Two novel outer membrane proteins involved in intrinsic aminoglycoside resistance in *Pseudomonas aeruginosa*

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ABSTRACT

The expression of tripartite multi-drug efflux pumps such as MexA-MexB-OprM in *Pseudomonas aeruginosa* contributes to intrinsic resistance to a wide variety of antimicrobials, including β-lactams, chloramphenicol, macrolides, quinolones, and tetracycline. MexX-MexY are the only linker and pump efflux system components in *P. aeruginosa* that have been shown to confer intrinsic resistance to aminoglycosides. While a number of studies suggest that OprM, the main efflux outer membrane protein (OMP), forms a functional channel with the MexX-MexY proteins, other data suggests that another OMP is the native channel for the MexX-MexY efflux system.

Fifteen functionally uncharacterized OprM-homologues identified in the recently-sequenced genome of *P. aeruginosa* were possible candidates for the role of the native outer membrane channel for MexX-MexY. Insertional inactivation of OpmG resulted in an 8-fold decrease in MIC to streptomycin, kanamycin, and gentamicin, while inactivation of OpmH resulted in 4- to 8-fold decreases in MIC to kanamycin and streptomycin. When reintroduced into *P. aeruginosa* on multicopy plasmids, both OpmG and OpmH were able to complement the susceptibility of their respective mutants. Changes in MIC due to pseudo-reversion through compensatory mutations were not a factor, as demonstrated by mini-microarray hybridization analysis of the OprM-homologues. This study demonstrates that the two novel outer membrane proteins OpmG and Opmh play a role in aminoglycoside resistance, and that OpmG is likely the main aminoglycoside efflux channel.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Acr	acriflavin
ApR	ampicillin-resistant
bp	base pair
Cb	carbenicillin
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
Cm	chloramphenicol
Clin	clindamycin
Ctax	ceftriaxone
Ctzd	ceftazidime
CV	crystal violet
DEPC	diethylpyrocarbonate
Ery	erythromycin
Fus	fusidic acid
Gm	gentamicin
HP	hypothetical protein
Imi	imipenem
Km	kanamycin
LB	Luria broth
LBLS	Luria broth (low salt)
LBNS	Luria broth (normal salt)
MDR	multi-drug resistant
Mer	meropenem
MF(S)	major facilitator (superfamily)
MIC	minimal inhibitory concentration
Nal	nalidixic acid
Nor	norfloxacin
OD	optical density
OMP	outer membrane protein

Opm	outer membrane protein of the OprM family
ORF	open reading frame
PCR	polymerase chain reaction
Pmb	polymixin B
RND	resistance/nodulation/cell division
RT	reverse transcription
Sm	streptomycin
SDS	sodium dodecyl sulfate
SSC	sodium acetate and sodium chloride
Tc	tetracycline
TcR	tetracycline-resistant
Tm	tobramycin
TMD	transmembrane domain
UV	ultraviolet

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INTRODUCTION

1.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a common Gram negative bacterium, found ubiquitously in the environment and often in association with plants and animals (Beinlich *et al.,* 2001). In healthy humans, *P. aeruginosa* presents no significant threat to an individual's health; however, in immunocompromised persons, *P. aeruginosa* has been known to cause severe infections (Hancock and Speert, 2000). Almost all patients with Cystic Fibrosis (CF), in whom *P. aeruginosa* often colonizes the thick mucous layer in the lungs, eventually suffer from chronic respiratory tract infections that end in death (Hancock and Lam, 1998; Hancock and Speert, 2000). It is also a common pathogen of patients with burn wounds or those who have received immunosuppressive drugs for surgery (Hancock and Lam, 1998). Indeed, *P. aeruginosa* is responsible for about 10% of all nosocomial (hospital-acquired) infections (Hancock and Speert, 2000). *P. aeruginosa* possesses several virulence factors that aid in colonizing the human host, including mucous production, exotoxins (such as exotoxin A), extracellular proteases, extracellular lipases, endotoxin, and antibiotic resistance determinants (Hancock and Lam, 1998).

Antibiotic resistance in particular is a major obstacle for treatment of *P. aeruginosa* infections. This bacterium has been shown to have high-level intrinsic resistance to nearly all major classes of antibiotics, including quinolones, aminoglycosides, macrolides, β-lactams, tetracyclines, and chloramphenicol (Masuda *et al.,* 2000b). Antibiotic-modifying enzymes, such as an inducible chromosomally-encoded periplasmic β-lactamase, contribute to this resistance (Hancock and Woodruff, 1998; Nakae *et al.*,

1999; Ciofu *et al.,* 2000). However, the broad-spectrum resistance seen in this organism is conferred largely by a combination of low outer membrane permeability and multi-drug efflux systems (Nikaido, 1996). The multi-drug efflux systems of *P. aeruginosa*, and indeed of many Gram negative bacteria, are noted for their ability to transport several structurally-unrelated compounds as substrates for efflux.

1.2 Multi-Drug Efflux Systems of Gram Negative Bacteria

It was originally proposed that the intrinsic resistance to antibiotics seen in *P. aeruginosa* and other Gram negative bacteria was solely a result of low outer membrane permeability. However, a poorly permeable outer membrane cannot account for this resistance on its own, since internal drug concentrations would nevertheless reach equilibrium levels without the aid of another compensatory mechanism (Li *et al.,* 1994; Nikaido, 1996). One such mechanism is the multi-drug efflux system, which functions to transport the antibiotic from the cell back into the extracellular environment. Thus, the low rate of drug influx due to an outer membrane of low permeability acts synergistically with active antibiotic export in maintaining a low intracellular drug concentration.

Energy-dependent efflux in Gram negative bacteria uses a proton antiport system where the energy from protons diffusing across the cytoplasmic membrane down their concentration gradient is coupled to the active movement of the drug from the intracellular to the extracellular environment. Therefore, an energy uncoupler, such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which dispels the energy gradient by shuttling protons back into the cell, serves to abolish efflux, leading to the accumulation of antibiotic inside the cell (Li *et al.,* 1994; Nikaido, 1996; Poole *et al.,* 1996; Kohler *et al.,* 1997).

Escherichia coli possesses one constitutively expressed multi-drug efflux system, AcrA-AcrB-TolC, which was initially implicated in resistance to the dye acriflavin, and now known to play a role in resistance to dyes, detergents, erythromycin, and fusidic acid (Nikaido, 1996). The constitutively expressed MexA-MexB-OprM multi-drug efflux system of wild-type *P. aeruginosa* is chromosomally encoded as a three-gene operon, and accounts for the broad-spectrum resistance to many antibiotics including β-lactams (but not imipenem), chloramphenicol, macrolides, quinolones, and tetracycline (Li *et al.,* 1995; Nikaido, 1996, Masuda *et al.,* 2000b).

It is known that *P. aeruginosa* possesses at least two other multi-drug efflux systems, MexC-MexD-OprJ and MexE-MexF-OprN, both of which are also encoded chromosomally as three gene operons. Although neither of these systems account for intrinsic resistance in this organism since they are not normally expressed in wild-type *P. aeruginosa.* They can nevertheless account for mutational and acquired resistance. MexC-MexD-OprJ is negatively regulated by the NfxB repressor, and *nfxB* mutants show increases in resistance to fourth-generation cephalosporins, chloramphenicol, macrolides, quinolones, and tetracycline (Nikaido, 1996; Poole *et al.,* 1996; Masuda *et al.,* 2000b). MexC-MexD-OprJ was later shown to be inducible by subinhibitory concentrations of ethidium bromide, rhodamine 6G, and acriflavin (Morita *et al.,* 2001). In contrast, MexE-MexF-OprN is positively regulated by the MexT protein, and *nfxC* mutants, which express MexE-MexF-OprN, are resistant to chloramphenicol, quinolones, and trimethoprim, as well as the carbapenem subclass of β-lactams, (Nikaido, 1996; Kohler *et al.*, 1997) due to an incident downregulation of the carbapenem porin OprD (Ochs *et al.*, 1999). In addition, mutations in the *mexR* gene, encoding the repressor for the *mexA-*

mexB-oprM operon, can result in overexpression of the MexA-MexB-OprM proteins and confer the hyper-resistant *nalB* phenotype (Saito *et al.,* 1999; Ziha-Zarifi *et al.,* 1999; Srikumar *et al.,* 2000).

However, the mechanisms by which these additional pumps are regulated *in vivo* are not yet well understood. Interestingly, it has been shown that the inactivation of one efflux system can result in the upregulation of another. Indeed, *mexA-mexB-oprM* and *oprM* deletion mutants were found to upregulate expression of both MexC-MexD-OprJ and MexE-MexF-OprN (Li *et al.,* 2000). Since the outer membrane components of an efflux system are interchangeable (Srikumar *et al.,* 1997; Gotoh *et al.,* 1998), and since many efflux systems have overlapping substrates specificities, this sort of compensation might mask phenotypic differences between mutants, particularly in terms of drug resistance and susceptibility.

1.3 RND-type Transport Systems

Transport of compounds across the Gram negative double membrane is significantly more complex than across the Gram positive single membrane (Nikaido, 1996). Not only are there two biological barriers to be traversed, but the energy needed for transport is localized to the cytoplasmic membrane. The efflux pumps of Gram negative organisms overcome this difficulty by utilizing a multi-protein transport system that facilitates movement of the compound across both membranes in a single step (Nikaido, 1996; Zgurskaya and Nikaido, 2000b). *E. coli* AcrA-AcrB-TolC and the three *P. aeruginosa* systems Mex-A-MexB-OprM, MexC-MexD-OprJ, and MexE-MexF-OprN, all belong to this class of transport systems, called the Resistance/Nodulation/Cell Division (RND)–

family, so named for the original proteins placed in this group (for review see Saier Jr. *et al.*, 1994), and whose members share a common tripartite structural make-up (Figure 1).

The first and defining component of the RND-type transport system, designated AcrB in *E. coli* and MexB/MexD/MexF in *P. aeruginosa*, is the RND-pump. It resides in the inner membrane and acts as a proton antiporter, actively transporting compounds using the proton motive force. Analysis of the amino acid sequence suggests a characteristic transmembrane topology for these inner membrane transporters (Saier *et al.,* 1994; Zgurskaya and Nikaido, 2000b). It was later experimentally confirmed (Gotoh *et al.,* 1999; Guan *et al.,* 1999) that this topology consisted of twelve transmembrane domains (TMD), where the first and fourth extracytoplasmic loops (between TMD's 1 and 2, and TMD's 7 and 8) are largely hydrophilic and extend into the periplasmic space (Figure 2). It is believed that these two extended loops may form the basis of the interaction between the inner membrane component of the system and the other components of the efflux system (Zgurskaya and Nikaido, 2000b).

A second component (TolC and OprM/OprJ/OprN) is the outer membrane protein (OMP), and is the pore through which the drug finally exits the cell into the extracellular milieu. Porins play several roles in bacterial outer membranes, and the existence of a large number of characterized OMPs and uncharacterized homologues in *P. aeruginosa* is suggests that they collectively have many functions, such as the export of toxic compounds and virulence factors and the import of catabolites such as amino acids and alternative carbon sources (for a review, see Hancock and Brinkman 2002). Many porins present in the bacterial outer membrane are substrate-specific, such as FhuA of *E coli*, a gated porin that specifically mediates the transport of iron-siderophores, or are general

Figure 1. Model of the structure of the tripartite Gram negative RND efflux system. The proton gradient across the inner membrane energizes the RND pump which moves the drug from the cytoplasm into the outer membrane pore (OMP) that spans the periplasm and the outer membrane.

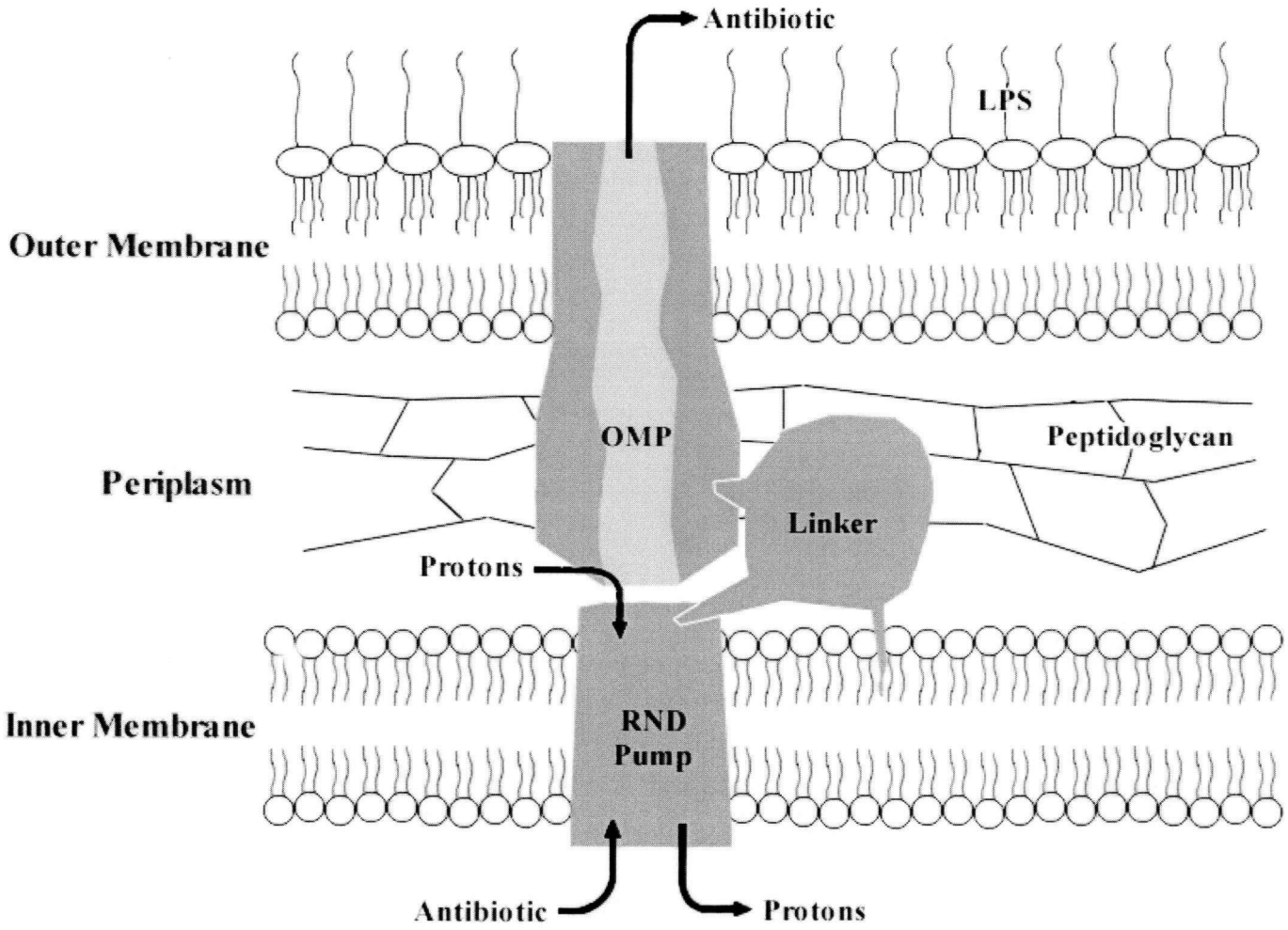
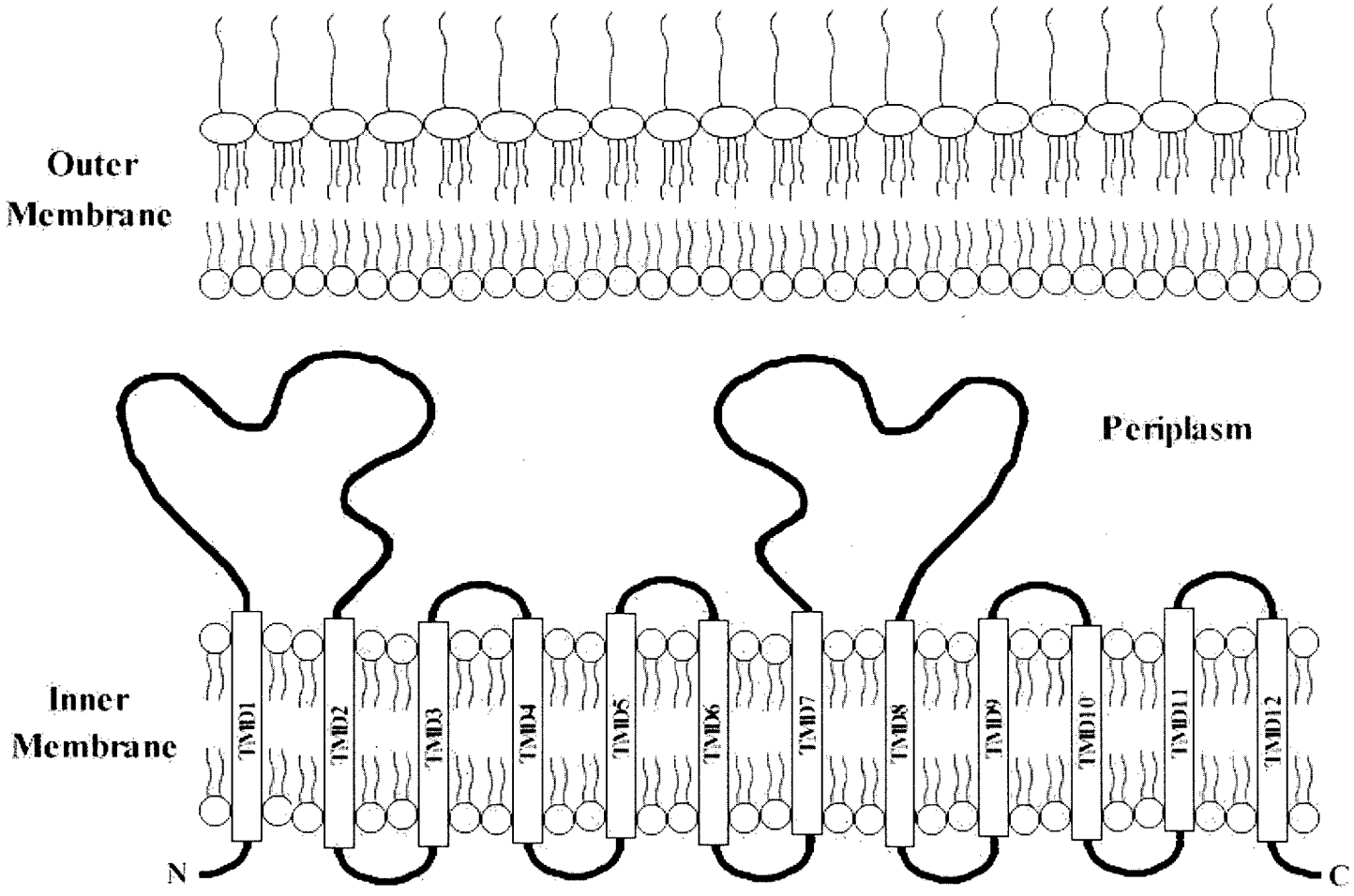


Figure 2. Topological model of an inner membrane RND pump. The distinguishing characteristic are the two periplasmic loops between transmembrane domains (TMD) 1 and 2 and TMDs 7 and 8.



diffusion porins, like OmpF of *E. coli*, which are selective on the bases of size and charge. Multi-drug efflux pumps, by their nature, accommodate many, often structurally dissimilar, antimicrobial agents, and thus the OMP component must also accommodate a broad range of substrates. This includes compounds that are both hydrophobic and hydrophilic, are of many different sizes, including proteins in the case of TolC and other porins involved in type I secretion, which is .a substrate range that is greater than that of general diffusion porins but which is still able to distinguish those substrates from normal and necessary cellular constituents (Zgurskaya and Nikaido, 2000b). Although it has been suggested that the channel-forming efflux OMPs are gated and contain multiple substrate binding sites, so that engagement of the substrates and/or the inner membrane components of the pump opens the gate, allowing the substrate to pass through (Andersen *et al.,* 2000; Koronakis *et al.,* 2000), the mechanism of substrate selectivity is still unknown (Zgurskaya and Nikaido, 2000b). It is believed that the general mechanism involves the recognition of broad physical characteristics of the substrates, such as charge or hydrophobicity, instead of a recognition of structure (Paulsen *et al.,* 1996).

