Review

Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions

Herbert P. Schweizer

Department of Microbiology, Immunology and Pathology
Colorado State University, Fort Collins, CO 80523-1682A, USA
Corresponding author: H.P. Schweizer
E-mail: Herbert.Schweizer@colostate.edu

ABSTRACT. *Pseudomonas aeruginosa* is an opportunistic human pathogen exhibiting innate resistance to multiple antimicrobial agents. This intrinsic multidrug resistance is caused by synergy between a low-permeability outer membrane and expression of a number of broadly-specific multidrug efflux (Mex) systems, including MexAB-OprM and MexXY-OprM. In addition to this intrinsic resistance, these and three additional systems, MexCD-OprJ, MexEF-OprN and MexJK-OprM promote acquired multidrug resistance as a consequence of hyper-expression of the efflux genes by mutational events. In addition to antibiotics, these pumps export biocides, dyes, detergents, metabolic inhibitors, organic solvents and molecules involved in bacterial cell-cell communication. Homologues of the resistance-nodulation-division systems of *P. aeruginosa* have been found in *Burkholderia cepacia*, *B. pseudomallei*, *Stenotrophomonas maltophilia*, and the nonpathogen *P. putida*, where they play roles in resistance to antimicrobials and/or organic solvents. Despite intensive studies of these multidrug efflux systems over the past several years, their precise molecular architectures, their modes of regulation of expression and their natural functions remain largely unknown.

Key words: Efflux pumps, Antimicrobials, Multidrug resistance, Drug tolerance, *Pseudomonas*

INTRODUCTION

The causes for antimicrobial resistance are multifactorial. In the case of antibiotics, it has been well documented that resistance is mainly caused by continued over reliance on and imprudent use of these antibacterial agents (Davies, 1996), and increasing evidence is being obtained suggesting that the same may be true for the emergence of biocide resistance (Russell, 2001; Schweizer, 2001). Of particular concern is the possible cross-resistance of antibiotics and biocides due to common resistance mechanisms (Schweizer, 2001; Levy, 2000, 2002; Poole, 2002). Metal resistance is being observed as the result of polluted environments (Hassan et al., 1999). The consequence of continued exposure to antibacterials is an enrichment of bacteria that are intrinsically resistant to antimicrobials or have acquired resistance mechanisms to these substances (Lerner, 1998; Hellinger, 2000; Livermore, 2000; Poole, 2002). *Pseudomonas aeruginosa*, *Burkholderia* ssp. and *Stenotrophomonas maltophilia* are of clinical significance because of their innate multidrug resistance and their ability to acquire high-level multidrug resistance (Poole, 2001). Although *P. putida* is not commonly found to be a pathogenic bacterium, it is known for its high-level solvent tolerance.

EFFLUX AS A MECHANISM OF ANTIMICROBIAL RESISTANCE

Bacterial resistance mechanisms have been mostly determined for antibiotics and include: i) exclusion from the cell, e.g., by the outer membrane of Gram-negative bacteria (Hancock, 1997, 1998); ii) enzymatic inactivation, e.g., by β-lactamases (Thomson and Smith, 2000), by aminoglycoside-modifying enzymes (Davies and Wright, 1997), by chloramphenicol acetyltransferases (Murray and Shaw, 1997), as well as by tetracycline- (Chopra and Roberts, 2001) and by macrolide- (Nakamura et al., 2000) inactivating enzymes; iii) target alterations, e.g., fluoroquinolone resistance (Hooper, 2000) and enterococcal vancomycin resistance (Cetinkaya et al., 2000), and iv) active efflux from the cell, e.g., for fluoroquinolones (Poole, 2000a,b) and for tetracycline (Chopra and Roberts, 2001). Similar resistance mechanisms are also involved in biocide resistance (Russell, 2001; Schweizer, 2001; Levy, 2002; Poole, 2002). Although exclusion from the cell due to reduced outer membrane impermeability was thought to play a key role in the intrinsic resistance of *P. aeruginosa* and related bacteria to many antimicrobial compounds, this is now attributed to synergy between a low-permeability outer membrane and active efflux from the cell (Poole, 2000a; Poole and Srikumar, 2001). To date, five families of bacterial efflux systems have been identified (Putman et al., 2000). These include: i) the small multidrug resistance family (Chung and Saier Jr., 2001); ii) the resistance-nodulation-division (RND) family (Zgurskaya and Nikaido, 2000), which is part of the larger RND permease superfamily (Tseng et al., 1999); iii) the major facilitator superfamily (Pao et al., 1998); iv) the ATP-binding cassette family (van Veen and Konings, 1998); and v) the multidrug and toxic compound extrusion family (Brown et al., 1999). Well-characterized representatives of these families from Gram-positive and Gram-negative bacteria are shown in Figure 1. All of these transporters catalyze active drug efflux and therefore require energy, mostly in the form of proton motif force, but some also in the form of ATP (Paulsen et al., 1996). In *P. aeruginosa* and related bacteria, the RND family is the best characterized (Poole, 2001) and is of clinical significance (Poole and Srikumar, 2001), and therefore this review focuses on this important family of efflux pumps. The prevalence of RND pumps in bacteria

