DISSERTATION

STUDIES ON MULTIDRUG EFFLUX SYSTEMS AND TRICLOSAN RESISTANCE IN *Pseudomonas aeruginosa*

Submitted by

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WE HEREBY RECOMMEND THAT THIS DISSERTATION PREPARED UNDER OUR SUPERVISION BY RUNGTIP CHUANCHUEN ENTITLED "STUDIES ON MULTIDRUG EFFLUX SYSTEMS AND TRICLOSAN RESISTANCE IN *Pseudomonas aeruginosa*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

STUDIES ON MULTIDRUG EFFLUX SYSTEMS AND TRICLOSAN RESISTANCE IN *Pseudomonas aeruginosa*

In this dissertation, the role of RND multidrug efflux system in triclosan resistance was studied in *Pseudomonas aeruginosa*. Infections with *P. aeruginosa* are notoriously known for being very difficult to treat because of its high intrinsic drug resistance and its ability to develop resistance to a wide range of antimicrobial agents. This high resistance primarily results from synergy of its low outer membrane permeability and the expression of multidrug efflux pumps. Genomic DNA sequence analysis revealed that there are as many as 37 efflux systems representing all known efflux pump families. Twelve of these belong to the resistance nodulation division (RND) family and these have emerged as clinically significant in Gram-negative bacteria, including P. *aeruginosa*. Four RND efflux systems – MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY - had been described in wild-type strain PAO1 when this dissertation research was started. Two additional ones were found since - MexGHI-OpmD and MexVW - and this research identified a seventh, MexJK. Only MexAB-OprM is constitutively expressed at low levels in PAO1 and the other RND efflux systems are only expressed in strains containing regulatory mutations. The characterization of new efflux pumps therefore awaits identification of regulatory mutations expressing these pumps and these can usually only be obtained after identification of the respective pump substrates or the fortuitous discovery of clinical isolates expressing the pumps after antibiotic exposure. *P. aeruginosa* is well known for its high intrinsic resistance to triclosan, a broad spectrum bisphenolic biocide that is the active ingredient in many consumer

products with antimicrobial properties. Although wild-type *P. aeruginosa* strains express the triclosan target enoyl-acyl carrier protein reductase (FabI), a crucial enzyme of the bacterial fatty acid biosynthetic cycle, they are triclosan-resistant due to constitutive expression of the MexAB-OprM efflux system. In this study, triclosan was proven to be a good substrate for most of characterized multidrug efflux systems including MexAB-OprM, MexCD-OprJ and MexEF-OprN, but not MexXY. In fact, experiments presented in this dissertation showed that RND pumps are solely responsible for high-level triclosan resistance of *P. aeruginosa*, which enables this bacterium to survive in the presence of triclosan concentrations in excess of 1000 μg/ml. Since triclosan is a good and perhaps universal RND efflux pump substrate, it was reasoned that it could be used as a tool to isolate regulatory mutants expressing hitherto unidentified efflux pumps and to use these mutants to study efflux pump function, molecular architecture and regulation. Proof-of-concept was obtained by exposure of a triclosan-susceptible Δ(*mexAB-oprM*) *P. aeruginosa* strain to triclosan. This procedure selected triclosan-resistant bacteria at high frequencies and these bacteria became simultaneously multidrug resistant (MDR) due constitutive overexpression of MexCD-OprJ caused by mutations in its regulatory gene, *nfxB*. The types of mutations obtained were similar to those previously obtained after exposure to fluoroquinolones in laboratory and clinical settings. These experiments supported the notion that triclosan and antibiotics can cause drug resistance via similar mechanisms and that a link between antiseptic, triclosan, and antibiotic resistance does indeed exist. The next step was as to prove that triclosan is also an excellent tool for selection of regulatory mutants expressing normally silent multidrug efflux pumps. By exposing a susceptible □(*mexAB-oprM*) □(*mexCD-oprJ*) strain to triclosan, a new RND efflux pump, MexJK, was discovered and characterized. It was found that expression of the *mexJK* operon is negatively governed by the product of a regulatory gene, *mexL*, located upstream of and transcribed divergently from the *mexJK* operon. The regulatory mutants obtained by tricosan exposure constitutively expressed MexJK due to an alanine to aspartate change in the

putative helix-turn-helix motif of MexL. Gene fusion analysis verifed the negative effect of *mexL* on *mexJK* expression, indicated that the degree of *mexJK* repression/derepression is dependent on the growth medium and showed that MexL autoregulates its own expression. To determine modes of regulation of *mexL* and *mexJK* expression at the molecular level, MexL was overexpressed and purified as a fusion protein containing a carboxy-terminal hexahistidine peptide tag. The purified protein showed an apparent molecular weight of 25,000 daltons. Biochemical and genetic experiments showed that MexL oligomerizes and exists in solution as a tetrameric protein. Gel mobility shift and footprinting assays demonstrated that MexL is a specific DNA binding protein and that MexL binds to both DNA strands of the 94 bp *mexL-mexJ* intergenic region. The protected region encompassed two inverted repeats of the GTATTT hexamer sequence, which may be recognized by MexL as part of its operator site. The *mexL* and *mexJK* promoter regions were also localized to the MexL protected region. These two promoter regions overlap and share a common -10 region. The *mexL* promoter was verified by mapping the *mexL* transcript start site by RNase protection assays and the *mexJ* promoter was localized using *mexJ-lacZ* gene fusions. In summary, the studies presented in this dissertation verified that triclosan is a substrate for most *P. aeruginosa* RND efflux pumps, can be used to isolate regulatory mutants expressing known and unknown RND efflux pumps, and is therefore an excellent tool for efflux pump discovery and characterization. The materials and tools developed during these studies will be useful for further studies, especially those aimed at understanding efflux pump function – e.g., pump assembly and substrate recognition – and efflux pump inhibitor discovery.

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Dedicated to

my entire family

especially to my parents

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ABBREVIATIONS

A_{540nm}	absorbance at 540 nm
A_{600nm}	absorbance at 600 nm
ABC	ATP-binding cassette
AIDS	acquired immune deficiency syndrome
Ap	ampicillin
apr	alkaline protease encoding gene
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
att	locus for phage attachment
B.	*Burkholderia*
β-Gal	β-galactosidase
bla	β-lactamase encoding gene
bp	base pair(s)
C_4-HSL	N-butyryl homoserine lactone
Cb	carbenicillin
°C	degree(s) Celcius
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
Cip	ciprofloxacin
Cl^-	chloride ion

CNS	central nervous system
cm	centimeter(s)
Cm	chloramphenicol
CTX	cytotoxin
Da	Dalton(s)
DHPs	dihydropteroate synthetase
DNA	deoxyribonucleic acid(s)
dNTP	deoxyribonucleoside triphosphate(s)
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylenediamine tetraacetic acid
EF	elongation factor
ER	endoplasmic reticulum
ERCF	European Registry for Cystic Fibrosis
Ery	Erythromycin
FRT	Flp recombinase target
GI	gastrointestinal
Gm	gentamicin
His_6	hexahistidine-containing peptide
HIV	human immunodeficiency virus
HS	heparan sulfate
HSL	homoserine lactone(s)
HSPG	heparan sulfate proteoglycan
HTH	helix-turn-helix
h	hour(s)
int	phage CTX integrase gene

IPTG	isopropyl-β-D-thiogalactopyranoside
K.	*Klebsiella*
kb	kilobase(s) or 1000 bp
kDa	kilodalton(s)
Km	kanamycin
l	liter(s)
lacI	*E. coli lac* repressor structural gene
lacZ	□-galactosidase encoding gene
lacY	lactose permease encoding gene
lasA	elastase encoding gene
lasB	elastase encoding gene
LasI	*P. aeruginosa N*-[3-oxododecanoyl]-L-HSL synthase
LasR	*N*-3-oxo-C_{12}-HSL-binding regulatory protein
LB	Luria-Bertani medium
LD_{50}	lethal dose killing 50 percent of specimens
log	logarithmic growth phase
LPS	lipopolysaccharide(s)
M.	*Mycobacterium*
M	molar
Mb	megabasepairs
Mex	multidrug efflux
mM	millimolar
MATE	multidrug and toxic compound extrusion family
MBC	minimal bactericidal concentration
MCAC	metal chelation affinity chromatography
MCS	multiple cloning site(s)

mEq	milli-Equivalent(s)
MFP	membrane fusion protein
MFS	major facilitator superfamily
mg	milligram(s)
MIC	minimal inhibitory concentrations
min	minute(s)
ml	milliliter(s)
M_r	relative molecular weight
MSD	membrane-spanning domain
NBD	nucleotide-binding domain
NEC	necrotizing entercolitis
ng	nanogram(s)
nm	nanometer(s);
NNIS	National Nosocomial Infections Surveillance
nt	nucleotide(s)
omlA	outer membrane lipoprotein A
OMP	outer membrane protein
ori	origin of replication
oriT	origin of transfer
P.	*Pseudomonas*
PABA	*p*-aminobenzoic acid
P_{lac}	*E. coli lac* operon promoter
P_{lacUV5}	*E. coli lac* operon UV5 promoter
P_{trc}	*E. coli trp-lac* hybrid promoter
PAGE	polyacrylamide gel electrophoresis
PAI	*Pseudomonas* autoinducer

PBS	phosphate-buffered saline
PBP	penicillin binding protein(s)
PCR	polymerase chain reaction
PIA	*Pseudomonas* isolation agar
PLC-H	hemolytic phospholipase
PLC-N	non-hemolytic phospholipase
PMF	proton motive force
PQS	*Pseudomonas* quinolone signal
R	regulatory domain
r	resistance/resistant
RBS	ribosome-binding site
Rep	replication protein
RhlAB	rhamnosyltransferase subunits A and B
RhlI	*P. aeruginosa* C_4-HSL synthase
RhlR	C_4-HSL binding regulatory protein
Rif	rifampin
RND	resistance –nodulation-division
RT	room temperature
S.	*Staphylococcus*, *Sternotrophomonas* or *Salmonella*
s	sensitive/susceptible
sacB	*Bacillus subtilis* gene encoding levansucrase
SCV	small colony variants
SDS	sodium dodecyl sulfate
Sm	streptomycin
SMR	small multidrug resistance
Std	standard(s)

TBE	tris-borate-EDTA
TG	tris-glycine
TLM	thiolactomycin
Tc	tetracycline
tet	tetracycline-resistance encoding gene
TMS	transmembrane segment
toxA	exotoxin A encoding gene
Tri	triclosan
Ts	temperature sensitive
u	unit(s)
µl	microliter
µM	micromolar
µg	microgram(s)
UTI(s)	urinary tract infection(s)
V.	*Vibrio*
v/v	volume by volume
w/v	weight by volume
wt	wild-type
XGal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
xcpP	gene encoding a protein of the general secretory pathway
xcpR	gene encoding a protein of the general secretory pathway

LIST OF PUBLICATIONS

Parts of this dissertation have been published in the following articles:

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CHAPTER 1

INTRODUCTION

1.1. MICROBIOLOGY OF *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, non-spore forming, non-acid fast, rod-shaped bacterium. It is 0.5 to 1.0 μm in width by 1 to 3 μm in length and is motile due to one polar flagellum (22). *P. aeruginosa* has characteristic grape-like odor that can be determined by experienced microbiologists. This bacterium is oxidase and catalase positive. As a mesophile, its optimal temperature for growth is 37°C and it can grow at temperatures as high as 42°C (99). *P. aeruginosa* is normally strictly aerobic but it can grow in the absence of oxygen if nitrate (NO_3) is provided as electron acceptor. *P. aeruginosa* does not require large amounts of organic growth factors, so it is often observed growing in distilled water, which is evidence for minimal nutrient requirements. Although the organism lacks a complete Embden-Meyerhoff pathway, it can metabolize a diversity of carbon sources, e.g. mannose, glucose, fructose, ribose, xylose, acetate, and C_4 to C_{10} fatty acids by the Entner-Doudoroff pathway, the pentose phosphate cycle, the tricarboxylic acid cycle and β–oxidation. However, it cannot produce acid from lactose or sucrose (229). *P. aeruginosa* commonly occurs in soil, water, sewage, regularly on the surfaces of plants and occasionally on the surfaces of animals. Since *P. aeruginosa* is ubiquitous in the environment, it is also called a cosmopolitan bacterium. The bacterium has been recognized as an infectious agent transmitted by food and water (156) and is particularly known as an important

bacterial contributor to spoilage of conventionally pasteurized milk products (245). Currently, legislation in many countries demands that bottled water products be free of *P. aeruginosa* (156). *P. aeruginosa* has a dimorphic lifestyle. It can be naturally found in a planktonic form, as an actively motile single cell or a sessile biofilm form, i.e. as aggregated cells enclosed in slime and attached to surfaces. In general, *P. aeruginosa* isolates may produce three colony types. Most natural isolates from soil or water typically produce a small, rough colony. Clinical isolates yield two types of smooth colonies. One of the two types has a fried-egg appearance, which is large, smooth, with flat edges and an elevated appearance. The other type has a mucoid appearance, which is attributed to the production of alginate slime and is frequently obtained from respiratory and urinary tract secretions. Most *P. aeruginosa* isolates are able to produce pigments of which the three major types are pyocyanin, pyoverdine and pyochelin. Pyocyanin is a blue-green, non-fluorescent, soluble phenazine pigment. This pigment yields a distinctive blue-green color that is the characteristic of suppurative infections caused by the bacterium. *P. aeruginosa* also produces and secretes pyochelin and pyoverdine, two low molecular weight iron-chelating agents known as siderophores that are produced in response to iron-limiting conditions. These siderophores form a complex with iron and deliver the cofactor to the cells through specific membrane receptors. Both pyoverdine and pyochelin are fluorescein (green-yellow, fluorescent). All siderophores have been shown to contribute to the virulence of *P. aeruginosa*.

P. aeruginosa has a large (6.3 Mb) genome, which is currently one of the largest and most complex bacterial genomes completely sequenced. Its genome encodes 5,570 genes, and its GC content ranges from 55-70 % (62.5 % average).

1.2. *P. aeruginosa* VIRULENCE FACTORS

Falkow (1988) described bacterial virulence factors as i) being encoded by a gene of the bacterium causing disease but it is usually absent or inactivate in the etiological strains, ii) being

required for the bacterial virulence, therefore, loss of the gene will diminish the virulence, and iii) being usually expressed during infections (48). *P. aeruginosa* is an opportunistic pathogen, however, its infections are often fatal. This bacterium possesses a large number of virulence factors that contribute to the pathogenesis of clinical infections. A variety of *P. aeruginosa* virulence factors have been studied and they include cell-associated and secreted factors.

1.2.1. Cell-associated virulence factors. These virulence factors contribute to colonization and virulence. They include lipopolysaccharide (LPS), alginate, flagella and pili (45). *P. aeruginosa* uses flagella and pili for its adherence. Whereas *P. aeruginosa* flagella contribute to tissue invasion at the early stage of infections, the pili contribute to adherence to epithelial cells and mucosal surfaces. The pili of *P. aeruginosa* are type IV pili or *N*-methyl-phenyl-alanine pili. They form a multifunctional retractable structure providing a unique form of motility called twitching motility (99).

Production of alginate is a unique virulence property of *P. aeruginosa*. Alginate is a linear polymer composed of D-mannuroic acid linked to L-guluronic acid by β (1-4) linkages. It forms a viscous gel around bacterial cells and such colonies are referred to as mucoid. Mucoid *P. aeruginosa* colonizes the pulmonary tracts of cystic fibrosis (CF) patients leading to blockage of lung airways (99). Lipopolysaccharide (LPS) of *P. aerugionosa* is another virulence factor. Whereas lipid A is endotoxic, O-antigen is an immunogen. *P. aeruginosa* produces 2 types of LPS: type A and type B. The A-type LPS consist of repeating units of D-rhamnose, and is antigenic. The B-type LPS determines the serotype of the bacterium (99).

1.2.2. Secreted virulence factors *P. aeruginosa* secretes several exoproteins that contribute to its virulence and are identified by testing in various isogenic and non-isogenic strains. The bacterium utilizes three types of protein export systems including type I, type II and type III

systems. Some factors are secreted by membrane blebbing. However, most of *P. aeruginosa* exoproteins are secreted by type II pathways.

Alkaline protease is encoded by the *aprA* gene and is essential for colonization during corneal infections (99). The secretion of AprA requires three accessory proteins including AprD (ATPase), AprE (translocase) and AprF (outer membrane protein). The proteins form a type I export system (65, 234). *P. aeruginosa* also produces an inhibitor of AprA, which is AprI (99).

Elastase is a extracellular protease accounting for 90% of *P. aeruginosa* protease activity (82). It degrades elastin and also many other proteins required for maintaining the structural integrity of the host cell. Elastase, or LasB, is encoded by the *lasB* gene. The *lasA* gene encodes for a serine protease that nicks elastin so that it is more susceptible to proteolysis by elastase and other proteases (45). There is another protease homologous to LasA which is called LasD. While LasA is active at a broad pH range, LasD is active at higher pH (99). LasA and LasB are subject to regulation by the LasR regulator, and are under the control of the LasR-LasI quorum sensing system (181).

Exotoxin A is encoded by the *toxA* gene and is the most toxic exoprotein produced by *P. aeruginosa* (99). It catalyzes the ADP-ribosylation of the eukaryotic translation factor EF-2 to form ADP-ribosyl-EF-2 and thus inhibits the protein synthesis of the host cells (244). Production of exotoxin A is regulated by quorum sensing and iron starvation (99).

Phospholipase C molecules in *P. aeruginosa* are classified into 2 types including hemolytic (PLC-H) and nonhemolytic (PLC-N) phospholipases which are encoded by *plcH* and *plcN*, respectively. PLC-H and PLC-N are homologous proteins. PLC-H is a phosphatidyl cholinase and sphingomyelinase that breaks down human and mouse erythrocytes. PLC-N acts on phosphatidylcholine and phosphatidylserine. Both phopholipases contribute to tissue damage, especially in lungs (99).

Exoenzymes S, T, U, and Y are encoded by the *exoS*, *exoT*, *exoU* and *exoY* genes, respectively. They are the effector molecules of the only type III secretion pathway identified in

P. aeruginosa. In CF patient isolates, ExoS is much more common than in other isolates. ExoS exhibits ADP-ribosylating activity. This activity is activated by an eukaryotic protein called FAS (Factor for activating exoenzymes S). ExoT is 75% identical to ExoS (45). Both ExoS and ExoT exhibit cytotoxic activity by inhibition of bacterial internalization into epithelial cells and macrophages, leading to disruption of the actin cytoskeleton, and prevention of wound healing (56). ExoU causes epithelial and macrophage cell death by necrosis (74). ExoY is an adenylate cyclase. Its role as virulence factor remains unclear (45).

Other secreted virulence factors produced by *P. aeruginosa* include rhamnolipids, cytotoxin, lectins and pyocins. Rhamnolipids are biosurfactants containing rhamnose and fatty lipid. They facilitate emulsification of water-insoluble substrates. Therefore, they may solubilize phospholipids on lung surfaces, making them more accessible to phospholipase C. Production of rhamnolipids is controlled by the Rhl quorum sensing pathway, but their secretory pathway remains unknown (45). *P. aeruginosa* produces cytotoxin with activity against leukocytes and other eukaryotic cells, the CTX toxin. The CTX toxin is encoded by a lysogenic CTX phage and causes pores in the lipid layers of the host cells (246). Two types of lectins, specific sugar-binding proteins, are found in the bacterium. PA-IL recognizes D-galactose, while PA-IIL binds specifically to L-fucose and D-mannose. Lectins facilitate bacterial cell adherence. Expression of both is regulated by the quorum sensing system (246). Lastly, *P. aeruginosa* produces bacteriocins called pyocins. Pyocins are secreted into the medium and kill other bacteria, and the lysed bacteria are degraded and utilized as nutrient sources (33).

1.3. MEDICAL PROBLEMS CAUSED BY *P. aeruginosa*

P. aeruginosa is an opportunistic pathogen that can cause severe infections, particularly in immunosuppressed individuals. Such immunocompromised people include patients undergoing immunosuppressive therapies, e.g. cancer treatment, patients with severe burns and cancer,

patients with human immunodeficiency virus (HIV) infections and AIDS, and CF patients. *P. aeruginosa* is primarily a nosocomial pathogen that is very common within the hospital setting (9). The overall incidence of *P. aeruginosa* infections in the US hospitals averages about 0.4 % (11), and the bacterium is the fourth most commonly-isolated nosocomial pathogen accounting for 10.1 % of all hospital-acquired infections (11). Although the prevalence of colonization of healthy individuals outside the hospital is relatively low, *P. aeruginosa* is sometimes present as part of the normal flora of humans (227). Unfortunately, the exact sources and modes of transmission of this pathogen are often unclear because of its ubiquitous presence in the environment. Although *P. aeruginosa* causes diseases that range from acute and septicemia to chronic, infections with this pathogen are particularity common in CF patients. Therefore, the following paragraphs review will discuss in more detail *P. aeruginosa* infections in CF patients, and then give some detail about other infections caused by the pathogen.

1.3.1. *P. aeruginosa* infections in Cystic Fibrosis (CF) patients

Cystic fibrosis (CF), earlier also called fibrocystic disease of the pancrease or mucoviscidosis, is a chronically autosomal-recessive disease that has been recognized as a collection of various clinical syndromes of the respiratory, gastrointestinal and reproductive systems (129). CF symptoms are characterized by thick, dehydrated airway mucus, pancreatic insufficiency, bile duct obstruction, high Cl^- sweat, intestinal obstruction and chronic sinusitis (195).

CF is the most frequent inherited lethal disease in Causasians with an incidence of one in 2,500 live births in white Americans and Europeans (113, 128). It is the most common life-shortening genetic disease among white people (about 1:3,300) in the United States. The incidence in nonwhite people is much less, e.g. approximately 1:15,300 in African Americans and 1:32,000 in Asian Americans (151). An estimated 30,000 people in the United States have CF

(24) and 3% of the white population carries this recessive gene (151). CF is also the most prevalent hereditary disease among white children in the United States. Approximately 1 in 2,500 U.S. children is born with CF each year. The Cystic Fibrosis Foundation (2002) reported an increased incidence from 19,987 in 1995 to 22,732 in 2001. Ninety-six, 6.0, and 3.8% of CF patients in 2001 were Caucasian, Hispanic, and African Americans, respectively, and 449 CF patients died in that year (24).

CF arises from mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CFTR is initially synthesized as a 135-140 kDa core-glycosylated immature protein in the endoplasmic reticulum (ER) membrane. A fraction of 20-40% of immature CFTR matures to the Golgi apparatus. Mature CFTR is 150 to 160 kDa on SDS-PAGE gels and contains endoglycosidase H susceptible oligosaccharide chains (111). The majority (60–80%) of immature CFTR that fails to mature is rapidly degraded (111). It is evident that degradation of the misfolded CFTR is mediated by proteasome, which is found abundantly in the cytoplasm and nucleus of eukaryotic cells. However, it was suggested that the additional nonproteasomal proteolytic systems must also contribute to CFTR degradation (111). CFTR is a member of the ATP-binding cassette (ABC) family of transporters. It is comprised of a six membrane-spanning peptide containing domain (MSD), followed by a nucleotide binding domain (NBD), a regulatory (or R) domain, and a tandem repeat of the MSD-NBD motifs (129, 217). Both NBD-1 and NBD-2 of CFTR are responsible for binding and hydrolysis of ATP supplying the energy for opening and closing the ion channel. They function in a sequentially coordinated fashion. The N-terminal NBD-1 hydrolyses one molecule of ATP to open the channel and then the C-terminal NBD-2 hydrolyzes another molecule of ATP to close the channel (129). The R domain was proposed to be a regulatory domain. When dephosphorylated, the R domain will interact with NBD-1 and block the ATP binding site on the NBD. In contrast, the phosphorylated R domain interacts with the NBD in a different way, such that its ATP-binding site is available (133). However, the role of regulation by the R domain needs further investigation.

The *CFTR* gene was mapped to the long arm of chromosome 7 (199). From a physiological perspective, the CFTR gene mutations are classified into 5 classes based on the primary responsible mechanism for reduced CFTR chloride channel function (183). In class 1, i.e. mutations leading to no synthesis, there is failure of CFTR translation, typically due to stop codons or frameshifts, e.g. G542X. In class 2 mutants, CFTR fails to mature and is degraded by protease in the endoplasmic recticulum (ER). This class includes the ΔF508 mutation, which is the most common CF mutation. Class 3 mutations causes defective regulation, i.e. CFTR is fully processed and inserted in the membrane, but the mature protein is insensitive to activation. This class is exemplified by the G551D mutation. In class 4 mutations, decreased conductance is observed, the mature protein is normally activated, but chloride conductance is reduced, as for example in R347P mutants. Lastly, class 5 mutation lead to decreased CFTR abundance. Mutations are in splice sites that affect the efficiency of normal mRNA splicing. Therefore, these reduce CFTR in the cell membrane. This class includes C to T transitions. Class 4 and 5 mutations are expected to be associated with mild disease. Although there have been over 1,000 mutations identified in the CFTR gene, the most common mutation, accounting for 70% of all the *CFTR* mutant alleles, is the ΔF508 mutation (88). According to DNA analysis in CF patients reported by Cystic Fibrosis Foundation (2001), 88.9% of 16,713 genotyped patients have ΔF508 mutations (24). The ΔF508 mutation is a single deletion of CTT containing the third C nucleotide of the ATC isoleucine codon at position 507 and the first two TT nucleotides of the TTT phenylalanine codon at position 508, which are both located in the nucleotide binding domain of CFTR (24). Data suggested that this 508 position of the *CFTR* gene is a mutational hot spot. Besides ΔF508, the identified mutations include ΔI506, ΔI507, I506C, and F508C (24). Like other integral membrane glycoproteins, CFTR is required to traffic to the Golgi apparatus for membrane localization. However, the ΔF508 CFTR protein is improperly folded, mostly retained in ER, and eventually delivered to the degradation apparatus (129). To date, it remains unclear if

the major problem with the ΔF508 CFTR protein is derived from the defective intracellular delivery of mature protein to the plasma membrane or the instability of the mature ΔF508 CFTR at the surface. What is known is that the mutation causes in the reduced levels of CFTR in the plasma membrane (129).

CFTR was shown to regulate ion transport via several mechanisms. Whereas its innate function was proven to be a chloride channel, the R domain regulates the activity of the potassium channel. CFTR mediates the secretion of bicarbonate ions and the suppression of the sodium channel (242). Since the chloride channel plays a crucial role in fluid secretion by epithelial tissues, the malfunction of CFTR results in the abnormality of the transport of electrolytes across epithelial cells in all affected tissues (128). CF patients with defective CFTR have an abnormally high electrolyte sweat, which is called salty sweat. For this reason, the sweat test has been used for diagnosis of the disease (37, 129). Sweat in dermis is isotonic and contains 105 mEq of chloride as is present in serum. Under normal conditions, chloride is absorbed out of the sweat at the skin surface. Therefore, the chloride concentration in normal sweat is below that formed in serum. In general, less than 40 mEq is considered normal and less than 20 mEq is considered typical. In CFTR mutations, chloride is not absorbed out of sweat. This results the higher chloride containing sweat (more than 60 mEq) (129, 215).

P. aeruginosa plays an important role as the most prevalent pathogen in the airway of CF patients (72, 129, 182). According to the CF annual report, 59, 48, 3.1 and 8.4 % of respiratory cultures from CF patents were positive for *P. aeruginosa*, *Staphylococcus aureus, Stenotrophomonas maltophilia* and *Burkholderia cepacia*, respectively (24). Most CF patients suffer from recurrent and chronic *P. aeruginosa* infections. Sputum from these patients can contain up to 10^7 to 10^8 *P. aeruginosa* colony-forming units per ml (225). A report showed that approximately 6 to 20 % of CF patients have *P. aeruginosa* in their gastrointestinal tract without any symptoms and display a significant immune response to the pathogen (221). It was long

thought that CF patients were generally infected by unique lineages of environmental acquired *P. aeruginosa*. Recently, Australian researchers reported cross-infection of *P. aeruginosa* from patient to patient (8). This implied that cohort segregation of CF patients into those that are carrying *P. aeruginosa*, and those that are not, may be necessary. So far, the mode of transmission of *P. aeruginosa* is not completely clear. The infection in CF may result from social contacts and/or may be hospital acquired. Since *P. aeruginosa* is ubiquitous, it is believed that most clinical isolates emerge from the environment (242). To date, the pathogenesis of *P. aeruginosa* infection in CF patients is not fully understood but seems to be due to the combination of the unusual mucoidy of the organism and the thick-viscous mucus on the apical surface of the lung due to the defective chloride transport. CF airway secretions contain abnormal chloride concentrations and this fact is always cited as the major host factor that promotes chronic colonization of the bacterium. It was proposed that such abnormal ionic-strength secretions compromise several host defense mechanisms e.g. the phagocytosis by macrophages and neutrophils. In the airway, the major innate defense is the mucociliary action of the airway epithelium. The clearance mechanisms include the ciliated apical surface of the airway epithelium and the biphasic mucus layer lining the airway lumen. The mucus consists of an upper, viscous layer that is responsible for trapping foreign bodies and microorganisms, and a lower, more fluid layer where the cilia beat to generate movement. It was suggested that the CFTR mutations may alter either mucus secretion or mucus reabsorption or both. This causes the mucous to become a uniform, viscous monolayer, therefore, cilial beating is no longer effective and *P. aeruginosa* colonies persist in the airway (129). Park and his colleagues reported that *P. aeruginosa* can promote the secretion of heparan sulfate proteoglycan (HSPG) named syndecan-1 on the airway epithelial cell surface to resist the host innate immunity (176, 177). Syndecan-1 is the most abundant among four types of mammalian syndecans. It is a major source of heparan sulfate (HS) that can be released in a soluble form known as an ectodomain. This ecotodomain can compromise the function of many innate immunity molecules (e.g. neutrophil, elastase, cathepsin

G and chemokines) by binding and inactivating them. *P. aeruginosa* elastase encoded by *lasA* was proven to be an inducer of the shedding of the syndecan-1ectodomain (177). Another reason that CF lungs may be sensitive to colonization by *P. aeruginosa* is the lower level of defensin, a low molecular weight antimicrobial agent in the airway (76). Even though many theories were proposed about the innate immunity deficiency, none of them could explain why *P. aeruginosa* is dominant in CF lungs.

It is well known that chronically infected CF lung provide the environment for the diversity of *P. aeruginosa* morphology varients. *P. aeruginosa* is capable of changing to a mucoid phenotype, which results from the overproduction of polysaccharides called alginate and mucoid exopolysacchride (MEP). The mucoid phenotye is usually unstable outside the CF lung and can be converted to a non mucoid phenotype (16, 211). Alginate was shown to scavenge hypochlorite produced by phagocytic cells and to reduce chemotaxis of polymorphonuclear leukocytes (PMN) to the lungs. Therfore, mucoid cells are resistant to both PMNs and macrophages. In addition, *P. aeruginosa* is capable of producing type III secretion products, e.g. ExoS, that can inhibit phagocytic cell motility (129) and ExoU that is cytotoxic to eukaryotic cells (31, 49, 52, 252). For all of the reasons mentioned above, mucoid *P. aeruginosa* cells are difficult to eliminate. Besides the mucoid phenotype, another phenotype, dwarf cells or small colony variants (SCV), does exist. The SCV morphotype showed an increased capacity to form biofilms and to attach to host cells, both considered to be major virulence factors (75).

It has been suggested that increased antibiotic use promoted the emergence of *P. aeruginosa* prevalence due to the suppression of other bacteria by antibiotics (114). In the course of CF lung infection, *S. aureus* is usually the initial bacterial pathogen. Unfortunately, it has been suggested that continuous antistaphylococcal usage prevents pneumomonia but converts patients from *P. aeruginosa*-negative to *P. aeruginosa*-positive by an unknown biological mechanism (197). It is still questionable if there is indeed a progression from *S. aureus* to *P. aeruginosa* infections. Ratjen et al. (2001) investigated if continuous antistaphylococcal therapy increased the

risk of acquiring *P. aeruginosa*. This was a retrospective study performed by using data from the German Centers of the European Registry for Cystic Fibrosis (ERCF). Out of 639 CF patients, those receiving continuous broad spectrum antibiotics had a significantly higher rate of *P. aeruginosa* acquisition compared to patients receiving only intermittent or no antibiotic therapy. The data confirmed that prophylactic antistaphylococcal therapy promoted a higher rate or earlier onset of *P. aeruginosa* colonization (197). How such treatment provoked the clinical outcome remains to be determined. It was also suggested that early infection primes for later *P. aeruginosa* infection in the CF airway. This suggestion was supported by Burns et al. (2001) who studied the rate of *P. aeruginosa* infections in CF children. According to serological results, 97.5% of CF patients are infected with *P.aeruginosa* by age 3. This suggested that *P. aeruginosa* infection occurs earlier in CF than was previously reported, and may be intermittent or not detectable by culture because these early infections are due to nonmucoid strains that are potentially easier to treat. In addition, most of the inflammation in CF airways is the result of prior or concurrent *P. aeruginosa* infections (20).

1.3.2. Other *P. aeruginosa* infections

Besides lung infections in CF patients, *P. aeruginosa* can cause a variety of infections in most organ systems. Even though *P. aeruginosa* is an opportunistic pathogen, it can cause infections in previously healthy and immunologically competent people. The majority of such infections involve the skin, soft tissue, bone, joint, urinary tract and external ear cavities. Many outbreaks of *P. aeruginosa* infections in previously healthy individuals are associated with exposure to public swimming pools and hot tubs. An outbreak of *P. aeruginosa* hot-foot syndrome was recently reported in 40 children who were exposed to a wading pool coated with an abrasive grit (50). The syndrome is characterized by the extremely painful erythematous nodules on the soles. Cultures from pustules of the infected children and the pool revealed

identical *P. aeruginosa* strains. Mocan and Karaguzel (1997) reported cystitis associated with *P. aeruginosa* infection in healthy girls and women who were previously exposed to hot tubs or whirpools (153). Near-drowning in rivers, swimming pools or hot tubs has been shown to cause *P. aeruginosa* pneumonia in healthy adults (43). In HIV infected patients, *P. aeruginosa* bacteremia, pneumonia, sinusitis and urinary tract infections are very common. In a case control study, 58 of 73 HIV–infected patients were afflicted with complicating *P. aeruginosa* infections, of which 62% were bacteremic with the mortality rate being as high as 22% (41). In a retrospective study of 233 HIV infected patients who died between 1985 and 1996, *P. aeruginosa* was the most dominant isolate from patients with proven bacterial pneumonia (4).

1.3.2.1. *P. aeruginosa* infections in patients with wound and trauma

P. aeruginosa infections are very common in patients with wound and trauma. The organism was the second most common infective agent in patients with burn wounds over a period of 20 years as reported by the National Nosocomial Infections and Surveillance (NNIS) Networks (169, 209). *P. aeruginosa* found in burn wounds may pre-exist in the gastrointestinal tract. It can then cause infection by fecal contamination or from bacterial translocation from the gut to lymphatic and blood vessels (227). It was also shown that consumption of vegetables promoted the subsequent colonization, and therefore limitation of vegetables consumption was suggested in patients with burn wounds (109, 190). *P. aeruginosa* can cause infections in patients with traumatic wounds. However, there are four types of injuries that are nearly always followed by *P. aeruginosa* infection. These include nail-puncture injuries of the foot, ear piercing, traumatic injuries occurring in fresh water and burns (227). Post-traumatic bone and joint infections, e.g. osteomyelitis, osteochondritis and septic arthritis feet usually occur following a nail-punctured foot. *P. aeruginosa* perichondritis of cartilage of the ears may occur following ear-piercing and accupuncture (30). *P. aeruginosa* otitis externa is very common in patients with traumatic injuries

when exposed to fresh water. Interestingly, up to 74-94% of diabetic patients have malignant otitis externa but the pathogenesis of this disease in these patients is still unclear (227).

1.3.2.2. *P. aeruginosa* eye and ear infections

P. aeruginosa ocular infections including conjunctivitis, corneal ulcers, corneal abscesses and cellulites are usually associated with eye trauma, topical use of steroids, surgery, and hospitalization. The main cause for corneal ulcers and acute keratitis is the use of contact lenses. *P. aeruginosa* is the most frequently isolated bacterial species from contact-lense wearers with keratitis (127). Approximately 15 to 50% of people using contact lenses will develop corneal ulcers or acute keratitis at some point in their life, and more than 40% of these cases involves *P. aeruginosa*. Irive et al. (1994) also reported incidences of *P. aeruginosa* endophthalmitis, which is a diffuse infection of the vitreous body, retina and uveal layer, and can be a cause of permanent vision loss (89).

P. aeruginosa also causes ear infections including otitis externa, acute otitis media, chronic supparative otitis media, sinusitis and perichondritis. The most severe ear infection caused by *P. aeruginosa* is malignant external otitis in which the infection proceeds into the cartilage bone of the external auditory canal, mastoid and the base of cranium of the skull leading to osteomyelitis of the skull, and cranial neuropaties (198). These result in facial paralysis, meningitis and abscess formation. Interestingly, Sundstorm and his colleges (1996) showed that *P. aeruginosa* isolates from otitis externa produced less pyocyanin and urease in comparison to those isolated from other infections. This may be influenced by nutrition factors or properties of the particular strain isolated. However, further studies are needed to understand the pathogenesis of the disease and answer why *P. aeruginosa* is dominant in otitis externa (228).

1.3.2.3. *P. aeruginosa* bacteremia

P. aeruginosa bacteremia is a serious, possibly fatal condition, and usually a secondary infection following the primary infection site, e.g. gastrointestinal, urinary and respiratory tracts, skin and soft tissues (9). From a clinical analysis of 58 patients with AIDS, 62% of infections were bacteremias primarily associated with venous catheters (16), pneumonia (12), soft tissue (4) and urinary tract (4, 41). In another study, the respiratory and pancreatobiliary tracts were the most common sources of the bacteremia, and hypertension, higher age (more than 60 years), and the presence of an underlying malignancy were independently associated with significantly increased mortality, with mortality rates ranging from 38% to greater than 50% (26). From a retrospective study of cancer patients, *P. aeruginosa* bacteremia occured in about 4.7 per 1,000 patients. It was most common in patients with acute leukemia (55 per 1,000) (25). Mensa et al. (1996) reported that in 189 patients, *P. aeruginosa* bacteremia represented 6.9% of the total number of bacteremias, 6.9% of nosocomial bateremia and 23.6% of nosocomial gram-negative bacteremia (239).

1.3.2.4. *P. aeruginosa* endocarditis

P. aeruginosa endocarditis is usually predisposed by host conditions, e.g. a prosthetic heart valve, intracardiac tubing, leukemia, cancer, anticancer therapy and host behavioral practices. The major predisposing cause is intravenous drug use and up to 90% of *P. aeruginosa* endocarditis cases are associated with intravenous drug addicts (198). Left-sided endocarditis shows more serious symptoms including severe congestive heart failure, cardiac valve abscesses and septic metastasis, and needs aggressive antimicrobial therapy and surgery (91). In contrast, right-side endocarditis results in fever, septic pulmonary infarction and pleural effusion. *P. aeruginosa* endocarditis of the tricuspid valve is the mildest disease with a high probability of survival in comparison to infections of the mitral and aortic valves (91). *P. aeruginosa* infection

of mitral and aortic valves is always followed by bacteremia and embolism, and therefore valvulectomy, valve replacement and antibiotic treatment are immediately required (110).

1.3.2.5. *P. aeruginosa* infections of skin, soft tissues, bone and joints

The most notorious cutaneous infection caused by *P. aeruginosa* is folliculitis or dermatitis and ecthyma gangrenosum. *P. aeruginosa* folliculitis appears as prulitic, maculopapular, or vesiculopustular rashes on the extremities and the trunk. The infections are usually associated with exposure to public swimming pools, hot tubs, spas, whirpools or hydropatherapy pools. Fortunately, the disease is often self-limited without treatment. However, it may progress to severe lesions, especially in immunocompromised patients. *P. aeruginosa* ecthyema gangrenosum is the most remarkable appearance of *P. aeruginosa* sepsis. It occurs in about 1 to 6% of patients with *P. aeruginosa* bacteremia (198). *P. aeruginosa* ecthyema gangrenosum can happen on any part of the body but the most common areas are the buttocks, perineum, extremities and underarm. Eventhough the cutaneous lesions are small, serious necrosis and hemorrhage can develop.

P. aeruginosa infections of bone and joints are usually involved in acute and chronic osteomyelitis, acute arthritis and osteochondritis. The pathogen has a particular predilection for fibrocartilaginous tissue. Predispositions include intravenous drug abuse, as well as *P. aeruginosa* endocarditis and bacteremia. In general, *P. aeruginosa* complicates infections of small joints and bones of foot. *P. aeruginosa* osteochondritis was found in children and adults with puncture wounds on the foot and the bacterium was isolated from worn soles (92). Recently, Knouse et al. (2002) reported a *P. aeruginosa* infection in a patient who had a molar extraction. The patient had an evident infection of a root canal and *P. aeruginosa* infection was introduced at the time of surgery. The patient had persistent *P. aeruginosa* bacteremia, painful and swelling around soft tissue of his neck, and finally a rare right carotid artery aneurysm (103).

1.3.2.6. *P. aeruginosa* infections of the gastrointestinal (GI) tract

Colonization of the gastrointestinal tract by *P. aeruginosa* is very common in immunocompromised people, including patients with HIV, those having undergone surgery or those receiving broad-spectrum antibiotics (227). Although *P. aeruginosa* is not part of the normal flora of the intestine, it can survive in the GI tract and can be isolated from stool. It has been isolated as a unique pathogen or among other bacteria from perirectal and anorectal infections (198). *P. aeruginosa* infections of the GI tract can result in severe enteritis and necrotizing enterocolitis (NEC) caused by toxin production. The latter is fatal in newborns. From the GI tract, *P. aeruginosa* can spread to the blood causing bacteremia and septic shock, and to subcutaneous tissues and fascia resulting in severe necrotizing gangrene (198).

