Characterization of RND Efflux Pumps of

Serratia marcescens

by

Ayush Kumar

A Thesis Submitted to
The Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree
of

Doctor of Philosophy

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba, R3T 2N2

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

DOCTOR OF PHILOSOPHY

To my Mom and Dad

Abstract

Serratia marcescens is a prominent opportunistic pathogen in clinical settings, responsible for serious infections in immunocompromised individuals. This organism has been shown to be resistant to several classes of antibiotics, making the treatment of infections very difficult. Recently, *S. marcescens* has been shown to be resistant to fluoroquinolone, a relatively new group of antibiotics. The major mechanism for fluoroquinolone resistance in various Gram-negative pathogens is the active efflux of the antibiotic molecule mediated by inner membrane efflux pumps belonging to the Resistance-Nodulation-Cell Division (RND) family. RND pumps work in conjunction with a periplasmic protein and an outer membrane protein in Gram-negative organisms. In order to design effective chemotherapy to cure *S. marcescens* infections, identification and characterization of efflux pump(s) in this organism is essential. This thesis establishes active efflux as a resistance mechanism in *S. marcescens*, and presents molecular characterization of two different efflux pumps in this organism. Clinical isolates of *S. marcescens* were examined, and results established active efflux as a mechanism of resistance in *S. marcescens*. Two RND pump-encoding genes were identified by using the PCR targeting conserved motifs found within RND pump-encoding genes. To clone the complete genes of these two putative RND complexes, a *S. marcescens* genomic DNA library was constructed and screened with DNA probes synthesized from the PCR products. Both operons were isolated and sequenced. Sequence analysis revealed that both operons contained RND complex-encoding genes. The operons were named *sdeAB* and *sdeCDE* (*sde*, *Serratia* drug efflux). Functional analysis of the *sdeAB* and *sdeCDE* gene products revealed that SdeAB is in fact a

multidrug efflux pump with a wide range of substrates. The *sdeAB* locus was also found to be over-expressed in laboratory-derived fluoroquinolone resistant mutants, suggesting that it is a major pump in *S. marcescens*. Substrate specificity could not be ascertained for SdeCDE, as it was not found to efflux any of the compounds tested. A gene encoding the outer-membrane component was also identified, cloned, and characterized. Functional analysis of the outer-membrane component revealed that it is involved in energy-dependent efflux of antimicrobial agents, and could therefore be acting in conjunction with the active efflux pump(s) in *S. marcescens*. A MarA homolog was also identified upstream of the *sdeAB* locus and the gene was named *sdeR*. SdeR may be involved in the over-expression of *sdeAB* in multidrug resistant isolates of *S. marcescens*. A putative binding site for SdeR upstream of *sdeAB* was identified.

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Very special thanks to Kari for her tremendous support in making this work possible. To my sister Ritu, who kept reminding me that I could. Finally, to my mom and dad, for always being there for me and showing me the way.

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List of abbreviations

Å	angstrom
ABC	ATP-Binding Cassette
Ap	ampicillin
ATCC	American Type Culture Collection
Cb	carbenicillin
CCCP	carbonyl cyanide m-chlorophenylhydrozone
cfu	colony forming unit
Chl	chloramphenicol
Cip	ciprofloxacin
CPTC	13-cyclopentylthio-5-OH tetracycline
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine-tetra-acetic acid
EPI	efflux pump inhibitor
ESBL	extended spectrum β-lactamases
EtBr	ethidium bromide
hrs	hours
HTH	helix-turn-helix
kb	kilobase pairs
kDa	kiloDaltons
Km	kanamycin
L	litre(s)
Mar	multiple antibiotic resistance
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFP	membrane fusion protein
MFS	major facilitator superfamily
mg	milligram(s)
MIC	minimum inhibitory concentration
min	minute(s)
mM	millimolar
mm	millimeters
Nal	nalidixic acid
ng	nanogram(s)
Nor	norfloxacin
Nov	novobiocin
Ofx	ofloxacin
OMF	outer membrane factor
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PQS	*Pseudomonas* quinolone signal
QRDR	quinolone resistance determining region
RND	resistance-nodulation-cell division
RR	relative resistance
SDS	sodium dodecyl sulfate
Sm	streptomycin
SMR	small multidrug resistance
Tc	tetracycline
TMS	transmembrane spanner
μg	microgram(s)
μl	microlitre(s)
v/v	volume/volume
w/v	weight/volume

ONE

Introduction and Literature Review

You see things and say, 'Why'?
But I dream things that never were and I say, 'Why not'?
-G. B. Shaw

1.1. Introduction

To date very little has been reported on the mechanisms involving fluoroquinolone resistance in *Serratia marcescens.* The majority of studies describe target mutation as the mechanism of resistance to fluoroquinolones; however, with active efflux now being recognized as the major mechanism of resistance to fluoroquinolones, it is highly likely that such a mechanism is also present in *S. marcescens*. Identification of active efflux as a resistance mechanism in this organism will contribute to a better understanding of the intrinsic resistance mechanisms of *S. marcescens*. The sequences of RND pumps obtained in this thesis will contribute to the RND pump sequences data from other organisms and will be helpful to determine regions that contribute to the assembly and function of these complexes, which is still not very well understood.

In addition, identification of regulatory region(s) that play a role in the over-expression of these pumps and possible interactions of regulatory protein(s) with antibiotics in *S. marcescens* will help in a better understanding of the role of antibiotics in the generation of MDR phenotype.

1.2. *Serratia marcescens*

Serratia marcescens is a Gram-negative bacillus belonging to the *Enterobacteriaceae* family. It is found in various ecological niches, including soil, water, air, plants, and animals and is defined as an oxidase-negative Gram-negative bacillus producing DNAse (Grimont & Grimont, 1984). It is motile by peritrichous flagella.

S. marcescens was considered a non-pathogenic aquatic saprophytic organism until late in the 20th century. Although the first instance of its pathogenicity to humans was reported in 1913 (Woodward & Clarke, 1913), it was not until a nosocomial outbreak by this organism in 1951 that *S. marcescens* was identified as a threat in clinical settings (Wheat *et al*, 1951). Since then, infections caused by this organism have been frequently reported in a variety of clinical settings, and *S. marcescens* is now considered to be a prominent opportunistic pathogen. Presently it is implicated in respiratory- and urinary tract infections, septicemia, meningitis, and endocarditis. It is commonly isolated from the urine of patients with indwelling catheters, patients with serious underlying diseases, especially neonates, patients requiring treatments in intensive care units, neutropenic patients, and those with disseminated malignancies (Johnson *et al*, 1998). Different environmental sources have been identified as reservoirs for this organism, including disinfectants (Archibald *et al*, 1997; Vigeant *et al*, 1998), pressure transducers (Beck-Sague & Jarvis, 1989), bronchoscopes (Vandenbroucke-Grauls *et al*, 1993), and airconditioner ducts (Uduman *et al*, 2002). *S. marcescens* can be transmitted to neonates through feeding, use of soaps (Archibald *et al*, 1997), contaminated antiseptics (Bosi *et al*, 1996), breast pumps (Gransden *et al*, 1986), and via contact with other patients. One recent study reported that neonatal lower respiratory tract infections were caused most often by *S. marcescens* (Albers *et al*, 2001).

S. marcescens has also been implicated as a major cause of lower respiratory tract infection in elderly patients (Byrne *et al*, 2000). Bacteraemia as a result of *S. marcescens* infection occurs in surgical patients or those in intensive care units, while

endocarditis is particularly common in intravenous drug users (Brouqui & Raoult, 2001). Although *S. marcescens* does not form the normal conjuctival flora, its capability to survive in contact lens solutions can lead to a variety of ocular infections, including purulent conjunctivitis, keratitis, corneal ulcers, and endophthalmitis (Atlee *et al*, 1970; Duffey, 1995; Johnson *et al*, 1992).

Environmental isolates of *S. marcescens* are typically red in color due to the production of the pigment prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosin) and early methods for identification of *S. marcescens* were based on pigment production by the isolates. However, clinical isolates of *S. marcescens* are usually unpigmented, which has led to many instances of misdiagnosis of the causative agent for different infections. In addition, it has been observed that non-pigmented isolates are more resistant to antibiotics (Gargallo-Viola, 1989) than pigmented isolates.

Treatment of infections caused by *S. marcescens* is difficult due to high resistance shown by this organism to a variety of antibiotics. It is intrinsically resistant to ampicillin, cefuroxime, and tetracycline (Lambert & O'Grady, 1992). Acquired resistance follows closely the patterns of the usage of new antibiotics. For example, the introduction of third-generation cephalosporins and quinolones led to the reduction in the use of aminoglycosides and penicillins, explaining the lower frequency of strains resistant to latter two antibiotics in 1989/1990 than in 1980 (Aucken & Pitt, 1998). Resistance to quinolones was reported in *S. marcescens* almost immediately after they came into use (Fujimaki *et al*, 1989). At present, resistance has been reported in *S. marcescens* to all major classes of antibiotics used, including β-lactams, aminoglycosides, and quinolones.

1.3. Antibiotic Resistance in *S. marcescens*

1.3.1. β-lactam Resistance

1.3.1.1. β-lactamases

β-lactams are the most widely used antibiotics for the treatment of *S. marcescens* infections. Penicillin was the first β-lactam to be used clinically with new varieties still being evolved today. Resistance to β-lactams is caused primarily by β-lactamases, enzymes that cleave the β-lactam ring of the antibiotic molecule. *S. marcescens* is known to produce a variety of β-lactamases belonging to three broad categories: metallo-β-lactamases, extended spectrum β-lactamases (ESBLs), and other β-lactamases.

S. marcescens has been shown to have metallo-β-lactamases and ESBLs. Metallo-β-lactamase enzymes contain two zinc atoms at the active site and are distinguished by EDTA-mediated inhibitions. The presence of metallo-β-lactamases has been investigated in *S. marcescens* (Ito *et al*, 1995), and bla_{IMP}-like (imipenem degrading β-lactamases) metallo-β-lactamases were found to be encoded by plasmids. The bla_{IMP}-encoded metallo-β-lactamase can efficiently hydrolyze both carbapenems and cephalosporins. Chromosomally encoded serine-based β-lactamases, SME-1 and SME-2, have also been reported in *S. marcescens* (Naas *et al*, 1994; Queenan *et al*, 2000). These enzymes are known to confer resistance to carbepenems, aztreonam, cefamandole, and cephalothin.

Extended-spectrum β-lactamases (ESBLs), variants of β-lactamase initially reported as a result of increased use of oxyimino-cephalosporins in the 1980s, found in different species of members of the family *Enterobacteriaceae, P. aeruginosa*,

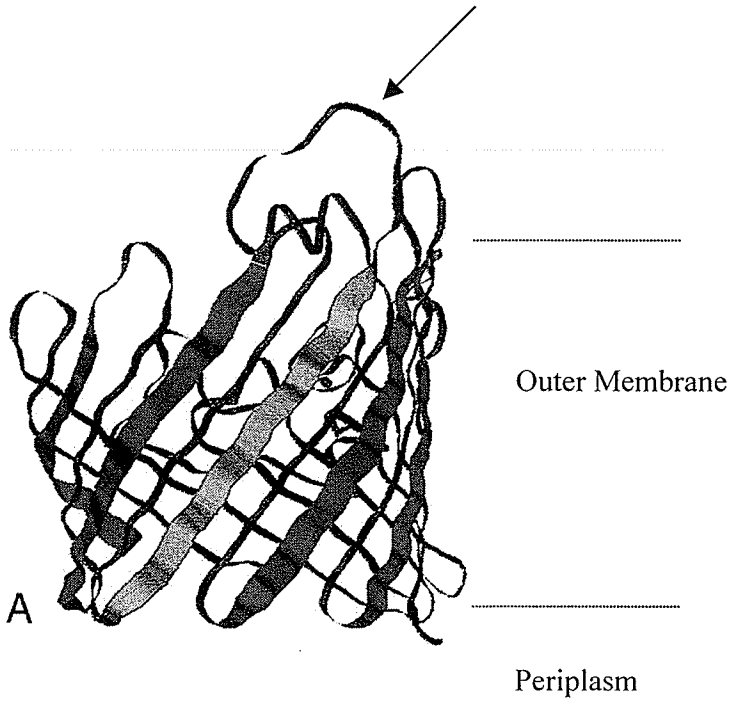
Haemophilus influenzae, and *Neisseria gonorrhoeae* (Bradford, 2001), have also been reported in *S. marcescens* (Luzzaro *et al*, 1998). This group of β-lactamases is characterized by inhibition by clavulanic acid (Bush *et al*, 1995).

