Drug Efflux Pump Classification and Their Functional Characteristics

Bacterial genomes encode a large number of transporters that are usually proportional to the sizes of their genomes. Over the last decade, Saier and colleagues developed a database based on functional and phylogenetic classification of membrane transporters from a variety of living organisms [23]. Approximately 60 superfamilies and >800 transporter families of membrane transporters are currently documented in this database (http://www.tcdb.org; accessed on December 31, 2015). These transporters are involved in various cellular functions including the

transport of various small/large molecules both to and from cells, as well as metabolism. Transporters involved in drug resistance mainly fall into these five superfamilies: (i) the resistance-nodulation-cell division (RND) superfamily [24], (ii) the major facilitator superfamily (MFS) [25, 26], (iii) the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily, (iv) the drug/metabolite transporter (DMT) superfamily [27], and (v) the ATP-binding cassette (ABC) superfamily [28]. While drug efflux pumps of the MOP exporter superfamily includes the multidrug and toxic compound extrusion (MATE) family [29], those within the DMT superfamily contains the small multidrug resistance (SMR) family [30]. Additional drug transporter families have been described, including the AbgT family for antimetabolite transporters (e.g., involved in sulfonamide resistance) [31] and the proteobacterial antimicrobial compound (PACE) family (e.g., involved in disinfectant resistance) [32]. The structural and mechanistic characteristics of transporters from these superfamilies and their representative pumps in different microorganisms of clinical relevance are described throughout the book (see Chaps. 1, 2, 3, 4, and 5). Here, a brief summary of the functional characteristics is considered necessary to facilitate an overview of the role of drug efflux pumps in antimicrobial development.

Drug efflux transporters are widely distributed in bacteria and can be divided based on their composition into multicomponent or single-component pumps [18]. The RND pumps found in Gram-negative bacteria are a typical tripartite efflux complex, as represented by the AcrAB-TolC efflux system in *Escherichia coli* and the MexAB-OprM system in *Pseudomonas aeruginosa* [18, 33, 34]. These multicomponent domains together span the entire cytoplasmic (inner) membrane, periplasm, and outer membrane and can function for efficient extrusion of antimicrobial agents [33]. Pumps of the MFS and ABC superfamilies can be either of multicomponents (such as EmrAB-TolC or MacAB-TolC of *E. coli*) or a single component (such as Tet pumps) [18]. Pumps within the MATE and SMR families are generally single component (including paired pumps; see Chap. 3) [29, 30]. These pumps can directly extrude substrates out of the cell in Gram-positive bacteria [35]. In contrast, single-component pumps in Gram-negative bacteria mainly expel substrates into the periplasm, and in turn other multicomponent pumps may aid to collaboratively efflux substrates out of the cell [18, 36].

The substrate specificity of drug efflux pumps also varies from pump to pump. Certain drug efflux pumps, such as the first-identified plasmid-encoded Tet efflux pumps, are drug specific and confer resistance to tetracycline agents [37]; others, such as the aforementioned RND-type AcrAB-TolC and MexAB-OprM efflux systems, accommodate a wide range of structurally unrelated agents including various classes of clinically used conventional antibiotics, biocides, detergents, dyes, and organic solvents [18].

Drug efflux pump expression is critical to their role in intrinsic or acquired drug resistance. If a drug efflux pump is well expressed in a wild-type strain, it is usually involved in intrinsic resistance (e.g., the AcrAB-TolC and MexAB-OprM pumps). Expression of multiple drug efflux pumps must be well controlled by their regulators, as changes to these regulators (i.e., through mutations or by induction/inhibition)

can alter drug efflux pump expression and subsequently affect drug susceptibility phenotypes [18, 38]. Acquired resistance has been widely recognized in clinical isolates with mutations in regulatory genes involved in efflux pump expression. As such, effective intervention of pump gene expression may also serve as an important strategy in drug discovery [39].

It is important to note that despite of the presence of numerous proven and putative drug exporters in bacteria, only a few efflux pumps have demonstrated compelling clinical significance. For example, there are dozens of multidrug efflux exporters in *E. coli* that provide resistance when expressed in plasmid vectors [40], but there are only a very limited number of pumps (such as AcrAB and EmrE) whose inactivation or overexpression can dramatically affect drug resistance phenotypes, thus being considered clinically relevant [41]. Similarly, while the MexAB-OprM system in *P. aeruginosa* is the major pump that mediates high-level intrinsic and/or acquired MDR [42, 43], several others (such as MexXY, MexCD-OprJ, and MexEF-OprN) are mainly involved in acquired resistance in clinical isolates [18]. Thus, when targeting cell efflux, the pumps of clinical importance would be considered as the primary drug targets.

28.3 Drug Access to Cellular Targets: Influx and Efflux of Antimicrobial Agents and Implications in Antimicrobial Development

The ease for antimicrobial agents to access their cellular targets is highly dependent on its intracellular concentration and thus both the influx and efflux of these agents become critical. The balance between these opposite processes will ultimately affect the drug steady states within the cell [19, 44]. Furthermore, the influx and efflux status of these agents is highly dependent on outer membrane and cell wall permeability against the agents. In bacteria where outer membrane permeability is low (such as *A. baumannii* and *P. aeruginosa* [discussed in [18] and Chaps. 13 and 14]), efflux pumps play a predominant role in both intrinsic and acquired resistance. For other Gram-negative bacteria with relatively high outer membrane permeability (such as *E. coli*, *Enterobacter*, *Klebsiella*, and *Neisseria* spp.), a loss of outer membrane channel-forming protein(s) is often needed to enhance drug efflux pump efficiency and to achieve clinically relevant resistance [45]. Small and relatively hydrophilic antimicrobial agents such as β-lactams, fluoroquinolones, and tetracyclines typically penetrate the cell via porin channels [46–48] (although transmembrane diffusion can be more complex when considering the protonation and deprotonation of the agents under various pH conditions [47]). Gram-negative organisms also feature lipopolysaccharide (LPS) on the outer leaflet of the outer membrane barrier (while the inner leaflet contains phospholipids, like the cytoplasmic membrane) [49]. The role of the LPS in influencing the drug access should never be underestimated as the physical and chemical properties of drug candidates become highly relevant with regard to porins and LPS [46]. Several basic parameters, including molecular weight as well as partition and distribution coefficient (P or D)

values of antimicrobial compounds, need to be considered in drug discovery [14, 50–53]. In 1976, Nikaido showed the impact of molecular size and hydrophobicity/hydrophilicity of antimicrobial agents on their activity against the wild-type and LPS-deficient bacterial strains. Small hydrophilic agents (<650 Da) penetrate through the porins, while hydrophobic antimicrobials including dyes (some with molecular weights of >1,200 Da) diffuse by dissolving into the hydrocarbon interior of the outer membrane, together presenting evidence on the pathways of drug transmembrane diffusion [54]. During the early stages of drug efflux characterization in the 1990s, we assessed the relationship between physical properties, such as molecular weights and logP (logD) values of extracytosolic targeting agents (e.g., β-lactams) and susceptibility phenotypes to understand how these agents were subjected to drug efflux by pumps located in the inner membrane [55, 56]. Efflux becomes more evident with agents containing more lipophilic side chains. The latter facilitates the diffusion of the agents which are, nevertheless, captured by drug efflux pumps [56].

O'Shea and Moser highlighted the importance of physicochemical properties of agents in antimicrobial discovery, particularly in relation to the outer membrane and drug efflux pumps of Gram-negative bacteria [51]. A recent article by Brown et al. [57] has examined the trends (and exceptions) of physical properties for approximately 3,200 antibacterial project compounds and their activity against Gram-positive and Gram-negative bacteria. This analysis showed that agents least susceptible to efflux are typically either highly polar and small in molecular weight (e.g., faropenem) or very large and zwitterionic (e.g., ceftazidime) [57]. Ceftazidime was previously noted to not be affected by *E. coli* AcrAB [58] or *P. aeruginosa* Mex pumps [59]. In *P. aeruginosa*, low efflux compounds typically have molecular weights of either less than 300 Da or greater than 650 Da; in contrast, compounds with molecular weights between 350 and 650 Da are highly effluxed [57]. Overall, small agents show good penetration rates through porins (particularly hydrophilic compounds) and large molecules can penetrate through the diffusion across the lipid bilayer, thus together it is not surprising that both are hardly accommodated by efflux pumps [46, 54, 60]. To note, other properties such as chemical charges and hydrophobicity also significantly affect influx/efflux rates [57].

A gene inactivation approach was used to construct various genetically defined mutants to assess drug efflux system impact (particularly the AcrAB pump) and LPS on drug susceptibility (see Chap. 9). (These mutants were used for high-throughput screening of antimicrobial candidates as well as efflux pump inhibitors [61]). LPS deficiency significantly rendered mutants hypersusceptible to hydrophobic agents such as azithromycin, erythromycin, novobiocin, crystal violet, and sodium dodecyl sulfate (see Chap. 9); its impact on hydrophilic agents was less evident. Chemical agents that disrupt cell membrane integrity (by acting on the LPS) also display similar effects on antimicrobial susceptibility [62–64]. Overall, the limited drug access issue generally offers an explanation for the anti-*S. aureus* activity (but not anti-*E. coli* or anti-*P. aeruginosa* activity) of fusidic acid, lincosamides, macrolides, mupirocin, pleuromutilins, oxazolidinones, and rifamycins [65]. As such, drug access greatly contributes to the activity spectrum of antimicrobial agents.

Drug access to cellular targets in mycobacteria also merits particular attention for the development of antimycobacterial agents. The high-lipid content within the mycobacterial cell wall forms a formidable permeation barrier [66, 67], which interplays with the increasingly appreciated role of drug efflux mechanisms in mycobacterial resistance [68–72]. A study assessing the contribution of physicochemical space (e.g., molecular weights, lipophilicity, and polar surface) in antituberculosis drugs has developed a mathematic model to identify regions of physicochemical space with potential antimycobacterial activity [52]. This helps to consider drug influx and efflux processes under the unique mycobacterial membrane structure [38, 52].

28.4 Impact of Drug Efflux on Antimicrobial Discovery

28.4.1 Tools for Screening Antimicrobial Candidates and Efflux Pump Inhibitors

Our increasing understanding of multidrug transporters has certainly facilitated antimicrobial discovery and development [22, 38]. Identification of antimicrobial drug candidates requires the initial screening of agents with potential antimicrobial activity. Cell-based growth inhibition assays have been a conventional tool for screening antimicrobial candidates. However, if wild-type strains are only used, the assays may not have sufficient sensitivity to uncover antimicrobial candidates. Our knowledge of intrinsic resistance-related major efflux pumps allows us to exploit antimicrobial-hypersusceptible drug efflux pump-deficient mutant cells for such screening [18, 38, 41, 73]. For example, AcrAB-TolC-deficient strains have uncovered hypersusceptibility to antimicrobials including anti-Gram-positive agents such as macrolides and novobiocin [41]. These strains provide a more sensitive tool for antimicrobial screening [57, 74]. (Also, lower amounts of candidate compounds can be tested.) Brown et al. [57] have shown that the use of TolC-deficient efflux pump mutants have aided the identification of the more active agents than any other screenings of potential antibacterial compounds. Another recent study carried out by Nayar et al. [75] similarly used *tolC* mutant for a high-throughput cell wall reporter assay to identify novel antibacterial targets and agents. Two structurally different compounds, sulfonyl piperazine and pyrazole, were found to inhibit the lipid A synthesis of LPS and the release of lipoprotein from the inner membrane, respectively. While the *tolC* mutant is hypersusceptible to these agents with MIC values of 0.25 and 0.125 μg/ml, respectively, wild-type *E. coli* require MIC values of >64 and 8 μg/ml for the two compounds – highlighting the benefits of drug efflux pump mutants in antimicrobial discovery [75].

Hypersusceptible strains had been obtained for antimicrobial characterization even prior to the discovery of multidrug efflux pumps (as in the case of the frequently used Z61 strain of *P. aeruginosa*, which was generated by chemical mutagenesis) [76–78]. *P. aeruginosa* Z61 was initially called as a “true permeation mutant” [77] and was

later found to be deficient in LPS integrity and devoid of a functional MexAB-OprM pump [42, 79, 80]. This strain continues to be used in the characterization of antimicrobial agents [81, 82]. In summary, hypersusceptible mutants deficient in major RND pumps and/or LPS [41, 49, 83–86] are optimal model organisms for screening potential agents against Gram-positive and Gram-negative organisms.

Currently, conventional antimicrobial susceptibility testing using mutants with known pump expression (i.e., lacking or differential expression) can readily be applied to quantitatively assess whether the antimicrobial agents in question are the substrates of a drug efflux system by looking at their MIC data [84, 87, 88]. These mutants can also be used for functional efflux assays to screen for efflux pump inhibitors. Known efflux pump substrates such as fluorescent molecule dyes (e.g., ethidium bromide) serve as good efflux indicators in whole cell assays of potential pump inhibitors [61, 68]. Accumulation levels of the indicator dye suggest whether the compound of interest possesses activity as a pump inhibitor. Furthermore, a dose-response relationship can also be measured. These assays can be easily conducted using conventional fluorometers and plate readers, as well as through fluorescence microscopy and high-resolution flow cytometry [61, 89]. Recently, a technical method has been reported to quantify the time scale of antimicrobial accumulation at the single bacterial cell level based on tunable ultraviolet excitation of antimicrobials in combination with microscopy [90].

28.4.2 Targeting Drug Efflux Pumps

Since the initial discovery of drug efflux pumps, the drug efflux process has been considered as a potential drug target for antimicrobial development. Over the last two decades, major efforts have been made to develop bacterial drug efflux pump inhibitors to combat antimicrobial resistance [21, 91–93]. (In fact, similar efforts have been made to develop mammalian cancer cell efflux transporters such as P-glycoprotein and multidrug resistance proteins [94–97].) The significance of RND pumps in clinically relevant resistance has made these pumps an attractive target for developing RND pump-specific inhibitors [18, 98]. AcrAB-TolC and MexAB-OprM are the two mostly targeted efflux systems used in antimicrobial synergistic discovery platform [18]. In Gram-positive bacteria, the MFS pump NorA has been a model pump for the development of efflux pump inhibitors. A recent study showed the structure-activity relationship for indole-based compounds as effective inhibitors of NorA [99]. Efflux pump inhibitors are extensively discussed in Chap. 29, with an examination of both natural and synthetically derived compounds. To note, agents with potential to inhibit efflux pump expression are also potential candidates for therapeutic intervention. Several efflux component-specific antisense peptide nucleic acids (i.e., synthetic peptide nucleic acid oligomers covalently coupled to a cell-penetrating peptide) were shown to repress CmeA and CmeB expression in *Campylobacter jejuni* and thus render cells more susceptible to ciprofloxacin and erythromycin [39, 100].

Optimal efflux pump inhibitors should be used in the combination with antimicrobials that are substrates of the target efflux pumps to (i) specifically repress target drug efflux activity and (ii) simultaneously allow the antimicrobial to exert its action. Together, this combinatorial approach mimics the β-lactam-β-lactamase inhibitor combination in the treatment of resistant infection. (β-Lactam-β-lactamase inhibitor combination continues to show its importance in antimicrobial therapy as exemplified by the new entry of ceftazidime-avibactam and ceftolozane-tazobactam into the clinical market [101, 102].) RND pump inhibition has demonstrated to help an Ftz-directed antistaphylococcal prodrug (TXY436) to be active against *A. baumannii*, *E. coli*, and *K. pneumoniae*, by increased drug access [103]. Interestingly, a clinically used agent thioridazine (an antipsychotic agent) has also demonstrated its inhibition of mycobacterial efflux pumps at clinically reachable levels [104–106]. Although the last two decades of efflux pump inhibitor development have not produced any agents ready for clinical trial, our growing knowledge of RND pump-type crystal structures as well as transporters (alone and complexed with antimicrobial ligands/efflux pump inhibitors) [93, 107–111] is expected to provide a springboard for improved design of novel efflux pump inhibitors for their potential use as antimicrobial adjuvants.

28.4.3 Bypassing Drug Efflux Pumps

Bypassing drug efflux pumps is another strategy for antimicrobial discovery [11, 112]. In this regard, tetracycline-specific efflux pumps have been targeted by a new design type of agents that circumvent tetracycline resistance mechanisms including efflux [113]. Several tetracycline derivatives are not subjected to Tet efflux pumps. For instance, tigecycline is active against strains expressing Tet efflux pumps [114] (although this agent is a typical substrate of RND pumps; reviewed in [18]). Similarly, a novel aminomethylcycline derived from chemical modifications of minocycline, omadacycline, is highly active against *tet* gene-containing strains of *S. aureus*, enterococci, streptococci, and *E. coli* (omadacycline MIC values are reduced by 32- to 516-fold against when compared with those of tetracycline) [115, 116]. The activity of a novel fluorocycline, eravacycline, is also minimally impacted by *tet* resistance determinants [117]. Unlike many cephalosporin agents, ceftazidime and ceftolozane activities are little impacted by *E. coli* AcrAB and/or *P. aeruginosa* Mex pumps [58, 59].

Another strategy to combat multidrug-resistant Gram-negative bacteria involves disrupting the structural integrity of the outer membrane. This approach is not only to select another drug target (since membrane disruption itself could have a lethal effect on bacteria) but also increases target access for the drugs and thus efficiently counter against drug efflux. Indeed, outer membrane permeabilizers are able to compromise the impact of drug efflux by enhancing drug influx. Cationic antimicrobial peptides target cell membranes [118, 119] and thus show antibacterial activity against multidrug-resistant pathogens, often independent of major drug efflux

pumps [120] (although certain efflux pumps can accommodate antimicrobial peptides [121, 122]). Targeting the LPS biosynthetic pathways also merits attention since this can also adversely affect outer membrane permeability. For example, sulfonyl piperazine and pyrazole compounds inhibit lipid A synthesis for LPS and the release of lipoprotein from the inner membrane, respectively; together, these actions compromise the integrity of the cell envelope [75]. Ciclopirox is an antifungal agent that also possesses antibacterial activity against Gram-negative bacteria [123]. This agent at 5–15 μg/ml was shown to inhibit drug-resistant *A. baumannii*, *E. coli*, and *K. pneumoniae* by targeting galactose metabolism and LPS biosynthesis [124]. Ciclopirox can be further assessed for clinical values in combination with conventional antimicrobial agents [125–127]. Another example of an LPS-targeting agent for drug discovery is POL7080, a synthetic cyclo-peptide (peptidomimetic) which acts on the LPS-assembly protein, LptD, and displays an impressive *in vitro* activity against *P. aeruginosa* (MIC_{90} of 0.25 μg/ml for 100 isolates tested; MIC of 0.06 μg/ml for PAO1 strain) [128].

28.5 Impact of Efflux Pumps on the Newly Developed Antimicrobial Agents

28.5.1 New Antimicrobials of Existing Classes as Efflux Pump Substrates

Modification of existing antimicrobial classes to create newer and improved agents has always been an important approach in antimicrobial discovery and development [129]. However, new derivatives of antimicrobials often continue to be substrates of drug efflux pumps. The clinical significance of this phenomenon is dependent on the role of the particular pumps in given clinical isolates.

β-Lactam-β-lactamase Inhibitor Combinations In recent years, several new cephalosporin-β-lactamase inhibitor combination products have been authorized for clinical use or clinical trials (e.g., ceftazidime-avibactam, ceftaroline-avibactam, and ceftolozane-tazobactam), a major advancement in antimicrobial product development [101, 102, 130–132]. Nonetheless, like other β-lactams and β-lactamase inhibitors [43, 84], these combinatorial agents are subjected to the impact of drug efflux and the membrane permeation barrier. Avibactam, a non-β-lactam-β-lactamase inhibitor, cannot reverse efflux-mediated ceftazidime resistance [133], and the outer membrane permeability barrier has a major impact on activity of ceftazidime-avibactam [64]. Ceftaroline is still affected by efflux pump- and/or porin-related resistance mechanisms, although ceftolozane appears less impacted by Mex pumps in comparison with many other β-lactams [134–137]. An early study showed *in vitro* activity of ceftolozane against *P. aeruginosa* with an MIC_{90} value of 1 μg/ml against 193 isolates; this value was 8- to 16-fold lower than those of three traditional anti-*P. aeruginosa* agents, ceftazidime, imipenem, and ciprofloxacin.

This result is likely attributable to the little impact of several major RND pumps, including MexAB-OprM, on ceftolozane activity [59]. Aztreonam-avibactam is another combination in clinical trial that displays good activity against *Enterobacteriaceae* producing serine β-lactamases and metallo-β-lactamases [138]. However, activity against *A. baumannii* or *P. aeruginosa* is lacking or limited [138]; the robust RND efflux pumps in these species can be partially to blame [55]. The contribution of drug efflux pumps can also illustrate reduced or no synergistic activity of several combinations against multidrug-resistant *A. baumannii* and *P. aeruginosa* [132, 139, 140].

Eravacycline and Omadacycline These two different tetracycline-related agents were specifically developed to overcome *tet*-mediated resistance mechanisms (drug efflux and ribosome target protection). Eravacycline has impressive *in vitro* activities against a number of Gram-negative species, with an exception for its limited activity against *Burkholderia cenocepacia* and *P. aeruginosa* isolates (with MIC_{90} values of 32 μg/ml) [13, 117], which highly suggests this agent as a substrate of RND pumps for these two species.

Omadacycline possesses strong *in vitro* activity against a range of Gram-positive and Gram-negative bacteria with *tet* resistance determinants [115, 116]. Its activity against non-*tet* gene-containing strains (such as *S. aureus*, enterococci, streptococci, and *E. coli*) is similar to that of tetracycline. However, this agent is a substrate of RND-type AcrAB-TolC and MexAB-OprM pumps [11].

Ketolides and Oxazolidinones Newer ketolides (e.g., cethromycin and solithromycin) and oxazolidinones (posizolid, radezolid, and tedizolid) continue to be the agents mainly targeted against Gram-positive bacteria [11]. Their lack of potency for Gram-negative bacilli is likely attributable to the mechanisms of drug efflux and the outer membrane permeability barrier (as shown with the earlier members of these antimicrobial classes) [141–145]. Cethromycin was still found to be a substrate of the AmrAB-OprA pump [146].

Neoglycosides A new aminoglycoside derivative of sisomicin, plazomicin (a neoglycoside), was developed by circumventing aminoglycoside-modifying enzymes [147, 148]. It displays strong activity against a range of Gram-positive and Gram-negative bacteria (including multidrug-resistant isolates) [149–151]. However, this activity is compromised by increased drug efflux in *A. baumannii* and *P. aeruginosa* [147]. Inactivation of *P. aeruginosa* drug efflux pumps was found to reduce the plazomicin MIC value by 32-fold and increased expression of the MexXY pump raised the MIC value by twofold [147].

Newer Quinolones or Fluoroquinolones Quinolones are the typical substrates of multidrug efflux pumps of both Gram-positive and Gram-negative bacteria [38]. The activity profiles of several newer quinolones in clinical trials (e.g., fluoroquinolones delafloxacin, finafloxacin, JNJ-Q2, non-fluorinated nemonoxacin, and an isothiazoloquinolone ACH-702) [11, 152–158] suggest that these agents are substrates of RND

pumps. Nevertheless, although still a substrate of AdeABC and AdeM pumps of *A. baumannii*, the broad-spectrum fluoroquinolone agent DS-8587 was found to have a better activity against AdeABC- or AdeM-overproducing multidrug-resistant mutants than ciprofloxacin and levofloxacin [159, 160], further supported by its efficacy in an animal model [161]. An anti-*S. aureus* tricyclic fluoroquinolone, WCK 771, is not considered as a substrate of the NorA pump with good activity against quinolone- or glycopeptide-resistant staphylococci [162, 163]. Besifloxacin is another newer fluoroquinolone less affected by multidrug/fluoroquinolone efflux pumps of Gram-positive and Gram-negative bacteria [164].