A third component of the tripartite RND efflux systems, is a linker protein, which in some manner facilitates a one-step transport event across both the inner and outer membranes of the Gram negative bacteria (Zgurskaya and Nikaido, 2000b). Linker proteins were once referred to as membrane fusion proteins, so named for sharing C-terminal sequence homology with a group of paramyxoviral fusion proteins (Zgurskaya and Nikaido, 2000b), and it was once thought that they fulfilled their role by fusing the inner and outer membranes of the bacterial cell envelope. Recent crystallization of the outer membrane protein TolC of *E. coli* (Koronakis *et al.,* 2000) has forced researchers to

re-evaluate this hypothesis (Putman *et al.,* 2000). It is now believed that the linker somehow facilitates interaction between inner and outer membrane components. Designated AcrA in *E. coli* and MexA, MexC, or MexE in the *P. aeruginosa* multi-drug efflux systems, the linker is largely periplasmic. A number of linkers contain a lipid modification consensus sequence in their N-terminal regions, followed by an invariant cysteine residue that marks the site of cleavage and modification for lipid attachment (Dinh *et al.,* 1994). Experimental evidence has shown that both MexA of *P. aeruginosa* (Yoneyama *et al.,* 2000) and a cell division linker protein, EnvC, of *E. coli* (Seiffer *et al.,* 1993) are in fact lipoproteins each anchored in the inner membrane by their lipid components.

Each of the three components has previously been shown to be necessary for efflux since the absence of any one of the three components of the efflux system abrogates efflux activity. It has been shown through cross-linking studies that the inner membrane RND-pump and the linker protein are closely associated in complexes (Zgurskaya and Nikaido, 2000a) and are non-interchangeable, such that MexA forms a functional complex only with MexB, but not with MexD, for example (Yoneyama *et al.,* 1998). In contrast, engineering *P. aeruginosa* to express chimeric MexA-MexB-OprJ and MexC-MexD-OprM efflux pumps has demonstrated that outer membrane components are interchangeable (Srikumar *et al.,* 1997; Gotoh *et al.,* 1998). Since the MexA-MexB-OprJ chimera retains the substrate specificity of the native MexA-MexB-OprM pump, and similarly, MexC-MexD-OprM mediates the efflux of the same antibiotics as MexC-MexD-OprJ, it is in fact the inner membrane components that determine the substrate selectivity of the efflux systems (Srikumar *et al.,* 1997; Gotoh *et al.,* 1998).

The crystal structure of TolC was recently determined (Koronakis *et al.,* 2000). It was originally thought that TolC and other efflux-related OMPs would have a similar structure to other bacterial outer membrane porins, such as OmpF of *E. coli*, in which the pore is formed from a multi-stranded β-barrel (Andersen *et al.,* 2000; Andersen *et al.,* 2001) and exists in the outer membrane as a homotrimer (Koebnik *et al.,* 2000). Interestingly, the crystal structure of the trimeric TolC protein showed a single pore with an α-helical domain spanning the entire periplasm and a β-barrel domain in the outer membrane, whose entire structure was formed from three TolC monomers that each formed a third of the overall barrel structure (Koronakis *et al.,* 2000).

This new structure for TolC has provided some insight into the gating mechanism for these channel-forming OMPs. The periplasmic α-helical domain consists of three coiled-coil regions consisting of four helices each, an inner pair and an outer pair. The possible rotation of the inner pair of helices around the outer pair helices would serve to dilate and constrict the periplasmic end of the barrel (Koronakis *et al.,* 2000). It is now believed that the direct interaction of inner and outer membrane components, most likely while the transporter is engaged by a specific substrate, triggers the movement of the helices and the dilation of the entrance of the barrel (Andersen *et al.,* 2000; Koronakis *et al.,* 2000). This new information has also called the role of the membrane fusion protein into question, since it is now apparent that a membrane fusion event is not necessary to facilitate one-step transport across two membranes. The current model suggests that the linker protein somehow promotes the interaction between the inner membrane transporter and the outer membrane pore but is not involved in any form of inner and outer membrane fusion thus making the term "membrane fusion protein" somewhat of a

misnomer (Andersen *et al.,* 2000). The trimeric nature of the outer membrane components likely has a role in this interaction since it has been previously shown that the inner membrane translocases of these transport systems also act in groups of three (Thanabalu *et al.,* 1998).

1.4 Aminoglycoside Uptake and Efflux

Aminoglycosides are a class of polycationic, polysaccharide-based antibiotics that contain two or three amino sugars attached to a cyclitol ring (Davis, 1987). Aminoglycosides are routinely used to treat serious bacterial infections despite their toxicity (Davies and Wright, 1997). Since their discovery, there has been much controversy among researchers surrounding the mechanisms of uptake and action of aminoglycosides since they are incredibly pleiotropic in terms of their effects.

Because aminoglycosides are cationic molecules, they compete with divalent cations such as magnesium for the polyanionic portions of the lipopolysaccharide (LPS) molecule on the outer leaflet of the Gram negative outer membrane (Mao *et al.,* 2001), an observation supported by the well-characterized antagonism of aminoglycosides by salts (Hancock, 1981; Mingeot-LeClercq *et al.,* 1999; Mao *et al.,* 2001). Displacement of divalent cations by the much larger aminoglycoside molecule destabilizes and permeabilizes the outer membrane for aminoglycoside entry, a process known as self-promoted uptake (Mingeot-LeClercq *et al.,* 1999; Mao *et al.,* 2001). Aminoglycosides bypass the inner membrane in a two-step, energy-dependent manner characterized by an initial slow period of uptake (phase 1) followed by a rapid intracellular accumulation of the drug (phase 2) (Hancock, 1981; Mingeot-LeClercq *et al.,* 1999). Early studies established that both functional electron transport and protein synthesis were absolutely

required for aminoglycoside uptake and action (Davies and Wright, 1997; Mingeot-LeClercq *et al.,* 1999; Mao *et al.,* 2001), which was supported by data that showed that energy uncouplers like carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and protein synthesis inhibitors such as chloramphenicol were sufficient to prevent killing by aminoglycosides (Hancock, 1981; Davis, 1987). Since mutations to genes encoding ribosomal proteins such as *rpsL* result in high-level mutational resistance to some aminoglycosides, a ribosomal interaction most certainly plays a role (Moat and Foster, 1995; Davies and Wright, 1997; Mao *et al.,* 2001), and there is strong evidence to suggest that membrane damage is also plays a role (Davies and Wright, 1997).

Resistance to aminoglycosides takes many forms, many of which are various aminoglycoside-modifying enzymes. In fact, more than fifty enzymes have been shown to inactivate aminoglycosides according to three general modes of action: 1) ATP-dependent phosphorylation, 2) ATP-dependent adenylation, and 3) acetyl-CoA-dependent *N*-acetylation (Davies and Wright, 1997; Mingeot-LeClercq *et al.,* 1999). Although certain combinations of these enzymes can confer resistance to a variety of aminoglycosides, broad aminoglycoside resistance in the absence of these modifying enzymes has been noted in several clinical isolates (Westbrock-Wadman *et al.,* 1999). The observation of broad-spectrum intrinsic aminoglycoside resistance in clinical isolates suggests the existence of an efflux pump capable of transporting aminoglycoside antibiotics.

1.5 MexX-MexY and Intrinsic Aminoglycoside Resistance

Clinical isolates of *P. aeruginosa* are often resistant to aminoglycosides (Hancock and Speert, 2000). Curiously, despite empirical evidence of intrinsic aminoglycoside

resistance in this organism, none of the previously characterized efflux systems of *P. aeruginosa*, or of Gram negative bacteria at large, have been implicated in aminoglycoside efflux (Nikaido, 1996). Based on homology studies of the proposed aminoglycoside efflux pump and linker protein, AmrA and AmrB, identified in *Burkholderia pseudomallei* (Moore *et al.,* 1999), an *E. coli* efflux pump protein, AcrD (Rosenberg, 2000), and a fourth multi-drug efflux system of *P. aeruginosa*, MexX-MexY, were recently identified and when overexpressed were shown to confer resistance to aminoglycosides (Aires *et al.,* 1999; Mine *et al.,* 1999; Westbrock-Wadman *et al.,* 1999) as well as to many β-lactams, chloramphenicol, macrolides, quinolones, and tetracycline (Masuda *et al.,* 2000b). The MexX-MexY system, like MexA-MexB-OprM, is expressed under normal laboratory conditions (Aires *et al.,* 1999) and is responsible for conferring intrinsic aminoglycoside resistance in wildtype *P. aeruginosa*. Its expression is negatively regulated by the repressor MexZ, whose gene is encoded immediately upstream of *mexXY*. In addition, MexX-MexY was the first of the four characterized efflux systems of *P. aeruginosa* that has been shown to be induced by the presence of subinhibitory concentrations of some of its substrates, namely tetracycline, erythromycin, and gentamicin (Masuda *et al.,* 2000a; Morita *et al.,* 2001), although MexCD-OprJ was later identified as being inducible (Morita *et al.,* 2001).

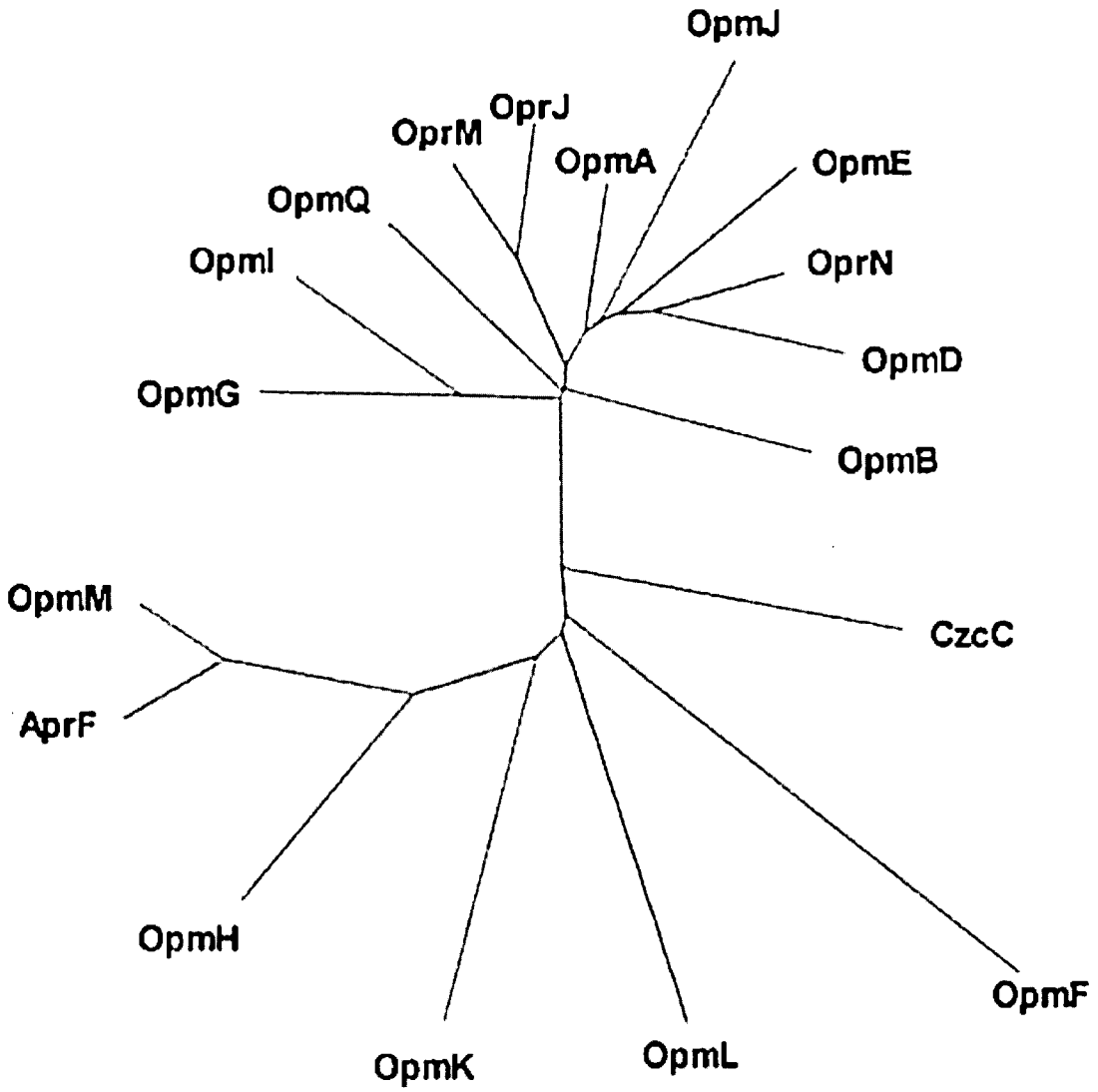
Unlike the other three, previously-characterized, multi-drug efflux systems of *P. aeruginosa*, the three components that constitute the MexX-MexY pump are not encoded as a three-gene operon. Indeed, although *mexX* and *mexY* are co-transcribed, the outer membrane protein component is not contiguous with the two-gene *mexXY* operon, nor is there an outer membrane protein encoded immediately upstream or downstream of the

two genes (Westbrock-Wadman *et al.,* 1999). This has led to some controversy as to the identity of the outer membrane component of MexX-MexY. While evidence showed that *mexXY* and *oprM* introduced on multicopy plasmids into *E. coli* (Mine *et al.,* 1999) and *P. aeruginosa* (Aires *et al.,* 1999; Masuda *et al.,* 2000a) could constitute a working efflux pumps in both organisms, the observation that some broadly aminoglycoside resistant clinical isolates of *P. aeruginosa* have upregulated levels of MexX-MexY but downregulated levels of OprM (Westbrock-Wadman *et al.,* 1999) suggests that the phenotype might result from overexpression of plasmid-borne genes and that the identity of the native OMP is still unknown. It is however not unlikely that OprM could function as the outer membrane component for more than one efflux system, as there is already a precedent for multi-functional outer membrane proteins. The well-studied outer membrane pore TolC of *E. coli* forms active transport complexes for Colicin V (CvaA-CvaB-TolC), hemolysin (HlyB-HlyD-TolC) (Postle *et al.,* 2000) secretion, as well as with AcrA-AcrB for multi-drug efflux.

1.6 Outer Membrane Proteins of *Pseudomonas aeruginosa*

A BLAST homology search of the recently completed *Pseudomonas aeruginosa* genome sequence revealed a total of seventeen homologues of OprM. A tree showing the eighteen members of the OprM family is shown in Figure 3. The proteins fall into two general clusters, those more closely similar to OprM and a second, looser cluster that comprises homologues of type I protein secretion and cation efflux pathways. The first cluster has eleven members and includes the three previously-characterized multi-drug efflux OMPs of *P. aeruginosa*, OprM, OprJ, and OprN. The second cluster contains seven members, including AprF, an OMP implicated in the transport of alkaline protease,

Figure 3. Phylogenetic analysis of the 18-member OprM family. The tree was constructed using the Neighbour-joining method from PHYLIP and Tree View. Uncharacterized OprM homologues are designated Opm, for probable **o**uter membrane **p**rotein Opr**M** family. (Drawn by Fiona Brinkman)



OpmN, which is highly homologous to CzcC in *Ralstonia eutropha*, responsible for cadmium and zinc efflux, as well as the closest *P. aeruginosa* homologue of *E. coli* TolC, OpmH. Fourteen of these homologues, including OpmH and OpmN (CzcC), remain functionally uncharacterized.

1.7 Aims of this Study

Despite the discovery of MexX-MexY in *P. aeruginosa*, the overall mechanism of intrinsic aminoglycoside resistance in this organism is still unclear. The identity of the outer membrane component for the MexX-MexY system is still under debate. Furthermore, it is not known whether outer membrane impermeability and active efflux by MexX-MexY are alone responsible for intrinsic resistance to aminoglycosides in *P. aeruginosa* (Mao *et al.*, 2001). However, it is clear that impermeability-type resistance to aminoglycosides is a common feature in *P. aeruginosa* and active efflux is likely a major component (Mao *et al.*, 2001). Therefore, in order to understand this phenomenon, it is essential that the identity of the OMP for MexX-MexY be determined. Given the large number of uncharacterized OprM homologues present in the genome sequence of *P. aeruginosa*, the goals of this thesis were (a) to screen knock-out mutants of all the uncharacterized OMP genes, using minimal inhibitory concentration (MIC) assays and identify candidate OMPs that may be responsible for mediating efflux of aminoglycosides; (b) to clone and express candidate OMPs and complement the aminoglycoside susceptibility of each knock-out mutant; and (c) to determine if compensatory upregulation of other OMPs in the OprM family in response to insertional inactivation of the OMP of interest played a role in determining the resistance phenotype in this organism.

