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

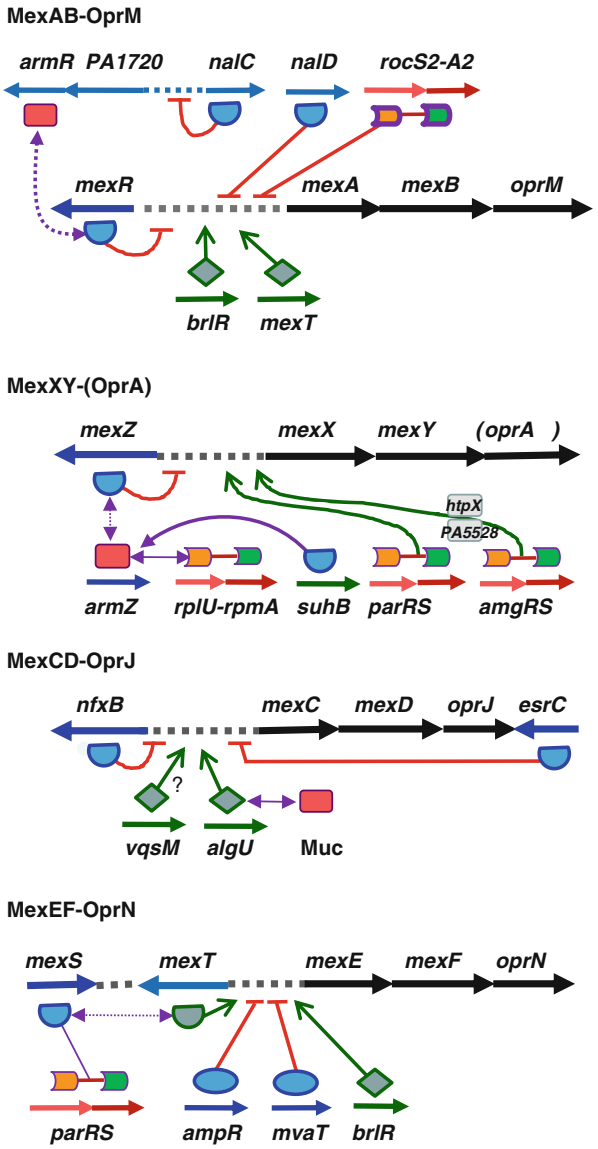

Fig. 14.1 Regulation of the expression of four RND multidrug efflux systems of *P. aeruginosa*. These pumps are controlled by a local regulator (mostly by a repressor [MexR, NfxB, EsrC, or MexZ] or by an activator [MexT]). Positive and negative regulations of the relevant gene transcriptions are, respectively, 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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