1.3.2.7. *P. aeruginosa* infections in patients with indwelling-medical devices and transplantation

P. aeruginosa biofilms are commonly present on indwelling medical devices, e.g. urinary catheters, vascular or peritoneal dialysis catheter and show very high antibiotic resistance (40). *P. aeruginosa* infections due to colonization of catheters are particularly common in patients in intensive care units and the bacterium is the most common gram-negative bacterium isolated from patients with continuous ambulatory peritoneal dialysis (227). Urinary and vascular catheters are responsible sources for infection in up to 15–20 % of *P. aeruginosa* bacteremia in hospitalized patients (239). Urinary tract infections (UTIs) caused by *P. aeruginosa* are generally associated with renal surgery and genitourinary instrumentation (169). *P. aeruginosa* may occur in patients with pacemakers, aortofemoral stents and grafts, and implanted blood pumps (19, 116, 208). Clinical manifestations of *P. aeruginosa* UTIs are indistinguishable from those of other UTIs. However, very common clinical signs of *P. aeruginosa* UTIs include ulcerative lesions

of the urinary tract mucosa with hematuria and sloughing necrotic tissues in the urine and renal infarcts (115). Recently, Yardy (2001) reported that 7 patients with urodynamic equipment to measure bladder pressure developed *P. aeruginosa* infections in the urinary tract and died due to the reuse of the device (253). Transplant patients may suffer from *P. aeruginosa* infections and the sites of infection depend on the type of transplant. For example, infections of the urinary tract, abdominal wounds and bile ducts are most common in patients with kidney and liver transplantations, respectively. In addition, pneumonia is the most common complication in patients with lung transplantation. These primary infections may be followed by secondary *P. aeruginosa* bacteremia (240).

1.4. MECHANISMS OF ANTIMICROBICAL RESISTANCE IN BACTERIA

The current problem of multidrug resistance in many pathogens, including *P. aeruginosa*, directly results from the inappropriate usage and over-prescribing of antimicrobials (1). As bacteria are efficient in adaptation and development of new resistance mechanisms to antimicrobial agents, antibiotic resistance will continue to be a clinical problem. Mechanisms of antimicrobial resistance in bacteria are fairly well understood and can be either intrinsic or acquired. The term "intrinsic resistance" refers to insensitivity originating from natural or inherent features of the cells without acquisition of any resistance factors (203, 204). Intrinsic resistance is usually encoded by genes on the chromosome and results from physiological properties of bacterial cells, for example, low permeability of the outer membrane and the constitutively-expressed MexAB-OprM efflux system of *P. aeruginosa*. In contrast, acquired resistance occurs in previously susceptible populations. Bacteria acquire resistance as a result of chromosomal mutations or acquisition of genetic elements (plasmids and transposons) (145, 203). In clinical settings, acquired resistance is usually associated with exposure to antibiotics during therapy. The basic mechanisms of antimicrobial resistance are classified into four categories

including drug inactivation, target modification, permeability alteration and active efflux (241). Bacteria can posses one or all of these mechanisms simultaneously.

1.4.1. Drug inactivation. By this mechanism, drugs are destroyed or inactivated, mostly by modification enzymes, so that they no longer posses antimicrobial activity. Enzymatic inactivation is the most common mechanism of resistance to aminoglycosides, β–lactams (especially penicillins and cephalosporins) and chloramphenicols in pathogenic bacteria (34). The best-known resistance mechanism in this category is β–lactam resistance, which is mediated by β–lactamases that inactivate the antibiotics by hydrolyzing the β–lactam ring of the molecules. β–lactamases are widely distributed in bacteria, and classified as penicillinases, cephalosporinases, metallo-β–lactamases and oxacillinases (232). *P. aeruginosa* contains a chromosomally encoded β–lactamase. Enzyme production is normally low, but can be induced to higher levels after exposure to β–lactams. Bacterial resistance to aminoglycosides is often due to chemical modification of the antibiotic by modifying enzymes, i.e. phosphotransferases, adenyltransferases and acetyltransferases, which compromises the binding of the antibiotics to the ribosomal target (241). Aminoglycoside-modifying enzymes have been found in both gram-positive and gram-negative bacteria such as *S. aureus*, *S. pyogenes* and *P. aeruginosa*. Resistance to chloramphenicol is typically mediated by the modifying enzyme chloramphenicol transacetylase, which acetylates the hydroxyl group on the chloramphenicol molecule. This acetylated chloramphenicol fails to bind its target (34). Other instances of drug resistance in this category include resistance to macrolides and tetracyclines.

1.4.2. Target modification. Modifications that change the antimicrobial activity of antibiotics include 1) target sites that become insensitive to antibiotics but still perform their normal physiological function, 2) overproduction of targets so that higher concentrations of drugs are

needed to inhibit bacterial growth, and 3) duplication of target sites such that the second site is not sensitive to antibiotics. Vancomycin resistance is catalysed by the product of the *vanA* genes that alters the peptidoglycan D-ala-D-ala side chain to abnormal termini (23). This altered D-ala-D-ala does not bind vancomycin but still allows normal peptidoglycan polymerization to occur. *S. pneumoniae* becomes resistant to sulphonamides via mutation of the enzyme dihydropteroate synthetase (DHPS). In certain organisms, resistance to sulphonamides can result from hyperproduction of *p*-aminobenzoic acid (PABA). β–lactam resistance due to alteration of penicillin-binding proteins (PBP) is also common. Methicillin resistance in *S. aureus* is usually due to the production of PBP2a that has a low affinity for the antibiotic and is the only functional PBP in the presence of β–lactams. Resistance to fluoroquinolones is mediated by mutations in *gyrA* and *parC* encoding for DNA gyrase and topoisomerase, respectively (51).

1.4.3. Permeability alteration. This mechanism impairs drug uptake resulting in decreased drug accumulation. Therefore, the intracellular drug concentration of drugs is not enough to hit the targets. Examples of antibiotics possessing this resistance mechanism include tetracycline and aminoglycosides, and many other drugs. In *E. coli*, tetracycline enters the cells by diffusion. Chromosomal mutations that reduce the expression of OpmF outer membrane protein, through which the antibiotic normally diffuses, confer low resistance to tetracycline (204). Foscomycin resistance is also mediated by impaired uptake. The antibiotic enters the cells by means of either a glycerol-3-phosphate (G-3-P) or glucose-6-phosphate (G-6-P) transport system. Therefore, chromosomal mutations in the G-6-P or G-3-P uptake systems will also inhibit foscomycin transport and cause resistance to the antibiotic (204). Another example is aminoglycoside resistance. Aminoglycosides may be modified by periplasmic-modification enzymes, i.e. phosphotransferases, adenyltransferases and acetyltransferases. The modified aminoglycosides usually fail to cross the cytoplasmic membrane (251).

1.4.4. Active efflux. Drugs can be actively pumped out of the cell resulting in a reduction of intracellular drug accumulation. Both drug-specific and multidrug efflux systems have emerged as a major problem in clinical settings, especially multidrug efflux pumps that confer multidrug resistance (119). Efflux pumps are now proven to play important roles in antibiotic resistance in both gram-positive and gram-negative bacteria, including *P. aeruginosa*, *E. coli*, *N. gonorrhoea*, *H. influenzae* and *B. cepacia*. The most common antibiotic resistance associated with efflux is resistance to tetracyclines mediated by TetA to TetE, TetG and Tet H in gram-negative bacteria, and TetK and TetL in gram-positive bacteria (51). In *P. aeruginosa*, the Mex systems act as transporters for a variety of antimicrobial agents including tetracycline, fluoroquinolones, macrolides, tetracycline, β-lactams, chloramphenicol and aminoglycosides (189). Mutations in repressors cause overpression of these *mex* operons. In *E. coli*, the AcrAB efflux system transports tetracycline, ciprofloxacin, β-lactams and novobiocin (172). In some clinical isolates of *S. aureus*, it was noted that the significant level of fluoroquinolone resistance is partly due to overexpression of the *norA* gene encoding an efflux transporter (96, 256).

1.5. UNIQUE PROPERITIES OF *P. aeruginosa* RELATED TO ANTIMICROBIAL RESISTANCE

1.5.1. Low permeability outer membrane. It has been known for quite a long time that *P. aeruginosa* possesses a low outer membrane permeability (168). (The outer membrane permeability of *P. aeruginosa* and other related bacteria in comparison to that of *E. coli* is shown in table 1.1. As *P. aeruginosa* lacks the classical high permeability porins that are present in most other gram-negative bacteria. In constrast, it contains low efficiency porins that allow the diffusion of small molecules at about one-hundredth of the rate obtained with the classical *E. coli*

porin channels. Therefore, hydrophilic molecules can diffuse across the outer membrane of this organism only slowly (67). The outer membrane permeability of *P. aeruginosa* is only 1-8% of that of *E. coli* (68) but it has a large exclusion limit (67). This large exclusion limit is due to OprF, which is the major porin in the outer membrane of the bacterium. Despite its high copy number, OprF represents an inefficient uptake route for antibiotics (71, 250). It was shown that

Table 1.1. Outer membrane permeability of *Pseudomonas aeruginosa* and other nonfermentative bacteria in comparison to *Escherichia coli* (modified from (70)).

Bacteria	Relative outer membrane permeability (%)
Escherichia coli	100
Pseudomonas aeruginosa	1-8
Bukholderia cepacia	11
Stenotrophomonas maltophilia	3-5
Acinetobacter baumanii	1-3

It was shown that only a small fraction of OprF up (as low as 400 molecules per cell) forms large channels, whereas the remaining OprF fraction forms small channels that are impermeable to antibiotic. It was proposed that such a small portion of large channels per cell is the main basis of the low permeability of *P. aeruginosa* (250). Since the intracellular concentration of drugs is the result of the balance between their influx and efflux rate, it is most likely that the slow influx of drugs due to the low outer membrane permeability helps efflux systems work more efficiently (167). In additon, the cell wall of this bacterium contains a high lipid content that acts as permeability barrier. *P. aeruginosa* lipopolysaccharide (LPS) contains 12-18 phosphates per molecules, compared to *B. cepacia* which has only 2 phosphates per LPS molecules and also a

high arabinosamine content (69). This is why *B. cepacia* does not show self-promoted uptake and is resistant to all polycationic antibiotics including aminoglycosides and polymyxin (70, 155).

1.5.2. *P. aeruginosa* biofilms. A biofilm is a cell population that grows on a surface and is enclosed in an exopolysaccharides matrix. *P. aeruginosa* is frequently found growing in biofilms with distinct mushroom and stalk-like structures (36). The growing biofilms prevent *P. aeruginosa* from being attacked by phagocytes, antibodies, complement and antibiotics. Biofilms are infamous for difficult eradication due to the high resistance to many anibiotics including tetracycline, erythromycin, quinolones, β-lactams, etc. (39). It was believed that biofilms have a much higher resistance than the planktonic cells do. Spoering and Lewis (2001) argued that this was not the case, at least not for *P. aeruginosa*. They suggested that in fact biofilms and the planktonic cells had similar sensitivity to killing by antibiotics, and tolerance to antibiotics in stationary-phase or biofilm cultures was largely dependent on the presence of persister cells. The majority of biofilm cells was shown to be killed within a clinically achievable range of antibiotic concentrations but there are the remaining small populations that are insensitve to future even increasing drug concentration. These remaining resistance cells are called the persisters (17). However, the true role that persister cells play in biofilms needs further investagation. Biofilms have been proposed to promote antibiotic resistance in *P. aeruginosa* by many mechanisms, i.e. reduced drug penetration, reduced growth rate or expression of possible biofilm–specific resistance genes. i) The exopolymer matrix enveloping biofilms acts as diffusion barrier against antibotics. Since this exopolysaccharide matrix contains negative charges, it efficiently protects cells from positively charged antibiotics, e.g. aminoglycosides. The slow diffusion decreases concentration of antibiotics in biofilms, and facilitates degradation of incoming antibiotics by host enzymes. However, many studies showed that some antibiotics can penetrate through biofilms completely, albeit with a reduced diffusion rate. ii) Cells in the deeper layers of biofilms are slow-growing or dormant cells. Since most antibiotics are more effective in killing actively

metabolizing cells, these non-growing cells are not sensitive to antibiotics and contribute to resistance of biofilms. This is similar to what happens to planktonic cells during stationary phase; these are also insentitive to antibiotics (121). iii) Since biofilms may not be more resistant to antibiotics than planktonic cells, it is possible that biofilm-specific resistance genes may be expressed (121). It is not understood why biofilms may need to express other resistance mechanisms than planktonic cells. Therfore, whether biofilms express specific genes to become highly resistant remains unclear.

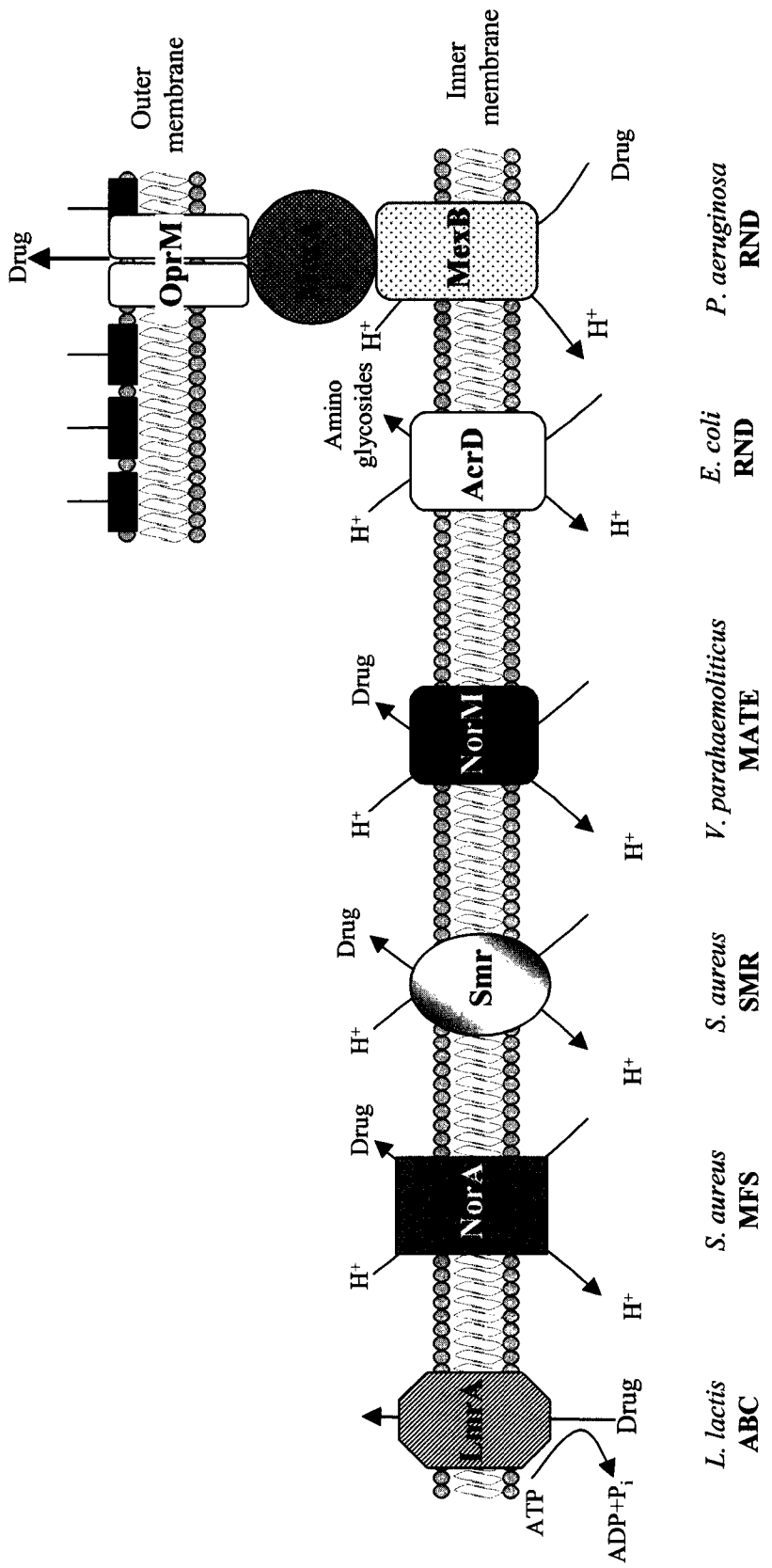
Multidrug efflux systems were believed to play an important role in the intrinsic resistance of *P. aeruginosa* biofilms since antibiotic resistance patterns of bioflims are similar to the substrate specificity of MexAB-OprM (35). Recently, De Kievit and colleages (35) proved that mulitdrug efflux systems including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY do not play a role in the antibiotic resistance of *P. aeruginosa* biofilms. As MexAB-OprM and MexCD-oprJ are not hyperexpressed during *P. aeruginosa* biofilm growth, expression of both efflux pumps in biofilm and planktonic cells are not different. Biofilms formed by a MexAB-OprM overexpressing mutant and a MexAB-OprM deletion mutant both exhibited high antibiotic resistance (35).

1.6. EFFLUX SYSTEMS

The low permeability of the outer membrane in *P. aeruginosa* alone cannot completely shut out the influx of small molecules and produce significant antimicrobial resistance. Therefore, other contributing mechanisms are needed for this purpose (167). In the past two decades, drug specific and multidrug efflux systems have emerged as major medical problems (132, 166). To date, we face the possibility that many, if not all, pathogenic bacteria will be soon be resistant to

all known antibiotics. Therefore, it is essential to understand the structure, function and origin of these multidrug systems. Although initially outer membrane impermeability that was thought to be the main reason for the intrinsic antimicrobial resistance of *P. aeruginosa*, it is now accepted that the main reason is synergy between a low-permeability outer membrane and active efflux from the cell. Since efflux systems catalyze active drug efflux, they require energy. Most bacterial multidrug efflux systems are secondary transporters using proton motive force (PMF) to mediate the extrusion of toxic compounds from the cells by a coupled exchange with protons (H^+) but some of them utilize ATP to drive extrusion (120). Bacterial drug efflux pumps have been classified into five families (192) including the ATP-Binding Cassette (ABC) Superfamily (237), the Major Facilitator Superfamily (MFS) (174), the Small Multidrug Resistance (SMR) family (29), the Resistance-Nodulation-Division (RND) family (236), and the Multidrug and Toxic Compound Extrusion (MATE) family. Well-characterized representatives of these families in gram-positive and gram-negative bacteria are shown in figure 1.1. The ABC and MFS superfamilies are large and ancient, while the SMR, RND, and MATE family are smaller and recently developing families. Of these five families, only the efflux pumps in the ABC superfamily are ATP dependent and the other four are secondary multidrug transporters driven by proton-motif-force (PMF). Secondary transporters are more common in gram-negative bacteria, for example, *E. coli* is predicted to have 17, 3, 6, and 3 of transporters belonging to the MFS, SMR, RND, and ABC families, respectively (15). Saier et al. (206) summarized the occurrence of known and putative multidrug efflux systems in six bacteria (i.e. *E. coli, H. influenzae, M. genitalium, B. subtilis, M. janneschii,* and *Synechocystis PCC6803*) of which extensive sequence data were available at the time of the study. They observed that the numbers of chromosomally encoded drug efflux pumps are approximately proportional to genome size and roughly proportional to the total number of transport systems identified in these organisms (206).

Figure 1.1. Schematic illustration of the main types of drug efflux pumps in bacteria. Illustrated from left to right are Lmr, a member of the ATP-binding cassette (ABC) family; NorA, a member of the major facilitator superfamily (MFS); Smr, a member of the small multidrug resistance (SMR) family; NorM, a member of the multidrug and toxic compound extrusion (MATE) family; and AcrD and MexAB-OprM, two members of the resistance-nodulation-division (RND) family. All system extrude drugs in an energy-dependent manner, using either ATP or proton motif force. The efflux systems in MFS, SMR, and MATE are structurally similar. They are designated as distinct families by phylogenetic relations or size.



In addition, both pathogenic and non-pathogenic bacteria carry a comparable number of chromosomally encoded multidrug efflux systems. These data proved that the multidrug efflux sytems did not arise recently in response to extensive drug use. Instead, they have long been encoded by the bacterial genome and may play a physiological role in the extrusion of toxic substances in various habitats (206).

1.6.1. ABC-type transporters. These are found in bacteria, archeae and eukaryotes. They generally consist of more than 1,000 amino acid residues and two highly hydrophobic domains that usually have six transmembrane segments (TMS) each, and two nucleotide binding domains (NBDs) that are energy-coupling domains localized to the cytoplasmic side of the plasma membrane. There are more than 300 sequenced bacterial ABC drug transporters and most of them extrude specific substrates. The first and best characterized bacterial ABC multidrug transporter is LmrA from *Lactococcus lactis* (14, 238). Other known bacterial ABC drug transporters include MsrA that effluxes erythromycin in *Staphylococcus* (201), DrrAB and TlrC that effluxe daunorobucin and tylosin, respectively, in *Streptomyces* sp. (100, 202). The best characterized eukaryotic ABC multidrug efflux pump is P-glycoprotein encoded by the Mdr1 gene in humans and rodents. P-glycoprotein also mediates resistance to antimalarial agents in *Plasmodium falciparum* and iodoquinol in *Entamoeba histolitica* (44), (173).

1.6.2. Major Facilitator Superfamily (MFS). This family consists of membrane transporters found in bacteria, archeae and eukaryotes, and are involved in the symport, antiport or uniport of diverse sustrates, e.g. sugars, oligosaccharides and antibiotics (136). They generally consist of more than 400 amino acid residues. There are up to 17 families of which 2 families encompass multidrug efflux transporters. These two drug resistance families include one cluster with 12 TMS and the other with 14 TMS. Sequence analyses revealed that the 14-TMS drug transporter arose from a primordial 12-TMS protein (174). The studies of Griffith et al. (62) and Marger and

Saier (136) showed that the 12- and 14 TMS families share significantly higher sequence similarity in their N-terminal halves than in their C-terminal halves. They proposed that the N terminal regions of MFS transporters are involved in the energy transport and that the C-terminal regions are involved in determination of substrate specificity (62, 136). The best characterized drug efflux protein within the MFS transporter family is TetB, a tetracycline transporter of *E. coli,* which is closely related to TetK and TetL. TetB is an inner-membrane protein possessing 12 TMS and it was shown to exchange a tetracycline-Mg^{++} complex with a proton (119).

1.6.3. Small Multidrug Resistance (SMR) family. Members of this family are much smaller than the MFS proteins and typically contain between 100 to 110 amino acid residues arranged into four α-helical TMS. They are the smallest secondary drug transporters known. They are proposed to function as homooligomeric complexes because of their very small size (178, 180). It was found that SMR efflux catalyzed the exchange of 2 or 3 H^+ per drug cation. Multidrug efflux pumps of the SMR family are found only in prokaryotes. The first gene encoding a protein of the SMR family, *qacC*, was identified on conjugative and non-conjugative plasmids in clinical *S. aureus* isolates. It was also known as *qacD* and *ebr*, but is now renamed as *smr*. QacC mediates resistance to diverse organic cations, e.g. quaternary ammonium compounds, dyes and ethidium bromide (18, 178).

1.6.4. Multidrug and Toxic Compound Extrusion (MATE) family. This is a novel family of bacterial multidrug transporters (18). Morita et al. (158) identified two putative proteins, NorM in *Vibrio parahaemolyticus* and its homoloque YdhE in *E. coli* that confer resistance to fluoroquinolones, dyes and aminoglycosides. NorM consists of 450 amino acid residues and has a molecular weight of 49,422 Da (157, 158). Since NorM contains 12 hydrophobic regions, it was first suggested to be a member of the MFS. From sequence analyses, however, NorM and YdhE do not share significant sequence similarities with any members of the MFS and thus are not

members of this family. Therefore, Brown and his colleague classified them into a new family of transporters named the MATE family (18). The functions and modes of regulation of the MATE protein family remain to be elucidated.

1.6.5. The Resistance Nodulation and Division (RND) family. The name of RND family was first recognized by Saier et al. in 1994 (205) when they found that the cobalt, zinc, cadmium (CzcA) and the cobalt, nickel (CnrA) resistance efflux systems of *Alcaligenes eutrophus* had characteristics different from the well-characterized ATPase translocator family (205). The CzcA and CnrA proteins are homologous to a protein (EnvD) that functions by an unknown mechanism in cell division and to a set of three proteins (NolGHI) that are involved in the nodulation of legumes by *Rhizobium meliloti*. They therefore referred to this novel family of protein as the resistance/nodulation/division family (205). Although transporter proteins of the RND family are ubiquitous, the RND multidrug efflux systems are unique to gram-negative bacteria. From phylogenetic analysis, the RND efflux systems are classified into 3 clusters including i) heavy metal efflux, e.g. Czr; ii) secretion of nodulation factors, e.g. NolFGH; and iii) multidrug efflux, e.g. Acr, Mex, and Mtr (206). Most RND-type drug efflux operons are chromosomally encoded.

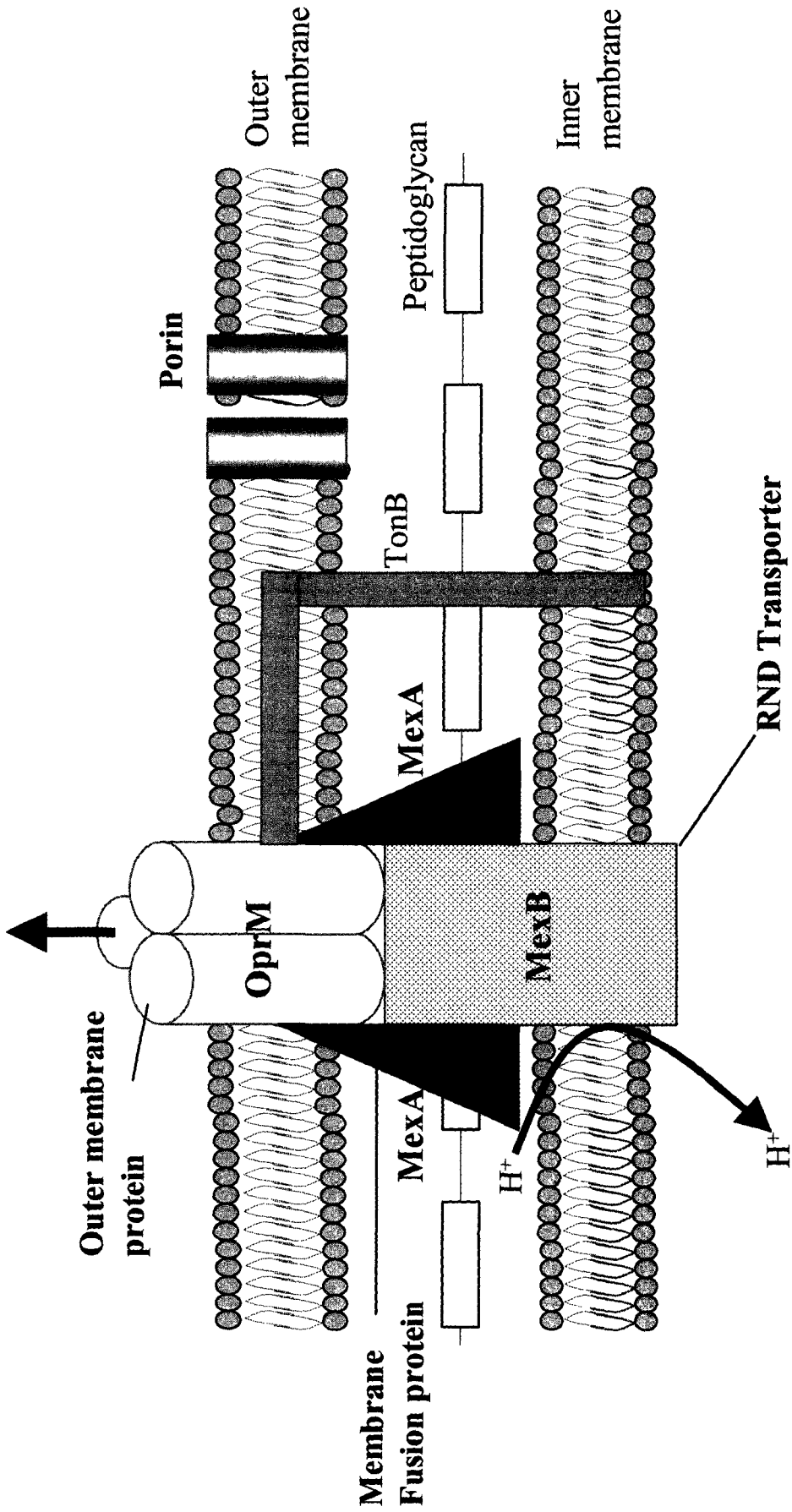
1.6.6. Components of RND efflux systems

The structure of RND-type drug efflux systems is most complex because they catalyze direct drug efflux across two membranes. It is now generally accepted that the RND multidrug efflux systems function as tripartite systems consisting of a cytoplasmic membrane-associated RND transporter (MexB, MexD, MexY, MexK and MexH), periplasmic membrane fusion protein (MFP) (MexA, MexC, MexE, MexX, MexJ and MexI of *P. aeruginosa*; and AcrA of *E. coli*) and an outer membrane protein (e.g. OprM, OprJ, OprN and OpmH of *P. aeruginosa*). A schematic

model of a RND transporter, represented by MexAB-OprM of *P. aeruginosa*, is presented in figure 1.2. For function, it requires a linker protein, MFP, although its exact function is yet unclear. However, variants of this tripartite system have been observed, e.g. the AcrD efflux system of *E. coli* that has only the cytoplasmic membrane-associated RND transporter and is involved in aminoglycoside efflux. The existence of this single-component transporter can be explained by the hydrophilic property of aminoglycosides because the main permeability barrier for such hydrophilic drugs is the cytoplasmic membrane (200).

1.6.6.1. RND transporters. The secondary structure of the RND transporters was proposed to consist of ~300-1000 amino acids, and 12 TMS with two large loops between TMS1 and TMS2 and between TMS7 and TMS8 (figure 1.3) (178, 206). These two big loops are extracytoplasmic hydrophilic domains and their exact function is unknown. Sequence analysis showed that the N-terminal and C-terminal halves of RND protein share sequence similarity implying that they may arise from an ancestral protein with 6 TMS (206). The membrane topology of MexB was analyzed by using alkaline phosphatase gene fusions and verified to have 12 TMS, of which the N-and C-termini are attached to the cytoplasmic inner membrane. MexB contains two large hydrophilic loops of 311 and 314 amino acid residues, respectively (64). These results are consistent to those from topological analyses of MexD conducted by using alkaline phosphatase and β-lactamase gene fusions (57). These studies confirmed that this topological model can probably be applied to other RND efflux transporters. The RND proteins contain 4 highly conserved motifs including motif A, B, C and D (178). Such conservation implies that they may be essential for their structure and function, even though the potential function of these motifs has not been elucidated. However, the recent topological analysis of MexB and MexF showed that the conserved charge residues located in TMS 2, 4 and 10 are essential for pump function, which is probably also true for other RND systems (5).

Figure 1.2. Schematic model of MexAB-OprM, a member of the resistance-nodulation-division (RND) family. MexAB-OprM consists of MexA, a membrane fusion protein; MexB an inner membrane RND transporter; and OprM, an outer membrane channel protein. Drug efflux is energized by the proton motif force such that the pump functions as drug:H^+ antiporter. It is believed that the energy transducer TonB also participates in the process.



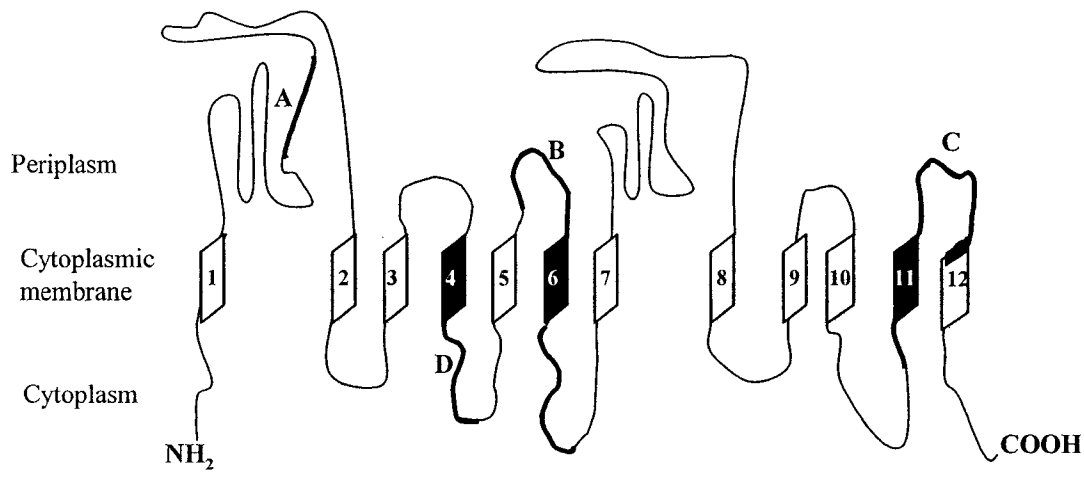


Figure 1.3. Topology model of multidrug transporters of the resistance-nodulation-division (RND) family. The inner membrane transporters of RND type efflux systems consist of 12 TMS and 2 large-periplasmic loops between TMS 1 and 2 and TMS 7 and 8. The conserved motif A, B, C, and D are highlighted in black and denoted as A, B, C, and D, respectively.

1.6.6.2. Membrane fusion proteins. The MFP proteins were first identified by Dinh et al. (38) and were found to be significantly homologous to the membrane fusion protein (F protein) of the paramyxo simian virus 5 (38). Therefore, the name MFP is derived from the virus MFP. Like virus F protein, the MFP proteins contain two strongly hydrophobic regions near the N and C terminal ends. Dinh and his colleagues (38) also suggested that these regions may serve as the interaction regions with the inner and outer membrane since they are present universally among the MFP proteins (38). These findings support the hypothesis that MFP link or "fuse" the inner and outer membranes of gram-negative bacteria. No homologs of MFPs have been reported in gram-positive bacteria and eukaryotes so far. Although the MFP proteins were shown to be anchored in the inner membrane by either a single N-terminal TMS spanning segment, e.g. HlyD (210), or by a lipid moiety, e.g. AcrA, AcrE (131) and MexA (188), their function remains

unclear. Studies of alignment and structure prediction by Johnson and Church (94) indicated that the MFP proteins including MexC and MexE, contain two copies of highly conserved motifs at the N- and C- terminal ends, and two regions of highly coiled-coil regions separated by a gap of 5 to 10 residues. This symmetry suggested that the MEP molecule could simply fold back on itself at the gap between the helical regions (94). From the prediction, two hypothetical models for formation and function of the MFP have been proposed (94). In the first model, the MFP proteins bring the inner and outer membrane into close opposition by the formation of a α-hairpin or intramolecular lipoyl domain. In the second model, they form a channel-like structure spanning the periplasmic space, connect both inner and outer membrane and enable substrate transport. Many, but not all, MFPs are lipoproteins. Ma et al. (131) reported that AcrA is a lipoprotein, which is anchored in the inner membrane by its N-terminal lipid moiety and most of the mature protein is exposed to the periplasm (131). Later, Zgurskaya and Nikaido (1999) showed that the lipid-modification at the N-terminus is not essential for AcrA function because the non-lipidated mature form of AcrA can function with similar efficiency (258). This finding is consistent with the fact that many of the MFPs lack a lipoprotein signal sequence (38). Similar experiments were conducted with MexA of the MexAB-OprM system of *P. aeruginosa* (254). Substitution of cysteine 24 with phenylalanine or tyrosine did not alter the function of MexA. Therefore, lipid modification is not required for MexA function, which is consistent with previous observations made with AcrA (254). Based on these findings, two other topological models were proposed for MexAB-OprM. In the first model, MexA is associated with two large periplasmic domains of MexB with an intimate interaction. This tight interaction of MexA and MexB facilitates the interaction with OprM. In the second model, MexA is located over the two hydrophobic loops of MexB and may form a hollow tube to OprM. These data also raised a doubt about the hypothesis that MFP connects the inner and outer membranes (254). Even though many models have been proposed, the structure and topology of efflux systems still needs further investigation. Up to date, it is known that in some ABC transporters, e.g. HlyBD-TolC, the formation of inner

membrane transporter-periplasmic MFP-outer membrane protein complex would be in the order: substrate binding to the inner membrane transporter, which then binds periplasmic membrane fusion, which then binds outer membrane protein (117). It was also shown that in some efflux systems, the inner membrane transporter-periplasmic MFP complex will interact with the outer membrane protein when the substrate is present, e.g. the HlyB-HlyD complex is preformed and binds TolC in the presence of substrates (230).

1.6.6.3. Outer membrane channel proteins. The outer membrane protein (OMP) encoding genes of the efflux systems in gram-negative bacteria are often found in the same operons as the genes encoding for the inner membrane transporters and the MFP, e.g. MexAB-OprM and MexCD-OprJ of *P. aeruginosa* (179). However, some outer membrane protein genes are not always present in the same gene clusters as the other efflux component genes, e.g. *opmH* of *P. aeruginosa* and *tolC* of *E. coli* (165). The OMPs are required for substrate export across the outer membrane. The most extensively studied OMP is TolC, a multifunctional protein that serves as the channel protein for more than one export system (259). TolC is believed to assemble as a trimeric protein, and to adopt a β–barrel structure similar to that of outer membrane porins (112). It was shown to form channels in lipid bilayers and to span the outer membrane as a β–barrel and the periplasm as a α–helical barrel (112). In constrast to the RND transporters and MFPs, which stably associate *in vivo*, OMPs do not appear to permanently associate with the other efflux components. This is supported by the function of OprM and TolC as components of many multidrug efflux systems. Therefore, OMPs cannot be impounded by any one system. It is possible that OMPs connect to the other components only when a substrate is actively pumped (189). In addition, an OMP can be substituted with other proteins of similar function (58). For example, OprM could replace OprJ (58) and OprN (138). MexCD (152) and MexXY (223) could functionally associated with TolC of *E. coli* (223). MexAB-OprM was shown to function *in E. coli* but TolC could not complement the function of OprM (233).

1.6.6.4. Recent developments. Among the RND efflux systems, the most extensively-studied is the AcrAB-TolC system of *E. coli*. Recently, AcrA and AcrB were demonstrated to stably associate *in vivo*, independently of substrates and TolC (260). The crystrallographic structures of AcrB (161) and TolC (112) have been reported. From the crystal structure, yet another model of RND efflux pump function was proposed (figure 1.4). AcrB is a trimer consisting of 12 TMS per monomer and has large priplasmic domains forming a funnel. The top of the funnel diecrtly contacts the periplasmic α-barrel portion of TolC, and a very large central cavity located between 3 TMS domains. The openings located between protomers and just outside the external surfaces of phospholipids were called vestibules connect the outside to the cavity (257). It is hypothesized that substrates from the outside lipid layer of the membrane bi-layer travel through the vestibule and bind to the wall of the central cavity. Although it is obvious that the first step of the transport process is binding of the substrates to the central cavity, it remain unclear how the substrates can reach the top of the central cavity (257). It seems possible that other RND pumps, including those of *P. aeruginosa*, catalyze the transport of substrates via similar mechanisms.