28.5.2 New Classes of Antimicrobials as Efflux Pumps Substrates

The discovery of new classes of antimicrobials with different modes of action from existing antimicrobials constitutes a major effort to combat antimicrobial resistance. However, both direct and indirect data suggest that many of new classes of antimicrobials are indeed substrates of drug efflux pumps.

Cell Wall Inhibitors Teixobactin, an antibiotic of a newly described class, was identified from a soil microorganism and displays good activity against Gram-positive bacteria that include enterococci, staphylococci, streptococci, *Clostridium difficile*, and *Mycobacterium tuberculosis* (with MIC values in a range of 0.005 to 0.5 μg/ml) [165]. This agent inhibits cell wall synthesis by binding to a highly conserved motif of the lipid II precursor of peptidoglycan and the lipid III precursor of teichoic acid. Although indicated (unconvincingly) in the article's title for "no detectable resistance," teixobactin lacks activity against Gram-negative bacteria (with MIC values of 25 μg/ml for *E. coli* and >32 μg/ml for *K. pneumonia* and *P. aeruginosa*) [165]. This intrinsic resistance in Gram-negative bacteria is highly expected due to the membrane permeability barrier and/or drug efflux pumps. Additionally, as mentioned earlier, an *E. coli tolC* mutant was successfully used to discover two classes of agents, sulfonyl piperazine and pyrazole compounds, that ultimately affect cell wall structure. However, these compounds are likely strong substrates of TolC-dependent drug efflux pump(s) [75].

Metalloamidase LpxC Inhibitors LpxC is zinc-dependent UDP-3-O-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase involved in the biosynthesis of LPS, thus serving only as an anti-Gram-negative target [57, 166, 167]. Although an inhibitor of LpxC, CHIR-090, displays good *in vitro* activity against *P. aeruginosa* with an MIC value of 0.5 μg/ml, this agent is also subjected to extrusion by *P. aeruginosa* MexAB-OprM, MexCD-OprJ, and MexEF-OprN pumps [168]. While both the MexAB-OprM or MexCD-OprJ producer is eightfold more resistant to CHIR-090 than the wild-type PAO1 strain, mutants deficient in MexB and MexY pumps are eightfold more susceptible than the PAO1 strain [168].

Peptide Deformylase Inhibitors These agents target an essential bacterial metalloenzyme which deformylates the *N*-formylmethionine of newly synthesized polypeptides. However, one such inhibitor, LBM415, was found to be a substrate of the AcrAB-TolC pump in *Haemophilus influenzae* with a 16-fold MIC difference between the wild-type strain and *acrB*-deficient mutant [88, 169]. Intrinsic resistance to LBM415 in *P. aeruginosa* (MIC of 128 μg/ml for wild-type strain) is mediated by MexAB-OprM and MexXY-OprM pumps with a 32-fold MIC difference between the wild-type strain and pump-deficient mutant [170]. Antibacterial activity of several other peptide deformylase inhibitors (including actinonin and indole derivatives) was also significantly compromised by the membrane barrier and efflux pumps (outer membrane disruption and AcrAB inactivation increased actinonin activity by a 256-fold MIC reduction in *E. coli*) [171, 172]. Methionine aminopeptidase is an enzyme involved in protein N-terminal processing for the removal of the N-terminal methionine from new polypeptides. Inhibitors of this enzyme, several hydroxamic acid derivatives, are also adversely impacted by the membrane barrier and AcrAB pump [173].

Type IIA Topoisomerase Inhibitor GSK299423 A new class of type IIA topoisomerase inhibitors, piperidinylalkyquinolones (such as GSK299423), was developed [174]. As demonstrated through the co-crystal structure of DNA gyrase and DNA in complex with GSK299423, this agent displays a novel mode of inhibition that overcomes fluoroquinolone resistance. However, Despite its broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria, the half-maximum inhibitory concentration of GSK299423 against *S. aureus* was eightfold lower than that required against *E. coli*. There were also impressive differences between the MIC values against several *S. aureus* (i.e., only ≤0.016 μg/ml) and those for *Enterobacter cloacae*, *K. pneumoniae*, *Proteus mirabilis*, *P. aeruginosa*, and *Stenotrophomonas maltophilia* (0.5–8 μg/ml) [174]. These data most likely suggest a possibility for GSK299423 as a substrate of Gram-negative efflux pump(s).

28.6 Concluding Remarks

The current antimicrobial resistance crisis fuels an urgent need for new antimicrobial agents, particularly those against multidrug-resistant Gram-negative bacilli. Significant efforts are being made from many stakeholders including governments, academia, and the pharmaceutical industry to promote antimicrobial discovery and development [7, 10, 16, 126, 175–177]. However, among many factors affecting antimicrobial development, a major scientific hurdle to face is the impact from clinically relevant multidrug efflux pumps and the outer membrane permeability barrier of Gram-negative bacteria [11, 35, 38, 65, 178, 179]. With the appreciation of drug efflux pumps in resistance, diverse natural and synthetic chemicals (including natural secondary metabolites) have been screened for either the

inhibition of drug efflux pumps or the bypass of this pump action. From a drug efflux point of view, reassessment of the existing clinically used drugs or compound libraries can be helpful in identifying agents that overcome efflux-mediated drug resistance. As discussed, an inability to reach cellular targets explains our lack of new classes of antimicrobial agents against Gram-negative bacilli for several decades [175, 176]. New antimicrobial pipelines are often only available for Gram-positive species as well as Gram-negative species lacking significant drug efflux activity and an effective outer membrane barrier [11, 13, 15]. Thus, even with the increasing emphasis and discovery of novel antimicrobial targets to combat drug-specific resistance mechanisms [167], the challenge for drug penetration into the cell needs to be tackled. Moreover, the complexity of antimicrobial development is not only limited to bacterial killing but also needs to consider the safety of potential drug candidates. Effective development of several efflux pump inhibitors has adversely been hindered by their toxicities to mammalian cells [18]. Optimizing and balancing the various factors that influence effectiveness and safety of drug leads are critical for successful antimicrobial discovery and development. Finally, one must emphasize the phenomenon that the novel antimicrobials including those intervening efflux mechanisms are fully expected to select resistance as shown in the evolution of drug efflux mechanisms. There will always be an arms race between microorganisms and antimicrobial development. While efforts are needed to find new antimicrobials, reduced exposure of antimicrobials to microorganisms through prudent antimicrobial use is the major driver to minimize resistance emergence and spread.

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Chapter 29
Antimicrobial Drug Efflux Pump Inhibitors

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Abstract Bacterial multidrug resistance is on the rise, and bacterial efflux pumps make a major contributor to this development. Efflux pump inhibitors (EPIs) may be a solution to this problem, since they can reverse resistance to clinically administered antimicrobial agents. Multiple EPIs with activity against Gram-positive and Gram-negative bacteria have been described over the past decades. However, none of the synthetic EPIs have currently reached a stage of clinical applicability. Investigators have evaluated certain drugs already in clinical use for their ability to potentiate the activity of antimicrobials via inhibition of drug efflux pumps. Most of the compounds reported to date do not reach clinical concentrations for EPI activity at the *in vivo* target site, with a notable exception, i.e., thioridazine shown to potentiate the killing of *Mycobacterium tuberculosis* in human macrophages. Our understanding of the molecular mechanisms for efflux pump inhibition has been considerably advanced, mainly because of the availability of crystal structures of multidrug transporters, including those in complex with substrates or EPIs (in particular with the AcrAB-TolC efflux system of *Escherichia coli*). Crystallographic data as well as results from mutagenesis and computational chemistry studies have laid the foundation for rational EPI design.

Keywords Gram-positive bacteria • Gram-negative bacteria • *Mycobacterium* • Multidrug resistance • Efflux pump inhibitors • MFS • RND • Bmr • NorA • AcrAB-TolC • MexAB-OprM • P-glycoprotein

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29.1 Introduction

Bacterial multidrug resistance (MDR) has reached alarming proportions over the past years, severely compromising treatment options against life-threatening diseases in the clinic. While most of the clinically observed mechanisms of resistance are somewhat specific with regard to the target or compound involved (e.g., modification of penicillin-binding proteins or presence of a β-lactamase), some more universal mechanisms of resistance are also known and involve reduced influx and/or enhanced efflux leading to reduced concentration of an antimicrobial at bacterial target site [1, 2]. These universal mechanisms can also facilitate the acquisition of more specific antimicrobial resistance, e.g., by target mutations. Reduced influx (e.g., due to the porin loss in Gram-negative bacteria or alteration of the cell envelope) generally works in synergy with efflux pumps. Hence, highly reduced cell envelope permeability as observed in many non-fermenting bacteria may even render a less-expressed efflux pump capable of dramatically reducing the accumulation of a given drug. However, under the circumstances of a highly permeable cell envelope, even a highly active efflux pump may have a hard time in reducing the intracellular concentration of such a drug.

Depending on the species, the relative contribution of efflux pumps to the degree of MDR may also vary considerably in the presence of other factors like β-lactamases, altered penicillin-binding-proteins, or ribosomal target modifications. Reduced intracellular accumulation of an antimicrobial agent caused by decreased influx or enhanced efflux is such a threat to antimicrobial treatment options that the use of compounds which reverse these mechanisms of resistance appears to be an attractive strategy. For example, the polymyxins, bactericidal antimicrobials known as drugs of last resort for the treatment of multidrug-resistant Gram-negative infections, are powerful permeabilizers of the outer membrane and can thus be used in combination with β-lactams or tetracyclines to potentiate their antimicrobial activity [3, 4]. While such a combination therapy can reverse resistance, side effects of polymyxins, mainly nephrotoxicity, are often hindering therapy [5]. It is difficult to separate the desired effect (permeabilization of the cell envelope) from the unwanted side effects, since both are mediated by the polycationic structure and the fatty acid tails of polymyxins. Although new permeabilizers with reduced nephrotoxicity have been reported [6], none of them have yet found their way beyond preclinical testing. This fact makes a strong case for exploring the other possible route which leads to elevated intracellular accumulation of a drug: use of drugs that inhibit bacterial efflux pumps and thus potentiate the activity of “old” antimicrobials against bacteria which have developed specific mechanisms of resistance. In addition, they could also be used to reduce the emergence of resistance and to expand the spectrum of antibacterial activity [7].

If these so-called efflux pump inhibitors (EPIs) act against MDR efflux pumps with a wide substrate spectrum, they are expected to not only reverse resistance to a single drug but to multiple clinically beneficial antimicrobials (e.g., β-lactams, fluoroquinolones, and tetracyclines) at the same time. However, while such

broad-spectrum activity may render many clinically administered antimicrobials more active, practical pharmacokinetic considerations and synergistic limitations (e.g., futility of reversing β-lactam resistance in the presence of highly active β-lactamases) suggest that only certain EPI/antimicrobial combinations may be clinically useful in certain bacteria. For example, it has recently been described that extended-spectrum β-lactamase-mediated ceftazidime and cefepime resistance could be reversed by an EPI in clinical *Pseudomonas aeruginosa* isolates but not in *Enterobacteriaceae* [8]. Moreover, in spite of many advances made in the pursuit of EPIs, none of the drugs described below have yet been clinically approved for inhibiting bacterial efflux pumps. Many newly discovered EPIs have either failed the preclinical trials (e.g., due to toxicity problems) or their development has been halted for other reasons.

This dilemma has led some researchers to repurpose clinically approved drugs for bacterial efflux pump inhibition. In fact, reserpine, one of the EPIs discussed below, was one of the earliest discovered drugs that displayed activity against efflux pumps of Gram-positive bacteria. However, the clinical applicability of these approved drugs is counterbalanced by the fact that they are relatively weak EPIs, which do not reach the plasma levels required to inhibit bacterial efflux pumps. Moreover, many of these compounds interfere with mammalian efflux pumps, leading to unintended consequences in combination therapy.

The only clinically approved drug, which has been demonstrated to act as an EPI and achieve sufficient concentrations at the target site (in the macrophage), is thioridazine. Nevertheless, many of the agents discussed below in detail can still be regarded as attractive model compounds and have thus the potential to help researchers gain insight into the molecular mechanisms behind EPI activity and possibly allow for the design of more potent and less cytotoxic compounds in the not-too-distant future. This chapter provides a comprehensive review of the current status on antimicrobial drug efflux pump inhibitors.

29.2 Identification of EPIs

Screening for agents with EPI activity is nowadays typically carried out using commercially available compound libraries prepackaged in microtiter plates. Normally, efflux pump-overproducing bacteria and subinhibitory concentrations of the desired antimicrobial are added to the wells of the microtiter plates containing individual screening compounds, and growth is assessed after a period of incubation (e.g., overnight) in nutrient broth. Fluoroquinolones have often been used as the antimicrobial combination partner of choice for this purpose. Fluoroquinolone resistance at clinical breakpoints has been shown to be fully reversible by an EPI addition despite resistance by classical target-specific mechanisms [9, 10]. Alternatively, fluorescent dyes that are substrates of efflux pumps (e.g., ethidium bromide) can be used to demonstrate enhanced intracellular accumulation produced by a putative EPI.

In the case of growth inhibition or increased intracellular fluorescence, potentiation of the antimicrobial activity is assumed. However, this effect could be due to mechanisms of action other than efflux pump inhibition (e.g., membrane permeabilization). To prove that the potentiation effect is solely due to efflux pump inhibition, one can test if the putative EPI reduces the minimal inhibitory concentration (MIC) of the given antimicrobial or enhances dye accumulation in an efflux pump-deficient strain at concentrations which potentiate the activity of the antimicrobial in an efflux pump-overproducing strain, which should not occur if the compound is an "ideal EPI," meaning that it does not possess any synergistic activity besides efflux pump inhibition. A classic example of an almost "ideal EPI" would be 1-(1-naphthylmethyl)-piperazine (NMP) [11]. While such a definition might be highly satisfactory from an EPI research point of view, it negates the fact that compounds displaying multiple mechanisms of action against bacterial resistance might be quite useful from a clinical point of view.

But how can efflux pump inhibition be detected in the presence of additional mechanisms, if the MIC or dye accumulation assays cannot be used for this purpose? The solution to this problem is the usage of real-time efflux assays in whole cells, which are capable of directly measuring efflux pump activity and dose-dependent inhibition by an EPI. In Gram-negative bacteria, most of the published assays have relied on relatively hydrophobic environment-sensitive fluorescent dyes (e.g., *N*-phenyl-1-naphthylamine [NPN], Nile red, and 1,2′-diphenylamine), which were used to preload bacteria de-energized by uncouplers, and efflux was typically triggered by addition of glucose or other energy sources after a washing step [12–16]. Since these hydrophobic dyes rely on partitioning into the cell envelope and removal from the periplasm by efflux pumps of the resistance-nodulation-cell division (RND) superfamily, they generally do not work in Gram-positive bacteria. Thus, in Gram-positive species, the DNA intercalator ethidium bromide is typically used as a dye for efflux assays [17, 18]. It can also be used in Gram-negative bacteria; however, ethidium efflux is somewhat slower in Gram-negative cells than Gram-positive cells when comparing the abovementioned hydrophobic dyes [19], presumably because ethidium has to be released from double-stranded DNA and has to cross both the cytoplasmic (inner) and the outer membranes. Using these fluorescent assays, (semi-)quantitative data on substrate transport can be obtained which allows for ranking the EPIs by their potency.

Real-time fluorescent efflux assays may also seem to be the perfect tool to derive efflux rates and thus kinetic constants, which would in turn allow for learning about the type of pump inhibition by an EPI, e.g., if it is a competitive inhibitor. Unfortunately, due to the nonlinearity between dye-loading concentrations and fluorescence intensity – maybe due to self-quenching – such conclusions cannot currently be drawn [14, 15]. However, a β-lactam real-time efflux assay has been published, which could overcome these problems. Briefly, by determining the difference between β-lactam influx rate and its spectrophotometrically measured hydrolysis rate, the authors could calculate the corresponding efflux rate and derive classic Michaelis-Menten constants [20, 21].

In the following sections on individual compounds, we will mainly consider a compound as an EPI if it fulfills at least one of the two criteria suggestive of efflux pump inhibition: (i) if it is an "ideal EPI" in the sense of the above definition – basically does not enhance antibiotic susceptibility or dye accumulation in an efflux pump-deficient strain; (ii) if it does inhibit substrate efflux in a real-time efflux assay.

29.3 EPIs Against Gram-Positive Bacteria

29.3.1 Inhibitors of P-Glycoprotein: Reserpine, Verapamil, and Other Synthetic Compounds

Reserpine (Fig. 29.1), a plant alkaloid isolated from *Rauwolfia serpentina* (used for centuries in India against various ailments), has been used as an antihypertensive/antipsychotic drug after its introduction to the market in 1954 [22]. Due to numerous side effects, it is rarely used today. However, reserpine is still an important agent for experimental studies, and its pharmacological activity is based on inhibition of the human vesicular monoamine transporter, a protein belonging to the major facilitator superfamily (MFS). It is capable of transporting monoamine neurotransmitters into synaptic vesicles of presynaptic neurons for later release into the synaptic cleft.

In 1981, Saturai and colleagues discovered that reserpine [23] and the calcium channel blocker verapamil [24] were capable of inhibiting vincristine and vinblastine efflux in multidrug-resistant leukemia cells using radioactively labeled drugs. In 1988, Neyfakh simplified these experiments by demonstrating that reserpine and verapamil enhanced intracellular accumulation of various fluorescent dyes in transformed hamster fibroblasts [25]. In the same year, another group discovered that reserpine inhibits photoaffinity labeling of the mammalian ABC transporter P-glycoprotein, which mediates MDR against numerous xenobiotics including anticancer drugs [26], revealing that reserpine was in fact an inhibitor of P-glycoprotein.

Shortly thereafter, Neyfakh and colleagues laid the foundation for the field of bacterial efflux pump inhibition. They extended their work with reserpine to a prokaryotic organism, namely, a *Bacillus subtilis* strain, which developed an MDR phenotype upon exposure to the cationic dye rhodamine 6G [17]. Using ethidium bromide accumulation and efflux assays, it was demonstrated that reserpine reversed the bacterial MDR phenotype and that ethidium-sensitive strains transformed with a plasmid containing the newly discovered *bmr* gene developed the same MDR phenotype. In fact, this was the first time that the feasibility of inhibition of a bacterial efflux pump (the Bmr transporter) by a drug had been demonstrated. It is intriguing to note that Bmr is an MFS transporter like the vesicular monoamine transporter, markedly increasing the possibility that reserpine is capable of inhibiting both transporters, although the sequence identity is somewhat low. However, unlike these two proton-driven MFS transporters, P-glycoprotein belongs to a completely unrelated

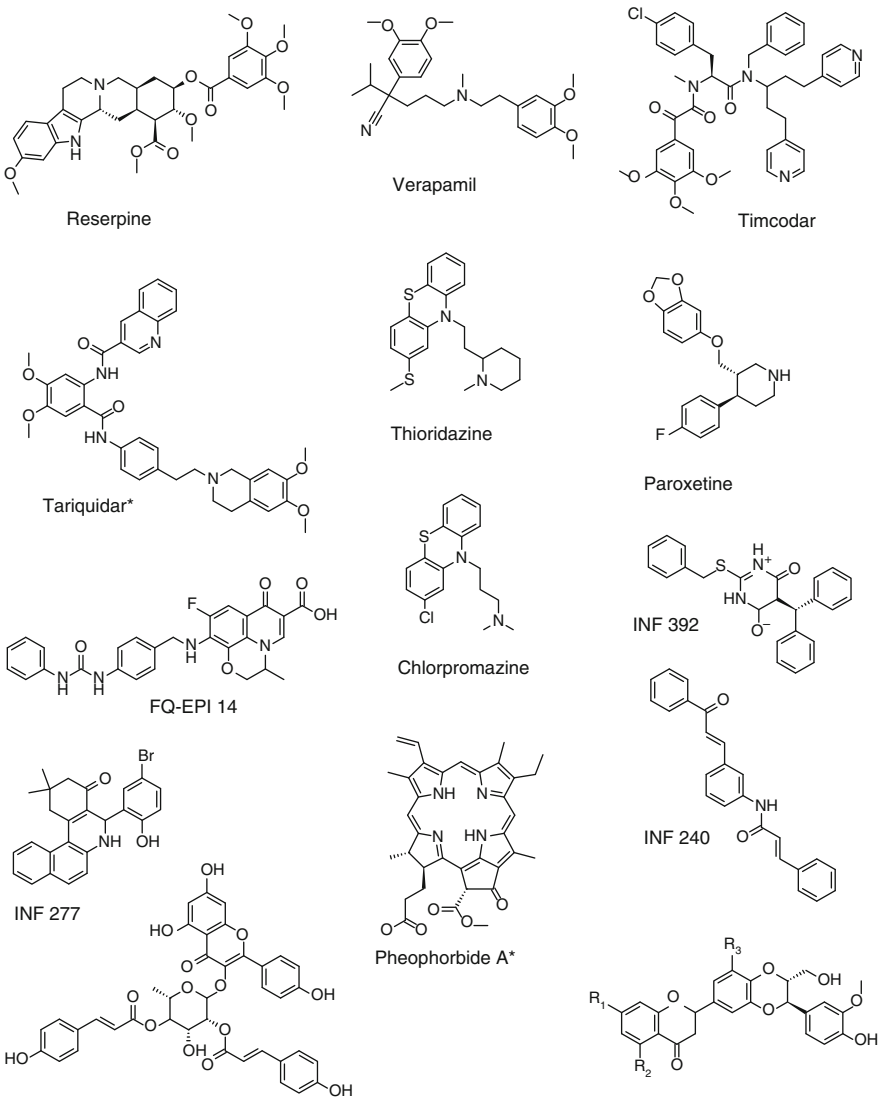

Fig. 29.1 Chemical structures of proven or suggested (*) efflux pump inhibitors with activity against Gram-positive bacteria

transporter of the ATP-binding cassette (ABC) superfamily, making use of ATP hydrolysis for substrate transport. Thus, it remained a mystery why all three transporters could not only recognize a broad range of substrates (although Bmr and other related bacterial MFS transport proteins have a preference for cationic substrates) but were also all inhibited by the same drug. Since reserpine has historically

been such an interesting model EPI and is still widely used today, we now focus in more detail on its role in Gram-positive MDR efflux research, especially in *B. subtilis* and *Staphylococcus aureus*.

The next logical step to learn more about the mechanism of efflux pump inhibition was to select for Bmr-mediated resistance to the EPI reserpine. Such a strain was obtained by reserpine and ethidium bromide co-selection, and it was found that the single amino acid substitution Val286Leu was responsible for the tenfold hyposensitivity to the inhibitory effect of reserpine while the potency of the structurally similar EPI rescinnamine was only weakly affected [27]. Since the amino acid leucine is larger than valine (caused by an additional methylene group), it was assumed that binding of reserpine was hindered by the more marked protrusion of this residue into the substrate-binding pocket, and using radioactively labeled reserpine, it could be demonstrated that its binding to the Bmr transporter was in fact diminished. Conversely, mutating leucine to the smaller glycine had the opposite effect, namely, hypersensitivity to the effects of reserpine and enhanced binding of this drug to Bmr [27].