varies widely, ranging from none in *Mycobacterium tuberculosis*, 1 in *Bacillus subtilis*, 4 in *Escherichia coli* to 12 in *P. aeruginosa* (Stover et al., 2000).
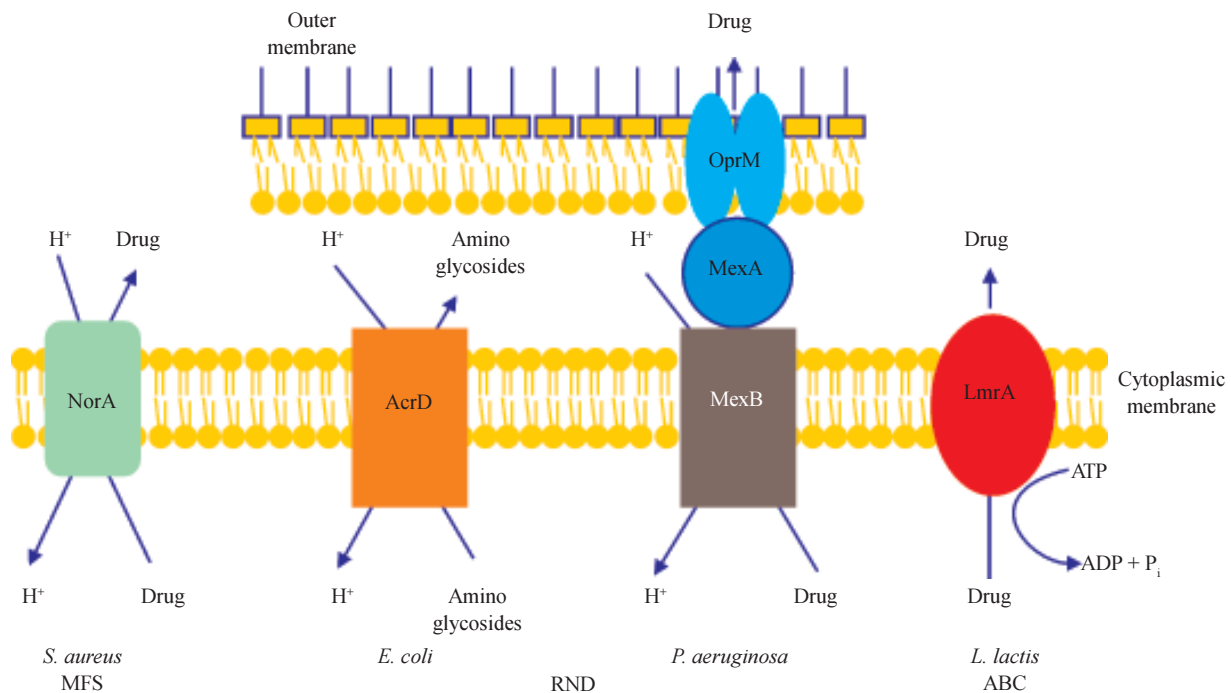

Figure 1. Schematic illustration of the main types of bacterial drug efflux pumps shown in *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Lactobacillus lactis*. Illustrated are NorA, a member of the major facilitator superfamily (MFS); AcrD and MexAB-OprM, two members of the resistance-nodulation-division (RND) family and LmrA, a member of the ATP-binding cassette (ABC) family. All systems extrude drugs in an energy-dependent manner, either by using proton motif force or ATP. The two other types of efflux systems found in bacteria, multidrug and toxic compound extrusion (MATE), and small multidrug resistance (SMR), look structurally similar to the MFS but are designated as distinct families, based on phylogenetic diversity (MATE) or size (SMR).

ARCHITECTURE OF RND EFFLUX PUMPS

Most lipophilic and amphiphilic drugs can cross the cytoplasmic membrane spontaneously, and their accumulation in the periplasm would accelerate their reentry into the cytoplasm. To take full advantage of the above-mentioned exclusion properties of the cell wall in these bacteria, and to bypass the periplasm, it has therefore been proposed that tripartite efflux systems, consisting of a cytoplasmic membrane-associated drug-proton antiporter of the RND family, a periplasmic membrane fusion protein (MFP) and an outer membrane channel-forming protein are required to expel drugs from the cytoplasm to the surrounding medium (Zgurskaya and Nikaido, 1999a,b, 2000). However, variants of this tripartite system have been observed. The AcrD system of *E. coli* consists of only the RND-type cytoplasmic membrane-associated transporter involved in efflux of aminoglycosides (Rosenberg et al., 2000). The existence of a single-component RND transporter for aminoglycosides can be rationalized because the main permeability barrier for hydrophilic drugs, like aminoglycosides, is the cytoplasmic membrane and therefore expulsion into the periplasm achieves its purpose.