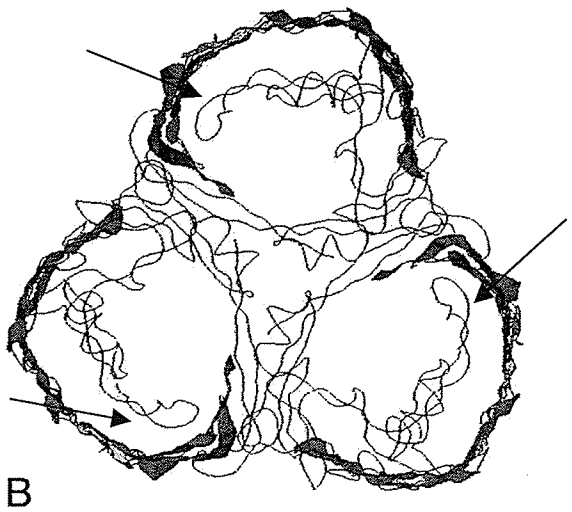
1.3.1.2. Outer Membrane Permeability

Penicillins and cephalosporins are the most commonly used classes of β-lactam antibiotics against *S. marcescens* infections. For β-lactam antibiotics to be effective in Gram-negative bacteria, they must penetrate the outer membrane. The Gram-negative outer membrane is a semipermeable lipid bilayer that acts like a molecular sieve to allow the passage of small hydrophilic molecules such as nutrients, waste products, and β-lactam antibiotics (Nikaido & Nakae, 1979) into and out of the cell. The degree of permeability of this membrane depends on the presence of pore-forming proteins called porins. Porins are membrane-spanning molecules that form water-filled channels. Although very few porins have strong amino acid homologies, most have strikingly similar physical features. Porins generally form a trimer of identical monomers. A major structural feature of porins is the high degree of β-sheet structure that zigzags through the membrane to form the stable pore-forming structure (Jeanteur *et al*, 1991). Crystal structures for porins of *Rhodobacter capsulatus* and *E. coli* (Fig.1.1) reveal a conserved 16-strand anti-parallel β-barrel structure for each monomer (Cowan *et al*, 1992; Garavito *et al*, 1983; Walian & Jap, 1990; Weiss & Schulz, 1992), implicating the role of a long loop, or eyelet region,

Fig. 1.1. Three-dimensional structure of the OmpF porin of *E. coli* (Neuwald *et al*, 1995). (A) β-barrel structure of the OmpF porin. (B) Porin trimer as seen from the top. Arrows indicate the eyelet loop (Loop 3) that is responsible for the forming the constricting zone in the channel.

Fig. 1.1.





which extends into the porin channel directing the channel size and ion selectivity (Cowan *et al*, 1992; Struyve *et al*, 1993).

Porins are classified as either non-specific or specific. Non-specific porins act as general diffusion pores, while specific porins possess binding sites for specific solutes (Hancock, 1991; Nikaido, 1992). In general, it is usually the non-specific porins that are involved in the passage of β-lactam antibiotics (eg. *E. coli* OmpF/C (Zimmermann, 1980), OprF of *Pseudomonas aeruginosa* (Angus *et al*, 1982) and changes in porin copy number, size, selectivity, or function can alter the rate of diffusion of hydrophilic β-lactam antibiotics (Nikaido & Rosenberg, 1981).

The role of porins in β-lactam resistance is well known in a variety of Gram-negative organisms. *E. coli* produces two non-specific porins, OmpF and OmpC, which are synthesized in differing amounts in response to osmolarity (Kawaji *et al*, 1979). OmpF has the wider pore diameter and thus, a higher rate of permeability to β-lactams (Nikaido & Rosenberg, 1983). Mutant strains deficient in OmpF are more resistant to β-lactam antibiotics due to slower penetration through the narrower OmpC channel. The slower rate of entry also enhances the ability of the periplasmically-located β-lactamases to hydrolyze antibiotics, while in the wild-type strains, β-lactam entry is too fast for effective degradation (Nikaido, 1988). The role of porins in β-lactam resistance in the other members of the *Enterobacteriaceae* family has also been reported. These include, for example, *Klebsiella pneumoniae* (Hernandez-Alles *et al*, 1999), *Enterobacter aerogenes* (De *et al*, 2001), *Proteus mirabilis, Proteus vulgaris, Morganella morganii, Providencia rettgeri*, and *Providencia alcalifaciens* (Mitsuyama *et al*, 1987). Non-specific diffusion porins

have been identified in many other Gram-negative organisms (Hancock, 1986), some of which have been shown, *in vitro*, to allow the diffusion of β-lactams (egs. *Campylobacter coli* and *Campylobacter jejuni* (Page *et al*, 1989), and *Salmonella typhimurium* (Oppezzo *et al*, 1991).

1.3.1.3. *Serratia marcescens* Porins

Two porins, OmpC and OmpF (named due to their similarity to *E. coli* porins), have been identified in the outer membrane of *S. marcescens* (Hutsul & Worobec, 1994; Hutsul & Worobec, 1997), and their role in antibiotic resistance of organism has also been recently demonstrated (Ruiz *et al*, 2003). OmpC has a molecular weight of 40 kDa while OmpF is a 41-kDa protein. The PEFGG(D) motif is conserved in enterobacterial porins and forms a turn at the tip of the third external loop (Cowan *et al*, 1992; Jeanteur *et al*, 1991). This loop forms the eyelet region, which is responsible for pore size and selectivity. Although, the OmpC porin of *S. marcescens* is approximately 60% amino acid homology to the enterobacter porins, a notable difference was found in the its eyelet region, which instead has the sequence $PEFD^{112}GM^{114}$. With this in mind, D^{112} and M^{114} were selected for individual alteration to glycine (G) and aspartic acid (D), respectively, to reflect the enterobacterial consensus sequence (Hutsul, 1996). The relative permeability of *S. marcescens* wild-type OmpF and OmpC and the mutant *S. marcescens* OmpC porins to cephaloridine, cephalothin, cefotaxime and glucose was tested using the liposome swelling assay. Results of liposome swelling assays demonstrated that the *S. marcescens* the OmpC porin with the 112 residue changed allowed more rapid diffusion of uncharged solutes than wild type OmpC porin. This mutation appeared

to increase the permeability of OmpC to that detected for enterobacterial porins with the consensus eyelet sequence (Hutsul, 1996). Similar results have been reported for *Enterobacter aerogenes* (De *et al*, 2001) and *E. coli* (Simonet *et al*, 2000). However, there was no significant difference in the rate of penetration of larger solutes, suggesting that the amino acid at position 112 may play an important role in pore constriction but not selection. No change in the permeability of the porin resulted from the change of methionine to aspartate at 114 position, therefore the significance of $M^{114}D$ substitution in the eyelet region remains unclear in *S. marcescens*.

1.3.2. Aminoglycoside Resistance

Common aminoglycosides used to treat bacterial infections are gentamicin, tobramycin, amikacin, and streptomycin. These antibiotics act against both Gram-positive and Gram-negative organisms by interfering with protein synthesis as a result of ribosome binding. Resistance to aminoglycosides is widespread, with more than 50 aminoglycoside-modifying enzymes identified to date (Fluit *et al*, 2001). These enzymes confer resistance to aminoglycosides by modifying their structures in a way to prevent ribosome binding. Depending on the type of modification offered by these enzymes, they are classified as aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT), or aminoglycoside phosphotransferases (APH). A number following the name with or without either a prime or double prime indicates the position of the modification on the substrate. Several aminoglycoside-modifying enzymes have been reported in *S. marcescens* and include AAC(3)-V (conferring resistance to gentamycin) (Barg, 1988) and AAC(6`)-I which is found exclusively in *S. marcescens* (Snelling *et al*, 1993).

1.3.3. Quinolone Resistance

DNA gyrase is the major target for quinolone antibiotics in Gram-negative organisms. DNA gyrase is a type II topoisomerase that relieves the supercoiling of DNA during the replication process. The enzyme alters the topological state of the DNA molecule by cleaving both strands, passing a double strand of DNA through the gap and resealing the ends (Champoux, 2001). DNA gyrase has an A_2B_2 structure, with the A and B subunits encoded by *gyrA* and *gyrB* respectively. The GyrA subunit of the DNA gyrase consists of residues responsible for DNA binding and cleavage and also the Quinolone Resistance Determining Region (QRDR), while the GyrB subunit is responsible for ATP hydrolysis and capturing of the DNA strand (Heddle & Maxwell, 2002). Resistance to quinolones generally arises from various mutations in the QRDR of *gyrA* (Yoshida *et al*, 1990a). In *E. coli*, mutations affecting Ser-83 and Asp-87 codons have been found in a vast majority of quinolone resistant clinical isolates (Vila *et al*, 1994).

DNA gyrase-mediated resistance to quinolones has also been reported in *S. marcescens* (Fujimaki *et al*, 1989). Among the *Enterobacteriaceae*, fluoroquinolone-resistant clinical isolates of *S. marcescens* have been shown to be displaying the greatest diversity of mutations in the *gyrA* gene. Three mutations in *gyrA* have been identified in this organism: Gly-81 to Cys, Ser-83 to Ile or Arg, and Asp-87 to Asn (Weigel *et al*, 1998).

Another mechanism of quinolone resistance observed in various organisms is the active efflux of antibiotic.

1.4. Active Drug Efflux

Emergence of multidrug resistant bacteria is one of the most challenging problems faced by antibiotic therapy today. The term **m**ulti**d**rug **r**esistance (mdr) is used to describe intrinsic mechanisms of resistance mediated by genes that are a part of the normal genome of the cell. MDR mediated by drug efflux pumps was first described by Levy and coworkers in 1980 (McMurry *et al*, 1980), when they showed that energy-dependent efflux was responsible for tetracycline resistance in *E. coli*.