METHODS AND MATERIALS

2.1 Strains, Plasmids, and Growth Conditions

All strains used in this study are listed in Table I, and all plasmids used in this study are listed in Table II. Strains were grown at 37°C in Luria Broth (LB) medium {1.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl for *E. coli* (LBNS) or 0.05% (w/v) NaCl for *P. aeruginosa* (LBLS)}, or on LB agar containing 2% (w/v) agar. All media components were obtained from Difco Laboratories (Detroit, MI, USA). Antibiotics were supplied at the following concentrations for plasmid maintenance in *E. coli*, ampicillin 100 μg/ml; for plasmid maintenance in *P. aeruginosa*, carbenicillin 200 μg/ml, tetracycline 100 μg/ml; for maintenance of insertion mutations in H956-H969, tetracycline 100 μg/ml; and for maintenance of the insertion mutation in K613, $HgCl_2$ 15 μg/ml.

2.2 General Techniques

Protocols for general DNA techniques such as DNA isolation and agarose gel electrophoresis were found in Sambrook *et al.* (1989). DNA restriction and modifying enzymes were purchased from Invitrogen Life Technologies (Burlington, ON, Canada) or New England BioLabs (Mississauga, ON, Canada) and were used according to manufacturers' protocols. Plasmid DNA was isolated by alkaline lysis (Sambrook *et al.*, 1987) or by using a QIAprep spin miniprep kit (Qiagen Inc., Chatsworth, California, USA), and PCR products were purified using a Qiaquick PCR purification kit (Qiagen Inc.).

Table I. Bacterial strains used in this study.

Strain	Description	Reference/Source
E. coli		
DH5α	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 Δ(*lac*ZYA-*arg*F)U169*deo*R(ϕ80Δ*lac*ZdM15)	Hanahan, 1983
P. aeruginosa		
H103	Wild type PAO1	Nicas and Hancock, 1980
H911	Wild type PAK	Chiron (Pathogenesis Corp.)
H956	H911 *opmK*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H957	H911 *opmA*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H958	H911 *opmG*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H959	H911 *aprF*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H960	H911 *opmI*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H961	H911 *opmE*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H962	H911 *opmL*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H963	H911 *opmD*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H964	H911 *opmJ*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H965	H911 *opmN*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)

To be continued…

Table I. Bacterial strains used in this study.

Strain	Description	Reference/Source
P. aeruginosa		
H966	H911 *opmH*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H967	H103 *opmF*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H968	H911 *opmB*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H969	H911 *opmM*::*miniTn5*-Tc^R	Chiron (Pathogenesis Corp.)
H730 (K372)	PAO6609(*met*9011 *amiE*200 *rpsL pvd*9) Pch$^-$, deficient in pyochelin production and ferripyochelin receptor production	Poole *et al.*, 1991
H743 (K613)	K372 *oprM*::ΩHg, OprM-deficient	Poole *et al.*, 1993

Table II. Plasmids used in this study.

Plasmid	Description	Reference/Source
pCR2.1	TA cloning vector; Ap^R	Invitrogen
pJJ104	pCR2.1*opmG*; Ap^R	This study
pJJ101	pCR2.1*opmH*; Ap^R	This study
pUCP21	*Escherichia – Pseudomonas* shuttle vector; Ap^R	Schweizer, 1991
pUCP27	*Escherichia – Pseudomonas* shuttle vector; Tc^R	Schweizer, 1991
pJJ106	pUCP27*opmG*; Tc^R	This study
pJJ105	pUCP27*opmH*; Tc^R	This study
pJJ107	pUCP21*opmG*; Ap^R	This study
pJJ109	pUCP21*opmH*; Ap^R	This study

2.3 PCR

All PCR was performed on an MJ Research Minicycler (MJ Research Inc., Waltham, MA, USA). PCR primers were synthesized by AlphaDNA (Montreal, PQ, Canada). PCR reactions were performed according to the manufacturer's protocol for Platinum Pfx DNA polymerase (Invitrogen Life Technologies). All PCR reactions, performed using PAO1 genomic DNA, included 5% DMSO in the reaction mixtures. PCR primers used in this study are shown in Table III.

2.4 DNA Sequencing

DNA plasmids for sequencing were isolated using a QIAprep spin miniprep kit (Qiagen Inc.). PCR primers were used for DNA sequencing. Sequencing reactions contained 3.2 pmol of primer, at least 200 ng of template DNA, and components from the BigDye Terminator Cycle Sequencing kit, according to manufacturer's protocols. Sequencing reactions were carried out in an MJ Research Minicycler (96°C for 30 sec, 50°C for 15 sec, 60°C for 4 min; 29 cycles), run on an Applied Biosystems 373 DNA Sequencer, and analysed using ABI 373 Data Collection and Analysis programs. PCR products to be sequenced were purified using a Qiaquick PCR purification kit (Qiagen Inc.). One-half volume sequencing reactions were carried out in the manner previously described and were run on the MJ Research Basestation 51 Automated DNA Fragment Analyzer. Sequence data was analyzed using the B.C.S. (Basestation Control Software) and Cartographer Analysis software.

2.5 Transfer of DNA into *E. coli* and *P. aeruginosa*

Electrocompetent *E. coli* cells were prepared by growing cells to an OD_{550} of 0.4 – 0.6 in LB with 0.5% w/v NaCl (LBNS). Cells were pelleted by centrifugation at 7000

Table III. Oligonucleotide primers used for the amplification of *oprM* homologues.

Primer	Sequence/Description
opmG forward	AAA GGA TCC ATG CCG TTC CCT CTT C
opmG reverse	AAA CTG CAG GTA GAA GAA CTC CCA CGC
opmH forward	AAA GGA TCC CAC ATC GAT CCG GAC
opmH reverse	AAA CTG CAG CGA GCA GGA TGT ACC
oprM-left	CAC CAT GAG CCG CCA ACT GTC C
oprM-right	TGG GTC ACG GTC TGC TGG TTC C
oprJ-left	CGC AAC CTG CGG CAG AAA CAG C
oprJ-right	CTT GGC CAG CTT CAG GGC TTC G
oprN-left	GCG CGA GAA GAT TGC CCT GAG C
oprN-right	CTC CTG GCG CTT GCC ATA GTC G
uvrD-left	AAC GCC CTG ATC GCC AAC AAC C
uvrD-right	AGC GGC ATG TCC ATG ACC TTC G
opmA-left	CAG TTG CGC GGC GAA CAG AAC C
opmA-right	GTG CAT CGC GTC GTC GAC TTC C
opmB-left	GAT CCG CCT GCT CAA CGA CAC G
opmB-right	CCG CCG TCA AAC AGG GTC ATG G
opmD-left	TTC GGT GCT CGC TGC CAG TTG C
opmD-right	TCG ACC AGT TGC CGC AGG TCA C
opmE-left	GAT GGT CGA ACG CCT GGT CAG C
opmE-right	GCA GCG CCA TAG CCG TTG AAG G

To be continued...

Primer	Sequence/Description
opmG-left	TCG ACC AAC TGA TCG GCG AAG C
opmG-right	ATC GCG CCG AGA TTG AGG TTG G
opmI-left	CGG CAC GCA TTT CGA GGT CAG C
opmI-right	TTG CGC GGC GAA GTC CTT CTG G
opmJ-left	CGC TTG CCA GTC ATC TGC GTT G
opmJ-right	TCG AGG CGG ACG GTG TGT TTC C
opmQ-left	GGC CTG GTG TTC GGC TTC ATG C
opmQ-right	CTC GCT GGC CTT GAG GCT TTC C
aprF-left	CAG CAC CGG CAA GTC CAA GTC C
aprF-right	GAA GTT CGC CGC CAC CAG TTC C
czcC-left	AGG ACA CCC GCC AAG GCA ATC G
czcC-right	TCG TCG AGA TGG CGC AGC AAG G
opmF-left	CAA CGG ATC GCG CAG AAG TTC G
opmF-right	GCC TTG AGT TGC AGG CGA TTG G
opmH-left	GCC AGC AAC TAC GCG GTC AAC G
opmH-right	CTG CTT CAG GCG CAG GGT ATC G
opmK-left	GCG ACC CTG CAG AAC ACC TTC G
opmK-right	CTA TGG CTG CGT GCC AGG TTG G
opmL-left	AGC GAT ATG GCG CGG GTG ATC C
opmL-right	AGT TGC CGC TCT GCC TCG AAC C
opmM-left	CAA CAA GGC GCG CAA CGA CTC C
opmM-right	CCA GTT CGC GAT CCT CCA GTG C

RPM at 4°C for 10 min. The pellet was then resuspended in cold sterile dH_2O and the process repeated. After resuspension and a second centrifugation, the pellet was resuspended in 10% v/v glycerol and pelleted by the same procedure. Two washes in 10% v/v glycerol were performed, and the pellet was finally resuspended in 3 ml 10% v/v glycerol and dispensed into sterile Eppendorf tubes in 100 µl aliquots. The aliquoted cells were then snap-frozen in a dry ice–EtOH bath and stored at -70°C. Electrocompetent *P. aeruginosa* cells were grown to a similar OD in LB with 0.05% w/v NaCl (LBLS) and washed using an identical preparation procedure as above, except that washes were performed in ice-cold magnesium electroporation buffer (MEB – 1mM $MgCl_2$, 1mM HEPES, [pH 7.0]). Cells were resuspended in ice-cold MEB, aliquoted, snap-frozen and stored at -70°C.

Electroporation was performed by adding approximately 100 ng of DNA to 100 µl of cells in an ice-cold electroporation cuvette. The mixture was incubated on ice for 45 min, and then the cuvette was placed in the electroporator and subjected to 2.5V (25µFD, 200Ω). 900 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was added to the cells and the mixture incubated at 37°C for 1 hour for ampicillin/carbenicillin selection or 3 hours for tetracycline selection. Following the incubation, cells were plated onto selective media and incubated at 37°C overnight.

2.6 Determining Minimal Inhibitory Concentration

Minimal inhibitory concentration (MICs) of strains to antibiotics were determined according to the protocol for two-fold broth microdilution described by Amsterdam (1991). *P. aeruginosa* cells were grown in LB media with low salt (0.05% w/v NaCl).

After overnight incubation at 37°C, MICs were scored by noting the absence of bacteria in a dilution series of antibiotic, representing the well containing the lowest concentration of antibiotic needed to inhibit bacterial growth. Reported MICs were always the result of at least three independent trials, and changes in MIC of at least 4-fold are considered by convention to be significant.

2.7 Mini-microarrays

2.7.1 Microarray Construction

Microarrays were constructed according to the protocol developed by Brazas and Hancock (unpublished data). Amplicons corresponding to 600 bp internal fragments of each of the OprM family homologues were amplified from PAO1 genomic DNA using the gene-specific primers shown in table 4. The PCR reaction mixture (50 µl total volume) contained: 5 µl of 10 X amplification buffer, 1 mM $MgSO_4$, 50 µM of each dNTP, 200 µM of each of the forward and reverse primers, 5% DMSO, 10ng DNA template, and 1.25 U of Platinum Pfx DNA polymerase (Invitrogen Life Technologies). The reactions were carried out for 30 cycles using a Minicycler (MJ Research Inc.) and each cycle included a denaturation step (94°C for 30 sec), followed by an annealing step (62-65°C for 45 sec), followed by an extension step (72°C for 1 min). The cycling was preceded by an initial denaturation step (94°C for 5 min) and followed by a final extension step (72°C for 5 min).

Amplicons were purified using the Qiagen PCR Purification Kit and quantitated using an Ultrospec 2000 UV spectrophotometer (Amersham Pharmacia Biotech Piscataway, NJ, USA), and then resuspended in spotting solution (0.4 M NaOH, 10 mM EDTA [pH 8.2] in RNase free dH_2O) at a concentration of 20 ng/µl. The amplicons were denatured

at 100°C for 10 min and then immediately placed on ice and recollected by a brief centrifugation at 4°C. Samples were then transferred to a 96-well microtitre plate and spotted onto positively charged nylon membranes (Boehringer Mannheim Laval, PQ, Canada) in 0.5 µl spots using a 96-well groove-pin replicator (V & P Scientific San Diego, CA, USA). After air drying, the membranes were soaked in alkaline denaturing solution (1.5 M NaCl, 0.5 M NaOH in RNase free dH_20) for 10 min and then transferred to neutralizing solution (1 M NaCl, 0.5 M Tris HCl [pH 7.0] in RNase free dH_20) for 5 min. Membranes were allowed to air dry and then were baked for 30 min at 80°C in a Tek Star Jr. hybridization oven (Bio/Can Scientific). Membranes were then wrapped in transparent plastic wrap and exposed to UV light for 30 seconds to crosslink the DNA to the membrane. The membranes were stored between filter papers at 4°C.

2.7.2 RNA Isolation

Cultures of *P. aeruginosa* strains H911, H958, and H966 were grown overnight with appropriate selection and then subcultured to fresh media and allowed to grow to an OD of 0.5, corresponding to 5 x 10^8 cells/ml. All manipulations of RNA were carried out in designated RNase-free areas, and all solutions were treated overnight with 1% diethylpyrocarbonate (DEPC) and then autoclaved to inhibit RNases. RNA was isolated using the RNeasy mini RNA isolation kit (Qiagen): Pelleted cells were resuspended in 100 µl of TE buffer containing 400 µg/ml lysozyme and allowed to incubate at room temperature for 2.5 min. Buffer RLT (350 µl) containing β-mercaptoethanol was added and the sample vortexed. Ethanol (250 µl of 100%) was added and mixed by gentle pipetting. The mixture was applied to an RNeasy mini spin column. Following a 30 sec spin at 13000 rpm, 700 µl buffer RW1 was applied to the column and the mixture spun

again for 30 sec at 10000 rpm. The column was then washed twice with 500 ml RPE buffer containing ethanol and centrifuged once for 30 sec. Waste ethanol was removed from the collection tube, followed by a second 2 min centrifugation at 10,000 rpm to dry the column of ethanol. The column was then transferred to a new collection tube, spun at 13000 rpm for 1 min to clear all traces of RPE buffer and then transferred to another new collection tube. RNA was eluted in 40 μl of RNase free water (supplied) by a final spin at 13000 rpm.

Contaminating genomic DNA was removed from the RNA sample using DNA-*free* kit (Ambion): 0.1 volume of 10 X DNase I buffer and 2 U of DNase were added to the RNA sample and the mixture incubate at 37°C of 30 min. After resuspending the DNase Inactivation Reagent by vortexing, 5 μl of the slurry was added to the tube and mixed by gentle pipetting. Following a 2 min incubation at room temperature, the sample was centrifuged at 13000 rpm for 1 min to pellet the DNase Inactivation Reagent. Purified RNA was quantitated in the Ultrospec 2000 UV spectrophotometer (Amersham Pharmacia Biotech) and stored at -20°C.

2.7.3 Reverse Transcription

Reverse transcription reactions were performed according to the protocol for the use of Superscript II RNase H$^-$ reverse transcriptase (Invitrogen Life Technologies): 2 μg of RNA and 2 μl of 10 μM 5' reverse primer pool is added to the initial reaction mixture (12 μl), which is then heated to 70°C to denature the RNA and primers. Following a quick chill on ice and a brief centrifugation to collect the pellet, 4 μl of 5 X First Strand Buffer , 2 μl of 100 mM dithiothreitol, and 1 μl of 40 mM dNTP mix (10 mM each dNTP) were added to the reaction and heated to 42°C for 2 min. 200 U of Superscript II was added

and the mixture heated at 42°C for 50 min. The reaction was inactivated by heating for 15 min at 70°C. cDNA was stored at –20°C.

2.7.4 Radiolabelling DNA Probe and Hybridization

α^{32}P-dCTP was incorporated into DNA during quantitative PCR, carried out as follows: the PCR reaction mixture (50 µl total volume) contained: 5 µl of 10 X Amplification buffer, 1 mM $MgSO_4$, 50 µM of each dATP, dGTP, and dTTP, 50 µCi α^{32}P-dCTP (Amersham Pharmacia Biotech), 200 µM of each of the forward (5') and reverse (3') primer pools (containing a mixture of the 19 primers used in the original amplification), 5% DMSO, 10 ng cDNA template, and 1.25 U of Platinum Pfx DNA Polymerase (Invitrogen Life Technologies). The reactions were carried out for 15 cycles using a Minicycler (MJ Research Inc.) and each cycle included a denaturation step (94°C for 30 sec), followed by an annealing step (63°C for 45 sec), followed by an extension step (72°C for 1 min). The cycling was preceded by an initial denaturation step (94°C for 5 min) and followed by a final extension step (72°C for 5 min).

Unincorporated α^{32}P-dCTP was purified from labeled PCR product using MicroSpin G-50 columns (Amersham Pharmacia Biotech). Incorporation of radioactive nucleotide was confirmed by adding 1 µl of PCR product to 5.5 ml scintillation fluid and measuring radioactivity on a Beckman 6000 IC Scintillation Counter.

Prior to hybridization using labeled DNA probe, membranes were placed in glass hybridization tubes and incubated at 42°C for 3 hours with 5 ml of prehybridization buffer (5X SSC, 5X Denhardt's solution, 50% w/v formamide, 1% w/v sodium lauryl sulfate [SDS]), and 100 µg/ml denatured salmon sperm DNA to block nonspecific binding to the membranes. α^{32}P-labeled DNA probe was denatured at 100°C for 5 min

and then chilled on ice. 14 µl of radioactive probe was added to the hybridization tube. Following an overnight incubation at 42°C, membranes were washed twice in 5 ml of each of 2X SSC/0.1% SDS and 0.2X SSC/0.1% SDS while rotating at room temperature and then twice in 0.2X SSC/0.1% SDS while rotating at 42°C. Membranes were subsequently blotted dry using filter paper and wrapped in transparent plastic wrap.

2.7.5 Autoradiographic Imaging

Membranes were placed into MD Storage Phosphor Screen (Molecular Dynamics) for 72 hours. Autoradiographic imaging was performed on the Molecular Dynamics PhosphorImager SI. Quantification of hybridization spots was performed using the ImageQuant version 1.1 (Molecular Dynamics) software. A circular box was drawn around the location of each spotted amplicon (regardless of hybridization signal), and local background (calculated as the intensity of the pixels surrounding each box) was subtracted from the intensity of the hybridization signal (calculated as the intensity of the pixels inside the box). The PCR-amplified *uvrD* gene, encoding a constitutively active DNA repair enzyme, was placed on the array as an internal control, since its expression had been previously shown to remain constant under a number of conditions (M. Brazas, personal communication). The calculated spot-density values were normalized by dividing by the density value for *uvrD*. Comparisons between conditions (wildtype and mutant strains) were performed by taking the ratio of spot density values of the wildtype over the mutant. Fold changes greater than 2-fold were determined to be significant.