Although the exact molecular architecture of a tripartite RND efflux system still remains a mystery, some recent progress has been made regarding the topologies of the outer membrane protein component, OprM, and the RND transporter component, MexA, of one *P. aeruginosa* efflux pump, MexAB-OprM. Functional OprM is a trimeric protein, which besides the typical barrel configuration of the outer membrane-embedded portion of the protein exhibits long periplasmic extensions capable of spanning the periplasm (Wong et al., 2001). This led to the proposal that OprM is capable of directly interacting with the RND transporter, MexB. A topology analysis of MexB using alkaline phosphatase fusions not only predicted 12 transmembrane domains typical of such transporter proteins, but also several extensive periplasmic loops (Guan et al., 1999). Therefore, it is conceivable that OprM directly interacts with MexB. What role the MFP MexA plays in this process remains a mystery although it is clear that it is required for the functioning of RND efflux pumps (Zgurskaya and Nikaido, 1999a,b).

RND EFFLUX PUMPS OF *PSEUDOMONAS AERUGINOSA*

An analysis of the genome sequence of this bacterium reveals the existence of 12 potential RND efflux systems (Figure 2) (Stover et al., 2000). Of these, only the function of six - MexAB-OprM (Poole et al., 1993), MexCD-OprJ (Poole et al., 1996), MexEF-OprN (Köhler et al., 1997), MexGHI-OpmD (Aendekerk et al., 2002), MexJK (Chuanchuen et al., 2002b) and MexXY (Mine et al., 1999; Westbrock-Wadman et al., 1999) - have been experimentally confirmed. Known substrates for these pumps are listed in Table 1. From these listings it is clear that although the substrate spectrum for many of these pumps is wide, some selectivity exists and, perhaps not too surprising, it rests with the RND transporters of the Mex pumps (Srikumar et al., 1997; Maseda et al., 2000b).

Table 1. Substrate profiles of characterized *Pseudomonas aeruginosa* efflux pumps.