A few years later, additional substitutions of Bmr residues Phe143 and Phe306 were published by the same group using co-incubation of *B. subtilis* with reserpine and either ethidium bromide, rhodamine 6G, or tetraphenylphosphonium [28]. This time, unlike Val286Leu, the substitutions did not only reduce sensitivity to the inhibitory action of reserpine on drug efflux but also affected Bmr substrate specificity differently. All Phe143 substitutions caused marked reductions in norfloxacin, ethidium bromide, and acriflavine resistance without affecting resistance to rhodamine 6G or tetraphenylphosphonium. Conversely, all Phe306 substitutions reduced resistance to various substrates but not to ethidium bromide [28]. It seemed thus that at least in the case of the Val286Leu mutation, the impact on the EPI effects of reserpine could be clearly separated from the impact on Bmr substrate specificity mediated by the two phenylalanine substitutions. These results were an important step in bacterial EPI research. The authors also concluded from the experimental data that different drugs interacted with different domains of a substrate recognition site of the Bmr transporter and suggested that the old mystery of how transporters could recognize such a wide variety of different substrates, yet their amino acid sequences were so different, might merely be explained by the large size and/or chemical complexity of the substrate recognition site of an MDR efflux pump [28, 29]. Later, when the same group managed to crystallize the Bmr regulator BmrR in complex with tetraphenylphosphonium, it turned out that binding of the substrate was on one side mediated by van der Waals contacts of the phenyl rings of tetraphenylphosphonium with multiple hydrophobic residues, namely, tyrosines, of the binding pocket. On the other hand, electrostatic interactions between the positively charged tetraphenylphosphonium and a negatively charged glutamate suggested a clear structural basis for the observed preference of this regulator for cationic compounds [30]. This landmark study gave a first hint of how the bacterial multidrug recognition mystery could be solved using these two principles. The authors suggested that similar binding principles might be used by other multidrug-binding proteins. In retrospect, such a model offered a brilliant explanation, why the above

Phe143 and Phe306 substitutions of the Bmr efflux pump had such a profound impact on substrate recognition, since they dramatically reduced the hydrophobic interactions between substrates and binding pocket. Yet it was still entirely speculative what the structural properties of an EPI had to be to interfere with substrate transport.

Neyfakh and colleagues [31] extended their work with reserpine to the NorA efflux pump of *S. aureus*, an MFS transporter with 44 % sequence identity to the Bmr protein and similar substrate spectrum (various dyes including ethidium bromide, tetraphenylphosphonium, and fluoroquinolones containing a positively charged piperazine ring and chloramphenicol). It was demonstrated that reserpine in this particular strain had a minimal effective concentration (MEC) of only 0.63 μg/ml in decreasing the ethidium bromide MIC, while at 20 μg/ml (still below reserpine's intrinsic MIC), ethidium bromide susceptibility was reaching the levels of the pump knockout strain, and ethidium efflux was completely abolished in both *bmr*- and *norA*-overexpressing *S. aureus* strains [31].

Subsequently, Mitchell et al. [32] showed that reserpine could also inhibit other *S. aureus* MFS efflux pumps like QacA and QacB using not only ethidium bromide but also other cationic dyes such as 4′,6-diamidino-2-phenylindole, pyronin Y, and trimethylamine-diphenylhexatriene in real-time efflux assays. Reserpine also inhibited the *S. aureus* transporter MepA of the multidrug and toxic compound extrusion (MATE) family [33]. From a clinical standpoint, *S. aureus* is obviously much more important than *B. subtilis*. However, the question arises as to whether the expression levels of the *S. aureus* efflux pumps described above are relevant in clinical isolates. Indeed, a study done by the Kaatz group revealed that roughly half of the clinical *S. aureus* strains tested overexpressed at least one efflux pump [33]; hence using an EPI to reverse resistance might be a feasible strategy. In this study, among strains overexpressing a single efflux pump gene, *norA* was the most abundant (43 %), followed by *norB* (23 %) and *mepA* (10 %). The *qacA* and *qacB* genes were only detected in about 3 % of all strains [33]. This points to the relative importance of the NorA pump in *S. aureus* and has important implications for future EPI testing and design. Additionally, reserpine was shown to be capable of inhibiting MFS transporters in *Streptococcus pneumoniae* (PmrA) [34], *Enterococcus faecalis* (EmeA) [35], *Listeria monocytogenes* (Lde) [36], and various mycobacteria [37–40].

Another EPI which was first tested with P-glycoprotein is the calcium channel blocker verapamil (Fig. 29.1) [24]. Like reserpine, this agent has been frequently used as a model EPI in most of the Gram-positive species mentioned above [32, 35, 38, 41]. Although both compounds are clinically approved drugs, severe side effects at the plasma levels necessary for potentiation of antimicrobial chemotherapy unfortunately do not allow their clinical use. However, although the same situation exists for verapamil and inhibition of P-glycoprotein in anticancer chemotherapy, a randomly controlled trial in patients suffering from advanced lung cancer demonstrated that verapamil at plasma concentrations that caused manageable side effects has led to dramatically improved survival rates in the verapamil arm (in theory verapamil should only have provided minor potentiation of standard

chemotherapy) [42]. While this may sound contradictory, there may actually be logic behind these observations. It is known from animal experiments that verapamil is considerably enriched in lung tissue [43]. Recently, it has been demonstrated that the antimicrobial activity of bedaquiline, a recently approved drug against multidrug-resistant *Mycobacterium tuberculosis*, can be dramatically potentiated by coadministration with verapamil [44, 45]. Verapamil at 50 μg/ml reduced the bedaquiline MIC by eightfold. While the maximal achievable plasma levels of verapamil were about 100 times lower, marked accumulation of this drug in the lungs might actually render the potentiation of bedaquiline activity possible. Overexpression of efflux pump genes from various superfamilies was observed in multidrug-resistant *M. tuberculosis* strains, suggesting that EPIs could help to reverse such drug resistance phenotypes [46].

Several clinically relevant efflux pumps have been identified in *M. tuberculosis* and *Mycobacterium bovis* that were inhibited by verapamil and reserpine, namely, the MFS transporter P55 (mediating resistance to aminoglycosides, rifampicin, and tetracycline), as well as the ABC transporter DrrAB (conferring resistance to ethambutol, streptomycin, and tetracycline) and another ABC transporter, capable of exporting fluoroquinolones and encoded by the *Rv2686c-Rv2687c-Rv2688c* operon [47].

More modern synthetic P-glycoprotein inhibitors like biricodar and timcodar (Fig. 29.1) were also shown to inhibit ethidium efflux in *S. aureus* and to potentiate the activity of various antimicrobials in *S. pneumoniae* and *E. faecalis* [48]. While biricodar was found to be a very weak *S. aureus* EPI with an MEC of >100 μg/ml, timcodar's MEC (1.6 μg/ml) was about fourfold lower than that of reserpine. Timcodar also increases the antimicrobial potency of bedaquiline, clofazimine, and rifampicin against *M. tuberculosis*, although it is still unclear if this effect is due to efflux pump inhibition [49]. Elacridar at 1 μg/ml inhibited ethidium efflux in a NorA-overproducing *S. aureus* strain by about 60 % [50]. Tariquidar (Fig. 29.1) was found to be a strong potentiator of ciprofloxacin accumulation in the same *S. aureus* strain (MEC of ca. 1 μg/ml); however no efflux assays or antimicrobial susceptibility assays in a reference strain with reduced or abolished *norA* expression were carried out to rule out any non-efflux mechanisms [51]. Timcodar, elacridar, and tariquidar have not yet been clinically approved. But safety and clinical achievement of plasma levels needed for potentiation of antibacterial activity have at least been demonstrated for tariquidar in humans [52]. While clinical trials of tariquidar against multidrug-resistant cancers have largely failed [53], it remains as one of a few agents in the pipeline for potential EPI coadministered with fluoroquinolones against Gram-positive bacteria.

Omeprazole, a proton pump inhibitor and inhibitor of P-glycoprotein, was found to possess only half the activity of reserpine in potentiating the antimicrobial activity of various fluoroquinolones in a NorA-overproducing *S. aureus* strain [54]. The anti-inflammatory drug celecoxib, an inhibitor of both cyclooxygenase-2 and P-glycoprotein, is another rather weak EPI reported to be capable of inhibiting ethidium efflux in a *norA*-overexpressing *S. aureus* strain [55].

29.3.2 Psychotropic Drugs: Neuroleptics and Antidepressants

A fascinating yet mysterious feature in medical pharmacology is the link between psychotropic drugs and their antimicrobial and/or synergistic activity. Chlorpromazine (Fig. 29.1), a dopamine antagonist and one of the first clinically available neuroleptics of the phenothiazine class, has been called a breakthrough in medicine; since after its introduction to the market in the 1950s, symptoms of psychotic patients could be improved dramatically [56]. It is a relatively weak neuroleptic and over the years has been replaced by more efficient drugs.

However, with the advent of bacterial MDR, chlorpromazine and other phenothiazine neuroleptics (e.g., thioridazine and promethazine) as well as some closely related thioxanthenes (e.g., clopenthixol) have experienced renewed interest from researchers mainly because of their antimicrobial activity [57–62]. Moreover, using standard ethidium efflux assays, several studies have recently been able to demonstrate that chlorpromazine and thioridazine (Fig. 29.1) are in fact EPIs capable of inhibiting efflux in NorA- or MepA-overproducing *S. aureus* strains [63, 64]. While thioridazine and another phenothiazine (prochlorperazine) were equipotent to reserpine in inhibiting ethidium efflux of a NorA-overproducing strain, chlorpromazine was about threefold less potent, and the thioxanthenes *cis*(Z)-flupentixol and *trans*(E)-flupentixol were about twofold less potent, based on the half-maximal inhibitory concentration (IC_{50}) values [63]. But even the more potent phenothiazines cannot achieve sufficient plasma levels for EPI activity. Hence, Kaatz and colleagues [65] synthesized 1,4-benzothiazine derivatives retaining the phenothiazine core structure and determined that one compound was twice as effective as reserpine in reducing norfloxacin MICs.

Phenothiazines have also been shown to inhibit ethidium efflux in various mycobacteria [18, 66, 67]. Especially, thioridazine has garnered considerable interest because a small compassionate use study has demonstrated favorable outcome in extensively drug-resistant tuberculosis patients when coadministered with linezolid and moxifloxacin [68]. It is currently unclear whether the EPI activity of thioridazine played a role in this result or whether other antimicrobial or synergistic mechanisms were responsible. Another compassionate use study demonstrated temporary improvement in extensively drug-resistant tuberculosis patients on thioridazine monotherapy, yet fatal outcome was not avoided [69]. This might have been due to the advanced stage of disease, yet it cannot be excluded that the outcome was due to the lack of combination therapy efficacy. However, even thioridazine monotherapy was capable of reducing bacterial lung loads in a tuberculosis mouse model ~2.5-fold after 2 months [70].

It has been documented by the Amaral group that thioridazine and chlorpromazine are equipotent antitubercular drugs against multidrug-resistant *M. tuberculosis* strains with maximal growth inhibition occurring at 12 μg/ml, yet the authors preferred thioridazine over chlorpromazine because of markedly lower side effects [71]. However, based on *in vitro* activity data, thioridazine should not reach sufficient plasma levels in humans. Nevertheless, another study suggested that thioridazine,

like chlorpromazine [72], might be concentrated inside human macrophages since it could kill intracellular multidrug-resistant strains below the maximal plasma levels (<0.5 μg/ml) [73]. As such, EPI activity of thioridazine seems to be feasible at clinically achievable concentrations.

A recent study using different multidrug-resistant *M. tuberculosis* strains (outside the macrophage) proved that ethidium efflux was completely inhibited by thioridazine at 7.5 μg/ml and that antimycobacterial activity of amikacin, isoniazid, and rifampicin was potentiated in most strains at 3.8 μg/ml [67]. It is interesting to note that phenothiazines have also been proposed to inhibit Ca^{2+} and K^{+} efflux from the phagolysosome of human macrophages, subsequently leading to acidification and enhanced killing of the bacteria [74]. So the inhibition of both bacterial and mammalian efflux pumps might make it almost impossible for intracellular bacteria to escape from the dual action of thioridazine, namely, enhanced antimicrobial activity and stimulation of phagolysosomal killing (the latter being completely independent of the bacterial resistance level). While this sounds in theory attractive, more studies are warranted to further prove whether these mechanisms actually take place in the human host and that patients can be cured by the addition of thioridazine as a second-line antitubercular drug. Nevertheless, thioridazine is obviously one of the most exciting compounds in the list of Gram-positive EPIs presented here, since no other agents come as close to the possibility of clinical applicability.

Another group of psychotropic drugs with EPI activity are the selective serotonin reuptake inhibitors (SSRIs). Paroxetine (Fig. 29.1) and other SSRIs were found to interfere with the NorA and MepA efflux pumps in *S. aureus* [75]. However, paroxetine and the equipotent SSRI NNC 20-7052, when compared with reserpine, displayed a twofold lower potency with regard to ethidium efflux inhibition in a NorA-overproducing strain. Subsequently, the Kaatz group tested a number of derivatives. One of the EPIs with improved activity (No. 16) was found to be equipotent to reserpine [76].

29.3.3 EPIs from Natural Sources

One of the most potent EPIs from natural sources or plant extracts discovered so far is kaempferol-3-O-α-L-(2,4-*bis*-E-*p*-coumaroyl)rhamnoside (Fig. 29.1) [77]. It was discovered in an ethanolic extract from *Persea lingue*, a plant used in traditional medicine by the Chilean Huilliche people. Based on the IC_{50} (2 μM), the EPI potency of this compound in an ethidium efflux assay is ca. fivefold higher than that of reserpine using a NorA-overproducing *S. aureus* strain. In the same strain, the MIC of ciprofloxacin was reduced eightfold at 1.56 μg/ml.

Another medical plant, *Berberis fremontii*, used traditionally by Native Americans, is a berberine producer. Berberine displays only weak antimicrobial activity, obviously due to MDR efflux. However, a potent EPI extracted from *Berberis fremontii*, namely, the flavolignan 5′-methoxyhydnocarpin-D (Fig. 29.1),

acted as a potentiator of berberine activity [78]. In a wild-type *S. aureus* strain, it reduced at 10 μg/ml the MICs of norfloxacin, ethidium bromide, and berberine by, respectively, four-, eight-, and 16-fold. In the Δ*norA* strain, 5′-methoxyhydnocarpin-D had no effect on the norfloxacin MIC, but reduced the MIC values of ethidium bromide and berberine by, respectively, 32- and 128-fold, leading the authors to propose that 5′-methoxyhydnocarpin-D has EPI activity against another efflux pump [78]. This agent at 30 μg/ml almost completely inhibited berberine efflux in a wild-type *S. aureus* strain. Interestingly, although NorA substrates are typically lipophilic cations, based on its pKa value, 5′-methoxyhydnocarpin-D was suggested by the authors to be actually an EPI which exists as a mixture of an anion and a neutral molecule at physiological pH and should hence not be a typical pump substrate [78].

The same group carried out systematic structure-activity relationship studies and discovered that a derivative, 5,7-deoxyhydnocarpin-D (Fig. 29.1), displayed a tenfold higher potency in reducing the berberine MIC [79]. Another ingredient from *Berberis* plants, the porphyrin pheophorbide A (Fig. 29.1), also exhibited considerable potency by reducing the berberine MIC for an *S. aureus* wild-type strain at 0.5 μg/ml [80]. Ciprofloxacin MICs were reduced by >16-fold [81], although it is unclear if this was due to efflux pump inhibition.

Two resin glycosides extracted from morning glory, orizabins IX and XV, were at a concentration of 5 μM almost twofold more potent than reserpine in inhibiting ethidium efflux in a NorA-overproducing *S. aureus* strain, and, for the same strain, orizabin IX at 1 μg/ml decreased norfloxacin MIC by 16-fold [82]. At higher concentrations, this agent showed poor solubility.

An EPI isolated from *Artemisia absinthium* is 4′,5′-O-dicaffeoylquinic acid, which at 10 μg/ml reduced the MIC values of ethidium bromide, berberine, and fluoroquinolone by, respectively, four-, >eight-, and two- to fourfold for a NorA-overproducing *S. aureus* strain [83]. At the same concentration, berberine efflux was almost completely abolished in *S. aureus* and *E. faecalis* [83]. Piperine, an alkaloid that can be extracted from black pepper (*Piper nigrum*), was shown to have moderate activity against a NorA-overproducing *S. aureus* strain [84]. This agent also inhibited ethidium efflux in *M. smegmatis* [85] and reversed rifampicin resistance in a *M. tuberculosis* strain overexpressing the *Rv1258c* MDR efflux gene [86]. In addition, piperine showed synergistic activity with rifampicin in a murine pulmonary tuberculosis model, where it stimulated proliferation of T and B cells, increased Th-1 cytokines, and enhanced macrophage activation [87]. Farnesol, a plant metabolite, is another compound capable of inhibiting ethidium efflux in *M. smegmatis* [88]. At 32 μg/ml, it abolished ethidium efflux almost completely and reduced the ethidium bromide MIC by eightfold, while reserpine at the same concentration only resulted in a twofold reduction [88].

Many more EPIs from natural sources have been reported; however, most of them were only moderately active and did not surpass the EPI activity of reserpine. Flavonoids with moderate EPI activity against the NorA efflux pump of *S. aureus* were reported from *Lupinus argenteus* (biochanin A) [89], green tea (epigallocatechin gallate) [90], and *Praxelis clematidea* (tetramethylscutellarein) [91]. Other rather modest NorA EPIs encompass a kaempferol glycoside (tiliroside) from

Herissantia tiubae [92], capsaicin from hot chili [93], and diterpenes from *Rosmarinus officinalis* [94].

29.3.4 Other Synthetic EPIs

A large number of synthetic EPIs against Gram-positive efflux pumps have been described in the literature. Here we only focus on the most potent ones.

FQ-EPI 14 (Fig. 29.1) belongs to a series of fluoroquinolone derivatives and is one of the most potent *S. aureus* EPIs discovered so far [95]. By linking a bisarylurea moiety to the ofloxacin core, FQ-EPI 14 at 0.5 μM was dramatically more potent in ethidium efflux inhibition than reserpine in strains overproducing NorA (>70 % inhibition) or MepA (>50 % inhibition). Among some quinolone ethyl ester derivatives, the compounds 8f, 12d, and 13d (sharing as key feature for NorA inhibition a thiopyranopyridine moiety in the C-7 position) displayed slightly more inhibition activity than reserpine in the standard ethidium assay [96], whereas some quinoline derivatives were equipotent (compound 28f) or slightly more potent (compound 29f) [97]. An innovative follow-up study by the same team focused on structural optimization of 2-phenylquinolines using ligand-based pharmacophore modeling, since molecular docking studies were not feasible due to the lack of a NorA crystal structure. This study was successful in identifying two compounds (3i and 3s) which possessed IC_{50} values for ethidium efflux that were about 2.5-fold lower than reserpine [98].

A high-throughput screening approach identified several compounds capable of potentiating ciprofloxacin activity in a NorA overproducer. INF 392 (Fig. 29.1) was the most potent one, reversing ciprofloxacin resistance in *S. aureus* by fourfold at 0.2 μg/ml, while other EPIs like INF 240 and INF 277 (Fig. 29.1) were about twofold and fourfold weaker in potency [99]. INF 55, a somewhat weaker nitro-indole-derived EPI discovered by the same group, was subsequently linked to the natural antimicrobial agent berberine to generate an antibiotic/EPI hybrid, hence avoiding typical pharmacokinetic problems arising with coadministration of such drugs for antimicrobial potentiation. One of these antibiotic/EPI hybrids, SS14, displayed dramatically enhanced antimicrobial activity in a NorA overproducer with its MIC of ca. 20 μM in comparison with berberine MIC of 650 μM [100]. Lastly, two piperine analogues, SK-20 and SK-56, were found to be potent EPIs with an MEC of 6.25 μg/ml [101].

29.3.5 Current Status and Future Outlook Regarding Gram-Positive EPIs

As described above, the last decades have witnessed the discovery of a lot of EPIs with activity against Gram-positive bacteria, including such with marked clinical importance, e.g., *S. aureus* and *M. tuberculosis*. While we do know that efflux

pumps, especially of the MFS-type, play a role in drug resistance and that this resistance can at least partly be reversed by the use of EPIs, their development for clinical use is currently hampered by several problems. First, the field is challenged by the lack of crystal structures of important efflux pumps, especially of MFS transporters like staphylococcal NorA. Although homologues of multidrug MFS transport proteins have been crystallized (mainly in Gram-negative bacteria [102, 103]), revealing the precise molecular mechanisms of efflux pump inhibition will have to rely on high-resolution crystal structures of relevant Gram-positive MFS proteins, including protein/substrate as well as protein/EPI complexes. Second, although the landmark studies by Neyfakh and colleagues [17, 27, 104, 105] have given us a hint on how an EPI like reserpine might inhibit substrate transport in an MFS transporter by interaction with certain amino acid residues, basically no EPI-relevant loss-of-function mutations have been reported in pump proteins of clinical importance like NorA. These two issues severely limit our ability for structure-based design of new powerful EPIs, due to the lack of insight into the molecular mechanisms behind efflux pump inhibition. Third, although a wealth of clinically approved drugs have been found to possess EPI activity, none of them achieve plasma levels needed for synergy with antimicrobials, as derived from *in vitro* checkerboard assays. However, as described above, thioridazine may still be of clinical relevance, since it may accumulate inside the macrophage as it markedly enhances intracellular killing of *M. tuberculosis* at clinically achievable plasma levels, although efflux may only partly contribute to the observed effects. Clearly, more clinical trials are needed to demonstrate if thioridazine as part of a second-line antimycobacterial regimen is beneficial to patients suffering from extensively drug-resistant tuberculosis. Accordingly, other clinically used drugs with only moderate EPI activity should also be checked more closely for accumulation in different body compartments. For example, verapamil might be another candidate EPI with *in vivo* activity against *M. tuberculosis* because of its marked accumulation in the lung tissue. Another EPI, tariquidar, has been tested in humans and achieves sufficient plasma levels, yet its fate regarding further clinical trials is unclear.

29.4 EPIs Against Gram-Negative Bacteria

As described above, EPI research in Gram-positive bacteria has due to their important role in MDR efflux mainly focused on MFS transporters and to a lesser degree on MATE and other transporter families. However, the picture looks completely different in Gram-negative bacteria, due to the special double-membrane structure of the cell envelope [106, 107], where the MFS transporters do play a role as single-component pumps within the cytoplasmic membrane, but RND efflux pumps are in fact the most important contributors to MDR. The tripartite RND pumps have double-membrane-spanning assemblies, and their main role is to immediately capture xenobiotics in the periplasm, before they can traverse the cytoplasmic membrane and cause harm to the cell [108, 109]. Hence, the RND pumps and the outer

membrane permeability barrier act in synergy and constitute an early defense line against potentially bactericidal agents. Since single-component efflux pumps in the cytoplasmic membrane can only transport substrates from the cytoplasm to the periplasm, they heavily rely on cooperation with the RND pumps, whose task is then to clean the periplasm from these substrates [109].

Contrary to clinically relevant MFS transporters like NorA in Gram-positive bacteria, whose substrate spectrum is mainly confined to lipophilic cations (e.g., positively charged fluoroquinolones), the substrate spectrum of typical RND efflux pumps is much wider, including neutral and acidic compounds [110]. Hence, the recognition of such a wide variety of substrates by an RND pump might seem to be more complicated than in the case of the more selective MFS transporters, where a combination of hydrophobic and electrostatic interactions (the latter being mediated by negatively charged amino acid residues) dominate the recognition process as brilliantly demonstrated by Neyfakh and colleagues when they crystallized the BmrR regulator in complex with tetraphenylphosphonium and adopted this binding mode as a basic model for drug binding by Bmr and other multidrug transporters [30]. Due to the suggested complexity of RND transporter substrate recognition, it seems thus that EPIs against the broad-spectrum RND efflux pumps might be hard to find.

29.4.1 Phenylalanine-Arginine β-Naphthylamide

Despite the challenge to identify inhibitors of RND pumps, a team of scientists at Microcide published in 1999 a report on an agent MC-207,110 (i.e., phenylalanine-arginine β-naphthylamide [PAβN]; Fig. 29.2), which was demonstrated to potentiate the activity of levofloxacin against *Pseudomonas aeruginosa* strains overproducing either the MexAB-OprM, the MexCD-OprJ, the MexEF-OprN, or the MexXY-OprM RND efflux pumps [111]. MexAB-OprM is typically constitutively produced in wild-type strains, whereas MexCD-OprJ, MexEF-OprN, and MexXY-OprM are often found to be overproduced in clinical isolates [109]. The discovery was enabled by high-throughput screening of a synthetic compound and fermentation extract library for potentiation of the antimicrobial activity of levofloxacin.