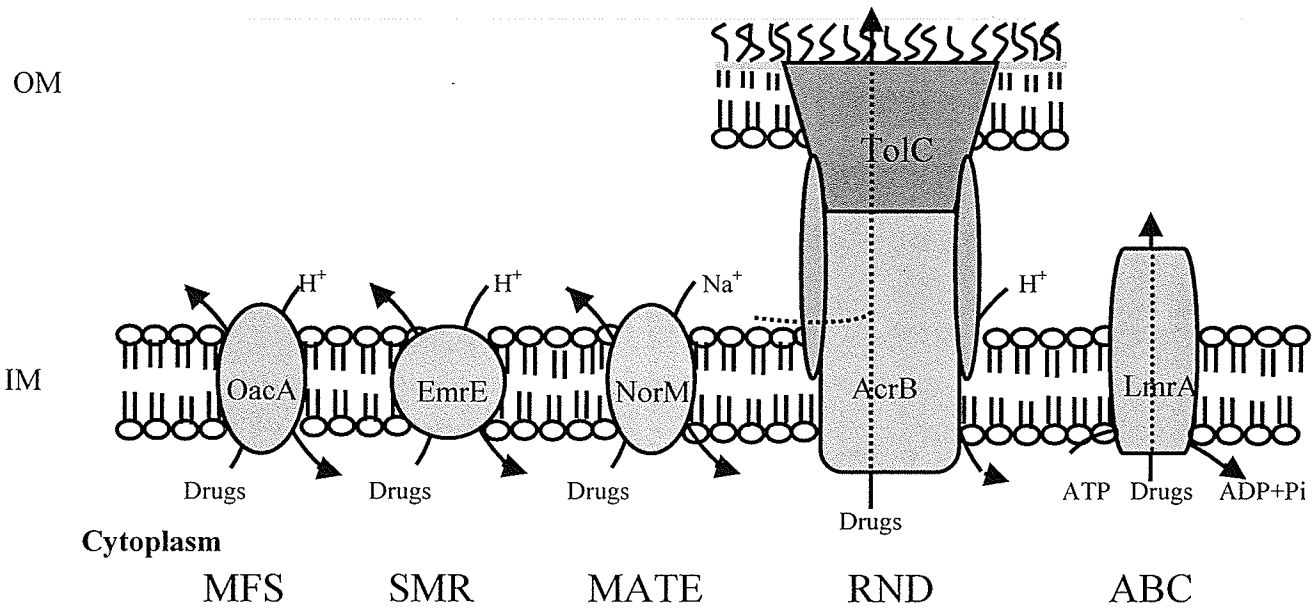
Antibiotic efflux is associated with the over-expression of transporters that pump out a broad range of structurally unrelated compounds from the cells in an energy-dependent manner, without any alteration or degradation of the drug. The ability of bacterial cells to pump out drugs is a complex phenomenon involving a combination of reduced influx and increased efflux within the cell envelope. Analysis of various available genome sequences has shown that known and putative drug efflux transporters constitute from 6% to 18% of all bacterial transporters (Paulsen *et al*, 1998). The most alarming aspect of this phenomenon is that the most advanced type of antibacterial agents, including the fluoroquinolone group of drugs like ciprofloxacin, are the compounds which seem to be selecting for multidrug resistant mutant strains capable of over-producing these multi-specific efflux pumps.

Bacterial antimicrobial efflux transporters are now classified into five families (Fig.1.2): 1) the major facilitator superfamily (MFS) (Marger & Saier, 1993); 2) the ATP-binding cassette (ABC) superfamily (van Veen & Konings, 1998); 3) the small

Fig. 1.2. Diagrammatic representation of multidrug efflux pumps belonging to 5 different families with their representatives (adapted from Paulsen, 2003).

IM, inner membrane; OM, outer membrane.

Fig. 1.2.


Environment
OM
IM
TolC
AcrB
OacA
EmrE
NorM
LmrA
H+
H+
Na+
H+
Drugs
Drugs
Drugs
Drugs
ATP
Drugs
ADP+Pi
Cytoplasm
MFS
SMR
MATE
RND
ABC

multidrug resistance (SMR) family (Paulsen *et al*, 1996b); 4) the resistance-nodulation-cell division (RND) superfamily (Saier *et al*, 1994); and 5) the multidrug and toxic compound extrusion (MATE) family (Brown *et al*, 1999).

These pumps can also be classified based on the number of components they have, either as single component transporters pumping out drug molecules across the inner membrane, or as multiple-component systems that contain the inner membrane transporters, along with an outer membrane factor (OMF) and a periplasmic membrane fusion protein (MFP), that pump out drug molecules across the inner and the outer membranes of Gram-negative bacteria.

1.4.1. Major Facilitator Superfamily (MFS)

The MFS is an ancient, large, and diverse superfamily that includes more than a thousand sequenced members. Members of this family catalyze uniport, solute:cation (H^+ or Na^+) symport, solute:H^+ antiport, or solute:solute antiport. This superfamily of transporters consists of proteins involved in transport of sugars, metabolites, anions, and drugs, and are driven by the proton-motive force. Six families of the MFS are known to export drugs, two of which are bacterial specific, two are eukaryotic specific, and two are ubiquitous (Saier & Paulsen, 2001). These transporters usually function as single-component pumps such as NorA pump of *Staphylococcus aureus* (Yoshida *et al*, 1990b), but in Gram-negative bacteria they function with the MFP and OMP components, an example being the EmrAB-TolC pump of *E. coli* (Lomovskaya & Lewis, 1992). Drug pumps belonging to this superfamily usually belong to families of proteins that consist of 12- or 14-transmembrane spanners (TMS) (Pao *et al*, 1998). The MFS proteins that catalyze

drug efflux are from three subfamilies, drug:H^+ antiporter (DHA) 1 (eg. Bmr of *Bacillus subtilis*), DHA 2 (eg. QacA of *Staphylococcus aureus*), and DHA 3 (eg. MefA of *Streptococcus pyogenes*). The DHA1 and DHA2 family proteins are ubiquitous and are known to efflux a very broad range of structurally distinct drugs. DHA1 members are also known to export sugars, polyamines, uncouplers, monoamines, acetylcholine, paraquat, and methylglyoxal. DHA2 family members have more restricted substrate specificity, but they are known to tranport bile salts and dyes. The DHA3 family is restricted to prokaryotes, and is known to efflux antibiotics including macrolides and tetracycline.

1.4.2. ATP-Binding Cassette (ABC) Superfamily

ABC transporters contain both uptake and efflux transport systems, and use ATP hydrolysis to transport a variety of substances including sugars, amino acids, ions, drugs, polysaccharides, and proteins (Fath & Kolter, 1993; Higgins, 1992). The transporters of the ABC superfamily consist of two integral membrane domains/proteins and two cytoplasmic domains/proteins. The bacterial ABC transporters generally consist of 6 transmembrane spanners and an ATP binding subunit localized on the cytoplasmic side of the inner membrane. Both the integral membrane channel constituent(s) and the cytoplasmic ATP hydrolyzing constituent(s) may be present as homodimers or heterodimers. Drug efflux pumps belonging to the ABC-superfamily are not very common in bacteria, although LmrA pump of *Lactococcus lactis* is one example (Bolhuis *et al*, 1996). The current ABC superfamily includes 21 procaryotic efflux systems (Saier & Paulsen, 2001).

1.4.3. Small Multidrug Resistance (SMR) Family

Transporters belonging to the SMR family contain only about 110 amino acid residues and 4 TMSs. These transporters utilize the proton-motive force as an energy source. Due to the small size of the proteins, they were believed to be functioning as trimers (Paulsen *et al*, 1996a); however, a recent study has shown that these proteins actually exist as tetramers (Ma & Chang, 2004). Some of the well-characterized pumps of this family include the Smr pump of *S. aureus* (Grinius *et al*, 1992), and EmrE pump of *E. coli* (Schuldiner *et al*, 1997) that are known to efflux dyes, drugs, and cations.

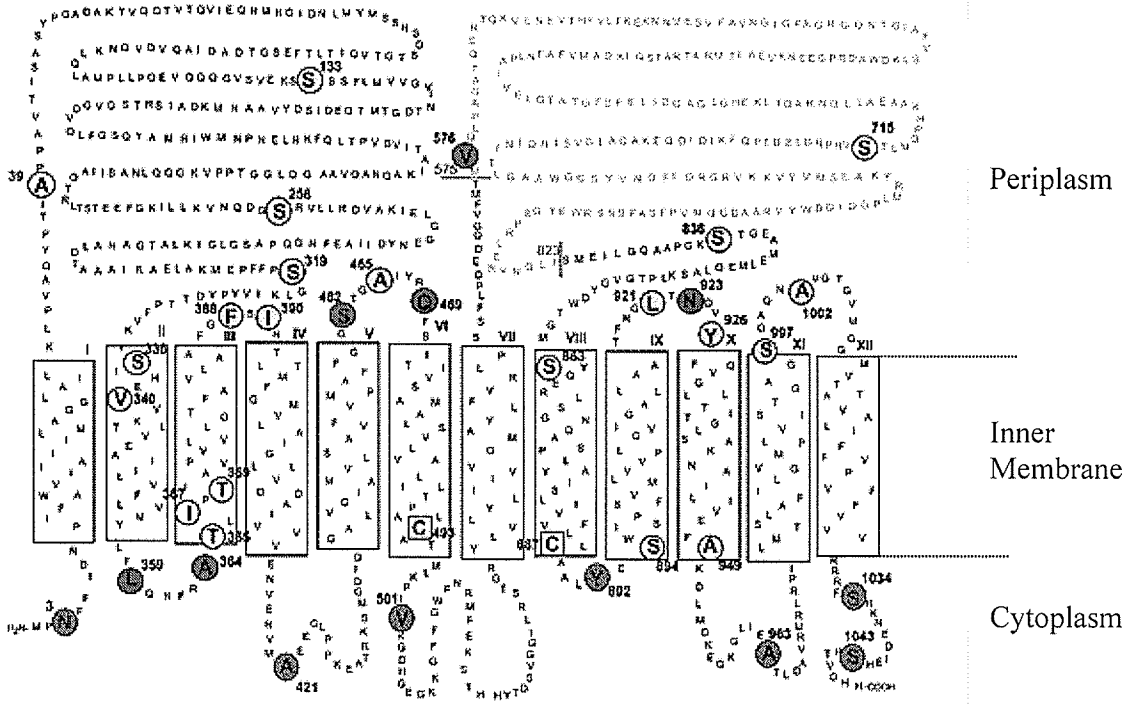
1.4.4. Resistance-Nodulation-Cell Division (RND) Superfamily

Proteins belonging to the RND superfamily were initially thought be present only in bacteria; however, they have now been reported in eukaryotes and archaea as well (Tseng *et al*, 1999). RND drug transporters are typically chromosomally-encoded, although one plasmid-encoded RND drug transporter has recently been reported (Droge *et al*, 2000). All characterized members of the RND family catalyze substrate efflux via an H^+ antiport mechanism. RND pumps play an important role in acquired and intrinsic resistance of Gram-negative bacteria to a variety of antimicrobials. All RND pumps known to date are multidrug transporters.

RND pumps function by forming complexes with a MFP and OMP in Gram-negative organisms, and consist of 12 TMSs. The characteristic feature of the topology is the presence of 2 large periplasmic loops between TMSs 1 and 2 and TMSs 7 and 8 (Fig. 1.3). N-terminal halves of RND family proteins are homologous to the C-terminal halves, and thus these proteins are believed to have risen from an

Fig. 1.3. Membrane topology of the AcrB protein of *E. coli* showing 12 transmembrane spanners (TMSs) and two large periplasmic loops between TMSs 1 and 2, and 7 and 8 (Fujihira *et al*, 2002).

Fig. 1.3.



intragenic tandem duplication event that occurred in the primordial system before the divergence of the family members. There is, however, one example of an RND homologue from *Mycobacterium jannaschii* that has only 6 TMSs, and no internal duplication. It is possible that this protein associates with another component and functions as a homo or heterodimer. The best-studied members of these pumps are the AcrAB-TolC system of *E. coli* (Ma *et al*, 1993; Ma *et al*, 1995), and the MexAB-OprM system of *Pseudomonas aeruginosa* (Poole *et al*, 1993) that are known to efflux antibiotics, heavy metals, dyes, detergents etc.

1.4.5. Multidrug and Toxic Compound Extrusion (MATE) Family

Previously thought be members of the MF superfamily, the proteins belonging to the MATE family are now classified into a separate family of transporters as, in spite of similar membrane topology, they show no sequence homology to members of MFS. Examples of proteins belonging to this family include NorM of *Vibrio parahaemolyticus* and YdhE of *E. coli* (Morita *et al*, 1998). These are of about 450 amino acyl residues in length and contain 12 putative TMS. Proteins belonging to this family use the Na^+ gradient as the energy source to pump out cationic dyes and fluoroquinolones (Morita *et al,* 1998).

1.5. General Features of the RND Superfamily

Efflux pumps belonging to the RND superfamily are most prevalent in Gram-negative bacteria, and are responsible for resistance of these organisms to various groups of antibiotics including quinolones, aminoglycosides, and β-lactams. This thesis describes two different novel RND pumps in *S. marcescens* and thus, the reminder of this review will focus on the RND family.