| Efflux pump	Substrates	References
MexAB-OprM	β-Lactams, β-lactam inhibitors, fluoroquinolones, tetracycline, chloramphenicol, novobiocin, macrolides, trimethoprim, triclosan (irgasan), ethidium bromide, SDS, aromatic hydrocarbons, thiolactomycin, cerulenin, acylated homoserine lactones	Reviewed in Poole, 2001
MexCD-OprJ	β-Lactams, fluoroquinolones, chloramphenicol, tetracycline, novobiocin, trimethoprim, macrolides, crystal violet, ethidium bromide, acriflavine, SDS, aromatic hydrocarbons, triclosan	Reviewed in Poole, 2001
MexEF-OprN	Fluoroquinolones, chloramphenicol, trimethoprim, aromatic hydrocarbons, triclosan, *Pseudomonas* quinolone signal (precursors)?	Köhler et al., 2001; Poole, 2001
MexGHI-OpmD	Vanadium; acylated homoserine lactones?	Aendekerk et al., 2002
MexJK	Tetracycline, erythromycin, triclosan	Chuanchuen et al., 2002b
MexXY	Fluoroquinolones, aminoglycosides, tetracycline, erythromycin	Westbrock-Wadman et al., 1999; Li et al., 2002
CzrAB-OpmN	Cadmium, zinc	Hassan et al., 1999
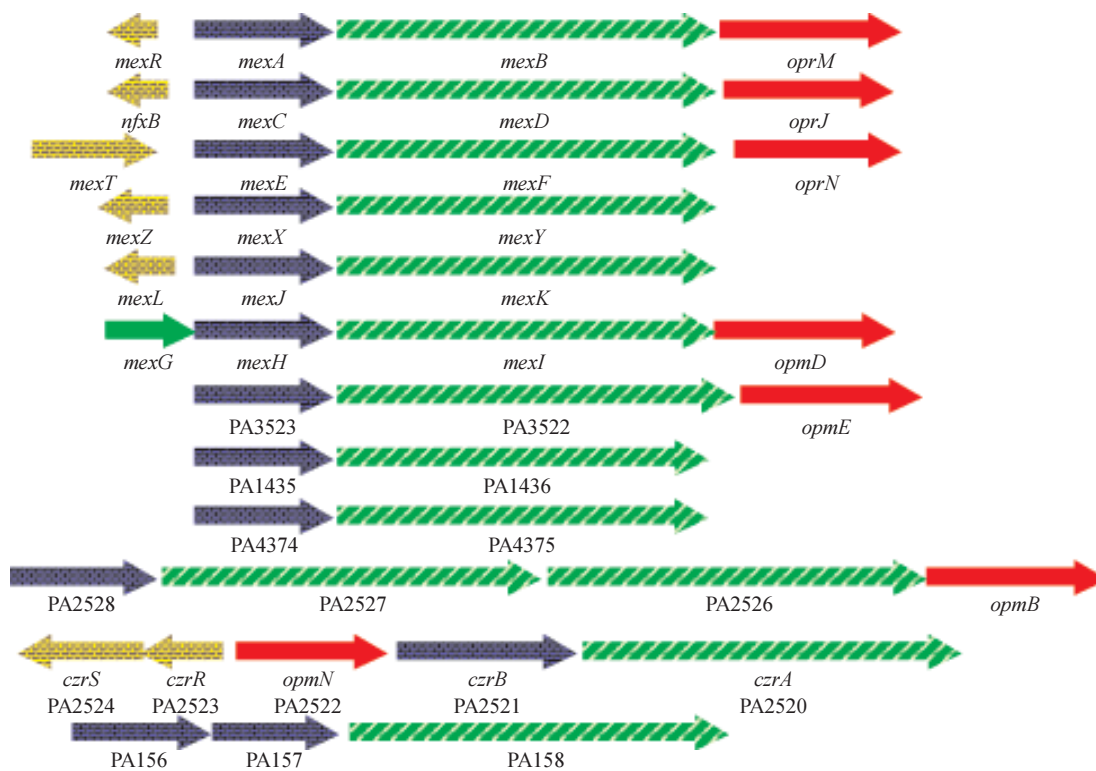
Figure 2. Proposed resistance-nodulation-division (RND) efflux pumps encoded by the *Pseudomonas aeruginosa* genome. Genes encoding protein components of characterized efflux pumps are denoted by gene names and genes encoding protein components of uncharacterized pumps are labeled with the PA numbers assigned in the annotated *P. aeruginosa* genome sequence. RND transporter-encoding genes are depicted as cross-hatched yellow/green arrows, major facilitator superfamily-encoding genes as blue arrows and regulatory genes as yellow arrows. An additional membrane protein-encoding gene required for function of the MexGHI-OpmD efflux pump is shown as a green arrow. Outer membrane channel protein designations are those proposed by Dr. R.E.W. Hancock from the University of British Columbia (http://cmdr.ubc.ca).

The genetic organization of the *P. aeruginosa* RND efflux operons (Figure 2) reveals several commonalities, as well as some significant differences. First, genes encoding the RND transporter and corresponding MFP, respectively, are always present. Second, genes encoding regulators and outer membrane channel proteins, respectively, are not always present, and, in fact, some operons neither contain a regulatory gene nor an outer membrane protein-encoding gene linked to the efflux operon. Third, some operons contain additional genes besides those encoding the RND transporter and MFP, respectively. For example, *mexGHI-opmD* contains *mexG*, which encodes a membrane protein that is required for pump function (Aendekerk et al., 2002). Similarly, PA2528-PA2527-PA2526-*opmB* (Figure 2) contains an additional RND transporter-encoding gene, PA2526. Several questions arise from these observations: i) does the absence of a regulatory protein equate with constitutive expression of the respective operon? and ii) do pumps always require an outer membrane protein channel for function and, if so, which outer membrane channel protein(s) do they use?

REGULATION OF EXPRESSION OF *PSEUDOMONAS AERUGINOSA* RND EFFLUX PUMPS

Table 2 summarizes what we know to date about regulators and inducers of *P. aeruginosa* efflux pumps. With the exception of MexAB-OprM, the expression of most of these efflux

systems is tightly regulated and this has recently been verified by transcriptional profiling using Affymetrix Gene Chips® (Lory, S., personal communication; Chuanchuen, R. and Schweizer, H.P., unpublished results). To date, the expression of only one RND system, MexXY, has been shown to be inducible in the presence of selected antibiotics (Masuda et al., 2000b) and expression of the MexGHI-OpmD pump is cell density dependent, i.e., regulated by the quorum-sensing regulatory circuit(s) (Aendekerk et al., 2002). Overexpression of the other RND pumps is observed as a consequence of exposure to antimicrobials *in vivo* and *in vitro* and this overexpression is usually caused by mutations in genes encoding regulatory proteins (Poole et al., 1996; Saito et al., 1999; Westbrock-Wadman et al., 1999; Ziha-Zafiri et al., 1999; Jalal et al., 1999, 2000; Maseda et al., 2000a; Join-Lambert et al., 2001; Chuanchuen et al., 2002b). These mutations are stable, since expression persists long after removal of selective pressure. For example, triclosan-resistant derivatives of a triclosan-susceptible *P. aeruginosa* mutant strain expressed the MexJK efflux pump due to a mutation in the cognate regulatory gene, *mexL* (Chuanchuen et al., 2000a,b). This single-base change led to an alanine to an aspartic acid change in the second helix of the putative helix-turn-helix putative DNA-binding domain of MexL (Figure 3). Therefore, this mutation was predicted to result in the production of an inactive MexL protein and, in fact, when the binding activity of purified MexL mutant protein was compared to that of the wild-type MexL protein it was unable to bind to its target DNA (Chuanchuen and Schweizer, 2002b). The hitherto best characterized *mex* regulatory protein is MexR, a regulator of MexAB-OprM expression. Although the *mexAB-oprM* operon is always transcribed at low but detectable levels, MexAB-OprM is overproduced in MexR mutants