PAβN at 10 μg/ml decreased levofloxacin MICs of the MexAB-OprM overproducer by eightfold [111]. Synthesis of the derivatives led to the discovery of compounds with an up to fourfold increase in potency. A follow-up study demonstrated that at 20 μg/ml PAβN was capable of massively potentiating the activity of a wide variety of antimicrobial drugs in the MexAB-OprM overproducer, including chloramphenicol (128-fold in MIC reduction), erythromycin (128-fold), and sparfloxacin (32-fold), in line with the behavior of a broad-spectrum EPI [9]. However, it came as a surprise that compounds like carbenicillin (fourfold) and tetracycline (eightfold) experienced only modest MIC reductions, whereas the MexAB-OprM-deficient strain produced a huge MIC drop (up to 512-fold). A mystery was however

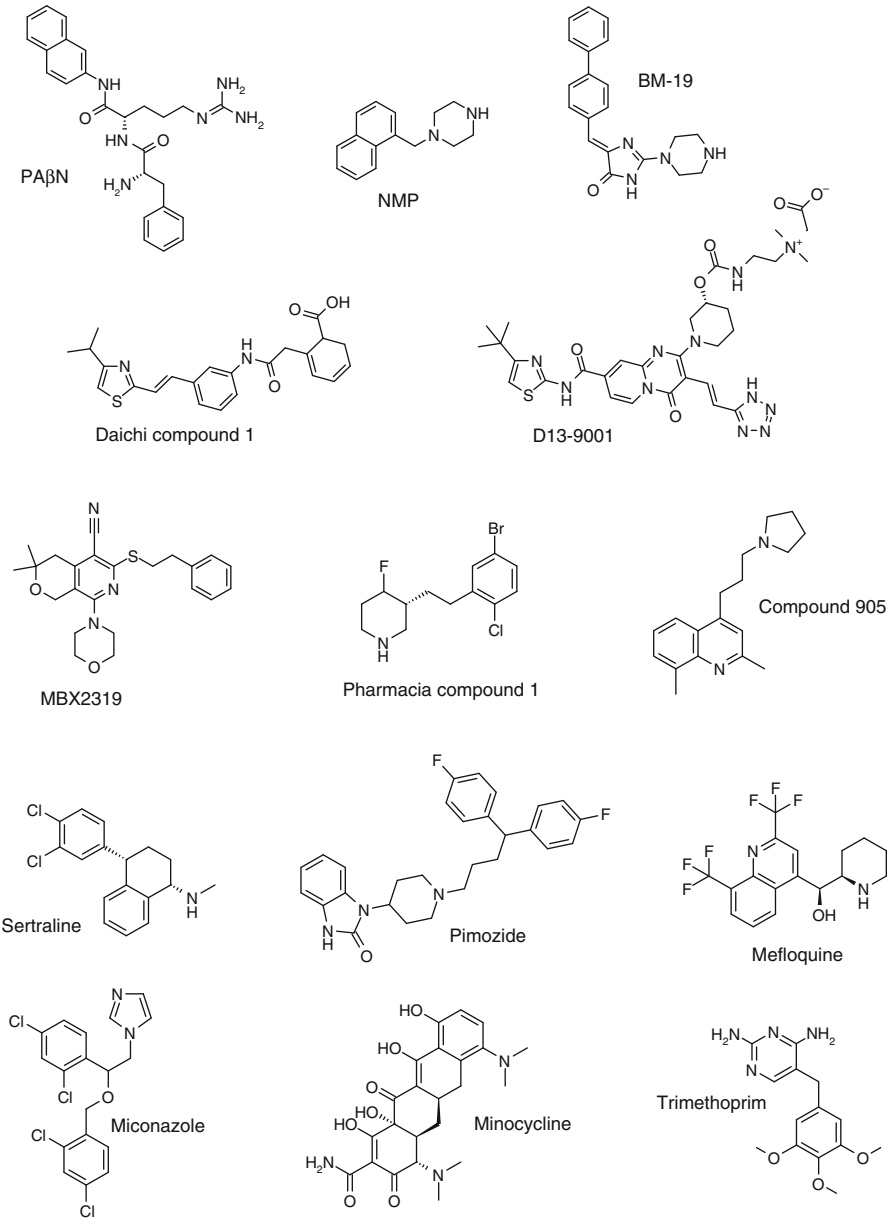

Fig. 29.2 Chemical structures of synthetic efflux pump inhibitors with activity against Gram-negative bacteria

concerned with ethidium bromide. Although the MIC difference between the MexAB-OprM overproducer and its genetically inactivated counterpart was large (32-fold), PAβN had no impact on the ethidium bromide MIC in the MexAB-OprM overproducer [9]. This clearly suggested that PAβN does not simply shut the efflux

pump down completely, but that it inhibits substrate transport in a more dynamic way dependent upon the chemical nature of the substrate. The authors who discovered PAβN also showed that PAβN does not affect the proton gradient of the cells, while a later study by the use of a LacY transport assay confirmed that proton gradient-dependent LacY transport was only marginally reduced in the presence of PAβN [14].

The Microcide group carried out the first fluorescent efflux inhibition assay in an AcrAB-TolC-overproducing *E. coli* strain using the environment-sensitive membrane probe NPN. After loading of these whole cells with the dye, efflux was triggered either by addition of glucose alone or by a combination of glucose with variable amounts of the EPI [9]. Instead of PAβN (which was described as unsuitable because of its intrinsic fluorescent properties), the EPI MC-002,595 was used and a dose-dependent inhibition of NPN efflux was recorded. The MEC was 32 μg/ml and efflux was almost completely inhibited at 128 μg/ml. Using a nitrocefin hydrolysis assay, the authors then showed that PAβN acted as a mild permeabilizer (half-maximal activity was observed at 70 μg/ml) of the outer membrane, but only in the absence of a functional MexAB-OprM pump [9]. However, the permeabilization effect was completely abolished by addition of 1 mM Mg^{2+} [9]. Accordingly, a recent study in *P. aeruginosa* PAO1 demonstrated that PAβN at 25 μg/ml was capable of potentiation of antimicrobial activity of the bulky antibiotic vancomycin (which cannot normally penetrate the outer membrane efficiently), an effect that was prevented by addition of 1 mM $MgSO_4$ [112].

What is the molecular mechanism behind PAβN efflux pump inhibition? A report was published in 2005 by Yu et al. [113] describing the crystallization of the *E. coli* AcrB Asn109Ala mutant protein in complex with PAβN in the R32 space group. However, only a year later, an asymmetric crystal structure derived from monoclinic crystals of AcrB was published by two research groups, and it was suggested that this was the physiologically relevant structure depicting a succession of distinct transport stages [114, 115]. While Murakami et al. [115] were capable of co-crystallizing AcrB and two substrates (doxorubicin and minocycline), no reports have been available to show the crystallization of AcrB in complex with PAβN in the asymmetric conformation. Although the physiological relevance of the AcrB crystal grown in trigonal space group R32 is currently unclear, it is interesting to note that in this conformation, PAβN was located inside a pocket close to an assembly of hydrophobic residues around Phe666 that was later named the "proximal binding pocket," after the two substrates rifampicin and erythromycin could be co-crystallized in this region [116]. PAβN was found by Yu and coworkers [113] to interact with Phe664 and Arg717. Site-directed mutagenesis of Phe664 and Phe666 to alanine reduced the MICs of substrates like acriflavine, ethidium bromide, sodium dodecyl sulfate, and taurocholic acid (but not of rhodamine 6G and tetracycline); in contrast the ARg717Ala mutation mainly impacted the novobiocin MIC. However, the impact of these mutations on the MIC of PAβN or its ability to potentiate the activity of different antibiotics or to inhibit dye efflux was not examined. It is thus unclear if the binding of PAβN to this proximal binding pocket has any relevance for the inhibition of substrate transport in AcrB.

On the other hand, evidence from another study examining a mutation in an MdtF (a close homologue to AcrB) pump overproducer suggests that the so-called distal substrate-binding pocket around Phe610 might actually play a role in the interaction between AcrB and PAβN [117]. In this study, two different *E. coli* strains were obtained after exposure of a Δ*acrB*Δ*acrF* strain to levofloxacin. Both strains overexpressed the *mdtEF* genes leading to an MDR phenotype. However, the strains differed in their substrate specificity. One strain was found to have acquired an MdtF Val610Phe mutation (homologous to Val612Phe in AcrB) and displayed four-fold lower PAβN MICs as well as lower macrolide MICs and a dramatically higher (16-fold) linezolid MIC compared with the strain with the wild-type MdtF sequence [117]. Making use of the fact that PAβN is an MdtF substrate and is intracellularly cleaved to yield the highly fluorescent β-naphthylamine, intracellular PAβN accumulation could be followed over time. And indeed, the Val610Phe mutant demonstrated considerably more intracellular PAβN accumulation than its wild-type counterpart, suggesting that transport of PAβN by the MdtF pump protein was obviously markedly inhibited by this mutation. In comparison, the homologous Val612Phe AcrB mutation caused an eightfold reduction in the PAβN MIC; however, its EPI activity was not altered (J. Bohnert, S. Schuster, and W. V. Kern, unpublished data).

Site-directed mutagenesis of the phenylalanine residues within the distal binding pocket (called the hydrophobic trap [118]) has revealed that Phe610Ala is the mutation that has the most dramatic impact on substrate MICs including PAβN [119]. Since most substrate MICs are almost reduced to the level for an *acrB* deletion strain, the change in EPI efficacy of PAβN can unfortunately not be assessed. More structural and site-directed mutagenesis data are needed to gain a better insight into the molecular mechanisms behind efflux pump inhibition by PAβN. The innovative Neyfakh strategy of generating resistance to the effects of an EPI by exposing bacteria to an EPI/substrate combination has failed with PAβN [119]. Even using random mutagenesis of AcrB has not been a successful strategy so far [120].

Although a number of PAβN derivatives with reduced toxicity have been synthesized, and one of the improved compounds, MP-601,205, had reached phase I clinical trials [7], it seems that the further development of this series has been put on hold.

29.4.2 1-(1-Naphthylmethyl)-Piperazine

This agent (NMP; Fig. 29.2), besides PAβN, is another EPI, widely used as a reference compound in efflux research studies. It was discovered by our group after screening of a compound library of small *N*-heterocyclic aromatic compounds for EPI activity [11]. Since it is presumably a strong serotonin receptor agonist based on structural similarity with another piperazine [121], it will not find clinical application as an EPI. Although its potency in reducing the levofloxacin MIC is weaker than that of PAβN (NMP concentrations of 100 μg/ml and PAβN

concentrations of 25 μg/ml are about equipotent, reducing levofloxacin MICs eightfold in an AcrAB-TolC-overproducing *E. coli* strain) [11], it has several features that make it a useful model EPI. First, compared with PAβN, at typical working concentrations, it has almost no impact on antimicrobial MICs (the only exception being rifampicin, whose MIC is reduced by fourfold with NMP at 100 μg/ml) in RND efflux pump-deficient strains, coming close to what we have defined as an "ideal EPI." In contrast, PAβN at 25 μg/ml reduces most antimicrobial MICs at least twofold and the rifampicin MIC even more than 128-fold in RND efflux pump-deficient strains. Second, contrary to PAβN, NMP does markedly inhibit the efflux of ethidium and can thus easily be used as an EPI in standard ethidium bromide MIC or fluorescent assays. In an ethidium accumulation assay using an AcrAB-TolC overproducer, the MEC of NMP was found to be 25 μg/ml [11].

NMP has been demonstrated to potentiate the activity of various antimicrobials against AcrAB-, AcrEF-, and MdtEF-overproducing *E. coli* strains [11, 117] as well as in other *Enterobacteriaceae* [122, 123] and *Acinetobacter baumannii* [124]. Contrary to PAβN, however, it has no EPI activity against *P. aeruginosa*, which may be due to the differences in the structure of the outer membrane.

Furthermore, RND Efflux pumps have been implicated in playing a role in virulence [125–127] and biofilm formation [128], and NMP has been demonstrated to decrease the production of cholera toxin [129] and to decrease biofilm formation [130].

What do we know about the molecular mechanism by which NMP inhibits efflux pumps? AcrB has never been crystallized in complex with NMP; however, molecular dynamics simulations by Vargiu et al. [131] have suggested that both NMP and PAβN bind initially to the distal binding pocket of AcrB in contact with phenylalanine residues of the hydrophobic trap. Thereafter, both EPIs were found to move toward a loop around Phe617, which has also been called the switch loop and has been implicated in substrate transport [132]. In contrast to the EPIs, all regular substrates stayed within the distal binding pocket. Based on the simulation study, NMP was found to be the only EPI capable of crossing the switch loop. After the traverse, the naphthyl ring of NMP interacted with Phe617, Phe664, and Phe666 of the proximal binding pocket, whereas the positively charged piperazine ring interacted with waters and the negatively charged Asp681. Interestingly, in the co-crystallization of AcrB and PAβN in the R32 space group, published by Yu and coworkers [113], PAβN was found to bind very closely to this position in the proximal binding pocket, whereas the molecular dynamics simulations did not suggest such a position for PAβN. When NMP stayed on the side of the distal binding pocket in other simulation runs, the naphthyl ring interacted with Phe136, Phe615, and Phe617, whereas the piperazine ring interacted with waters and Gln89 and Arg620. Although site-directed mutagenesis has suggested a role of various phenylalanine residues within the hydrophobic trap of the distal binding pocket in substrate transport [15, 117], a specific effect on EPI activity has not been demonstrated. Moreover, none of the charged or polar residues implicated above have ever been examined for their role in NMP efficacy.

To gain more insight into the mechanisms of mode of action of NMP, our team exposed an AcrAB-TolC-overproducing *E. coli* strain to a combination of NMP and linezolid in the hope of selecting mutants that would point to the amino acid residues needed for NMP activity. In fact, a mutant was obtained by serial selection that was partially resistant to the effect of NMP, and sequencing of the *acrB* gene revealed a point mutation leading to a change of AcrB residue 288 from glycine to serine [120]. At the same time, however, this mutant displayed altered susceptibility to various other pump substrates, and since introduction of this mutation into the wild-type *acrB* gene could only reproduce part of the phenotype, it was decided to focus solely on random *in vitro* mutagenesis of *acrB* and single-step selection of mutants resistant to the action of NMP in combination with linezolid. Even so, the Gly288 residue of AcrB was found to be mutated in about 44 % of all sequenced *in vitro*-selected mutants, mostly to serine but some of them also to methionine or cysteine; however the effect on NMP efficacy was limited. In two mutants the combined appearance of the Gly141Asp and Asn282Tyr mutations was observed [120]. When this double mutation was introduced into the wild-type AcrB, this mutant demonstrated markedly reduced NMP efficacy in combination with linezolid. Typically, NMP at 100 μg/ml reduces the linezolid MIC of an AcrAB-TolC overproducer by 32-fold. In the case of the double mutant, however, NMP could potentiate the antimicrobial action of linezolid only by fourfold, while the susceptibility of the mutant to linezolid was unchanged [120]. In short-term (30 min) linezolid accumulation assays, the EPI effect of NMP was even almost completely lost. Interestingly, the NMP efficacy was not significantly reduced with regard to other substrates (apart from the fluorescent dye Hoechst 33342, whose accumulation was about 25 % reduced in the presence of NMP, compared with the wild-type strain, and apart from a 50 % reduced efficacy of NMP in short-term levofloxacin accumulation assays). Thus, this effect was highly dependent upon the substrate linezolid that had been used for selection of the random mutants with NMP. This makes a strong case for the hypothesis (already mentioned above in the context of the incapability of PAβN to inhibit transport of ethidium) that the action of many of our currently known EPIs does not involve a complete shutdown of an efflux pump, but rather a dynamic and flexible interaction among EPI, pump protein, and substrate.

Although mainly limited to a single substrate, this dramatic reduction in NMP efficacy allows us to map the observed mutations to AcrB and to come up with a model of how the interaction with NMP may take place. Interestingly, all four observed substituted residues line up next to the phenylalanine residues Phe178 and Phe610 within the distal binding pocket, suggesting that this region is the hot spot for interactions with NMP and/or linezolid [120]. This is also the area in which a gain-of-function mutation (Gly288Asp) was recently discovered that conferred increased ciprofloxacin resistance [133]. Curiously, Gly288Ser has also been demonstrated in our random mutagenesis study to be a gain-of-function mutation for ethidium transport [120].

It might be conceivable that NMP exerts its inhibitory action by an interaction of its naphthyl ring with Phe178 and Phe610 and of its piperazine ring with the polar Asn282. It is well known from our initial structure-activity relationship studies that the unsubstituted piperazine ring of NMP is essential for EPI activity and that this

activity is almost completely lost by any substitutions [11]. In the double mutant, the change of Gly141 to the negatively charged aspartic acid residue might cause a strong interaction with the positively charged piperazine ring of NMP. This pull from its original position may render NMP less likely of interfering sufficiently with the transport of linezolid, although it is currently not known, why the transport of other substrates – even small ones like chloramphenicol – is less affected. We also do not know how these mutations might interact with linezolid binding to the distal pocket – if anything they seem to rather improve linezolid transport than to slow it down. It seems that the interaction of NMP with the suggested residues might be relatively flexible, reflecting the fact that NMP is a rather small EPI with moderate activity, which, in comparison with other EPIs, has been predicted to have a lower AcrB binding affinity [134].

29.4.3 Other Piperazines

As previously described, various derivatives of NMP have been synthesized in our laboratory. While elongation of the spacer between the naphthyl core and the piperazine ring has been found to give slightly enhanced potency, none of these derivatives were capable of reaching the potency of PAβN [11]. However, in a recent study, we discovered among several piperazine derivatives of arylideneimidazolones an EPI, named BM-19 (Fig. 29.2), capable of reversing at a concentration of 50 μM (ca. 20 μg/ml) the levofloxacin MIC in an AcrAB-TolC-overproducing *E. coli* strain by eightfold, comparable with PAβN [135]. The levofloxacin MIC in a Δ*acrB* strain was not affected by addition of BM-19. In a Nile red real-time efflux assay, the IC_{50} of this compound was found to be 50 μM as well.

The biggest surprise was, however, the fact that BM-19 and some derivatives were acting as environment-sensitive fluorescent membrane probes, a feature which could be used in a classic real-time efflux assay [135]. This clearly demonstrated that BM-19 is in fact a substrate of the AcrAB-TolC efflux system. PAβN has fluorescent properties as well (due to the intracellular cleavage and production of β-naphthylamine); however it cannot be used as environment-sensitive dye. In contrast, BM-19 is the first EPI with a dual function as membrane probe, which can be used for influx as well as efflux assays. This makes it an excellent tool for exploring the impact of various AcrB mutations on both EPI and substrate transport [135].

29.4.4 Daichi Compound 1 and Optimization to D13-9001

The scientists of the Daichi company, in collaboration with Microcide scientists, discovered compound 1 (Fig. 29.2), a rather potent EPI which sensitized at 0.63 μg/ml a *P. aeruginosa* strain to the antimicrobial action of levofloxacin (eightfold MIC reduction) [136]. It selectively inhibited the MexAB-OprM efflux pump. Since the *in vivo* activity and water solubility were very poor, a long optimization process was

carried out, leading to a series of pyridopyrimidines, of which D13-9001 (Fig. 29.2) was revealed to be one of the most promising candidate agents [137]. D13-9001 still retained good potency (the levofloxacin MIC was decreased eightfold at 2 μg/ml in a *P. aeruginosa* strain overproducing the MexAB-OprM system), had good water solubility, and produced favorable survival rates in a rat pneumonia model in combination with aztreonam. Contrary to PAβN, D13-9001 refractory MexB mutants could be obtained; however details were not published [7].

Moreover, D13-9001 has garnered considerable interest, because it is still the only EPI which was co-crystallized with both the AcrB and MexB pump proteins in an asymmetric crystal conformation by the Yamaguchi group (Fig. 29.3) [118]. The authors showed that the hydrophobic core part of D13-9001 (a *tert*-butyl thiazolyl aminocarboxyl pyridopyrimidine moiety) interacted with the phenylalanines of the hydrophobic trap (Phe136, Phe178, Phe610, Phe615, and Phe628) at the bottom of the distal binding pocket of AcrB, whereas the tetrazole ring and the piperidine aceto-amino ethylene ammonioacetate moiety interacted with the charged and/or hydrophilic residues Asn274, Arg620, Gln176, and Ser180. Similar interactions were found for MexB. Binding assays demonstrated that D13-9001 bound with high affinity to the purified AcrB and MexB proteins. In subsequent molecular dynamics

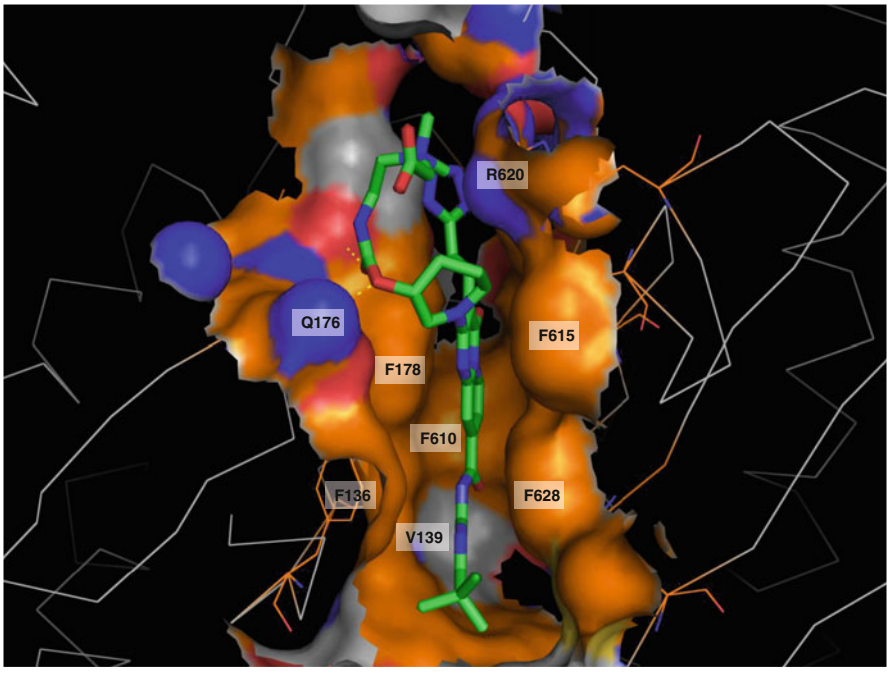

Fig. 29.3 Efflux pump inhibitor D13-9001 crystallized in complex with AcrB in the distal binding pocket of AcrB (The figure was created with PyMOL (http://www.pymol.org) using the structure coordinates deposited in the Protein Data Bank (PDB code 3W9H) [118])

simulations by Vargiu et al. [134], the high binding affinity was confirmed and the binding mode within the hydrophobic trap and the extension of this bulky drug to the upper part of the distal binding pocket were found to be remarkably similar to the crystal structure.