1.7. THE RESISTANCE NODULATION AND DEVISION (RND) MULTIDRUG EFFLUX SYSTEMS OF *P. aeruginosa*

Several RND multidrug efflux pumps have been characterized in *P. aeruginosa*. Since they have emerged as key mechanisms for antimicrobial resistance in this bacterium, they are of clinical significance. Genome analysis suggests the presence of genes for 12 potential RND efflux systems in the *P. aeruginosa* PAO1 wild type genome (figure 1.5) (226). To date, seven RND multidrug systems have been characterized. These include MexAB-OprM (185), MexCD-OprJ (186), MexEF-OprN (106), MexXY (243), MexJK (28), MexGHI-OpmD (3) and MexVW (125). Half of the RND efflux systems share similar genetic and structural organizations (figure 2),

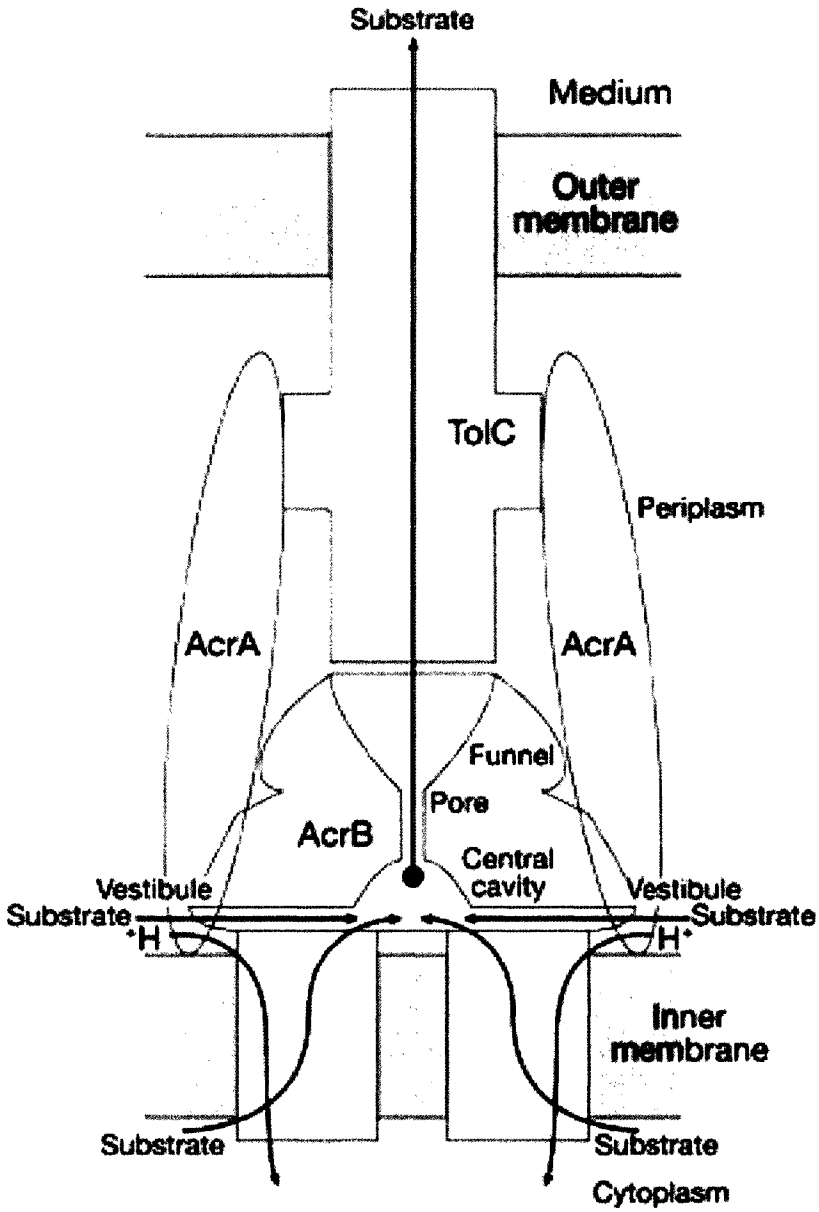
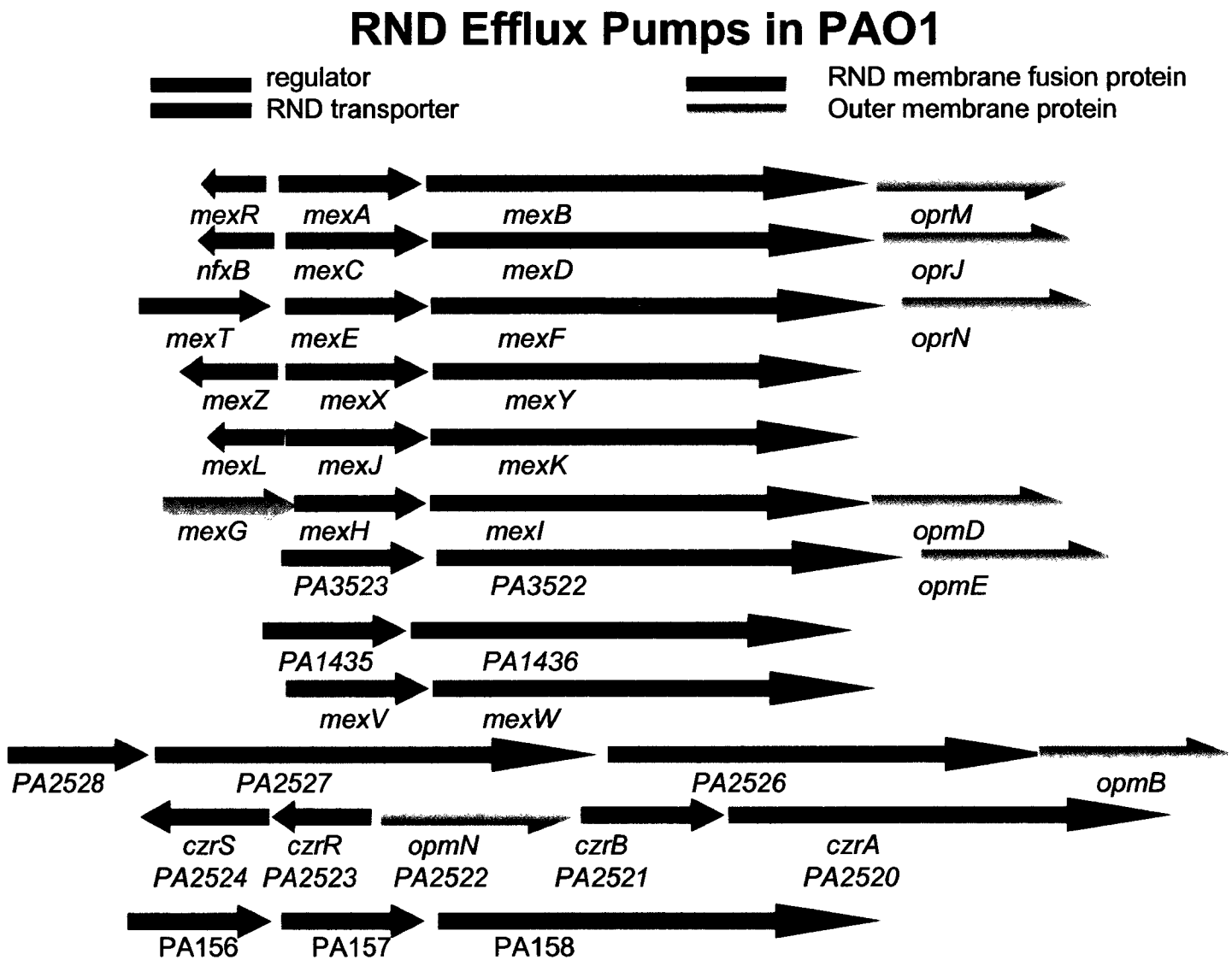


Figure 1.4. Structure-based model of the *E. coli* AcrAB-TolC multidrug efflux system. According to this model, AcrB and TolC interact directly. When the substrate is transported, AcrB might recruit TolC to form a direct exit pore from cytoplasm to the extracelluar medium. (modified from (161).

consisting of a cytoplasmic membrane–associated RND transporter (MexB, MexD, MexY, MexK and MexH), a periplasmic MFP (MexA, MexC, MexE, MexX, MexJ and MexI), and an outer membrane protein (e.g. OprM, OprJ, OprN and OpmH). The regulatory genes are frequently located upstream of the structural genes. In constrast to the genes coding for transporter and MFP proteins, which are always present in the operons, the genes encoding for regulatory and outer membrane are not always present. For example, MexXY and MexJK lack their own OMP-encoding genes (28, 243). The RND multidrug transporters show much broader drug specificity than the MFS and SMR multidrug transporters (178). They are capable of efflux of substrates that are different in classes, structure and size. Known substrates for these efflux pumps are listed in table 3. Some substrates can be pumped out by multiple RND efflux systems.

1.7.1. MexAB-OprM. MexAB-OprM is the only RND efflux pump that is constitutively expressed at low but detectable lavels, and thus contributes to the intrinsic drug resistance of PAO1. This system is overproduced in *nalB* mutants carrying mutations in *mexR*, its cognate repressor (222). In addition, overexpression of MexAB-OprM occurs without mutations in *mexR*, or the promoter regions of *mexR* and *mexA*. These mutants are called *nalC* (222). MexAB-OprM was first identified as the ORFABC operon and thought to export the siderophore pyoverdine (187). ORFA and ORFB showed significant homology to EnvC and EnvD of *E. coli* (187). It was thought to be MexAB-OprK until Gotoh et al. (59) proved that the outer membrane protein of the *mexAB* operon is OprM. OprK and OprM are distinct proteins with high homology and OprK is not encoded by the *mex* operon. Therefore, *mexAB-oprK* was renamed *mexAB-oprM* (59, 66). MexAB-OprM has the broadest substrate spectrum of all known efflux pumps. Its substrates include β-lactams, β-lactamase inhibitors, tetracycline, chloramphenicol, trimethoprim, fluoroquinolones, macrolides, rifampin, sulfamethoxazole and fusidic acid (122, 163, 188). Of the

Figure 1.5. Proposed efflux systems of the resistance-nodulation-division (RND) family encoded by the *Pseudomonas aeruginosa* genome. Genes encoding for the characterized and uncharacterized efflux systems are denoted by gene name and PA numbers as in the annotated *P. aeruginosa* genome, respectively. The RND-transporters are indicated as red arrows. Genes encoding for membrane-fusion protein, outer membrane channel proteins, and regulatory proteins are indicated as dark blue, yellow, and green arrows, respectively. An additional gene encoding for a membrane protein required for function of MexGHI-OpmD is depicted as a light blue arrow. This diagram is modified from that showing potential RND efflux systems of *P. aeruginosa* as originally proposed by R.E.W. Hancock from the University of British Columbia (http://cmdr.ubc.ca)



β-lactams, only carbapenems are poor substrates of MexAB-OprM (108, 139, 144). Besides antibiotics, MexAB-OprM pumps out other substances such as biocides (triclosan), detergents (SDS), dyes (acriflavine) and fatty acid biosynthesis inhibitors (thiolactomycin) (213). The export of β-lactams by MexAB-OprM is interesting because β-lactam resistance mediated by RND efflux system is not common. In contrast to other substrates that target cytoplasmic components, β-lactams have targets in the periplasm. OprM was first reported to be associated with multidrug resistance in *P. aeruginosa* by Masuda and Ohya (141). The *oprM* gene is highly conserved among clinical and environmental isolates of *P. aeruginosa* and broadly distributed among Pseudomonad and related bacteria, e.g. *P. syringae*, *B. pseudomallei*, *P. putida*, etc. (108). It contains a highly conserved amino acid sequence LGGGGW at the extreme C terminus that was confirmed to be important for its expression and activity (123). It is also a TolC homologue showing 20% similarity. Like TolC, OprM also contained a β–barrel of 12 TMS domains and adopts a similar conformation. This structural conformation is in contrast to a monomeric barrel consisting of 16 β-strands recently proposed by Wong et al. (247). However, studies of the secondary structures of OprM and TolC confirmed their α-helical nature and the placement of β–barrel regions (123, 249). The conformation of OprM is similar to the TolC-like conformation (123). MexAB-OprM probably uses TonB, a cytoplasmic membrane energy coupling protein, to facilitate the channel opening of OprM (263). OprM cannot serve as efflux pump independently of MexAB-OprM and cannot functionally replace TolC (248).

1.7.2. MexCD-OprJ. MexCD-OprJ is overexpressed in *nfxB* strains carrying mutations in the *nfxB* gene, which is a negative regulatory gene located upstream of the operon (171). The NfxB protein also negatively autoregulates its own expression (218). MexCD-OprJ is tightly regulated, thus it is not expressed in *P. aeruginosa* wild-type under laboratory conditions (86, 224). MexCD-OprJ was originally identified as a determinant of fluoroquinolone resistance (171). The

nfxB mutants were classified into two types according to the degrees of changes in their susceptibilities. These include type A strains expressing moderate changes and type B strains expressing large changes. Type A mutants are more resistant to ofloxacin and erythromycin. In contrast, type B mutants are more resistant to tetracycline and chloramphenicol (140). Both types showed resistance to new cephems, e.g. cefpirome, cefeprime, cefclidin and cefozopram. This efflux system is also responsible for the extrusion of novobiocin, chloramphenicol, tetracycline, fluoroquinolones, macrolides, some β-lactams (140) and sulphonamides (105). The β-lactams that are the substrates of MexCD-OprJ are limited to 4th generation cephems, e.g. cefpirome and cefepime (186) and the ordinary cephems e.g. cefoperazone and ceftazidime (60). MexCD-OprJ does not export carbenicillin and aztreonam (224), and aminoglycosides (186). MexCD-OprJ expression was recently shown to be inducible by tetraphenylphosphonium chloride, ethidium bromide, rhodamine 6G and acriflavin (159).

1.7.3. MexEF-OprM. MexEF-OprN is positively regulated by *nfxC* (or *mexT*) and overexpressed in *nfxC*-type mutants (106, 143). Like MexCD-OprJ, MexEF-OprM is not expressed in wild type (106). The nature of the mutations leading to the overexpression of MexEF-OprN has not been elucidated but they relate to MexT expression (104, 170). The *nfxC* mutants show cross-resistance to trimethoprim, chloramphenicol, tetracycline, fluoroquinolones and imipenem but hypersusceptibility to carbenicillin, cephems and aminoglycosides (53, 106, 138). MexEF-OprN is the only known efflux system that is involved in imipenem resistance (170). However, the role of MexEF-OprN as an imipenem transporter remains unclear because of a concomitant decrease in the OprD outer membrane protein (170). OprD is a channel-forming porin that facilitates the penetration of carbapenem antibiotics, e.g. imipenem and basic amino acids across the outer membrane protein of *P. aeruginosa* (194, 235). Loss of OprD reduces imipenem influx and promotes resistance to the antibiotic (54, 87, 108). Interestingly, expression of OprD declines when OprN is overproduced in the *nfxC* mutant (143). Introduction of MexT

into PAO1 wild type reduced the transcription of OprD and promoted the expression of MexEF-OprN. Therefore, MexT was proposed to function as a repressor and an activator (170). It was proposed that MexEF-OprN effluxes *Pseudomonas* quinolone signal (PQS), since MexEF-OprN mutants show reduced production of some virulence factors (107). There was a recent report that clinical isolates of *P. aeruginosa* expressed MexAB-OprM and MexEF-OprN simultaneously (191).

1.7.4. MexXY. MexXY, also called *amrAB* or *mexGH*, is not expressed in wild type cells (243). The MexXY operon is negatively regulated by *amrR* (also called *mexZ*). As it lacks its own outer membrane protein encoding genes, it apparaently uses OprM as an outer membrane protein to function as a tripartite efflux system (135, 142, 152). When expressed in *E. coli*, MexXY uses TolC and, in this case, OprM was shown to be functionally interchangeable with TolC (152). Although MexXY-OprM exports a wide range of antibiotics, including macrolides, quinolones and chloramphenicol, it has the distinctive ability to efflux aminoglycosides (135). It also confers higher resistance to fluoroquinolones that other Mex systems do (152). Expression of MexXY is inducible by the presence of specific substrates, i.e. tetracycline, gentamicin and erythromycin (142, 144) and induction is dependent on the drug concentration (84). It was also found that veterinary *P. aeruginosa* isolates also express both MexAB-OprM and MexXY concurrently (12).

1.7.5. MexJK. MexJK is not expressed under laboratory growth conditions. It displays the most restricted substrate profile of any of the hithereto characterized *P. aeruginosa* efflux systems, effluxing only triclosan, erythromycin and tetracycline. It co-functions with more than one OMP. Even though MexJK uses OprM to efflux antibiotic erythromycin, it uses OpmH to efflux triclosan. It is still a puzzle how this multidug efflux pump chooses one OMP over the other for drug efflux (28).

1.7.6. MexGHI-OpmD. MexGHI-OpmD expression was shown to be quorum-sensing dependent (3). The expression of the pump contributes the reduced production of quorum sensing regulated molecules, e.g. elastase, rhamnolipid, pyocyanin and pyoverdine. MexGHI-OpmD confers resistance to vanadium but not to other heavy metals and antibiotics. So far, a possible role of MexGHI-OpmD as a multidrug efflux pump is unclear (3).

1.7.7. MexVW. MexVW is the most recently characterized RND efflux system in *P. aeruginosa*. The *mexVW* operon contains neither an upstream regulatory gene nor an OMP gene downstream of the operon. MexVW is normally silent in wild type and its regulation of expression is unknown. MexVW cooperates with OprM to function as a tripartite efflux system. Overexpression of MexVW elevates resistance to norfloxacin, ofloxacin, chloramphenicol, tetracycline, ethidium bromide and acriflavin, but not to gentamicin (125).

1.7.8. Other RND systems. Besides the above memtioned RND efflux systems, five additional putative efflux pumps have been revealed by the *P. aeruginosa* genome sequence. The PA3523-PA3522-PA3521 operon has no upstream transcriptional regulatory gene and PA3521 is OpmE, which is an OMP in the OprM family. The PAO1435-PA1436 operon has neither an upstream transcriptional regulatory gene nor an associated OMP. The PA2528-PA2527-PA2526-*opmB* operon contains an additional RND transporter-encoding gene, PA2526. The last two RND efflux systems including PA2522-PA2521-PA2520 (*czcCBA*) and PA156-PA157-PA158, are involved in the efflux of divalent metal cations, i.e., Cd, Zn and Co (73).

1.8. SUBSTRATE SPECIFICITY OF THE RND MULTIDRUG EFFLUX SYSTEMS OF *P. aeruginosa*

The RND multidrug systems of *P. aeruginosa* are capable of effluxing a variety of substrates. Known substrates of the RND multidrug efflux systems in *P. aeruginosa* and related bacteria are summarized in table 1.2. Even though many experiments about RND efflux systems have been conducted and many models have been proposed, it remains unclear how these efflux pumps can posses such broad substrate specificities. We face the fact that one model cannot explain the mode of recognition for all substrates. A number of studies showed that the inner membrane transporters play important roles in the substrate recognition. So far, it is not clear if only the inner membrane transporters are responsible for substrate recognition or the MFPs or OMPs also participate in this process (189). A genetic analysis of MexAB-OprM and MexCD-OprJ done by Yoneyama and colleages (255) showed that the inner membrane components of the RND systems are not interchangeable and that the periplasmic MFPs are specific for each RND transporter (255). In contrast, another study demonstrated that RND transporters were interchangeable (162). Analysis of a chimeric AcrB/MexB protein expressed in a single operon with MexA and AcrA showed that the specificity determinants for multidrug recognition and efflux are located in the T60-V612 region of the large periplasmic loop of the RND transporter (233). This is consistent with a study performed in *E. coli* by Elkins and Nikaido (42). Replacement of the two large periplasmic loops of AcrD by those of AcrB conferred resistance to AcrB substrates at similar level to native AcrB. In another experiment, the two large periplasmic loops of AcrB were replaced by those of AcrD. The latter chimera showed resistance to only the typical AcrD substrates, namely aminoglycosides (42). Murata et al. (162) also showed that MexB and MexD are responsible for substrate recognition of MexAB-OprM and MexCD-OprJ, respectively (162). The chimeric MexAD-OprM and MexCB-OprJ system conferred resistance to the typical substrates

Table 1.2. Substrate profile of RND multidrug efflux pumps in *P. aeruginosa* and related bacteria.

Organisms	Efflux components	Substrates
Pseudomonas aeruginosa	MexAB-OprM	β-lactams, β-lactamase inhibitors, chloramphenicol, fluoroquinolones, macrolides, novobiocin, tetracycline, triclosan, trimethoprim, ethidium bromide, SDS, aromatic hydrocarbons, thiolactomycin, cerulenin, acylated homoserine lactones
	MexCD-OprJ	β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, tetracycline, triclosan, trimethoprim, ethidium bromide, SDS, aromatic hydrocarbons, crystal violet, acriflavine
	MexEF-OprN	Chloramphenicol, fluoroquinolones, triclosan, trimethoprim, aromatic hydrocarbons, *Pseudomonas* quinolone signal
	MexXY-OprM	Fluoroquinolones, aminoglycosides, tetracycline, erythromycin
	MexJK-OprM	Erythromycin, tetracycline
	MexJK-OpmH	Triclosan
	MexGHI-OpmD	Vanadium, acylated homoserine lactone precursors
	MexVW-OprM	Chloramphenicol, fluoroquinolones, erythromycin, trimethoprim, ethidium bromide, acriflavine

Table 1.2. Substrate profile of RND multidrug efflux pumps in *P. aeruginosa* and related bacteria (cont.).

Organisms	Efflux components	Substrates
Burkholderia cepacia	CeoAB-OpcM	Chloramphenicol, ciprofloxacin, trimethoprim
Burkholderia pseudomallei	AmrAB-OprA	Aminoglycosides, macrolides
Stenotrophomonas maltophilia	SmeABC	β-lactams, aminoglycosides, fluoroquinolones
	SmeDEF	Erythromycin, tetracyclines, fluoroquinolones, ethidium bromide
Pseudomonas putida	MepABC	β-lactams, tetracycline, novobiocin, erythromycin, aromatic hydrocarbons
	SrpABC	aromatic hydrocarbons
	TtgABC	Chloramphenicol, ampicillin, tetracycline, toluene
	TtgDEF	tetracycline, erythromycin, fluoroquinolones, ethidium bromide

Compiled from references (3, 126, 186, 214).

of MexCD-OprJ and MexAB-OprM, respectively (162). Recently, spontaneous mutants of *P. aeruginosa* containing mutations in the large periplasmic loops of MexD were isolated. These spontaneous mutants alter the substrate specificity of the MexCD-OprJ pump to include the normally non-transported β-lactam carbenicillin. This confirmed a role of the large periplasmic loops in substrate recognition (134).

1.9. REGULATION OF EXPRESSION OF THE RND MULTIDRUG EFFLUX SYSTEMS IN *P. aeruginosa*

Even though RND efflux systems in *P. aeruginosa* share similar genetic organizations and molecular architecture, they differ in their regulation of expression, of which the mechanisms are not yet well understood. The known and suggested regulatory components of RND efflux operon expression in *P. aeruginosa* are summarized in table 1.3. Expression of the best characterized RND efflux systems is due to mutations in their regulatory genes selected by exposure to antimicrobials *in vivo* and *in vitro*. For example, overexpression of MexAB-OprM, MexCD-OprJ, MexXY, and MexJK is due to mutations in *mexR* (46, 207), *nfxB* (86, 143) (218), *mexZ* (243) and *mexL* (28), respectively. These mutations are stable because constitutive expression of these efflux pumps persists after removal of selective pressure. Only expression of MexXY has been shown to be inducible in the presence of certain substrates (84, 144). Expression of MexGHI-OpmD is cell density-dependent (3). The most extensively studied *mex* regulatory protein in *P. aeruginosa* is MexR, a negative regulator of MexAB-OprM expression (2, 46, 126, 207, 222). Although MexAB-OprM is always transcribed at low but detectable levels, it is overexpressed in *mexR* mutants. MexR is a member of the MarR family in *E. coli*. MexR was purified and shown to be a DNA-binding protein that binds to two binding sites within the *mexR-mexA* intergenic region (46). Besides MexR, NfxB was also purified and shown to bind upstream of *mexC* (218). At least one of the RND efflux systems, MexEF-OprN has a positive

Table 1.3. Regulatory components of the RND–type efflux systems in *Pseudomonas aeruginosa*

Efflux pumps	Regulators	Family	Function	Inducers
MexAB-OprM	MexR	MarR	Repressor	Unknown
MexCD-OprJ	NfxB	LacI/GalR	Repressor	Unknown
MexEF-OprN	MexT	LysR	Activator	Unknown
MexXY	MexZ	TetR	Repressor	Pump sunstrates
MexJK	MexL	TetR	Repressor	Unknown
MexGHI-OpmD	LasR or RhlR?	Unknown	Unknown	Cell density
MexVW	Unknown	Unknown	Unknown	Unknown
CzrAB-OpmN	CzrSR	Two-component system	Sensor/kinase	Unknown
PA156-PA157-PA158	Unknown	Unknown	Unknown	Unknown
PA1435-PA1436	Unknown	Unknown	Unknown	Unknown
PA2528-PA527-PA2526-OpmB	Unknown	Unknown	Unknown	Unknown
PA3525-PA3522-OpmE	Unknown	Unknown	Unknown	Unknown

Compiled from references (63, 73, 192, 212).

regulatory gene, i.e. *mexT*. MexT belongs to of the LysR family of transcriptional activators (104). Interestingly, with the exception of the *mexT*$^{+}$ Geneva type strain, most PAO1 wild-type strains and their derivatives contain *mexT* mutations prohibiting the expression of MexEF-OprN in these strains (104). It was proposed that there are 3 types of *mexT*-mediated regulation of MexEF-OprN in *P. aeruginosa*. Type-I: *mexT* of wild type encodes inactive MexT, and *nfxC*-type mutants derived form this parent have an additional mutation in *mexT* converting MexT from the inactive to the active form. Type-II: *mexT* in wild type have an 8-bp insertion producing inactive MexT and *nfxC*-type mutants that are from this parent have a deletion of the 8-bp insert converting MexT from the inactive to the active form. Type-III: Both wild type and their *nfxC* mutant derivatives have identical and active MexT proteins. *nfxC*-type mutants have an additional mutation in another gene named *mexS* that negatively regulates expression of MexT. However, *mexS* remains unidentified (137).

Beside theses known regulatory genes, the presence of other negative regulatory factors was proposed because many clinical isolates constitutively express efflux pumps in the absence of identifiable mutations in the regulatory genes or regulatory regions. It was reported that the expression of MexAB-OprM in clinical veterinary *P. aeruginosa* isolates is not due to mutations in *mexR* (12). In *nalC* mutants, overexpression of MexAB-OprM is independent from mutations in *mexR*. This suggests that there is an unidentified regulator of the *mexAB-oprM* operon (222). In our previous study, we found the overexpression of MexXY in clinical isolates of *P.aeruginosa*. This mutant has two base pair changes in *mexZ* but they are silent mutations without an animo acid change. Thus, there may be some other mechanism that is involved in regulating the expression of MexXY (12). This finding exemplified the need to conduct research on efflux pumps *in vivo*, since results from *in vitro* experiments do not necessarily correlate with what happens during infection.

The role of a global regulator has been shown in *E. coli* efflux pump expression. MarR is a negative regulator of the *marRAB* operon and MarA is a global activator of the expression of

multiple genes including *ompF* and the *acrAB* genes (216). The function of MarB is not yet known. AcrR acts as a secondary regulator to prevent excessive expression of AcrAB (130). There has not been a global regulator reported in *P. aeruginosa.* However, PA4878 is a putative transcriptional regulator sharing 50% identity with TipA and MtaA, two global transcriptional regulators of efflux pump expression in *S. lividans* and *B. subtilis*, respectively (10, 85). It may have a similar function in *P. aeruginosa*.

1.10. PHYSIOLOGICAL ROLES OF THE RND EFFLUX SYSTEMS IN *P. aeruginosa*

Even though the RND efflux systems in *P. aeruginosa* have been intensively studied and are well known for their clinical importance, their physiological roles remain largely unclear. Neyfakh (164) proposed two hypotheses for the natural function of bacterial multidrug transporters. First, multidrug transporters have evolved to transport specific physiological compounds and drugs are incidental substrates. Those drugs are recognized because they structurally mimic the natural substrates of transporters. Second, mulitidrug transporters have evolved to protect the cells from various environmental toxins (164). Even there are some experimental arguments supporting each hypothesis, it is still hard to judge which hypothesis will ultimately prove correct. Similar numbers of chromosomally–encoded multidrug efflux systems are found in pathogens and non-pathogens (206). This suggests that these transporters have not arisen recently in pathogens in response to antimicrobial exposure but already existed for some other physiological purpose. Current knowledge suggests that efflux pumps are part of the natural defense mechanisms against toxic compounds in various habitats. For example, the AcrAB system effluxes bile salts in the gastrointestinal tract, therefore it is essential for survival of *E. coli* in this environment (231). Besides their roles as defense mechanisms against toxic environmental agents, most of the efflux systems are thought to efflux uncharacterized cellular products (108). Up to date, physiological roles of only two *P. aeruginosa* RND efflux systems, MexGHI-OpmD

and CzrAB-OpmN, which are metal efflux systems, are known (3, 73). *P. aeruginosa nalB* mutants overexpressing MexAB-OprM were shown to produce reduced levels of the *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (PAI-1), as well as reduced levels of many virulence factors that are regulated by quorum sensing, e.g. pyocyanin, casein protease and elastase. The proposed explanation is that MexAB-OprM is involved in direct export of PAI-1 (47). Similarly, in *nfxC* nutants overexpression of MexEF-OprN reduces the production of virulence factors controlled mainly by the *las* and *rhl* signaling systems, e.g. elastase, pyocyanin and rhamnolipids (107). In addition, MexGHI-OpmD may be able to efflux acylated homoserine lactones or precursor metabolites. Therefore, these data support the notion that at least some RND efflux systems may play an important role in quorum-sensing signal homeostasis.

1.11. RND EFFLUX SYSTEMS IN OTHER RELATED BACTERIA

Besides *P. aeruginosa*, other related bacteria, e.g. *B. cepacia*, *B. pseudomallei* and *S. maltophilia*, have also emerged as clinically significant pathogens because of their innate resistance to multiple antibiotics and their ability to develop high resistance. RND efflux systems are also found in other bacteria that exhibit similar cell wall properties to that of *P. aeruginosa* (185). See table 1.2 for an overview of sustrates effluxed by some of the pumps found in each bacterium.

B. cepacia was originally identified as a plant pathogen. Its clinical importance as a human pathogen has been increasing, especially in CF patients (61). Like other gram-negative bacteria, this pathogen is intrinsically resistant to multiple antibiotics and the most likely cause for such multidrug resistance is outer membrane impermeability and efflux (193). The *B. cepacia* chromosome encodes the *ceoAB-opcM* efflux operon, which is homologous to *mexAB-oprM* of *P. aeruginosa*, and confers resistance to chloramphenicol, trimethoprim and fluoroquinolones (21)). However, the role of the CeoAB-OpcM efflux system in intrinsic resistance is still unclear (185).

So far, a regulatory gene of this efflux system has not been identified. Since expression of CeoAB-OpcM is inducible by the presence of salicylate, it is suggested that its regulator may be similar to MarR (185).

B. pseudomallei is of clinical significance as an causative agent of meliodosis (32). Meliodosis is difficult to treat because *B. pseudomallei* is intrinsically resistant to many antimcrobial agents, including β-lactams, macrolides, polymyxin and aminoglycosides (219). The AmrAB-OprA efflux system, an RND-type system, has been recently characterized in the pathogen (154). This efflux system confers resistance to aminoglycosides and macrolides. Expression of AmrAB-OprA is regulated by AmrR, which is encoded by the upstream *amrR* gene (154).

S. maltophilia has emeraged as an increasingly important nosocomial pathogen in debilitated and immunosuppressed people (193). This pathogen is intrinsically resistant to multiple antimicrobial agents, e.g. aminoglycosides, ciprofloxacin, β-lactams and trimethoprim (262). Like *P. aeruginosa,* such intrinsic resistance is probably due to the combination of reduced permeability and expression of efflux pumps (261). The porins of *S. maltophilia* are proposed to be similar in size to those of *E. coli* but present in much lower copy number causing the overall low outer membrane permeability of the bacterium (193). Two RND type efflux systems, SmeABC and SmeDEF, have been identified in *S. maltophilia* (6, 124). SmeDEF is the first efflux system identified in *S. maltophilia* (6) and was shown to be expressed in clinical isolates (7). It may contribute to multidrug resistance (tetracycline, chloramphenicol, erythromycin and fluoroquinolones) in clinical isolates (7). SmeABC expressing strains display resistance to fluoroquinolones, β-lactam and fluoroquinolones (124). Expression of SmeABC is regulated by the SmeRS two-component regulatory system encoded by the upstream *smeRS* genes (124).

P. putida is generally not a human pathogenic bacterium. It is well known for its resistance to toluene which is mediated by active efflux systems that may or may not transport

drugs (90). The SrpABC system of *P. putida* S12 is a member of the RND family and shows the most homology to MexAB-OprM of *P. aeruginosa*. It exports many organic solvents but not clinical relevant antimicrobials (101). The MepABC system of *P.putida* strain KT2442 effluxes both organic solvents and antimicrobial agents (55). In *P. putida* DOT-T1E, there are at least two sets of genes encoding for toluene efflux systems, TtgABC and TtgDEF. The TtgABC system is constitutively expressed and confers resistance to both toluene and antimicrobial agents (ampicillin, tetracycline and chloramphenicol) (196). In contrast, TtgDEF effluxes only toluene but not antimicrobials (160). Expression of SrpABC (102), TtgABC (196), and TtgDEF (160) is inducible by aromatic hydrocarbons; therefore their primary function is solvent transport.

1.12. TRICLOSAN

1.12.1. Chemical properties and mode of action

Triclosan, 2,4,4′- tricloro-2′-hydroxydiphenyl ether (figure 1.5), was first synthesized by Ciba-Geigy Company, Switzerland, under its trade name Irgasan DP300 (13). It is a synthetic, non-ionic broad-spectrum antimicrobial agent belonging to the bis-phenol group that has been used extensively for over 30 years for its antibacterial and antifungal activity, as well as its safety (95, 146). Triclosan is partly insoluble in water, moderately soluble in alkaline solution and most soluble in organic solvents. It is thermally stable and can be heated to 200°C for up to 2 hours (13). Due to its extreme stability, triclosan has been formulated in variety of products as diverse as soap, toothpaste, hand lotions, mouthwashes, underarm deodorants, carpets, fabrics and plastics (95). Triclosan is recognized by the United States Food and Drug Administration (FDA) as either a non-prescription or a prescription drug (95). It is also approved for fungistatic, fungicidal and bacteriostatic usages. Under its name Irgasan CH3565, it is provided as the active ingredient in Bacto *Pseudomonas* isolation agar, at a concentration of 25 μg /ml, and used for the

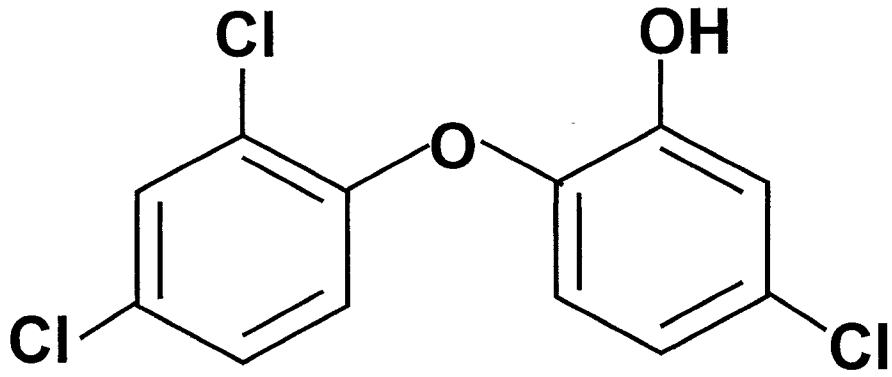


Figure 1.6. Structure of triclosan. Triclosan (2,4,4′-tricloro-2′-hydroxydiphenyl ether) is a biocide of the bisphenol family.

specific selection of *Pseudomonas* sp. This is because *P. aeruginosa* is naturally resistant to triclosan (214).

Triclosan was long thought to be a non-specific biocide disrupting cell membrane structure and function until McMurry et al. (149) showed that it acted as a specific and very potent inhibitor of the enzyme enoyl-acyl carrier protein reductase (ENR or FabI) of the fatty acid biosynthetic pathway in *E. coli* (149). Indeed, alterations of cell membrane permeability and function is a secondary outcome resulting from the inhibition of FabI and inhibited phospholipid synthesis (81). Many subsequent studies supported that FabI (or its homolog) is the intracellular target of triclosan in *P. aeruginosa* (83), *M. smegmatis* (147), *M. tuberculosis* (175, 220) and *S. aureus* (77). Triclosan is a slow-binding inhibitor that inactivates FabI by the formation of a stable ternary FabI-NAD^+-triclosan complex. X-ray crystallography of the complex showed that the binding site of triclosan is close to the nicotinamide ring of the NAD^+ nucleotide cofactor in the active site of the enzyme (79). ENR catalyzes the terminal reaction in the fatty acid elongation cycle by transfering H^+ from the C_4 of NADH to the C_3 of an enoyl moiety covalently linked to the phosphopantetheine arm of the acyl carrier protein (ACP) and then the substrate is rearranged

to produce an enolate anion. Triclosan structurally mimics an enolate anion intermediate and results in misplacement of the enoyl C3 carbon and the enolate oxygen to the phenol ring of triclosan (118). Although ENR is essential for survival, the FabI gene is absent from some bacterial genomes, e.g. *S. pneumoniae*, and *Clostridium acetobutyliticum*. Therefore, strains lacking FabI must posses a different ENR-encoding gene and the substitute of FabI was identified as FabK (78). Some bacterial genomes including *P. aeruginosa* and *M. tuberculosis*, contain both a *fabI* and a *fabK* gene. In *S. pneumoniae*, FabK could restore the function of FabI but it is not a target of triclosan (78). In *P. aeruginosa*, *fabI* null mutants are resistant to triclosan. Therefore, it was suggested that *P. aeruginosa* contains a FabK homologue as a second, triclosan-resistant ENR (83). An additional triclosan target termed FabL was reported in *B. subtilis* (80). The *fabI* knockout was as sensitive as the wild-type *B. subtilis* strain to triclosan, whereas the *fabL* knockout was 250-fold more sensitive to the biocide (80).

1.12.2. Mechanisms of triclosan resistance

Mechanisms of triclosan resistance have been intensively studied in the past three to four years to evaluate a possible link between antibiotic and biocide resistance. It is already evident that bacteria use multiple mechanisms to develop triclosan resistance (214). These mechanisms are classified into three categories that include detoxification via active efflux pumps, target mutations and increased target expression, and degradation by enzymes.

1.11.2.1. Detoxification via active efflux pumps. *P. aeruginosa* is well known for its extreme resistance to triclosan. This is mainly due to the combination of the relative impermeability of the outer membrane protein and active efflux systems. Of the 12 RND efflux systems, triclosan is an excellent substrate for MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexJK-OpmH. Since

Table 1.4. Regulatory mutations selected by triclosan and fluoroquinolones (modified from (214))

Selecting antimicrobial	Regulatory gene	Efflux pumps expressed	Amino acid changes in regulatory	References
Triclosan	*nfxB*	MexCD-OprJ	E28K (HTH[a])[b] L29W (HTH)[b] R42H (HTH) L88P	(27)
Triclosan	*mexL*	MexJK	A47D (HTH)	(28)
Norfloxacin	*nfxB*	MexCD-OprJ	R42G (HTH)	(171)
Ciprofloxacin	*nfxB*[c]	MexCD-OprJ	R82L	(93)

[a] HTH affects putative helix turn-helix DNA binding domain
[b] These mutation were found in the same isolates.
[c] Isolates from cystic fibrosis patients with long term exposure to ciprofloxacin

MexAB-OprM is constitutively expressed in *P. aeruginosa,* it is primarily responsible for intrinsic resistance to triclosan in the pathogen. We have shown that triclosan exposure selects for regulatory mutants expressing various efflux pumps. Regulatory mutations obtained by exposure to triclosan and fluoroquinolones that lead to expression of efflux pumps are similar in nature (see table 1.4). This finding is of particular medial concern because it was proven for the first time that exposure of bacterial cells to triclosan could select for regulatory mutants expressing efflux systems. These mutants are resistant to multiple antimicrobial agents because the efflux systems are multidrug transporters (27, 28). In a triclosan-susceptible *P. aerginosa* containing Δ(*mexAB-oprM*), triclosan could select for mutations in *nfxB*, causing overexpression of MexCD-OprJ (27). These mutations were similar to the mutations obtained by exposure to fluoroquinolones. This data confirmed that a link between antibiotic and biocide resistance does exist. As shown by the

studies presented in this dissertation, triclosan is indeed an excellent tool for selecting mutants overexpressing multidug efflux pumps. Mutants containing mutations in *mexL* were isolated by exposure to triclosan. Expression of MexJK is the only responsible mechanism for triclosan in the mutants because deletion of *mexJK* results in triclosan-susceptible derivatives (28). Triclosan was also found to be a substrate of the AcrAB efflux system in *E.coli*. Deletion of the *acrAB* gene lowered the minimal inhibitory concentration (MIC) for triclosan from 0.17 μg/ml in wild type to 0.019 μg/ml in AcrAB mutants (148).

1.12.2.2. Target alteration. This includes target mutations and increased target expression. In general, most biocides appear to have multiple cellular targets, therefore mutations of target sites are rare in biocide-resistant organisms (146). However, triclosan resistance seems to be an exception (184). In *E. coli*, exposure to triclosan caused *fabI* mutations close to the NADH-cofactor binding site and promoted resistance to triclosan and other antimicrobial agents (81, 149). This observation is consistent with findings of several studies in *M. smegmatis* and *M. tuberculosis*. In *M. smegmatis*, triclosan selects for spontaneous mutations affecting InhA expression, a FabI homologue, and caused cross-resistance to isoniazid, a drug used for clinical treatment of *M. tuberculosis* infection (79). In contrast, triclosan-resistant mutants of *M. tuberculosis* contain a T to G point mutation the putative ribosomal-binding site upstream of *mabA*, which is located upstream of *inhA* (220).

1.12.2.3. Degradation by enzymes. Triclosan degradation by enzymes is another mechanism of resistance. This mechanism was shown to operate in *P. putida* TriRY and *A. xylosoxidans ssp. denitrificans,* which were isolated from soil and grow on medium containing 1% triclosan as sole carbon and energy source (150). In addition, triclosan degradation was shown in *Sphingomonas*

strain RD1. The latter bacterium is capable of mineralizing a part of triclosan and loss of the ability to mineralize triclosan resulted in the increased susceptibility to the biocide (97, 98).

1.13. REFERENCES

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1.14. HYPOTHESIS, AIMS AND PREVIEW OF CHAPTERS

Even before the beginning of the studies presented in this dissertation, efflux pumps were shown play an important role in bacterial resistance to a multitude of natural and man-made compounds, including antibiotics and biocides. *P. aeruginosa* is unique in that it contains no fewer than 37 efflux pumps, including 12 putative RND-type systems. Of the five RND efflux pumps characterized to date, all play a major role in multidrug resistance and many of these are constitutively expressed in clinical isolates. However, only a fraction of the 12 RND pumps has been studied in some detail thus far since no general tool had been available for isolating mutants expressing normally silent RND pumps. Just before the present studies were initiated research in the Schweizer laboratory discovered triclosan as such a tool. **The hypothesis therefore was that triclosan can be used as a substitutive tool for antibiotics for the discovery of mutants that exhibit deregulated expression of normally silent pumps in laboratory mutant isolates and that these mutants can then be used to answer several important and yet unresolved questions**, including:

- Are all of these pumps drug efflux systems?
- What are the regulatory circuits and metabolic factors that govern regulation of efflux pump expression?
- Do all RND efflux pumps require an OM channel protein for function?
- What are the physiological roles of RND efflux pumps?