Three major criteria (energy source, phylogenetic relationship, and substrate specificity) were used to divide the RND superfamily into seven families, namely the Heavy Metal Efflux (HME) family, the Hydrophobe/Amphiphile Efflux-1 (HAE-1) family, the Nodulation Factor Exporter (NFE) family, the SecDF family, the HAE-2 family, the Eukaryotic Sterol Transporter (EST) family, and the HAE-3 family (for details see, http://www.biology.ucsd.edu/~msaier/ transport/). All seven members of this superfamily function via substrate:proton antiport (Nies & Silver, 1995).

Proteins belonging to the HME family export heavy metals (Rensing *et al*, 1997). These proteins have been found only in Gram-negative bacteria thus far, and are known to efflux heavy metals including Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Ag^{2+}. Members of the HAE-1 family show specificity for drugs (egs. AcrB, AcrD pumps of *E. coli*, and MexB of *P. aeruginosa*), bile salts (e.g. MtrD of *Neisseria gonorrhoeae*), and other hydrophobic compounds (Saier *et al*, 1998). NFE family proteins are believed to secrete lipooligosaccharides that function as signaling molecules in rhizobial nodulations in leguminous plants (Gottfert, 1993). The SecDF family is comprised of bacterial and archeal proteins that function together as auxillary consitituents of the universal (Type II) secretory pathway (Bolhuis *et al*, 1998). The HAE-2 family includes about 20 proteins that are exclusive to Gram-positive bacteria (e.g. ActII3 of *Streptomyces coelicolor* exports the antibiotic actinorhodin, which it produces) (Fernandez-Moreno *et al*, 1991). The EST family is, as the name suggests, exclusive to eukaryotic cells and includes the human Niemann-Pick C disease protein (NPC), believed to be playing a role in cholesterol homoestasis (Loftus *et al*, 1997). A homolog of NPC identified in the yeast *Saccharomyces cerevisiae*, YMP

(YPL006w), is ~30% identical to NPC, with identities spread throughout the length of the protein (Nelissen *et al*, 1997). The last family, HAE-3, includes proteins from archaea and spirochetes (Tseng *et al*, 1999). These proteins have not yet been functionally characterized.

1.5.1. RND pumps in *E. coli*

Analysis of the *E. coli* genome has revealed the presence of seven putative and known RND transporters (Nishino & Yamaguchi, 2001). The AcrAB-TolC system has been identified as the predominant drug efflux pump of this organism, and it is the best-characterized RND pump to date. This system demonstrates a very broad substrate specificity, that includes acriflavine, β-lactams, bile salts, chloramphenicol, crystal violet, ethidium bromide, fatty acids, macrolides, organic solvents, fluoroquinolones, and SDS. Other pumps belonging to the RND family in *E. coli* include AcrEF, AcrD, YhiUV, and MdtABC that use a variety of antimicrobials as their substrates. All *E. coli* RND pumps studied use TolC as the outer-membrane component. Knock-out experiments with *acrEF*, *yhiUV*, and *mdtABCD* did not change drug susceptibilities of the wild-type *E. coli* cells, suggesting that these pumps do not play a significant role in the antimicrobial resistance of this organism (Sulavik *et al*, 2001). The AcrD pump is believed to function as a single component pump to efflux aminoglycosides (Rosenberg *et al*, 2000); however it was shown to require AcrA and TolC to efflux bile salts and novobiocin (Elkins & Nikaido, 2002). When overexpressed, YhiUV-TolC pump is responsible for resistance to doxorubicin, erythromycin, deoxycholate, and crytal violet, while MdtABC system confers resistance to bile salts and novobiocin

(Baranova & Nikaido, 2002; Nagakubo *et al*, 2002). Interestingly, the MdtABC system contains two different RND transporters, MdtB and MdtC, and both are required for drug extrusion. Interestingly, an MFS transporter gene, *mdtD*, has been found downstream of the *mdtABC* operon. However, it does not appear to play any role in the antibiotic resistance (Baranova & Nikaido, 2002).

1.5.2. RND pumps of *P. aeruginosa*

P. aeruginosa is notorious for causing a variety of infections, particularly in immuno-compromised individuals. This organism shows a high degree of intrinsic resistance to different antibiotics, making effective drug therapy very difficult. One of the major contributing factors to antibiotic resistance of this organism is low outer membrane permeability. However, with the discovery of efflux pumps in *P. aeruginosa*, active efflux is now considered a very important mechanism of antibiotic resistance. The first efflux pump to be reported in this organism is the MexAB-OprM, an RND-type pump (Poole *et al*, 1993). To date, seven RND-type drug efflux pumps have been found in this organism: MexAB-OprM, MexCD-OprJ (Poole *et al*, 1996a), MexEF-OprN (Kohler *et al*, 1997), MexXY-OprM (Mine *et al*, 1999), MexJK-OprM (Chuanchuen *et al*, 2002), MexGHI-OpmD (Aendekerk *et al*, 2002), and MexVW-OprM (Li *et al*, 2003). Unlike *E. coli*, where only one outer membrane protein component has been found to be working in conjunction with different pumps, at least 17 homologs of OprM are known to be expressed in *P. aeruginosa* (Jo *et al*, 2003).

1.5.2.1. MexAB-OprM

The MexAB-OprM system of *P. aeruginosa* is responsible for intrinsic resistance of this organism, as it has been shown to be responsible for antimicrobial resistance of even the wild-type cells. The MexAB-OprM system has the broadest range of substrate specificity amongst all known efflux pumps of *P. aeruginosa*. Substrates of this system include β-lactams, quinolones, macrolides, tetracyclines, chloramphenicol, novobiocin, sulfonamides, trimethoprim, and thiolactomycin (Kohler *et al*, 1996; Li *et al*, 1994a; Li *et al*, 1994b; Li *et al*, 1995), as well as non-antibiotic compounds including dyes, detergents, triclosan, and organic solvents (Li *et al*, 1998; Schweizer, 1998; Srikumar *et al*, 1998).

The export of β-lactams by MexAB-OprM system is intriguing, as efflux-mediated resistance to this group of antibiotics in not very common. In some instances it has been shown to play a more important role than the AmpC β-lactamase of *P. aeruginosa* (Masuda *et al*, 1999; Nakae *et al*, 1999). Of the β-lactams, only carbapenems appear to be poor substrates for MexAB-OprM.

OprM can function with various efflux pumps. It not only operates with MexAB, but also MexXY (Mine *et al*, 1999; Mao *et al*, 2001), MexJK (Chuanchuen *et al*, 2002), and MexVW (Li *et al*, 2003). It has also been shown to complement OprJ of MexCD-OprJ (Gotoh *et al*, 1998) and OprN of MexEF-OprN systems (Maseda *et al*, 2000). This replacement of the native outer-membrane component by OprM does not affect the substrate specificity of the RND pump.

It has been suggested that MexAB-OprM system is responsible for efflux of physiological compounds synthesized inside the cells, as it has been shown to efflux

homoserine lactone from *P. aeruginosa* cells (Pesci *et al*, 1999). However, this seems to be a controversial topic as another group has shown that a *mexAB-oprM* overexpressing strain of *P. aeruginosa* was not able to produce a quorum-sensing response (Evans *et al*, 1998). Another study has shown that MexAB-OprM system is required for the invasiveness of *P. aeruginosa* in mice models (Hirakata *et al*, 2002).

1.5.2.2. MexCD-OprJ

MexCD-OprJ is not expressed in wild-type *P. aeruginosa* (Poole *et al*, 1996a). Overexpression of this system in strains carrying mutations in *nfxB* results in resistance to quinolones, tetracycline, chloramphenicol, acriflavine, ethidium bromide, triclosan, and organic solvents (Poole *et al*, 1996a; Chuanchuen *et al*, 2001; Li *et al*, 1998). *P. aeruginosa nfxB* mutants can be classified in two categories, A and B. A-type mutants are resistant to ofloxacin, erythromycin and some new cephalosporins, and type B mutants are resistant to tetracycline and chloramphenicol as well, in addition to the agents mentioned for type A mutants. However, type B mutants are four to eight times more susceptible to many penicillins, carbapenems, and aminoglycosides than the wild-type strain of *P. aeruginosa* (Masuda *et al*, 1996). This could be due to the down-regulation of the MexAB-OprM system (Li *et al*, 2000b) and AmpC β-lactamase (Masuda *et al*, 2001) in MexCD-OprJ-overexpressing mutants.

1.5.2.3. MexEF-OprN

The MexEF-OprN system is not expressed in wild-type strains of *P. aeruginosa* under normal laboratory growth conditions, and disruption of the *mexEF-oprN* genes does not alter the antibacterial susceptibility of this organism (Kohler *et*

al, 1997). This system is overexpressed in *nfxC*-type multidrug resistant strains, where it is responsible for resistance to fluoroquinolones, tetracycline, chloramphenicol, and trimethoprim (Kohler *et al*, 1997). The *nfxC* mutants also show resistance to imipenem, although this is known to be due to a decrease in the number of molecules of the outer membrane protein OprD (Ochs *et al*, 1999). The hypersusceptibility of *nfxC* strains to β-lactams and aminoglycosides (Fukuda *et al*, 1990) may result from decreased expression of MexAB-OprM and MexXY-OprM, as suggested for the *nfxB* mutants. Upregulation of MexEF-OprN system has also been shown to play a role quorum sensing in *P. aeruginosa* by affecting the intracellular levels of PQS (*Pseudomonas* quinolone signal) (Kohler *et al*, 2001).

1.5.2.4. MexXY-OprM

In contrast to the MexAB-OprM, MexCD-OprJ, and MexEF-OprN systems, the *mexXY* operon does not have a linked gene for an outer membrane component (Mine *et al*, 1999). MexXY system utilizes OprM as its outer membrane component (Aires *et al*, 1999, Mine *et al*, 1999). Deletion of *mexXY* genes results in increased susceptibility of *P. aeruginosa* strains to aminoglycosides, tetracycline, and erythromycin (Aires *et al*, 1999). Interestingly, when *mexXY* is expressed in *E. coli*, it promotes resistance to fluoroquinolones, although this efflux system does not contribute to intrinsic resistance to these agents in *P. aeruginosa* (Aires *et al*, 1999; Mine *et al*, 1999). It appears that fluoroquinolones are not able to induce MexXY expression in wild-type *P. aeruginosa*, although mutants hyperexpressing MexXY demonstrate enhanced fluoroquinolone resistance (Masuda *et al*, 2000). Overexpression of the MexXY-OprM pump has been observed in several

impermeability type aminoglycoside-resistant strains of *P. aeruginosa*, and deletion of *mexY* compromises this resistance, thus confirming the role of MexXY-OprM system in the aminoglycoside resistance of these mutants (Wesbrock-Wadman *et al*, 1999)

1.5.2.5. MexJK-OprM

The MexJK pump is not expressed in wild-type strains of *P. aeruginosa* (Chuanchuen *et al*, 2002). One interesting feature of this pump, in strains overexpressing this system, is that it has been shown to require OprM for extruding ciprofloxacin, erythromycin, and tetracycline, but functions independently of OprM for triclosan resistance (Chuanchuen *et al*, 2002).

1.5.2.6. MexGHI-OpmD

The MexGHI-OpmD system contains an MFP, MexH, an RND transporter (MexI) and an OM channel (OpmD). This pump also contains a small integral membrane protein of unknown function, MexG (Aendekerk *et al*, 2002). This system is active in the wild-type cells and is responsible for resistance to vanadium (Aendekerk *et al*, 2002). Interestingly, deletion mutants of *mexGHI-ompD* exhibit an increase in the resistance to tetracycline, netilmicin, and ticarcillin. It is suspected that this could be due to the overexpression of other pumps (Aendekerk *et al*, 2002) (Li *et al*, 2000a).