RESULTS

3 OprM family outer membrane proteins from the *P. aeruginosa* genome

3.1 Introduction

At the time of writing, the complete 6.3 megabase genome sequence of *P. aeruginosa* was among one of the largest known bacterial genomes, containing 5570 predicted ORFs (Stover *et al.,* 2000). Members of the *Pseudomonas* genus are well-known for their ability to survive in several environments as well as their ability to catabolise almost every carbon source, so it is not surprising that almost 10% of these genes encode putative regulatory proteins. A search of the entire genome sequence also revealed the presence of a large number of outer membrane proteins (Hancock and Brinkman, 2002), 17 of which are related to OprM. These genes were given the designation *opm*, standing for probable **o**uter membrane **p**rotein of the Opr**M** family. Table IV gives summary data on the homology of these OMPs. The following chapter will explore the phylogenetic relationships of the members of this gene family.

3.2 Homology of Multi-Drug Efflux Pumps of *Pseudomonas aeruginosa*

Following the completion of *Pseudomonas aeruginosa* genome sequence (Stover *et al.,* 2000), three large families of outer membrane proteins were identified. One such group is the 18-member OprM family of OMPs, which includes a cluster of eleven OMPs closely related to OprM and most likely involved in the transport of small molecules, as well as seven members in a second more distantly related cluster that includes AprF, the outer membrane protein involved in alkaline protease secretion. Figure 3 demonstrates the phylogenetic relationship between the OprM and the 17 homologues. Further

Table IV. Homology of the OprM homologues.

Protein	PAID#[a]	Closest non-*P. aeruginosa* homologue	Closest *P. aeruginosa* homologue
Efflux cluster			
OprM	0427	Major intrinsic multi-drug resistance outer membrane efflux protein (*P. aeruginosa*)	58% similar to OprJ
OprJ	4597	Multi-drug resistance outer membrane efflux protein, expressed in *nfxB* mutants (*P. aeruginosa*)	58% similar to OprM
OprN	2495	Multi-drug resistance outer membrane efflux protein, expressed in *nfxC* mutants (*P. aeruginosa*)	56% similar to OpmD
OpmA	2837	63% similar to TrpC, OMP of toluene-induced toluene exclusion pump (*P. putida*)	53% similar to OprN
OpmB	2525	54% similar to OpcM, OMP of MDR system CeoA-CeoB-OpcM (*B. cepacia*)	50% similar to OprM
OpmD	4208	45% similar to TrpC, OMP of toluene-induced toluene exclusion pump (*P. putida*)	56% similar to OprN
OpmE	3521	44% similar to TrpC, OMP of toluene-induced toluene exclusion pump (*P. putida*)	52% similar to OprN
OpmG	5158	47% similar to putative outer membrane multi-drug resistance lipoprotein (*Ralstonia solanacearum*)	46% similar to OpmI

To be continued...

Table IV. Homology of the OprM homologues.

Protein	PAID#[a]	Closest homologue	Closest *P. aeruginosa* homologue
OpmI	3894	64% similar to FusA, fusaric acid resistance protein (*B. cepacia*)	46% similar to OpmG
OpmJ	1238	33% similar to putative outer membrane multi-drug resistance lipoprotein (*Ralstonia solanacearum*)	51% similar to OprN
OpmQ	2391	43% similar to TrpC, OMP of toluene-induced toluene exclusion pump (*P. putida*)	48% similar to OprM
Secretion cluster			
AprF	1248		
CzcC	2522	63% similar to CztC, OMP of RND proton/cation antiport system (*P. fluorescens*)	39% similar to OpmQ
OpmF	4592	40% similar to cyclolysin secretion OMP CyaE (*Bordetella pertussis*)	35% similar to CzcC
OpmH	4974	54% similar to TolC, OMP involved in efflux/secretion (*E. coli*)	47% similar to AprF
OpmK	4144	49% similar to cyclolysin secretion OMP CyaE (*Bordetella pertussis*)	18% similar to AprF
OpmL	1875	43% similar to putative outer membrane efflux protein (*Salmonella typhimurium*)	41% similar to AprF
OpmM	3404	71% similar to HasF, OMP involved in secretion (*P. fluorescens*)	68% similar to AprF

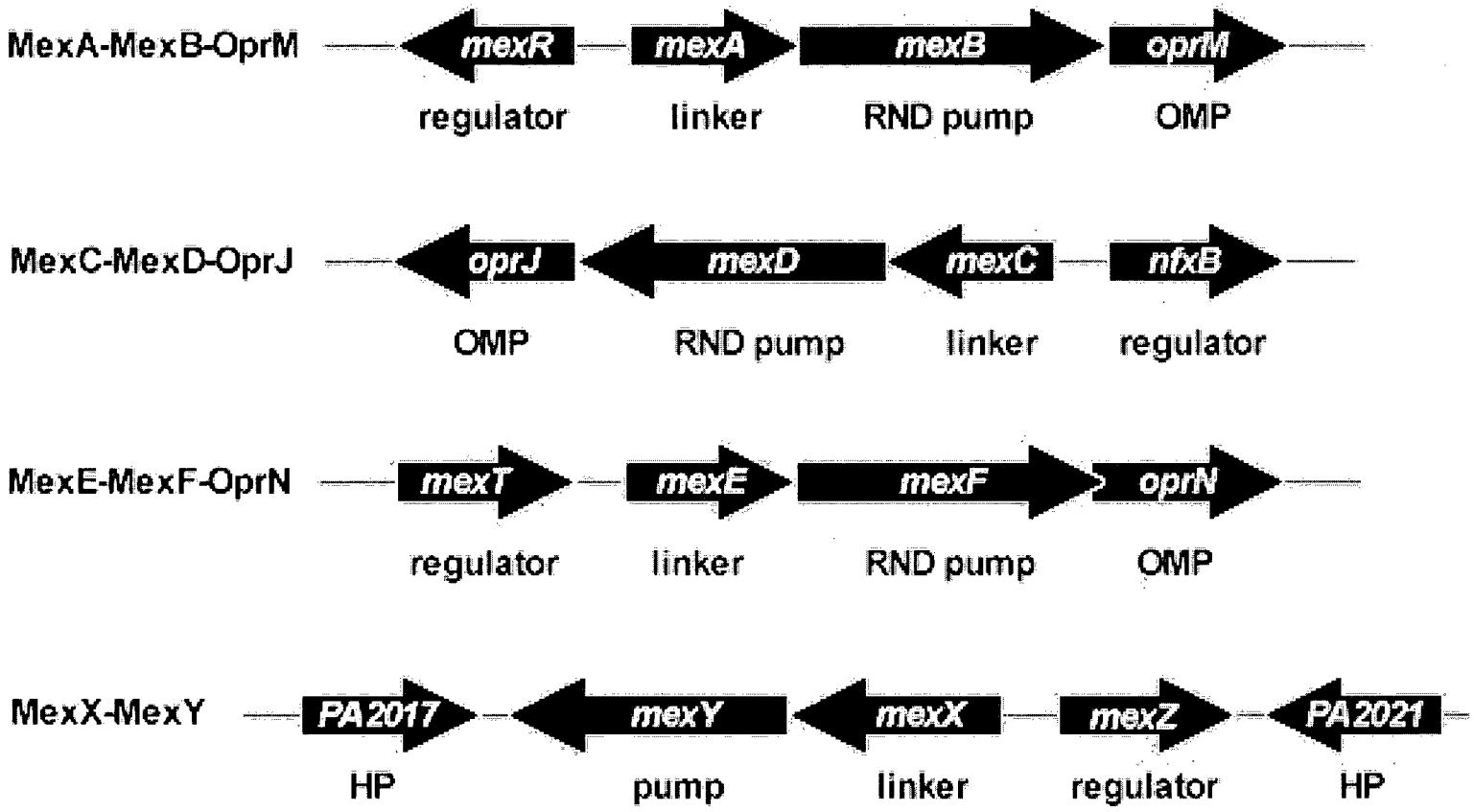
[a] The PAID# (*Pseudomonas aeruginosa* ID #) is the number of the ORF in the completed genome sequence.

examination of the genome revealed that each OprM homologue except OpmH was encoded as part of an operon containing genes for inner membrane proteins (either RND pumpds or ABC transporters) and/or linker proteins. While many of these operons share the same linker-pump-OMP gene organization as MexA-MexB-OprM, transposition of the genes within the operon is common. Interestingly, in three cases, the linker-pump-OMP gene organization is not preserved. The gene encoding *opmH*, which lies between a homologue of an *E. coli* thiamine biosynthesis gene and a gene bearing homology to a human NAD(P)H oxidoreductase, has no contiguous pump or linker proteins, while, in contrast, the genes encoding *opmB* and *opmF* are part of operons encoding two and three putative pump proteins respectively (Figure 4). This data is summarized in Table V.

Table V also shows that the transport systems involved in efflux tend to encode inner membrane transporters of the RND- or the major facilitator superfamilies (the exceptions being *opmI* and *opmQ*), while transport systems involved in secretion usually encode traffic ATPases (ABC transporter) proteins (the sole exception being *czcC*). Transport systems are usually categorized on the basis of their energy source, structure, and mode of transport (Saier, 2000).

The four characterized efflux systems of *P. aeruginosa* are all members of the RND-family of efflux transporters. RND transporters, found only in Gram negative bacteria, are proton antiport systems known for their unusually broad substrate specificities. They contain twelve transmembrane domains (TMDs), with both N and C termini within the cytoplasm (Putman *et al.*, 2000). The structural feature that distinguishes RND transporters from other transmembrane transporters is the presence of two large extracytoplasmic loops that project into the periplasm between TMDs 1 and 2, and TMDs

Figure 4. Gene organization of the OprM homologues. Most of the OprM homologues are encoded as three gene operons involving an inner membrane pump (of the RND– ABC– or MF– families), a periplasmic linker, as well as the outer membrane pore (OMP). Some operons include proximately-encoded putative regulators, though apart from the four characterized efflux systems of *P. aeruginosa*, the functions of the regulators have not yet been established. HP denotes a hypothetical protein.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mexR
mexA
mexB
oprM
regulator
linker
RND pump
OMP
MexC-MexD-OprJ
oprJ
mexD
mexC
nfxB
OMP
RND pump
linker
regulator
MexE-MexF-OprN
mexT
mexE
mexF
oprN
regulator
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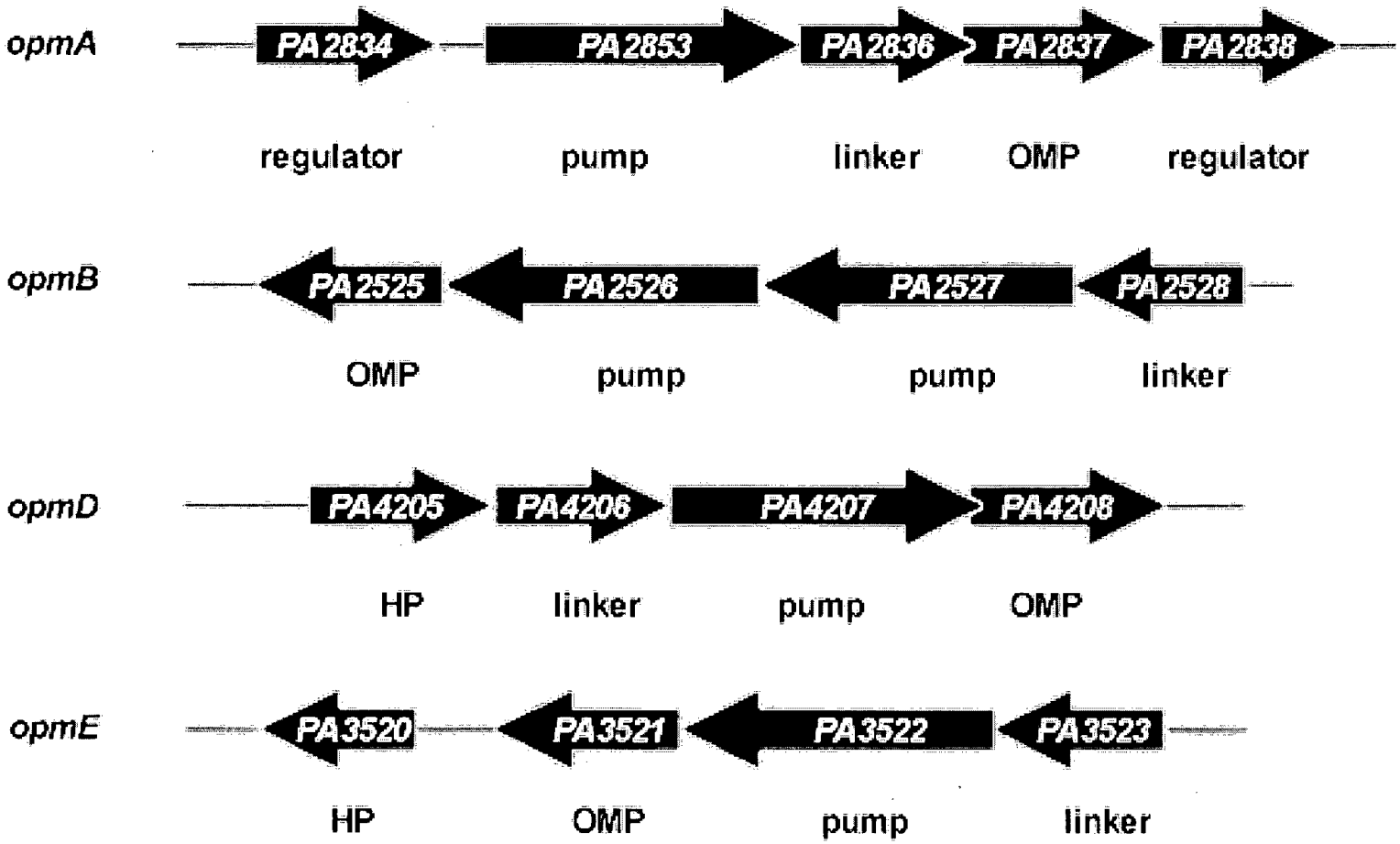
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PA4207
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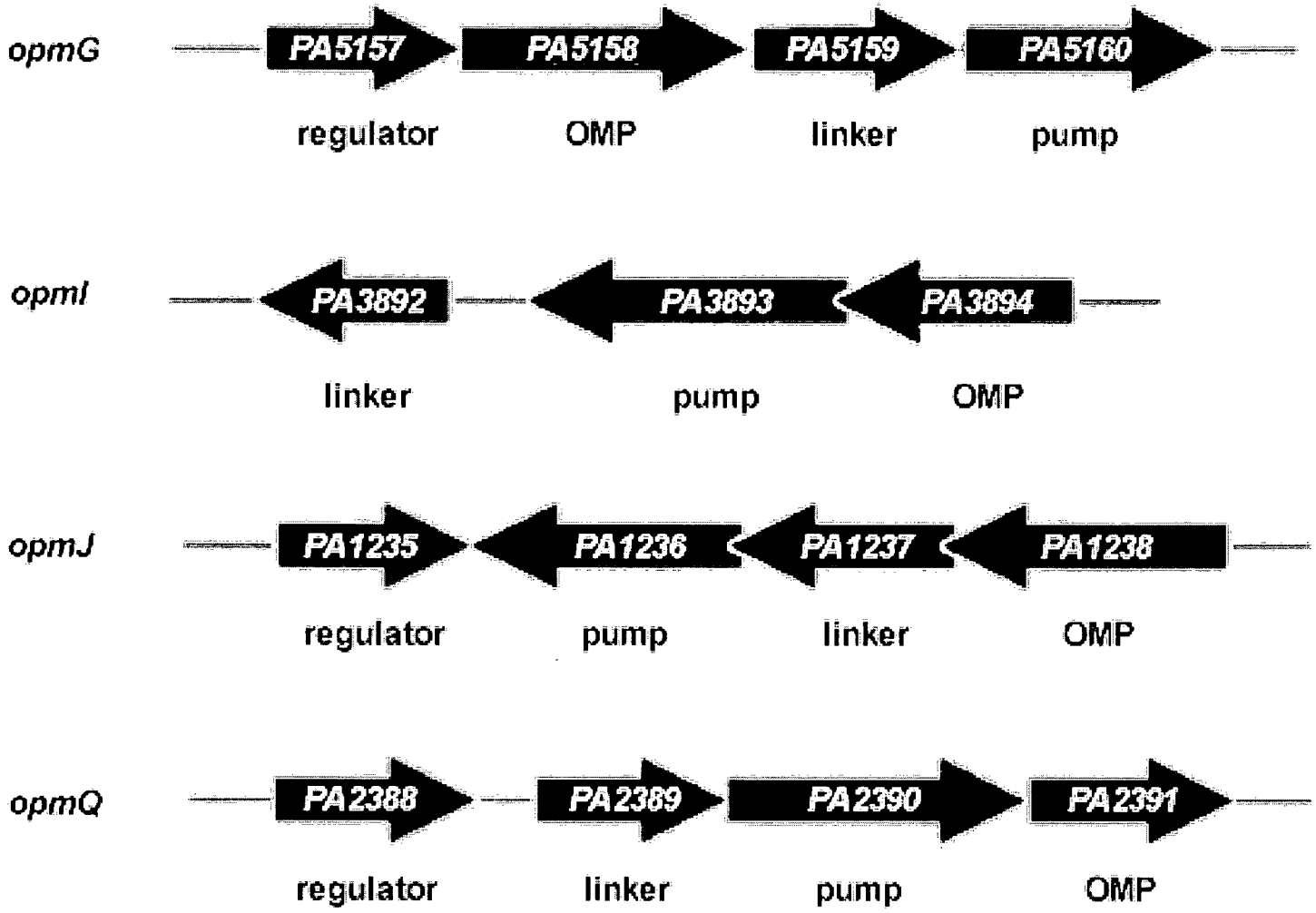
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regulator
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pump
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pump
OMP
opmJ
PA1235
PA1236
PA1237
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linker
OMP
opmQ
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regulator
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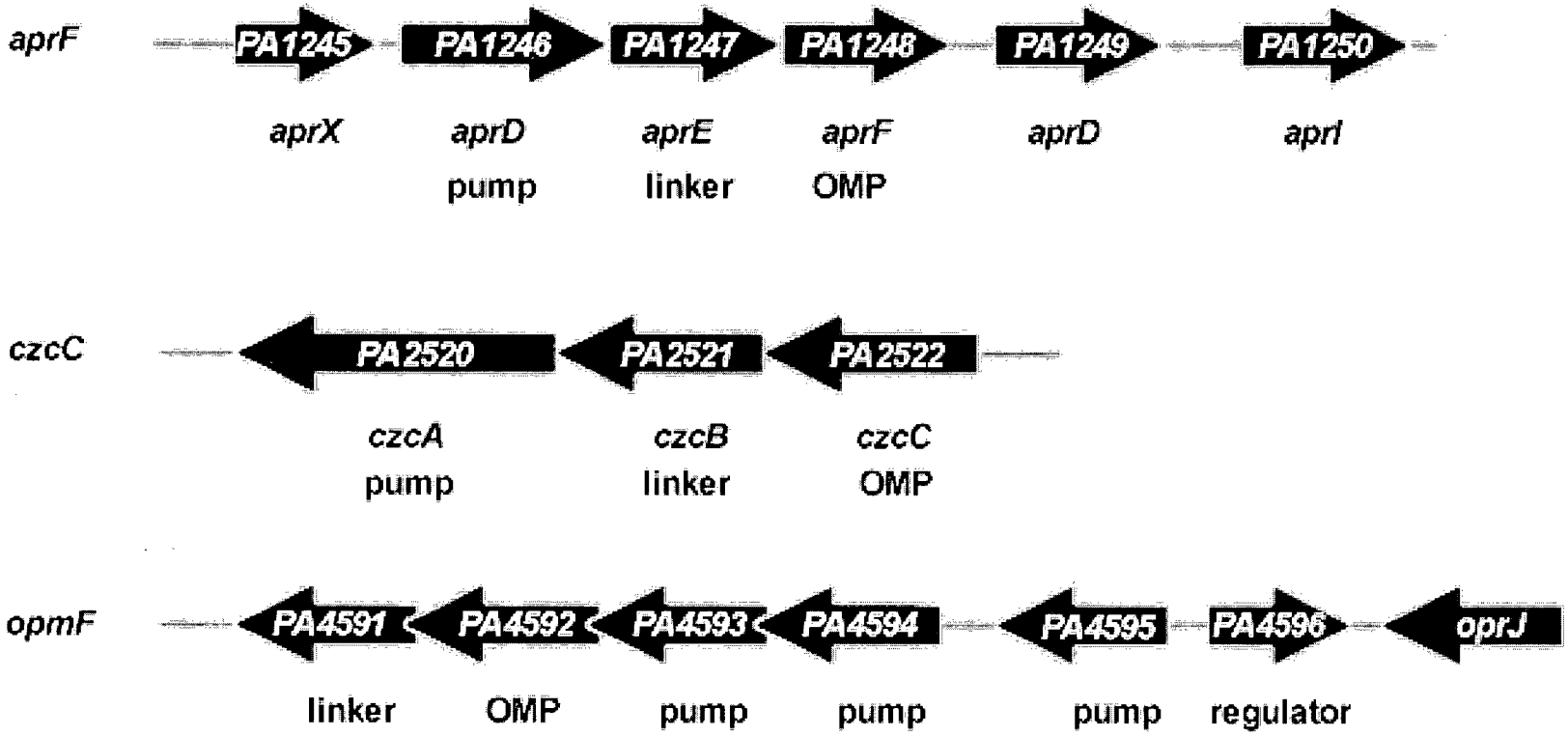
OMP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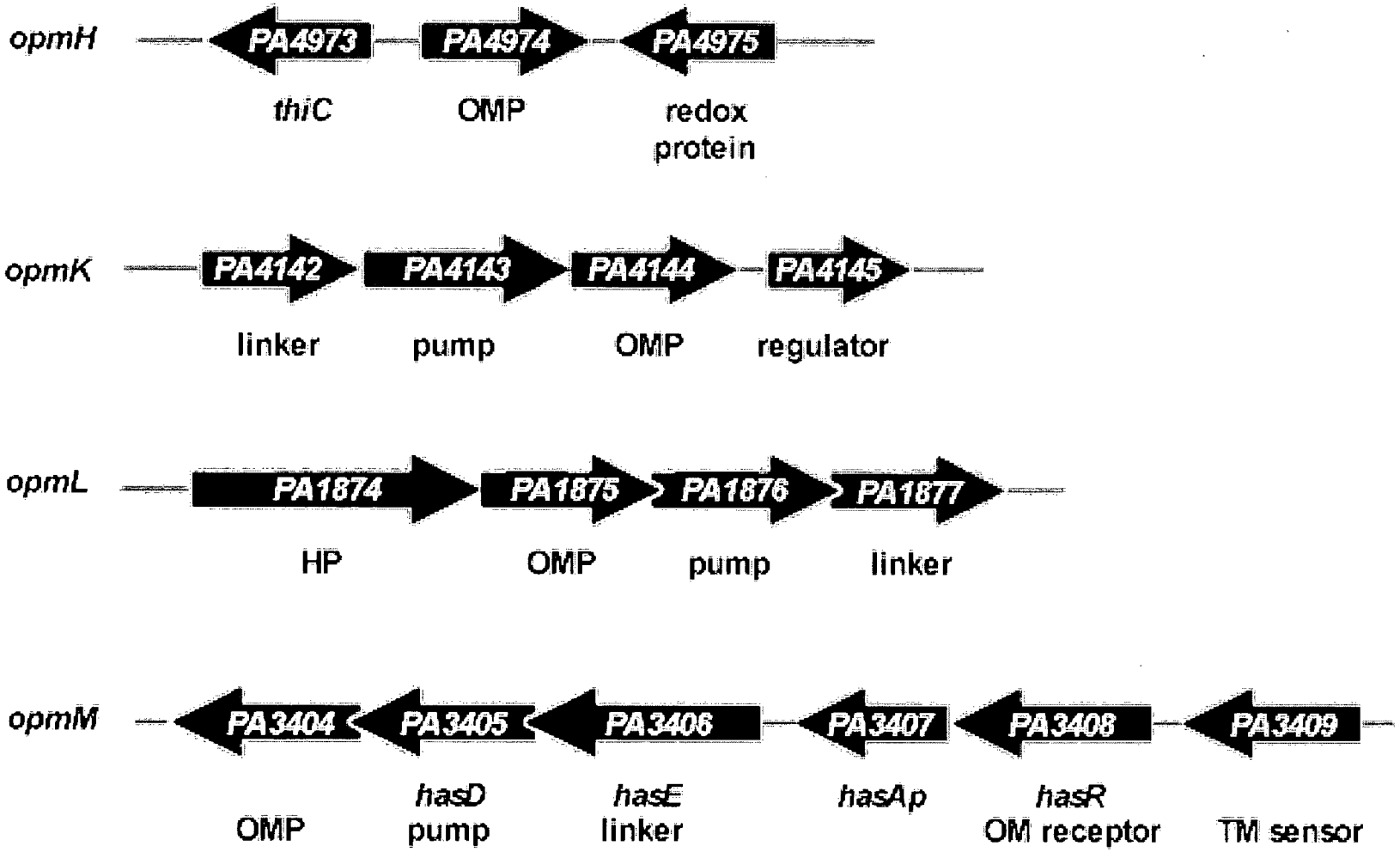
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redox protein
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linker
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OMP
regulator
opmL
PA1874
PA1875
PA1876
PA1877
HP
OMP
pump
linker
opmM
PA3404
PA3405
PA3406
PA3407
PA3408
PA3409
OMP
hasD pump
hasE linker
hasAp
hasR OM receptor
TM sensor