When the Yamaguchi group [118] constructed a homology model for MexY, it was suggested that one possible reason why D13-9001 was not capable of inhibiting this pump was a protruding Trp177 residue, homologous to Phe178 in AcrB and MexB, which hindered the proper binding of this EPI. When a site-directed Phe178Trp mutation was introduced, it was shown that D13-9001 failed to cause doxorubicin accumulation in the case of the AcrB mutant but unexpectedly not in the MexB mutant. Subsequent crystallization of both mutant proteins revealed that the Val139 residue of AcrB (homologous to MexY Ile138) hindered repositioning of D13-9001 and subsequent binding, whereas such steric hindrance did not occur in MexB. In fact, the Phe178Trp mutant MexB protein was crystallized in complex with D13-9001. Accordingly, in the Phe178Trp Val139Ala-double AcrB mutant, the EPI activity of D13-9001 was restored. Finally, the MexY Trp177Phe mutant was shown to be rendered susceptible to the EPI effects of D13-9001[118].

Without doubt, this study [118] is a major breakthrough in the field of RND EPI research. It suggests that tight binding of this particular EPI to the distal binding pocket is the central feature in inhibition of substrate transport. While this key message is encouraging for structure-based EPI designers, it also highlights the fact that designing broad-spectrum EPIs that are potent and can inhibit more than one RND pump at the same time is a considerable challenge. While PAβN is a weaker EPI than D13-9001, it nevertheless features an advantageous trait: contrary to D13-9001, it is capable of inhibiting all clinically relevant RND efflux pumps of *P. aeruginosa*, including MexXY. Since PAβN is a less bulky drug than D13-9001, its binding mode within the distal binding pocket may be more flexible, and steric hindrance by Tyr177 of MexY may be much less of a problem.

29.4.5 MBX2319 and Other Pyranopyridine EPIs

MBX2319 (Fig. 29.2), a pyranopyridine EPI, was discovered by scientists at Microbiotix in a high-throughput screening program, testing more than 180,000 compounds for their ability to sensitize cells to the antimicrobial action of ciprofloxacin [10]. This compound at an MEC of 1.56 μM potentiated the activity of ciprofloxacin, levofloxacin, and piperacillin by, respectively, four-, four-, and two-fold in an AcrAB-TolC-overproducing *E. coli* strain, but not in efflux-deficient strains. The levofloxacin MIC was reduced eightfold at 3.13 μM, making it a considerably more potent EPI than PAβN. Curiously, like PAβN, MBX2319 was not capable of reducing the MIC values of ethidium bromide, acriflavine, and novobiocin. At 25 μM, MBX2319 markedly potentiated the antimicrobial activity of fluoroquinolones and β-lactams in a wide variety of *Enterobacteriaceae*, but had mostly

weak activity against *P. aeruginosa* strains, with the exception of cefotaxime whose MIC was reduced sixfold in one strain [10].

The authors then demonstrated that MBX2319 inhibits nitrocefin efflux at concentrations as low as 0.2 μM and found that most of the inhibition occurred through a large increase in the K_m value, suggesting that this drug is a competitive inhibitor. They further confirmed in a nitrocefin influx assay that MBX2319 does not perturb the outer membrane and in a LacY transport assay that it does not interfere with the proton gradient [10]. Using molecular dynamics simulations, MBX2319 was predicted to bind to the lower part of the distal binding pocket of AcrB [134]. The pyridine ring of MBX2319 interacted with Phe136 and Phe628, whereas the morpholine ring was close to Tyr327. MBX2319 exhibited strong affinity for AcrB in the simulations, although it was lower than that of D13-9001.

Recently, the Microbiotix scientists tested 60 novel MBX2319 derivatives for structure-activity relationship and came up with several compounds that had considerably improved activity and water solubility [138]. When the morpholinyl group of MBX2319 was changed to 2,6-dimethylmorpholinyl and some amides were introduced, the resulting compounds 22i and 22f were found to be ca. 30-fold more potent than MBX2319 in their ability to potentiate the antimicrobial activity of levofloxacin and piperacillin. None of the compounds had any marked impact on nitrocefin influx or LacY transport [138]. It will be interesting to see if the improved activity can be attributed to higher binding affinity for AcrB in molecular dynamics simulations and if such increased AcrB affinity will lead to decreased EPI activity against *P. aeruginosa* RND pumps.

29.4.6 Other Synthetic EPIs

Pharmacia scientists reported on a high-throughput assay which identified a halogenated 3-arylpiperidine (Pharmacia compound 1; Fig. 29.2) capable of enhancing the accumulation of linezolid in *E. coli* (IC_{50} ~90 μM) [139]. Subsequent modifications of this compound failed to improve its activity.

Pagès and colleagues reported on the ability of a series of quinoline derivatives to inhibit the AcrAB-TolC efflux system in *Enterobacter aerogenes* and other *Enterobacteriaceae* [140]. Compound 905 (Fig. 29.2), an alkoxyquinoline with the highest potency, was able to increase chloramphenicol accumulation and to decrease chloramphenicol MICs in an AcrAB-TolC-overproducing *E. aerogenes* strain. The MEC was 100 μM, and full inhibition was only seen at concentrations as high as 1 mM [141]. The activity was later improved with a series of chloroquinoline compounds [142]. Moreover, the Pagès group explored a series of 4-alkylaminoquinazolines [143]. Compound 1167, a 4-(3-morpholinopropylamino)-quinazoline, potentiated the activity of chloramphenicol and various gyrase inhibitors not only in AcrAB-TolC but also in MexAB-OprM overproducers. The same group also discovered that some amine-alkyl derivatives of 5-arylidenehydantoin at

63 μM rendered a multidrug-resistant *Enterobacter cloacae* strain up to fourfold more susceptible to nalidixic acid, sparfloxacin, and chloramphenicol [144].

Zeng and colleagues [145] synthesized various indoles with an idea of using them as TolC-specific inhibitors. They chose as primary target the residues Asp153 and Tyr362 inside the aperture of the TolC barrel. Indeed, compound 2c at 0.5 mM demonstrated marked potentiation of chloramphenicol (32-fold MIC reduction), ciprofloxacin (16-fold), erythromycin (fourfold), and tetracycline (fourfold) against a multidrug-resistant *E. coli* strain, selected on increasing concentrations of chloramphenicol, whereas the potentiation in the wild-type strain was much less pronounced.

29.4.7 Clinically Used Drugs as EPIs Against Gram-Negative Bacteria

Like in Gram-positive bacteria, psychotropic drugs have received some attention as potentiators of antimicrobial agents. Chlorpromazine and thioridazine (Fig. 29.1), the phenothiazine neuroleptics discussed above, have been demonstrated by the Amaral group, in collaboration with our laboratory, to be capable of inhibiting ethidium efflux in an *E. coli* K-12 strain, constitutively expressing the AcrAB-TolC system [19]. Thioridazine (MEC of 5 μg/ml) and chlorpromazine (MEC of 10 μg/ml) fully inhibited ethidium efflux at 15 μg/ml, whereas PAβN even at 40 μg/ml had no impact. Likewise, Amaral and coworkers [146] showed that some newly synthesized phenothiazine derivatives were capable of enhancing the accumulation of ethidium in *E. coli* and AcrAB-TolC-overproducing *Salmonella enterica* serovar Enteritidis strains. However, none of the compounds were capable of potentiating the activity of antibiotics against these Gram-negative species, suggesting that the EPI effect of these phenothiazines may be highly specific to ethidium efflux. Nevertheless, the Piddock group showed chlorpromazine to potentiate the activity of ciprofloxacin (fourfold MIC decrease), chloramphenicol (267-fold), and tetracycline (eightfold) in *Salmonella enterica* serovar Typhimurium; however, a relatively high concentration of 200 μg/ml had to be used [147].

Clinically approved phenothiazines and the structurally related thioxanthenes are typically found to display far less potency in reversing antimicrobial resistance in Gram-negatives compared with Gram-positives, as demonstrated by Kristiansen and coworkers [148], and their maximal achievable plasma levels are not capable of exerting any synergistic activity, let alone intrinsic antimicrobial activity.

Pimozide (Fig. 29.2), a compound belonging to the diphenylbutylpiperidine class, is another neuroleptic agent that was recently discovered by our group to act as an EPI in an *E. coli* strain overproducing the AcrAB-TolC efflux pump [149]. The MEC and IC_{50} for inhibiting Nile red efflux were 25 μM and 50 μM, respectively. At 100 μM, only the MICs of levofloxacin, oxacillin, and ethidium bromide, but not of tetracycline, chloramphenicol, and linezolid, were reduced by twofold, making pimozide a rather weak and selective EPI [149].

We also recently published that antidepressants acting as SSRIs are capable of inhibiting Nile red efflux in AcrAB-TolC-, AcrEF-TolC-, MdtEF-TolC-, and MexAB-OprM-overproducing *E. coli* strains with sertraline (Fig. 29.2) being the most potent compound, displaying MEC and IC_{50} values comparable to pimozide [150]. Like in the case of pimozide, the activity against a panel of clinically applied antimicrobials was very limited. One reason for this may have been the induction of *marA* and *acrB* overexpression by 100 μM sertraline as demonstrated by quantitative reverse transcription-PCR assay. The induction of the *marRAB* system and subsequently enhanced efflux pump activity upon exposure to clinically used drugs has already been demonstrated in *E. coli* [151, 152], as well as overexpression of *ramA* in *Salmonella* upon exposure to chlorpromazine [153]. However, EPIs have not been systematically examined for such an effect, although it could certainly decrease the activity of these inhibitors. Curiously, when MIC assays were carried out in minimal medium, potentiation of antimicrobial activity by sertraline was markedly improved for clarithromycin, linezolid, and tetracycline (≥fourfold), although the reason for this observation is currently unclear.

The anti-malaria drug mefloquine (Fig. 29.2) inhibited *E. coli* ethidium efflux (MEC of 25 μM) and enhanced the ability of *E. coli* and *P. aeruginosa* to accumulate resazurin (MEC of 25 μM) [154]. At 50 μM, mefloquine was about 1.7-fold more potent than PAβN with regard to resazurin accumulation in *E. coli*. The calcium channel blocker verapamil (Fig. 29.1), a standard model EPI in Gram-positives, has been found to enhance ethidium accumulation in an *E. coli* strain (MEC of 40 μg/ml) [155]. Several other clinical drugs, which are nowadays often called "nonantibiotics," with either intrinsic and/or synergistic activity in combination with antimicrobial drugs, have been reported [156–158]; however, data with regard to the action of these compounds on RND efflux pumps are largely lacking.

However, one study by Ejim and coworkers is quite remarkable because of its high-throughput approach of examining the ability of almost 1,100 previously approved drugs to enhance the antimicrobial efficacy of minocycline in *S. aureus*, *E. coli*, and *P. aeruginosa* [159]. Minocycline is a known substrate of AcrB and one of the few drugs that have been co-crystallized with this protein in the distal substrate-binding pocket [115]. Although the group did not check for EPI activity in efflux pump-overproducing versus pump-deficient strains, the hits with regard to *E. coli* were remarkable, including both many previously unreported clinically used drugs and known EPIs like the SSRIs sertraline and paroxetine.

29.4.8 Antimicrobials as EPIs Against Gram-Negative Bacteria

We as well as others have demonstrated that some antimicrobial agents have the ability to inhibit MDR efflux pumps in Gram-negative bacteria, a feature that may sometimes be overlooked due to the fact that this activity can take place at concentrations above the MIC of the particular antimicrobial and hence may be difficult to

be demonstrated in simple checkerboard MIC assays. However, the availability of real-time efflux assays has made it possible to explore such EPI activity. For example, tetracyclines, including the well-known AcrB substrate minocycline (Fig. 29.2), have been shown by two groups to inhibit Nile red efflux in AcrAB-TolC-overproducing *E. coli* strains [14, 16]. In the first study, carried out in the Nikaido laboratory, minocycline was found to inhibit Nile red efflux with an MEC of 50 μM and an IC_{50} of 100 μM [14]. Chlortetracycline was another equipotent inhibitor of Nile red efflux. However, severely compromised LacY transport (accumulation of the LacY substrate down to 13 % of the control, compared with 45 % in the case of minocycline) suggested that this may be due to a marked disruption of the proton gradient. Another study carried out by Takatsuka et al. [160] also demonstrated that both tetracyclines inhibited nitrocefin efflux.

It may be argued that these assays were carried out at concentrations much higher than the MICs for these strains. However, tetracyclines are regarded as bacteriostatic agents, and even a bactericidal compound like ciprofloxacin was found to have no impact on Nile red efflux at concentrations as high as 1 mM which is three orders of magnitude higher than its MIC [14]. A study confirmed that chlortetracycline (IC_{50} of 92 μM) and minocycline (IC_{50} of 124 μM) were the most potent tetracycline inhibitors of Nile red efflux [16]. In addition, it was shown that erythromycin was incapable of inhibiting Nile red efflux [16]. However, erythromycin weakly inhibited efflux of the lipid-sensitive probe $DiBAC_4$-(3) (MEC of 62.5 μM), suggesting that the binding sites for these two compounds do overlap, in contrast to Nile red. Interestingly, this study also measured a considerable activity of antifungal azoles against Nile red and $DiBAC_4$-(3) efflux [16]. Miconazole (Fig. 29.2) was the most potent $DiBAC_4$-(3) efflux inhibitor with a stunning IC_{50} of 2.5 μM, whereas the IC_{50} for Nile red efflux inhibition was markedly lower at 20 μM [16]. However, miconazole is not available for systemic administration in humans due to its side effects and poor bioavailability; otherwise it may potentially have become a potent EPI against Gram-negative bacteria. This agent is commonly applied topically to the skin and mucous membranes to treat fungal infection, and we cannot rule out the possibility that it might find use as a topical EPI to potentiate the activity of local antiseptics or antibiotics, although careful studies on useful combinations will have to be conducted first.

In addition, trimethoprim (Fig. 29.2), a known inhibitor of dihydrofolate reductase, was shown to potentiate at 10 μg/ml the activity of ciprofloxacin by two to fourfold against various *Enterobacteriaceae*, including *E. coli*, but not in *Salmonella* serovar Typhimurium strains lacking the *acrB* or *tolC* gene [161]. Trimethoprim weakly potentiated tetracycline activity and had no impact on chloramphenicol MICs. It also led to increased accumulation of the dye Hoechst 33342 in *Salmonella* serovar Typhimurium; however, no efflux assays have been performed. Synergism between ciprofloxacin and trimethoprim was also described earlier in another study for various *Salmonella enterica* bloodstream isolates [162]; however it was unclear that this effect was due to efflux pump inhibition. Since maximal trimethoprim plasma levels in humans can reach close to 10 μg/ml when administered during high-dose antimicrobial therapy in *Pneumocystis jirovecii* pneumonia [163], this

antimicrobial could be a potential candidate for future EPI research against *Enterobacteriaceae*. However, due to the fact that trimethoprim is a very small, yet potent, molecule, it is unclear how this agent would interfere with binding of ciprofloxacin to the distal binding pocket of AcrB.

To conclude, none of the above clinically used drugs, with the notable exception of trimethoprim, can reach sufficient plasma levels in humans for EPI activity against Gram-negative bacteria. However, it is highly likely that only a very small fraction of the available drugs have been screened for EPI activity, and more research is definitely needed.

29.4.9 EPIs from Natural Sources

Plant extracts with EPI-like activity that generally display moderate-to-low potency in Gram-negative bacteria have been previously described [164–169]. Here, we mainly focus on reports that have extracted and/or used chemically defined natural compounds. Those reports dealing with uncharacterized extracts are excluded. However, it is interesting to at least briefly note that one plant extract study has demonstrated that even cruciferous vegetables, which are consumed by many of us as part of our daily diet, contain ingredients that are capable of potentiating, albeit moderately, the *in vitro* activity of various antimicrobials against multidrug-resistant Gram-negative strains, such as *K. pneumoniae* and *E. aerogenes* [168]. Hence, the possibilities for interference with transport of other biologically active molecules in our gut microbiota are manifold, and the consequences for human health are completely unclear.

One of the most potent EPIs was discovered while screening a large library of about 85,000 natural microbial fermentation extracts [170]. Two new compounds, EA-371α and EA-371δ (Fig. 29.4), produced by a new strain of *Streptomyces* spp., potentiated the activity of levofloxacin in a MexAB-OprM-overproducing *P. aeruginosa* strain by eightfold at concentrations of 2.5 and 1.25 μg/ml, respectively. Interestingly, these agents resemble the synthetic EPI D13-9001 in that they are selective for MexAB-OprM pump inhibition, are comparable in potency, and are relatively bulky compounds. It may thus be expected that they bind with high affinity to MexB. Strikingly, when the structurally highly related compounds benastatin A (Fig. 29.4) and B (lacking only an SO_3 group on C-11) were tested in potentiation assays, their activity had completely vanished.

Pheophorbide A (Fig. 29.1), present in several *Berberis* species and a natural product of chlorophyll breakdown, is probably the strongest known potentiator of ciprofloxacin activity ever extracted from a plant [81]. This compound at 0.5 μg/ml reduced the MICs of several wild-type strains of *E. coli* by only twofold; however ciprofloxacin-resistant clinical isolates had up to 16-fold reductions in ciprofloxacin MICs. Similarly, *P. aeruginosa* wild-type strains were unaffected by pheophorbide A; however, a highly ciprofloxacin-resistant strain had its MIC reduced by 128-fold. While there is a certain likelihood that this effect might be due to efflux pump inhibition, this hypothesis was not examined.

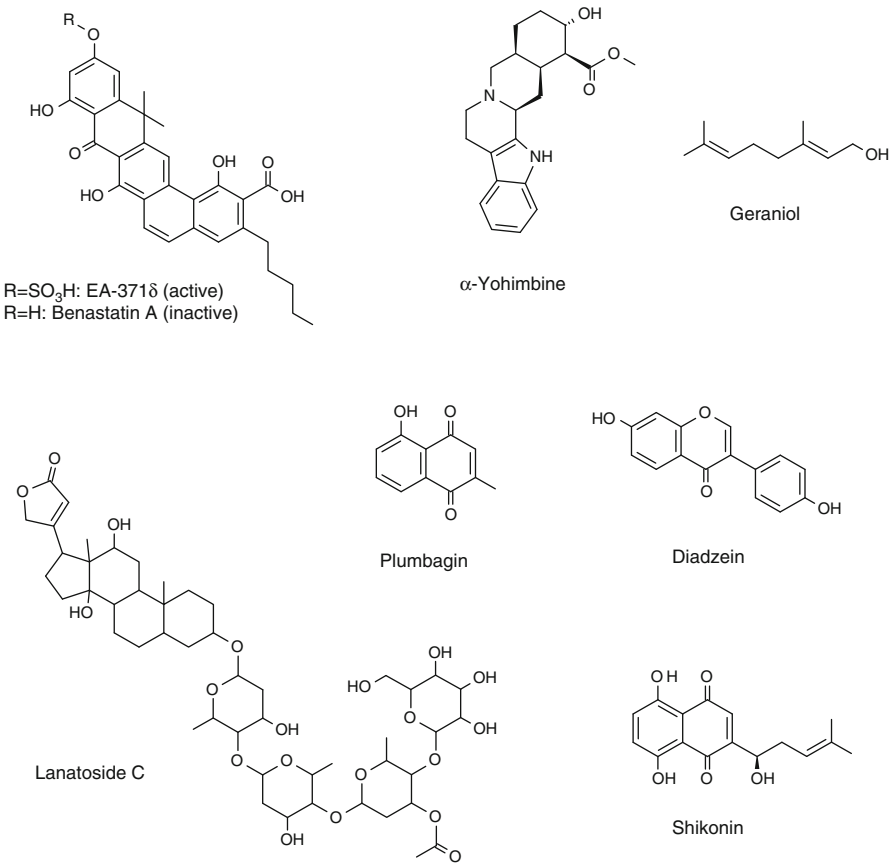

Fig. 29.4 Chemical structures of efflux pump inhibitors from natural sources with activity against Gram-negative bacteria

Several indole alkaloids from *Rauwolfia* plants, including α-yohimbine (Fig. 29.4), reserpine, and 10-methoxytetrahydroalstonine, were shown to inhibit ethidium efflux (MEC of 12.5 μg/ml) in an *E. coli* strain overexpressing *acrAB* and to reverse tetracycline and nalidixic acid resistance by eightfold at 10 μg/ml in this one as well as another multidrug-resistant *E. coli* strain [171]. Interestingly, reserpine (Fig. 29.1), another well-known *Rauwolfia* alkaloid that has already been extensively discussed above, was found to have the same potency against these *E. coli* strains.

Artesunate, an anti-malaria drug extracted from *Artemisia annua*, was demonstrated by Li et al. [172] to enhance accumulation of daunorubicin in an AcrAB-TolC-overproducing *E. coli* strain (MEC of 64 μg/ml), but not in the efflux-deficient strain. At 256 μg/ml, it markedly potentiated the activity of various penicillins and cephalosporins, presumably by inhibition of *acrAB* expression.

Lorenzi et al. discovered that extracts from *Helichrysum italicum* as well as the monoterpenoid geraniol (Fig. 29.4) produced by this plant were both potentiators of

β-lactam and fluoroquinolone agents in an AcrAB-TolC-overproducing *E. aerogenes* strain [173]. Ampicillin, penicillin and norfloxacin MICs were reduced by at least three orders of magnitude in the presence of geraniol at ¼ MIC (6.25 mM), however the effect of lower concentrations was not reported. The same group later synthesized derivatives retaining a monoterpenoid backbone (geranic acid) linked to polyamines and discovered that the most potent compound (No. 11) at 31 μM was capable of enhancing the susceptibility of *E. aerogenes* and *Salmonella* strains to chloramphenicol and nalidixic acid (four- to 32-fold MIC reduction) [174]. Compound 11 also efficiently inhibited 1,2′-dinaphthylamine real-time efflux with an MEC of 38 μM.

High-throughput *in silico* screening of a database of phytochemicals against the distal binding pockets of AcrB and MexB revealed lanatoside C and daidzein (Fig. 29.4) as putative candidate EPIs. Subsequent ethidium efflux assays in *E. coli* and *P. aeruginosa* demonstrated marked EPI activity by both compounds at only 16 μg/ml [175]. Curcumin, an ingredient from *Curcuma longa*, at 50 μg/ml increased susceptibility of 27 % of various clinical multidrug-resistant *P. aeruginosa* strains to carbenicillin, although the RND efflux pump overexpression profiles were not examined [176] (carbenicillin is indeed a major substrate of *P. aeruginosa* MexAB-OprM system [177]). Curcumin is typically used for food coloring and has been tested in various clinical anticancer trials, although its bioavailability is poor and would have to be markedly improved for synergistic activity [178].

Several phytochemicals were selected as putative EPIs using *in silico* docking of a small compound library to AcrB and subsequently found to markedly inhibit Nile red efflux in *E. coli* in the following order of potency: shikonin (25 μM; Fig. 29.3), plumbagin (50 μM; Fig. 29.4), nordihydroguaiaretic acid (100 μM), quercetin (200 μM), and mangiferin (500 μM). No membrane permeabilization effects were observed in the Δ*acrB* strain; however, the synergistic effects with various antimicrobials against the *acrAB*-expressing strains were also very limited [179].

29.5 Concluding Remarks

Ever since Neyfakh and colleagues created the field of bacterial EPI research, a lot of progress has been made. Especially, the availability of crystal structures and complementary site-directed mutagenesis studies of MDR efflux transporters have dramatically improved our understanding of substrate transport and their inhibition. However, this success is largely confined to Gram-negative bacteria, the prototype being the AcrAB-TolC RND transporter of *E. coli*. The Yamaguchi group has even managed to find the Holy Grail of EPI research, namely, to co-crystallize the potent EPI D13-9001 in an asymmetric conformation of AcrB, which is suggested to be the physiologically relevant one. This has opened up the road to structure-based EPI design, aided by computational chemistry. Whether and how soon this will lead to identify novel clinically applicable EPIs remains to be seen. Moreover, there are definitely potential candidate drugs in the pipelines of pharmaceutical companies, although none has reached clinical applicability yet.