1.6. Structure of RND efflux pumps

In Gram-negative bacteria, RND efflux pumps form a three-component complex traversing both the inner and outer membranes (Lomovskaya & Watkins, 2001b). The tripartite complex consists of the RND protein as the inner membrane

pump; a periplasmic protein, also referred to as the membrane fusion protein (MFP); and an outer membrane component called the outer membrane factor (OMF). The presence of a three-component membrane-traversing structure facilitates direct passage of the substrate from the cytoplasm into the external medium. All three components of RND efflux pumps can either be encoded by one gene cluster, e.g. MexAB-OprM of *P. aeruginosa* (Poole *et al*, 1993) or, in other cases, including the AcrAB pump of *E. coli* (Ma *et al*, 1993) and the MexXY pump of *P. aeruginosa* (Mine *et al*, 1999), the gene encoding the outer membrane component is not present in the gene cluster encoding these pumps. AcrAB, identified as the dominant efflux pump in *E. coli* (Sulavik *et al*, 2001), uses TolC as the outer membrane component (Fralick, 1996), while MexXY is known to share OprM of MexAB as the outer membrane component (Aires *et al*, 1999).

1.6.1. Inner membrane pump components

The inner membrane component of RND efflux pumps is responsible for the identification of the molecule to be effluxed (i.e. the drug molecule) (Zgurskaya & Nikaido, 1999). Two of the best-studied RND transporters, AcrB of *E. coli* and MexB of *P. aeruginosa*, pump out lipophilic and amphiphilic compounds (Nikaido, 1996) and can recognize a variety of substrates. However, an extensive study has shown that all substrates have a hydrophobic domain capable of insertion into the phospholipid bilayer (Nikaido, 1996). Inner membrane pumps consist of 12 transmembrane spanners (TMSs) connected by cytoplasmic and periplasmic loops. The periplasmic loops present between TMSs 1 and 2, and TMSs 7 and 8 are very large, consisting of about 300 amino acid residues each (Fujihira *et al*, 2002). Recent

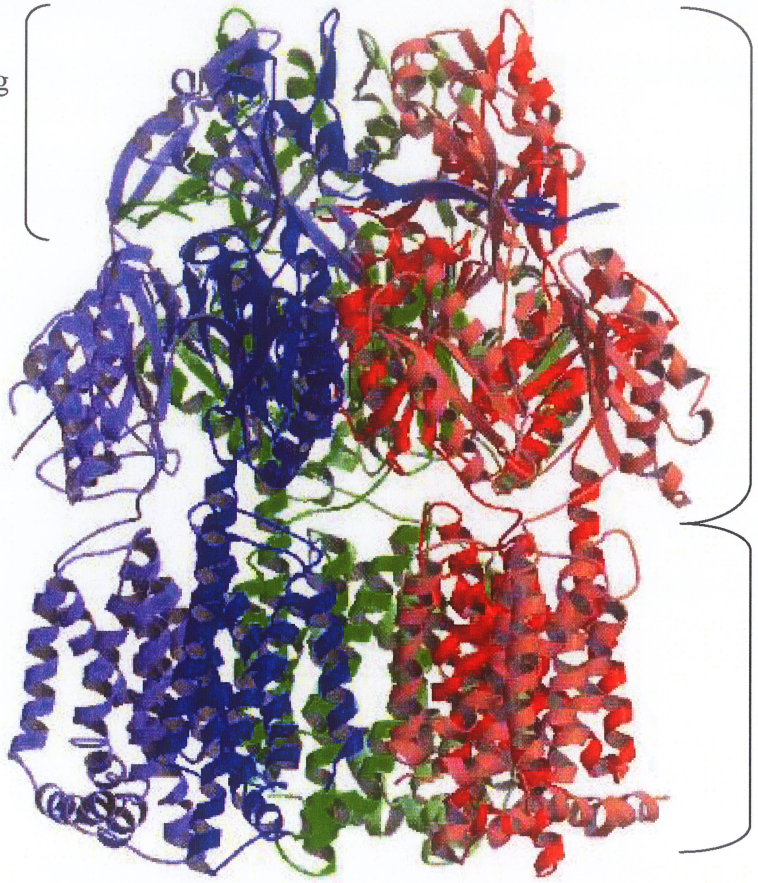
studies on the MexD pump of *P. aeruginosa* and AcrB and AcrD pumps of *E. coli* suggest that the large periplasmic loops have a role in substrate specificity (Elkins & Nikaido, 2002; Mao *et al*, 2002).

The recently-published crystal structure of the inner membrane component AcrB at 3.5 Å resolution (Murakami *et al*, 2002) shows that the AcrB protein is arranged as a homotrimer forming a jelly fish like structure (Fig.1.4). Each protomer is composed of a 50 Å thick transmembrane region and a 70 Å protruding headpiece which can be divided into two parts: TolC docking domain (30 Å) and the pore domain (40 Å).

The headpiece is formed by two large periplasmic loops, with the loops from one protomer interacting with loops of the other two protomers to form a trimer. The inter-monomeric interaction is also mediated by TMS 1 and TMS 8. The top of the headpiece is shaped like a funnel. The cavity present at the center of this funnel-shaped headpiece has three vestibules leading into the periplasm. This structure suggests that the substrates translocated from the cytoplasm or the periplasm are collected in the central cavity and from there they are actively transported through the pore into the channel formed by TolC in the outer membrane. It is expected that many other RND exporters would share a similar structure, as there is very high amino acid sequence similarity among RND transporters. The folding topology of MexB and MexD pumps of *P. aeruginosa* was found to be similar to that of *E. coli* AcrB, containing 12 TMS with two very large periplasmic loops between TMS 1 and 2 and also TMS 7 and 8 (Gotoh *et al*, 1999; Guan *et al*, 1999).

Fig. 1.4. Three-dimensional structure of the AcrB trimer at 3.5 Å (Murakami *et al*, 2002).

Fig. 1.4.


TolC-docking domain
70 Å Head Piece
Periplasm
50 Å Transmembrane region
Inner Membrane

The inner membrane transporter is responsible for the identification of substrates. This has been shown by various studies that combined different components of the tripartite system. For example, the hybrid system MexAB-OprJ still retains the drug specificity of the MexAB-OprM system (Srikumar *et al*, 1999). Some recent studies have conclusively shown that the two large periplasmic loops of the RND pumps are responsible for substrate recognition (Mao *et al*, 2002; Eda *et al*, 2003; Tikhonova *et al*, 2002; Elkins & Nikaido, 2002). These studies were performed using chimeric constructs of different RND pump proteins from *E. coli* and *P. aeruginosa*. It was observed that the chimeric protein containing the periplasmic loops of one protein retained the substrate specificity of that particular protein. This is consistent with the crystal structure of AcrB, where the vestibules between the periplasmic domains of the neighbouring protomers are believed to be the site of entry and possibly the recognition of substrates (Fig. 1.4) (Murakami *et al*, 2002). This mechanism might also explain the efflux of β-lactams and aminoglycosides by AcrD and MexY pumps, as these drugs are known not to enter the cytoplasm by spontaneous diffusion.

Recent developments in understanding the functional properties of RND pumps are the crystal structures of the AcrB protein bound with different substrates, namely ethidium bromide, rhodamine 6G, dequalinium, and ciprofloxacin (Yu *et al*, 2003). The structures show that multiple molecules of ligands bind simultaneously to the extremely large central cavity of 5000 cubic Å, primarily by hydrophobic, aromatic stacking and van der Waals interactions. Different substrates were found bound at different binding sites within the cavity. It was also observed that the drug

molecules are bound to the walls of the large internal cavity, and are located roughly at the level of the outer layer of the inner membrane bilayer, suggesting that the drug molecules diffuse through the vestibules between the AcrB monomers. Interestingly, there are no negatively charged amino acid residues near the substrate binding sites, leading to the hypothesis that the positive charges of the substrates are neutralized by the anionic head groups of the phospholipids in the cavity (Yu *et al*, 2003). This means that the substrate binding sites in the AcrB are composed of phospholipids from the outer layer of the inner membrane bilayer and protein side-chains.

Highly conserved amino acid residues have been identified in the transmembrane region of RND transporters (Tseng *et al*, 1999), for example Asp 407, Asp408 in the TMS4, and Lys940 in the TMS10 of AcrB pump. Replacement of these amino acids through site-specific mutagenesis renders these pumps non-functional. It is possible that these three residues, shown to form ion pairs (Murakami *et al*, 2002), function in proton translocation.

1.6.2. Periplasmic pump components

The periplasmic proteins of the RND multidrug complex belong to the membrane fusion protein (MFP) family (Dinh *et al*, 1994). These proteins are quite common in Gram-negative transport systems and have not been identified in Gram-positive bacteria, archaea, or eukaryotes, with one exception reported in *B. subtilis* (Johnson & Church, 1999). MFPs are generally of uniform size (about 380-480 amino acyl residues) and contain two hydrophobic domains, each near the N- and C-termini, which are believed to interact with the inner and outer membrane

components of the RND complexes (Zgurskaya & Nikaido, 2000a), thus facilitating the transfer of substrates across the periplasm.

The best-studied periplasmic component protein, AcrA of the *E. coli* AcrAB pump, is a highly asymmetric molecule approximately 20 nm in length (Zgurskaya & Nikaido, 2000a). AcrA consists of two regions of coiled-coils separated by a short stretch of amino acid residues with the coiled-coil region flanked by two lipoyl arms. The N-terminal region has a covalently linked lipid extension, while the C-terminal region is found to have a β-domain, which seems to be conserved in proteins belonging to the MFP family. Presence of the β-domain on the C-terminal end has led to speculations that perhaps this end is inserted in the outer membrane in a porin-like fashion. However, the recently-solved crystal structures of MexA fail to show this kind of arrangement (Akama *et al*, 2004; Higgins *et al*, 2004). Instead the structure by Higgins and colleagues shows that the C-terminus of MexA is positioned in close proximity to the N-terminus.

The periplasmic components of RND pumps are exclusive for each inner membrane component and cannot be interchanged among the inner membrane pumps, even when two inner membrane pumps are highly homologous (Yoneyama *et al*, 1998). However, one study has shown that the AcrD pump of *E. coli* can function with the AcrA protein of the AcrAB complex (Elkins & Nikaido, 2002). The study showed that the efflux of amphiphilic substrates like bile acids and novobiocin by AcrD was completely dependent on AcrA.