Table V. Efflux genes of the MexA-MexB-OprM homologues.

OMP gene	Genes order[a]	PAID#[b]	Strand	Transporter family[c]	Cluster
oprM	ABM	0425-0426-0427	-	RND	Efflux
oprJ	ABM	4599-4598-4597	+	RND	Efflux
oprN	ABM	2493-2494-2495	-	RND	Efflux
mexX-mexY[d]	AB	2019-2018	+	RND	Efflux
opmA	BAM	2835-2836-2837	-	MFS	Efflux
opmB	ABBM	2528-2527-2526-2525	+	RND	Efflux
opmD	ABM	4206-4207-4208	-	RND	Efflux
opmE	ABM	3523-3522-3521	+	RND	Efflux
opmG	MAB	5158-5159-5160	-	MFS	Efflux
opmI	MBA	3894-3893-3892	+	ABC	Efflux
opmJ	MAB	1238-1237-1236	+	MFS	Efflux
opmQ	ABM	2389-2390-2391	-	ABC	Efflux
aprF	BAM	1246-1247-1248	-	ABC	Secretion
czcC	MAB	2522-2521-2520	+	RND	Secretion
opmF	BBBMA	4595-4594-4593-4592-4591	+	ABC	Secretion
opmH	M	4974	-	N/A	Secretion
opmK	ABM	4142-4143-4144	-	ABC	Secretion
opmL	MBA	1875-1876-1877	-	ABC	Secretion
opmM	BAM	3406-3405-3404	+	ABC	Secretion

[a] The order of the linker, pump, and OMP proteins in the operon with respect to the MexA-MexB-OprM system, where A denotes a linker, B denotes a pump, and M denotes an OMP.
[b] The PAID# (*Pseudomonas aeruginosa* ID #) is the number of the ORF in the completed genome sequence.
[c] The family to which the pump protein(s) belong, where RND = resistance/nodulation/cell division, MFS = major facilitator superfamily, and ABC = ATP binding cassette transporter.
[d] MexX-MexY has no known cognate OMP to be named.

7 and 8 as shown in figure 2 (Gotoh *et al.,* 1999; Guan *et al.,* 1999). While their significance is not known, it is believed that these periplasmic extensions contact and interact with periplasmic domains of the OMPs, perhaps to initiate gating functions (Koronakis *et al.*, 2000; Andersen *et al.*, 2001).

Transporters of the major facilitator superfamily (MFS) are found in a variety of organisms, including Gram positive bacteria and eukaryotes. They can have symport or antiport functions and are not dependent on periplasmic and outer membrane components for their function. Like RND pumps, their substrate specificities can be quite broad, including such diverse compounds as antibiotics, oligo– and polysaccharides, and intermediary metabolites (Paulsen *et al.,* 1996). MFS are structurally divided into two groups, those with 12 transmembrane segments (TMS) and those with 14 TMS. They lack the large extracytoplasmic loops that distinguish RND transporters (Putman *et al.,* 2000). Among the most well-known examples of MFS transporters are the *E. coli* TetB tetracycline efflux protein, part of the 12 TMS family (Paulsen *et al.,* 1996) and the *emrAB* system of *E. coli* where EmrB is an MFS transporter of the 14 TMS family, while EmrA is a periplasmic protein that shares homology with other linker proteins (Paulsen *et al.,* 1996; Nikaido, 1998).

Traffic ATPases or ABC transporters, as their names imply, couple the energy of transport to the hydrolysis of ATP instead of the proton gradient. Present in many organisms including Gram negative bacteria, Gram positive bacteria, and eukaryotes, they are transmembrane proteins that usually have twelve transmembrane helices as well as a large cytoplasmic domain containing the Walker A and Walker B motifs as well as the nucleotide binding domain. Since ATP binding and hydrolysis occurs at the Walker

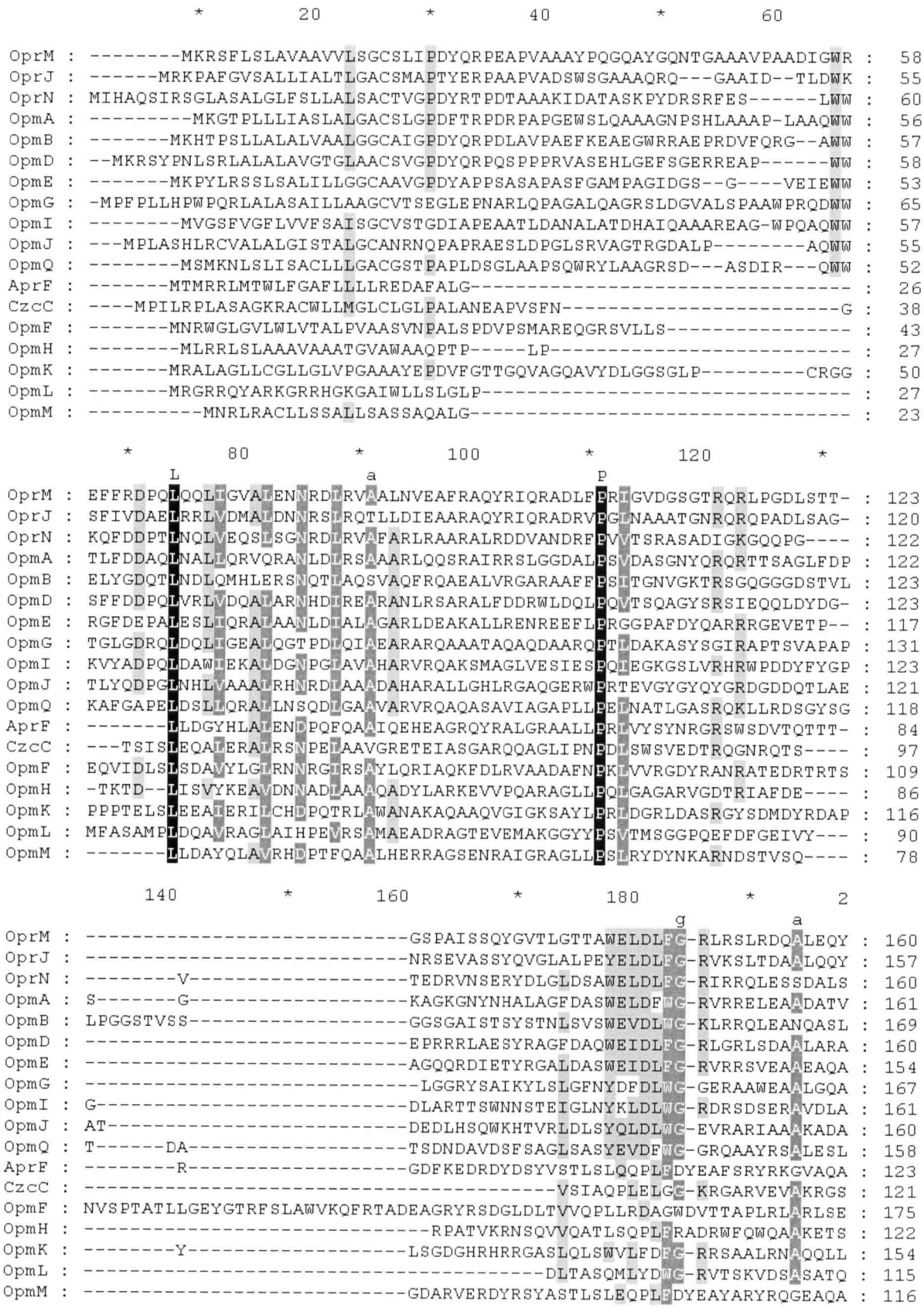
boxes and the nucleotide binding domain, these are the characteristic features of ABC transporters. ABC-based efflux systems are generally more specific than those of the RND– or MFS– families, though some broad spectrum ABC transporters have been identified (Putman *et al.,* 2000). Perhaps the best known multi-drug transporter of this type is the human P-glycoprotein encoded by the *mdr* gene, which can export various cytotoxic drugs often used for cancer therapy (Paulsen *et al.,* 1996; Mao *et al.,* 2001), and bacterial homologues of P-glycoprotein exist, such as LmrA of *Lactococcus lactis*, an ABC-family multi-drug transporter that confers resistance to a wide range of structurally dissimilar amphiphilic compounds (van Veen *et al.,* 1999; Margolles *et al.,* 1999).

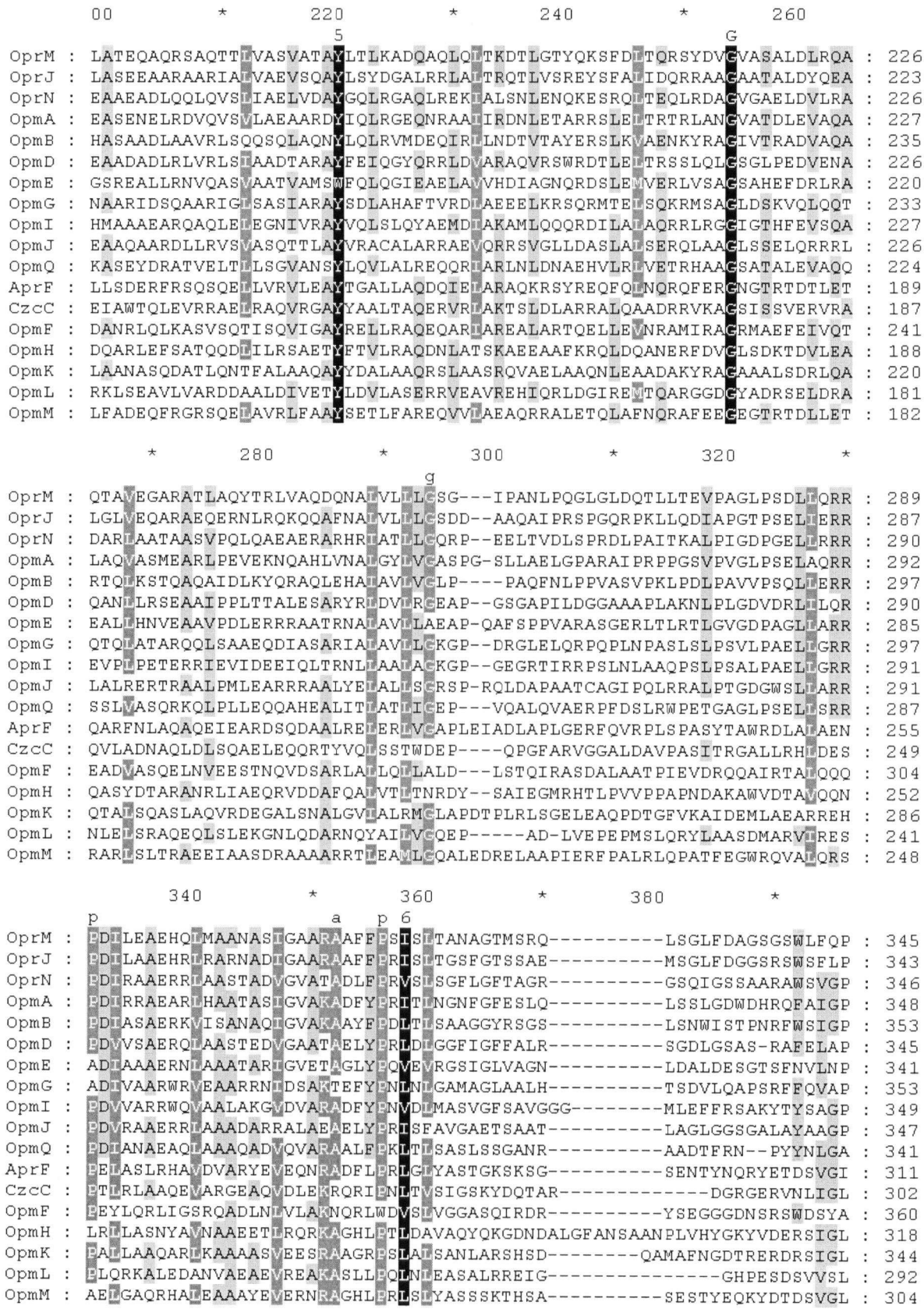
Type I protein secretion involves a three-component secretion apparatus similar to the typical multi-drug efflux. Type I secretion systems, which are often responsible for the export of proteases, lipases, toxins, and other secreted protein factors, are comprised of inner membrane ABC transporters in conjunction with linker and outer membrane proteins (Thanassi and Hultgren, 2000). This structure facilitates secretion of whole proteins without a periplasmic intermediate, in much the same manner as efflux allows for one-step transport across both the inner and outer membranes.