Table 2. Regulatory components of efflux pump operon expression in *Pseudomonas aeruginosa*.

Efflux pump[1]	Regulators	Function	Linked to operon	Inducer/Signal	References
MexAB-OprM	MexR	Repressor	Yes	Unknown	Saito et al., 2001; Evans et al., 2001; Lim et al., 2002
MexCD-OprJ	NfxB	Repressor	Yes	Unknown	Okazaki and Hirai, 1992; Chuanchuen et al., 2001
MexEF-OprN	MexS MexT	Repressor Activator	No Yes	Unknown	Köhler et al., 1999; Maseda et al., 2000a
MexGHI-OpmD	LasR? RhlR?	Unknown	No	Cell density	Aendekerk et al., 2002
MexJK	MexL	Repressor	Yes	Unknown	Chuanchuen et al., 2002a,b
MexXY	MexZ	Repressor	Yes	Selected pump substrates	Westbrock-Wadman et al., 1999
CzrAB-OpmN	CzrRS	Sensor/kinase	Yes	Cd, Zn	Hassan et al., 1999
PA156-PA157-PA158	Unknown	Unknown	No	Unknown	
PA1435-PA1436	Unknown	Unknown	No	Unknown	
PA2528-PA2527-PA2526-OpmB	Unknown	Unknown	No	Unknown	
PA3523-PA3522-OpmE	Unknown	Unknown	No	Unknown	
PA4374-PA4375	Unknown	Unknown	No	Unknown	

[1]For characterized efflux pumps, protein names are given and for uncharacterized pumps, PA numbers assigned during genome annotation are listed.
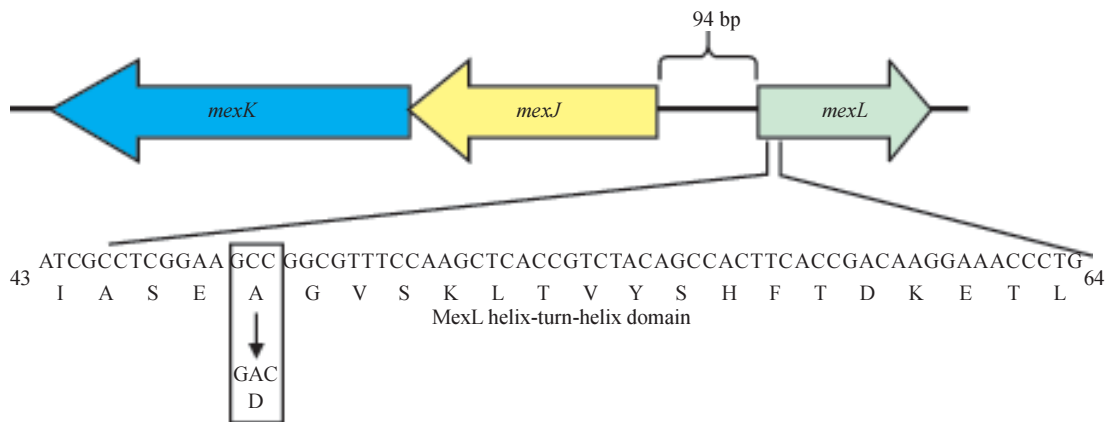
Figure 3. Organization of the *mexJK* operon and *mexL*, encoding a repressor of *mexJK* expression. The nucleotide and amino acid sequences of the predicted helix-turn-helix DNA-binding domain of MexL are shown, including the single-base change and resulting A47D amino acid change in a spontaneous *mexL* mutant that was obtained by triclosan selection. Such regulatory mutants are typically obtained after exposure to antibiotics or biocides (Schweizer, 2001).