The situation with regard to EPI research is different for Gram-positive organisms. While we do have crystal structures of MDR transporter regulators like BmrR and QcaR, even in complex with substrates, none of the Gram-positive MFS transporters themselves have been crystallized. Thus, a lot of work remains to be done before we can consider structure-based EPI design. Various synthetic EPIs with good activity against NorA of *S. aureus* have been reported, but no follow-up studies regarding generation of EPI resistance and scanning for mutations have been published in analogy to the early days of Bmr research. Finally, on a positive note, the EPI thioridazine might see possible clinical use in the treatment of multidrug-resistant or extensively drug-resistant tuberculosis, but more clinical studies are needed before this clinically used drug can be included in antituberculosis therapy regimen.

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Chapter 30
Multidrug Efflux Pumps and Their Inhibitors Characterized by Computational Modeling

Venkata Krishnan Ramaswamy, Pierpaolo Cacciotto, Giuliano Malloci, Paolo Ruggerone, and Attilio V. Vargiu

Abstract Antimicrobial resistance is a key public health concern of our era due to an ever-increasing number of drug-resistant pathogens, including several Gram-negative bacilli. The latter are endowed with a low permeable outer membrane and with numerous chromosomally encoded multidrug efflux pumps, which are not only ubiquitous but also polyspecific, thus recognizing a broad range of compounds. Efflux pumps are a major defense mechanism of these organisms against antimicrobials as they can significantly increase the levels of resistance by allowing time for the organisms to develop specific resistance mechanisms. One of the potential strategies to reinvigorate the efficacy of antimicrobials is by joint administration with efflux pump inhibitors, which either block the substrate binding and/or hinder any of the transport-dependent steps of the pumps. In this chapter, we provide an overview of multidrug resistance efflux pumps, their inhibition strategies, and the important findings from the various computational simulation studies reported to date with respect to the rational design of inhibitors and on deciphering their mechanism of action.

Keywords Antimicrobial resistance • Efflux pump • ABC • MATE • RND • P-glycoprotein • Efflux pump inhibitor • Molecular dynamics • Molecular docking

30.1 Introduction

Decades ago, when the incidents of bacterial resistance were not widespread and newer antimicrobial agents were continually being discovered, it was not surprising to hear that the era of infectious diseases caused by microbes was virtually over [1]. However, over the last two decades, there has been a dramatic surge in the number

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of multidrug-resistant bacteria, yet paradoxically the number of pharmaceutical companies developing new antimicrobial drugs has dwindled. These coincidences have collectively made antimicrobial resistance one of the world's most demanding health problems [2, 3].

With continuous efforts to develop better antimicrobial agents against such resistant microbes, successful milestones are being reached in the case of infections caused by Gram-positive organisms [4], while the Gram-negative pathogens (e.g., *Enterobacteriaceae*, *Acinetobacter*, and *Pseudomonas*) still prove to be a major challenge due to their very high intrinsic drug resistance. This intrinsic resistance is largely attributed to the permeability barrier imposed by the outer membrane (OM) and to the expression of chromosomally encoded drug efflux pumps [5].

Drug efflux pumps are ubiquitously expressed protein complexes residing in the membrane to expel a wide range of structurally diverse antimicrobials and toxins, thereby lowering their concentration inside the cell to sub-toxic levels [6–9]. They also enjoy a special status of being considered a part of the primary survival kit of microorganisms as these polyspecific pumps remove most of the xenobiotics from the cell interior to give the organism time to acquire resistance to agents through more specific adaptive mechanisms [10, 11]. This way the efflux mechanisms likely contribute to a rapid emergence of resistance in the presence of antimicrobial selection pressure. Efflux mechanism also interplays with other resistance mechanisms to significantly increase the levels and profiles of resistance [12].

The current shortage of new antimicrobials in the development pipeline to replace the ineffective ones adds to the urgency to protect the efficacy of existing drugs. One possible way of reinvigorating the previously effective drugs attenuated by bacterial efflux mechanism is by the combinatorial use of efflux pump inhibitors (EPIs). This chapter provides an overview of the various multidrug resistance (MDR) efflux pumps with particular emphasis on computational studies of their inhibitors that have been reported to date. After a brief introduction on the importance of efflux pumps for MDR, we describe the major families of multidrug transporters, their mechanism of function, and the various inhibition strategies. Moreover, we summarize the molecular modeling studies that facilitate our progress in developing efficacious inhibitors for better management of efflux-mediated MDR. For any family of MDR pumps, we focus here mostly on studies including the data from molecular dynamics (MD) simulations when they are present (either in addition to docking or exploiting experimental information about structures of receptor-inhibitor complexes). The reader is referred to the relevant literature on studies making exclusive use of molecular docking and other computational methods that are cheaper compared to MD simulations [13–42].

30.2 Efflux-Mediated Resistance and MDR

Bacteria have evolved a multitude of mechanisms that in solitude or in combination with each other function to counter the effectiveness of drugs and overcome the deleterious effect of any antimicrobial agents, thus making the bacteria

resistant to multiple drugs. These mechanisms [43–45] include (i) the alteration of the macromolecular drug target either through chemical modification or by mutation to insensitive variants (e.g., alteration of penicillin-binding protein in methicillin-resistant *Staphylococcus aureus* [46]); (ii) the protection of the target via the production of immunity proteins, alteration of metabolic pathways (e.g., elimination of the requirement of *para*-aminobenzoic acid in sulfonamide-resistant bacteria for the synthesis of folic acid and nucleic acids); (iii) the direct chemical modification or inactivation of the antibiotics (e.g., enzymatic inactivation of β-lactams by β-lactamases); (iv) the altered transport of the compounds into the cell (e.g., reduced membrane permeability barrier with decrease in production of porins); and (v) the increased active efflux of drugs out of the cell through efflux pumps.

Among the aforementioned resistance mechanisms, the efflux-mediated approach, where pumps actively export substrate molecules from the cytoplasm to the external medium in an energy-dependent manner, is the predominant one in MDR [47], working in synergy with the low permeability of the OM in Gram-negative bacteria to keep a tight check on the entry of unwanted toxic compounds. Indeed, drug molecules that have gained access to the periplasmic space can further penetrate the cytoplasmic (inner) membrane via diffusion, but they can be expelled out of the cell either by single-component pumps (e.g., Tet pumps [48]) or by multicomponent pumps (e.g., AcrAB-TolC of *Escherichia coli* and MexAB-OprM of *Pseudomonas aeruginosa* [47, 49, 50]).

The wide distribution and overlapping functions of MDR efflux pumps in bacteria hint at their probable role in physiological functions in addition to mediating intrinsic and acquired MDR [51]. A few of these functions include virulence, stress response, bacterial cell communication, colonization, fitness and intracellular survival, and transport of toxic compounds (as in the case of MacAB-TolC which is involved in exporting an extracellular peptide enterotoxin produced by enterotoxigenic *E. coli*) [52].

MDR pumps also function as either a preexisting mechanism or an activated resource in response to numerous cellular stresses caused by antibiotics and other chemical substances such as bile salts, fatty acids, and ethanol that are often substrates of pumps relevant for drug resistance. For instance, AcrAB, the major pump belonging to the resistance-nodulation cell division (RND) superfamily of transporters in enteric bacteria living within the intestinal tract, is upregulated under such stress conditions enabling the bacterial survival in host organisms [43]. The major facilitator superfamily (MFS) pump, MdtM, also functions with AcrAB-TolC in a synergistic manner to protect *E. coli* from bile salt stress [53]. Also, NorM [54], a multidrug and toxic-compound extrusion (MATE) family transporter, and MacAB [55], a macrolide-specific ATP-binding cassette (ABC) superfamily exporter, protect the bacteria against oxidative stress. In the case of *P. aeruginosa*, several Mex pumps are upregulated in response to various stress triggers like membrane-damaging or ribosome-disrupting agents, reactive oxygen species, and/or nitrosative stress [47, 56, 57].

Apart from the previously mentioned functions, MDR pumps have also been identified to play a substantial but varying role in the formation and survival of

biofilms in different species. For instance, the loss or inhibition of any of nine MDR pumps or the TolC OM protein in *Salmonella* impairs its biofilm-forming ability with reduced production of curli [58]. Similarly, *E. coli* mutants with a genetic deletion of one of the MDR pump genes results in reduced biofilm formation [59].

30.3 Classification of Drug Efflux Pumps

The transport proteins have been successfully classified by Milton Saier's group in over 800 families on the basis of functional and phylogenetic information (Transporter Classification Database: http://www.tcdb.org) [60]. The transporter genes identified in hundreds of sequenced bacterial genomes have also been documented in Ian Paulsen's database (http://www.membranetransport.org) [61]. Among the numerous families of transporters, the prominent ones responsible for MDR can be divided into two major groups based upon bioenergetical and structural features [51]: (i) Primary active transporters belonging to the ABC superfamily hydrolyze ATP as a source of energy. (ii) Secondary active transporters utilize the proton (or sodium) gradient as a source of energy (the proton motive force is an electrochemical gradient in which the movement of hydrogen ions drives transport of the substrate [62]) and are classified into four superfamilies/families (MFS, MATE, RND, and the small multidrug resistance [SMR]) on the basis of conserved consensus motifs and functional similarities. While the major clinically relevant efflux systems in Gram-positive bacteria are usually non-RND pumps and often the singleton protein pumps belonging to the MFS, MATE, SMR, or ABC, the RND efflux systems are by far the most important in Gram-negative bacteria [12].

30.4 Structural and Functional Mechanisms of Drug Efflux Pumps

30.4.1 ABC Pumps

ABC transporters are ubiquitous membrane systems involved in the efflux of toxins, metabolites, and drugs. These transporters are typically composed of two cytoplasmic nucleotide-binding domains (NBDs) and two hydrophobic transmembrane domains (TMDs) [63]. In some transporters, the TMDs, responsible for drug recognition and transport, are fused to highly homologous NBDs, where ATP is hydrolyzed. The NBDs possess the Walker A and B motifs, common to all ATP-binding proteins, and a signature motif, specific to ABC transporters [64]. It has been proposed that ABC efflux pumps were derived from secondary active transporters by superimposition of NBD onto the transporter during evolution [64]. These transporters are found to house multiple drug-binding sites, which is compatible with

their broad substrate specificity and multidrug binding capabilities. Ligand-binding and transport assays have shown that P-glycoprotein (P-gp, ABCB1, MDR1), the most extensively studied ABC member, has at least four pharmacologically distinct binding sites that are allosterically coupled [65, 66]. This family of exporters function with a mechanism termed the ATP switch model [67], where the nucleotide-driven interaction of the NBDs causes reorientation of the TMDs and reduces drug affinity, thereby transporting the substrate (Fig. 30.1a) [70, 71]. Ominous examples of ABC transporters are the mammalian P-glycoprotein active against cytotoxic compounds used in chemotherapy, LmrA of *Lactococcus lactis*, MsbA conferring resistance to erythromycin in Gram-negative bacteria, and MacAB-TolC of *E. coli* able to expel macrolides.

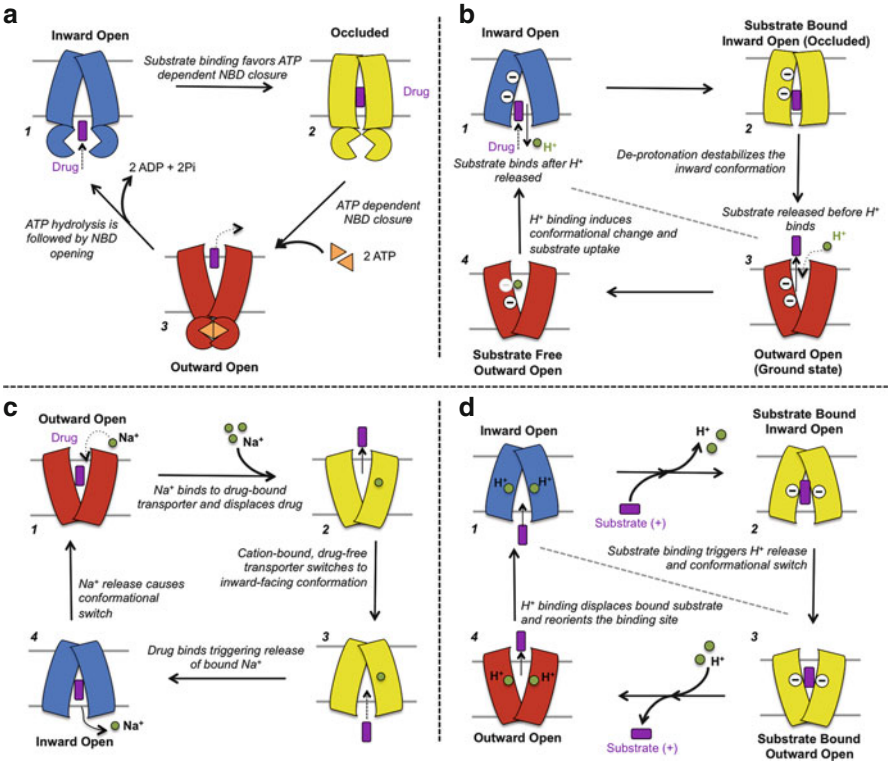


Fig. 30.1 Transport mechanisms proposed for members of four major families of MDR efflux pumps. (**a**) Simplified drug transport cycle of ABC efflux pumps showing the inward-facing, occluded, and outward-facing states. (**b**) Indirect competition mechanism in MFS multidrug/proton antiporters. (**c**) Na^+/multidrug antiport mechanism in transporters of the MATE family. (**d**) Alternating site transport mechanism of EmrE of the SMR family (Transport mechanism of the RND transporters is omitted here but described in detail in Chap. 1 of this book. Obtained with modification and permission from Refs. [63, 68, 69]. (**a**, **b**), are derivatives of figures from Du et al. [63] used under the Creative Commons Attribution License (CC BY). (**c**, **d**) are adapted from Lu et al. [68] and Schuldiner [69], respectively)

30.4.2 MFS Pumps

The MFS pumps belong to the largest group of secondary active membrane transporters [72]. They are omnipresent systems that transport sugars, intermediate metabolites, and drugs and are the major contributors of MDR in Gram-positive bacteria. Most of these pumps are singlet transporters belonging either to 12- or 14-transmembrane segment (TMS) members of the drug/H^+ antiporters. In Gram-negative bacteria, they are located in the cytoplasmic membrane and transport drugs from the cytosol to the periplasm from where constitutive RND pumps, such as AcrAB-TolC and MexAB-OprM, may capture and efflux the drug molecules to the external medium, thereby synergistically boosting the activity of these singlet pumps in producing resistance [73, 74]. These transporters operate through an alternating access mechanism (Fig. 30.1b) in which drug-binding sites are alternately exposed to the outside or inside of the cell to uptake and release substrates. Similar to P-glycoprotein, the MFS pumps also contain several distinct (possibly overlapping) allosterically coupled binding sites [75]. There exists an indirect competition between the substrates and protons for binding to their respective different locations, as shown in MdfA of *E. coli*, which might likely play a key role in their transport mechanism [76]. The most studied pumps of this family are NorA of *S. aureus* and its homologs Bmr and Blt of *Bacillus subtilis*, Tet pumps (12-TMS in Gram-negative bacteria and 14-TMS in Gram-positive bacteria) [48], and MdfA [77].

30.4.3 MATE Pumps

Efflux pumps of the MATE family are mainly 12-TMS Na^+/drug antiporters that pump substrates from the cytoplasm to the periplasmic space [78]. These transporters are widespread in bacteria and are also found in higher animals and plants. The common substrates of these pumps are cationic dyes, fluoroquinolones, and aminoglycosides. All MATE pump structures show a similar 12-TMS helix topology with an internal twofold sequence similarity reflected in the tertiary structure [68, 79, 80] as N-terminal and C-terminal lobes. These pumps exhibit distinct binding sites for cation and drug enabling their simultaneous binding. The cation binding (with an unusual cation-π interaction with an aromatic ring) and release promote the interconversion between the drug-free and cation-bound configuration and drug-bound configuration as shown in the case of NorM of *Neisseria gonorrhoeae* (Fig. 30.1c) [68].

30.4.4 SMR Pumps

Transporters in the SMR family [81] are the smallest drug efflux proteins known with just 100–120 amino acids folded into four relatively short transmembrane α-helices. They form either a homo- or heterodimer to exchange H^+ for pumping out

either monocationic (e.g., ethidium and tetraphenylphosphonium) or dicationic (e.g., methyl viologen) compounds into the periplasm. The orientation of monomeric subunits in the dimer was a long-debated issue with crystallographic data showing antiparallel arrangement of EmrE dimer while chemical cross-linking favoring a parallel arrangement [82]. Although the structure was withdrawn [83], this issue concluded on grounds that it can exhibit a dual topology and that the direction of insertion of the monomeric unit really does not matter for the efflux function [69, 84]. Structural plasticity and flexibility are the basis of multidrug recognition and transport in EmrE, the well-studied pump of this family [85]. This transporter shows functional symmetry where conformational changes in the two monomers result in an interconversion between inward- and outward-facing states [86]. A fixed stoichiometry of two protons is exchanged per substrate molecule, and this results in an electrogenic state for transport of monovalent cations but an electroneutral state for divalent cations [87]. The conserved membrane-embedded glutamate residue (Glu14) in each monomer is essential for proton and substrate binding. Hence, these transporters show an apparently simple, competitive, alternating site mechanism (Fig. 30.1d) in which all substrates bind to the same site [85] and compete with protons for binding [82, 86].

30.4.5 RND Pumps

Efflux pumps of the RND superfamily [88] are the major clinically relevant efflux systems in Gram-negative bacteria also due to their extremely wide substrate specificity [47]. Indeed, some of these transporters are able to recognize hundreds of antimicrobials belonging to various classes, and the different RND efflux systems in one species are altogether able to export a wide set of substrates ranging from lipophilic to amphiphilic molecules and finally to toxic divalent cations [89–91]. Several examples of pumps belonging to this family are AcrAB-TolC and AcrAD-TolC of *E. coli* and MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM of *P. aeruginosa* [47, 92]. They span the entire periplasmic space from the cytoplasmic membrane to the OM by forming tripartite efflux complex systems [93] comprising an RND transporter protein (e.g., AcrB) embedded in the inner (cytoplasmic) membrane, a periplasmic adaptor protein (a.k.a. membrane fusion protein; e.g., AcrA) located in the periplasmic space, and an OM protein resembling a long helical tunnel (e.g., TolC). Recently, a small cytoplasmic membrane protein known as AcrZ was found to be associated with AcrB of *E. coli* and might have potential role in enhancing the transport activity of AcrB for specific antimicrobials like chloramphenicol, puromycin, and tetracycline [94]. No trace of any such protein or its homologs has been found in other RND transporters. Du et al. [95] presented a pseudo-atomic structure of this entire tripartite system AcrABZ-TolC to explain the quaternary organization and key domain interactions and also proposed a cooperative process for channel assembly and opening. The RND transporter protein structurally resembles a jellyfish with each protomer comprising a total of three domains

[96, 97]: (i) TMD consisting of 12 α-helices embedded in the inner cytoplasmic membrane is the region where energy conversion via proton conduction takes place; (ii) pore (porter) domain in the periplasm where substrate recruitment and transport mainly occur; and (iii) OM protein docking domain also in the periplasm, which couples the RND transporter to the OM protein or to the hexameric assembly of membrane fusion proteins in the constituted pump.

The drug-binding sites for the RND family are within the periplasmic domain of the protein, in contrast to other MDR pumps discussed above [8] as evident from the drug-bound structures in the asymmetrical trimer configuration [97–99]. It has been postulated that the "resting state" of these transporters (i.e., the structure in the absence of substrates) corresponds to a symmetric structure in which each monomer assumes the same conformation, while the presence of substrates or inhibitors triggers conformational changes leading to an asymmetric configuration [49] (also see Chap. 1 of this book). The latter is characterized by three possible structures of each monomer in the trimer, which were indeed interpreted as reaction cycle intermediates: *loose* (a.k.a. "access") in which substrates become associated by loosely binding to a proximal (access) pocket, *tight* (a.k.a. "binding") in which substrates bind tightly to a more distal (deep) binding pocket, and *open* (a.k.a. "extrusion") which corresponds to the drug-released state of a functionally rotating mechanism (see Fig. 30.1; also see Fig. 1.6 of Chap. 1) [90, 98, 104–106]. A recent study [107] has put forward the hypothesis that high molecular mass substrates (and low molecular mass dimers as well [108]) are actually recognized by the proximal pocket of the *loose* monomer, while low molecular mass compounds are recognized by the distal pocket of the *tight* monomer instead.

According to the functional rotation mechanism, a concerted but not necessarily synchronous [105, 109] cycling of the monomers occurs through any of the asymmetric states: *loose*, *tight*, *open*, and back to *loose*. During a complete functional cycle, occlusions and constrictions inside the pore domain propagate from external gates toward the central funnel, driving the unidirectional transport of substrate ("peristaltic pump mechanism" [104]). In other terms, the substrate would gain access to the pore domain of the transporter via the *loose* and/or *tight* monomer, either from open clefts in the periplasm or through grooves between helices at the interface between pore and TMD [110, 111]. The substrate would then get accommodated into a large binding pocket when the monomer assumes the *tight* state and moved out toward the TolC docking domain upon a subsequent change to the *open* conformation.

These tripartite multidrug transporters are highly efficient in creating detectable resistance to antimicrobials as they export the drug substrates directly from the periplasm or the inner leaflet of the cytoplasmic membrane into the external medium, making the reentry of drugs through the low permeable OM cumbersome. The efficiency of RND pumps is synergistically associated with the presence and ability of single-component pumps located in the cytoplasmic membrane to flush out substrates from the cytoplasm [73, 74].

The past decade has seen numerous structural studies performed on various representative proteins from all five aforementioned multidrug transporter families (see

Chaps. 1, 2, 3, and 4), thus providing valuable data forming the foundation to explore similarities and differences in drug recognition and drug export mechanisms and for the future therapeutic inhibition of these transporters [112].

30.5 Inhibition Strategies for Efflux Pumps

Since active drug efflux plays a major role in intrinsic and acquired drug resistance in Gram-negative bacteria, inactivation of such pumps may open up a wide arena of possibilities for better antimicrobial adjuvant therapy. This strategy has several advantages [113] such as (i) elevation of the intracellular concentration of antimicrobials, (ii) reduction in the efflux-mediated intrinsic bacterial resistance, (iii) reversal of the acquired resistance associated with efflux pump overexpression, (iv) reduction in the frequency of emergence of highly resistant mutant strains by reducing the adaption time for development of additional mechanisms of resistance like target-based mutations [114], and (v) prevention of the export of endogenous microbial virulence factors, thus inhibiting microbial invasiveness [115, 116].

To revive the activity of an efflux-susceptible drug, efflux-mediated MDR can be inactivated by any of the following methods:

(i) Targeting the regulatory network involving activators and repressors that control the expression of efflux pumps [117] (e.g., altering the expression of AcrB from *Salmonella enterica* [11]; regulating efflux pump expression in *P. aeruginosa* [118–122]; targeting local repressor EmrR to alter the expression of EmrAB, a MFS transporter in *E. coli* [123]).

(ii) Altering the molecular design of existing susceptible antimicrobials to make them devoid of the chemophore recognized by efflux pump (e.g., chemically modified taxol escapes the action of P-glycoprotein [124]; tigecycline circumvents MFS pumps specific for tetracyclines [125]; telithromycin bypasses MefA/E and AcrAB systems [126]; among fluoroquinolones, gatifloxacin, levofloxacin, and moxifloxacin are not affected by NorA and PmrA pumps [127]). However, resistance against new compounds developed by this strategy was described shortly after their deployment [8].