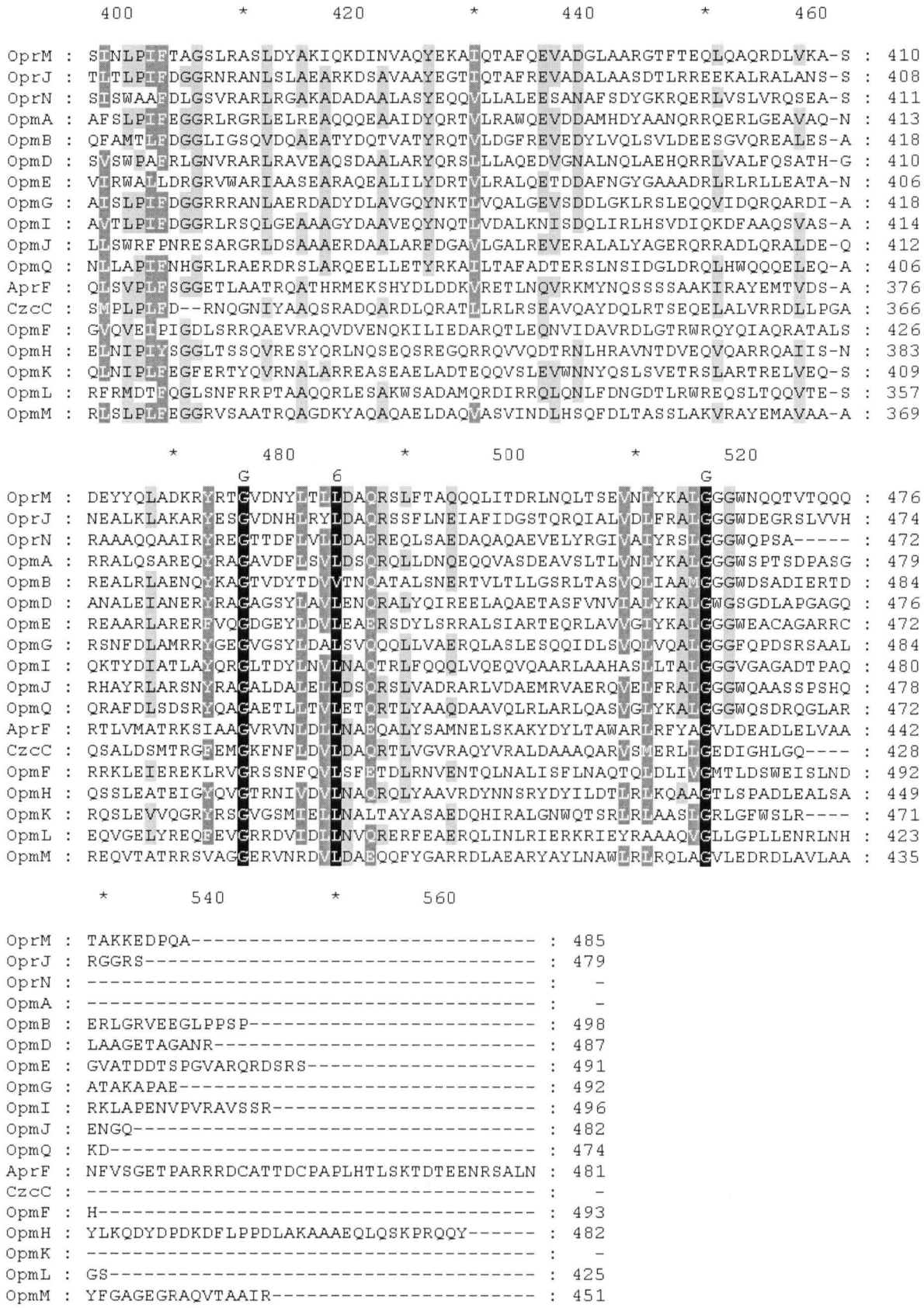
3.3 The OprM Family of Outer Membrane Proteins

The genome of *Pseudomonas aeruginosa* encodes a number of families of outer membrane proteins, including the 18-member OprM family. Table IV shows that the majority of these homologues, four from the efflux cluster and six from the secretion cluster, share their highest homology with outer membrane proteins from other bacteria, including PA4974, the OpmH protein which is 54% similar to the TolC protein of *E. coli.*

Figure 5. An alignment of the amino acid sequences of OprM and its seventeen homologues. The alignment was performed using the Clustal X (version 1.8) sequence alignment program and viewed using the GeneDoc software. Highly conserved residues are shaded in black with the single-letter amino acid code appearing in upper case above the alignment to denote those residues that are completely conserved among all eighteen members. This alignment was used to produce the unrooted tree shown in Figure 3.






400 * 420 * 440 * 460
OprM : SINLPIFTAGSLRASLDYAKIQKDINVAQYEKALQTAFQEVADGLAARGTFTEQLQAQRDLVKA-S : 410
OprJ : TLTLPIFDGGRNRANLSLAEARKDSAVAAYEGTLQTAFREVADALAASDTLRREEKALRALANS-S : 408
OprN : SLSWAAFDLGSVRARLRGAKADADAALASYEQQVLLALEESANAFSDYGKRQERLVSLVRQSEA-S : 411
OpmA : AFSLPIFEGGRLRGRLELREAQQQEAAIDYQRTVLRAWQEVDDAMHDYAANQRRQERLGEAVAQ-N : 413
OpmB : QFAMTLFDGGLIGSQVDQAEATYDQTVATYRQTVLDGFREVEDYLVQLSVLDEESGVQREALES-A : 418
OpmD : SVSWPAFRLGNVRARLRAVEAQSDAALARYQRSLLAQEDVGNALNQLAEHQRRLVALFQSATH-G : 410
OpmE : VTRWALLDRGRVWARIAASEARAQEALILYDRTVLRALQETDDAFNGYGAAADRLRLRLLEATA-N : 406
OpmG : ALSLPIFDGGRRRANLAERDADYDLAVGQYNKTLVQALGEVSDDLGKLRSLEQQVIDQRQARDI-A : 418
OpmI : AVTLPIFDGGRLRSQLGEAAAGYDAAVEQYNQTLVDALKNISDQLIRLHSVDIQKDFAAQSVAS-A : 414
OpmJ : LLSWRFPNRESARGRLDSAAAERDAALARFDGAVLGALREVERALALYAGERQRRADLQRALDE-Q : 412
OpmQ : NLLAPIFNHGRLRAERDRSLARQEELLETYRKALLTAFADTERSLNSIDGLDRQLHWQQQELEQ-A : 406
AprF : QLSVPLFSGGETLAATRQATHRMEKSHYDLDDKVRETLNQVRKMYNQSSSSAAKIRAYEMTVDS-A : 376
CzcC : SMPLPLFD--RNQGNIYAAQSRADQARDLQRATLLRLRSEAVQAYDQLRTSEQELALVRRDLLPGA : 366
OpmF : GVQVEIPIGDLSRRQAEVRAQVDVENQKILIEDARQTLEQNVIDAVRDLGTRWRQYQIAQRATALS : 426
OpmH : ELNIPIYSGGLTSSQVRESYQRLNQSEQSREGQRRQVVQDTRNLHRAVNTDVEQVQARRQAIIS-N : 383
OpmK : QLNIPLFEGFERTYQVRNALARREASEAELADTEQQVSLEVWNNYQSLSVETRSLARTRELVEQ-S : 409
OpmL : RFRMDTFQGLSNFRRPTAAQQRLESAKWSADAMQRDIRRQLQNLFDNGDTLRWREQSLTQQVTE-S : 357
OpmM : RLSLPLFEGGRVSAATRQAGDKYAQAQAELDAQVASVINDLHSQFDLTASSLAKVRAYEMAVAA-A : 369
* 480 * 500 * 520
G 6 G
OprM : DEYYQLADKRYRTGVDNYLTLLDAQRSLFTAQQQLITDRLNQLTSEVNLYKALGGGWNQQTVTQQQ : 476
OprJ : NEALKLAKARYESGVDNHLRYLDAQRSSFLNEIAFIDGSTQRQIALVDLFRALGGGWDEGRSLVVH : 474
OprN : RAAAQQAAIRYREGTTDFLVLLDAERQLSAEDAQAQAEVELYRGIVALYRSLGGGWQPSA----- : 472
OpmA : RRALQSAREQYRAGAVDFLSVLDSQRQLLDNQEQQVASDEAVSLTLVNLYKALGGGWSPTSDPASG : 479
OpmB : REALRLAENQYKAGTVDYTDVVTNQATALSNERTVLTLLGSRLTASVQLIAAMGGGWDSADIERTD : 484
OpmD : ANALEIANERYRAGAGSYLAVLENQRALYQIREELAQAETASFVNVLALYKALGWGSGDLAPGAGQ : 476
OpmE : REAARLARERFVQGDGEYLDVLEAERSDYLSRRALSIARTEQRLAVVGLYKALGGGWEACAGARRC : 472
OpmG : RSNFDLAMRRYGEGVGSYLDALSVQQQLLVAERQLASLESQQIDLSVQLVQALGGGFQPDSRSAAL : 484
OpmI : QKTYDIATLAYQRGLTDYLNVLNAQTRLFQQQLVQEQVQAARLAAHASLLTALGGGVGAGADTPAQ : 480
OpmJ : RHAYRLARSNYRAGALDALELLDSQRSLVADRARLVDAEMRVAERQVELFRALGGGWQAASSPSHQ : 478
OpmQ : QRAFDLSDSRYQAGAETLLTVLETQRTLYAAQDAAVQLRLARLQASVGLYKALGGGWQSDRQGLAR : 472
AprF : RTLVMATRKSIAAGVRVNLDLLNAEQALYSAMNELSKAKYDYLTAWARLRFYAGVLDEADLELVAA : 442
CzcC : QSALDSMTRGFEMGKFNFLDVLDAQRTLVGVRAQYVRALDAAAQARVSMERLLGEDIGHLGQ---- : 428
OpmF : RRKLEIEREKLRVGRSSNFQVLSFETDLRNVENTQLNALISFLNAQTQLDLIVGMTLDSWEISLND : 492
OpmH : QSSLEATEIGYQVGTRNIVDVLNAQRQLYAAVRDYNNSRYDYILDTLRLKQAAGTLSPADLEALSA : 449
OpmK : RQSLEVVQGRYRSGVGSMIELLNALTAYASAEDQHIRALGNWQTSRLRLAASLGRLGFWSLR---- : 471
OpmL : EQVGELYREQFEVGRRDVIDLLNVQRERFEAERQLINLRIERKRIEYRAAAQVGLLGPLLENRLNH : 423
OpmM : REQVTATRRSVAGGERVNRDVLDAEQQFYGARRDLAEARYAYLNAWLRLRQLAGVLEDRDLAVLAA : 435
* 540 * 560
OprM : TAKKEDPQA------------------------------ : 485
OprJ : RGGRS------------------------------------ : 479
OprN : -- : -
OpmA : -- : -
OpmB : ERLGRVEEGLPPSP-------------------------- : 498
OpmD : LAAGETAGANR----------------------------- : 487
OpmE : GVATDDTSPGVARQRDSRS--------------------- : 491
OpmG : ATAKAPAE-------------------------------- : 492
OpmI : RKLAPENVPVRAVSSR------------------------ : 496
OpmJ : ENGQ---------------------------------- : 482
OpmQ : KD------------------------------------ : 474
AprF : NFVSGETPARRRDCATTDCPAPLHTLSKTDTEENRSALN : 481
CzcC : -- : -
OpmF : H-------------------------------------- : 493
OpmH : YLKQDYDPDKDFLPPDLAKAAAEQLQSKPRQQY------ : 482
OpmK : -- : -
OpmL : GS------------------------------------ : 425
OpmM : YFGAGEGRAQVTAAIR---------------------- : 451

Although there are few conserved residues among the family members, some regions have more conserved residues than others. For instance, those residues presumed present at the interfaces of the outer membrane are often aromatic amino acids. Figure 5 shows an alignment of the members of this family.

3.4 Conclusions

To date, four RND efflux systems have been identified in the *P. aeruginosa* genome, which together have been shown to be capable of conferring resistance to almost every major class of antibiotics. However, since these genes have been present in the genome much longer than antibiotics have been in widespread use (Nikaido, 1998), the presence of at least eight other putative RND efflux operons alone in this family suggests that the ability to export toxic compounds *en masse* has played some important role in the evolution of this organism. Perhaps the number of systems capable of toxin export has contributed to the ubiquity of this organism in the environment. The observation that inactivation of one efflux systems can result in the upregulation of another (Li *et al.*, 2000) suggests that the expression of the MDR systems is under a tight network of regulation that is not yet understood.

RESULTS

4 OpmG– and OpmH–mediated intrinsic aminoglycoside resistance

4.1 Introduction

In patients with cystic fibrosis, the utility of aminoglycosides cannot be underrated. Although many aminoglycosides are highly toxic compounds with narrow therapeutic dose ranges, their synergistic effects when combined with some β-lactam antibiotics makes them ideal agents against recalcitrant *Pseudomonas aeruginosa* infection (Hancock and Speert, 2000). In the past, resistance to aminoglycosides has largely thought to be the result of aminoglycoside modifying enzymes; however, it is now believed that impermeability and efflux mechanisms are the predominant factors leading to broad-spectrum aminoglycoside resistance (Westbrock-Wadman *et al.,* 1999) and are a major concern due to the already limited selection of available antibiotic therapies against *Pseudomonas*. The identification of the *mexXY* operon in *P. aeruginosa* has provided some insight into the specific mechanisms for broad aminoglycoside resistance, but, in the absence of a defined OMP component, our understanding is far from complete. From among the uncharacterized OprM homologues, three were found to play roles in aminoglycoside resistance. This chapter outlines the research into possible outer membrane components for this system.

4.2 Screening miniTn*5* insertion mutants

MiniTn*5* insertion mutants (H956-H969) in each of the genes encoding the OprM homologues (except *opmQ*) were provided by PathoGenesis Corporation (Chiron). There are several derivatives of the miniTn*5* transposon, largely differentiated on basis of the selectable marker encoded within the cassette. Mini transposons do not encode their own

transposases, which are instead supplied in *trans* from a donor suicide plasmid. Since the plasmid cannot be maintained inside cells, growth on the appropriate antibiotic selects for cells within which the transposon integrates into the chromosome (de Lorenzo *et al.,* 1990; Herrero *et al.,* 1990).

The minimal inhibitory concentrations (MIC) of *P. aeruginosa* strains H956-H969 were determined for a number of antibiotics. Included in the screen were hydrophobic and hydrophilic antibiotics, dyes, detergents, the energy inhibitor CCCP, and ethidium bromide (Table VI), all of which are compounds that have been previously characterized as substrates for active export by tripartite multi-drug efflux systems in Gram negative bacteria.

Only one set of mutants demonstrated a definite resistance pattern. These three mutants showed increased susceptibility to all four aminoglycoside antibiotics tested (Table VII). H958 (*opmG*) showed 8-fold decreases in MIC of kanamycin, gentamicin, and streptomycin, as well as a 4-fold decrease in the MIC of tobramycin, as compared to the wildtype H911 (PAK). H960 (*opmI*) showed an 16-fold decrease in the MIC of streptomycin, an 8-fold decrease in the MIC of kanamycin, and 4-fold decreases in the MICs of gentamicin and tobramycin. H966 (*opmH*) showed 8-fold decreases in MICs of kanamycin and tobramycin, a 4-fold decrease in MIC streptomycin, and a 2-fold change in the MIC of gentamicin. Tetracycline resistance was observed, confirming maintenance of the transposon insertion. Carbenicillin is not a substrate for MexX-MexY (Masuda *et al.,* 2000b) and all three mutants showed no change in the MIC of this β-lactam as compared to H911. For comparison, insertional inactivation of *opmA*, *opmD*, or *opmL*, all of which are relatively well expressed in H911 (see below), did not result in

aminoglycoside susceptibility. Indeed, none of the remaining eleven mutants showed altered susceptibility to any of the four aminoglycosides tested. Similarly, deletion of OprM, which some authors have proposed to be the cognate outer membrane pore of MexXY (Aires *et al.,* 1999; Mine *et al.,* 1999; Masuda *et al.,* 2000a), only caused a 2-fold change in aminoglycoside susceptibility, which by convention is considered insignificant.

Figure 3 shows that OpmG and OpmI are highly related and are, in fact, more related to each other and outer membrane efflux pores from other organisms than to any *P. aeruginosa* genes. While OpmG and OpmI are still part of the efflux cluster, OpmH is part of the secretion cluster (Figure 3), and its closest homologue both inside and outside of the *P. aeruginosa* genome, is the multifunctional *E. coli* OMP TolC. Interestingly, of the fifteen OprM homologues, OpmH is the sole member of the family that is not encoded as an operon (Figure 4), making it an attractive candidate for the role of the native OMP for *mexXY.*

4.3 Complementation of the *opmG*, *opmH*, and *oprM* mutants

If o*pmG*, *opmH*, and *opmI* do play a role in aminoglycoside efflux, MICs values should be restored upon reintroduction of the genes into their respective mutants. However, since it is in fact inner membrane components that determine substrate specificity (Srikumar *et al.,* 1997; Gotoh *et al.,* 1998), if OpmG, OpmH, and OpmI are indeed channel-forming OMPs, then each protein might also be able to complement an *oprM*⁻ defect.