(Saito et al., 1999; Adewoye et al., 2002). The MexR protein was purified, its binding characteristics were characterized (Evans et al., 2001; Saito et al., 2001), and its structure with the operator sites was determined (Lim et al., 2002). The other known regulatory genes (Table 2), *nfxB* (Okazaki and Hirai, 1992; Shiba et al., 1995; Poole et al., 1996), *mexZ* (or *amrR*) (Westbrock-Wadman et al., 1999) and *mexL* (Chuanchuen et al., 2002b) encode negative regulators, and mutations in these genes lead to overexpression of the *mexAB-oprM*, *mexCD-oprJ*, *mexXY* and *mexJK* operons, respectively. NfxB and MexL have also been purified and shown to bind to DNA upstream of *mexC* (Shiba et al., 1995) and *mexJ* (Chuanchuen et al., 2002a), respectively. The *mexEF-oprN* operon is positively regulated by the *mexT* product, encoding a transcriptional activator of the LysR family (Köhler et al., 1999). Interestingly, with the exception of the Geneva type strain, which is $mexT^+$, most PAO1 wild-type strains and their derivatives contain *mexT* mutations preventing the expression of MexEF-OprN in these strains in the absence of *mexT* reversions or suppressor mutations (Maseda et al., 2000a; Köhler et al., 2001).

Besides these known regulators, other negative regulatory factors must also be involved in efflux operon expression. Many clinical isolates constitutively express various efflux pumps in the absence of identifiable regulatory mutations, suggesting regulation by hitherto unidentified regulators (Ziha-Zafiri et al., 1999; Pumbwe and Piddock, 2000; Beinlich et al., 2001). Other regulatory factors are further suggested since most of the efflux systems of unknown function are tightly regulated, although they do not contain regulatory genes in the vicinity of their structural genes (Figure 2). To date, few inducers for any of the RND multidrug efflux operons have been identified. The CzrAB-OpmN divalent cation RND efflux pump (Hassan et al., 1999) is regulated by a two-component regulatory system in the presence of divalent cations. Some clinical isolates resisting antibiotic treatment in patients are susceptible to the same antibiotics when isolated and propagated in the laboratory, suggesting inducible components of resistance. It is not known whether this is due to inducible efflux pumps or other mechanisms. Efflux pumps may be involved because MexXY expression is induced in laboratory cultures in the presence of selected pump substrates but this "induction" is not mediated via *mexZ* (Masuda et al., 2000a). Clearly, much work has yet to be done to understand the regulatory circuits underlying RND efflux pump expression in *P. aeruginosa*.

OUTER MEMBRANE CHANNEL REQUIREMENT

Since Gram-negative bacteria possess two membranes separated by the intervening periplasmic space, it is now generally assumed that RND efflux systems in these bacteria function as tripartite systems, which enable the bacterium to extrude antibacterials from the cytoplasm all the way to the extracellular medium (Zgurskaya and Nikaido, 2000). The evidence that even RND systems missing their own outer membrane channels nonetheless require such a protein to function as an efflux pump solely rests on the observations that MexXY (Masuda et al., 2000a; Chuanchuen et al., 2001) and MexJK (Chuanchuen et al., 2002b) require OprM in order to form a functional pump for the export of antibiotics. OprM was proposed because it is always expressed at low but detectable levels from a secondary promoter independent of MexAB (Zhao et al., 1998). However, the identity of the cognate outer membrane channel components, if any, and their interchangeability among different efflux pumps remains under considerable debate. While OprM can function with all RND transporter/MFP complexes studied to date, including MexCD (Gotoh et al., 1998), MexEF (Maseda et al., 2000b), MexXY (Mine et al., 1999; Masuda et al., 2000a; Chuanchuen et al., 2001) and MexJK (Chuanchuen et al., 2002b), other outer membrane channel proteins are more discriminative. Whereas OprJ and OprN did not function with MexJK (Chuanchuen et al., 2002b), and OprN failed to interact with MexAB (Maseda et al., 2000b), OprJ restored almost complete function to a MexAB system without OprM (Srikumar et al., 1997). Further complicating the picture significantly is the fact that the OprM family of outer membrane proteins contains 18 members (Table 3), of which only 7 are associated with RND efflux pumps and we know very little about the functionality, if any, of these proposed outer membrane protein channels. Although MexJK requires OprM for the export of antibiotics and dyes, it seems capable of promoting efflux of the common biocide triclosan, as a two-component system, consisting only of the cytoplasmic membrane-associated MexK RND transporter and the periplasmic MexJ MFP (Chuanchuen et al., 2002b). The

Table 3. The OprM family of outer membrane proteins.