(iii) Blocking the cytoplasmic membrane proteins with a high affinity competitively binding substrate (an EPI) to trap the efflux pump in an inactive conformation. These EPIs are clinically significant as they help evade antimicrobial resistance by inhibiting these pumps, reverse the acquired resistance associated with the overexpression of efflux pumps, and also suppress the emergence of mutations leading to resistance [10, 128–131] (e.g., the EPI of AcrB and MexB pumps, phenylalanine-arginine β-naphthylamide [PAβN]) [132]. However, toxicity issues have withheld these EPIs from clinical applications [10], although new compounds are being developed that have minimal toxicity but strong inhibitory effects on AcrB [133–135].

(iv) Depleting proton gradient to deprive the cytoplasmic membrane proteins of the motive force needed to work (e.g., carbonyl cyanide *m*-chlorophenylhydrazone [136], valinomycin, dinitrophenol, and phenothiazines such as promethazine [113, 128, 131, 137]; verapamil inhibits the MDR pumps of cancer cells and parasites in addition to improving the activity of tobramycin; reserpine inhibits the activity of Gram-positive efflux pumps Bmr and NorA [138]). However, these inhibitors affect the entire energetics of bacterial and also of eukaryotic cells, which makes them less attractive for clinical implication [116, 139].

(v) Specifically in the case of RND efflux pumps, the following approaches may disrupt the pump functionality: preventing the functional tripartite assembly formation by targeting protein-protein interfaces (e.g., designed ankyrin repeat proteins [DARPins] inhibited AcrAB-TolC formation by obstructing AcrA and AcrB interaction [140]); disrupting the interaction between AcrB and AcrZ (e.g., the absence of AcrZ diminished the substrate pool of AcrAB-TolC [94]); and blocking the exit duct (the OMP) (e.g., indole derivatives designed based on the structure of TolC prevented the opening of the channel [141], large cations targeting the negatively charged aspartate-rich entrance of TolC in *E. coli* [142]).

Among these different strategies to combat efflux-mediated MDR, inhibition of efflux pumps is considered to be a viable one [113], because a single potent inhibitor capable of competitively binding to a pump and preventing expulsion of its substrate antimicrobials could in principle also bind and block other MDR pumps overlapping in their substrate profiles [133, 135, 143]. In addition to revitalizing the therapeutic potential of the antimicrobials, these EPIs could also contribute to antibacterial action by hindering the transport of compounds needed for the normal growth and/or maintenance of the microorganism.

Numerous studies to date have guided our understanding of the structural and functional aspects of drug transporters at a molecular level and have also unveiled several fundamental concepts regarding their substrate binding and transport (for recent reviews, see, e.g., [9, 47, 49, 88, 91, 109, 144, 145]). These findings are useful for the rational design of inhibitors that can competitively bind to the efflux pumps and prevent the efflux of their substrate antimicrobials (and are also useful for the design of more efficient drugs that can escape efflux pumps) [49, 112].

A compound must satisfy the following criteria as postulated by Lomovskaya et al. [10] to qualify as an ideal clinically significant EPI: (i) It must potentiate the activity of antimicrobials in resistant strains expressing functional drug efflux pump. (ii) It must not have a significant effect on susceptible strains lacking the specific drug efflux pump. Moreover, the inhibitor should be free of any pharmacological activity on eukaryotic cells [146]. (iii) It must not potentiate the activity of antimicrobials that are not effluxed. (iv) It must increase the level of accumulation and decrease the level of extrusion of substrates of the efflux pump. (v) It must not permeabilize the OM; and (vi) It must not affect the proton gradient across the cytoplasmic membrane.

30.6 Computational Studies on Drug Efflux Pumps and Their Inhibitors

30.6.1 Role of Molecular Modeling in Drug Discovery

Our understanding of the structural aspects of MDR pumps from crystallographic structures has been significant but not sufficient to fruitfully assist structure-based drug design. To address mechanistic knowledge gaps, computational techniques [147] are a great resource as they can highlight functional dynamics of biological systems. In particular, molecular docking and MD are increasingly being used both for rationalizing existing data and for various predictions, for instance, about drug recognition and binding, translocation mechanisms, and structural relations with the surrounding environment using three-dimensional structures.

Molecular docking tries to mimic the natural course of interaction of the ligand and its receptor via a lowest energy pathway and in doing so offers a great benefit in quick and efficient prediction of binding modes of small molecules to proteins but not necessarily the accurate binding energies [148–150]. The absence of a protein X-ray crystal structure creates a major hindrance in studies of ligand-protein interactions, but development of a suitable homology model [151–153] of a target protein that can then be used for molecular docking and other structure-based studies could provide an alternative approach. Pharmacophoric studies based on the receptor or the ligand are important to identify the most common structural moieties that contribute to drug recognition and can be exploited in drug design [154–156].

MD simulation is a powerful technique that can provide atomic level descriptions of molecular systems with high temporal resolution. It is also often employed to validate the stability of homology models. Atomic level simulations in the scale of several hundred nanoseconds are routinely performed to obtain a detailed insight into conformational changes and free energies of interactions and at the same time to identify drug-binding locations, translocation processes, and interactions with the surrounding lipid bilayer [157]. From such techniques, a normal-mode analysis and a functional mode analysis of the protein movements allow the comparison between simulations of the apo and holo structures, with one or several molecules inside the drug-binding pocket [158]. This could lead to the identification of the movements intimately related with the translocation process, aiming for a better understanding of the first steps of the efflux mechanism [159]. Recently, the use of "coarse-grained" simulations, where four atoms are typically combined into one particle, and biased MD simulations has increased dramatically. Such simulations allow sampling of large conformational changes that would normally be inaccessible because of the large free energy barriers between such conformations and the consequent limitations due to the lack of computational time [160–163].

Computational methods with improved algorithms now provide a higher level of understanding of biochemistry for better design of compounds and in economical use of the available biological/chemical resources [158, 159, 164]. Various computational studies on the EPIs of multidrug transporters, mainly those specific to RND

and ABC transporters, have been reported. Most of these studies are focused on identification of effective EPIs by high-throughput screening of compound databases and determination of the mechanism of inhibitor function by analyzing the molecular level inhibitor-pump interactions and the coupled conformational changes occurring in the transporters. In the following, we describe examples from the relevant literature for several families of MDR efflux pumps.

30.6.2 RND Pumps

Inhibitors of these pumps include both medicinal plant extracts [165, 166] and synthetic compounds [134, 167–169]. Regarding the former compounds, two such successful studies have been reported so far where potent EPIs have been identified by *in silico* screening of natural compound databases. In one case, Ohene-Agyei et al. [165] employed molecular docking based screening to predict the bioactivity of plant compounds as effective inhibitors of AcrB by comparing with the known EPI, PAβN. They identified six compounds from docking results, of which plumbagin and nordihydroguaiaretic acid were found to be promising EPIs based on further efflux inhibition assays. In another case, Aparna et al. [166] obtained hits which are non-substrates of AcrB and MexB efflux proteins by using high-throughput virtual screening of an in-house database of phytochemicals and subsequently performing an exclusion-based filtering with the common pharmacophore models generated on the basis of known substrates of these pumps. These hits were then subjected to extra-precision docking against AcrB and MexB proteins and *in vitro* efflux inhibitory activity testing which eventually helped in the identification of lanatoside C and daidzein as promising EPIs effective for use in combination therapy against drug-resistant strains of *P. aeruginosa* and *E. coli*.

Takatsuka et al. [167] performed molecular docking of about 30 compounds (including substrates and inhibitors) to predict their interaction with the binding pocket of the *tight* protomer of AcrB as a means of understanding the substrate selectivity of AcrB. This study showed the presence of two large sites within the binding pocket, of which a narrow groove at one end of the pocket was preferred to a wide cave present at the other end of the pocket (Fig. 30.2). This docking study was validated by competition assays using nitrocefin efflux and covalent labeling of Phe615Cys mutant AcrB with fluorescein-5-maleimide, which also confirmed that the presumed groove binders competed against each other but not with the cave binders.

The first EPI-based MD simulation on RND transporters was reported by Vargiu and Nikaido [168], who examined the binding of nine substrates, two inhibitors, and two non-substrates to the distal pocket of AcrB in the presence of explicit water. They found that both the inhibitors (PAβN and 1-(1-naphtylmethyl)-piperazine [NMP]) bind to the lower part of the distal pocket that is rich in phenylalanine residues. After identification of the binding site for the inhibitor D13-9001 by X-ray crystallography, this pocket was also named a “hydrophobic trap” [132]. Though PAβN and NMP showed a fairly high binding affinity to the distal pocket of AcrB in docking study [167], both inhibitors slightly moved out of the pocket toward the

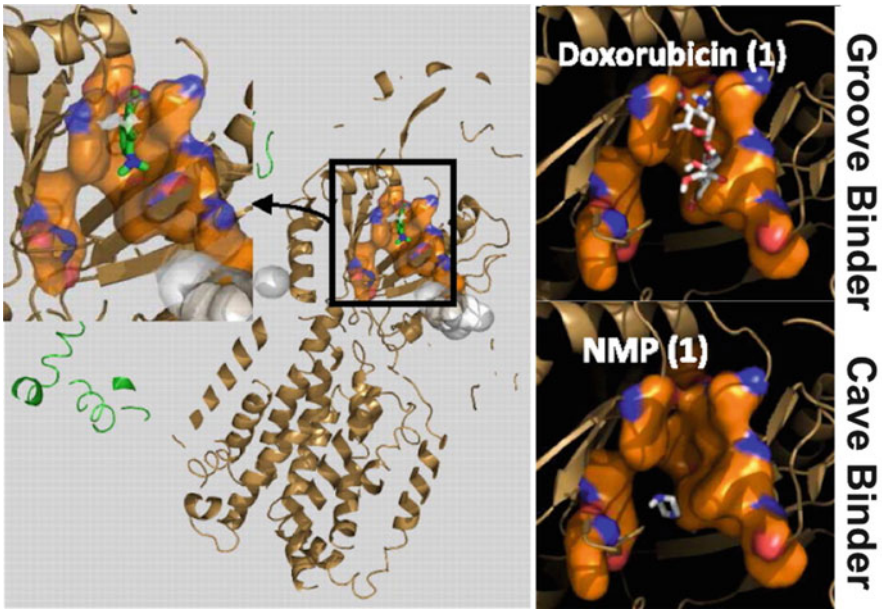


Fig. 30.2 (*Left*) Side view of the binding protomer of AcrB asymmetric trimer with the proximal portion clipped away to reveal the binding pocket shown as surface with carbons in *orange*. The co-crystallized minocycline (PDB code 2DRD) is shown as *green* sticks. (*Inset*) Enlargement of the binding pocket (*right*), predicted binding site of inhibitor 1-(1-naphtylmethyl)-piperazine (NMP) (cave binder) and substrate doxorubicin (groove binder) (Modified from Takatsuka et al. [167])

G-loop and straddled it during the course of the MD simulation (Fig. 30.3). This provided a possible explanation for the mechanisms of inhibition by PAβN and NMP. These inhibitors when bound to AcrB likely reduce the flexibility of the G-loop which is important for the smooth translocation of substrates between the proximal pocket and the distal pocket. This proposed explanation agrees well with findings from the recent experimental [108, 132] and MD simulation studies [170] of the Gly616Pro and Gly619Pro AcrB mutants, where mutations in the G-loop impaired the drug export [99, 107, 108].

A recent work by Vargiu et al. [169] identified the underlying molecular mechanism of inhibition of MBX2319 (a pyranopyridine EPI potent against RND pumps of the *Enterobacteriaceae* species) by comparing it with that of other inhibitors like D13-9001, PAβN, and NMP by molecular docking and MD simulations. They observed that D13-9001 and MBX2319 bound more tightly than the typical substrate minocycline to the distal pocket of the *tight* monomer. The binding mode of MBX2319 was comparable to that of doxorubicin in the Phe610Ala variant of AcrB [171, 172]. By binding to the lower part of the distal pocket in the *tight* protomer of AcrB, this inhibitor interacts in a manner similar to that of the hydrophobic portion of D13-9001 [132] with hydrophobic phenylalanine-rich cage branching off from the substrate-translocation channel (Fig. 30.4) [169]. Investigation of the minocycline

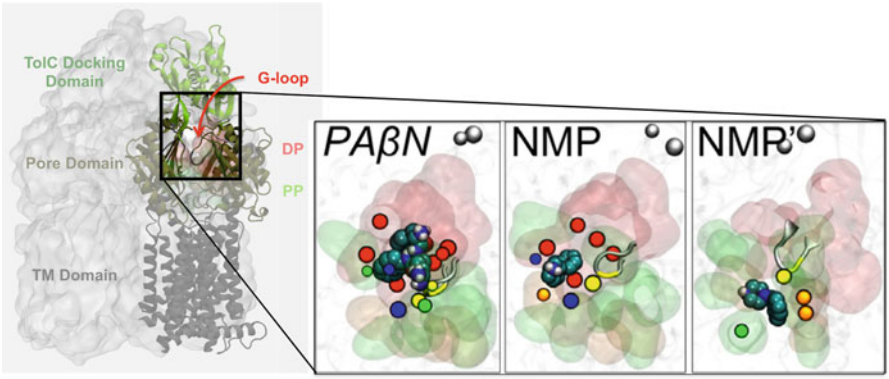


Fig. 30.3 Comparison among different binding modes of PAβN and NMP to the distal and proximal (NMP') binding pockets of AcrB showing straddling of G-loop by these inhibitors. Ligands are shown with spheres colored according to atom types (with nonpolar hydrogens removed). The distal pocket (DP), proximal pocket (PP), and the PC1/PC2 subdomain Cleft are shown with transparent *red*, *green*, and *orange* surfaces, respectively, while the G-loop is shown in *gray* cartoon. Residues within 3.5 Å from the ligand are shown as colored beads (*red*, *green*, *orange*, and *yellow* for those of DP, PP, Cleft, and G-loop, respectively). The residues common to both the pockets are colored *blue*. Residues defining the exit gate (far away from the ligand) are shown as *gray* beads (Modified from Refs. [49, 168])

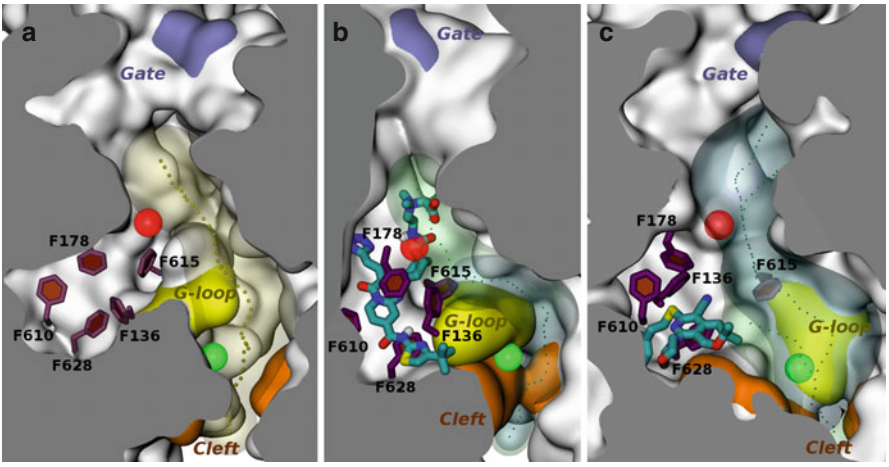
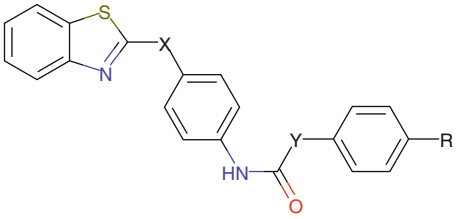


Fig. 30.4 Position of inhibitors D13-9001 (**b**) and MBX2319 (**c**) with respect to the hydrophobic trap in *tight* protomer of AcrB, as found in representative average structures of the complexes from MD simulations. The channel found in AcrB free of ligands (**a**) is also shown for reference. Ligands are shown in *thick sticks*; protein is shown with the molecular surface colored in *orange*, *yellow*, and *ice blue* at the PC1/PC2 cleft, the G-loop tip, and the exit gate, respectively, and *white* elsewhere (Adapted from Vargiu et al. [169])

(substrate) binding to such AcrB-inhibitor complexes supports the hypothesis that all these inhibitors (except D13-9001) could function by competitive binding. As MBX2319 neither contains any charged groups nor can utilize common specific channels to penetrate across the OM of *P. aeruginosa*, it does not remarkably inhibit efflux in this species [47, 133].

Continuing efforts are in progress to develop more potent broad-spectrum EPIs to effectively counter the efflux-mediated MDR in bacteria. One such success story is the development of potent derivatives of MBX2319, some of which are 30 times more potent than the original inhibitor, based on the potentiation of levofloxacin and piperacillin [134]. A very recent study combining the data from cellular, X-ray crystallographic analyses, and MD simulations allowed to unveil the molecular basis for pyranopyridine-based inhibition of AcrB [173]. Particularly, in this study [173], a soluble version of AcrB was engineered (essentially identical to the truncated model of AcrB previously used in MD simulations [168]), highly congruent in structure with the periplasmic part of the full-length protein and capable of binding substrates and potent inhibitors. All of the pyranopyridines included in the work [173] bind within the hydrophobic trap forming extensive hydrophobic interactions. Moreover, the increasing potency of improved inhibitors correlates with the formation of a delicate protein- and water-mediated hydrogen bond network. In addition to giving insights into the mechanism for AcrB efflux inhibition, the setup employed in this new study [173] provides a molecular platform for the development of novel combinational therapies against pathogenic *Enterobacteriaceae*.

One another successful development of EPIs was recently reported by Yilmaz et al. [174], where a modified docking approach named core-constrained docking was employed to identify and characterize the binding site of two-substituted benzothiazoles as potential EPIs with the ability to restore the antibacterial activity of ciprofloxacin in an AcrAB-TolC overexpressing mutant. In the core-constrained docking method, the ligand scaffold is constrained during the initial minimization and conformer generation stages, but is given flexibility during the final refinement stages. Among the compounds experimentally tested by them, BSN-004, BSN-006, and BSN-023 (Fig. 30.5) topped the list with clinically significant EPI activity and were found to bind similar to the co-crystallized AcrB substrates ciprofloxacin, minocycline, and doxorubicin in the distal pocket of the *binding* monomer. Also, the



Compound code	*R*	*X*	*Y*
BSN-004	OCH_3	-	CH_2
BSN-006	CH_3	-	CH_2
BSN-023	H	-	C_2H_4

BSN coded 2-substituted benzothiazoles

Fig. 30.5 Chemical structures of BSN coded 2-substituted benzothiazoles

higher calculated binding energies of BSN-006 and BSN-023 compared to that of ciprofloxacin indicated their possible role as competitive inhibitors in contrast to BSN-004, which with its lower binding energy might act as an uncompetitive inhibitor by simple steric hindrance.

Another very recent study was published by Nikaido and coworkers, who for the first time determined quantitatively the efflux transport kinetics of the EPI PAβN and its homologs Ala, Arg, and Phe β-naphthylamides [175]. In addition, they assessed the behavior of PAβN and its homologs as modulators of nitrocefin efflux through AcrB. These experiments demonstrated that PAβN is efficiently pumped out by AcrB with a sigmoidal kinetics and is able to change the nitrocefin kinetics into a sigmoidal one too. Furthermore, computational modeling showed that modulatory activity of PAβN and its homologs on the efflux of other substrates can be rationalized by inspecting their mode of binding to AcrB. Overall, the data support the hypothesis that PAβN inhibits the efflux of AcrB substrates by both binding to the hydrophobic trap and by interfering with the binding of other drug substrates to the upper part of the binding pocket.

A review on the reports that have brought an advancement in our understanding of the mechanism of functioning of several potent EPIs against RND pumps has been recently authored by Opperman and Nguyen [135].

30.6.3 ABC Transporters

The mammalian P-glycoprotein of the ABC transporters, whose bacterial homologs include MsbA and LmrA [176, 177], has been an efflux transporter of prime interest of this superfamily yielding valuable insights on the drug recognition and mechanism of transport [178]. We, therefore, have included important findings from this eukaryotic pump as they can be translated to their bacterial counterparts.

Vandevuer et al. [179] published the first computational study of EPIs of ABC transporters where they performed molecular docking of several first- and second-generation inhibitors (dexniguldipine, quinidine, quinine, S9788 [a lipophilic P-glycoprotein modulator], tamoxifen, and verapamil) of P-glycoprotein and evaluated the inhibitor interactions and binding positions in P-glycoprotein. The finding of different positions both for a single ligand and for different ligands corroborates the experimental evidence indicating the existence of multiple drug-binding sites. In agreement with a recently proposed pharmacophore model of P-glycoprotein ligands [180], several types of interactions including H-bonds, π-π, and cation-π were identified between P-glycoprotein and the docked ligands.

In order to identify the major differences in the behavior of substrate (colchicine and vinblastine) or inhibitor (latilagascene E, QZ59-SSS [cyclic-*tris*-(*S*)-valineselenazole], tariquidar, and verapamil) molecules inside the drug-binding pocket, Ferreira et al. [158] analyzed the type and number of contacts alongside the major residues involved in the ligand-protein interactions by docking and MD simulations. They found that with the exception of QZ59-SSS, all modulators exhibit a

higher number of nonbonded interactions especially with aromatic residues. They also observed that modulators frequently establish a higher number of simultaneous interactions. Their study identified several residues and at least two regions (Fig. 30.6 [Inset 1]) where interactions occur exclusively with modulators. The first region was located at the beginning of TMD6, comprising residues Leu328, Thr329,

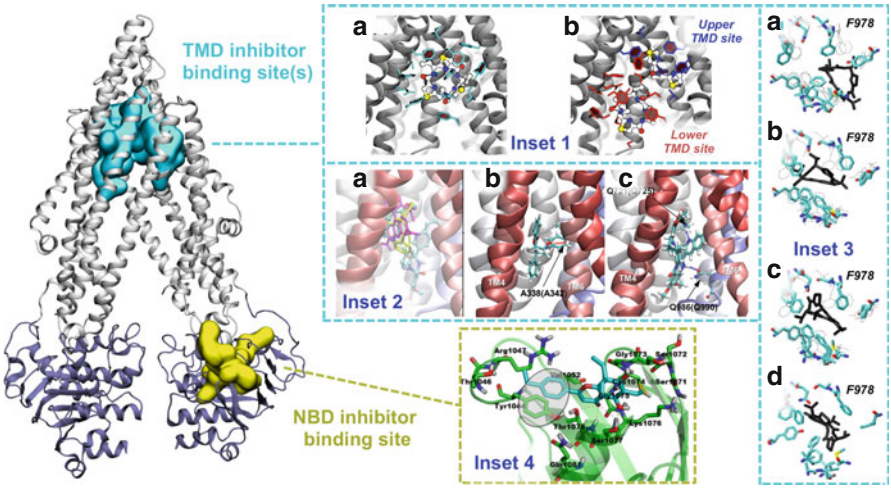


Fig. 30.6 Binding sites found for various inhibitors within the TMD and NBD regions of ABC transporters. The structure of P-glycoprotein (PDB code 3G60) is shown on the *left* side with cartoon representation and the TMD and NDB domains colored *white* and *ice blue*, respectively. The overall positions of binding sites are shown by means of surface representation (*cyan* and *yellow* surfaces, respectively, for the TMD and NBD binding sites) of residues participating in the binding to several inhibitors. The *insets* on the *right* side show the magnified residue level details of the binding of a few inhibitors at that site reported from different studies: (*inset 1*) binding site interactions of QZ59-RRR (**a**) and QZ59-SSS molecules each in the lower (*red*) and upper (*blue*) sites (**b**) in the P-glycoprotein internal cavity as seen in the co-crystallized structures (PDB codes 3G60 and 3G61 [100]). The inhibitors are shown with CPK representation and colored according to the atom type (C, N, O, and S atoms are colored *white*, *blue*, *red*, and *yellow*, respectively), while the side chains of residues within 4 Å of the ligand are shown with *sticks*. (*Inset 2*) (**a**) The docked structure of the low energy conformation of inhibitor XR9576 (C, N, O, and H atoms are colored *cyan*, *blue*, *red*, and *white*, respectively) superimposed on substrate rhodamine 123 (*yellow sticks*) and another inhibitor GP240 (*pink sticks*). Inhibitors GP240 (**b**) and XR9576 (**c**) are stabilized by formation of H-bond with specific residues of P-glycoprotein (Obtained with permission from Elsevier [101]). (*Inset 3*) (**a–c**) Different binding modes of the inhibitor QZ59-RRR (*black sticks*) to the TMD drug-binding pocket of P-glycoprotein as obtained by docking the compound on three different conformations of the protein extracted from MD simulations [102]. The three conformations of the binding site are all shown in each subfigure, with thicker sticks (colored according to atom type as in Inset 2) referring to the conformation used for that specific docking run, and the thin lines used for the two remaining conformations. The crystal structure of mouse P-glycoprotein with QZ59-RRR bound is shown in (**d**) (Adapted from Wise [102]). (*Inset 4*) Docked pose of desmosdumotin (sticks colored according to atom type as in *Inset 2*) in NBD2 (*green* transparent helices) highlighting the stacking interactions realized between the phenyl group of the ligand and Tyr1044. Residues within 4 Å along with the observed hydrogen bonds (*red dashed lines*) are shown (Modified from Gadhe et al. [103])

Phe332, Ser333, and Leu335 and corresponds to an intersection of the QZ59-RRR (cyclic-*tris*-(*R*)-valineselenazole) and QZ59-SSS sites defined by Aller et al. [100]. The other region included residues from TMD7 (Ser725 and Phe728), TMD10 (Glu871, Met874, and Leu875), and TMD11 (Phe934). Several other residues (Met68, Phe332, Leu335, and Tyr946) were also identified to interact with at least three modulators but not with substrates. However, in the case of tariquidar, though a large number of interacting residues common to substrates vinblastine and colchicine were found suggesting a possible competition for these residues, additional interactions with Met874, Leu875, and Phe934 in TMD10/11 not observed for any of the substrates were also identified. This could be well correlated with the increased modulatory effect of tariquidar and may also guide the development of more selective and potent modulators.