The *opmG* and *opmH* genes were PCR amplified from PAO1 genomic DNA and cloned. The identities of *opmG* and *opmH* were confirmed by sequencing; however, since

Table VI. Compounds tested in initial MIC screen of miniTn*5* insertion mutants.

Compound		
Chloramphenicol	Macrolides	β-Lactams
Fusidic Acid	Clindamycin	Penicillins
Polymixin B	Erythromycin	Carbenicillin
Rifamycin	Quinolones	Carbapenems
Tetracycline	Nalidixic Acid	Imipenem
Aminoglycosides	Norfloxacin	Meropenem
Gentamycin	Dyes and Detergents	Cephalosporins
Kanamycin	Acriflavin	Cefpirome
Streptomycin	Crystal Violet	Cefsulodin
Tobramycin	Ethidium Bromide	Ceftazidime
Energy Inhibitors	SDS	Ceftriaxone
CCCP	Dyes and Detergents	

Table VII. MICs to aminoglycosides, carbenicillin and tetracycline of *P. aeruginosa* mutants lacking selected outer membrane channel proteins.

Strain	Phenotype	MIC (μg/ml)[a]					
		Km	Gm	Sm	Tm	Cb	Tc
H911	Parent	100	0.8	8	0.25	50	1.3
H958	OpmG$^-$	13	0.1	1	0.063	50	25
H966	OpmH$^-$	13	0.4	2	0.031	50	25
H960	OpmI$^-$	13	0.2	0.5	0.063	100	13
H957	OpmA$^-$	100	0.8	8	0.25	100	13
H963	OpmD$^-$	100	0.8	4	0.25	100	25
H962	OpmL$^-$	50	0.8	8	0.5	100	25

[a] Abbreviations: Km, kanamycin; Gm, gentamicin; Sm, streptomycin; Tm, tobramycin; Cb, carbenicillin; Tc, tetracycline.

this strategy was unsuccessful in cloning the *opmI* gene, only the roles of OpmG and OpmH were explored in greater detail.

4.3.1 Complementation of an *oprM* mutant susceptibility phenotype

H730 is a derivative of *P. aeruginosa* PAO1 that has a number of mutations, including a mutation in the *rpsL* gene, the target of the aminoglycoside antibiotic, streptomycin. Consequently, H730 is highly resistant to the antibiotic streptomycin. H743 is an isogenic *oprM*::Hg mutant of H730, created by inserting a mercury cassette into the *oprM* gene of strain H730. Mutants deficient in OprM are super-susceptible to many β-lactams (but not carbapenems or cephalosporins), chloramphenicol, macrolides, quinolones, and tetracycline. Table VIII shows that there were significant (4-fold or greater) decreases in MIC of nalidixic acid, carbenicillin, erythromycin, clindamycin, chloramphenicol, and tetracycline between strains H730 and H743, in agreement with previous studies (Li *et al.,* 1995; Nikaido, 1996; Masuda *et al.,* 2000b). The MICs of fusidic acid and acriflavin also decreased, indicating that these compounds were also substrates of MexA-MexB-OprM. As noted above, aminoglycoside susceptibility was not significantly changed.

The PCR-amplified *opmG* and *opmH* genes were separately cloned behind the *lac* promoter on the pUCP27 *E. coli-Pseudomonas* shuttle vector to create pJJ106 and pJJ105, respectively. When multicopy plasmids bearing *opmG* (pJJ106) or *opmH* (pJJ105) were introduced into strain H743, antibiotic resistance was restored, indicated by the increased MICs of erythromycin, clindamycin, fusidic acid, chloramphenicol, nalidixic acid, and acriflavin. The MICs of erythromycin, chloramphenicol, and nalidixic acid were in fact higher in the complemented strains than those of the parent strain H730.

Table VIII. Complementation of an OprM$^-$ mutant with OpmG and OpmH.

Strain	Phenotype	MIC (μg/ml)[a]																
		Km	Gm	Sm	Ery	Clin	Fus	Cm	Tc	Cb	Imi	Mer	Ctzd	Ctax	Nal	Nfx	Pmb	Acr	CV
H730	Parent	50	3.1	>2500	125	>750	500	7.8	5	12.5	>5	0.63	0.032	>2.5	63	1	0.31	63	63
H743	OprM$^-$	25	1.6	1250	7.8	23	16	1	0.63	0.78	5	0.63	0.02	>2.5	16	1	0.31	7.8	63
H743 + pJJ106	OprM$^-$ + OpmG	25	0.8	2500	>500	>750	250	16	>20	0.78	5	0.63	0.04	>2.5	250	64	0.31	31	31
H743 + pJJ105	OprM$^-$ + OpmH	50	0.8	2500	>500	>750	250	31	10	0.78	5	0.63	0.04	>2.5	250	64	0.16	63	63

[a] Abbreviations: Km, kanamycin; Gm, gentamicin; Sm, streptomycin; Ery, erythromycin; Clin, clindamycin; Fus, fusidic acid; Cm, chloramphenicol; Tc, tetracycline; Cb, carbenicillin; Imi, imipenem; Mer, meropenem; Ctzd, ceftazidime; Ctax, ceftriaxone; Nal, nalidixic acid; Nfx, norfloxacin; Pmb, polymixin B; Acr, acriflavin; CV, crystal violet.

Although the MIC of tetracycline was also restored well above that in H730, it should be noted that this was probably due to the presence of a tetracycline resistance gene present in the vector pUCP27.

4.3.2 Complementation of *opmG* and *opmH* mutant susceptibility phenotypes

Since both H958 and H966 contain the miniTn*5* transposon encoding tetracycline resistance, plasmids pJJ106 and pJJ105, which also encode tetracycline resistance, could not be used to complement the original mutant strains. For this reason, the PCR-amplified *opmG* and *opmH* genes were separately cloned behind the *lac* promoter in the *E. coli-Pseudomonas* shuttle vector pUCP21, encoding ampicillin resistance, to create pJJ107 and pJJ109. Ampicillin-based selection in *Pseudomonas* is performed using the antibiotic carbenicillin, since the chromosomally-encoded β-lactamase gene of *P. aeruginosa* is ampicillin-inducible but not carbenicillin-inducible. Plasmid pJJ107 was introduced into strain H958, and pJJ109 was introduced into strain H966 by electroporation. Table IX summarizes the MIC data for the complementation experiments.

Strain H958 (*opmG*::miniTn*5*-Tc^R) demonstrated an 8-fold decrease in the MICs of the three aminoglycosides kanamycin, gentamicin, and streptomycin, as well as a 4-fold decrease in the MIC of fusidic acid, as compared to the parent wildtype strain H911. There were no other significant changes in MIC as a result of insertional inactivation of the *opmG* gene. Introduction of pJJ107 (*opmG*) into strain H958 resulted in only partial complementation of the MIC of kanamycin but full complementation of the MICs of gentamicin, streptomycin, and fusidic acid. Therefore, OpmG plays a role in aminoglycoside and fusidic acid efflux, but other factors might be involved in kanamycin

Table IX. Complementation of OpmG$^-$ and OpmH$^-$ mutants with OpmG and OpmH.

Strain	Phenotype	MIC (μg/ml)[a]																	
		Km	Gm	Sm	Ery	Clin	Fus	Cm	Tc	Cb	Imi	Mer	Ctzd	Ctax	Nal	Nfx	Pmb	Acr	CV
H911	PAK wildtype	100	0.8	8	125	>750	16	125	1.3	50	1.6	0.63	0.08	13	31	0.25	0.16	32	125
H958	OpmG$^-$	13	0.1	1	63	375	4	125	>20	50	1.6	0.63	0.08	13	31	0.25	0.16	32	125
H958 + pJJ107	OpmG$^-$ + OpmG	25	0.8	8	250	>750	16	125	>20	800	1.6	0.63	0.08	13	31	0.25	0.08	32	125
H966	OpmH$^-$	13	0.4	2	63	375	2	125	>20	50	0.8	0.15	0.08	6.3	31	0.25	0.16	4	16
H966 + pJJ109	OpmH$^-$ + OpmH	25	0.8	4	125	>750	8	250	>20	800	1.6	0.15	0.08	6.3	31	0.25	0.3	16	63

[a] Abbreviations: Km, kanamycin; Gm, gentamicin; Sm, streptomycin; Ery, erythromycin; Clin, clindamycin; Fus, fusidic acid; Cm, chloramphenicol; Tc, tetracycline; Cb, carbenicillin; Imi, imipenem; Mer, meropenem; Ctzd, ceftazidime; Ctax, ceftriaxone; Nal, nalidixic acid; Nfx, norfloxacin; Pmb, polymixin B; Acr, acriflavin; CV, crystal violet.

resistance, since resistance to this compound was not restored. The increase in carbenicillin resistance upon introduction of pJJ107 was due to the β-lactamase marker on pUCP21. Strain H966 (*opmH*::miniTn*5*-Tc^R) showed less dramatic decreases in the MICs of the three aminoglycosides. There was an 8-fold reduction in the MIC of kanamycin, but only 2- and 4-fold reductions in the MICs of gentamicin and streptomycin, respectively. However, there were 8-fold reductions in the MICs of the dyes acriflavin and crystal violet. Reintroduction of the *opmH* gene in pJJ109 resulted in only 2-fold increases in the MICs to the three aminoglycosides, but also 4-fold (partial but still significant) restoration of the MICs to acriflavin and crystal violet. The ability to efflux the dye acriflavin is of particular interest since this compound has previously been noted to be an inducer of MexX-MexY (Morita *et al.,* 2001).

Control pUCP21 and pUCP27 plasmids lacking cloned OpmH or OpmG did not result in any significant alterations to antibiotic resistance except for that conferred by plasmid-borne resistance markers.

4.4 Use of DNA mini-microarrays to assess compensation

It has been previously noted that the inactivation of one efflux pump can result in a compensatory increase in expression of another efflux pump (Li *et al.,* 2000) and it would appear to be a reasonable extrapolation that compensatory decreases in expression might also occur. This phenomenon, if present as a result of insertional inactivation of any of the OprM homologues, would be problematic in masking the specific mutant phenotype. DNA mini-microarrays were used to assess expression levels of each of the OprM homologues in the *opmG* and *opmH* knockout strains, to determine if the antibiotic susceptibility phenotypes of H958 and H966 might be due to, or affected by,

compensatory alterations in expression of another outer membrane channel.

Internal 600bp fragments of each OprM homologue and the *uvrD* gene were PCR amplified from PAO1 genomic DNA. The DNA repair enzyme *uvrD* was previously determined to be constitutively expressed under all tested conditions (Brazas, unpublished data) and was used as an internal standard. RNA was isolated from H911, H958, and H966 and reverse transcribed to cDNA using a 5' primer pool consisting of a mixture of the 5' primers of each amplicon. cDNA was used as template for low-cycle PCR during which α^{32}P-dCTP was incorporated. The resultant radioactive DNA, reflecting the amount of original mRNA, was used to probe the purified PCR amplicons that were spotted on positively charged nylon membranes using a replicator.

Figure 6 shows the hybridization pattern for H911 compared with H958, while Figure 7 shows the hybridization pattern for H911 compared with H966. Each figure is a representative example of three independent trials. Table X compares the fold changes in expression of each OprM homologue between strains H911 and H958 (refer to figure 6), while Table XI compares strains H911 and H966 (refer to Figure 7). After background subtraction, fold changes were calculated by dividing the density of each hybridization spot by the density of the *uvrD* hybridization spot on that membrane. The ratio of the normalized values for each gene between the wildtype and mutant then represent the fold change. Greater than 2-fold changes in gene expression were considered significant.

The spot densitometry results in Table X compares the quantified hybridization signals between the *opmG* mutant H958 (*opmG*::miniTn*5*-Tc^R) compared to the parent strain H911. When results were standardized to the level of expression of *uvrD*, the only significant changes were in the levels of *opmG* (as anticipated) and *oprN* message, which

were not observed in the mutant H958. Since it is known that OprN does not contribute to aminoglycoside resistance (Seiffer *et al.,* 1993; Saier *et al.,* 1994; Hancock and Speert, 2000), it is unlikely that the downregulation of OprN would be having any effect on the aminoglycoside resistance phenotype of strain H958.

Similar expression studies were performed on the mutant H966 (*opmH*::miniTn*5*-Tc^R) compared to the parents strain H911, shown in Table XI. As expected, *opmH* was not expressed in the mutant, but there were modest decreases in *opmG* (1.9-fold) and *opmI* (2-fold) that may have contributed to the H966 resistance phenotype. No alteration in the expression of *oprM* was observed in either H958 or H966.

4.5 Conclusions

By screening transposon insertion mutants of the fifteen uncharacterized homologues of OprM in the *Pseudomonas aeruginosa* genome, three were found to play a role in aminoglycoside resistance. When each of *opmG*, *opmH*, and *opmI* were inactivated, the mutant strains were between 4– and 32–fold more susceptible to aminoglycosides. When *opmG* and *opmH* were each introduced into the antibiotic susceptible *oprM*⁻ strain H743, broad spectrum drug resistance was restored to H743, indicating that both OpmG and OpmH are channel-forming OMPs that are capable of functioning with RND pumps for drug efflux. In addition, OpmG was able to complement the aminoglycoside susceptiblility of H958. OpmH was able to fully restore resistance to the dyes acriflavin and crystal violet, but only partially restore resistance to aminoglycosides.

Mini-microarray experiments were designed to monitor gene expression of each of the OprM homologues, and based on these experiments, compensatory upregulation of other *oprM* family OMPs is not a factor in masking the effects of knockouts in these

Figure 6. Comparison of expression of OprM homologue genes in wildtype PAK (H911; top panel) and the *opmG* mutant (H958; bottom panel) using mini-microarrays. RNA isolated from each strain was reverse transcribed to cDNA, radiolabelled with [α^{32}P]dCTP, and hybridized to a nylon membrane containing 10 ng spots of 600 bp internal fragments corresponding to each of the OprM homologues. Spot densitometry revealed an absence of *oprN* expression as well as the expected absence of *opmG* expression.

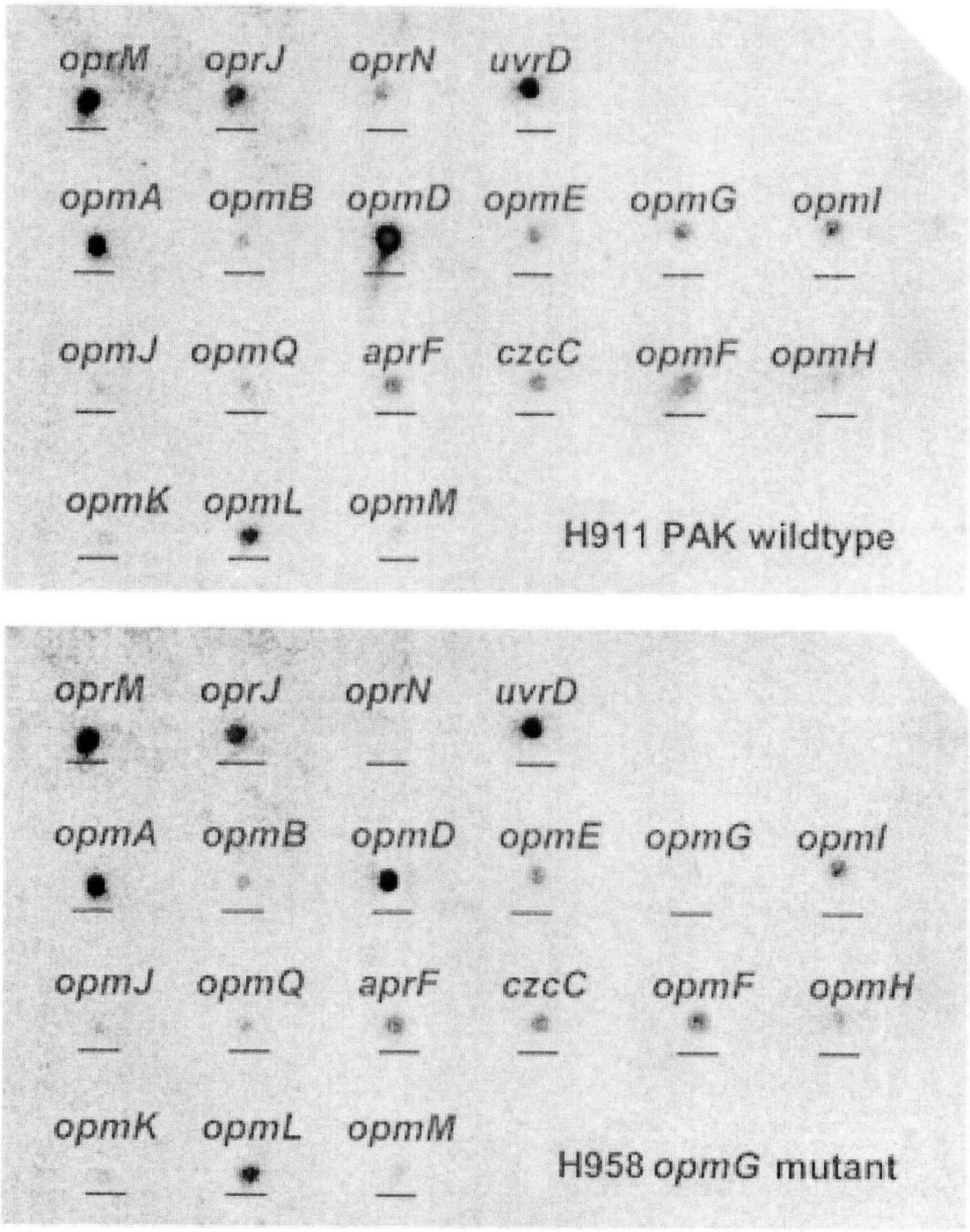


Table X. Quantitated spot densitometry values for strains H911 and H958.