1.6.3. Outer membrane components

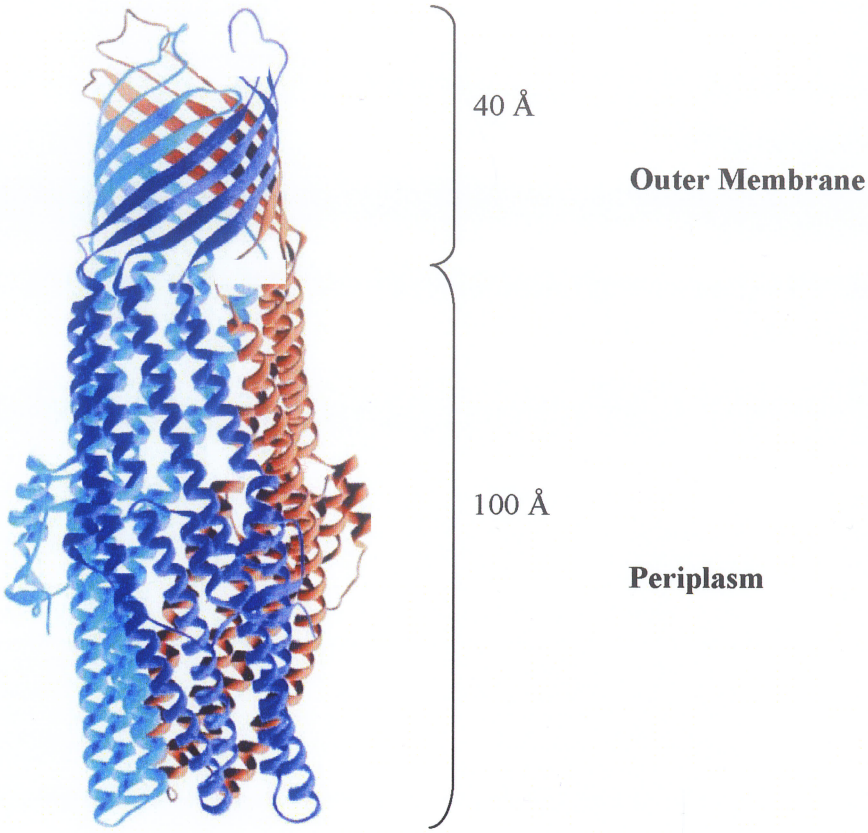
Outer membrane components are required in order to export drug molecules directly into the external medium. These components are of fairly similar size ranging from approximately 400 to 500 amino acyl residues. In some instances, a single outer-membrane protein has been shown to function with different pumps (Zgurskaya & Nikaido, 2000b). For example, TolC, the outer membrane component of the AcrAB pump can also function with the protein transporter HylB (Holland *et al*, 1990). Being the only functional outer-membrane component identified so far, TolC is believed to work with all drug efflux systems in *E. coli* that require an OMF. TolC has also been shown to act with the MexCD and MexXY pumps of *P. aeruginosa* when they are expressed in *E. coli* (Srikumar *et al*, 1998).

Two of the best-studied examples of outer membrane components of RND pumps are TolC and OprM. TolC is a trimeric outer membrane protein (Koronakis *et al*, 1997). The crystal structure of the protein (Koronakis *et al*, 2000) reveals the presence of an α-helical barrel which forms a ~100 Å long tunnel, through the periplasm. This barrel is anchored by the contiguous 12-stranded outer membrane β-barrel part of the protein (Fig.1.5). OprM has also been found to have a similar structure with both α-helical and β-barrels present (Wong *et al*, 2001).

It is proposed that both the TolC and OprM proteins have amphipathic β-strands with hydrophobic residues in the β-barrel facing the hydrophobic core of the bilayer. A ring of aromatic residues at the lipid-water interface is present between the outer membrane β-barrel and the periplasmic α-helical barrel (Wong *et al*, 2001).

Fig. 1.5. Three-dimensional structure of the outer membrane component, TolC, of the AcrAB-TolC efflux pump of *E. coli* at 2.1 Å (Koronakis *et al*, 2000).

Fig. 1.5.



This arrangement of aromatic amino acids is found on the periplasmic side of all known outer membrane β-barrels.

The TolC homotrimer is 140 Å in length, with the periplasmic α-helical barrel 100 Å, and the outer membrane β-barrel 40 Å. The α-barrel consists of 12 α-helices packed in an antiparallel arrangement to form a hollow cylinder. Karonakis and coworkers (2000) propose that the α-helical barrel of the TolC protein is virtually uniform in diameter near the β-barrel; however, the long helices are tapered at the proximal end (Koronakis *et al*, 2000). At this end, helices are arranged in inner and outer pairs, with inner pair helices forming an antiparallel coiled-coil. It is believed that the opening of the channel is achieved by inner coils re-aligning themselves with the outer coils, thus enlarging the channel opening (Andersen *et al*, 2002a). This "channel-tunnel" structure is long enough to span both the outer-membrane and the periplasm. When the end of the TolC tunnel contacts the top of the periplasmic domain of AcrB, a 170 Å long channel is created which is long enough to cover the entire periplasmic depth, allowing the substrate molecule to pass through directly into the external medium (Koronakis *et al*, 2000).

In addition to TolC, genes of three other homologs (*yjcP*, *yohG*, and *ylcB*) have been found in the *E. coli* genome, although none are genetically linked to genes for efflux pumps. Knock-out experiments with these three genes have indicated that it is unlikely that the corresponding proteins are involved in drug efflux (Sulavik *et al*, 2001).

In contrast to *E. coli*, in which TolC seems to be the only OM component involved in drug efflux, *P. aeruginosa* has been found to have 17 homologs of OprM

(Jo *et al*, 2003). Of these 17 proteins, OmpG and OmpH were shown to complement the OprM deficiency in the MexAB-OprM system. This finding also suggests that the outer membrane proteins of RND systems share the same structure and mechanism of action for efflux of various substrates even when they might share very low identity at the amino acid level.

Reconstitution studies with TolC and OprM in the black lipid bilayer system or proteoliposomes revealed only weak pore-forming activities (Benz *et al*, 1993; Yoshihara *et al*, 2002). This is consistent with the closed channel found in the crystal structure of TolC, where it is believed that the channel-tunnel structure remains closed under normal conditions, and opening is likely triggered by interaction with the inner membrane pump (Koronakis *et al*, 2000).

1.7. Natural Function of RND Pumps

The natural function of RND pumps is still a topic of debate. It was originally believed that these pumps were developed by Gram-negative pathogens as a defense mechanism to counter the increasing antibiotic concentrations in their environment. However, with phylogenetic studies pointing to the presence of RND pump homologs in Gram-positive bacteria, archaea, and even humans, it has been established that proteins belonging to RND pumps are part of an ancient family of proteins with representation in all major kingdoms (Tseng *et al*, 1999).

The AcrAB pump of *E. coli* was found to have the highest affinity for bile salts, as shown by the hypersusceptiblilty of genetically-constructed AcrAB knock-out strains to bile salts (Thanassi *et al*, 1997). The natural habitat of *E. coli* is the enteric tract (which is rich in bile salts) so the presence of this type of protective

mechanism is feasible. Similarly, a protective function has been attributed to the MtrCDE system, which provides resistance to faecal lipids in rectal isolates of *N. gonorrhoeae* (Shafer *et al*, 1995). Additional natural functions suggested for efflux pumps include: removal of toxins, removal of end products of metabolism (e.g. fermentation end products and toxins directed towards other organisms) and buffering the organisms against infrequent surges in pools of potentially toxic metabolites (Helling *et al*, 2002).

It has been suggested that the natural function of RND pumps might also include efflux of quorum-sensing signal molecules required for cell-to-cell signaling (Rahmati *et al*, 2002). *P. aeruginosa* has been shown to secrete a quinolone, designated PQS (Pseudomonas quinolone signal), which acts as a quorum signal (Pesci *et al*, 1999). This is indeed a very interesting finding as quinolones constitute a widely used group of antimicrobials and are substrates for RND pumps. Another study on the newly characterized MexGHI-OmpD pump of *P. aeruginosa* suggests that it is involved in the homeostasis of the quorum sensing regulatory molecule, N-acyl-homoserine lactone (Aendekerk *et al*, 2002). In addition, it has also been reported that SdiA, a quorum-sensing regulator from *E. coli*, also controls multidrug resistance by positively regulating the AcrAB pump (Rahmati *et al*, 2002). One can easily envision the efflux of certain antibiotics that structurally resemble these signaling molecules.

One study has recently suggested a correlation between the presence of AcrAB pump in *E. coli* and calcium transport (Jones *et al*, 2003). It is suspected that

AcrAB pump could be playing a role in the transport of the calcium-channel components to the membrane in *E. coli*.

1.8. Mechanism of Action of RND Efflux Pumps

The generalized transport reaction catalyzed by functionally-characterized RND proteins is:

$$Substrate_{in} + nH^{+}_{out} \rightarrow Substrate_{out} + nH^{+}_{in}$$

In spite of extensive research, the basic mechanism of multidrug transporters is not well understood. It is still not known how the driving force of these pumps is coupled to the export process. RND-type transporters are very versatile as far as substrate recognition is concerned, as they can pump out hydrophobic cations, neutral, zwitteronic, and negatively charged compounds. A common feature of all of these substrates seems to be large hydrophobic domains which ensure the partition of drug molecules into the phospholipid bilayer, favoring the notion that substrate binding occurs in the membrane environment. Direct evidence for binding within the membrane was obtained in reconstitution studies of purified AcrB (Zgurskaya & Nikaido, 1999), and subsequent crystal structures of the AcrB pump (Murakami *et al*, 2002; Yu *et al*, 2003) has established this as a fact.

The structure of AcrB suggests two possible pathways for the entry of substrates. Substrate molecules from the periplasm or the outer leaflet of the membrane could come in through the vestibules opening into the periplasm, while substrates from the cytoplasm or the inner leaflet could be transported through the transmembrane groove at the periphery of each transmembrane domain (Zgurskaya & Nikaido, 2000a). It appears that the substrates are collected in the central cavity and

they are actively transported through the pore in TolC. The ion pairs between Lys 940, Asp 407, and Asp 408 are the likely candidates for the transmembrane proton translocation site.

Two models have been proposed to describe interaction of three components (IM-MFP-OMF) of these pumps. One model suggests that all three components form a closed channel by oligomerization, while a second model suggests that the periplasmic components simply bring the outer and inner membranes together facilitating a tighter interaction of the outer and inner membrane components. Studies on the AcrA protein revealed that it can be cross-linked to monomeric AcrB, and support the latter model (Zgurskaya & Nikaido, 2000a). However, from the crystal structures of AcrB and TolC it can be deduced that the closing of the gap between the two membranes might not be required, even though there might still be some interaction between these two components.

A mechanical model for the functioning of these pumps has been hypothesized (Zgurskaya & Nikaido, 2000a) based on the crystal structures of AcrB and TolC proteins of *E. coli*. The α-helical tail of the TolC protein is long enough to traverse through the periplasm and thus could physically reach the AcrB protein located in the inner membrane, forming a continuous channel through the periplasm with the opening present towards the extracellular side. This interaction could be facilitated by the AcrA protein, which is anchored to the cytoplasmic membrane through its N-terminal lipid extension. The C-terminal region of AcrA, due to the presence of a conserved β-domain, is believed to be embedded in the outer membrane in the form of β-barrels in a fashion similar to outer membrane porin proteins. This

model predicts that the interaction of the substrate with AcrB results in conformational changes in the protein, which in turn causes folding back of the AcrA protein upon itself to bring the two membranes close to each other. This could result in the interaction of the α-helical tail of TolC with the funnel shaped headpiece of AcrB, which may serve as a signal for the opening of the TolC channel. Given that the diameter of the top of the headpiece of AcrB (~40 Å) is almost equal to the diameter of the helical tail of TolC, these two proteins might form a continuous channel once TolC is brought together with AcrB. Further, it has been shown that there is a ring of aspartate residues present in the channel opening of the TolC protein on the periplasmic side that play a role in the cation binding of the protein (Andersen *et al*, 2002b). This suggests that, once TolC interacts with the AcrB, the substrate is somehow translocated from the cavity of AcrB to TolC channel, then TolC guides the substrate to the extracellular medium.

