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

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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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X.-Z. Li et al. (eds.), *Efflux-Mediated Antimicrobial Resistance in Bacteria*,
DOI 10.1007/978-3-319-39658-3_12

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-

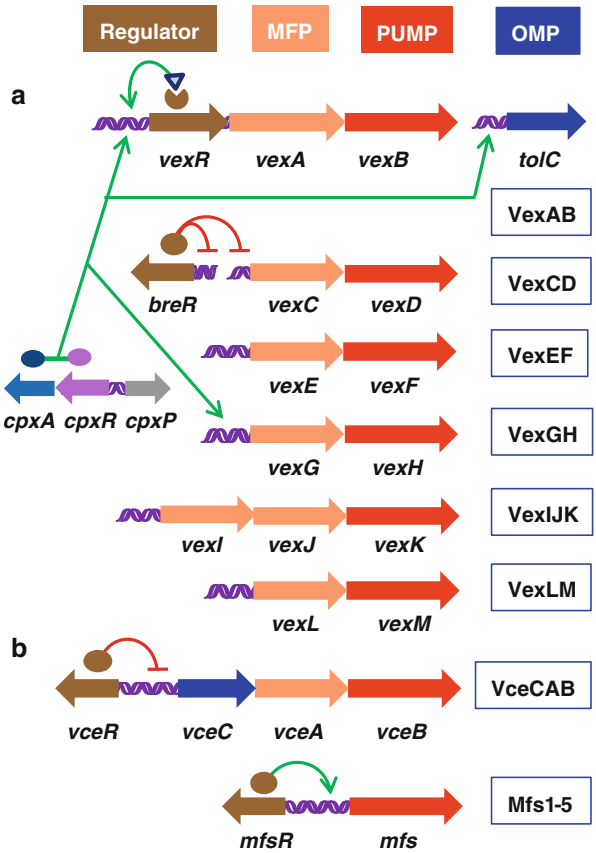

Fig. 12.1 Genetic organization of the known and putative chromosomally encoded RND (**a**) and MFS (**b**) efflux pumps in *V. cholerae* strain El Tor N161962. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *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-*

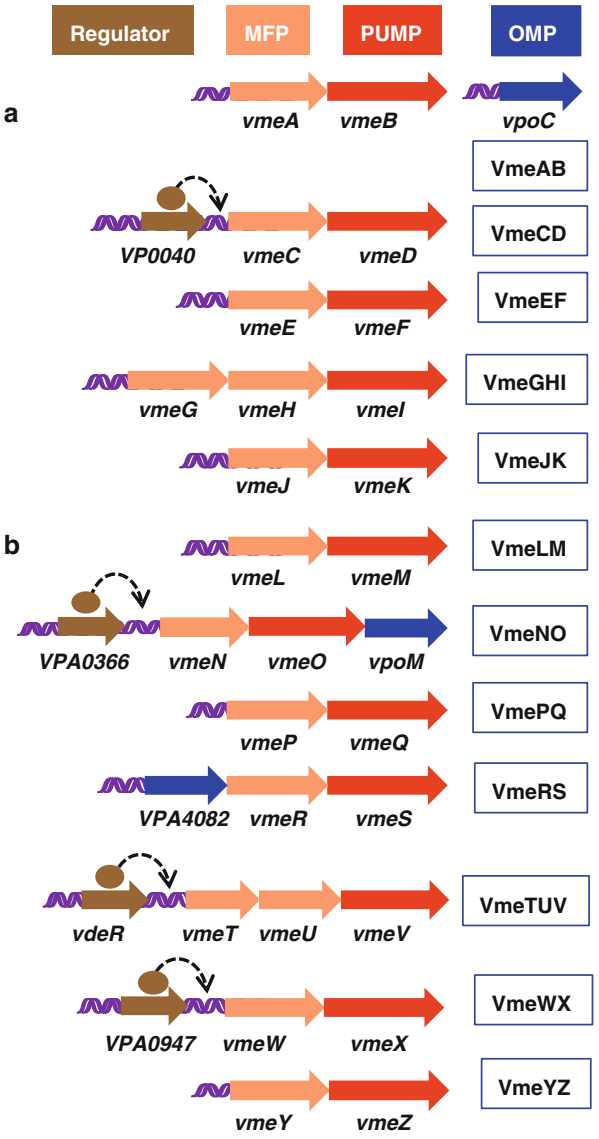

Fig. 12.2 Genetic organization of 12 known and putative RND efflux pumps encoded by chromosome 1 (**a**) and chromosome 2 (**b**) of *V. parahaemolyticus* strain RIMD2210633. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *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

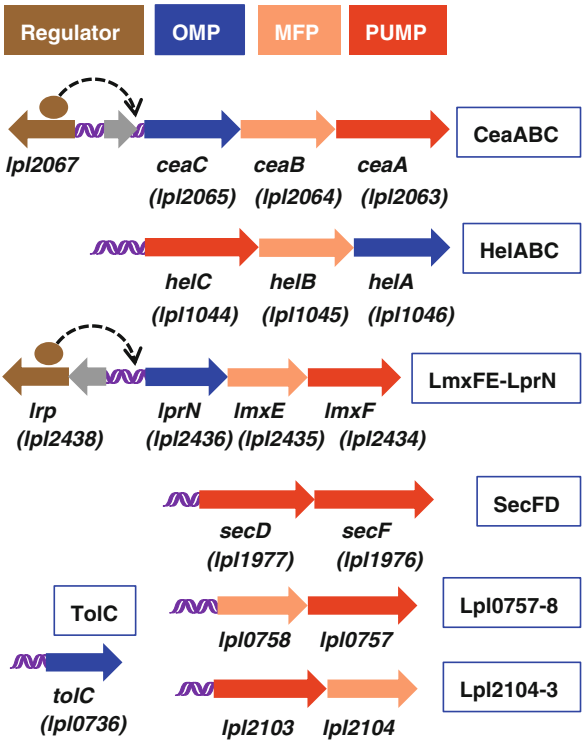

Fig. 12.3 Genetic organization of six known and putative chromosomally encoded RND efflux pumps in *L. pneumophila* strain Lens. The efflux pump operons or genes are presented with *arrows* showing their gene transcriptional directions. Three colors (*orange*, *red*, and *blue*) correspond to their roles 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.

Acknowledgments The views expressed in this chapter do not necessarily reflect those of Xian-Zhi Li's affiliation, Health Canada.

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