The following specific aims were pursued to test the hypothesis and to answer some of these important questions:

I) To show that triclosan can be used as a substitutive tool for antibiotics for selection of regulatory mutants expressing a clinically significant multidrug RND efflux

system, and that the types of regulatory mutants selected by triclosan and clinically significant antibiotics are similar (chapter 2).

II) To prove the hypothesis that triclosan can be used as a general tool to isolate regulatory mutants overexpressing uncharacterized RND multidrug efflux systems (chapter 3).

III) To demonstrate that regulatory mutants selected by triclosan exposure can be used to study the regulatory circuits and perhaps metabolic factors that govern the regulation of expression of normally silent efflux pumps (chapter 4).

IV) To further validate the usefulness of triclosan for the study of RND efflux pumps by demonstrating that efflux pump-mediated efflux is the sole mechanism responsible for the observed high-level triclosan resistance in *P. aeruginosa* (chapter 5).

CHAPTER 2

Cross-Resistance between Triclosan and Antibiotics in *Pseudomonas aeruginosa* Is ediated by Multidrug Efflux Pumps: Exposure of a Susceptible Mutant Strain to Triclosan Selects *nfxB* Mutants Overexpressing MexCD-OprJ

(Presented by Rungtip Chuanchuen, Kerry Beinlich, Tung T. Hoang, Anna Becher, RoxAnn R. Karkhoff-Schweizer and Herbert P. Schweizer. 2001. *Antimicrobial. Agents Chemother.* 45: 428-432)

The work presented in this article demonstrated that the link between antibiotics and biocides does exist. I acknowledge the contribution of everyone for this work. Kerry Beinlich performed growth experiments with MexCD-OprJ and MexEF-OprN. Tung Hoang assisted with characterization of *nfxB* mutants. Anna Becher assisted with triclosan selection.

2.1. ABSTRACT

Triclosan is an antiseptic frequently added to items as diverse as soaps, lotions, toothpaste, and many commonly used household fabrics and plastics. Although wild-type *Pseudomonas aeruginosa* expresses the triclosan target enoyl-acyl carrier protein reductase, it is triclosan resistant due to expression of the MexAB-OprM efflux system. Exposure of a susceptible Δ(*mexAB-oprM*) strain to triclosan selected multidrug-resistant bacteria at high frequencies. These bacteria hyperexpressed the MexCD-OprJ efflux system due to mutations in its

regulatory gene, *nfxB*. The MICs of several drugs for these mutants were increased up to 500-fold, including the MIC of ciprofloxacin, which was increased 94-fold. Whereas the MexEF-OprN efflux system also participated in triclosan efflux, this antimicrobial was not a substrate for MexXY-OprM.

2.2. INTRODUCTION

Pseudomonas aeruginosa is a clinically significant pathogen, that inficts disease on immunocompromised hosts (35). Infections caused by this bacterium are difficult to treat due to its highly intrinsic and its ability to develop acquired antibiotic resistances during therapy. Intrinsic resistance is mostly attributable to the expression of several multidrug resistance (MDR) efflux systems. The *P. aeruginosa* genome (34) contains structural genes for at least 12 resistance nodulation type efflux systems, of which only 4, i.e., MexAB-OprM (26), MexCD-OprJ (25), MexEF-OprN (13), and MexXY (1, 21, 36), have been characterized. Exposure to selected substrates can select for their upregulated or constitutive expression (13, 14, 25, 36).

2-hydroxyphenylethers are class of compounds that exhibit broad-spectrum antimicrobial activity. Triclosan is the most potent and widely used member of this class. (2, 5) and is used in hand soaps, lotions, toothpastes, and oral rinses, as well as in fabrics and plastics. It was long thought to act as a nonspecific "biocide" (28), but recent biochemical and genetic studies have shown that triclosan acts on a defined bacterial target in the fatty acid biosynthetic pathway, enoyl-acyl carrier protein (ACP) reductase (FabI) (7, 9, 10, 12, 18, 20) or its homolog InhA in mycobacteria (18). Some bacteria possess triclosan-resistant enoyl-ACP reductase homologs (FabK), and to date *P. aeruginosa* is unique among gram-negative bacteria in that it possesses both triclosan-sensitive and -resistant enzymes (8). Alterations in FabI active-site residues confer resistance to triclosan (9, 10, 20). Of particular concern is that such amino acid changes selected by exposure to triclosan lead to cross-resistance with other antimicrobial agents (9), including

clinically used front-line drugs, since some mutations leading to triclosan resistance in *Mycobacterium smegmatis* also caused resistance to isoniazid (18). Moreover, triclosan is a substrate of a multidrug efflux pump in clinical and laboratory *Escherichia coli* strains (19). We have recently shown that *P. aeruginosa* strain PAO1 is intrinsically resistant to triclosan by virtue of expression of the MexAB-OprM efflux pump (31), and the same is true for all strains of this species tested to date (our unpublished results).

While the contribution of antibiotic exposure to development of MDR due to efflux pump expression has clearly been documented in vitro and in vivo, little is known about antiseptic resistance mechanisms (29) and their possible contribution to MDR. In this paper we present results that triclosan is a substrate for multiple *P. aeruginosa* efflux pumps and that it is capable of selecting not just for mutants resistant to this particular antiseptic but, perhaps more importantly, also for MDR bacteria.

2.3. Materials and Methods

2.3.1. Bacterial strains, culture conditions, and molecular biology techniques. The bacterial strains used in this study are shown in table 2.1. Unless otherwise noted, bacteria were grown at 37°C in Luria-Bertani (LB) medium or on LB agar (30) or in Mueller-Hinton broth (MHB; Difco, Detroit, Mich.). For plasmid maintenance, *P. aeruginosa* media were supplemented with 200 μg of carbenicillin/ml. Unmarked efflux pump-negative mutants were derived using a previously described Flp/*FRT* recombinase technology (11). The sources for the mutant alleles were pPS952 for Δ(*mexAB-oprM*) (31), pPS1008 for Δ(*mexCD-opJ*) (derived by deletion of a 6,138-bp region encompassing three *Cla*I fragments from pKMJ002 (26), and pPS1128 for Δ(*mexXY*) (derived by deletion of a 2,868-bp DNA fragment encompassing several *Sal*I-*Xho*I fragments from pAMR-1 (36).) The chromosomal deletions were verified by PCR and genomic

Table 2.1. Bacterial strains used in this study

Strain	Relevant genotype or characteristic	Source or reference
PAO1	Wild type; produces MexAB-OprM	37
PAO200	Δ(*mexAB-oprM*)	32
PAO200-2	PAO200 *nfxB*; overproduces MexCD-OprJ	This study
PAO200-3	PAO200 *nfxB*; highly overproduces MexCD-OprJ	This study
PAO200-4	PAO200 *nfxB*; highly overproduces MexCD-OprJ	This study
KG3056	*nfxB*; overproduces MexCD-OprJ	6
KG2239	PAO1 with Δ(*mexR-mexAB-oprM*)	16
N103	KG2239 with Δ(*mexXY*)	16
PAO-7H	Overproduces MexEF-OprN	13
PAO3579	PAO1 with Δ*amrR* (Δ*mexZ*)[a]	38
PAO238	PAO200 with Δ(*mexCD-oprJ*)	This study
PAO253	PAO-7H with Δ(*mexAB-oprM*)	This study
PAO255	PAO253 with Δ(*mexEF-oprN*)	This study
PAO267	PAO3579 with Δ(*mexAB-oprM*)	This study
PAO280	PAO267 with Δ(*mexXY*)	This study

[a] amrR is identical to *mexZ* (1).

Southern analyses. Standard molecular biology methods were used (30). Plasmid pKMM128 is pAK1900 (27) expressing *oprM* (16).

2.3.2 Antimicrobial susceptibility testing. MICs were determined by the twofold broth microdilution technique according to National Committee for Clinical Laboratory Standards guidelines (22) or by the E-test system and the protocols provided by the supplier (AB Biodisk, Piscataway, N.J.) (ciprofloxacin and tetracycline only).

2.3.3 Selection and characterization of triclosan-resistant mutants. For isolation of triclosan-resistant derivatives of Δ(*mexAB-oprM*) strain PAO200, cells were grown in LB medium to stationary phase (A_{540}, ~2.6). Dilutions of these cells were plated on *Pseudomonas* isolation agar (PIA; Difco) whose formulation contained 25 μg of triclosan/ml. After an overnight incubation at 37°C, the colonies growing on the PIA plates were counted. For PCR amplification of the *nfxB* coding region from genomic DNA templates, two primers were designed: *nfxB*-up (5'-ACAATCtAGAAAAACCAACCGGG), which contained a single base mismatch (lowercase t) and which introduced an *Xba*I site (underlined) 27 bp upstream of the *nfxB* start codon, and *nfxB*-down (5'-CCGGAATTCCTGGGGGAGGTG), which primes to a region centered 236 bp downstream of *nfxB* containing an *Eco*RI site (underlined). PCRs were performed using *Taq* DNA polymerase (Qiagen, Santa Clarita, Calif.). The 828-bp PCR fragments were cloned as *Xba*I-*Eco*RI fragments into pUCP21T (32). Nucleotide sequences were determined by automated sequencing in the University of Colorado at Boulder sequencing facility. Extensions were primed utilizing the commercially available 24-nucleotide pUC/M13 reverse and forward sequencing primers for sequencing the cloned PCR fragments and the *nfxB*-up primer for the direct sequencing of PCR fragments. Computer-assisted sequence analyses were performed utilizing the SeqEd (Applied Biosystems, Foster City, Calif.) program.

2.3.4 Detection of outer membrane proteins. Cells of various *P.aeruginosa* strains were grown in LB medium to log phase (A_{540}, ~1.0). Samples of cells (1 ml) were harvested, centrifuged, and resuspended in the appropriate volumes of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 5% β-mercaptoethanol) to adjust for differences in cell densities. The resuspended cells were boiled for 4 min, and samples corresponding to ~25 μg of protein were analyzed by electrophoresis on 0.1% SDS-10% PAGE gel (pH 9.2) (15). The electrophoretically separated proteins were electroblotted onto nitrocellulose membranes, and the blots were processed as previously described (33). Hybridizing antibodies were detected using an antimouse antibody conjugated to horseradish peroxidase (HRP), and bound HRP activity was detected by exposure to luminogen substrate and X-ray film, according to the manufacturer's (Amersham, Arlington Heights, Ill.) protocol.

2.4. Results and Discussion

2.4.1. Triclosan is a substrate for multiple MDR efflux pumps. Our previous study (31) indicated that triclosan is a substrate for MexAB-OprM. Since MDR efflux systems export a variety of structurally unrelated substrates (23), we hypothesized that triclosan may be a substrate not only for MexAB-OprM but also for other *P. aeruginosa* efflux pumps. Defined mutants were obtained, and their triclosan susceptibilities were assessed by MIC determinations (table 2.2). Triclosan was a substrate for all tripartite efflux pumps analyzed in this study, including MexAB-OprM , MexCD-OprJ, and MexEF-OprN (figures 2.1, 2.2 and 2.3). Deletion mutants defective in these pumps all became triclosan susceptible. Mutant strain PAO267, expressing only MexXY, was triclosan susceptible and behaved the same as a strain (PAO280) expressing neither of the hitherto-characterized efflux pumps (figure 2.3). Since it has been proposed that MexXY requires

Table 2.2. Antimicrobial susceptibilities of *P. aeruginosa* strains used in this study

Strain (Plasmid)	Efflux protein(s) expressed	MIC (μg/ml)[a] of:					
		TRI	TET	CIP	TMP	ERY	GEN
PAO1	MexAB-OprM	>128[b]	16	0.064	512	256	1.6
PAO200	None[c]	24[e]	0.5	0.008	32	8	0.2
PAO200-2	MexCD-OprJ	>128	40	0.375	1,024	1,024	0.2
PAO200-3	MexCD-OprJ	>128	>256	0.75	>1,024	>1,024	0.1
PAO200-4	MexCD-OprJ	>128	>256	0.75	>1,024	>1,024	0.1
PAO238	None	20[e]	0.75	0.006	32	32	0.2
PAO253	MexEF-OprN	>128	6	2	>1,024	16	0.2
PAO255	None	24[e]	0.5	0.012	16	16	0.2
PAO3579	MexXY	>128	16	0.025	512	512	>3.2
PAO267	MexXY	32	0.5	0.016	16	32	0.2
PAO280	None	32	0.19	0.012	16	16	0.2
PAO267(pAK1900)	MexXY	32	0.5	0.012	16	32	0.2
PAO267(pKMM128)	MexXY-OprM	64	48	0.19	128	512	>3.2
PAO280(pAK1900)	None	32	0.25	0.008	16	16	0.2
PAO280(pKMM128)	OprM	64	0.5	0.012	16	32	0.2
KG2239(pAK1900)	MexXY[d]	32	0.5	0.008	16	32	0.20
KG2239(pKMM128)	MexXY-OprM	32	16	0.047	32	256	1.6
N103(pAK1900)	MexXY	32	0.25	0.008	16	16	0.2
N103(pKMM128)	MexXY-OprM	32	0.5	0.012	16	32	0.2

[a] The MICs of triclosan (TRI), tetracycline (TET), ciprofloxacin (CIP), trimethoprim (TMP), erythromycin (ERY), and gentamicin (GEN) were determined by either the microdilution method (TRI, TMP, ERY, and GEN) or the E-test method (CIP and TET). Values shown represent the averages of at least two experiments. Cells containing pAK1900 and pKMM128 were pregrown in MHB medium with 200 μg of carbenicillin/ml; no carbenicillin was present during incubation with triclosan.

[b] Triclosan is insoluble in aqueous solutions at concentrations >128 μg/ml.

[c] None implies that neither of the hitherto-characterized efflux systems, i.e., MexAB-OprM, MexCD-OprJ, MexEF-OprN, or MexXY, is expressed. The expression status of any other chromosomally encoded efflux systems in these mutants is unknown.

[d] Recent data indicate that MexXY is not expressed at detectable levels unless cells are grown in the presence of certain antibiotics (16).

[e] When determined by the twofold serial dilution method, this value was 32 μg/ml; to obtain the indicated value, cells were grown in MHB containing triclosan increasing in 2-μg/ml increments, starting at 16 μg/ml.

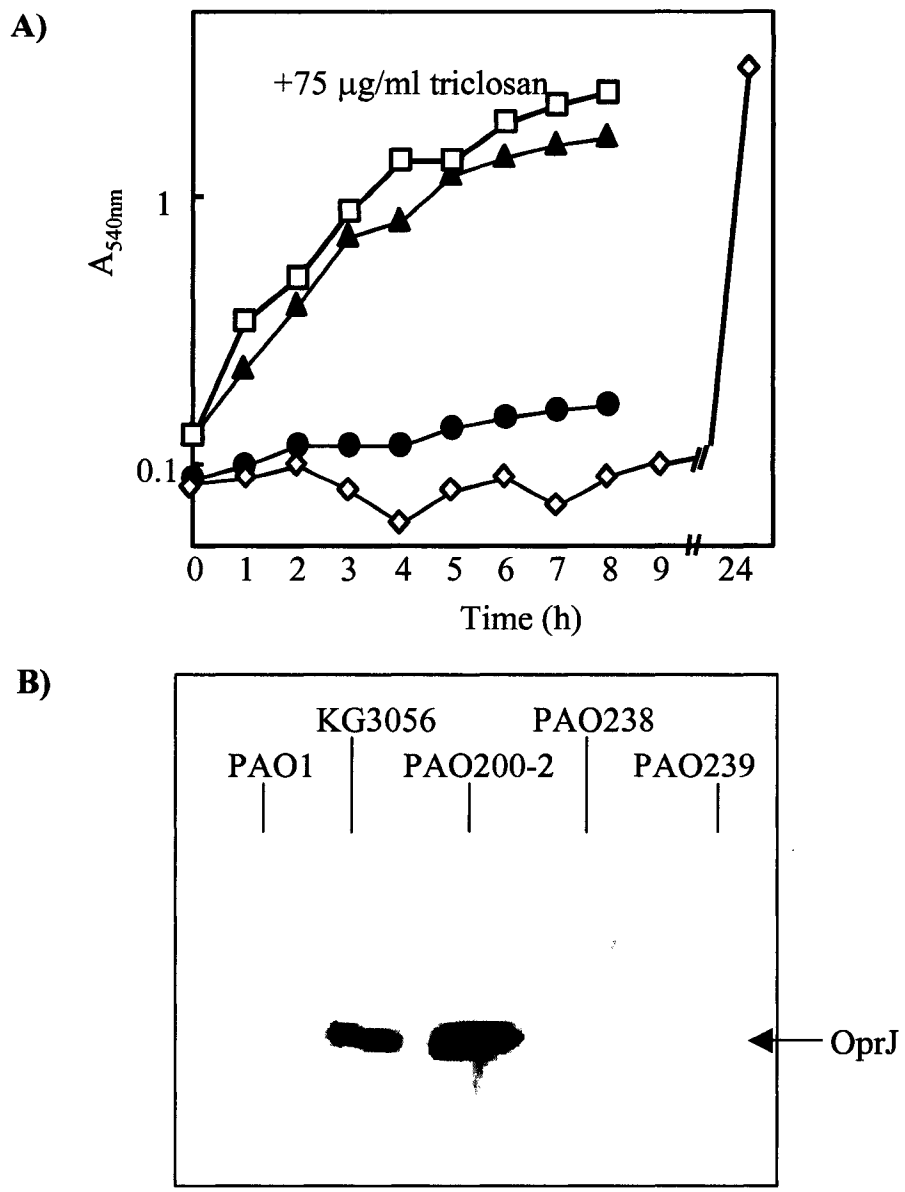

Figure 2.1. Triclosan is a substrate for MexAB-OprM and MexCD-OprJ. **A)** Bacteria were grown at 37°C with shaking in LB medium containing 75 μg/ml triclosan. The optical density was recorded after measuring the absorbance of the culture at 540 nm. The strains analyzed were PAO1(□) ($MexA^+B^+$-$OprM^+$), PAO200-2 (▲) ($MexC^+D^+$-$OprJ^+$), PAO200 (◇) (a Δ(*mexAB-oprM*)derivative of PAO1) and PAO238 (●) (Δ(*mexCD-oprJ*) derivative of PAO200-2, the latter two strains expressing no known efflux pumps. Note the rapid emergence of triclosan resistant cells in the PAO200 culture as indicated by the high density after 24 h. **B)** Anti-OprJ monoclonal antibiodies were used to probe cell lysates of strain PAO1, $OprM^+$; KG3056, $OprJ^+$; PAO200-2; OprJ+; and PAO238 and PAO239, OprJ null mutants (Δ(*mexCD-oprJ*) of PAO200-2.

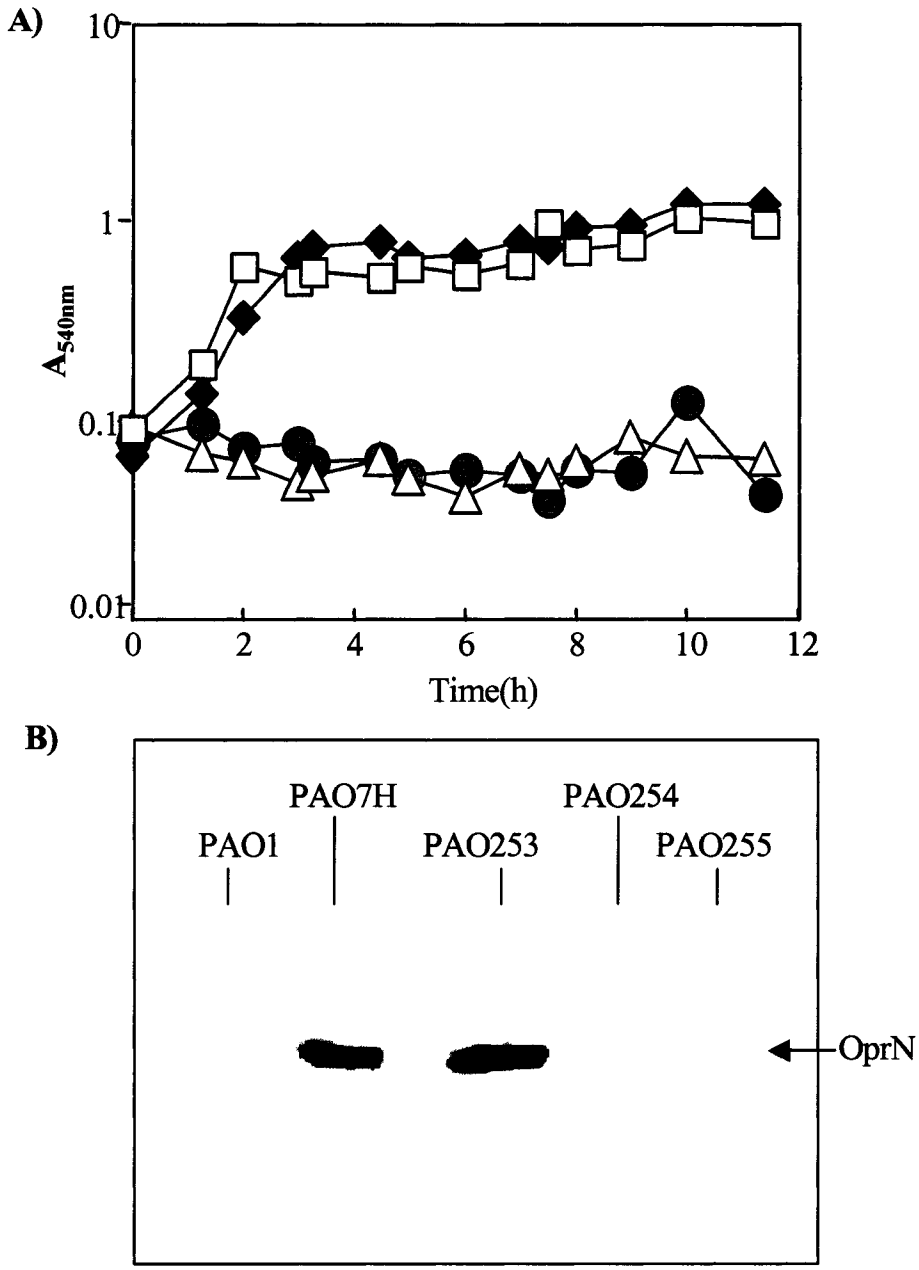

Figure 2.2. Triclosan is a substrate for MexEF-OprN. **A)** Bacteria were grown and cell densities measures as described in figure 1. The strains tested were PAO7H (□) ($MexA^+B^+$-$OprM^+MexE^+F^+$-$OprN^+$), PAO253 (◆) ($MexE^+F^+$-$OprN^+$; a Δ(*mexAB-oprM*) derivative of PAO7H, PAO254 (Δ), and PAO255 (●)(both Δ(*mexEF-oprN*) derivatives of PAO253 and expressing no known efflux pumps). **B)** Anti-OprN monoclonal antibodies were used to probe cell lysates of strain PAO1, $OprM^+$; PAO7H, $OprM^+OprN^+$; PAO253, $OprN^+$; and PAO254 and PAO255, OprN null mutants (Δ(*mexEF-oprN*))of PAO7H.

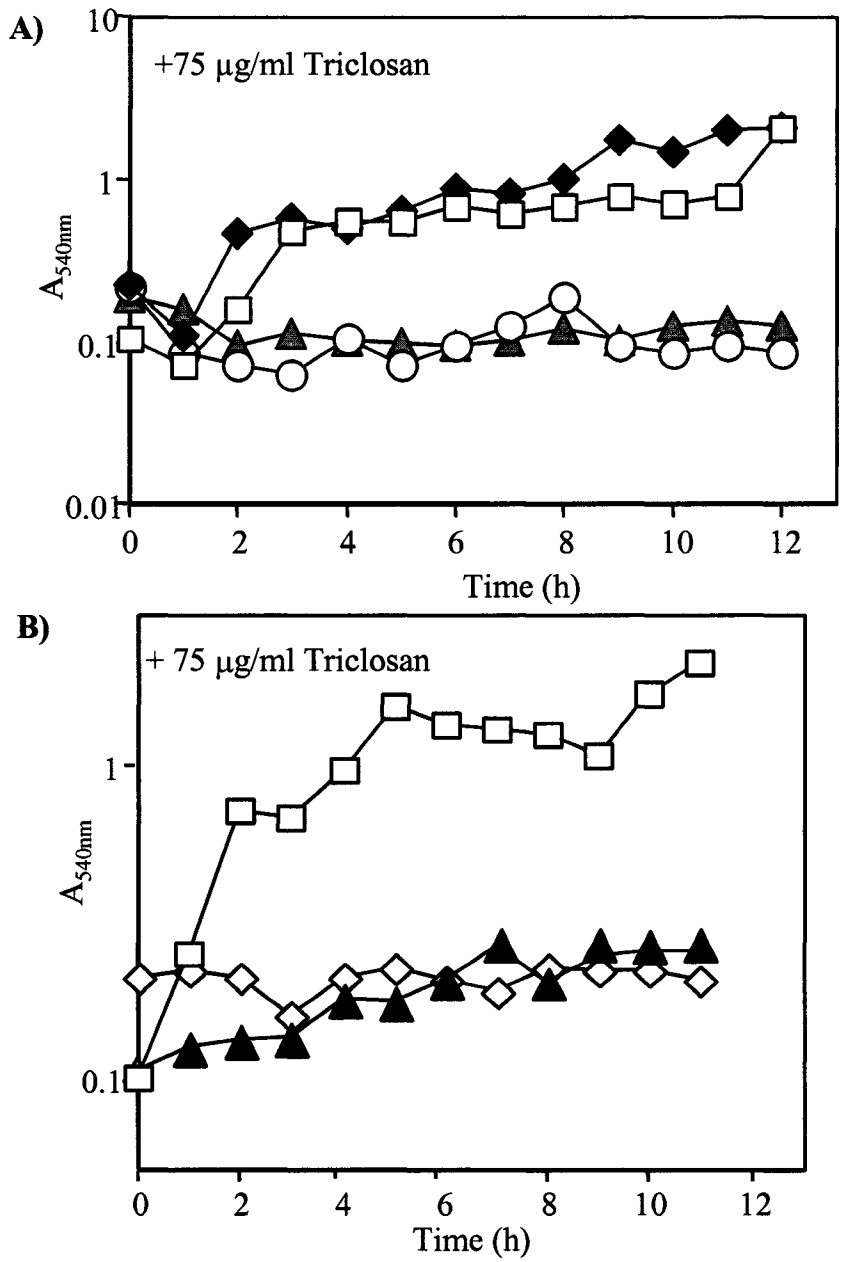

Figure 2.3. Triclosan is not a substrate of MexXY. A) Bacterial growth was monitored as described in figure 1. The strains analyzed were PAO1 (□) ($MexA^+B^+$-$OprM^+$), PA3579 (◆) ($MexA^+B^+$-$OprM^+MexX^+Y^+$), PAO267 (▲) ($MexX^+Y^+$, Δ(*mexAB-oprM*) derivative of PA3579 and PAO280 (○)Δ(*mexXY*) derivative of PAO267 expressing no known efflux pump) were grown and cell densities measured as indicated in figure 1. B) PAO267 cells harboring plasmids pRK415::Ap (◇) (vector control or pRSP08::AP (▲)(expressing OprM) were pregrown in LB medium containing 500 g/ml carbenicillin; no carbenicillin was present during growth on triclosan.

OprM for function (1, 16, 21), we considered the possibility that strain PAO267 was not triclosan resistant because it lacks OprM.

To test this hypothesis, we electroporated OprM-expressing pKMM128 and its vector control into PAO267 and its Δ(*mexXY*) derivative, PAO280. Only PAO267 containing pKMM128 effluxed tetracycline, gentamicin, erythromycin, trimethoprim, and ciprofloxacin (table 2.2), indicating that it expressed a functional MexXY-OprM system. However, this strain did not efflux triclosan (figure 2.3). The observed twofold increase in MIC from 32 μg/ml in the vector control to 64 μg/ml in the OprM-expressing strain was the same as the one observed in strain PAO280 harboring the same plasmids but lacking the MexXY system. We also tested KG2339/pKMM128, a strain known to express a functional MexXY-OprM system (16), and obtained similar results (table 2.2).

The MICs were slightly higher in the PAO267 background since MexXY expression is constitutive in this strain but inducible in KG2339 (16). These data conclusively demonstrated that triclosan was not a MexXY-OprM substrate.

2.4.2. Triclosan selects for multidrug-resistant *P. aeruginosa.* When susceptible cells of Δ(*mexAB-oprM*) strain PAO200 were exposed to triclosan, resistant mutants were readily obtained resulting in fully grown cultures after overnight incubation (figure 2.1A). To assess the frequency with which triclosan-resistant mutants were derived, we plated PAO200 cells on PIA medium and selected spontaneous triclosan-resistant mutants. Such mutants were obtained at a frequency of 10^{-6}. Three randomly picked triclosan-resistant derivatives, PAO200-2 to PAO200-4, were further analyzed, and all of them exhibited an MDR phenotype (table 2.2.), including resistance to the clinically administered drug ciprofloxacin, whose MIC for two of the three mutants analyzed was increased 94-fold.

Probing whole-cell extracts with anti-OprJ- and anti-OprN-specific monoclonal antibodies revealed that all three triclosan-resistant derivatives of PAO200 hyperexpressed OprJ but not OprN, demonstrating that their MDR phenotype was due to expression of the MexCD-OprJ efflux system (figure 2.4A). Although reference strain KG3056 was previously described as an OprJ type B hyperproducer (6), OprJ production in this strain was only a fraction of its expression in the three triclosan-resistant strains (figure 2.4A). To genetically verify that the MexCD-OprJ efflux system was expressed in response to exposure of PAO200 to triclosan, we isolated two *mexCD-oprJ* deletion mutants, PAO238 and PAO239. These mutants no longer expressed OprJ (not shown), were triclosan susceptible, and lost their MDR phenotype (table 2.2).

2.4.3. Triclosan selects for *nfxB* mutations. Expression of multidrug efflux systems is the result of exposure to antibiotics in both laboratory (25, 27) (6) (13) and clinical settings (37). Exposure of *P. aeruginosa* to norfloxacin selects for mutants which express MexCD-OprJ due to mutations in regulatory gene *nfxB* (6, 24, 25). Nucleotide sequence analysis of the PCR-amplified *nfxB* gene from strain PAO200 and its triclosan-resistant derivatives demonstrated that expression of the MexCD-OprJ efflux system in the triclosan-resistant mutant strains was indeed due to *nfxB* mutations (Fig. 2.1B). One strain, PAO200-4, contained a mutation that affected the helix-turn-helix DNA binding domain of NfxB, and strain PAO200-2 contained a mutation elsewhere in *nfxB*. The third strain, PAO200-3, contained two mutations in the helix-turn-helix region, and one of them also caused a frameshift and early termination at codon 35 of *nfxB*. Some of the mutations previously isolated by exposure to norfloxacin affected similar regions of NfxB; an Arg-to-Gly change at amino acid residue 42 caused by norfloxacin (24) corresponded to an Arg-to-His change caused by triclosan. To confirm that triclosan resistance was solely caused by *nfxB* mutations, we transformed a plasmid expressing a wild-type *nfxB* gene into the three mutant strains. In all three transformed strains, the MICs were similar to the ones observed with strain PAO200 (data not shown).

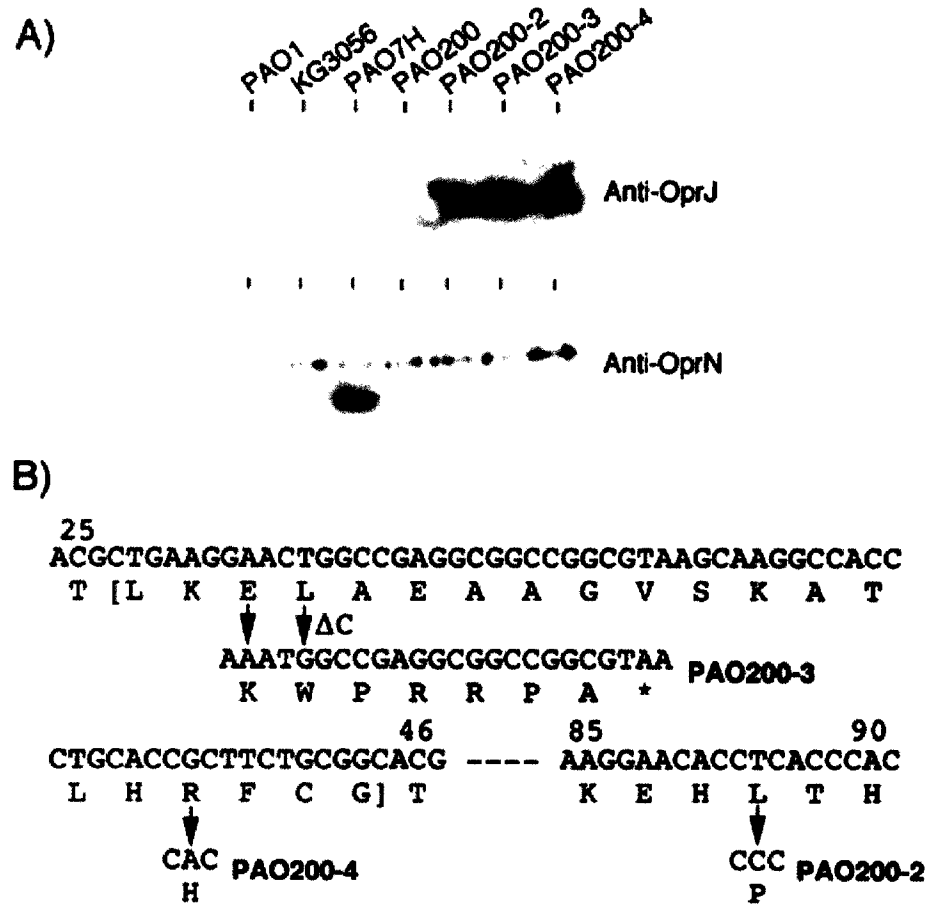

Figure 2.4. Western blots of *P. aeruginosa* cell lysates and mutations causing triclosan resistance. **A)** Standardized amounts of whole-cell lysates were separated on a 0.1% SDS-10% PAGE gel and electroblotted on nitrocellulose membranes, and the membranes were probed with monoclonal antibodies against OprJ and OprN. The strains analyzed were PAO1 $OprM^+$; KG3056 $OprJ^+$; PAO7H $OprN^+$; PAO200, an OprM null PAO1 mutant (Δ[*mexAB-oprM*]); and PAO200-2, PAO200-3, and PAO200-4, spontaneous triclosan-resistant *nfxB* derivatives of PAO200. **B)** Mutations leading to triclosan resistance. The *nfxB* genes from PAO200 and its three triclosan-resistant derivatives, PAO200-2, PAO200-3, and PAO200-4, were amplified by PCR from genomic DNA templates and sequenced. The *nfxB* sequence from each strain shown is the consensus obtained from six separate sequencing reactions; it was determined in duplicate from two separate clones, as well as in duplicate by directly sequencing the PCR products. Only portions of the *nfxB* sequence are shown, and codons are numbered as previously described (24). Arrows, changes from the PAO200 sequence. Amino acid residues constituting the putative helix-turn-helix DNA binding domain of NfxB are bracketed.

2.4.4. Implications of efflux-mediated triclosan resistance. Our results show that *P.aeruginosa* possesses multiple triclosan resistance mechanisms. These include efflux via the MexAB-OprM, MexCD-OprJ, and MexEF-OprN systems and probably FabI target mutations (12). However, in contrast to that in *E. coli*, where exposure to triclosan readily selects *fabI* mutants and overproduction of FabI leads to increased triclosan resistance (9, 10, 20), the first line of defense against triclosan in *P. aeruginosa* seems to be efflux and/or other hitherto-unknown resistance mechanisms, e.g., decreased outer membrane permeability (17). Whereas in *P. aeruginosa* overexpression of efflux pumps increased triclosan MICs by more than sixfold, overexpression of the AcrAB pump in *E. coli* increased the MIC only twofold (19). The MexXY system did not efflux triclosan, even in the presence of OprM.

Although possible links of cross-resistance between antiseptics and antibiotics due to efflux have been suggested before (19, 30), our studies demonstrate for the first time that exposure of a clinically significant bacterium to the antiseptic triclosan efficiently can select for MDR derivatives, including high-level resistance to an antipseudomonas drug. Exposures to antibiotics and triclosan select for similar regulatory mutations leading to expression of a multidrug efflux system. Although MexEF-OprN exports triclosan, we have not yet observed MexEF-OprN-expressing triclosan-resistant derivatives when plating either Δ(*mexAB-oprM*) strain PAO200 or Δ(*mexAB-oprM*) Δ(*mexCD-OprJ*) strain PAO238 on triclosan-containing medium. Since we have not systematically searched for MexEF-OprN-expressing derivatives of these strains, we cannot yet explain the apparent lack of such mutants. MDR *P. aeruginosa* is of foremost clinical importance since it is the leading cause of death in many hospital-acquired infections because of its intrinsic resistance to many antibiotics (35). Furthermore, most cystic fibrosis patients succumb to the debilitating effects of chronic *P. aeruginosa* infections due to eventual therapeutic failures caused by MDR-resistant bacteria (25). It has been well established that the massive prescription of antibiotics and their nonregulated and extensive usage are the

main causes for the development of extensive antibiotic resistance in bacteria (3, 4). Since antimicrobial agents provide the selective pressure for the development of resistance, the control of antibiotic usage is essential to prevent the development of resistance to antibiotics. Our results raise the notion that widespread and unregulated use of triclosan may promote the selection of MDR bacteria and thus compound antibiotic resistance.

2.5. ACKNOWLEDGMENTS

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CHAPTER 3

The MexJK Efflux Pump of *Pseudomonas aeruginosa* Requires OprM for Antibiotic Efflux but not for Efflux of Triclosan

(Presented in Rungtip Chuanchuen, Craig T. Narasaki and Herbert P. Schweizer. 2002 *Journal of Bacteriology* 184:5034-5044.)

The research presented in the paper shows that triclosan is an excellent tool for selection of mutations in regulatory genes of multidrug efflux systems. This results in mutants overexpressing multidrug efflux pumps. We were able to select mutants overexpressing a normally silent multidrug efflux pump, MexJK. In this paper, we present a molecular characterization of MexJK and describe that this system requirs OprM for efflux of antibiotics but not triclosan. I acknowledge Craig T. Narasaki for construction of *mexJ-lacZ* fusion and determination of the role of *mexL* in its expression.

3.1. ABSTRACT

Using the biocide triclosan as a selective agent, several triclosan resistant mutants of a susceptible *Pseudomonas aeruginosa* strain were isolated. Cloning and characterization of a DNA fragment conferring triclosan resistance from one of these mutants revealed a hitherto uncharacterized efflux system of the resistance nodulation cell divison (RND) family, which was named MexJK, and which is encoded by the *mexJK* operon. Expression of this operon is

negatively regulated by the product of *mexL*, a gene located upstream of and transcribed divergently from *mexJK*. The triclosan resistant mutant contained a single nucleotide change in *mexL*, which caused an amino acid change in the putative helix-turn-helix domain of MexL. The MexL protein belongs to the TetR family of repressor proteins. The MexJK system effluxed tetracycline and erythromycin, but only in the presence of the outer membrane protein channel OprM; OprJ and OprN did not function with MexJK. Triclosan efflux required neither of the outer membrane protein channels tested but necessitated the MexJ membrane fusion protein and the MexK inner membrane RND transporter. The results presented in this study suggest that MexJK may function as a two-component RND pump for triclosan efflux but must associate with OprM to form a tripartite antibiotic efflux system. Furthermore, the results confirm that triclosan is an excellent tool for the study of RND multidrug efflux systems and that this popular biocide therefore readily selects mutants which are cross-resistant with antibiotics.

3.2. INTRODUCTION

Pseudomonas aeruginosa is intrinsically resistant to many antimicrobial agents, including antibiotics, biocides and, to some extent, heavy metals. This intrinsic resistance can be attributed to synergy between an outer membrane with low permeability (11) and other contributing mechanisms, most notably drug efflux (32, 57).

Genome analyses revealed that *P. aeruginosa* encodes as many as twelve possible efflux systems of the resistance nodulation cell division (RND) family alone (50). Of these, only four, MexAB-OprM (34), MexCD-OprJ (33), MexEF-OprN (20) and MexXY (1, 52) have been characterized. It is now generally assumed that these efflux systems function as tripartite systems, i.e., they require an inner membrane RND family transporter (MexB, MexD, MexF, MexY), a periplasmic membrane fusion protein (MFP; MexA, MexC, MexE, MexX) and an outer membrane translocase channel (OprM, OprJ, OprN). The MexXY system, whose operon

does not encode an outer membrane protein (Opr) component requires OprM for function (25). In contrast to OprJ and OprN, whose expression is tightly regulated along with their associated Mex proteins, OprM is always expressed at low levels and there is evidence that its structural gene may be transcribed from a promoter independent of *mexAB-oprM* transcription (58). Unlike MexAB-OprM, which is always expressed at low but detectable levels, the expression of the other hitherto characterized efflux systems is tightly regulated. They are only expressed in mutants obtained by antibiotic exposure *in vitro* or *in vivo* (18, 20, 33, 38, 48, 59). Many of these mutants contain mutations in adjacent regulatory genes, e.g. *mexR*, *nfxB* or *mexZ*. In contrast, the *mexEF-oprN* operon is positively regulated by the *mexT* product, encoding a transcriptional activator of the LysR family (20) although the involvement of a putative negative regulator, MexS, has also been proposed (23). Since many patient isolates of animal and human origin constitutively express these efflux systems without displaying mutations in known regulators or regulatory regions, other hitherto unidentified regulatory mechanisms governing effux operon expression must exist (3, 36, 59). It has recently been shown that expression of at least one RND efflux system, PA4206-PA4207-PA4208 or *qsc,* is regulated by quorum sensing (53). Expression of the Czr system, a divalent cation efflux pump, is regulated by a two-component sensor kinase regulatory system (12). Furthermore, a ribosomal mutation has been implicated in MexXY expression in a tobramycin resistant clinical *P. aeruginosa* isolate (52).