In another study, Wise et al. [102] performed molecular docking and targeted MD simulations to evaluate the binding of inhibitors to P-glycoprotein. Ensemble docking was performed by taking 26 catalytically relevant non-redundant structures as receptors against 21 known transport ligands or inhibitors. In addition, the authors examined the transitions of the apo form from conformations that were wide open to the cytoplasm to transition state conformations that were wide open to the extracellular space and observed coupled movement of NBDs and TMDs that form the drug-binding cavities. NBDs showed pronounced twisting as the two domains approached each other, and this movement resulted in opening of the TMDs to the extracellular space as the ATP hydrolysis transition state was reached [102]. The largest movements of drug-binding site helices were observed for the pairs of helices 4/10 and helices 5/11. As the ATP hydrolysis transition state (fully opened outward conformation) approached, drug docking in the extracellular half of the transmembrane domains seemed to be destabilized as transport ligand exit gates opened to the extracellular space. The side chain of Phe978 (top of the binding site) was found to move out of the way in conformations close to the fully opened inward conformation thereby allowing QZ59-RRR analogue access to binding pocket (Figs. 30.5 and 30.6 [Inset 3]) [100, 102]. This supports the postulation of putative aromatic gating structures in the drug-binding sites of P-glycoprotein. The authors proposed that the destabilization of ligand binding in the extracellular half of the drug-binding site, coupled with denied access to ligand binding on the cytoplasmic side, would effectively force a release of the ligand to the extracellular space. They also suggested that there is no specific "inhibitor-binding site" located within the drug-binding domain of P-glycoprotein and that the mode of inhibition by these compounds, if binding occurs at the locations deduced from these docking studies, may be through the competition with substrate drugs.

In a similar study on EPIs for P-glycoprotein, Jara et al. [101] identified a common binding site for rhodamine 123 and modulators (derivatives of propafenone and XR9576 [tariquidar, one of the best modulators known at present]) with different modulation activity by performing molecular docking over the crystal structure of the mouse P-glycoprotein (Fig. 30.6 [Inset 2a]). The presence of a common binding site would suggest a competitive scheme for these inhibitors and the substrate rhodamine 123. Preliminary classical MD simulations on selected P-glycoprotein/

modulator complexes highlighted the importance of hydrophobic interactions and molecular flexibility of the modulator to fit the aromatic rings inside the TMD. It was found that the binding of two modulators (XR9576 and GP240 [a propafenone derivative]) was energetically more stable in P1 site than rhodamine 123 due to more favorable contributions of van der Waals interactions (hydrophobicity) and nonpolar solvation (Fig. 30.6 [Inset 2b, c]). Several interacting residues were found to be common to substrates and modulators in the region between transmembrane helices 4, 5, and 6 (Ser222, Ile306, Val338, Leu339, Ala342, and Phe343), with the aromatic residues contributing largely to the increase in the modulators' binding affinity. Binding of the inhibitor to this site could reduce the mobility of transmembrane helices (especially TM6) affecting the subsequent ATP hydrolysis. The interaction of TM12 (Val982) at a second site close to P1 was also observed with other inhibitors such as GP240. The molecular docking results in this study were concordant for some members of the GPxx family as reported by Klepsch et al. [181].

In order to investigate the role of P-glycoprotein flexibility in polyspecific drug binding, Liu et al. performed comparative MD simulations of inward-facing P-glycoprotein with/without inhibitor ligands (QZ59-RRR or QZ59-SSS) in explicit lipid and water environment [182]. They found that the flexibility of the binding pocket in P-glycoprotein, which is composed of the TMSs from both halves of P-glycoprotein, especially transmembrane helices 4, 5, and 6 and 10, 11, and 12, is essential for its polyspecific drug binding. Namely, while TM4 and TM5 are rigid and stabilize the whole structure, TM6 and TM12 show high flexibility, and the flexibility of the side chains of aromatic residues (Phe and Tyr) in the binding pocket allows them to form rotamers with different orientations, which is critical for the poly-specificity of the drug-binding cavity of P-glycoprotein. The authors found indeed the binding pocket of P-glycoprotein to be flexible and also to undergo ligand-induced conformational changes thus facilitating the residues lining the pocket to interact with multiple drugs. Finally, MD simulations illustrated the twisted conformational change of transmembrane regions in the outward-facing structure of P-glycoprotein, which might be important to export the substrate molecules, and the translational conformational change in the inward-facing structure, which regulates the opening/closing of the binding cavity of P-glycoprotein.

In order to have a comprehensive understanding of EPI action and conformational dynamics of desmosdumotin, an anticancer agent, Gadhe et al. explored its inhibition mechanism against P-glycoprotein (NBD2) by performing molecular docking and MD simulations [103]. Molecular docking showed that van der Waals and electrostatic interactions predominantly stabilize desmosdumotin binding to NBD2. MD simulations further indicated the involvement of Lys1076 and Ser1077 in hydrogen bonding and Tyr1044, Val1052, Gly1073, Cys1074, and Gly1075 in hydrophobic interactions. The π-π stacking hydrophobic interaction between the B-ring of desmosdumotin and side chain of Tyr1044 (encircled in gray in Fig. 30.6 [Inset 4]) identified in docking and stable during MD seems to be particularly important for inhibitor binding.

Recently, Ma et al. [183] carried out a systematic characterization and comparison of substrate (daunorubicin) and an inhibitor (QZ59-RRR and QZ59-SSS) effects on

NBD and TMD conformational dynamics using apo murine P-glycoprotein. Their simulation systems included the apo form of P-glycoprotein, the co-crystals with inhibitor QZ59 (QZ59-RRR and QZ59-SSS) bound (PDB codes 3G60 and 3G61, respectively [100]), and docking-generated complexes with the substrate daunorubicin bound to each of the two sites where inhibitor QZ59 was found. In six independent MD simulations of the apo protein embedded in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine bilayer, the authors observed an asymmetrical association of the NBDs where one of the two putative nucleotide-binding sites is further dissociated than the other, similar to what has been observed in other ABC transporter proteins. In the ligand bound complexes, this degree of association and the conformations of the nucleotide-binding site (Fig. 30.7) were dependent on the presence and the position of a substrate or an inhibitor bound in the TMD binding cavity. Namely, daunorubicin bound at the upper site triggers P-glycoprotein to undergo a closure event similar to that observed in apo simulations and also leads to the formation of the nucleotide-binding sites competent to bind ATP. The presence of an inhibitor (QZ59-RRR and QZ59-SSS) inside the drug-binding pocket kept the NBD site 2 open (maintaining crystallographic distances) with ATP-protein interaction energies significantly higher than the ones reported for substrates. This suggests that these inhibitors function by keeping the NBDs apart, thus preventing ATP hydrolysis. Moreover, the inhibitor QZ59-RRR exhibited higher affinities compared to that of the substrate, daunorubicin, owing to much more favorable van der Waals interactions.

Summarizing the findings of reference [183], a closure of the P-glycoprotein's internal binding pocket occurred only in the presence of the substrate bound at a

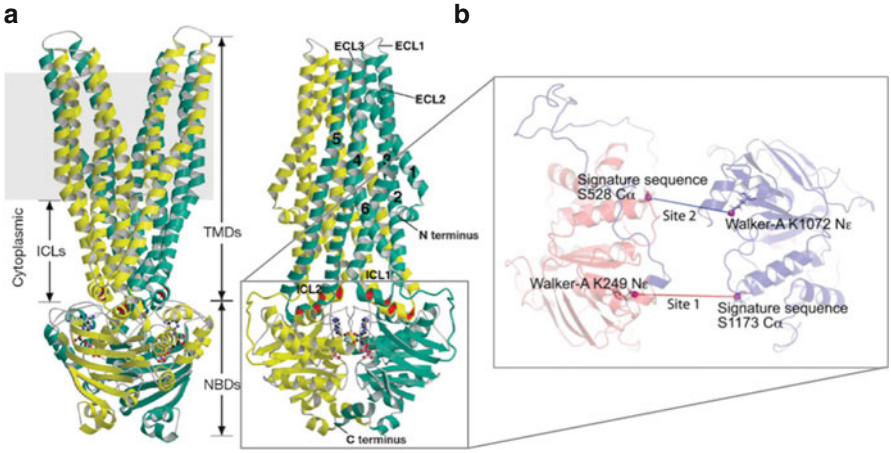


Fig. 30.7 (**a**) The representative structure of ABC transporter Sav1866 with the subunits colored *yellow* and *turquoise* and highlighting the important domains. (**b**) Schematic illustration of the nucleotide-binding sites 1 and 2. Here, the N-terminal Walker A motif and the C-terminal signature sequence form "site 1," whereas the C-terminal Walker A motif and the N-terminal signature sequence form "site 2" (Adapted with permission from Macmillan Publisher Ltd: *Nature* [183, 184])

certain site in the binding pocket, while the inhibitor kept the two NBDs far apart. A greater number of ligand-protein interactions were formed by ligands docked at the lower site compared to the upper site during the unrestrained simulations, presumably reflecting the ability of P-glycoprotein to "wrap up" the ligands and suggesting a substrate-dependent behavior for P-glycoprotein efflux in which the ligand-induced fit seems to play a key role in drug recognition.

In another study aiming to elucidate the mechanism of translocation by and inhibition of P-glycoprotein, Prajapati et al. [185] modeled this transporter in three different catalytic states (inward open [IO] [NBDs are far apart], intermediate open [IIO], and outward open [OO] [NBDs are in close proximity]) and studied a total of 17 systems including eight substrates, eight inhibitors, and one without ligand by multi-targeted MD. Substantial details on the changes occurring in TMDs, the role of intracellular coupling helices, and the displacements and conformational changes in the residues lining drug-binding pocket during the catalytic transition of P-glycoprotein from its inward open to outward open state were traced. Though no distinct site for substrate and inhibitor binding was noticed, significant difference in substrate and inhibitor-binding interactions and stability was observed during the simulation from IO to OO state. The authors clearly showed how the loss of stable binding interactions destabilized the substrate binding in the active site of P-glycoprotein and dislodged it during the IO to OO transformation. In contrast, the inhibitors maintained stable interactions with drug-binding residues Phe303, Ile306, Phe343, Phe728, Ile868, Phe942, Thr945, and Ala985, posing possibility of inhibition of the conformational change in P-glycoprotein structure (Fig. 30.8).

In addition to studies on understanding the mechanism of action of existing inhibitors of ABC pumps, attempts have been made to improve their activity or to design new ones. One such study was performed by Tardia et al. who reported a new series of total 21 polymethoxy benzamides with the P-glycoprotein inhibitory activity. The submicromolar IC_{50} level was reached through modulated lipophilicity of compounds and by establishment of an intramolecular hydrogen bond [186]. Eleven out of 21 of these compounds were active against both P-glycoprotein and MRP1. MD simulations and density functional theory calculations on these compounds advocated the presence of a unique conformation of the hit 4b (Fig. 30.9), which was characterized by a very stable intramolecular hydrogen bond. The authors claim that this conformational difference is the reason for the differential activities reported for the regioisomers 4a and 4b. They also state the strength of such intramolecular hydrogen bond interaction to be a sensitive parameter for soft modulation of the P-glycoprotein response as evident from 2,4,5-trimethoxybenzamide derivatives 3b, 4b, and 5b which display the highest activity and also the strongest intramolecular hydrogen bond.

Singh et al. [187] designed inhibitors of the transporter P-glycoprotein/MDR1 in *Leishmania*, responsible for the extrusion of miltefosine, a drug to treat leishmaniasis. Together with a series of activators of P4-ATPase protein to enhance import of miltefosine, a series of peptide inhibitors (Fig. 30.10a) of the P-glycoprotein-like ABC transporter were designed to overcome miltefosine resistance. The inhibitors were designed considering specificity to the target protein and also surface

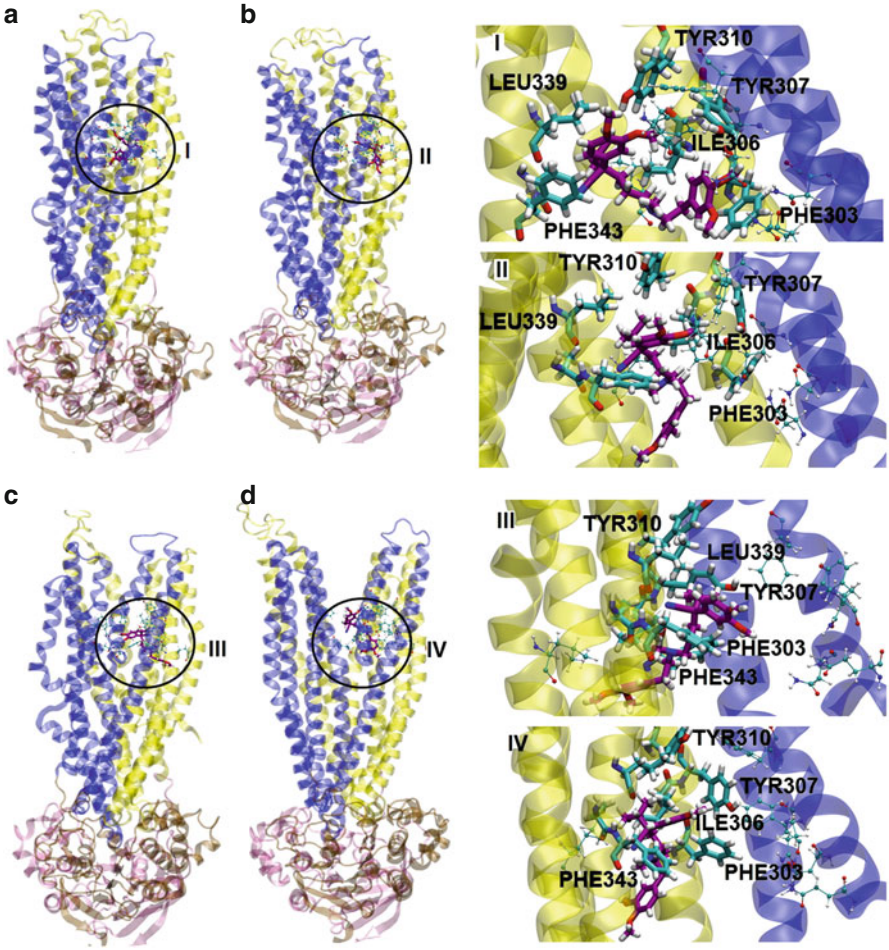


Fig. 30.8 Changes in molecular interactions of verapamil (inhibitor) observed during multi-targeted molecular dynamics simulation; (**a**–**d**) represent the P-glycoprotein transition states: initial inward open, at starting of intermediate open, after intermediate open and outward open, respectively. The magnified images of corresponding encircled regions are shown as I, II, III, and IV, respectively (Obtained with permission from Elsevier [185])

orientation and flexibility. The molecular docking of these designed inhibitors confirmed the high affinity of inhibitor-9 having the sequence "QFIYYSAYALCFWY" and interacting with Asp1029, Ala1022, and His55 of the transporter (Fig. 30.10b). This study provided insights into the possibility of targeting P4-ATPase (important for the import of alkylphospholipid drugs into the parasite) and ABC transporters for improving the therapeutic efficiency of antileishmanial agents.

The results from the various computational studies on inhibitors of ABC pumps summarized above reflect the importance of copious nonbonded interactions to be formed by an inhibitor molecule to compete and establish itself strongly in the binding site of the pump, thenceforth impeding the required conformational changes for

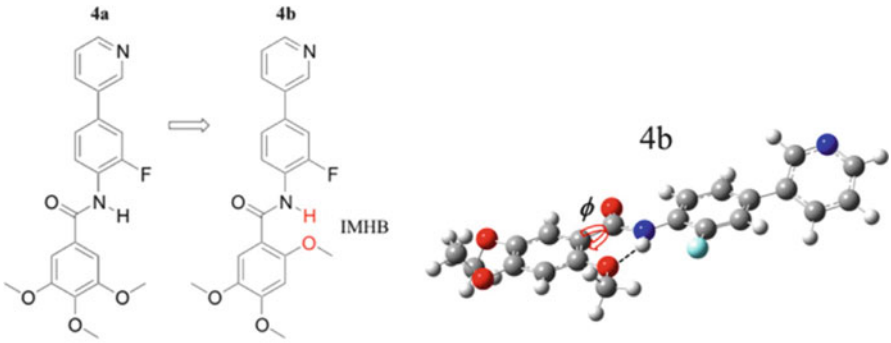


Fig. 30.9 2D structural representation of the regioisomers 4a and 4b highlighting the location of the intramolecular hydrogen bond (IMHB) (Obtained with permission from the American Chemical Society [186])

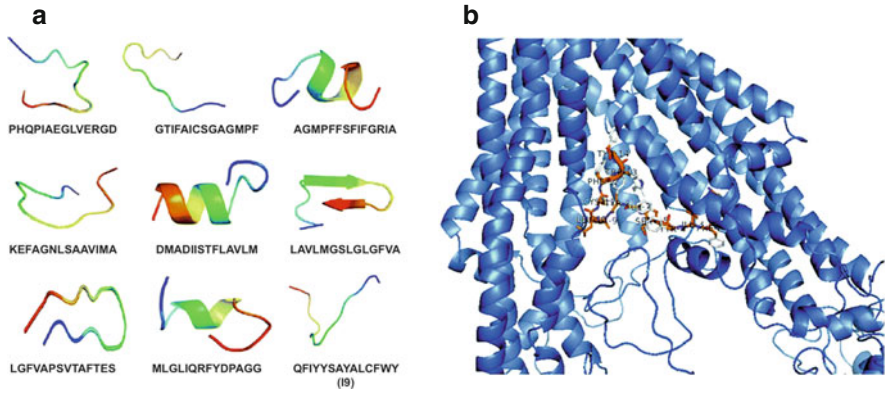


Fig. 30.10 (**a**) Designed peptide inhibitors of the ABC transporters along with their amino acid sequence. (**b**) Docked complex of ABC transporter with peptide inhibitor I9 (Modified from Singh and Mandlik [187] with permission from the Royal Society of Chemistry)

substrate transport. These studies showcase the substantial success achieved so far in identification of putative binding sites of inhibitors, the interacting protein residues and the nature of predominant interactions, and the inhibition mechanism, all of which can be collectively exploited to develop novel and potent inhibitors as done by Tardia et al. [186] and Singh et al. [187].

30.6.4 MATE Transporters

MD simulations were also employed in the study of EPIs for the MATE transporter NorA. The three isomeric hybrid compounds, SS14, SS14-M, and SS14-P, contain berberine, an antibacterial alkaloid known to be a substrate of NorA, fused at different positions of INF55 (5-nitro-2-phenylindole), an inhibitor of NorA. Tomkiewicz et al.

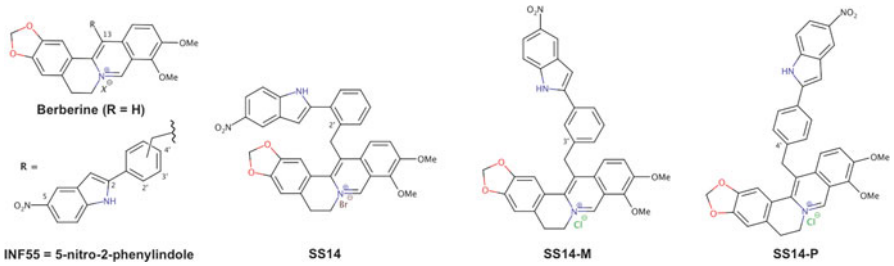


Fig. 30.11 Chemical structures of berberine and INF55 moieties as well as the isomeric hybrid compounds (SS14, SS14-M, and SS14-P)

[188] analyzed the effects of varying the relative orientation of the antibacterial and EPI components in these three isomeric hybrids. They found that a subtle repositioning of the pump-blocking INF55 moiety in berberine-INF55 hybrids has a minimal effect on their antibacterial activities of the hybrids but has a significant effect on their inhibitory action against MDR pumps. Based on the experimental results, authors reported all three hybrids to have a very similar activity against *S. aureus* and *Caenorhabditis elegans*, though SS14 showed a slightly higher potency than its isomers against the wild-type and NorA-knockout strains. Also, the SS14 hybrid showed only a minor inhibitory effect on MDR pumps when compared to that of SS14-M and SS14-P. Through MD simulations, authors identified that the hybrid SS14 prefers to adopt a more compact globular conformation with the INF55 moiety folded back over the berberine unit, whereas in SS14-M and SS14-P, the INF55 moiety extends away from berberine (Fig. 30.11) [188]. The unique conformation for SS14 identified here may explain why it shows different bacterial cell uptake kinetics and reduced inhibitory effects on MDR pumps relative to those of SS14-M and SS14-P.

30.7 Concluding Remarks

MDR is an unavoidable natural phenomenon and needs to be effectively countered with highest priority to prevent the advent of a post-antibiotic era with untreatable life-threatening infections. Efflux transporters like those of the MFS members in Gram-positive bacteria and RND members in Gram-negative bacteria are the primary saviors in clinically important pathogens. These transporters, if inhibited, can hinder the normal physiology as well as the MDR exhibited by pathogens toward numerous drugs, eventually reviving the era of antibiotic treatable infections. The recent reports on computational studies significantly contributing toward the development of several EPIs of such transporter systems and a better understanding of the structure and function of efflux transporter have provided a positive ray of hope toward development of better EPIs and novel antimicrobial agents that can bypass efflux. It would definitely be interesting to improve these molecules to widen their spectrum of activity, even if attainment of a universal prokaryotic EPI might not be

pragmatic. In addition to focusing solely on the competitive inhibitors of the MDR pumps, scientists are now considering inhibition of transcription of the genes coding for efflux pumps or inhibition of other members of tripartite complexes as possible alternatives.

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