The crystal structures of MexA of *P. aeruginosa* (Akama *et al*, 2004; Higgins *et al*, 2004) do not support the above model of mechanism but they do shed some light on the role of the periplasmic protein in the functioning of the RND complex. Both crystal structures suggest that MexA winds around the MexB-OprM interaction site, and that 13 MexA molecules might be needed to wind around a MexB-OprM trimer. Both structures further show that 13 monomers of MexA form a complex cylinder resulting from two arcs of 6 and 7 molecules respectively. However, the interior space of the sleeve appears to be too small to accommodate the MexB and OprM junction. Higgins and coworkers (Higgins *et al*, 2004) further suggest that this kind of arrangement is unlikely *in vivo* and that probably MexA molecules form a

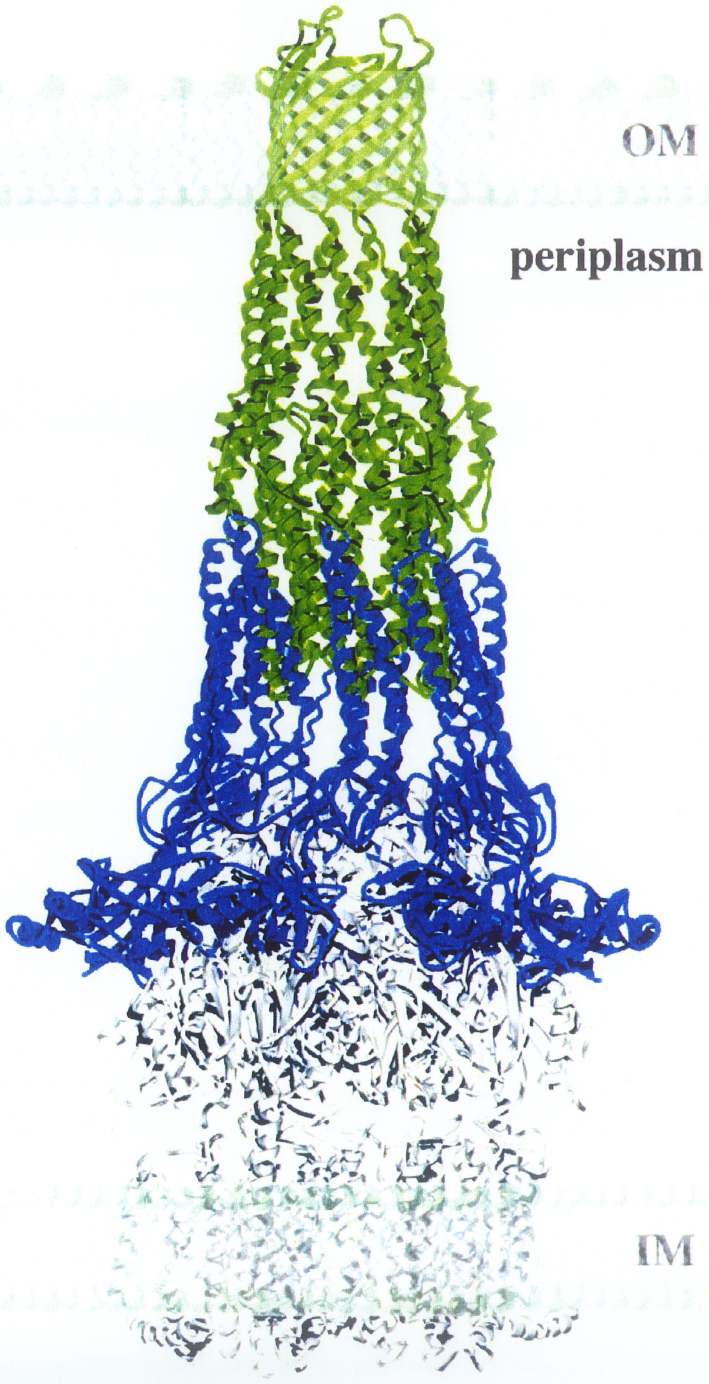
ring of 9 monomers, and this ring is sufficiently large to form a sheath around MexB-OprM trimer and provide a seal to stabilize the complex (Fig.1.6). From available crystal structures it is clear that the MFP plays a role in the stabilization of the RND-OMF complex, but the actual mechanism still remains elusive.

With the identification of MFP protein homologues in Gram-positive bacteria (Harley *et al*, 2000), there could be a more complex function for these proteins beyond bringing the two membranes together in Gram-negative bacteria. This is also in agreement with the close phylogenetic relationships of these proteins with the inner membrane component proteins (Dinh *et al*, 1994).

One feature of RND pumps that continues to amaze investigators is the broad substrate specificity. Interesting insights regarding substrate recognition came from the crystal structures of the regulatory proteins BmrR of *B. subtilis* (regulator of an MFS protein-encoding gene) (Zheleznova *et al*, 1999) and QacR of *S. aureus* (regulator of QacA pump) (Grkovic *et al*, 1998), which revealed the presence of a very large substrate- binding pocket, flexible binding sites, and the role of hydrophobic interactions. In addition, elucidation of the crystal structures of AcrB with different ligands has also provided a great deal of information regarding the mechanism of substrate binding by this protein (Yu *et al*, 2003). Yu and coworkers have crystallized the AcrB protein bound to rhodamine 6G, ethidium, dequalinium, and ciprofloxacin. The resulting structures suggest that different ligands bind to different positions within the central cavity via a different subset of residues. The ligand-binding cavity is very large with an area of approximately 5000

Fig. 1.6. Possible interaction of three components of the MexAB-OprM pump of *P. aeruginosa* (Higgins *et al*, 2004). It is suggested that 9 MexA molecules wrap around the complex formed by the MexB and the OprM proteins, stabilizing the interaction. The ring of 9 MexF molecules is shown in blue, the outer membrane protein (in open state) is shown in green, and the inner membrane pump is shown in white. OM, outer membrane; IM, inner membrane.

Fig. 1.6



$Å^3$. The upper part consists of many hydrophobic residues, suggesting that the substrate binding is primarily of hydrophobic nature.

While the details of substrate binding by AcrB are similar to those observed for BmrR and QacR, there are several salient features observed in the study of AcrB (Yu *et al*, 2003). Firstly, there can be several molecules of substrate bound to the central cavity simultaneously. There was no major change in the substrate-binding cavity size observed upon binding of substrate, as seen in the case of the QacR protein. Electrostatic interactions do not seem to play a major role in the substrate binding, again as seen for QacR protein, with substrate binding involving hydrophobic, aromatic, and van der Waal interactions. It was also observed that binding of substrate to the central cavity induces a rotation of 1° in each subunit, and that this motion enlarges the diameter of the periplasmic domain by ~2.5 Å. This rotation could be responsible for interaction of AcrB with AcrA, and the conformational changes in AcrA, which may be responsible for efflux of drug molecules through the α-helical tunnel of TolC. It is believed that the route of drug efflux is through the central pore formed by the pore helix in the periplasmic domain. The residues from each subunit interact at the center of the pore resulting in the pore being closed. It is proposed that the proton flux following the drug binding produces a large conformational change in AcrB that could lead to the opening of the pore and extrusion of the drug (Yu *et al*, 2003).

Recent elucidation of the crystal structure of the EmrE pump of *E. coli* (Ma & Chang, 2004) has also provided important insights about the mechanism of efflux pumps. The EmrE pump belongs to the SMR family, and is a small 12 kDa protein

with 110 amino acids and four predicted α-helices. It is a hydrophobic protein with only eight charged residues, including Glu-14, which is conserved throughout the SMR family and absolutely required for drug transport (Yerushalmi & Schuldiner, 2000). The structure of EmrE reveals a tetramer composed of two conformational heterodimers. One of the unusual features of the EmrE structure is the inverted orientation of polypeptides forming the conformational heterodimers (Ma & Chang, 2004). This conformation supports possible gene-fusion events producing transporters of other families such as drug/metabolite transporters, whose homologous subunits are thought to be inverted in the lipid bilayer (Jack *et al*, 2001).

Based on the crystal structure of the EmrE protein, a general mechanism for the efflux of cationic hydrophobic substrates has been proposed. It is believed that Glu-14 not only plays an important role in the binding of the cationic hydrophobic substrates, but also couples the proton-transport during the substrate translocation. The crystal structure shows two pairs of Glu-14 residues, one set near the proposed drug-binding pathway at the interface between structural heterodimers, and a second set located near the exterior of the protein close to the outer membrane side (Ma & Chang, 2004). In the high-affinity state, the drug-binding Glu-14 residues are negatively charged, and can bind cationic hydrophobic substrates from either the cytoplasm or from the inner membrane leaflet side of the cell membrane. Neighboring residues may form hydrophobic interactions with the drug molecule to stabilize the binding. In the presence of an electromotive force, the Glu-14s near the cell surface are protonated, resulting in conformational changes that are propogated through the heterodimer. This movement presents the substrate to the periplasmic

side of the cell membrane and forms a closed juncture, preventing the substrate from travelling back into the cytoplasm. The release of protons results in the transition to the low-affinity drug-binding state, resulting in the release of the drug molecule in the periplasm and return of EmrE to its high-affinity drug-binding state (Ma & Chang, 2004). The exact mechanism of proton passage through EmrE to the cytoplasm is not yet clear.

1.9. Regulation of RND Pump Gene Expression

Understanding the regulation of RND pump gene expression is very important for tackling the problem of multidrug resistance. Extensive progress has been made towards the understanding of the mechanisms of the regulatory pathways that govern the expression of drug transporters. The antimicrobial pumps known to be subject to regulatory controls typically belong to the MFS and RND superfamilies. The requirement for regulatory controls is required to prevent excessive production of the inner membrane component of pumps, as overexpression of these pumps has been shown to have a deleterious effect on cells. In fact, overexpression of the AcrAB pump was found to be toxic for *E. coli* (Ma *et al*, 1993), and a similar observation was reported upon overexpression of the TetA pump in *E. coli* (Lee & Edlin, 1985). This toxicity likely explains why there is an elaborate mechanism for regulation for the expression of many of these pumps. An exception to this is the members of the SMR family, which do not appear to be subject to any regulatory controls that can alter the level at which these proteins are synthesized.

Among the bacterial drug efflux genes that are inducible, there are only a few known examples in which translational controls have been shown to be the primary

level at which expression is controlled (e.g. TetA regulation) (Speer *et al*, 1992). The expression of the majority of bacterial drug transporter genes is controlled by transcriptional regulatory proteins. These regulatory proteins consist of repressors and activators of the target gene, and function at local and/or global levels.

1.9.1. Local Regulators

Local regulators, mostly repressors, are known to control the expression of many multidrug pumps. For example, *acrR* of *E. coli*, transcribed divergently from *acrAB* genes, encodes a repressor of the TetR repressor family, as it possesses a helix-turn-helix (HTH) DNA binding domain. TetR, the regulatory protein of the Tet pump, binds as a homodimer to its target operator, and represses the production of TetA (Orth *et al*, 2000). The repression by TetR persists until it is bound by tetracycline (Orth *et al*, 2000). This mechanism of inducible *tetA* expression suggests that other pumps that are repressed by TetR-like repressor also possess the same mechanism of expression. This was confirmed with the observation that MexXY (Masuda *et al*, 2000) and TtgABC (Duque *et al*, 2001) pump expression is induced by the substrates they pump out.

AcrR is known to repress both its own and *acrAB* transcription. It appears that the primary function of AcrR is to modulate *acrAB* expression, thus preventing excessive production of the AcrAB pump. Another example of a local repressor belonging to the TetR family is AcrS, that regulates the expression of the AcrEF pump and is encoded divergently from *acrEF* (Ma *et al*, 1994). There are several other examples of local repressors of multidrug efflux pumps that belong to the TetR

family, e.g. MexL (Chuanchuen *et al*, 2002), MexZ (Wesbrock-Wadman *et al*, 1999), and SmeT (Zhang *et al*, 2001).

All of the RND multidrug efflux systems of *P. aeruginosa* that have been studied are regulated by a linked regulatory gene, coding for either a repressor or an activator. The *mexAB-oprM* operon has the divergently transcribed gene, *mexR* located upstream. MexR encodes a transcriptional repressor of the MarR family (Li *et al*, 1995). Inactivation of *mexR* results in the overexpression of MexAB-OprM system (Poole *et al*, 1996b). Strains that overexpress the MexAB-OprM system often carry mutations in the *mexR* gene (Adewoye *et al*, 2002; Saito *et al*, 1999). MexR has been shown to bind to the promoter regions of *mexAB-oprM* and *mexR* (Evans *et al*, 2001). The crystal structure of the MexR protein (Lim *et al*, 2002) revealed that four MexR dimers in the asymmetric unit can be found in multiple conformations. This high degree of flexibility in MexR is different from a rather rigid MarR protein, which suggests that it could have a different mechanism of repression. To date, efforts to identify potential ligands that may act as inducers and bind MexR have been unsuccessful.