Although antibiotics have clearly been documented in laboratory and clinical settings as selective agents leading to upregulated efflux operon expression, our recent findings showed that triclosan, a broad-spectrum biocide, can do the same (5, 43). Prior to identification of enoyl [acyl carrier protein] reductase (FabI) as the intracellular triclosan target (15, 17, 27), it was thought that its antibacterial action resulted from nonspecific disruption of cellular membranes. Triclosan is now used in a wide range of consumer products, including cosmetics, toothpastes, lotions, soaps, cutting boards, mattress pads and many other products (4). Other antibacterial compounds with structures similar to triclosan include hexachlorophene and dichlorophene.

Both compounds contain the hydroxyphenyl moiety that is essential for the activity of triclosan and related hydroxydiphenylethers (14, 15). Hexachlorophene is used in disinfectants, surgical scrubs (19) and other consumer materials (47). It has a more limited spectrum of biological activity than triclosan, being more active against Gram-positive bacteria, but less effective against Gram-negative organisms (10). Although hexachlorophene inhibits FabI activity, this inhibition is not a component of its biocidal activity (13). Dichlorophene is also used as a broad-spectrum biocide, including as an agricultural fungicide, and an antimicrobial in soaps and shampoos. Its cellular target has not yet been defined.

Even though *P. aeruginosa* expresses a triclosan-sensitive FabI (17), wild-type strains are triclosan resistant due to expression of the MexAB-OprM system (42). Deletion of this efflux system generates triclosan susceptible mutants and exposure of these to this biocide selected MexCD-OprJ hyperexpressing mutants due to mutations in its negative regulatory gene, *nfxB* (5). Since the amphiphilic triclosan seems to be a substrate for most RND efflux systems, we reasoned that it might be a useful tool for the study of efflux systems whose substrate profiles and regulatory mechanisms have not yet been identified. In this report we describe a new efflux system, MexJK, which is constitutively expressed in *mexL* mutants obtained through exposure of a susceptible mutant strain to triclosan and describe the properties of this efflux pump.

3.3. Materials and Methods

3.3.1. Bacterial strains and media. Bacterial strains and plasmids used in this study are shown in table 3.1. LB (Luria-Bertani) medium from Gibco (Gaithersburg, MD) was routinely used as the rich medium for all bacterial strains. The minimal medium used was M9 (28) supplemented with 0.2% casamino acids (Difco, Detroit, MI). Concentrations of antibiotics used

Table 3.1. Bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant genotype or characteristic	Source/Reference
Strain		
PAO1	wild type; produces MexAB-OprM	(51)
PAO238	PAO1 derivative with Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*)[a]	(5)
PAO238-1	spontaneous Tri^{r} [b] derivative of PAO238	This study
PAO298	PAO238-1 with *mexJ*::Tn<TET-1>	This study
PAO314	PAO238-1 with Δ*mexJKL*::*FRT*	This study
PAO315	PAO314 with chromosomal *mexJ'-'lacZ* transcriptional fusion integrated at *attB*	This study
PAO318	PAO238 with Δ*mexL*::*FRT*	This study
PAO325	PAO314 with Δ(*mexXY*::*FRT*)	This study
PAO327	PAO238-1 with Δ(*mexXY*::*FRT*)	This study
Plasmid		
pADD948	Cb^{r}; broad-host-range *in vivo* cloning vector	(6)

Table 3.1. Bacterial strains and plasmids used in this study (cont.)

Strain/Plasmid	Relevant genotype or characteristic	Source/Reference
pAK1900	Cb^r; broad-host-range cloning vector	(35)
pBluescript SK(-)	Ap^r; cloning vector	Stratagene, La Jolla, Calif.
pBSP II SK(-)	Cb^r; broad-host-range cloning vector	(45)
pUC18	Cb^r; cloning vector	(54)
pPS856	Cb^r; Gm^r; source of *FRT*-Gm^r cassette	(16)
pKMM128	Cb^r; pAK1900 expressing *oprM* from $P_{lac}{}^c$	(25)
pTZ110	Cb^r; broad-host-range *lacZ* fusion vector	(44)
pUCP20T	Cb^r; broad-host-range cloning vector	(46)
pVLT35	Sm^r, Sp^r; broad-host-range expression vector	(7)
pJ22	Cb^r; pADD948 carrying the triclosan-resistance determinant from PAO238-1 on a ~32 kb chromosomal DNA fragment	This study

Table 3.1. Bacterial strains and plasmids used in this study (cont.)

Strain/Plasmid	Relevant genotype or characteristic	Source/Reference
pJ22::Tn<Tet 1>	Cb^r, Tc^r; pJ22 with *mexJ*::Tn<TET-1>	This study
pJZ110	Cb^r; pTZ110 with 655-bp *Bam*HI-*Xho*I fragment from pPS1176 containing the *mexJK* operon regulatory region	This study
pPS1150	Cb^r; pBSP II SK(-) carrying the *mexL-mexJK* genes on a 6,945-bp *Not*I fragment from pJ22	This study
pPS1151	Cb^r, Tc^r; pBSP II SK(-) with a *Not*I fragment from pJ22::Tn<TET-1> carrying *mexJ*::Tn<TET-1>	This study
pPS1152	Ap^r, Tc^r; pBluescript SK(-) with a *Not*I fragment from pJ22::Tn<TET-1> carrying *mexJ*::Tn<TET-1>	This study
pPS1153	Cb^r; pUCP20T with 816-bp *mexL* fragment	This study
pPS1162	Cb^r; pAK1900 expressing *oprN* from P_{lac}	This study
pPS1163	Cb^r; pAK1900 expressing *oprJ* from P_{lac}	This study
pPS1168	Cb^r; pUC18 with a 2,145-bp *Bgl*II-*Hin*dIII fragment from pPS1150 carrying the 5' end of *mexL*, all of *mexJ* and the 5' end of *mexK* in pUC18	This study
pPS1169	Cb^r; pPS1168 with 1,414-bp *Nco*I-*Xho*I deletion removing the 5' end of *mexL*, all of *mexJ* and the 5' end of *mexK* replaced with *FRT*-Gm^r fragment from pPS856	This study

Table 3.1. Bacterial strains and plasmids used in this study (cont.)

Strain/Plasmid	Relevant genotype or characteristic	Source/Reference
pPS1175	Cb^r; pBluescript SK(-) with 1,342-bp *Eco*RI-*Cla*I fragment from pPS1150 carrying all of *mexL* and the 5' end of *mexJ*	This study
pPS1176	Cb^r; deletion of a 725-bp *Xho*I fragment internal to *mexL* from pPS1175	This study
pPS1177	Gm^r; mini-CTX3-*lacZ* with 643-bp *Xho*I-*Pst*I fragment from pPS1176 containing the *mexJK* regulatory region	This study
pPS1180	Sm^r, Sp^r; pVLT35 expressing *oprM* from P_{tac} [d]	This study
pPS1234	Cb^r; pUCP20T with 1,231-bp *Kpn*I-*Pst*I PCR fragment expressing *mexJ* from P_{lac}	This study
pPS1235	Sm^r, Sp^r; pVLT35 with 3,759-bp *Eco*RI-*Sal*I fragment from pPS1150 expressing *mexK* from P_{tac}	This study

[a]This strain and its derivatives contain a 1.58 Mb chromosomal inversion between the *mexAB-oprM* and *mexCD-oprJ* operons (2). This inversion has no effect on efflux pump function nor any other phenotype since there is no net loss or gain of genetic information.
[b]Abbreviations: Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Gm^r, gentamycin resistance; Sm^r/Sp^r, streptomycin/spectinomycin resistance; Tri^r, triclosan resistance.
[c]P_{lac}, *E. coli lac* operon promoter.
[d]P_{tac}, *E. coli lac* operon and *trp* operon hybrid promoter.

in selection media were: *E. coli*, Ap, 100 μg/ml; spectinomycin (Sp), 150 μg/ml; and tetracycline (Tc), 15 μg/ml; *P. aeruginosa*: carbenicillin (Cb), 200 μg/ml; Sp, 400 μg/ml; Tc, 25 μg/ml and triclosan (Tri), 25 to 50 μg/ml. Spontaneous triclosan resistant mutants were selected by plating dilutions of PAO238 cells on *Pseudomonas* isolation agar (PIA; Difco; this formulation contains 25 μg/ml Tri), as previously described (5). Antibiotics were from commercial sources. Triclosan was a gift from KCI Chemicals (Armonk, NY), hexachlorophene and dichlorophene (2,2'-methylenebis (4-chlorphenol)) were purchased form Sigma (St. Louis, MO) and TCI America (Portland, OR), respectively.

3.3.2. Antimicrobial susceptibility testing. Minimal inhibitory concentrations (MICs) were determined by the two fold broth microdilution technique according to National Committee for Clinical Laboratory Standards guidelines (30) or the E-test method (AB Biodisk, Piscataway, NJ; ciprofloxacin and tetracycline only).

3.3.3. General DNA and mutagenesis procedures. All routine DNA procedures were performed using previously described methods (16, 39). For disruption of the *mexJ* gene, pPS1152 was electroporated (8) into strain PAO238-1 and Tc resistant (Tc^r) colonies were selected. The *mexJ*::Tn<TET-1> mutants were identified as Tc^r Cb susceptible (Cb^s). Gene replacement at the *mexJ* locus was confirmed using the inserts from pPS1150 and a 627-bp *Hin*dIII-*Sal*I fragment from pBR322ΔAP (41), respectively, as *mexJK*- and *tet*-specific probes. A chromosomal Δ*mexJKL* deletion was isolated by deleting a 1,414-bp *Xho*I-*Nco*I fragment from pPS1168 and replacing it with a Gm^r-*FRT* cassette from pPS856 (16). The resulting pPS1169 was electroporated into PAO238-1 and Gm^r colonies were selected. The Δ*mexJKL* colonies possessed a Gm^r Cb^s phenotype. Similarly, a chromosomal Δ*mexL* deletion strain was isolated by deletion of a 371 bp *Xho*I fragment from pPS1175 and replacing it with a Gm^r-*FRT* cassette from

pPS856, following by return of the deletion into the PAO238 chromosome via conjugation using the gene replacement vector pEX18Ap and sucrose counterselection (17). Flp recombinase-mediated excision was achieved utilizing a previously described procedure (16). The mutant genotypes were confirmed by PCR and Southern analysis using the inserts from pPS1168 (*mexJK'L*), pPS1175 (*mexL*) and pPS856 (Gm^r-*FRT*) as the probes. Genomic Southerns using biotin-labeled probes were performed as previously described (16). Mutants containing unmarked chromosomal Δ(*mexXY*) deletions were isolated using previously described plasmid constructs and procedures (5), and were verified by PCR analysis.

3.3.4. Cloning and analysis of the *mexJK*-containing region. For the cloning of DNA fragments carrying the Tri^r-conferring chromosomal region from PAO238-1, the bacteriophage mini-D3112-based *in vivo* cloning method of Darzins and Casadaban (6) was used with the following modifications. Strain PAO238-1 was lysogenized with D3112*c*ts (6). The resulting strain was transformed with pADD948 and a lysate was prepared by heat induction (6). Cells of recipient PAO238 were grown overnight and infected with the mixed lysate as previously described (40). The samples were spread on LB plates containing 30 μg/ml Tri and incubated at 30°C. Colonies growing on these plates after 24 to 36 h were purified on the same medium and analyzed for the presence of recombinant plasmids. Cb^r and Tri^r-conferring plasmids were confirmed by electroporation of PAO238. One plasmid was retained and named pJ22. The Tri^r-encoding region on this plasmid was localized by *in vitro* transposon mutagenesis using the EZ::TN™<TET-1> kit from Epicentre (Madison, WI) and the manufacturer's protocol. An aliquot of the mutagenized plasmid pool was electroporated into PAO238 and Cb^r Tc^r transformants were selected and screened for loss of Tri^r. DNA templates were prepared using the Qiagen mini-prep kit and the transposon isertion sites were determined by automated nucleotide sequencing. Sequencing reactions were primed using the TET-1 FP-1 and TET-1 RP-

1 primers from the EZ::TN™ <TET-1> mutagenesis kit. Homologous sequences were identified using online BLAST searches of the National Library of Medicine databases.

3.3.5. Cloning and sequencing of *mexL*. For PCR amplification of the *mexL* coding region from genomic and plasmid DNA templates two primers were designed. Primer *mexL*-up (5'-CGTTCGAaTTCTTATACTGGGCGG) contained a single base mismatch (lower-case a) and introduced a *Eco*RI site (underlined) 70 bp upstream of the *mexL* start codon. Similarly, *mexL*-down (5'-ACTGGGTCGAcCACTGGGACATC) contained a single mismatch (lower case c) and introduced a *Sal*I site (underlined) 108 bp downstream of *mexL*. PCR reactions were performed using *Pfu*-DNA polymerase (Stratagene). Reaction mixtures (50 µl) contained 1x *Pfu* buffer (Stratagene), 100 ng of each primer, 10 ng of plasmid or 100 ng of chromosomal DNA and 2.5 U of *Pfu*. The reaction mixture was subjected to the following thermal cycles: one cycle at 96°C for 5 min; 30 cycles for plasmid DNA or 35 cycles for chromosomal DNA (96°C, 45 s; 60°C for 45 s; 72°C for 1 min) and a final extension at 72°C for 10 min. Sequences of PCR fragments were determined using the same primers employed for amplification. For complementation analyses, the PCR fragments were digested with *Eco*RI and *Sal*I, gel-purified and then cloned between the same sites of pUCP20T (46) to yield pPS1153.

3.3.6. Construction of *oprM*, *oprJ* and *oprN* expression vectors. The *oprM*, *oprJ* and *oprN* genes were PCR amplified from genomic DNA templates using primers containing base mismatches (indicated by lower case letters in each primer sequence) that introduced new restriction sites after PCR amplification. For *oprM*, the forward primer *oprM*-up (5'-CGAGGaaTtCAAGCAGCAGGCGTCCGT) introduced an *Eco*RI site (underlined) upstream of the *oprM* start codon and ribosome-binding site. The reverse primer *oprM*-down (5'-ACGCCAaGcTtAGGGTCGGCGTTCTTG) introduced a *Hin*dIII site (underlined) downstream of *oprM*. For *oprJ*, the forward primer *oprJ*-up (5'-GCAGCAAGCttGCACCCATCGAAC)

introduced a *Hin*dIII site (underlined) upstream of the *oprJ* start codon and ribosome-binding site. The reverse primer *oprJ*-down (5'-CACCGgaTCCCACACGTTTACC) introduced a *Bam*HI site (underlined) downstream of *oprJ*. The forward primer for *oprN*, primer *oprN*-up (5'-CGCGAAGCttGCCGCGCCGCCA), introduced a *Hin*dIII site (underlined) upstream of the *oprN* start codon and ribosome-binding site. The *oprN* reverse primer, *oprN*-down (5'-GAGTGGtCGAcTTCCATCGGCCG) introduced a *Sal*I site (underlined) downstream of *oprN*. PCR reactions were performed using *Taq*-DNA polymerase (Gibco). Reaction mixtures (50 □l) contained 1x *Taq* buffer (Gibco), 30 pmoles of each primer, 100 ng of chromosomal DNA and 2.5 U of *Taq*. For amplification of *oprM*, the reaction mixture was subjected to the following thermal cycles: one cycle at 96°C for 5 min; 35 cycles (96°C, 45 s; 71°C, 45 s; 72°C, 2 min) and a final extension at 72°C for 10 min. The *oprJ* and *oprN* genes were amplified using the same denaturation and extension conditions, except that the annealing conditions were changed to 60°C, 45 s and 70°C, 45 s for *oprJ* and *oprN*, respectively. The PCR fragments were digested with the appropriate enzymes, purified from an agarose gel, and either ligated to *Eco*RI-*Hin*dIII digested pVLT35 for *oprM*, to *Hin*dIII-*Bam*HI digested pAK1900 for *oprJ* or *Hin*dIII-*Sal*I digested pAK1900 for *oprN*. This procedure generated pPS1180, pPS1162 and pPS1163 which express *oprM*, *oprJ* and *oprN*, respectively, from P_{tac} (pVLT35) or P_{lac} (pAK1900). OprM, OprJ and OprN expression was verified in *E. coli and P. aeruginosa* by Western blot analysis using specific antibodies, as previously described (3, 5).

3.3.7. Fluorescence measurements in cell suspensions. Cells for fluorescence measurements were grown overnight in M9 medium (28) supplemented with 1% thiamine and 0.2% casamino acids. The cells were washed in 100 mM NaCl, 50 mM sodium phosphate buffer (pH 7.0) as previously described (31). The washed cells were adjusted to an optical density at 540 nm of 0.1 in the wash buffer containing 0.05% of glycerol and used for fluorescence measurements within 2 h. The substrate used in fluorescence measurements was 2-(4-diethylaminostyryl)-1-

methylpyridinium iodide (DMP) and was added to cell suspension to a final concentration of 25 μM. Fluorescence emission intensity at room temperature was measured with a Fluorolog-3 fluorometer (Instruments S.A., Inc., Edison, NJ) and data were recorded and analyzed with DataMax and Igor Pro software. Excitation and emission wavelengths for DMP were, respectively, 467 and 557 nm. Slit widths were set at 5 nm for excitation and at 10 nm for emission.

3.3.8. Construction of a *mexJ-lacZ* fusion. A plasmid-borne fusion was constructed by ligating a 655-bp *Bam*HI-*Xho*I fragment from pPS1176 between the same sites of pTZ110 (44) to form pJZ110. The fused fragment contained 470 bp of *mexJ*, the 94 bp *mexJ-mexL* intergenic region and 71 bp of *mexL* coding sequence. The fusion plasmid was transferred to *P. aeruginosa* by electroporation. β-Galactosidase activity was measured and activity units calculated as decribed by Miller (28).

3.4. RESULTS

3.4.1. Isolation and characterization of triclosan resistant mutants. When triclosan susceptible cells of Δ(*mexAB-oprM*) Δ*mexCD-oprJ*) strain PAO238 (Table 3.1) were exposed to 25 μg/ml triclosan, Tri^r derivatives were obtained at a frequency of 10^{-8}. Four randomly picked Tri^r derivatives, PAO238-1 to PAO238-4, were further analyzed and all of them were highly Tri^r (MICs at least 128 μg/ml). None of them was resistant to hexachlorophene (MIC 8 μg/ml) or dichlorophene (MICs 32-64 μg/ml), two other antimicrobials with similar structure to triclosan. For comparison, in PAO1 dichlorophene was found to be a good substrate for MexAB-OprM (MIC 128 μg/ml) but hexachlorophene (MIC 4 μg/ml) was not. In addition, none of the mutants were resistant to any of the antibiotics tested (see footnote *a* to table 3.2), presumably due to lack

Table 3.2. Antimicrobial susceptibilies of efflux pump mutant strain.

Strain	Mex Efflux (outer membranes Protein)Epressed	MIC (μg/ml)[a]			
		Tri[a]	Tc	Cip	Ery
PAO1	MexAB (OprM) MexX^{i}Y^{ib}	>128[c]	16	0.064	512
PAO238	MexX^{i}Y^{i}	20	0.75	0.006	32
PAO238-1	MexJK MexX^{i}Y^{i}	128	1	0.004	16
PAO298	MexX^{i}Y^{i}	20	ND[d]	0.004	32
PAO314	MexX^{i}Y^{i}	20	0.75	0.006	32
PAO238-1 (pVLT35)	MexJK MexX^{i}Y^{i}	>128	0.75	0.004	16
PAO238-1 (pPS1180)	MexJK MexX^{i}Y^{i} (OprM)	>128	16	0.047	256
PAO314 (pVLT35)	MexX^{i}Y^{i}	32	0.38	0.006	16
PAO314 (pPS1180)	MexX^{i}Y^{i} (OprM)	32	10	0.016	128
PAO327 (pVLT35)	MexJK	128	0.19	0.004	8
PAO327 (pPS1180)	MexJK (OprM)	128	1.0	0.008	64
POA325/pVLT35	None	16	0.19	0.004	16
PAO325 (pPS1180)	(OprM)	16	0.25	0.006	16
PAO238-1 (pUCP20T)	MexJK MexX^{i}Y^{i}	>128	0.75	0.004	16
PAO238-1 (pPS1153)	MexJK MexX^{i}Y^{i}	20	0.75	0.006	16
PAO318	MexJK MexX^{i}Y^{i}	>128	0.75	0.006	16
PAO318 (pUCP20T)	MexJK MexX^{i}Y^{i}	>128	0.5	0.004	16
PAO318 (pPS1153)	MexJK MexX^{i}Y^{i}	20	0.5	0.004	16

[a]Abbreviations: Cip, ciprofloxacin; Ery, erythromycin; Tc, tetracycline; Tri, triclosan. All mutants were additionally tested for their susceptibilities to acriflavine, dichlorophene, carbenicillin, fusidic acid, gentamycin, hexachlorophene and trimethoprim, most of which are substrates of other efflux systems but not MexJK.
[b]MexX^{i}Y^{i}, induced MexXY
[c]at concentrations >128 μg/ml, triclosan is insoluble in aqueous solutions.
[d]ND, not done.

of an Opr channel (see below). Analysis of total outer membrane proteins did not reveal overproduction of any novel proteins (data not shown). Although the MexEF-OprN system supports the efflux of triclosan, in Western blots none of the four mutants expressed detectable levels of OprN (data not shown). This finding was not surprising since MexEF-OprN is not derepressible in PAO238 because this PAO background harbors an 8-bp insertion in the *mexEF-oprN* activator *mexT* (23). These preliminary results indicated that the system(s) responsible for the Tri^r observed in these mutants was novel and most likely did not involve overproduction of a new outer membrane protein. Since all four Tri^r PAO238 derivatives behaved similar in preliminary analyses, we decided to concentrate on one isolate, PAO238-1, and to decipher the mechanism(s) resonsible for its Tri^r phenotype.

3.4.2. Cloning of the triclosan resistance determinant from PAO238-1. The phage D3112-based *in vivo* cloning technique was used to clone a ~32 kb chromosomal DNA fragment from PAO238-1. The resulting plasmid, pJ22, conferred Tri^r when transferred the susceptible parent strain PAO238. *In vitro* transposon mutagenesis was used to localize the Tri^r determinant in the chromosomal insert of pJ22. The relatively high frequency, ~13%, by which the transposon mutagenized plasmid population lost their ability to confer Tri^r indicated that a relatively large region on pJ22 was required for the Tri^r phenotype. To further localize the Tri^r-encoding determinant to the *mexJK* region, the entire operon with its flanking DNA sequences was subcloned on a 6,945-bp *Not*I fragment (figure 3.1). As a control, the same fragment contain the 1,674-bp Tn<TET-1> insertion in *mexJ* was also subcloned. When transformed into PAO238, the plasmid containing the unmutagenized *Not*I fragment conferred Tri^r but the vector control and the plasmid containing *mexJ*::Tn<TET-1> did not.

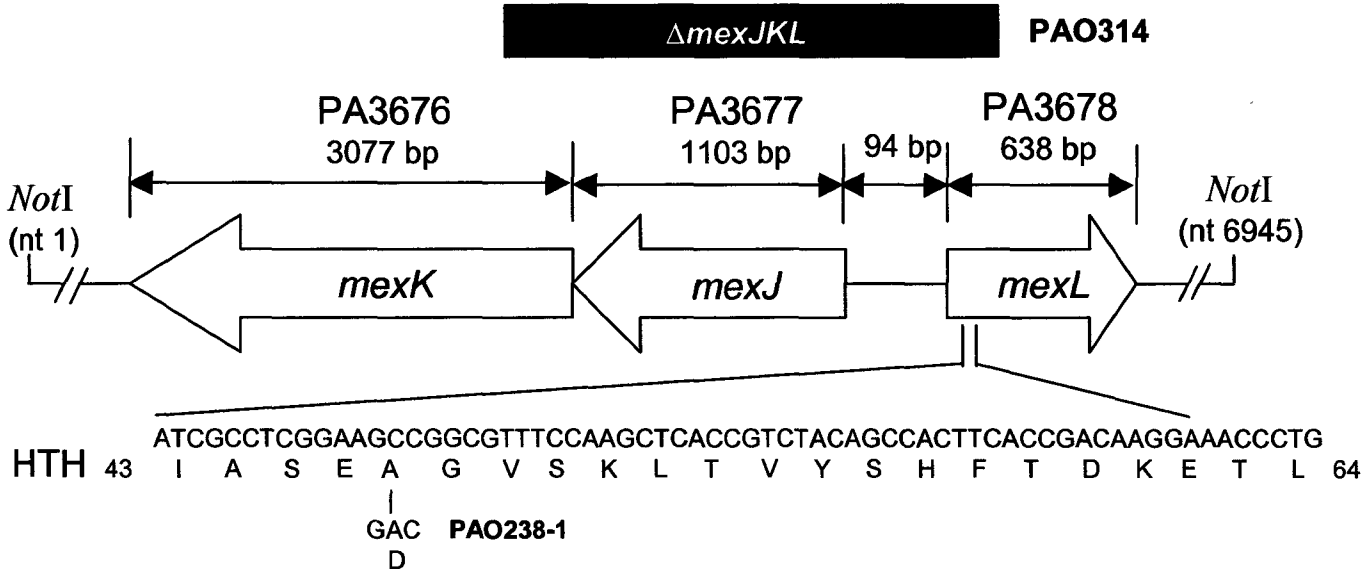

Figure 3.1. Organization of the *mexJK* operon and *mexL*, encoding a repressor of *mexJK* expression. The sizes of the genes and the corresponding PA annotations were taken from the published *P. aeruginosa* genome sequence (www.pseudomonas.com). The approximate extent of the *mexJKL* deletion in strain PAO314 is indicated by the black bar. The nucleotide and amino acid sequences of the predicted helix-turn-helix (HTH) DNA binding domain of MexL are also shown, including the single base change and resulting amino acid change in the *mexL* mutant PAO238-1.

3.4.3. A novel efflux system is responsible for triclosan resistance in PAO238-1. Nucleotide sequence analysis of one of the Tri^s transposon pJ22 insertion mutants revealed that it contained a single transposon insertion in a gene that was annotated as PA3677 in the published genome sequence (50). This gene (1,103 bp), which we named *mexJ* (figure 3.1), encodes the periplasmic MFP of a RND-type efflux system. The transposon in pJ22 is inserted between codons 110 and 111 of *mexJ*. Separated by 4 nt from *mexJ* and in the same transcriptional orientation is another 3,077 bp gene, *mexK*, which encodes PA3676, a putative transmembrane protein sharing significant homology with other inner membrane transporter proteins of RND type efflux systems of the acriflavine family. The proposed *mexJK* operon lies at 4.12 Mb on the PAO1 genome.

MexJ is 37-51% similar to MFPs from other *P. aeruginosa* RND efflux systems and most similar (51% and 48%) to PA156 and PA157, two MFPs shared by the same efflux pump with divalent cation transporter homology (50). Like other MFPs of the RND efflux protein family, MexJ is most likely a lipoprotein since it contains a signal peptidase II cleavage site (FLAACGNG) at the end of a 20 amino acid NH_2-terminal signal sequence. The role of acylation and membrane anchoring in MFP function remains unclear because many MFPs, including *P. aeruginosa* MexA (55) and *E. coli* AcrA (56), are functional as non-acylated proteins. The inner membrane transporter protein MexK is 41-64% similar to transporters from other *P. aeruginosa* RND systems and most related (64% similar) to PA158, the transporter component of an efflux system with divalent cation transporter homology. Based on amino acid comparisons, MexJK is thus most related to PA156-PA157-PA158 than to the other known or proposed RND-type drug efflux systems in this bacterium. Although PA156-PA157-PA158 and the related CzrCBA divalent cation efflux pump were previously excluded from the list of putative RND-type drug efflux systems (50), our data suggest that they are even more related to MexJK than some of the known Mex proteins and therefore should be included in this list.

To verify that the *mexJK* operon was indeed responsible for triclosan efflux in PAO238-1, a chromosomal *mexJ* mutant was constructed by transferring the *mexJ*::Tn<TET-1> to the PAO238-1 chromosome. The resulting mutant strain, PAO298, became Tri^s and behaved the same as the subsequently constructed Δ*mexJKL* mutant PAO314 (table 2).

3.4.4. The MexJK efflux system requires OprM for efflux of antibiotics but not triclosan. The molecular architecture of the MexJK operon is very similar to the MexXY system in that both systems do not contain their own Opr channel. Since it has been shown that MexXY requires OprM for antibiotic efflux (5, 25), we reasoned that PAO238-1 did not efflux antibiotics since it was lacking OprM. To test this hypothesis, PAO238-1 was electroporated with the OprM-expressing plasmid pPS1180 and its vector control, pVLT35. Only PAO238-1 containing pPS1180 effluxed tetracycline, erythromycin, ciprofloxacin (table 2), and to a lesser extent gentamycin and fusidic acid, but not carbenicillin, trimethoprim, dichlorophene and hexachlorophene (data not shown).

Because the *mexJK* overproducing strain PAO238-1 contains a wild-type *mexXY* operon, whose expression was recently shown to be inducible by several of its antibiotic substrates (25), we considered that inducible MexXY may have been at least partly responsible for the observed multidrug resistance phenotype observed in PAO238-1 expressing cloned OprM. To test this notion and to assess the true contribution of MexJK to multidrug resistance, we constructed three isogenic derivatives of PAO238-1, the Δ(*mexJKL*) strain PAO314 expressing only inducible MexXY, the Δ(*mexXY*) strain PAO327, which only expresses MexJK, and finally the Δ(*mexJKL*) Δ(*mexXY*) strain PAO325 expressing neither efflux system. These strains were then transformed with either the vector control pVLT35 or the OprM-expressing pPS1180 and MICs were determined for various drugs (table 2). As expected, PAO314 effluxed the known MexXY-OprM substrates ciprofloxacin, erythromycin, tetracycline and gentamycin (not shown) only when transformed with pPS1180 but did not efflux triclosan because it is not a MexXY-OprM

determined for various drugs (table 2). As expected, PAO314 effluxed the known MexXY-OprM substrates ciprofloxacin, erythromycin, tetracycline and gentamycin (not shown) only when transformed with pPS1180 but did not efflux triclosan because it is not a MexXY-OprM substrate (5). In contrast, PAO327 expressing OprM from pPS1180 no longer effluxed ciprofloxacin and gentamycin (not shown) but still effluxed tetracycline and erythromycin, albeit more weakly than PAO314. Erythromycin and tetracycline efflux in this strain was dependent on OprM. Levels of triclosan efflux were the same as those seen in PAO238-1 and were independent of OprM. Lastly, the Δ(*mexJKL*) Δ(*mexXY*) strain PAO325 neither effluxed triclosan nor any of the antibiotics tested. These results indicated that inducible MexXY, in concert with OprM, was mostly responsible for the multidrug resistance phenotype of PAO238-1, although MexJK contributed considerably to the triclosan, erythromycin and tetracycline resistance of this strain. Neither the MexJK nor the MeXY system did function with either OprJ or OprN, although the respective proteins were expressed in the cells used for MIC determinations, as indicated by Western blot analysis (data not shown).

The outer membrane channel requirement for MexJK was further assessed using 2-(4-diethylaminostyryl)-1-methylpyridinium iodide (DMP). This fluorescent probe fluoresces intensely when present in non-polar or hydrophobic environments but weakly in aqueous environments (31). As previously shown (31), DMP is efficiently extruded from cells by the MexAB-OprM system expressed in PAO1 (figure 3.2). Even cells overexpressing OprM in the absence of other known pump proteins (PAO314/pPS1180) showed some DMP efflux. Efficient extrusion of DMP via MexJK in PAO238-1 required OprM since a strain expressing MexJK alone (PAO238-1) behaved similarly to the Δ(*mexJK*) mutant PAO314. Although all of the cells studied for DMP efflux contained a functional MexXY system, its expression was not induced because the cells

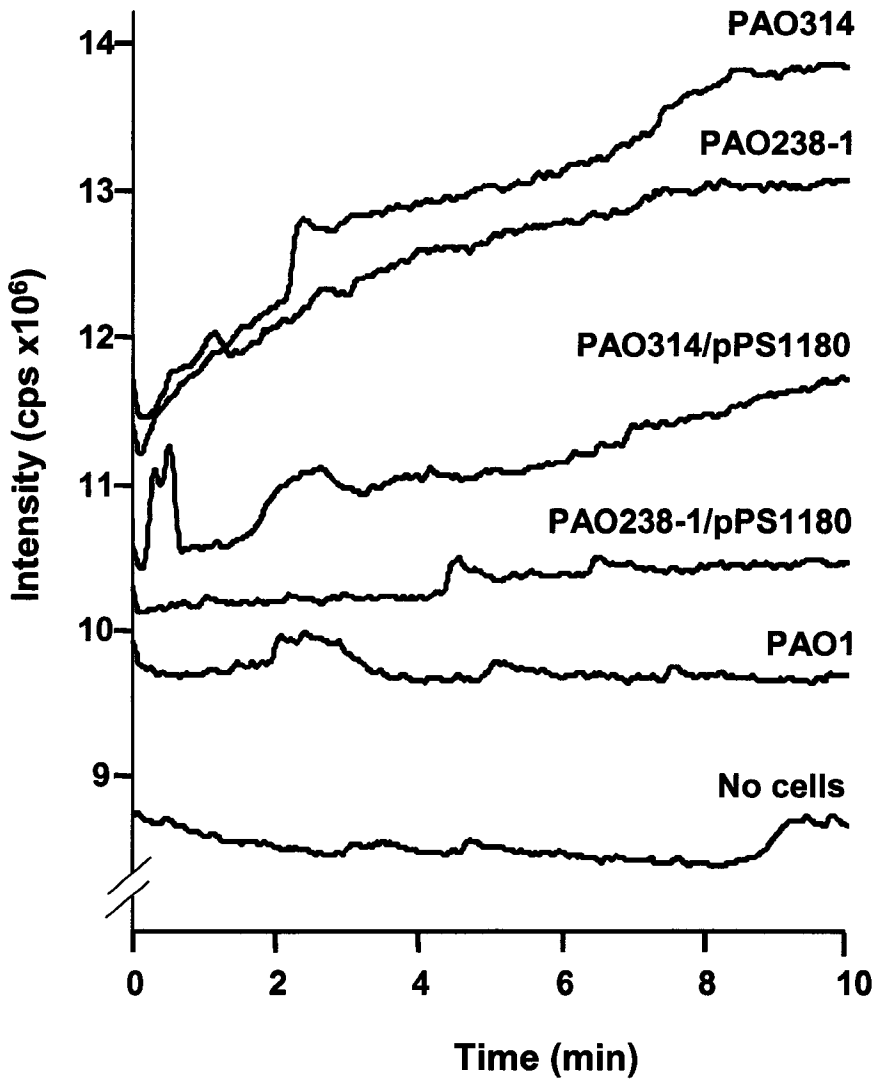

Figure. 3.2. Efflux of the fluorophore DMP from various mutant strains. The indicated strains were pregrown in M9 medium containing 1% thiamine and 0.2% casamino acids, then washed and adjusted to an optical density (600 nm) of 0.1. At T = 0 min, DMP was added to a final concentration of 25 μM and fluorescense was immediately recorded. The strains used were: PAO1, wild-type expressing MexAB-OprM; PAO238-1, a *mexL* mutant expressing MexJK; PAO314, Δ(*mexJKL*), expressing no efflux system. Strains containing pPS1180 express OprM.

3.4.5. MexJ and MexK are both required for a functional triclosan efflux system. Because MexJK effluxed triclosan independently of the expression of a known Opr channel, we reasoned that MexK may function in triclosan export independently of a MFP, similarly to AcrD, an RND aminoglycoside pump of *E. coli* (37). To test this hypothesis, plasmids overproducing MexJ and MexK were independently and in combination transformed into strain PAO325, which expresses no efflux pump, either because of deletion of the structural genes (MexAB-OprM, MexCD-OprJ, MexXY and MexJK) or because of a regulatory mutation (MexEF-OprN). PAO325 cells expressing MexJ or MexK alone did not efflux triclosan (table 3) and neither did uninduced cells cells containing both the MexJ- and MexK-expression plasmids. However, cells overproducing MexJ and MexK after induction with IPTG effluxed triclosan at levels observed in MexJK overproducing mutants PAO238-1 and PAO318 (table 2).

3.4.6. The *mexJK* operon is negatively regulated by the *mexL* gene product. The *mexJK* operon is separated by 94 bp from an upstream open reading frame, PA3678 or *mexL*, which is divergently transcribed from *mexJK* (figure 3.1). PA3678 was annotated as a putative transcriptional regulator of the tetracycline repressor (TetR) family since it contains the signature motif of this transcription regulator family. We therefore considered that *mexL* might encode a repressor of *mexJK* expression. If this were true, then *mexL* would most likely be mutated in PAO238-1 and on pJ22 but should be wild-type in PAO238. The *mexL* gene was PCR amplified from PAO238, PAO238-1 and pJ22. Whereas the *mexL* sequence from PAO238 was identical to the one established for PAO1, the *mexL* genes from PAO238-1 and pJ22 contained a single base change. This missense mutation replaced an alanine with a aspartic acid residue in the first helix of the putative helix-turn-helix domain (figure 3.1). The cloned *mexL* gene from PAO1 carried on pPS1153 restored Tri^s on PAO238-1 (table 2). Strain PAO318 containing a 371 bp chromosomal Δ*mexL*, which removes the helix-turn-helix coding sequence, constitutively expressed *mexJK*, as evidenced by high-level triclosan resistance (table 3.2) and high-level

expression of a *mexJ-lacZ* transcriptional fusion (figure 3.3). Cloned *mexL* restored triclosan susceptibility on PAO318 transformed with pPS1153 (table 3.2).

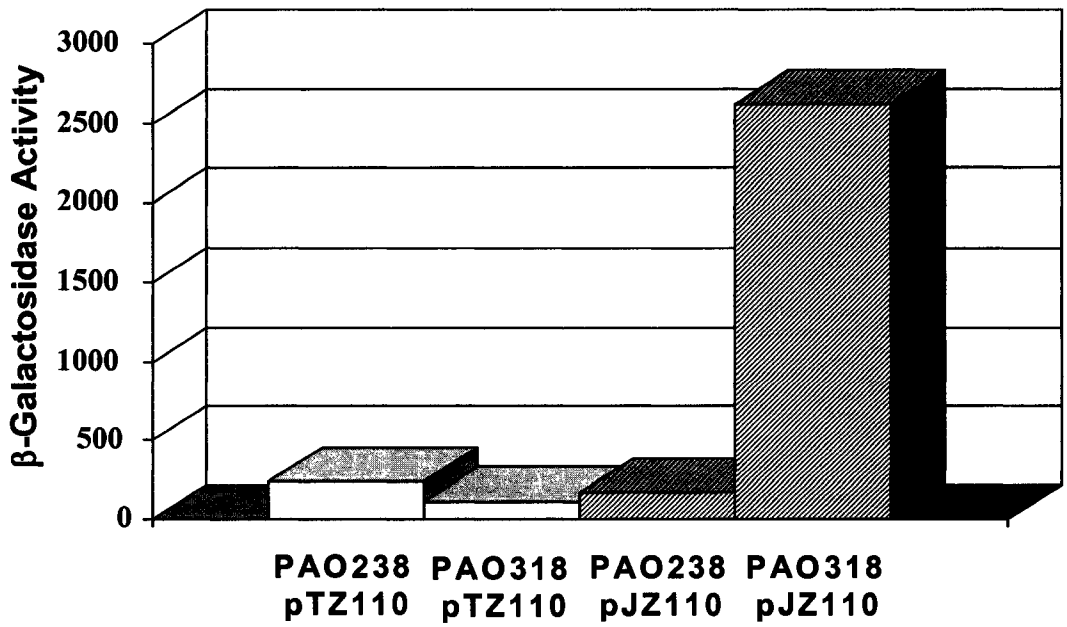

Figure 3.3. Repression of a *mexJ-lacZ* transcriptional fusion by MexL. Cells of strains PAO238 (*mexL* wild-type) or PAO318 (Δ*mexL*) containing a plasmid-encoded *mexJ-lacZ* transcriptional fusion (pJZ110) or the vector control (pTZ110) were grown to mid-log phase in LB medium supplemented with 100 μg/ml carbenicillin. β-Galactosidase actvity was measured in triplicate samples and the *t* distribution was used to establish 95% confidence limits.