Gene	PA number	RND component
oprJ	PA4597	Yes
oprM	PA0427	Yes
oprN	PA2495	Yes
opmA	PA2837	No
opmB	PA2525	Yes
opmD	PA4208	Yes
opmE	PA3521	Yes
opmF	PA4592	No
opmG	PA5185	No
opmH (*tolC*)	PA4974	No
opmI	PA3894	No
opmJ	PA1238	No
opmK	PA4144	No
opmL	PA1875	No
opmM	PA3404	No
opmN (*czcC*)	PA2522	Yes
opmQ	PA2391	No
aprF	PA1248	No

These assignments are according to Dr. R.E.W. Hancock, University of British Columbia (http://cmdr.ubc.ca).

question therefore is whether it is possible that RND pumps sometimes function as two-component pumps for the efflux of some substrates, possibly their natural substrates. This may also explain why 6 of 12 RND pumps did not maintain a gene encoding their own outer membrane channel proteins during evolution (Figure 2). From our current knowledge, we cannot rule out the possibility that a two-component RND efflux system is sufficient for the efflux of selected amphiphilic drugs, such as triclosan. If this were the case, then RND efflux pumps could be divided into three categories: i) single-component pumps consisting of an inner membrane RND transporter, as typified by AcrD of *E. coli*, for the efflux of hydrophilic drugs; ii) two-component pumps, consisting minimally of an inner membrane RND transporter and an MFP for the efflux of selected amphiphilic drugs, and iii) tripartite pumps consisting of an inner membrane RND transporter, an MFP and an outer membrane protein channel for the efflux of most lipophilic drugs (Figure 4).
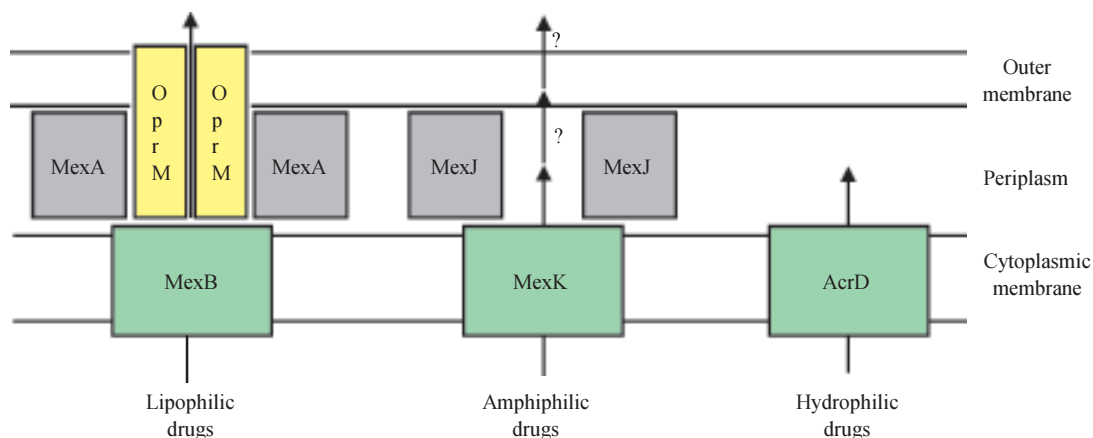

Figure 4. Potential types of bacterial resistance-nodulation-division (RND) efflux pumps. Based on the experimental evidence obtained to date, RND efflux pumps of Gram-negative bacteria could be divided into three categories. These are (from left to right): i) tripartite pumps consisting of an inner membrane RND transporter, a major facilitator superfamily (MFP) and an outer membrane channel protein for efflux of most amphiphilic and lipophilic drugs; ii) two-component pumps consisting minimally of an inner membrane RND transporter and an MFP for the efflux of selected amphiphilic drugs, and iii) single component pumps consisting of an inner membrane RND transporter for the efflux of hydrophilic drugs. While tripartite systems, such as *Pseudomonas aeruginosa* MexAB-OprM shown in this figure, and single-component systems, such as the one typified by AcrD of *E. coli*, have been experimentally verified, the existence of a two-component system is still speculative. The possible existence of such a system was suggested by the finding that MexJK of *P. aeruginosa* did not require any known outer membrane protein channel for triclosan efflux, but required both MexJ and MexK for the efflux of this biocide (Chuanchuen et al., 2002b). However, it cannot presently be ruled out that the MexJK proteins may interact with a hitherto unknown outer membrane channel protein and thus also may be part of a tripartite efflux system.

PHYSIOLOGICAL ROLE OF RND EFFLUX SYSTEMS IN *PSEUDOMONAS AERUGINOSA*

Despite rather intense investigations over the last few years, the physiological role(s) of the RND efflux pumps in *P. aeruginosa* remain largely unclear, with the exception of MexGHI-OpmD and CzrAB-OpmN, which function as metal efflux systems (Hassan et al., 1999; Aendekerk et al., 2002). Although RND pumps could function as efflux systems for undesirable metabolic by-products, they may also play a more active role in cellular metabolism. For example, strains hyperexpressing and/or lacking MexAB-OprM, MexEF-OprN and MexGHI-OpmD either exhibit altered levels of extracellular acylated homoserine lactones or other phe-

notypes tied to quorum sensing (Evans et al., 1998; Köhler et al., 2001; Aendekerk et al., 2002). Therefore, these systems may play a role in the efflux of quorum-sensing molecules or their metabolic precursors and thus play an important role in quorum-sensing signal homeostasis. However, more research is needed to establish a firm link between the expression of efflux pumps and bacterial quorum sensing.