	Gene	a) Spot density H911[a]	b) Normalized Values H911[b]	c) Spot Density H958[a]	d) Normalized Values H958[b]	e) Fold Change[c]
1	*oprM*	50357	1.3	50400	1.2	1.0
2	*oprJ*	25245	0.62	27874	0.66	0.93
3	*oprN*	12345	0.27	1364	0	N/A
4	*uvrD*	39803	1.0	41136	1.0	1.0
5	*opmA*	46138	1.2	39322	0.95	1.2
6	*opmB*	4682	0.071	6056	0.10	0.68
7	*opmD*	86457	2.21	51199	1.2	1.8
8	*opmE*	15846	0.37	16738	0.38	0.97
9	*opmG*	16352	0.38	1998	0	N/A
10	*opmI*	16739	0.39	20060	0.46	0.84
11	*opmJ*	6516	0.12	5671	0.094	1.3
12	*opmQ*	7390	0.14	7980	0.15	0.93
13	*aprF*	21691	0.52	17683	0.40	1.3
14	*czcC*	17018	0.40	13722	0.30	1.3
15	*opmF*	18690	0.44	31050	0.74	0.59
16	*opmH*	10998	0.24	10224	0.21	1.1
17	*opmK*	7804	0.15	6665	0.12	1.3
18	*opmL*	28289	0.70	27582	0.65	1.1
19	*opmM*	7731	0.15	8740	0.17	0.88

[a] Spot density quantities are arbitrary values.

[b] Normalized spot densities correspond to the spot density value for each gene divided by the spot density value of *uvrD*, i.e. a_n/a_4 for H911 and c_n/c_4 for H958.

[c] Fold change is calculated by taking the ratio of the normalized spot density values for the wildtype strain over the normalized spot density values of the mutant strain, i.e. b_n/d_n. Only differences greater than 2-fold were considered significant.

Figure 7. Comparison of expression of OprM homologue genes in wildtype PAK (H911; top panel) and the *opmH* mutant (H966; bottom panel) using mini-microarrays. RNA isolated from each strain was reverse transcribed to cDNA, radiolabelled with [α^{32}P]dCTP, and hybridized to a nylon membrane containing 10 ng spots of 600 bp internal fragments corresponding to each of the OprM homologues. Spot densitometry revealed the expected absence of *opmH* expression as well as 1.9 and 2-fold downregulations of *opmG* and *opmI* expression, respectively.

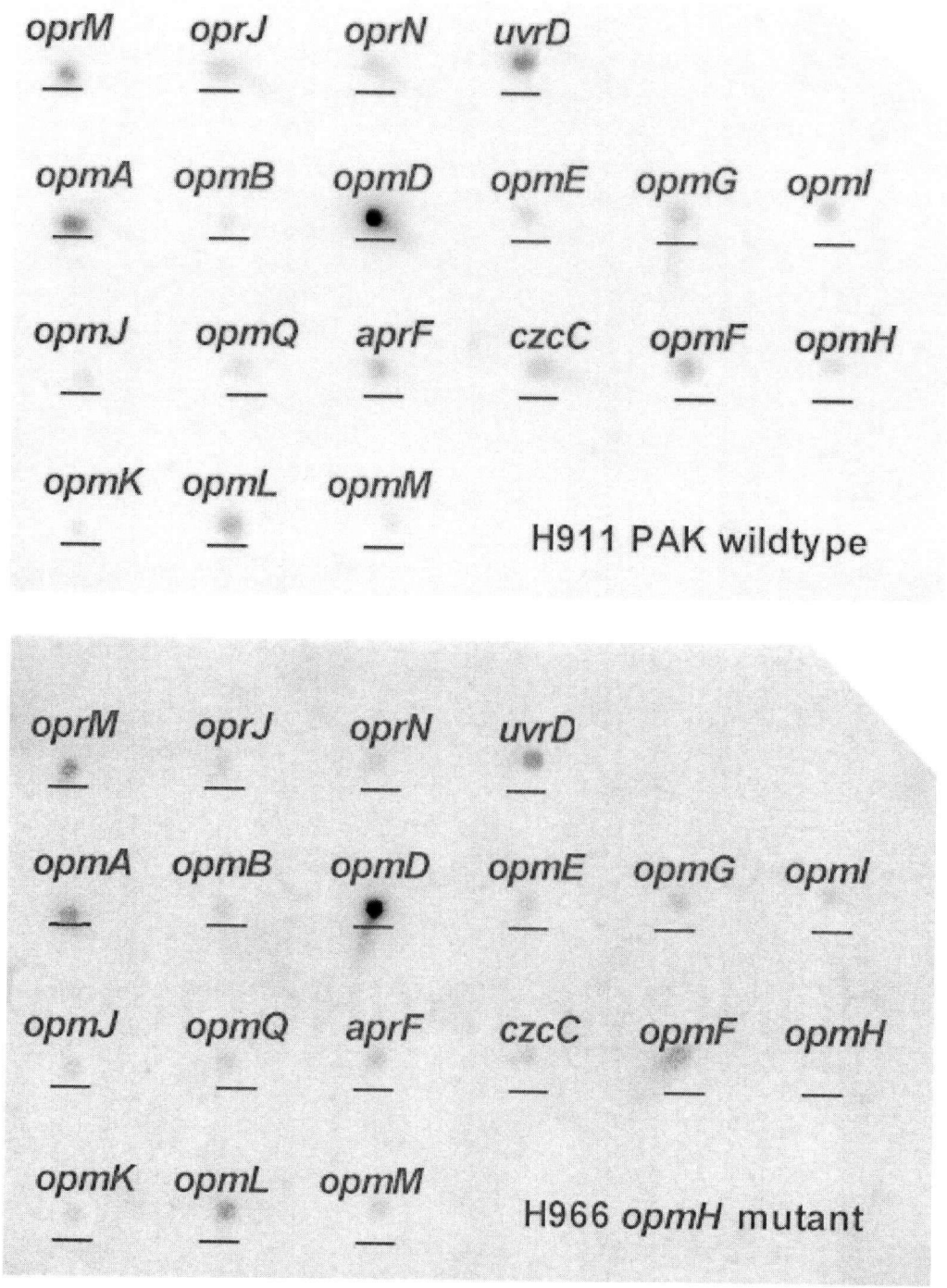


Table XI. Quantitated spot densitometry values for strains H911 and H966.

	Gene	a) Spot density H911[a]	b) Normalized Values H911[b]	c) Spot Density H966[a]	d) Normalized Values H966[b]	e) Fold Change[c]
1	*oprM*	757	0.83	1034	0.88	0.95
2	*oprJ*	421	0.37	403	0.25	1.5
3	*oprN*	271	0.17	520	0.37	0.46
4	*uvrD*	878	1.0	1158	1.0	1.0
5	*opmA*	1018	1.2	1871	1.7	0.70
6	*opmB*	264	0.16	410	0.26	0.62
7	*opmD*	2143	2.7	2180	2.0	1.4
8	*opmE*	410	0.36	435	0.28	1.3
9	*opmG*	650	0.70	424	0.37	1.9
10	*opmI*	517	0.51	401	0.25	2.0
11	*opmJ*	275	0.18	429	0.28	0.63
12	*opmQ*	317	0.23	435	0.28	0.82
13	*aprF*	667	0.71	645	0.49	1.4
14	*czcC*	574	0.58	485	0.33	1.7
15	*opmF*	714	0.78	716	0.56	1.4
16	*opmH*	259	0.15	147	0	N/A
17	*opmK*	286	0.19	413	0.26	0.72
18	*opmL*	565	0.57	840	0.69	0.83
19	*opmM*	206	0.13	435	0.25	0.52

[a] Spot density quantities are arbitrary values.

[b] Normalized spot densities correspond to the spot density value for each gene divided by the spot density value of *uvrD*, i.e. a_n/a_4 for H911 and c_n/c_4 for H966.

[c] Fold change is calculated by taking the ratio of the normalized spot density values for the wildtype strain over the normalized spot density values of the mutant strain, i.e. b_n/d_n. Only differences greater than 2-fold were considered significant.

strains. However, this does not rule out other secondary mutations, such as mutations in drug targets, that might be having an affect on recorded MICs. While secondary mutations would not alter the role of these OMPs in determining aminoglycoside resistance, assessing the degree of the effect of the mutation or the ability to complement the mutant would be more complicated. It is clear, however, that OMPs other than OprM play a role in intrinsic aminoglycoside resistance, though it is not possible to conclude from these studies if their role also involves the MexX and MexY proteins.

DISCUSSION

5.1 Introduction

At 6.3 Mb, *Pseudomonas aeruginosa* has one of the largest bacterial genomes. It is estimated that nearly 10% of the genes encode regulatory proteins, a testament to the environmental ubiquity of this organism (Stover *et al.,* 2000). Several large families of outer membrane proteins have also been identified. The eighteen members of the OprM family have been implicated in forming the outer membrane repertoire of proteins required for efflux of toxic compounds and ions and secretion of extracellular proteins. Eleven of the OprM family members are encoded as part of an efflux operon, containing genes for an inner membrane RND or MFS efflux pump and periplasmic linker protein, and another seven are encoded in operons with genes for ABC-type transporters as well as those for periplasmic linker proteins. Of the 18 members of the OprM family, only *opmH* is encoded without genes encoding a cognate transporter and linker, the characteristics components of the tripartite efflux and type I secretion systems used to move compounds across the Gram negative double membrane.

With seventeen homologous efflux/secretion systems (*opmH* is not part of an operon with any other components) present in the genome, it is clear that the ability to export harmful substances plays a large role in the ability of this organism to successfully colonize so many differing environments. The intrinsic resistance to antibiotic therapy that these efflux systems afford plays a large role in the success of *P. aeruginosa* at producing chronic infections in immunocompromised patients (Hancock and Speert, 2000). In particular, the aminoglycoside antibiotics are of particular interest since they are a common therapeutic drug for recalcitrant *Pseudomonas* infections (Davies and

Wright, 1997) and because it was believed until recently that hydrophilic molecules were not substrates for efflux (Nikaido, 1996). This project has examined two possible candidate outer membrane proteins that may function in conjunction with the MexY inner membrane RND efflux pump and the MexX linker in aminoglycoside efflux. Identifying the components of aminoglycoside efflux might provide leads to finding inhibitors of these systems, thus increasing the therapeutic value of currently available antibiotics.

5.2 Phylogenetic Analysis of OprM Homologues

Table IV shows that the eleven uncharacterized members of the efflux cluster share between 45 and 55% sequence similarity with OprM or OprN. Likewise, the members of the secretion cluster are highly homologous (over 40% similarity at the amino acid level) with AprF, the outer membrane protein involved in secretion of alkaline protease. Interestingly, when the amino acid sequences of the eighteen OprM homologues are aligned against each other (Figure 5), there are few residues that are conserved among the entire family. A more extensive alignment of OprM-like outer membrane proteins from Gram negative bacteria also reveals few conserved residues, but the observation has been made that many of the conserved regions occur at critical points in the structure, such as the glycine residues present at tight turns and the aromatic amino acids at the membrane interfaces (Andersen *et al.,* 2001). In addition, the phylogenetic analyses of these OMPs show a clustering of members according to the type of substrate exported: protein, cation, or antibiotic (Andersen *et al.,* 2001). Since not all of the channel forming OMPs are encoded as units with inner membrane and periplasmic members (OpmH and *E. coli* TolC for example), it seems likely that these channel-forming OMPs are adaptable enough to function with more than one pump, a phenomenon noted with both TolC and

P. aeruginosa OprM. It would not be surprising to learn that more than one OMP can function with MexX and MexY to confer intrinsic aminoglycoside resistance. While several researchers (Aires *et al.,* 1999; Masuda *et al.,* 2000a; Masuda *et al.,* 2000b) have shown that OprM can be co-expressed with MexX-MexY to confer aminoglycoside resistance, not all aminoglycoside-resistant isolates that highly express MexX and MexY also highly express OprM (Westbrock-Wadman *et al.,* 1999). Determining the identity of the native OMP in particular is crucial to understanding the process of aminoglycoside efflux as well as the intricacies of the efflux of both hydrophilic and hydrophobic compounds by the same system.

5.3 OpmG and OpmH can complement an OprM$^-$ defect

The genes encoding *opmG* and *opmH* were cloned behind the *lac* promoter of the pUCP27 vector to create the two recombinant vectors pJJ106 and pJJ105. Table VIII shows the ability of both the OpmG and OpmH proteins to complement the OprM$^-$ defect of strain H743. The ability to complement the antibiotic supersusceptibility associated with inactivation of *oprM* in strain H743, with the substrate specificity of MexA-MexB-OprM, demonstrates a number of important points: a) that both OpmG and OpmH are channel-forming outer membrane proteins capable of mediating efflux of antibiotics from the cell, b) that substrate specificity is indeed largely determined by the inner membrane components of the efflux pumps, as has been noted previously (Srikumar *et al.,* 1997; Gotoh *et al.,* 1998), c) that the ability of channel-forming OMPs to function with alternate pump and linker components (Gotoh *et al.,* 1998; Yoneyama *et al.,* 1998) is a common feature of the entire family, and d) that channel-forming OMPs, despite having

little sequence identity (Andersen *et al.,* 2001), still share the same structure and mechanism of action of gating necessary to function as efflux OMPs.

5.4 OpmG and OpmH can complement *opmG* and *opmH* mutants

OpmG was also able to complement the original mutant strains H958 when reintroduced on the pJJ107 vector (Table IX), a finding that supports the hypothesis that OpmG plays a role in aminoglycoside resistance. In contrast, complementation of aminoglycoside susceptibility by pJJ109 (*opmH*) in strain H966 (*opmH*::miniTn*5*-Tc^R) was only 2-fold. However, there was partial but significant complementation of the MICs to the two dyes acriflavin and crystal violet. The ability to complement an acriflavin susceptibility is a particularly interesting finding since the closest OpmH homologue (TolC of *E. coli*) was first implicated in the efflux of acriflavin (in conjunction with AcrA-AcrB), and because acriflavin was noted to be an inducer and a substrate for MexX-MexY (Morita *et al.,* 2001).

5.5 Mini-microarray analysis

MexC-MexD-OprJ and MexE-MexF-OprN have been shown to be upregulated in response to the loss of MexA-MexB-OprM by an unknown mechanism (10). The possibility that a miniTn*5* insertion mutation in any one of the OprM family homologues might result in compensatory up or downregulation of other homologues would prove problematic to assessing the roles of OpmG and OpmH in aminoglycoside resistance. Mini-microarrays were constructed to monitor expression of all members of this family in the wildtype and mutant strains. It should be noted that microarray analysis measures the amount of transcript as opposed to the amount of protein.

Figure 2 shows one representative experiment comparing the expression of the 18 OprM homologues in the parent strain H911 (top panel) and the *opmG* mutant H958 (bottom panel). Quantification and normalization of the hybridization signals to the *uvrD* internal standard (Table X) showed the loss of *opmG* expression (as expected) as well as the loss of OprN expression. Since OprN plays no known role in aminoglycoside resistance (Seiffer *et al.,* 1993; Saier *et al.,* 1994; Hancock and Speert, 2000), it is unlikely that this change in OprN expression is influencing aminoglycoside resistance in strain H958. Thus, I conclude that OpmG is the major outer membrane channel responsible for aminoglycoside efflux. As it is the first gene in its efflux operon (in contrast to OprM, which is the third gene in the *mexA-mexB-oprM*) , it seems possible that OpmG is made at larger levels than its cognate linker and pump proteins, providing an excess of OMP to potentially form efflux complexes with MexX and MexY.

Figure 3 shows one representative experiment comparing the expression of the 18 OprM homologues in the parent strain H911 (top panel) and the *opmH* mutant H966 (bottom panel). Similar quantification and normalization of the hybridization signals showed the expected loss of *opmH* expression. In addition there were modest decreases in *opmG* (1.9-fold) and *opmI* (2-fold) expression. It is thus possible that the effect of *opmH* on aminoglycoside resistance is being exaggerated due to the downregulation of two other outer membrane proteins which also play a role in aminoglycoside resistance (Table VII). Nevertheless, it is clear that OpmH does function as a channel forming outer membrane protein since it can complement a mutant lacking OprM (Table 3), and its ability to partially restore MICs of aminoglycosides, macrolides, fusidic acid, acriflavin,

and crystal violet (Table 4), demonstrates that OpmH may play a minor role in resistance to these compounds.

5.6 Summary

Despite the efforts of many separate research groups and the array of currently available pharmaceuticals, chronic infection by multi-drug resistant *Pseudomonas aeruginosa* remains a relevant clinical issue. The sheer number of proteins possessed by all bacteria that are dedicated to the modification, circumvention, and extrusion of toxic compounds, and the notorious ability of bacteria to horizontally transfer the genetic factors encoding many of these proteins, are major barriers to overcoming the problem of antibiotic resistance. Gram-negative tripartite multi-drug efflux systems are of particular concern since many RND- and MF- transporters have a broad spectrum of substrates that seems contrary to our traditional understanding of specific interactions between proteins and their substrates. The possibility exists that selective pressure applied to a population of bacteria in the form of a single antibiotic could result in the generation of a multi-drug resistant strain in a single step.

While four RND efflux systems have been characterized in *P. aeruginosa*, little is known about the signals that regulate their expression. Since the discovery and widespread use of antibiotics did not occur until the 20th century, it is obvious that efflux systems predate their inception into general use. The ability of bacteria to adapt and utilize pre-existing transport systems for the purpose of exporting novel toxic compounds underscores the plasticity that allows them to survive the effects of antimicrobials, both natural and human-made.

The demonstration that wildtype *P. aeruginosa* strain PAK appears to express all eighteen OprM family outer membrane proteins, some almost as strongly as OprM, despite studies that have shown that at least two, OprJ and OprN, are not expressed under normal growth conditions in strain PAO1, emphasizes the limited understanding we have about the control of these efflux systems. Cross-hybridization of the probes to multiple membrane-bound amplicons could account for this, and this possibility needs to be examined, possibly by using the amplicons themselves as probes for the membrane. Nevertheless, it is known that the ability to export harmful substances plays a large role in the ability of this organism to successfully colonize so many differing environments, and the intrinsic resistance to antibiotic therapy that these efflux systems afford plays a significant role in the success of *P. aeruginosa* at producing chronic infections in immunocompromised patients. In particular, the efflux of aminoglycoside antibiotics are of particular interest since they are a common therapeutic drug for recalcitrant *Pseudomonas* infections (Davis, 1987) and because it was believed until recently that hydrophilic molecules were not substrates for efflux (Saier *et al.,* 1994).

This project has examined the roles of two candidate outer membrane proteins, OpmG and OpmH, and identified one, OpmG, that may function in conjunction with the MexY inner membrane RND efflux pump and the MexX linker in aminoglycoside efflux. Further studies into the regulation of these outer membrane proteins, and the role of a third protein OpmI, will be required to evaluate their roles in determining resistance in *P. aeruginosa*. Identifying the components of aminoglycoside efflux might provide leads to finding ways to inhibit or circumvent these multi-drug efflux systems thus increasing the therapeutic lifespan and value of currently available antibiotics.

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