3.5. DISCUSSION

Although *P. aeruginosa* expresses FabI, the primary target for triclosan (15, 17, 27), mounting evidence now suggests that its primary defense mechanism against this biocide seems to be efflux via multiple RND systems. We previously showed that MexAB-OprM (42), MexCD-OprJ and MexEF-OprN, but not MexXY-OprM (5), efflux triclosan. Using triclosan as a selective

tool, a novel efflux system, MexJK, was identified in *mexL* mutants in a Δ(*mexAB-oprM*) Δ(*mexCD-OprJ*) background. The frequency (10^{-8}) by which triclosan resistant mutants were obtained was about two orders of magnitude lower compared to the frequency obtained when plating a Δ(*mexAB-oprM*) strain on the same medium (5). This observation is in agreement with results obtained by Lomovskaya et al. (21) who showed that the consequence of deletion of multiple pumps is a significant decrease in the frequency of spontaneously resistant mutants. In addition to MexCD-OprJ, MexJK is the second *P. aeruginosa* efflux system whose expression could be selected by exposure to triclosan, indicating that this biocide is a powerful tool for the study of efflux systems. In addition, this finding further underscores the previously raised concern that the widespread use of this antiseptic may compound antibiotic resistance. Although the originally isolated strain, PAO238-1, showed a multidrug resistance phenotype in the presence of OprM, a more careful analysis revealed that it was mostly due to inducible MexXY expression (25). This finding illustrates the importance of performing efflux pump characterization experiments in a MexXY mutant background.

Our results confirmed that *mexJK* transcription is governed by an upstream regulatory gene, *mexL*. This situation is similar to other negatively regulated *P. aeruginosa* efflux operons containing the repressor gene located upstream of the efflux operon structural genes (51). The missense mutation in strain PAO238-1 probably generated a non-functional MexL since it replaces and alanine with a aspartic acid residue in the first helix of the putative helix-turn-helix domain (Figure 1). Since this mutation could be complemented by wild-type *mexL*, MexL is a transcriptional regulator of *mexJK* expression. The 94 bp *mexL-mexJ* intergenic region is the smallest regulatory region identified thus far in any of the characterized, negatively regulated efflux operons. By comparison, the *mexR-mexA* intergenic region is 273 bp long, the *nfxB-mexC* intergenic region 159 bp and the *mexZ-mexX* intergenic region 236 bp.

Although the bisphenols analyzed in this study were similar in structure and size, and both triclosan and dichlorophene were substrates of the MexAB-OprM system, only triclosan

was a bisphenol substrate of the MexJK system. Since the substrate specificity of efflux systems seems to reside with the inner membrane transporters (24, 49), the differences found in the structures of the bisphenols, i.e., methane versus ether linkage of the rings and number and position of chlorines, may be indicative of the interaction, or lack thereof, of these substrates with the cytoplasmic membrane transporters.

Most lipophilic and amphiphilic drugs can cross the cytoplasmic membrane spontaneously and their accumulation in the periplasm would accelerate their reentry into the cytoplasm. It has therefore been proposed that tripartite efflux systems, consisting of an inner membrane transporter, a periplasmic MFP and an Opr channel, are crucial for their expulsion all the way into the medium (57). However, of the 12 potential RND-type efflux systems encoded by the chromosome, only seven operons encode their own Opr channel (50). Since the MexAB-OprM system is always expressed at low levels and since *oprM* can even be transcribed at low levels independently of *mexAB* from its own promoter (58), it has been speculated that OprM may serve as a universal Opr channel for efflux systems lacking their own Opr component. Indeed, it has been shown that OprM can functionally link with MexCD (9), MexEF (24) and MexXY (5, 25, 29). Moreover, since the *mexXY* operon does not encode its own Opr it is dependent on OprM for function (5, 25, 29). In this study we show that MexJK, like MexXY, also requires OprM for efflux of its antibiotic substrates and the DMP fluorophore (Figure 2). However, OprJ and OprN did not link functionally with the MexJK complex. It is currently not known why OprM can function with all inner membrane transporter/periplasmic MFP complexes studied to date, while other Opr's are more discriminative. Whereas OprJ and OprN did not function with MexJK (this study), and OprN failed to interact with MexAB (24), OprJ restored almost complete function to a MexAB system without OprM (49).

Interestingly, MexJK-mediated triclosan efflux did not require OprM although it required both the MexK transporter proteins and the MexJ MFP. There are two possible explanations to rationalize these observations. (i) for triclosan efflux, MexJK may interact with

another, hitherto unknown, Opr channel. This Opr channel would probably not function in antibiotic efflux because of the OprM requirement for this process. Besides OprM, the only known Opr channel to be expressed in wild-type cells is the quorum sensing-regulated PA4208, which was shown to be expressed in stationary phase using gene fusions (53), as well as by transcriptional profiling (22). However, it is currently unknown whether this Opr channel functions with other efflux systems or what role, if any, it may play in the extrusion of antimicrobials from the cell. (ii) Alternatively, triclosan may be an exceptionally good substrate for MexJK and may therefore be efficiently removed from the cytoplasm independently of an Opr component. Thus, triclosan may either accumulate in the periplasm, similar to tetracycline expelled from the cytoplasm by the action of tetracycline efflux pumps (26), or it may be expelled into and/or through the outer membrane by an unknown mechanism. Because triclosan is an amphiphilic drug and its periplasmic accumulation would favor spontaneous reentry into the cytoplasm, one would have to favor the hypothesis that MexJK interacts with an Opr channel to expel triclosan completely from the cell, although it is puzzling why this channel would not function in the efflux of antibiotics. Alternatively, MexJ may play a more active role in depositing triclosan into or through the outer membrane because MFPs are seemingly highly asymmetric proteins capable of spanning the periplasm, as previously shown for AcrA from *E. coli* (56). Thus, we cannot rule out the possibility that a two-component RND efflux system may be sufficient for efflux of selected amphiphilic drugs. If this were the case, then RND efflux pumps could possibly be divided into three categories. (i) single component pumps consisting of an inner membrane transporter, as typified by AcrD of *E. coli*, for efflux of hydrophilic drugs; (ii) two-component pumps consisting minimally of an inner membrane transporter and a MFP for efflux of selected amphiphilic drugs, and (iii) tri-partite pumps consisting of an inner membrane transporter, a MFP and an Opr channel for efflux of most amphiphilic and lipophilic drugs.

3.6. ACKNOWLEDGMENTS

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CHAPTER 4

Purification and characterization of MexL, the tetrameric transcriptional repressor of the *mexJK* multidrug efflux operon in *Pseudomonas aruginosa*

Rungtip Chuanchuen, Jared B. Gaynor, and Herbert P. Schweizer

(Parts of this chapter will comprise a manuscript in preparation by Rungtip Chuanchuen, Jared B. Gaynor, and Herbert P. Schweizer)

I thank Jared B. Gaynor for construction of MexL-LexA-DBD fusions for dimerization studies of MexL. The research presented in this chapter will help to understand the mechanisms of regulation by MexL that is a representative of the repressors of the normally silent RND efflux systems.

4.1. ABSTRACT

Previous results demonstrated that the *P. aeruginosa mexJK* efflux operon is only expressed in mutants with defects in the upstream *mexL* gene, which encodes a probable repressor of the TetR family. Using *lacZ* gene fusions not only confirmed regulation of *mexJ* expression by

MexL but also revealed negative autoregulation of *mexL* expression. Repression of *mexJ-lacZ* expression was most profound in cells grown in minimal glucose medium and least obvious in LB-grown cells. MexL was overexpressed in *Escherichia coli* and purified as a fusion protein with a carboxy-terminal extension containing a hexahistidine-tag (His_6). MexL-His_6 was purified to near homogeneity by Ni^{2+} affinity chromatography. Analysis of MexL-His_6 by native polyacrylamide gel electrophoresis and size exclusion chromatography revealed its tetrameric nature in solution. The multimerization capability of MexL was confirmed using a genetic approach and these studies revealed that the mutant phenotype of a $MexL_{A47D}$ protein was not due to an oligomerization deficiency. This approach was also used to determine domains important for oligomerization of MexL. Gel mobility shift and footprinting assays demonstrated that MexL binds specifically to the *mexL-mexJ* intergenic region to sequences located between –84 and –20 from the *mexJ* initiation codon. MexL protected about 60 nucleotides on each strand and the protected regions overlapped almost perfectly, a finding consistent with MexL regulating the expression of both *mexL* and *mexJK*. The protected region contains predicted –10 and –35 promoters sequences for both *mexL* and *mexJ*, and the –10 regions for both promoters are predicted to overlap. The *mexL* promoter assignment was verified by mapping the *mexL* transcription start site. Using *lacZ* fusions, the *mexJ* promoter was localized to the predicted regions although repeated attempts aimed at identification of the exact *mexJ* transcription start site failed. The MexL protected region contains two inverted repeats of the GTATTT hexamer sequence, which could be recognized by MexL. Although this was not yet definitively proven, their location in the protected region and their overlap with the *mexL* and *mexJ* promoter sequences strongly supports their participation in MexL binding. In summary, the results confirmed at the molecular level that MexL is a transcriptional repressor of the *mexJK* operon and also of *mexL*. The results of the studies also laid the foundation and provided the molecular tools necessary for discovery and characterization of MexL effector (or *mexJK* inducer) molecules.

4.2. INTRODUCTION

Pseudomonas aeruginosa has become of special concern because of its high intrinsic antibiotic resistance and its ability to develop high-level multidrug resistance, especially during drug therapy. Such high resistance was shown to be mainly due to the synergy between a low permeability outer membrane and active efflux. Of the several families of multidrug resistance systems found in *P. aeruginosa*, members of the resistance nodulation and division (RND) family of efflux systems are clinically the most significant. To date, six of the RND efflux systems have been characterized in some detail, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, and MexVW (1, 4, 19, 21, 34, 35, 50). While only MexAB-OprM is always expressed at low but detectable levels, the expression of other RND efflux systems is tightly regulated (33). Recently, evidence was obtained that MexCD-OprJ is also inducible and expressed at low but detectable levels in wild type cells (27). The expression of many of these efflux systems is governed by regulators, which are encoded by genes that are physically linked to the operons. The overexpression of most efflux systems *in vitro* and *in vivo* is caused by mutations in regulatory genes, e.g., *mexR* (22, 36, 38), *nfxB* (30, 45), *mexL* (4), or *mexZ* (50), which are the repressors of the *mexAB-oprM*, *mexCD-OprJ*, *mexJK* and *mexXY* operons, respectively. Only the expression of *mexEF-OprN* is governed by a positive regulator, which is encoded by the upstream *mexT* gene (18). Interestingly, most PAO1 strains possess a *mexT* insertional mutation that blocks the expression of MexEF-OprN (24). In addition, other regulatory factors also have an affect on the expression of RND efflux systems, e.g., MexGHI-OpmD is regulated by the quorum sensing systems (1). To date, regulation of MexAB-OprM expression by MexR has been most extensively studied (10, 22). However, MexAB-OprM is probably an atypical example of an RND efflux systems because of its low-level constitutive expression. Some clinical isolates express MexAB-OprM although they do not contain *mexR* mutations (46). Regulation of MexAB-OprM is quite complex and other regulator(s) besides MexR are involved.

Two types of MexAB-OprM overexpressing mutants have been characterized. The *nalB* type mutants contain mutations in *mexR*, whereas *nalC* type mutants do not. Recently, it was found that *nalC* mutants contain mutations in PA3721, a transcriptional regulator of the TetR family. PA3721 is encoded in the same operon as PA3719 and PA3720, two genes encoding proteins of unknown function (15). The operon may function like *marRAB* in *E. coli* and its role as a global regulatory genes should not be overlooked (15). Additionally, *nalD* type mutants expressing MexAB-OprM without mutations in either *mexR* or PA3721 were isolated (15).

Previously. the *P. aeruginosa mexL* mutant, PAO238-1, was isolated by exposure of a susceptible mutant, PAO238, to the broad-spectrum biocide triclosan. This mutant overexpresses MexJK, presumably because it encodes an inactive MexL repressor due to a single nucleotide change in *mexL* that causes an alanine 47 to aspartate change in the putative helix-turn-helix motif of MexL. Our initial studies showed that *mexL* is located 94 bp upstream of and transcribed divergently from *mexJK*. MexL belongs to the TetR repressor family that includes MexZ, a regulator of *mexXY* in *P. aeruginosa* (2), AmrR a regulator of *amrAB-oprA* in *Burkholderia pseudomallei* (26), QacR a repressor of *qacA/qacB* in *Staphylococcus aureus* (11), AcrR a repressor of *acrAB* in *E. coli* (23, 48), MtrR a repressor of *mtrCDE* in *Neisseria gonorrhea* (13), and SmeT, a repressor of *smeDEF* in *S. maltophilia* (41). Most of the TetR family repressors have similar monomer molecular weights (21-25 kDa) (17). Transcriptional regulators of the TetR family were shown to self-assemble into multimers, bind to the operators and repress the transcription of the downstream operons, as well as autoregulate the expression of their own structural genes (12). Since MexJK is normally silent, it is anticipated that an understanding of MexJK regulation by MexL will give a clearer picture of the mechanism(s) of regulation of normally silent RND efflux systems. In this study, we purified and characterized MexL, and investigated the molecular mechanisms of regulation of *mexJK* and *mexL* expression by this repressor. Our data support the notion that MexL is a repressor of *mexJK* transcription. The

protein acted as a specific DNA-binding protein and exhibited features common to most members of the TetR family.

4.3. Materials and Methods

4.3.1. Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids that were used in this study are described in table 4.1. Luria-Bertani (LB) medium from Difco (Detroit, MI) was used as a growth media for all bacterial strains, unless otherwise stated. The minimal medium used was M9 medium (39) supplemented with 0.2% casamino acids (Difco, Detroit, MI) or 0.2% glucose (Sigma, St. Louis, MO). Cultures were routinely incubated at 37°C with shaking (250 rpm). Where necessary, antibiotics were used in growth media as follows: ampicillin, 100-150 μg/ml; tetracycline, 15 μg/ml for *Escherichia coli*; and for *Pseudomonas aeruginosa*, tetracycline, 10-15 μg/ml, and carbenicillin, 100-200 μg/ml. Antibiotics were purchased from Sigma, with the exception of carbenicillin which was purchased from Fisher Scientific. Triclosan was a gift from KIC Chemicals (Armonk, NY).

4.3.2. Antimicrobial susceptibility testing. MICs were determined by the two-fold broth microdilution technique according to National Committee for Clinical Laboratory Standards guidelines (28). The E-test system was employed only for ciprofloxacin and tetracycline, and was used according to manufacturer's instructions (AB Biodisk, Piscataway, NJ).

4.3.3. General DNA methodology. Routine DNA manipulations were performed as previously described (14, 40). Preparations of chromosomal and plasmid DNA were performed using the ISOQUICK Nucleic acid extraction kit (ORCA Research, Bothell, WA) and QIAprep® Mini-spin kit (Qiagen, Valencia, CA), respectively, and these kits were used according to

manufacturer's instructions. DNA fragments were blunt-ended in restriction enzyme buffers by addition of 0.25 mM dNTPs and 2.5 U of T4 polymerase (Invitrogen, Carlsbad, CA), followed by incubation at 37°C for 15 min. T4 polymerase was removed by using the PCR purification protocol (Qiagen). PCR primers used for amplification of various fragments are listed in table 4.2.

4.3.4. Construction of *mexJ-lacZ* fusions. Plasmids pPS1239 and pPS1240 contain *mexJ-lacZ* transcriptional fusions carrying the entire *mexL* gene and 113-bp of *mexJ* on fragments derived from PAO238-1 and PAO238, respectively. Plasmid pPS1239 was constructed as follows. The *mexL* gene and a partial *mexJ* gene were PCR amplified from PAO238-1 chromosomal DNA by using primers *mexL*-up and LJ5D (see table 4.2 for primer sequences). The PCR reaction (50 µl) contained 1 u of *Taq* DNA polymerase (Invitrogen), 30 pmoles of each primer, 5% (vol/vol) dimethyl sulfoxide (DMSO), 0.2 µM of each deoxynucleotide (dNTP), 1.5 mM $MgCl_2$, 0.5 µg chromosomal DNA, and 1 x PCR buffer (Invitrogen). Cycle conditions were 95°C for 5 min followed by 30 cycles of 95°C for 45 s, 65°C for 45 s, and 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. The resulting 954 bp PCR product was digested with *Sal*I and *Pst*I, and cloned between the *Xho*I–*Nsi*I sites of pTZ120. Similarly, pPS1240 was constructed by amplifying the *mexL* and partial *mexJ* region from PAO238 using the same primers, cycle conditions and cloning strategy.

4.3.5. Construction of a *mexL-lacZ* fusion. Plasmid pPS1237 carrying a *mexL-lacZ* transcriptional fusion was constructed by inserting the 632 bp *Xho*I–*Eco*RI fragment from pPS1176 between the same sites of pTZ110 such that the *mexL* promoter (P_{mexL}) drives transcription of *lacZ*.

Table 4.1. Bacterial strains and plasmids used in this study

Strain (plasmid)	Relevance genotype or characteristic	Source or reference
Pseudomonas		
PAO1	Prototroph expressing MexAB-OprM	(49)
PAO238	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) derivative of PAO1 (encodes wild-type *mexL*)	(4)
PAO238-1	Spontaneous Tri^r *mexL* derivative of PAO238 (encodes mutant *mexL* with A47D change)	(4)
PAO314	PAO238-1 with Δ(*mexJKL::FRT*)	(4)
E. coli		
HPS1	A cloning host F^-Δ(*lac-proAB*) *endA*1 *gyrA*96 *hsdR*17 *supE*44 *relA*1 *recA*1 *thi* Rif^r zzx::mini-Tn5Lac4	(43)
SU101	A reporter strain with a wild-type *lexA* operator sequence upstream of *sulA-lacZ* in the chromosome	(8)

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
Plasmids		
pADD948	Cb^r; broad-host-range *in vivo* cloning vector	(6)
pCR2.1	Ap^r; TA cloning vector	Invitrogen
pET-21b	Ap^r; T7 expression vector allowing construction of COOH-terminal His_6 fusion proteins	Novagen
pTZ110	Cb^r; broad-host-range *lacZ* transcriptional fusion vector	(44)
pTZ120	Cb^r; broad-host-range *lacZ* transcriptional fusion vector pTZ110 with *Nco*I and *Nsi*I restriction sites	This study
pRK415	Tet^r; broad-host-range cloning vector containing P_{lac} [a]	(16)
pSR658	Tet^r; wild-type LexA-DBD fusion vector	(8)

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1150	Cb^r; pBSP II SK(-) carrying the *mexL-mexJK* operon on a 6,945-bp *Not*I fragment from pJ22	(4)
pJ22	Cb^r; pADD948 with Tri^r determinant from PAO238-1 on an ~32-kb chromosomal DNA fragment	(4)
pPS1153	Cb^r; pUCP20T with 816 bp fragment *mexL* from PAO1	(4)
pPS1166	Ap^r; pCR2.1 with a 640 bp PCR fragment from pPS1150 carrying *mexL* from PAO238-1 (expressing MexL with A47D change)	This study
pPS1167	Ap^r; pET-21b with a 624 bp *Nde*I-*Not*I fragment from pPS1166. Source of MexL-His_6 with A47D change	This study
pPS1175	Cb^r; pBluescript SK(-) carrying all of *mexL* and 466 bp of the 5' end of *mexJ*	(4)
pPS1176	Cb^r; pPS1175 with a deletion of a 371-bp *Xho*I fragment internal to *mexL*	(4)

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1173	Ap^r; pCR2.1 with a 205 bp PCR fragment from PAO1 including DNA 165 bp upstream and 36 bp downstream of the *mexJ* start codon. Source of the entire 94-bp *mexL-mexJ* intergenic region	This study
pPS1188	Ap^r; pCR2.1 with a 173 bp PCR fragment from PAO1 including DNA sequences between –244 and –71 from the *mexJ* start codon	This study
pPS1190	Ap^r; pCR2.1 with a 154 bp PCR fragment from PAO1 including DNA 41 bp upstream and 113 bp downstream of the *mexJ* start codon	This study
pPS1190	Ap^r; pCR2.1 with a 154 bp PCR fragment from PAO1 including DNA 41 bp upstream and 113 bp downstream of the *mexJ* start codon	This study
pPS1191	Ap^r; pCR2.1 with a 181 bp PCR fragment from PAO1 including DNA 68 bp upstream and 113 bp downstream of the *mexJ* start codon	This study

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1201	Cb^r; pTZ120 with a 173 bp *Eco*RI-*Pst*I fragment from pPS1188; carrying DNA sequences located between 244 and 71 bp of the *mexL-mexJ* intergenic region.	This study
pPS1202	Cb^r; pTZ120 with a 154 bp *Sal*I-*Pst*I fragment from pPS1190, carrying DNA 41 bp upstream and 113 bp downstream of *mexJ* start codon	This study
pPS1204	Cb^r; pTZ120 with a 205bp *Xho*I-*Eco*RV fragment from pPS1173 containing the entire 94-bp *mexL-mexJ* intergenic region	This study
pPS1209	Cb^r; pTZ120 with a 203bp *Eco*RI-*Pst*I fragment from pPS1232 carrying DNA 90 bp upstream and 113 bp downstream of *mexJ* start codon	This study
pPS1210	Cb^r; pTZ120 with a 184 bp PCR fragment from pPS1233; carrying DNA 71 bp upstream and 113 bp downstream of the *mexJ* start codon	This study

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1216	Ap^r; pCR2.1 with a 640 bp PCR fragment containing *mexL* from PAO1	This study
pPS1217	Ap^r; pET-21b with a 624 bp *Nde*I-*Not*I fragment from pPS1216. The source of MexL-His_6	This study
pPS1232	Ap^r; pCR2.1 with a 203 bp PCR fragment from PAO1 including 90 bp upstream and 113 bp downstream of the *mexJ* start codon	This study
pPS1233	Ap^r; pCR2.1 with a 184 bp PCR fragment from PAO1 including 71 bp upstream and 113 bp downstream of the *mexJ* start codon	This study
pPS1236	Cb^r; pTZ120 with a 49 bp DNA fragment including DNA between –87 and -36 of the *mexL-mexJ* intergenic region	This study
pPS1237	Cb^r; pTZ110 with *Xho*I–*Eco*RI fragment from pPS1176 This *Xho*I-*Eco*RI fragment containing the 5' end of *mexL* and *mexJ*. The promoter of *mexL* is in the same orientation as P_{lac}.	This study

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1239	Cb^r; pTZ120 with the sequence containing the entire *mexL* and 113 bp from the *mexJ* start codon from PAO238-1	This study
pPS1240	Cb^r; pTZ120 with the sequence containing the entire *mexL* and 113 bp from the *mexJ* start codon from PAO238	This study
pPS1245	Tet^r; pRK415 with a 816 bp *mexL* fragment from pPS1153	This study
pPS1284	Ap^r; pCR2:1 carrying a 728 bp *mexL* fragment from pPS1153 (carries wild-type *mexL*)	This study
pPS1285	Ap^r; pCR2:1 carrying a 728 bp *mexL* fragment from pPS1175 (carries PAO238-1 mutant *mexL*)	This study
pPS1286	Tet^r; pSR658 carrying a 718 bp *Sac*I-*Kpn*I containing *mexL* from pPS1284	This study
pPS1287	Tet^r; pSR658 carrying a 718 bp *Sac*I-*Kpn*I containing *mexL* from pPS1285	This study

Table 4.1. Bacterial strains and plasmids used in this study (cont.)

Strain (plasmid)	Relevant genotype or characteristic	Source or reference
pPS1312	Tet^r; pPS1286 with a 736 bp *Xho*I deletion (carries 22 *mexL* codons)	This study
pPS1463	Ap^r, pCR2:1 with a PCR fragment containing 147 bp of *mexL*, the entire *mexL-mexJ* intergenic region, and 150bp of *mexJ* from PAO1	This study

Abbreviations: Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Gm^r, gentamycin resistance; Rif^r, rifampin resistant; Sm^r/Sp^r, streptomycin/spectinomycin resistance; Tri^r, triclosan resistance.

[a]P_{lac}, *E. coli lac* operon promoter.

Table 4.2. Primers used in this study

Primers	Sequences
mexL-pET21b-up	5'-TAAGCatATGTCAGAATCCACCTCC-3'
mexL-pET21down	5'-TCAGgCGgCCGCGGCGAAGGC-3'
LJ1U	5'-CTTGGgAAttCCGGCTTCCGAGGC-3
LJ2D	5'-CATCAcTgCAGTATTTCAATTGTATAAG-3'
LJ4U	5'-TACCGtCgAcTATAAGAACTCGAACGC-3'
LJ5D	5'-GGGCTGcAgGACGATCGCCGG AC-3'
LJ6U	5'-TGGACccATGGGTTCTGTATTTTTACT-3'
LJ7U	5'-GCGCgaATtCAATTGAAATACTGGACT-3'
LJ8U	5'-GCGCgaATtCAATTGAAATACTGGACT-3'
LJ9Ulinker	5'-AATTCCAATTGAAATACTGGACTGATGGGTTCTGTA TTTTTACTATACCGCCCAGA-3'
LJ9Dlinker	5'-AGCTTCTGGGCGGTATAGTAAAAATACAGAACCCATCAGTC CAGTATTTCAATTGG-3'
LJ11U	5'-AACGCCGGCTTCCGAGGCGATGGC-3'
LJ11D	5'-GGGAAAGGCCTGGCTCACCTCCCC-3'
LJ14U	5'-TCGAGAATGGCTTCCCGTTTGG-3'

Table 4.2. Primers used in this study

Primers	Sequences
LJ16D	5'-CTAGATGCATGCTCGAGCGGCCG-3'
NcoI/NsiI linker 1	5'- GATCCCATGGTTATGCATG-3'
NcoI/NsiI linker 2	5'-GATCCATGCATAACCATGG-3'
MexLF	5'-AGCGCgaGctcGAATCCACCTCCTCCGTC-3'
MexLR	5'-CGACGGgtACCTGGCGCCGCGCGG-3'

Some primers contain mismatches (indicated by lowercase letters) that introduced new restriction sites (underlined) after PCR amplification.

For complementation experiments, pPS1245 carrying the *mexL* coding region in the same orientation as P_{lac} was constructed as follows. Plasmid pPS1153 was digested with *EcoR*I, blunt ended, and then digested with *Sal*I. The *mexL* fragment was cloned into pRK415 obtained by digesting with *Hin*dIII, blunt ending and then digesting with *Sal*I.

4.3.6. Purification of MexL protein. To produce hexahistidine-tagged MexL (MexL-His_6), the *mexL* gene was amplified from *P. aeruginosa* PAO1 genomic DNA using primers *mexL*-pET21b-up, which anneals at the 5' end of *mexL* and incorporates a *Nde*I restriction site and *mexL*-pET21b-down, which anneals at the 3' end of *mexL* and incorporates a *Not*I restriction site, while removing the *mexL* stop codon. PCR conditions were as described above, except that the elongation step was for 45 s at 72°C. The PCR fragment was gel-purified and cloned into the

pCR2:1 vector from the TA-cloning kit (Invitrogen) according to manufacturer's instructions to generate pPS1216. The 630 bp *Nde*I-*Not*I fragment from pPS1216 was cloned into *Nde*I and *Not*I restricted pET-21b (Novagen) to produce pPS1217. To generate a clone expressing a histidine-tagged PAO238-1 mutant $MexL_{A47D}$ protein, the mutant *mexL* gene was amplified from pPS1150 and cloned into pCR2:1 to yield pPS1166. Then, a *Nde*I-*Not*I fragment containing mutant *mexL* was cloned between the same sites of pET-21b to yield pPS1312.

To overexpress and purify MexL-His_6, *E.coli* BL21(DE3) carrying plasmid pPS1217 was grown at 37°C overnight in LB broth containing ampicillin (100 μg/ml). The culture was diluted 1:10 into 250 ml of LB broth with 100 μg/ml ampicillin and incubated at 37°C until it reached an OD_{600} of 0.3-0.5 (approximately 1-2 h). Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation was continued for 3-4 h. Bacterial cells were harvested by centrifugation at 15,000x*g* at 4°C for 10 min. The pellet was resuspended in 20 ml MCAC0 (20 mM Tris-Cl [pH 7.9], 0.5 M NaCl) and lysed by passing through a French Pressure cell. Broken cells were centrifuged at 4°C at 34,000x*g* for 30 min. The supernatant was collected and treated with 2,500 u DNase I for 30 min on ice. MexL was purified by methyl chelation affinity chromatography on a 2 ml disposable column (Clontech) containing 1 ml-Ni^{2+} NTA agarose (31). The column was washed in a stepwise fashion with 10 ml each of MCAC20 (20 mM Tris-Cl [pH 7.9], 0.5 M NaCl, 20 mM imidazole) and MCAC40 (20 mM Tris-Cl [pH 7.9], 0.5 M NaCl, 40 mM imidazole). The MexL-His_6 protein was then eluted by the addition of 5 ml MCAC200 (20 mM Tris-Cl [pH 7.9], 0.5 M NaCl, 200 mM imidazole) and 1 ml fractions containing mostly MexL (>95% pure) were collected. The purity of eluted fractions was assessed by electrophoresis on a 0.1% SDS- 10% PAGE, followed by Coomassie blue staining. Fractions containing MexL were dialyzed overnight at 4°C using 1.5 l dialysis buffer (20 mM Tris [pH 7.5], 10% glycerol) which was changed once during the dialysis period. The purified MexL-His_6

protein was stored in dialysis buffer at –70°C. $MexL_{A47D}$-His_6 protein from PAO238-1 was prepared from pPS1312-containing BL21(DE3) cells using the same procedures.

4.3.7. Protein determination. Protein concentrations were determined by using the Bradford assay (BioRad, Hercules, CA). Bovine serum albumin (Sigma) was used as the protein standard.

4.3.8 Non-denaturing gradient polyacrylamide gel electrophoresis. The native molecular size of MexL-His_6 was estimated by nondenaturing gradient polyacrylamide gel electrophoresis (9). The gel contained a 5–20% linear gradient of polyacrylamide stabilized with a 0-20% linear glycerol gradient. Ten micrograms of each sample were mixed with an equal volume of loading buffer (100 mM Tris-HCl, [pH. 7.5], 40% glycerol, 2 mg/ml bromophenol blue) and loaded on the gel. The electrophoresis was carried out at 80 V for 16-20 h at room temperature. The native molecular weight of MexL-His_6 was estimated by comparison of its migration distance to those of the standard proteins, which were urease (272 kDa for its trimer and 545 kDa for its hexamer), bovine serum albumin (BSA) (66 kDa for its monomer and 132 kDa for its dimer), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). Standard proteins were purchased from Sigma. Each protein standard was reconstituted in 1 ml of 50 mM NaCl, 1 mM sodium phosphate [pH 7.0] to obtain a final concentration of 1 mg/ml, except urease, which was dissolved in 5 ml of water to obtain a final concentration of 0.2 mg/ml.

4.3.9 Size exclusion chromatography. Gel-filtration chromatography of MexL-His_6 was performed at 4°C as previously described (20) with some modifications. Dextran (Sephadex G200) was packed into a 1.5 cm x 50 cm column (BioRad, Hercules, CA) at a flow rate of 10.5 ml/h using an ECONO gradient pump (BioRad). The packed column was equilibrated with 0.02

M Tris HCl [pH 7.5], 1 mM EDTA [pH 8.0] and 0.1 mM DTT at the packing flow rate. Aliquots (500 µl) containing 1 mg/ml of protein, 25 µg/ml of pUCP20T DNA, and 1mM ATP were loaded and chromatographed with 0.02 M Tris HCl [pH 7.5], 1 mM EDTA [pH 8.0], 0.1 mM DTT and 15% glycerol using a flow rate of 7 ml/h. The absorbance (280 nm) of the effluent was monitored using an ECONO UV monitor (BioRad) and recorded utilizing a BioRad chart recorder. The standard proteins included β-amylase, bovine serum albumin (BSA), chicken ovalbumin, alcohol dehydrogenase, and carbonic anhydrase (Sigma). The elution volume (V_e) of each protein was determined using the time at which the sample peak (absorbance at 280 nm) was detected and the operating flow rate. The void (V_0) and total (V_t) volumes were determined using plasmid DNA and ATP, respectively. The K_{av} was determined by using the following calculation: $K_{av} = (V_e - V_0)/(V_t - V_0)$.

4.3.10. Cloning of portions of the *mexL-mexJ* intergenic region for gel mobility shift assay.

The various portions of the *mexL-mexJ* intergenics regions were PCR amplified from genomic DNA templates by using different pairs of primers as follows: LJ1U - LJ2D, LJ4U - LJ5D, LJ6U - LJ5D, LJ7U - LJ5D, LJ8U-LJ5D and mexLJ-up - mexLJ-down. Each primer contained base mismatch(es) that introduced a restriction site suitable for directional cloning (table 4.2.). The PCR fragments were cloned into pCR2:1 vector to yield pPS1188, pPS1190, pPS1191, pPS1232, 1233 and pPS1173, respectively (table 4.3). An additional fragment was obtained by hybridizing two oligonucleotides, LJ9Ulinker and LJ9Dlinker, encompassing nucleotides -36 to -87 relative to the *mexJ* start codon.

4.3.11. Linker tailing technique. Introduction of the *Nsi*I and *Nco*I recognition sites into the polylinker of pTZ110 was achieved as previously described (42). Briefly, 1-2 µg of pTZ110 DNA were digested with *Bam*HI. Simultaneously, 0.5 µg of each of NcoI/NsiI linker 1 and

NcoI/NsiI linker 2 in water were pre-hybridized by boiling for 1 min, followed by cooling to room temperature over a period of 2-3 h. The *Bam*HI digested pTZ110 was ligated with the pre-hybridized oligonucleotides for 4 h, and the ligation mixtures were then heated at 65-70°C for 5 min. The ligation mixture was then electrophoresed on a 1% agarose gel and the linear, tailed fragment gel-purified. A 50 µl sample of the tailed pTZ110 was resuspended in an equal volume of 2x hybrization buffer (200 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA), heated at 80°C for 5 min and allowed to cool to room temperature for 3 h. Then, 10-20 µl of the mixture was transformed into *E. coli* DH5α competent cells. Transformants were selected on LB agar plates containing 100 µg/ml ampicillin and 20 µg/ml Xgal. Plasmid DNA was isolated and the presence of the *Nsi*I and *Nco*I sites was verified by restriction enzyme digestion and DNA sequence analysis. The result plasmid was named pTZ120.

4.3.12. Construction of *mexJ-lacZ* transcriptional fusions for *mexJK* promoter mapping.

Various portions of the *mexL-mexJ* intergenic regions were fused to *lacZ* in pTZ120. To do this, pPS1188, pPS1190, pPS1173, pPS1232, and pPS1233 were digested with *Eco*RI+*Pst*I, *Sal*I+*Pst*I, *Xho*I+*Eco*RV, *Eco*RI+*Pst*I, and *Eco*RI+*Pst*I, respectively. The digested DNA fragments were purified from an agarose gel, and then ligated to pTZ120 digested with *Eco*RI+*Nsi*I, *Sal*I+*Nsi*I, *Xho*I+*Sma*I, and *Eco*RI+*Nsi*I to produce pPS1201, pPS1202, pPS1204, pPS1209, and pPS1210, respectively. For construction of pPS1236, the LJ9U and LJ9D oligonucleotide linkers with *Eco*RI and *Hin*dIII overhangs was tailed into the same sites of pTZ120 (table 4.3). The inserts were PCR amplified from each plasmid and their nucleotide sequences determined. The fusion plasmids were transformed into *P. aeruginosa* by electroporation (7). β-galactosidase activity was measured and activity units were then determined as described by Miller (25) (see 4.3.17). For complementation experiments, pPS1245 (*mexL*$^+$) was transformed into Δ(*mexLJK*) strain PAO314 carrying the respective fusion plasmids.

Table 4.3. Plasmids used as sources of DNA fragments for gel mobility shift assays and gene fusion for promoter mapping

Plasmid	Source of DNA fragments (primers)	Description
pPS1173	PCR (mexLJup-mexLJdown)	PCR from genomic DNA; contains region between –165 upstream and +36 downstream of the *mexJ* start codon [a]
pPS1188	PCR (LJ1U-LJ2D)	PCR from genomic DNA; contains region between -244 and –71 upstream of the *mexJ* start codon [a]
pPS1190	PCR (LJ4U-LJ5D)	PCR from genomic DNA; contains region between –41 upstream and +113 downstream of the *mexJ* start codon [a]
pPS1191	PCR (LJ6U-LJ5D)	PCR from genomic DNA; contains region between –68 upstream and +36 downstream of the *mexJ* start codon [a]
pPS1232	PCR (LJ7U-LJ5D)	PCR from genomic DNA; contains region between –90 upstream and +36 downstream of the *mexJ* start codon [a]
pPS1233	PCR (LJ8U-LJ5D)	PCR from genomic DNA; contains region between –71 upstream and +36 downstream of the *mexJ* start codon [a]
pPS1201	pPS1188	Subcloning of an *Eco*RI-*Pst*I fragment from pPS1188 between the *Eco*RI-*Nsi*I sites of pTZ120
pPS1202	pPS1190	Subcloning of a *Sal*I-*Pst*I fragment from pPS1190 between the *Sal*I-*Nsi*I sites of pTZ120
pPS1204	pPS1173	Subcloning of a *Xho*I-*Pst*I fragment from pPS1173 between the *Xho*I-*Sma*I sites of pTZ120
pPS1209	pPS1232	Subcloning of an *Eco*RI-*Pst*I fragment from pPS1232 between the *Eco*RI-*Nsi*I sites of pTZ120
pPS1210	pPS1233	Subcloning of an *Eco*RI-*Pst*I fragment from pPS1233 between the *Eco*RI-*Nsi*I sites of pTZ120
pPS1236	Hybridized oligonucleotides, (LJ9U-LJ9D)	Tailing of hybridized linkers between the *Eco*RI-*Hind*III sites of pTZ120

[a] See figures 4.10 and 4.11 for nucleotide coordinates.

The transformants carrying the two compatible plasmids were selected on LB agar plates with 100 μg/ml carbenicillin and 10 μg/ml tetracycline.

4.3.13. Construction of LexA-DBD-MexL fusions. Wild-type *mexL* was PCR amplified from pPS1153 by using primer MexLF (introducing a *Sac*I site and engineered to allow in-frame fusion of the MexL start codon to LexA) and primer MexLR (introducing a *Kpn*I site downstream of *mexL*). PCR conditions were as described above except that annealing temperatures of 83°C and 1 min extension times were used. The 728 bp PCR product was cloned into pCR2:1 to form pPS1284. This plasmid was then digested with *Sac*I and *Kpn*I and the 718-bp fragment cloned between the same sites of pSR658 to form pPS1286 (5). This plasmid encodes a MexL-LexA-DBD fusion protein of 312 amino acids (102 amino acids from LexA-DBD and 210 from MexL; note that methionine and serine, the first two amino acids of the 212 amino acid MexL protein were changed to glutamic acid and leucine). Digestion of pPS1286 with *Sac*II eliminated a 331 bp internal *mexL* fragment and self ligation yielded pPS1294. This plasmid encodes a fusion protein with 97 amino acids of MexL and 51 amino acids encoded by *mexL* downstream and vector DNA. Deletion of a 736 bp fragment by *Xho*I digestion removed 566 bp of *mexL* plus 170 bp of *mexL* downstream and vector sequences, and yielded pPS1312. This plasmid encodes a fusion protein containing 22 amino acids of MexL and 36 amino acids encoded by vector DNA. A mutant $MexL_{A47D}$-LexA-DBD fusion was constructed in the same fashion. PCR was performed by using pPS1175 as template, and the MexLF and MexLR primers. The 728 bp PCR product was cloned into pCR2:1 to form pPS1285. Subcloning of the pPS1285 into pSR658 then formed pPS1287.

4.3.14. DNA binding assay and measurement of binding affinity. To obtain templates for MexL binding assays, pPS1173, pPS1188, pPS1190, pPS1191 and pPS1232 were digested with *Eco*RI+*Xho*I, *Eco*RI+*Pst*I, *Sal*I+*Pst*I, *Nco*I+*Pst*I and *Eco*RI+*Pst*I, respectively. The *Eco*RI-*Xho*I fragment of pPS1173 is a 205 bp DNA fragment containing the 94 bp *mexL-mexJ* intergenic region. The fragments were gel purified, end-labeled with [α-^{35}S]dATP (3,000 Ci mmol^{-1}; PerkinElmer life and Analytical Sciences, Boston, MA) using Klenow fragment (Invitrogen), and purified by using the QIAquick® Nucleotide Removal Kit (Qiagen, Valencia, CA) (39). The end-labeled DNA fragments (~10,000 cpm) were incubated with purified MexL-His$_6$ (~4.2 μM) in a binding reaction containing 1x binding buffer (10 mM Tris [pH 7.5], 150 mM KCl, 20% glycerol, 2 mM EDTA [pH 8.0], 2 mM DTT) and BSA (66 ng/μl) for 15 min at room temperature (32). The binding mixtures were immediately loaded on a 4% non-denaturing polyacrylamide gel in 1x TG buffer (25 mM Tris [pH 8.3], 192 mM glycine) and electrophoresed at 160-165 volts for 1.5-2 h using the same buffer. The gels were dried under vacuum and labeled fragments were visualized by autoradiography. To determine the specificity of MexL, an excess of the same unlabeled 205-bp fragment from pPS1173 was included as competitor DNA.

To estimate MexL binding affinity, the labeled 205 bp DNA fragment from pPS1173 and the same experimental conditions described above were used except that the binding reactions were performed with increasing concentrations of MexL (0.5 μM, 1.0 μM, 2.0 μM, 3.1 μM, 4.1 μM, 5.2 μM, 6.2 μM, 7.2 μM and 8.3 μM). After autoradiography, amounts of the remaining free DNA fragments were estimated by scanning the autoradiograph and analyzing band intensities with Scion Image software (NIH Image 1.6). The values of free DNA fractions were plotted against the log [MexL] to generate a Bjerrum plot (51). The MexL concentration at which 50% of the MexL binding sites were occupied (K_d) was estimated from the plot. The relative constant (K_b) for MexL binding was determined from the fraction of DNA bound (f_{bound}) at a given protein concentration (P) in the linear range of the graph by calculation of $K_b = f_{bound}/[P\ (1- f_{bound})]$ as

previously described (37). For all calculations it was assumed that the MexL used in theses assays was 100% active.