EFFLUX SYSTEMS IN OTHER BACTERIA

RND efflux systems are also found in bacteria that exhibit cell wall properties similar to *P. aeruginosa* (Poole, 2001).

Although originally identified as a plant pathogenic bacterium, *Burkholderia cepacia* is a human pathogen of increasing importance (Govan and Deretic, 1996). It is resistant to multiple antimicrobial agents (Quinn, 1998) and this resistance can be in large part attributed to the expression of the CeoAB-OpcM efflux pump, which confers resistance to chloramphenicol, trimethoprim and fluoroquinolones (Burns et al., 1996). The expression of CeoAB-OpcM is regulated by salicylate, presumably because it mimics iron starvation conditions (Burns and Clark, 1992).

Burkholderia pseudomallei is the causative agent of meliodosis (Dance, 1991) and is highly antibiotic resistant (Simpson et al., 1999). The AmrAB-OprA RND efflux pump confers resistance to aminoglycosides and macrolides, and a Δ(*amrAB-oprA*) mutant became highly susceptible to multiple antibiotics (Moore et al., 1999). The expression of this efflux pump is governed by the adjacent regulatory gene AmrR (Moore et al., 1999).

Stenotrophomonas maltophilia is an increasingly important nosocomial pathogen in debilitated and immunosuppressed individuals (Quinn, 1998; Valdezate et al., 2001). Like *P. aeruginosa* and *Burkholderia* ssp., it is also resistant to multiple antimicrobial agents (Denton and Kerr, 1998; Quinn, 1998). Thus far, two RND efflux systems have been identified, SmeABC (Li et al., 2002) and SmeDEF (Alonso and Martinez, 2000; Zhang et al., 2001), and SmeDEF was shown to be expressed in clinical isolates (Alonso and Martinez, 2001). Expression of SmeABC is probably positively regulated by the SmeRS two-component regulatory system, whose structural genes are located upstream of the *smeABC* operon (Li et al., 2002).

Although *P. putida* is rarely a pathogen, it is known for its high solvent tolerance (Inoue and Horikoshi, 1989) and this is in great part attributable to active efflux (Isken and de Bont, 1996). The responsible efflux systems may or may not efflux medically relevant antimicrobials. SrpABC from strain S12 (Isken and de Bont, 1998; Kieboom et al., 1998a) and TgtDEF from strain DOT-T1E confer toluene tolerance but not resistance to medically relevant antimicrobials (Mosqueda and Ramos, 2000). In contrast, MepABC from strain KT2442 (Fukumori et al., 1998) and *ttgABC* from strain DOT-1E (Ramos et al., 1998) efflux solvents and antimicrobials. Since the expression of TtgABC (Ramos et al., 1998), TtgDEF (Mosqueda and Ramos, 2000) and SrpABC (Kieboom et al., 1998b) is inducible by aromatic hydrocarbons, the primary physiological role of most of these efflux systems is probably to confer solvent tolerance.

CONCLUDING REMARKS

A major factor governing antimicrobial resistance in *P. aeruginosa* and related bacteria is synergy between reduced outer membrane permeability and active efflux from the cell via

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RND efflux pumps. In pathogenic bacteria these pumps are of clinical significance since they are expressed in clinical bacterial isolates and most of them are involved in the extrusion of clinically significant antibiotics. Although RND pumps have been studied in some detail, much research remains to be performed, particularly to advance our understanding of their regulation of expression, of their outer membrane channel protein requirements and of their physiological function. Most of our knowledge about RND efflux pump expression and function stems from work that has been performed with *E. coli* and *P. aeruginosa*, but efflux pumps in other medically, agriculturally and medically important bacteria are not yet well understood. The finding that *P. aeruginosa* mutants that express no efflux pumps are susceptible to most clinically useful antibiotics, including fluoroquinolones, β-lactams and aminoglycosides, prompted research into efflux pump inhibitors, and several such inhibitors have been identified (Renau et al., 1999; Lomovskaya et al., 2001). Some of these are broad-spectrum RND pump inhibitors, while others inhibit individual efflux pumps. Inhibition of efflux pumps significantly decreased the level of intrinsic resistance, reversed acquired resistance and resulted in decreased frequency of emergence of resistance to efflux pump substrates (Lomovskaya et al., 2001). Inhibition of efflux pumps is therefore an attractive approach to improve the efficacies of antibiotics that are substrates of these pumps.

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