4.3.15. DNase I footprinting assay. DNase I footprinting assays were carried out as described in the instructions of the SureTrack Footprinting kit (PerkinElmer life and Analytical Sciences) with some modifications. All solutions were prepared in the laboratory because the kit was no longer commercially available. For the *mexJ* coding strand, target DNA was the *Xho*I-*Not*I fragment (*mexL-mexJ* intergenic region) from pPS1173 labeled at the *Xho*I end. For the *mexL*-coding strand strand, target DNA was the *Xba*I fragment from pPS1173 that was end-labeled before digestion with *Bam*HI. The fragments were end-labeled with [α-^{35}S]dATP as described above. The labeled DNA fragments (~100,000 cpm) were incubated with purified MexL-His_6 at concentrations of 5, 7.5 and 10 μM in 50 μl reactions containing gel mobility shift assay buffer (10 mM Tris-HCl [pH 7.5], 150 mM KCl, 20% glycerol, 2 mM EDTA [pH 8.0], 2 mM DTT) and BSA (66 ng/μl). The mixtures were incubated for 15 min at room temperature. An ice-cold Ca^{2+}/Mg^{2+} solution (5 mM $CaCl_2$ and 10 mM $MgCl_2$) was added to achieve final concentrations of 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$, respectively. The mixtures were incubated for 1 min at room temperature. After addition of 2 μl of 0.0156 u/μl DNase I (Invitrogen) in Ca^{2+}/Mg^{2+} solution, the mixtures were incubated for 2 min at room temperature and the reactions were stopped by addition of 140 μl Dnase I stop solution (192 mM sodium acetate [pH 5.2], 32 mM EDTA [pH 8.0], 0.14 % SDS, and 64 μg/ml yeast tRNA). The mixtures were subjected to phenol:chloroform extraction (1:1. vol/vol) and ethanol precipitation. The pellets were washed successively with absolute ethanol and 70% ethanol, and then resuspended in 3 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The DNA fragments were heated at 80°C for 3 min, quick cooled on ice, and immediately loaded on a 5% (wt/vol) polyacrylamide-8 M urea sequencing gel. Labeled DNA fragments were visualized

by autoradiography. Chain-termination sequencing reactions using the same labeled templates were performed by using the Sequenase Version 2.0 DNA sequencing kit (Amersham Bioscience) as described by the manufacturer. Primers LJ14U and LJ16D were used for sequencing of the *mexL* and *mexJ*-coding strands, respectively.

4.3.16. RNase protection assay. RNase protection analysis was performed as previously described (3, 29). Briefly, total RNA was isolated from *P. aeruginosa* PAO318 by the hot phenol method followed by DNase I treatment. A 20 μg sample of total RNA was ethanol-precipitated and resuspended in 25 μl of hybridization buffer (40 mM piperazine-*N*,*N*'-bis[2-ethanesulfonic acid], PIPES [pH 6.4], 400 mM sodium acetate [pH 7.0], 1 mM EDTA, 80% deionized formamide). A DNA fragment containing the *mexL* promoter was amplified from PAO1 genomic DNA by using primer LJ11U annealing 147 bp downstream of *mexL* start codon and primer LJ11D annealing 150 bp downstream of the *mexJ* start codon. The purified DNA fragment was cloned into pCR2:1 in such away that MexL transcription is opposite to the T7 promoter. The resulting plasmid, pPS1463 was digested with *Bam*HI and used for the synthesis of a ^{32}P *mexL* riboprobe by using the Riboprobe System-T7 (Promega, Madison, WI) following the manufacturer's instructions. The 20 μl labeling reaction containing 1-2 μg linearized DNA template; 1x transcription-optimized buffer; 50 μCi of [α–^{32}P]CTP (PerkinElmer life and Analytical Sciences); 20 u RNasin ribonuclease inhibitor; 10 mM DTT; 0.5 mM of each ATP, GTP and UTP; 12 μM CTP; and 10 u T7 RNA polymerase was incubated at 37°C for 1 h. After addition of 20 μg of yeast tRNA, the labeled riboprobe transcripts were treated for 15 min at 37°C with 1 u of RNase-free DNase I per μg of RNA template. Sixty μl of TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) was added to bring to the volume to 100 μl and the labeled RNA was then purified by phenol-chloroform-isoamyl alcohol extractions. To remove unincorporated nucleotides, 0.5 volume of 7.5 M ammonium acetate and 3 volumes of absolute ethanol were

added and mixed, and the mixture was placed at -70°C for 30 min. The riboprobe was collected by centrifugation for 5 min at 12,000x*g* at 4°C. The supernatant was removed and the riboprobe was dissolved in 25 µl of hybridization buffer (80% formamide, 40 mM PIPES [pH 6.4], 1 mM EDTA, 0.4 M NaCl). Approximtely 500,000 cpm of the labeled RNA probe were added to 25 µl of RNA preparation and hybridized overnight at 65°C. Next, RNA digestion reaction was performed with 40 µg/µl RNaseA and 500 u RNase T_1 at 37°C for 30 min. The reaction was stopped with 0.6% SDS and 300 µg/µl proteinase K by incubation at 37°C for 30 min. The digested product was subjected to phenol:chloroform extraction and ethanol precipitation. The pellets were dissolved in 8 µl of loading dye solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The RNase digested product was loaded on a 8 M Urea/5% polyacrylamide gel, electrophoresed and the labeled DNA fragments were visualized by autoradiography. A sequencing ladder was obtained using the chain-termination method performed by using LJ11U as the primer, pPS1463 as the template and the Sequenase Version 2.0 DNA sequencing kit. The sequencing reactions were run on the same gel as the RNase digested products. The integrity of total RNA was confirmed by RNase protection analysis using a riboprobe specific for the constitutively expressed *omlA* gene (29).

4.3.17. β-galactosidase activity assay β-galactosidase activity was measured in whole cells as previously described (25). To do this, 0.1 ml of cells corresponding to an OD_{540} 0.6 to 0.8 added into 0.9 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). Before use, 270 µl of β–mercaptoethanol (14.2 M) was added into 100 ml Z-buffer to obtain a final β–mercaptoethanol concentration of 38 mM. The cells were permeabilized by adding 10 µl of 0.1% SDS and 10 µl of chloroform, followed by vortexing at full speed for 10 s. Reactions were initiated by adding 200 µl of 4 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG) to the SDS-chloroform-treated cell samples. β-galactosidase activity was assayed at room temperature and

stopped by adding 500 µl 1 M Na_2CO_3. After removing cells by centrifugation in a microfuge for 2 min, the absorbances of supernatants were measured at 420 nm and β-galactosidase activity was calculated using the formula: β-galactosidase activity = 1000 x OD_{420}/ OD_{540} x T x V, where T is the reaction time (min) that yields sufficient yellow color and V is volume of cells assayed (ml). The activity is reported in Miller units.

4.4. Results

4.4.1. MexL is a repressor of *mexJK*. It was peviously shown (see chapter 3), that cloned *mexL* was able to repress *mexJ-lacZ* expression. Here, we fused the *mexL-mexJ* intergenic region to *lacZ*, with either a wild-type or a mutant *mexL* gene present on the same constructs. When transformed into a *mexL* mutant strain, β-galactosidase activity in cells containing pPS1239 carrying the mutant *mexL* gene from PAO238-1 was generally higher than those in cells carrying pPS1240 with wild-type *mexL* (figure 4.1). The degree of repression was dependent on the growth medium and was most pronounced (15-fold) in cells grown in M9-glucose medium. Repression was most modest in LB-grown cells. These results demonstrated that MexL is indeed a repressor of the *mexJK* operon.

4.4.2. MexL expression is autoregulated. Since MexL is a member of the TetR family of repressor proteins and several of these are subjected to autoregulation, it was ascertained if *mexL* expression is regulated by MexL. To do this, two experimental approaches were used. First, pPS1237 carrying a *mexL-lacZ* transcriptional fusion and pPS1245 containing the wild-type *mexL* gene under P_{lac} control were co-transformed into *E. coli* HPS1 and β-galactosidase activity was determined in the presence and absence of 1 mM IPTG. The expression of *mexL-lacZ* was repressed 2-fold in *E. coli* cells that co-expressed MexL (figure 4.2). Second, the same two

plasmids were co-electroporated into Δ(*mexLJK*) strain PAO314 and β-galactosidase activity was measured. In the presence of MexL, expression of *mexL-lacZ* fusion was reduced ~10 folds in *P. aeruginosa* PAO314. These data indicated that MexL negatively autoregulates its own expression in both *P. aeruginosa* and *E. coli*.

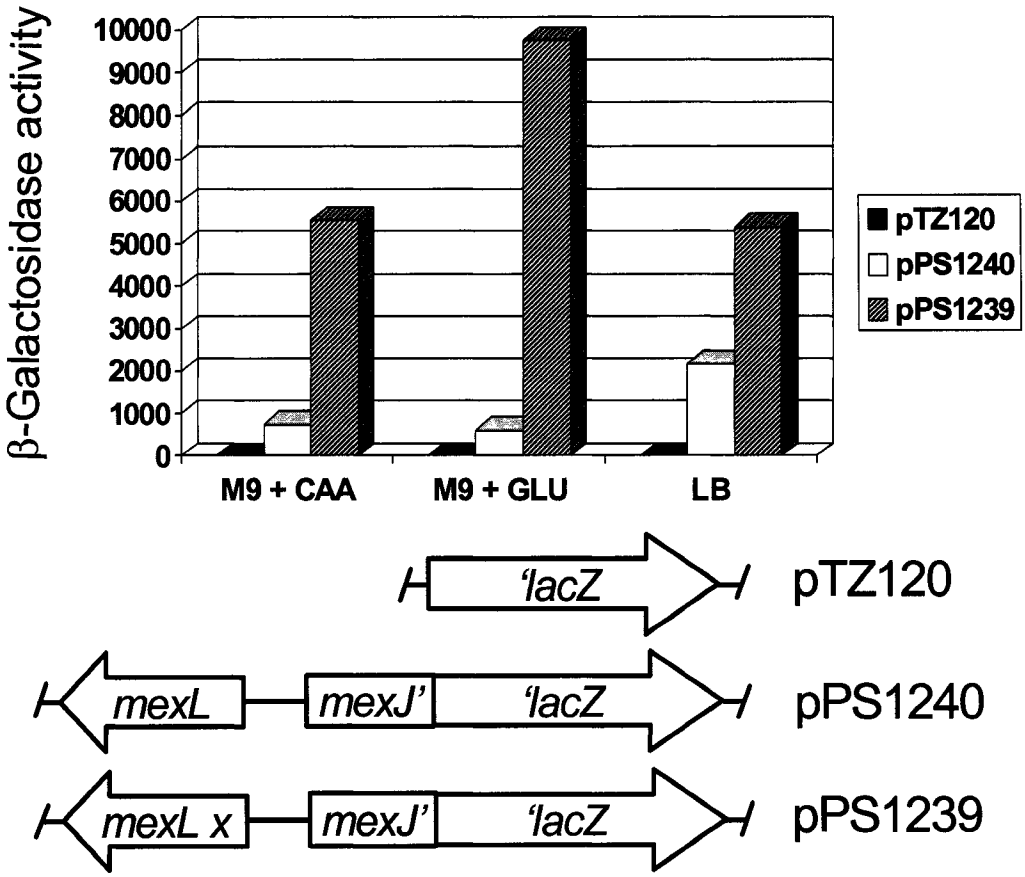

Figure 4.1. Repression of *mexJ-lacZ* expression by MexL. Plasmid pPS1240 contained wild-type *mexL*, the *mexL-mexJ* intergenic region and the *mexJ* 5' end. Plasmid pPS1239 contained the same DNA fragment but it was derived from PAO238-1 and thus contains a mutated *mexL* gene (indicated by the x). The plasmids were electroporated into Δ(*mexLJK*) strain PAO314 and β-galactosidase activities were measured in log-phase cells grown in the indicated media. Abbreviations: CAA, casamino acids; GLU, glucose.

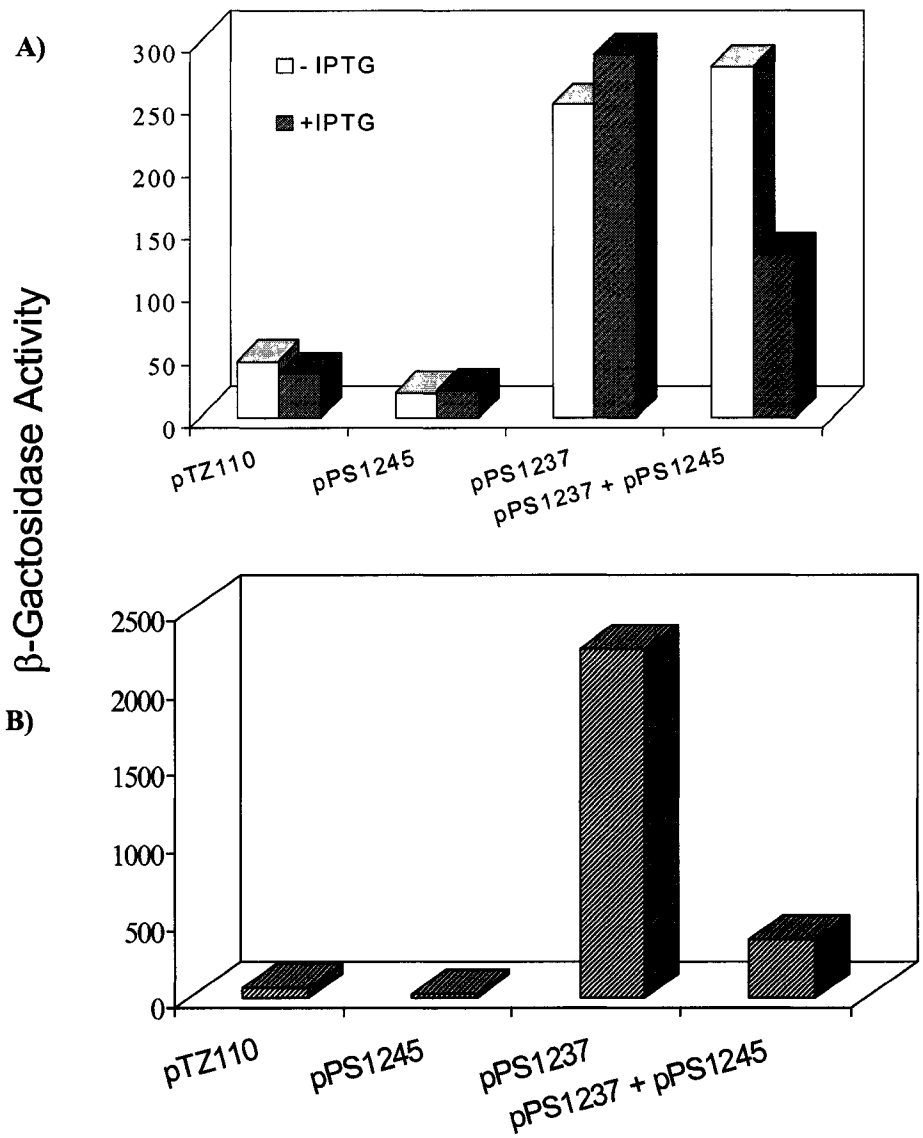

Figure 4.2. Autoregulation of *mexL* expression. Effect of MexL on *mexL-lacZ* expression was monitored **A)** in *E. coli* and **B)** in *P. aeruginosa*. **A)** *E. coli* strain HPS1 was transformed with the indicated plasmids and β-galactosidase activity was measured in cells where *mexL* expression from pPS1245 was either uninduced (-IPTG) or induced (+IPTG). The empty *lacZ* fusion vector pTZ120 and pPS1245 alone served as controls. **B)** β-Galactosidase expression was measured in cells of *P. aeruginosa* strain PAO314 (Δ[*mexLJK*]) containing the indicated plasmids. Cells were grown in M9 glucose.

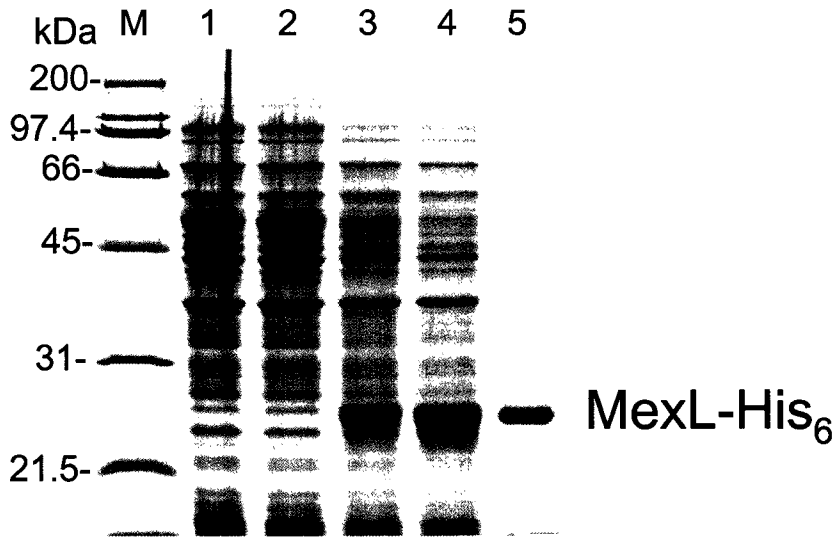

Figure 4.3. Overexpression and purification of MexL-His_6. MexL was overexpressed with a carboxy-terminal hexahistidine-containing extension in *E. coli* BL21(DE3). The expressed MexL-His_6 fusion protein was purified by Ni^{2+} NTA affinity chromatography and analyzed by SDS-PAGE. The gel was stained with Coomassie blue. Lane M, marker proteins (top-to-bottom: myosin, phosphorylase b, bovine serum abumin, ovalbumin, carbonic anhydrase, trypsin inhibitor); lane 1, uninduced cells containing pET-21b; lane 2, induced cells containing pET-21b; lane 3, uninduced cells containing pPS1216 expressing MexL-His_6; Lane 4, induced cells containing pPS1216; lane 5, purified MexL-His_6.

4.4.3. Purification of MexL. To study repressor action at the molecular level, an attempt was made to purify MexL. To facilitate purification, a MexL-His_6 fusion protein expression construct was derived, which allowed affinity purification of MexL with a carboxy-terminal hexahistidine by Ni^{2+} affinity chromatography (figure 4.3.). As judged by SDS-polyacrylamide gel electrophoresis, the MexL preparation contained a major protein of ~25,000 Da, which is close to the 24,242 Da calculated for the 220 amino acid fusion protein monomer. Purity was estimated at >95%.

4.4.4. Native MexL is a tetrameric protein. MexL belongs to the TetR family of proteins and members of this protein family function as multimeric proteins. To determine if MexL oligomerizes, the molecular weight of MexL-His_6 in solution was determined by native PAGE and size exclusion chromatography (figure 4.4). On native PAGE, the native size of MexL-His_6 was estimated at 97,600 Da and by gel filtration its native size was indicated as 83,500 Da. Both of these values are consistent with native MexL-His_6 being a tetrameric protein, with a calculated molecular weight of 96,968 Da.

4.4.5. Genetic evidence for MexL multimerization. Since the biochemical experiments indicated that MexL is a tetrameric protein, an attempt was made to verify this genetically by using the LexA-based system devised for this purpose (5, 8). The principle of this system lies in the fact that although a truncated LexA consisting of only the DNA binding domain (DBD) can recognize its operator sequence, it is functional as a transcriptional repressor only in dimeric form. Other domains can be fused in frame to the LexA-DBD and will restore the repressor's function if these domains interact. To this end, the entire MexL protein coding sequence was fused in-frame to LexA-DBD. The resulting plasmid construct, pPS1286, along with the vector control encoding only the LexA-DBD, was transformed into *E. coli* strain SU101, which contains a chromosomally integrated *sulA-lacZ* fusion, whose expression is under LexA control. Expression of LexA-DBD or LexA-DBD-MexL was induced by IPTG addition and the β-galactosidase activity of the *sulA-lacZ* reporter was measured. As a positive control a LexA-DBD-NeuD construct was used since NeuD, a protein involved in polysialic acid synthesis in *E. coli*, is known to mutlimerize in this system (5, 8). Induced cells expressing only LexA-DBD (pSR658) exhibited high levels of β-galactosidase activity since LexA-DBD cannot multimerize, and thus not bind to the *sulA* promoter region and repress *sulA-lacZ* transcription. In contrast, cells containing the positive control pSR658*neuD* expressing LexA-DBD-NeuD expressed lower

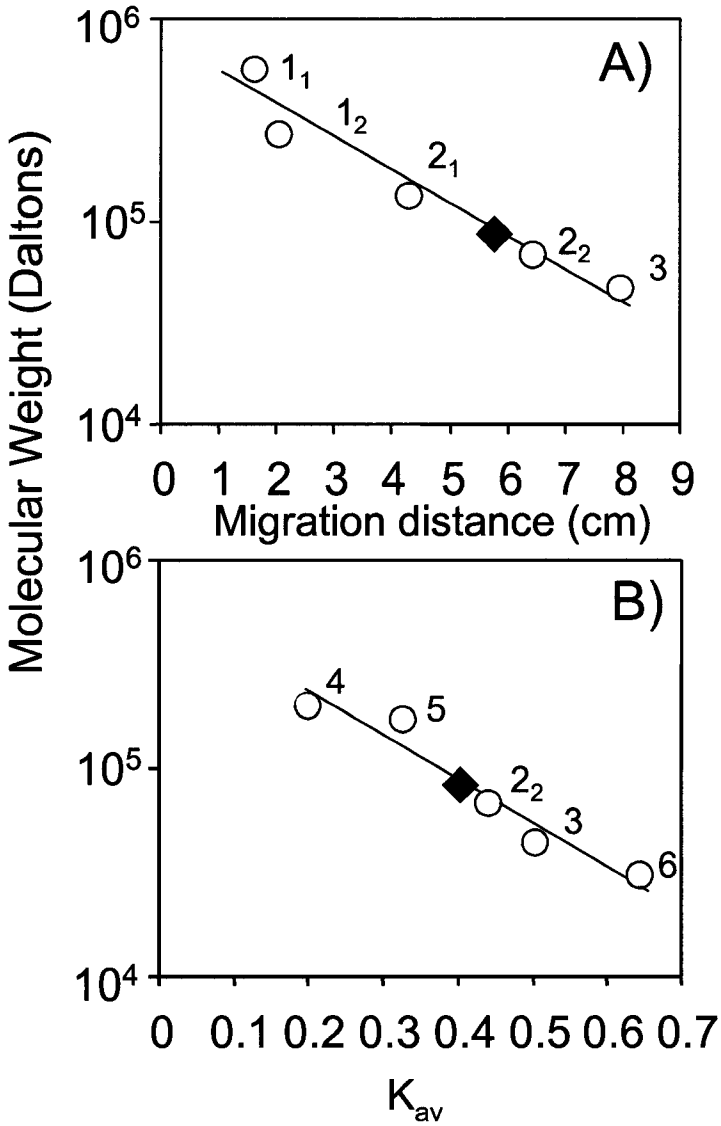

Figure 4.4. Native molecular weight of MexL-His_6. **A)** Native gradient polyacrylamide gel eletrophoresis. The standard proteins and their molecular weight (x 10^3) in daltons were: 1_1, the dimer of urease (545 kDa); 1_2, the monomer of urease (272 kDa); 2_1, the dimer of bovine serum albumin (132 kDa); 2_2, the monomer of BSA (66 kDa); and 3, chicken ovalbumin (45 kDa). The relative migration of MexL is indicated by the diamond. **B)** Size exclusion chromatography on Sephadex G-200. The numbers and symbols refer to some of the same proteins employed in **A)**. The additional standard proteins were: 4, β-amylase (202 kDa); 5, alcohol dehydrogenase (167 kDa); and 6, carbonic anhydrase (29 kDa). The diamond marks the K_{av} of MexL-His_6.

(repressed) levels of β-galactosidase. Levels of β-galactosidase in IPTG-induced SU101 cells containing either pPS1286 (LexA-DBD-MexL) or pPS1287 (LexA-DBD-$MexL_{A47D}$) were both greatly reduced indicating that both forms of MexL can assist in LexA-DBD multimerization. β-Galactosidase activity levels in uninduced cells containing the various plasmids were similar to those of uninduced cells containing pSR658 (data not shown). An attempt was made to roughly define regions involved in MexL multimerizaton. To this end, various portions of the MexL coding sequence were deleted. Two plasmids, pPS1294 and pPS1312 containing the first 97 or 22 MexL residues, respectively, were constructed and transformed into SU101. Although cells containing either plasmid still exhibited reduced levels of β-galactosidase activity, activity levels were substantially higher than in cells expressing a LexA-DBD fusion protein containing a full length MexL. These results indicated that although the first 97 residues of the 212 amino acid MexL protein can assist in its multimerization, efficient oligomerization requires additional residues located in the second half of the protein (figure 4.5).

4.4.6. MexL binds specifically to the *mexL-mexJ* intergenic region. To demonstrate that MexL binds to the *mexL-mexJ* intergenic region in a specific manner, gel mobility shift assays were performed using purified MexL-His_6. As evidenced from the results presented in figure 4.6, panel A, purified MexL-His_6 shifted an end-labeled 205-bp DNA fragment containing the 94 bp *mexL-mexJ* intergenic region. MexL-His_6 binding to this fragment was specific since the band shift was not observed in the presence of excess competitor DNA (same unlabeled 205 bp fragment). When the same labeled DNA fragment was incubated with $MexL_{A47D}$-His_6 mutant protein, no band shift was observed (panel B, lane 1) demonstrating that the A47D mutation indeed adversely affects the DNA binding capacity of the mutant protein. From figure 4.6, panel B, it is also evident that a fragment containing DNA from -97 to +113 (relative to the first nucleotide of the *mexJ* ATG start codon) showed the same band shift pattern as that observed

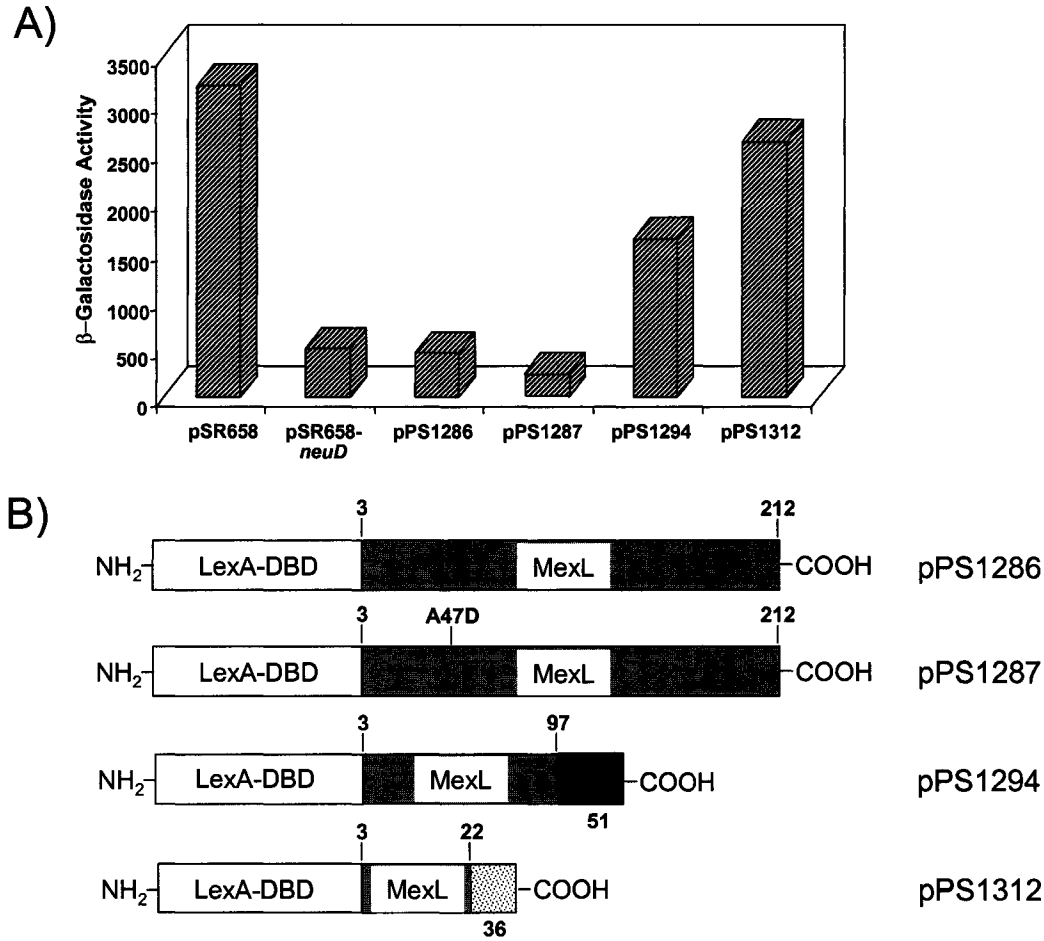

Figure 4.5. Genetic evidence for multimerization of MexL. **A)** Plasmids containing the LexA-DBD-MexL fusions shown in **B)** were transformed into *E. coli* SU101 carrying *sulA-lacZ* on the chromosome and β-galactosidase activity was measured in IPTG-induced cells. The negative control was pSR658 expressing LexA-DBD and the positive control was pSR658*neuD* expressing LexA-DBD-NeuD. **B)** Schematic representation of domains carried by various LexA-DBD-MexL expressing plasmids (note that the first residues of native MexL were changed during vector construction to allow in-frame fusion of the protein with LexA-DBD). Small numbers above the cross-hatched boxes indicate numbers of MexL residues contained in the respective fusion proteins. The position of the A47D change in the mutant MexL protein is also indicated. Black and stippled boxes indicate amino acids encoded by *mexL* down stream and plasmid sequences.

with the pPS1173 insert. To roughly localize the the MexL binding (operator) sites, PCR fragments containing various portions of the *mexL-mexJ* intergenic regions were generated and used as templates in band shift assays. These experiments (data not shown) localized the MexL

operator(s) to a region located between -90 and -41 upstream of *mexJ*. This was further verified by end-labeling a synthetic 51-bp oligonucleotides encompassing the –86 and –37 region from the *mexJ* start codon and use it in the band shift assay (figure 4.6, lanes labeled oligo). It is evident that MexL-His_6 band-shifted the oligonucleotide, although the shift was not as complete as those observed with the pPS1173 and pPS1232 inserts. This was probably due to incomplete labeling of the oligonucleotide leaving unlabeled "competitor DNA".

The affinity of MexL binding was estimated using a Bjerrum plot (figure 4.7). Using the data derived from this experiment, the apparent K_d was estimated to be 9×10^{-7} M and the relative binding constant (K_b) was estimated to be 10^{-7} M^{-1}.

4.4.7. Identification of the MexL operator sites. A closer analysis of the *mexL-mexJ* intergenic region (figure 4.10) revealed two inverted GTATTT repeats that are separated 16 nucleotides. To more precisely map the MexL operator sites with respect to these inverted repeats, the *mexL-mexJ* intergenic region was ended labeled on either the *mexL* or *mexJ*-coding strand and subjected to Dnase I footprinting. The two strands were analyzed because MexL

strand and subjected to DNase I footprinting. The two strands were analyzed because MexL expression is autoregulated and one would therefore expect MexL binding sites(s) on the *mexL* coding strand. The DNase I footprinting profile revealed a single protected area on either strand (figures 4.8 and 4.9) and these two protected areas overlapped almost perfectly. Whereas the

protected area on the *mexJ*-coding strand was located between -22 and -84 upstream of the *mexJ* start codon, that of the *mexL*-coding strand was situated between -20 and -81. The protected area encompassed the two inverted repeats, as well as the predicted -35 and -10 regions of the *mexJ* and *mexL* promoters.

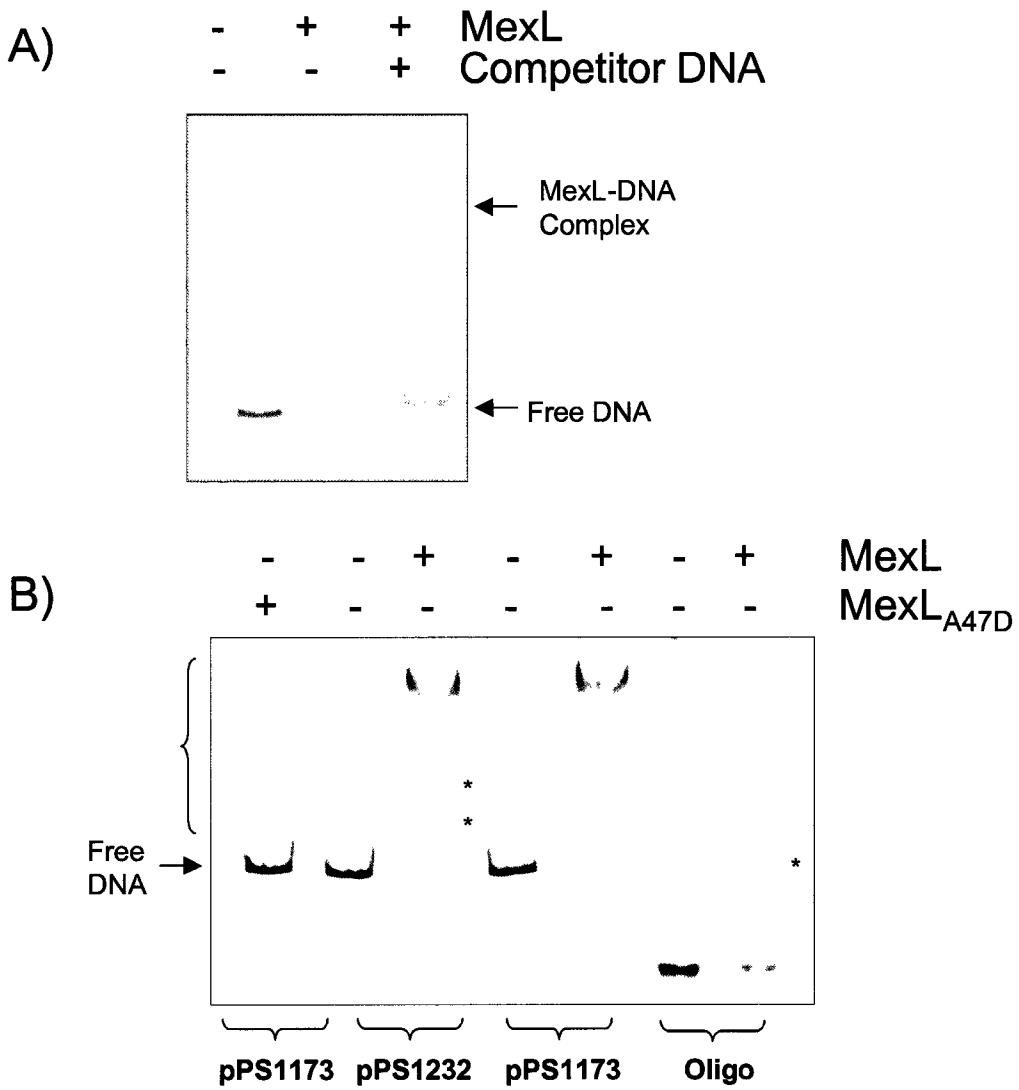

Figure 4.6. Gel mobility shift assays. **A)** An end-labeled 205 bp fragment from pPS1173 containing the entire 94 bp *mexL-mexJ* intergenic region was incubated with MexL-His_6 in the absence or presence of unlabeled competitor DNA (same unlabeled 205 bp fragment). **B)** Approximate localization of the MexL binding sites in the *mexL-mexJ* intergenic region. Plasmid pPS1173 contains DNA from –165 to +36, pPS1232 contains DNA from –90 to +113 and oligo denotes a synthetic oligonucleotide encompassing nucleotides located between –86 and –37 of the intergenic region (sequences are numbered relative to the first nucleotide of the *mexJ* ATG codon). All fragments were incubated with MexL-His_6, except for the fragment in the first lane which was incubated with $MexL_{A47D}$-His_6. The bracket to the left marks regions of band-shifted fragments observed with pPS1173 and pPS1232. Asterisks mark weak band-shifted fragments.

0 0.5 1.0 2.0 3.1 4.1 5.2 6.2 7.2 8.3 μM MexL

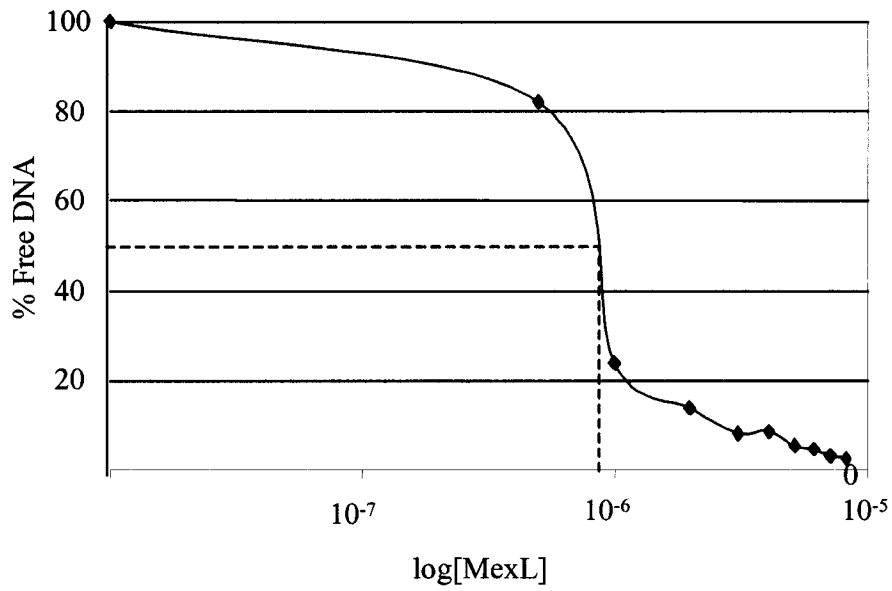

Figure 4.7. Estimation of the K_d for MexL using a Bjerrum plot. The 205 bp end labeled DNA fragment from pPS1173 was used as the template for gel mobility shift assay with increasing amounts of MexL. The amount of free DNA was estimated by scanning the autoradiograph and determination of band intensities using NIH Image software.

Several attempts were made to map the *mexJ* transcription start sites using either primer extension, or rapid amplification of complementary DNA ends (RACE) or RNase protection, but all attempts failed. However, the *mexL* transcription start was successfully mapped using RNase protection. The labeled riboprobe covered the *mexL-mexJ* intergenic region and started at 147 bp

downstream of the *mexL* initiation codon. The protected area was 176 bp in length, which corresponded to a transcriptional start at a T residue located 29 bp upstream of the *mexL* start codon (figure 4.11). To confirm this transcription start site of *mexL*, RT-PCR was performed on PAO238 total RNA using LJ11U-LJ17D and LJ11U-LJ18D primers. LJ17D annealed within the

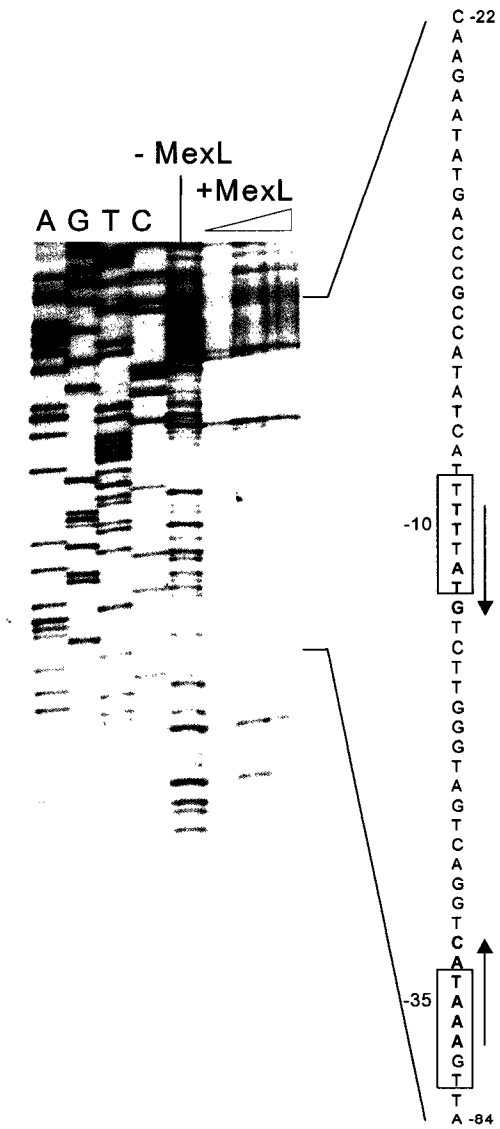

Figure 4.8. DNase I footprinting analysis of MexL-DNA interactions in the *mexL-mexJ* intergenic region. DNase I footprinting was performed with the *Xho*I-*Not*I fragment of pPS1173, which was labeled on the *mexJ*-coding strand. Labeled DNA fragments were treated with DNase I in the absence of MexL-His_6 (-MexL) or in the presence of increasing amounts (5 μM, 7.5 μM and 10 μM) of MexL-His_6 +MexL). The nucleotide sequence of the protected area is indicated on the right. Inverted repeats are marked with arrows and the predicted -10 and -35 regions of the *mexJ* promoter are boxed. A sequencing ladder of the same DNA template is shown on the left.

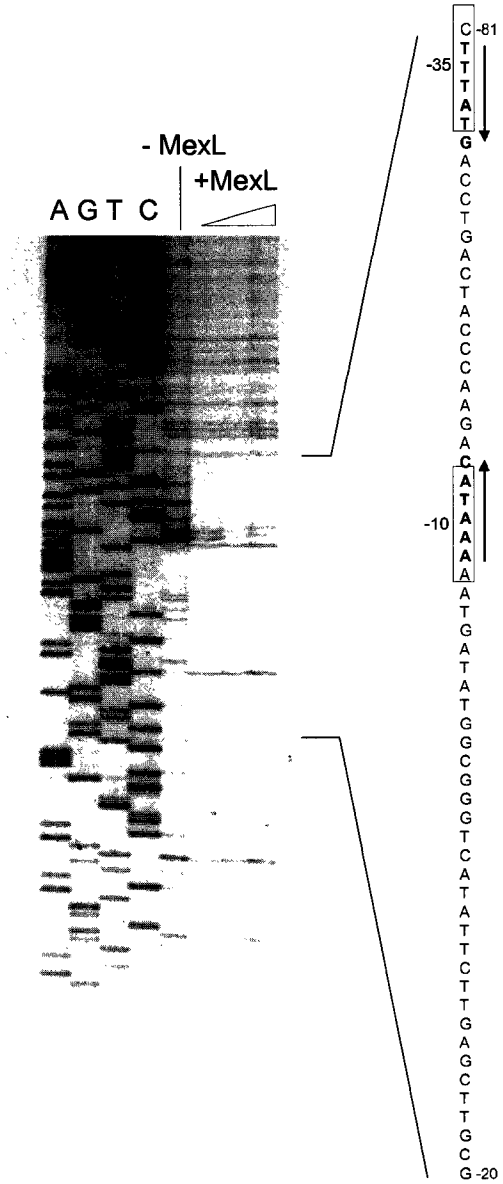

Figure 4.9. DNase I footprinting analysis of MexL-DNA interactions in the *mexL-mexJ* intergenic region. DNase I footprinting was performed with the *Xba*I-*Bam*HI fragment from pPS1173, which was labeled on the *mexL*-coding strand. Labeled DNA fragments were treated with DNase I in the absence of MexL-His_6 (-MexL) or in the presence of increasing amounts (5 μM, 7.5 μM and 10 μM) of MexL-His_6 (+MexL). The nucleotide sequence of the protected area is indicated on the right. Inverted repeats are marked with arrows and the predicted -10 and -35 regions of the *mexL* promoter are boxed. A sequencing ladder of the same DNA template is shown on the left.

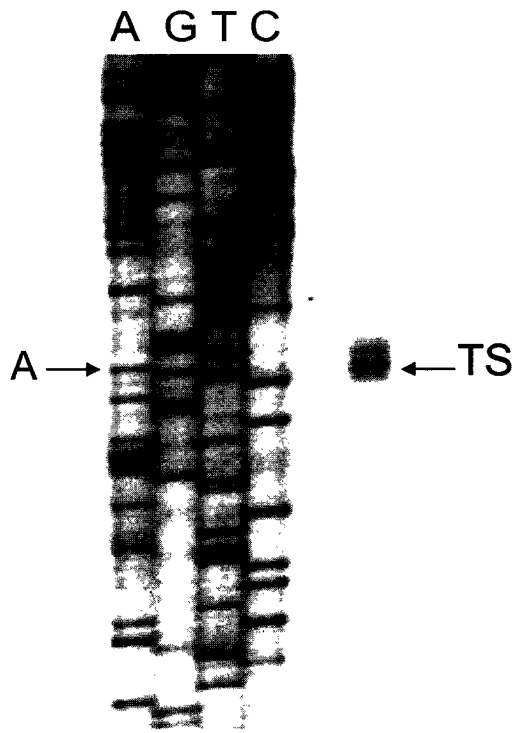

Figure 4.10. RNase protection analysis of the *mexL* promoter. The major transcription start site (indicated with TS) corresponded to an A in the AGTC sequencing reaction and the *mexL* transcript therefore starts with a T that is located 29 nucleotides upstream of the *mexL* start codon (see figure 4.11).

expected *mexL* transcript and LJ18D annealed outside of the expected *mexL* transcript. The expected 175-bp RT-PCR product was only obtained when LJ17D was used but no product was observed when LJ18D was used (data not shown). These data suggested that the assigned *mexL* transcription start site is probably correct.

4.4.8. Mapping of the *mexJ* promoter. Since we failed to obtain the *mexJ* transcriptional sites using conventional mapping technologies, transcriptional *lacZ* gene fusions carrying various portions of the *mexL-mexJ* intergenic regions were constructed and used to approximate *mexJ*

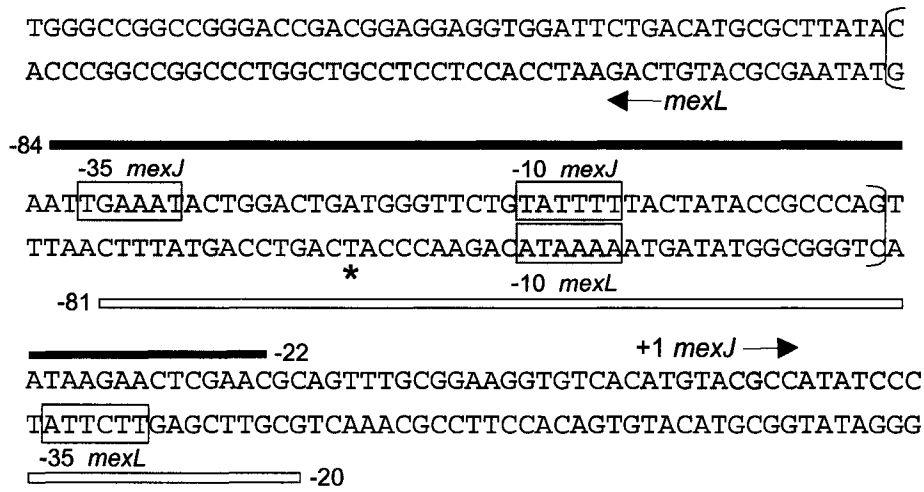

Figure 4.11. Regulatory elements within the *mexL-mexJ* intergenic region. The *mexL* and *mexJ* coding regions are shown on the respective strands and highlighted in grey. Shadowed boxes indicate the regions containing the inverted GTATTT repeat. The putative -10 and -35 regions of the *mexJ* and *mexL* promoters, as predicted by the promoter prediction program www.fruitfly.org/seq_tools/promoter.html, are boxed. The *mexL* transcription start site is marked with an asterisk. A black box indicates the -84 to -22 region on the *mexJ* coding strand that is protected by MexL and an open box indicates the -81 to -20 region on the *mexL* coding strand protected by MexL. All coordinates are relative to +1 which was arbitrarily chosen as the first nucleotide in the *mexJ* start codon The boundaries of a synthetic oligonucleotide used for band shift assays are indicated by brackets.

promoter location. The various portions of the *mexL-mexJ* intergenic region contained in the *mexJ-lacZ* fusion plasmids are shown in figure 4.12. The fusion plasmids were electroporated into Δ*mexLJK* strain PAO314 and β-galactosidase activities were measured. Cells containing either the vector pTZ120 or pPS1201, which contains the -244 to -71 region, did not show any detectable β-galactosidase activity, whereas cells containing pPS1204, which carries the entire intergenic region from -165 to +36, showed the highest activity, followed by pPS1236 (-86 to -37) and pPS1209 (-90 to +113) containing cells, which exhibited significant levels of β-galactosidase activities. MexL, co-expressed from a compatible plasmid, repressed *mexJ-lacZ* transcription from pPS1204, pPS1209 and pPS1236. Plasmids pPS1202 (-41 to +133) and

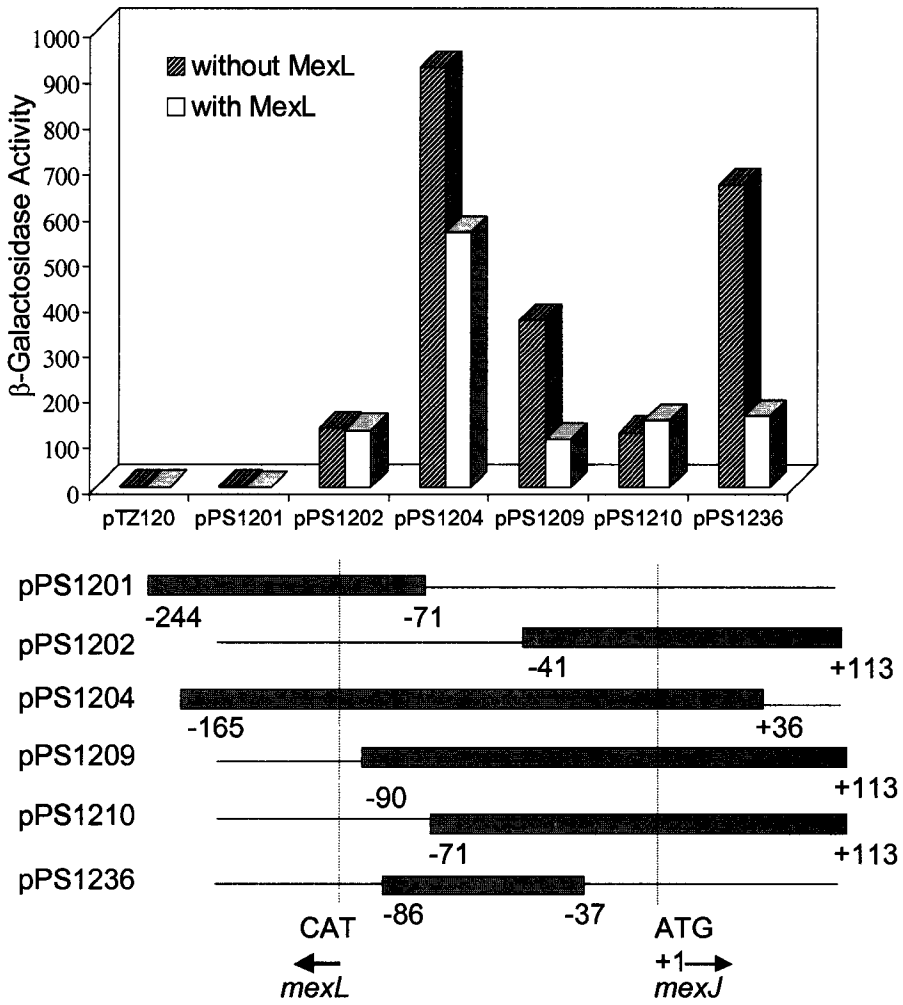

Figure 4.12. Localization of the *mexJ* promoter within the *mexL-mexJ* intergenic region using *lacZ* transcriptional fusions. PCR fragments containing the portions of *mexLJ* intergenic region indicated by the boxes were cloned in front of a promoterless *lacZ* on pTZ120 to form the respective *mexJ-lacZ* fusion plasmids pPS1201 to pPS1210; pPS1236 was obtained by ligating a synthetic oligonucleotide encompassing the sequences between –86 and –37 into pTZ120. The plasmids were transformed into Δ*mexLJK* strain PAO314 and β-galactosidase activities were measured. For expression of MexL. cells were co-transformed with pPS1245. Sequences are numbered relative to the first nucleotide of the *mexJ* start codon. Cells were grown in M9 glucose medium.

pPS1210 (-71 to +113) directed expression of lower levels of β-galactosidase activity; however, expression of this activity was not repressable by MexL. In summary, these results are consistent with the notion that the entire *mexJ* promoter and its associated regulatory sequences are located between nucleotides -86 and -37 upstream of *mexJ*, as presented in figure 4.11.

4.5. DISCUSSION

The MexJK system is one of the normally silent RND multidrug efflux systems of *P. aeruginosa*. By triclosan exposure of a susceptible mutant, we previously isolated a mutant derivative that constitutively expressed this efflux system due to a single base change in the upstream and divergently transcribed *mexL* regulatory gene. The mutation caused an A47D amino acid change located in the helix-turn-helix domain of the MexL protein. Sequence alignments confirmed that MexL belongs to the TetR family of repressor proteins and that the A47D amino acid change falls into a region that was previously shown to be required for DNA binding of other members of this family (figure 4.13). Thus, all previous indications were that MexL is a repressor of *mexJK* operon expression. In this study, we characterized MexL and its interaction with the *mexL-mexJ* intergenic region. This region is especially amenable for studies on regulation of efflux operon expression because at 94 nucleotides it is the shortest regulatory region of any of the *P. aeruginosa* RND efflux operons.

By gene fusion analysis we confirmed that MexL is not only a repressor of *mexJK* operon expression but that it also autoregulates its own expression in a negative manner. Repression of *mexJK* expression was dependent on the growth medium. Whereas *mexJ-lacZ* expression was efficiently (~15-fold) repressed by MexL in cells grown in minimal glucose medium, repression was less efficient in cells either grown in minimal casamino acids or LB medium (~2-fold). The

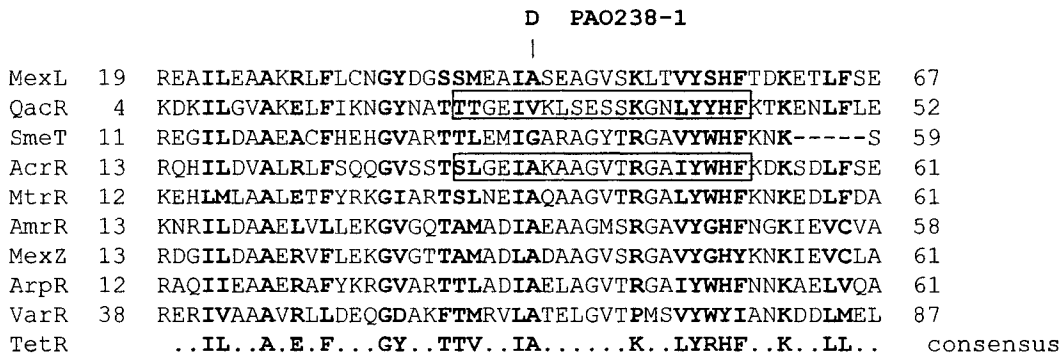

Figure 4.13. Partial sequence alignments of the TetR family of repressors of bacterial efflux systems. The alignment was performed using the NCBI-conserved-domain search program. Protein names are shown on the left and flanking numbers indicate the locations of the aligned amino acids in the respective proteins. Bold letters mark conserved residues corresponding to the TetR consensus sequence. Known DNA-binding domains of AcrR and QacR are boxed. The A47D change in MexL which abolishes DNA binding in the mutant protein from strain PAO238-1 is shown at the top.

possibility that LB medium or casamino acids may contain an effector of *mexJK* expression will necessitate further investigation.

To investigate the interaction of MexL with the *mexL-mexJ* intergenic at the molecular level, we purified MexL as a fusion protein with a carboxy-terminal peptide containing a hexahistidine domain. MexL was easily overexpressed in *E. coli* and accumulated even in uninduced cells to considerable levels. The Mex-His_6 protein was purified to near homogeneity using a Ni^{2+}-NTA agarose affinity column. Native polyacrylamide gel electrophoresis and size exclusion chromatography experiments indicated that MexL is a tetrameric protein in solution. This is not unexpected since many other DNA binding proteins are multimeric proteins (47). The ability of MexL to multimerize was further demonstrated using a genetic approach which showed

that a LexA-DBD-MexL fusion protein was able to repress a LexA-controlled *lacZ* fusion in *E. coli*. These experiments demonstrated that as few as 22 amino acids from the extreme amino terminus of MexL could assist in LexA-DBD multimerization, although efficient oligomerization required full-length MexL. A LexA-DBD-$MexL_{A47D}$ fusion protein was constructed and shown to be fully multimerization-proficient. Thus, the inability of $MexL_{A47D}$ to repress *mexJK* transcription is not due to lack of oligomerization of the mutant protein.

Band shift assays showed that purified MexL-His_6 specifically bound to the *mexL-mexJ* intergenic region. In contrast, purified $MexL_{A47D}$-His_6 protein did not bind to this region although it was stable after purification and multimerized in solution. Thus, the single A47D change in the helix-turn-region of the mutant protein affects the ability to bind to its target DNA. Under the conditions used in this study, the relative binding constant (K_b) estimated by band shift assays was 10^{-7} M^{-1} and the relative dissociation constant (K_d) was 9 x 10^{-7} M. Although this is acceptable for a DNA binding protein, the affinity to its target is not extraordinarily high since K_d's in the 10^{-8} to 10^{-9} M range have been observed for many DNA binding proteins (32). This may mean that (i) binding conditions still have to be optimized; (ii) that the techniques used for determining K_d and K_b were not optimal or, less likely, (iii) that an "assisting factor" was missing from the *in vitro* binding reactions.

Band shift experiments localized the MexL operator(s) to a region located between 86 and 37 nucleotides upstream of the *mexJ* ATG start codon (the first nucleotide of this codon was designated as +1). This region contains an inverted repeat of the hexanucleotide GTATTT which may form part of the MexL recognition sites. Dnase I footprinting experiments of the *mexL* and *mexJ* coding strand showed that MexL protected a single region on either strand. The protected regions covered 62 (*mexJ* coding strand) and 61 (*mexL* coding strand) nucleotides, and overlapped almost perfectly. A promoter prediction program revealed that the putative –10 and –35 regions for the *mexJ* and *mexL* promoters were located in these protected regions and that both

promoters share an overlapping –10 region. This is consistent with the observation that MexL negatively autoregulates its own expression. The *mexL* promoter was confirmed by mapping the *mexL* transcript start site to this region. Despite repeated attempts, we were unable to map the *mexJ* transcription start site by utilizing standard mapping techniques. However, by employing *lacZ* fusion technology the *mexJ* promoter was mapped to a region located between –86 and –37 nucleotides upstream of *mexJ*. This localization is consistent with the results of footprinting experiments. Although the involvement of the GTATTT hexanucleotide sequence in MexL binding has not yet been definitively proven, their central localizations within the protected regions, and overlap with the *mexL* and *mexJ* promoter regions strongly supports their involvement in MexL binding.

In summary, the results presented in this study elucidated the molecular modes of action of a repressor of a normally silent RND efflux system. They also laid the foundation for elucidation of the nature of the effector of MexL function, i.e., the cognate inducer(s) of *mexJK* operon induction, and provided the molecular tools required for its discovery and characterization.

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CHAPTER 5

High-level triclosan resistance in *Pseudomonas aeruginosa* is solely due to efflux

(Presented in Rungtip Chuanchuen, RoxAnn R. Karkhoff-Schweizer, Herbert P. Schweizer. 2003 *American Journal of Infection Control* 31: 124-127.)

The work in this article corroborated the notion that multidrug efflux systems are the sole responsible mechanism for high-level triclosan resistance in *P. aeruginosa*. It also demonstrated that triclosan is a substrate for most characterized RND efflux systems except MexXY-OprM and MexGHI-OpmD. It supported our previous study that a link between triclosan and antibiotics does exists via multidrug efflux systems.

5.1. ABSTRACT

Triclosan is a bis-phenolic biocide that is widely used in surgical scrubs, disinfectants, soaps, toothpastes and numerous other consumer products (2). Bacteria develop triclosan resistance via mechanisms that are well-documented for development of antibiotic resistance, namely target mutations, enzymatic modification and active efflux (13). Concern has been voiced about the widespread use of triclosan and the emergence of triclosan-resistant bacteria which may simultaneously become resistant to multiple antibiotics due to shared resistance mechanisms (8, 12, 13). Although it was previously noted that scientific evidence has not shown that a link exists between the use of triclosan-containing products and antibiotic resistance (5),

increasing experimental evidence is now being obtained that such a link does exist (8, 11-13). Moreover, some triclosan applications, especially triclosan-incorporated polymers, may provide the ideal setting for triclosan resistant bacteria to grow (6). *Pseudomonas aeruginosa* is resistant to triclosan (2), most likely due to the expression of a single multidrug efflux pump of the resistance nodulation and cell division (RND) family (13). We previously showed that exposure of triclosan-susceptible mutant strain to this biocide resulted in the emergence of strains expressing another RND multidrug efflux pump, MexCD-OprJ, which in broth-grown cells conferred medium-level (128 μg/ml) resistance to triclosan, and simultaneous resistance to multiple antibiotics (3). Although it was previously shown that most, if not all, *P. aeruginosa* strains tested exhibited high-level (~1000 μg/ml) triclosan resistance (2), these studies were performed with genetically undefined American Type Culture Collection or other laboratory collection strains and thus the resistance mechanism(s) causing high-level resistance remained unknown. The *P. aeruginosa* PAO1 genome encodes 12 proposed RND pumps and to date we showed that triclosan is a substrate for 4 out of the 5 RND pumps that are confirmed multidrug efflux systems (13). However, we never determined whether the high-level triclosan resistance of *P. aeruginosa* was due to expression of a single efflux system, or due to combination of efflux pump expression and other mechanisms. In this study, we used defined *P. aeruginosa* strains expressing various efflux systems and isogenic mutant strains to assess (i) whether high-level triclosan resistance was solely due to expression of efflux pumps and (ii) which of the hitherto characterized efflux pumps contributes to high-level triclosan resistance.

5.2. METHODS

The wild-type *P. aeruginosa* strain PAO1, several defined efflux pump-expressing PAO1 mutant derivatives and the cognate efflux pump deletion strains were employed (table 1). All strains,

except PAO238-1 and PAO314, were previously described (3). Strain PAO238-1 was derived from PAO238 as a spontaneous triclosan-resistant mutant that expresses a novel RND efflux system, MexJK (13). PAO314 is a *mexJK* deletion derivative of PAO238-1 and PAO346 an *opmD* deletion derivative of PAO238-1; these strains were constructed using previously described methods (3). To determine the maximal triclosan minimal inhibitory concentration (MIC), an agar incorporation method was employed. Triclosan was dissolved in 75% 2-methoxyethanol to 100 mg/ml and Mueller-Hinton agar plates were prepared that contained this biocide in two-fold serial dilutions ranging from 0 to 1024 μg/ml. Plates were inoculated with a 48 pin replicator and scored following National Committee for Clinical Laboratory Standards guidelines(10).

5.3. RESULTS

Using standard two-fold serial microdilution broth MIC determinations, we previously established the MIC for triclosan due to efflux pump expression as being ~128 μg/ml (3), the highest value testable in aqueous solutions since triclosan precipitates at about this concentration. In this report, we describe the contribution of individual RND efflux pumps to high-level (>1000 μg/ml) triclosan resistance using an agar incorporation method and the best-suited solvent for triclosan, 2-methoxyethanol. Employing this method, the MIC for the prototypic laboratory strain PAO1 was >1024 μg/ml (table 1) and this high-level resistance was due to active efflux via the MexAB-OprM pump from the cell since the MIC for the isogenic efflux pump mutant strain PAO200 was 16 μg/ml. Above 1024 μg/ml, triclosan precipitated and no accurate MIC values could be determined. The maximum solvent concentration in the plates was 0.77%, well below the MIC for 2-methoxyethanol, which for both strains on MHA plates was 6.25%. Similar high MIC levels were obtained with PAO1-derived mutants expressing the MexCD-OprJ and

Table 5.1. Minimum inhibitory concentration of triclosan *in vitro*

Strain	Relevant Genotype*	RND Efflux Pumps Expressed	MIC (μg/ml)
PAO1	*mexA*$^+$*B*$^+$*-oprM*$^+$ *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexAB-OprM MexGHI-OpmD	>1024
PAO200	Δ(*mexAB-oprM*) *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexGHI-OpmD	16
PAO200-2	Δ(*mexAB-oprM*) *nfxB mexC*$^+$*D*$^+$*-oprJ*$^+$ *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexCD-OprJ MexGHI-OpmD	>1024
PAO238	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexGHI-OpmD	16
PAO253	Δ(*mexAB-oprM*) *mexS mexE*$^+$*F*$^+$*-oprN*$^+$ *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexEF-OprN MexGHI-OpmD	>1024
PAO255	Δ(*mexAB-oprM*) *mexS* Δ(*mexEF-oprN*) *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexGHI-OpmD	16

Table 5.1. Minimum inhibitory concentration of triclosan *in vitro*

Strain	Relevant Genotype*	RND Efflux Pumps Expressed	MIC (μg/ml)
PAO267	Δ(*mexAB-oprM*) *mexZ mexX*$^+$*Y*$^+$*mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexXY MexGHI-OpmD	16
PAO280	Δ(*mexAB-oprM*) *mexZ* Δ(*mexXY*) *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexGHI-OpmD	16
PAO238-1	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) *mexL mexJ*$^+$*K*$^+$ *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexJK MexGHI-OpmD	128
PAO314	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexJKL*) *mexG*$^+$*H*$^+$*I*$^+$*-opmD*$^+$	MexGHI-OpmD	16
PAO346	Δ(*mexAB-oprM*) *nfxB* Δ(*mexCD-oprJ*) Δ(*mexXY*) *mexL mexJ*$^+$*K*$^+$ *mexG*$^+$*H*$^+$*I*$^+$	MexJK MexGHI**	64

*Only the genotypes relevant to the expression of the pumps under study are listed. Under the conditions employed in this study, all strains express the quorum sensing regulated MexGHI-OpmD system (1). The other efflux systems listed in this table are expressed due to regulatory mutations in *nfxB* (such mutants express MexCD-OprJ) (3), *mexL* (such mutants express MexJK) (4), *mexS* (such mutants express MexEF-OprN) (3) and *mexZ* (such mutants express MexXY) (3). Although strains PAO1, PAO200, PAO200-2, PAO238, PAO253, PAO255 and PAO238-1 are wild-type for *mexXY*, these genes are not expressed since triclosan does not function as an inducer for this system (4). **This strain expresses a dysfunctional MexGHI system due to insertional mutation of OpmD.

MexEF-OprN efflux pumps, respectively. A strain expressing only the MexJK efflux pump exhibited an MIC of 128 μg/ml. Since the corresponding efflux pump mutant strains all exhibited the same low MICs, high-level triclosan resistance in each case was due to the expression of a single RND drug efflux system. We previously reported that triclosan is not a substrate of the MexXY pump (3) and the current experiments confirmed this finding. Under the conditions employed in this study, all strains still express the quorum sensing-regulated MexGHI-OpmD system (1) but this system does not contribute to significant triclosan resistance since strains expressing only MexGHI-OpmD, i.e. PAO200, PAO238, PAO255, PAO280 and PAO314, all exhibit the same low MIC (16 μg/ml). Moreover, strain PAO346 expressing only MexJK, but a dysfunctional MexGHI system due to deletion of OpmD (1), exhibited a similar triclosan MIC (64 μg/ml) as strain PAO238-1 expressing MexJK and MexGHI-OpmD (128 μg/ml).

5.4. CONCLUSIONS

Previous studies showed that various genetically undefined culture collection *P. aeruginosa* strains exhibited triclosan MICs that were approximately 1000 μg/ml (2). In this report, we demonstrate for the first time that this high-level resistance in wild-type cells is due to expression of a single RND multidrug efflux pump, MexAB-OprM. However, the expression of any one of the other characterized, but normally silent RND pumps in this bacterium, including MexCD-OprJ and MexEF-OprN, can lead to high-level triclosan resistance, provided that this biocide is a substrate for the respective pump. Other efflux systems, such a MexJK, contribute to lesser amounts of resistance. The only other pump expressed in the strains studied, MexGHI-OpmD, does not significantly contribute to intrinsic triclosan resistance. In addition to the six RND pumps listed in table 1, *P. aeruginosa* contains six additional RND-type efflux systems but their contribution to triclosan resistance is unknown. We can be pretty certain, however, that they do

not play a significant role in intrinsic triclosan resistance since they are not expressed in wild-type strains, as assessed by transcriptional profiling employing *P. aeruginosa* Affymetrix GeneChips®. Therefore, an assessment of their roles, if any, in acquired triclosan resistance awaits the identification of regulatory mutants expressing these pumps. All wild-type *P. aeruginosa* strains examined to date express MexAB-OprM. Even if this efflux pump alone may not be sufficient to confer resistance to triclosan levels found in many consumer products, additional RND pumps may be readily expressed since it is well documented that the exposure of *P. aeruginosa* to this biocide readily selects for mutants expressing normally silent multidrug RND efflux pumps (3, 13). Plausible consequences of the expression of additional efflux pumps besides MexAB-OprM are (i) that the triclosan contained in many consumer products is probably completely ineffective against this bacterium and (ii) that the already high levels of intrinsic antimicrobial resistance conferred by MexAB-OprM are further increased by acquiring resistance via other pumps, possibly even resistance to antimicrobials that are not substrates of MexAB-OprM. Since many clinical isolates express multiple RND efflux systems (3, 13), and because interplay between RND efflux pumps was shown to provide either additive or multiplicative effects on drug resistance (7), the triclosan MICs of bacteria expressing multiple efflux pumps are probably well in excess of what can be experimentally measured. Therefore, the observed and probable MIC levels of such bacteria are well in the range of triclosan levels (0.1 to 1% or 1,000 to 10,000 μg/ml) found in many consumer products. Our experiments clearly demonstrate that the well-established high-level triclosan resistance is not due to the high lipid level of the *P. aeruginosa* cell membranes as previously reported (9) or other properties of the cell envelope, such as reduced permeation, but rather due to active efflux from the cell. A triclosan-insensitive enoyl-acyl carrier protein reductase (13) may be a factor contributing to the residual low-level resistance observed in the efflux pump mutant strains. RND pumps, many of which also efflux antibiotics, are found in diverse clinically significant bacteria. Since triclosan is an excellent substrate of RND pumps (13), a link between triclosan and antibiotics clearly exists, although its clinical

significance remains yet to be fully explored. At the very least, in environments where other bacteria are suppressed by triclosan, *P. aeruginosa*, and probably related bacteria, should be well equipped to tip the ecological balance in their favor.

* our unpublished results; GeneChips® were obtained from and subsidized by Cystic Fibrosis Foundation Therapeutics, Inc.

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CHAPTER 6

6.1. CONCLUDING REMARKS

Although multiple mechanisms contribute to the well-known high intrinsic drug resistance of *P. aeruginosa*, it is now mostly attributed to the synergy of a low-permeability outer membrane and the expression of multidrug resistance efflux pumps (11, 12, 16). Antimicrobial resistance due to multidrug efflux systems is more complicated than that caused by other mechanisms because it is multidrug resistance. The contribution of antibiotic exposure to development of MDR has clearly been documented, however little is known about biocide resistance mechanisms and their possible contribution to MDR (17, 18). Antimicrobial products constitute a large and hot new market for consumers concerned about infections or microbial contamination. One of the most commonly used antibacterials is triclosan (3, 7). Even though *P. aeruginosa* possesses the main triclosan target, enoyl-ACP reductase (FabI) it also encodes a second, triclosan-resistant enoyl-ACP reductase (FabK) (6). However, FabK alone cannot explain the extremely high-level (>1 mg/ml) triclosan resistance shown by *P. aeruginosa*, and therefore drug efflux had to be implicated. The studies in this dissertation were initiated to define the relationships of triclosan, MDR and multidrug efflux pumps. The findings have led to a better understanding of triclosan resistance mechanisms and its cellular effects, the function, molecular architecture and regulation of multidrug efflux pumps – particularly RND pumps - and shed some light on a possible link between antiseptics and antibiotic resistance. Specific progress was made in the following areas:

i) It was shown that triclosan is an excellent substrate for most characterized RND efflux systems (chapter 2). This is due to amphiphilic property of this bisphenolic biocide. Since RND pumps are usually expressed in regulatory mutants selected by substrate – i.e., antibiotic - exposure in laboratory and clinical isolates, we reasoned that exposure of triclosan-susceptible strains to this biocide should similarly select for efflux pump-expressing regulatory mutants. If this were the case, triclosan could be used as a substitute for antibiotics to study those pumps for which no substrates are known and no clinical isolates are available. To validate this approach, it was attempted to isolate regulatory mutants affectingexpression of a clinically relevant efflux system, MexCD-OprJ, for which triclosan is a good substrate. Triclosan readily promoted the emergence of triclosan-resistant, MexCD-OprJ pump-expressing bacteria that became simultaneously resistant to multiple antibiotics, including clinically significant fluoroquinolones, because it selected for mutants with mutations in the *nfxB* regulatory gene. Notably, triclosan selected for similar regulatory mutants as those found in laboratory and clinical isolates that were exposed to fluoroquinolones *in vitro* or during clinical therapy. This provided evidence that under the right circumstances a link between triclosan and antibiotic resistance may indeed exist. Of particular concern is that the widespread use of certain antiseptics – mostly triclosan - in home and clinical settings may lead to selection for MDR bacteria and thus compromise use of still valuable antibiotics. Since most characterized *P. aeruginosa* multidrug efflux pumps participate in triclosan efflux, the current picture may get even more complex given the possible, perhaps additive contributions of the as many as 37 efflux systems recently identified by *P. aeruginosa* genome sequence analyses.

ii) After validating triclosan as a selective tool for regulatory mutants, the biocide was used to select a mutation in a regulatory gene, *mexL*, expressing a normally silent pump, MexJK (chapter 3). The mutant was then used to establish that MexJK is a multidrug pump and it therefore may be of clinical relevance. However, this remains to be proven by analyzing various

multidrug resistance clinical *P. aeruginosa* isolates for MexJK expression, e.g. by RT-PCR or by using anti-MxJ or anti-MexK antibodies. Since the *mexJK* operon does not encode its own outer membrane channel protein, the question was asked whether it uses OprM for its function. It was shown that MexJK used OprM for antibiotic efflux but not for triclosan efflux (chapter 3). Although not described in this dissertation, the structural genes for all 18 members of the OprM family were individually deleted in the MexJK-expressing mutant background and the analysis showed that OpmH is required for efflux of triclosan by MexJK. Interestingly, construction of hybrid *mexJK-oprM* and *mexJK-opmH* showed that MexJK chooses its outer membrane protein channel in a substrate-dependent manner. For example, MexJK-OprM effluxes erythromycin but not tirclosan and, vice versa, MexJK-OpmH effluxes triclosan but not erythromycin. Thus, substrates may play an important role in efflux pump assembly and this is a new concept. A manuscript describing these interesting findings is in preparation.

iii) Regulatory mutants overexpressing multidrug efflux systems obtained by exposure to triclosan can be used to study regulation of the normally silent efflux systems. In his study, the *mexL* mutant was employed to establish the modes of regulation of MexJK by MexL at the molecular level using purified MexL protein (chapter 4). A current model for regulation of *mexL* and *mexJK* expression by MexL is presented in figure 6.1. These studies also indicated that the composition of the growth media had an influence on levels of *mexJK* repression by MexL. Follow-up studies may shed light possible *mexJK* effector/inducer molecules and therefore help solve another mystery in this area of research, namely, answer the question of what the physiological functions of the efflux pumps really are. Crystallization and elucidation of its structure of MexL may also assist these investigations.

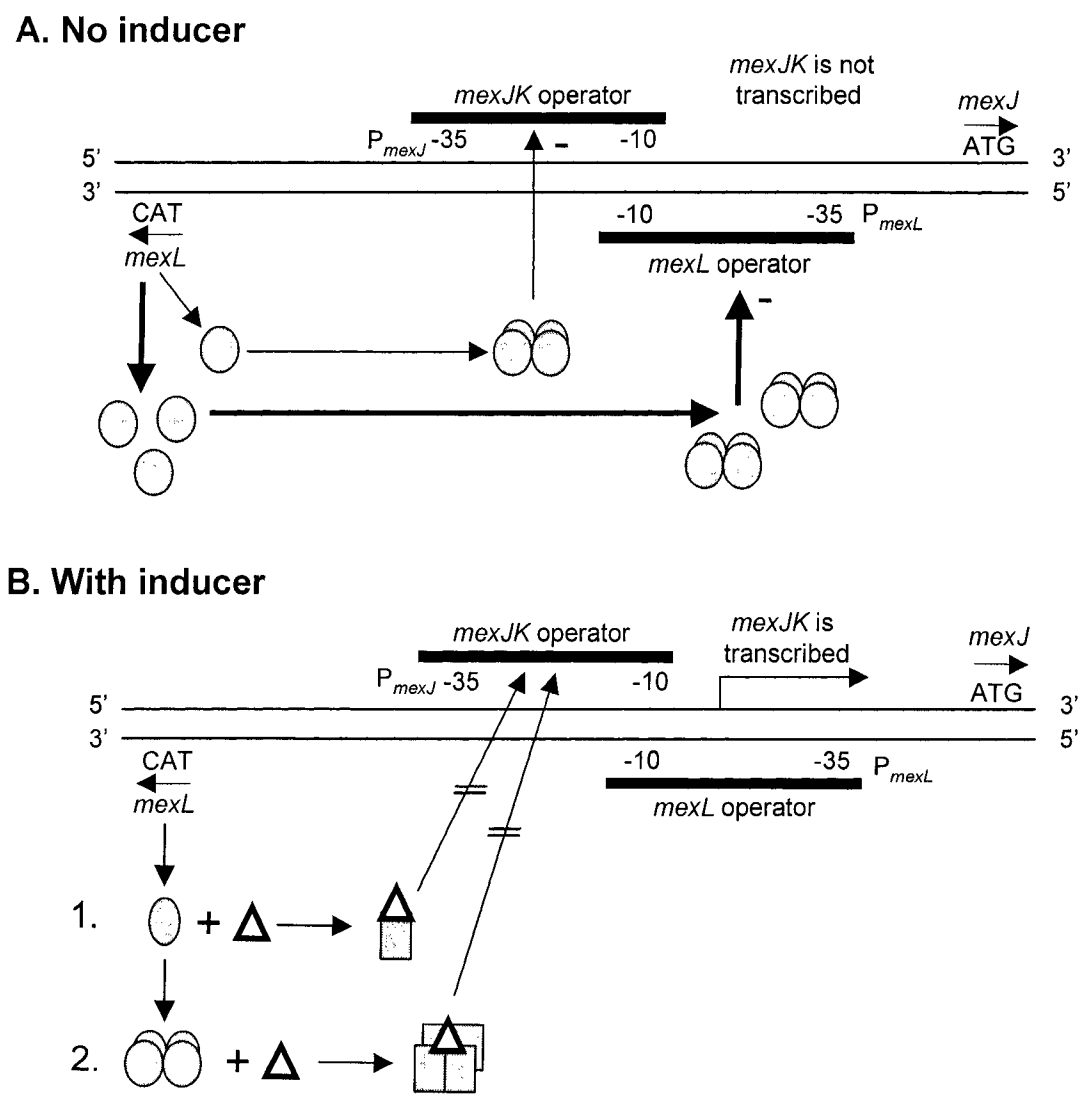

Figure 6.1. Model for *mexL* and *mexJK* regulation by MexL. **A.** In the absence of an inducer, MexL (grey ovals) will bind to the *mexJK* operator and suppress transcription of *mexJK* (thin arrows) since the biding affinity of MexL to its own operator is less than that to the *mexJK* operator. Once the *mexJK* operator is occupied and MexL accumulates to levels no longer required for *mexJK* repression, the concentration of MexL will be controlled by the autoregulation process: the excess MexL will bind to the *mexL* operator in the *mexL* promoter region and thus repress its own expression (thick arrows). **B.** In the presence of an inducer (open triangle), several initial scenarios can be envisioned: 1. an inducer-MexL monomer complex is formed which leads to a conformational change in MexL (grey rectangles) so that MexL cannot tetramerize; or 2. MexL does tetramerize and binding of the inducer causes a conformational change. In both cases, the MexL-inducer complex can no longer bind to the operator in the *mexJK* promoter region, resulting in *mexJK* transcription. Transcription of *mexL* is probably shut off because RNA polymerase binding to the *mexJK* promoter causes dissociation of the DNA strands in this region which most likely precludes simultaneous binding to and transcription from the *mexL* promoter.

iv) It has long been known that *P. aeruginosa* is highly resistant (> 1 mg/ml) to triclosan and this was attributed to the high lipid content of its cell wall and, more recently, due to the presence of a triclosan-resistant enoyl-ACP reductase (FabK) in addition to the triclosan-susceptible enzyme (FabI). However, this study showed that RND multidrug efflux pumps are exclusively responsible for this high-level triclosan-resistance (chapter 5) (4). Although we used the best known solvent for triclosan, i.e., 2-methoxyethanol, solubility in agar media is still limited to ~1 mg/ml. However, since MexAB-OprM-, MexCD-OprJ- and MexEF-OprN-expressing still grew in the presence of 1,024 μg/ml triclosan, the true MIC for these strains is probably much higher and actual MIC determinations will require improved solubilization methods. This may be important since many consumer products contain triclosan at concentrations between 0.1-1% (1-10 mg/ml) and *P. aeruginosa* may well be able to thrive at these concentrations.

The research presented in this dissertation made many important contributions to the field of efflux pump research. However, there are many questions about multidrug efflux systems that remain unsolved, especially in the areas of regulation of RND efflux operon expression and substrate recognition. For example, it is known that expression of MexEF-OprN is positively regulated by MexT, which in turn is negatively regulated by MexS. Is MexS a specific regulator of MexEF-OprN expression or is it a global regulator? Many clinical *P. aeruginosa* isolates express various efflux pumps in the absence of mutation in the regulatory genes (2, 13), this suggested that other negative regulatory factors must be involved in regulation of expression of efflux pumps (16). One of the most interesting question in this area of research remains how RND efflux systems recognize and capture the various, structurally diverse substrates. Even though many experiments, including co-crystallization of an RND transporter with bound substrates, have been performed aimed at elucidating mechanisms of substrate recognition by RND efflux pumps, many questions remain to be answered (1, 5, 10, 19, 20).

On a more practical note, the availability of defined multidrug efflux pump-expressing strains and their mutant derivatives will benefit the pharmacological industry as useful new tools for the drug discovery and evaluation process (9, 15). Inhibitors of multidrug efflux pumps may extend the spectrum and usefulness of existing drugs. Several inhibitors of efflux pumps have already been investigated (8, 14). Many of the mutants developed in this study are currently being used by several small pharmaceutical companies in their drug screening and evaluation efforts.

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