MexCD-OprJ of *P. aeruginosa* is regulated by the NfxB repressor encoded by a gene located upstream of the *mexCD-oprJ* operon (Poole *et al*, 1996a). Based on sequence similarity, the NfxB protein appears to belong to the LacI-GalR repressor family (Weickert & Adhya, 1992). NfxB binds to the upstream region of *nfxB*, indicating that it is involved in negative autoregulation of the *nfxB* gene (Shiba *et al*, 1995). Expression of the *mexXY* operon is controlled by MexZ, a protein belonging

to the TetR family. It is encoded upstream of the operon in the opposite direction (Aires *et al*, 1999) and represses the operon expression.

MexT, a member of the LysR family of transcriptional regulators, is an activator of *mexEF-oprN* (Kohler *et al*, 1999). This type of regulator is unique when campared to other RND operons of *P. aeruginosa*, which are negatively regulated. The *mexT* gene is located upstream of the operon and is transcribed in the same direction as the *mexEF-oprN* genes. Overexpression of *mexT* induces the expression of *mexEF-oprN*, and decreases the expression of outer membrane protein encoding *oprD* (Masuda *et al*, 2001).

1.9.2. Two-component regulatory systems

Some efflux pumps are known to be regulated by two-component systems. These systems are found extensively in bacteria and allow them to react to changes in their environment. These systems consist of a sensor kinase and a response regulator. In response to the environmental stimuli, the sensor kinase phosphorylates the response regulator, which in turn activates or represses the target genes (Stock *et al*, 2000). Examples of the two-component systems that are known to regulate RND pumps are BaeSR for the MdtABC pump of *E. coli* (Baranova & Nikaido, 2002; Nagakubo *et al*, 2002), EvgAS for the YhiUV pump of *E. coli* (Nishino & Yamaguchi, 2002), and SmeSR for the SmeABC pump of *S. maltophilia* (Li *et al*, 2002). In these systems, the genes encoding two-component systems are located in very close proximity to the efflux pump gene operons and their products function as activators.

A recent investigation has revealed that the EvgAS system is actually responsible for upregulation of a variety of drug effux genes: *emrKY*, *yhiUV*, *acrAB*, *mdfA*, as well as *tolC* (Eguchi *et al*, 2003). This is further evidence in support of the suggestion that expression of MDR systems is controlled by a complex regulatory network. The expression of *tolC* was also shown to be positively regulated by yet another two-component system, PhoPQ (Eguchi *et al*, 2003).

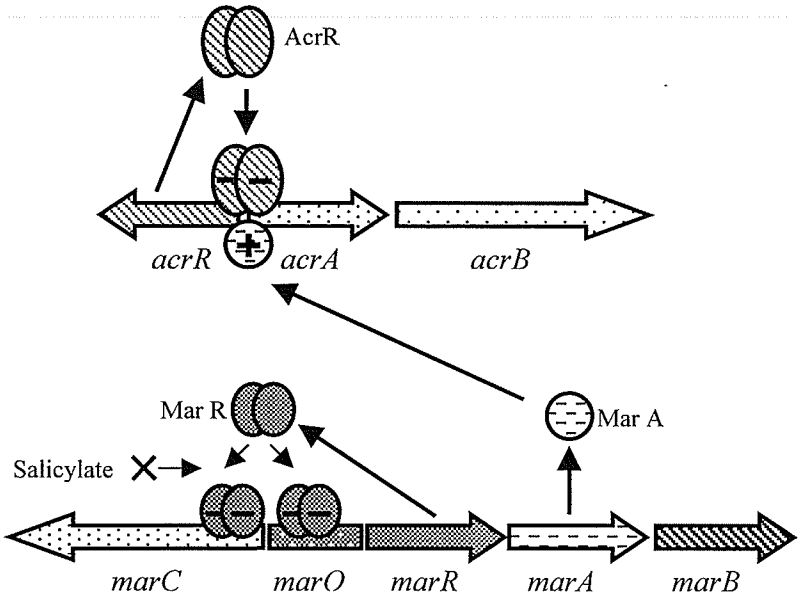
1.9.3. Global regulators

The role of global regulators in the expression of efflux pumps is best-studied for the *E. coli* AcrAB-TolC system (Fig.1.7). So far, four global transcriptional activators, MarA, SoxS, Rob, and SdiA, have been recognized to be regulating the expression of this system. The *mar* (multiple antibiotic resistance) locus consists of the *marRAB* operon and the divergently transcribed *marC*, both being expressed from a central operator/promoter region, *marO* (Alekshun & Levy, 1997). MarR is a repressor and MarA is an activator, while the functions of MarB and MarC remain unknown.

MarA is a member of the AraC family of transcriptional activators, and activates its own transcription as well as a large number of other genes by binding to 20-bp DNA sequences known as 'marboxes' that are located in the vicinity of the promoters for the target genes. A marbox is found next to the *acrAB* promoter region (Alekshun & Levy, 1997), and MarA has been shown to bind this region and activate transcription of the operon. The global nature of MarA has been demonstrated using gene array analysis, in which the protein was shown to regulate the expression of over

Fig. 1.7. Schematic representation of the regulation of the *acrAB* locus by AcrR and MarA. AcrR represses (-) transcription of the *acrRAB* genes, while MarA up-regulates (+) the expression of the *acrAB* genes. The expression of the *marRAB* operon is repressed by MarR, encoded by *marR*. However, anionic compounds, like salicylate, interact with MarR to prevent (X) its binding to DNA. This results in constitutive expression of the *marA* gene, thus up-regulating the *acrAB* operon (Schumacher & Brennan, 2002).

Fig. 1.7.


AcrR
acrR
acrA
acrB
Mar R
Mar A
Salicylate
marC
marO
marR
marA
marB

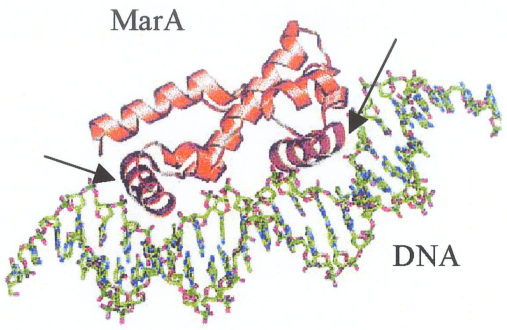
60 *E. coli* genes (Barbosa & Levy, 2000). One well-documented example of MarA activity is activation of transcription of *micF*, which produces an antisense RNA that downregulates the expression of *ompF*, a gene encoding an outer membrane protein that is responsible for entry of various antibiotics (Delihas & Forst, 2001). Reduction in the expression of *ompF*, the site for drug entry, combined with overexpression of *acrAB-tolC*, represents a highly effective mechanism by which MarA can coordinate a response to the presence of toxic antimicrobials.

The crystal structure of MarA bound to the *marRAB* operon has been solved (Rhee *et al*, 1998) (Fig.1.8). MarA binds to the DNA as a monomer and possesses two separate HTH DNA-binding domains linked by a long α-helix. An operator sequence typically consists of 11-12 bp for a DNA-binding protein to recognize it. As a typical HTH motif is capable of recognizing only 6 bp, the presence of 2 HTH motifs in a single polypeptide chain of MarA explains how this protein can function as a monomer.

The MarR repressor is the product of the first gene of the *marRAB* operon. MarR controls the intracellular levels of MarA and thus plays a crucial role in the marA-mediated activation of *mar* regulon promoters. MarR also binds to the *marO* region, but at a site distinct from the MarA binding site. MarR represses transcription of the *marRAB* operon by binding as a dimer to two distinct regions in *marO*, referred to as site I and site II. Binding of MarR to site I, located between the -35 and -10 boxes of the *marRAB* operon, is necessary for repression (Martin & Rosner, 1995). MarR-DNA interactions can be inhibited by several anionic

Fig. 1.8. Crystal structure of MarA bound to DNA (Rhee *et al*, 1998). Helix-turn-helix motifs that bind to the DNA are indicated by arrows.

Fig. 1.8.



compounds, including 2,4-dinitrophenol, plumbagin, menadione, and salicylate (Alekshun & Levy, 1999), though the mechanism of inhibition is not yet known.

The crystal structure of MarR in association with salicylate shows that it contains a DNA-binding domain belonging to the winged-helix family (Alekshun *et al*, 2001), as observed for the MexR protein of *P. aeruginosa* (Lim *et al*, 2002). Each subunit of the MarR dimer is an α/β protein and can be divided into two domains: the N/C-terminal domain, responsible for dimerization, and the central domain, containing the winged-helix DNA-binding motif. The structure shows two binding sites for salicylate, both within the DNA-binding motif, leading to speculations about the physiological role of salicylate in the induction of multidrug resistance phenotype. Overall, MarA activates expression of the *mar* regulon, including *acrAB*, *tolC*, and *marRAB*, while MarR acts by repressing the synthesis of MarA.

Some homologs of MarA have also been shown to increase the transcription of *marRAB* by binding to *marO* in *E. coli*. These are SoxS, the effector of the *soxRS* global superoxide response (*sox*) regulon, and Rob, that binds to the *E. coli* chromosomal origin of replication (White *et al*, 1997). Elevated levels of SoxS and Rob have been shown increase the transcription of *acrAB*.

For SoxS to have an effect on *acrAB-tolC* transcription in wild-type cells, the expression of the *soxS* gene has to be activated by superoxide-generating agents via the conversion of SoxR, a divergently transcribed local transcriptional activator, into an active form (Martin & Rosner, 1997; Nunoshiba *et al*, 1992). In contrast to MarA and SoxS, Rob is twice as large and is involved in the regulation of *acrAB* operon. Unlike MarA and SoxS, it is synthesized constitutively. Recently, the transcriptional

activation of *mar-sox* regulon genes by Rob was shown to occur through the binding of inducers like medium-chain fatty acids and bile salts (Rosenberg *et al*, 2003).

Also recently, SdiA, an *E. coli* protein that is homologous to the receptor of acyl homoserine lactone quorom-sensing signal family was found to positively regulate AcrAB expression (Rahmati *et al*, 2002). Although the physiological role of SdiA in *E. coli* is not clear as *E. coli* K-12 lacks the genes for the production of acyl homoserine lactone.

1.10. Synergism between efflux pumps and other resistance mechanisms

Widespread use of antimicrobial agents has given rise to a variety of resistance mechanisms in bacteria. Broadly these various mechanisms can be classified into the following categories:

1. Alteration of the target site
2. Decrease in the concentration of drug reaching the target site by altered rates of entry or removal of the drug
3. Degradation/modification of the antibiotic
4. Synthesis of resistant, or alternate pathways that are no longer susceptible to the antibiotic
5. Failure of the cell to metabolize the drug to its active state

Crosstalk between various mechanisms can result in increased resistance to a particular antibiotic.

1.10.1. Interaction between efflux pumps

Multiple efflux pumps in a cell that have overlapping substrate specificity increase the efflux capability of that cell, hence producing higher levels of resistance. A mathematical model for synergism between different efflux pumps was derived by Lee and colleagues (Lee *et al*, 2000), in which they showed that if two pumps in the same organism operate by the same mechanism (either single-component pumps that pump substrate into periplasm, or multicomponent pumps that pump substrate into the external medium) the net effect is additive. However, if there are two different kinds of pumps (a single-component and a multi-component type) working together, the effect is multiplicative. This model also explains how overexpression of the Tet pump (a single-component pump) can result in extremely high resistance to tetracycline in Gram-negative bacteria. As these pumps are single-component pumps, they are only able to pump antibiotics into the periplasm and there is always the possibility of the antibiotic diffusing back into the cytoplasm. However, multi-component pumps that are expressed constitutively expressed can perhaps synergize with Tet pumps and make the pumping more effective.

1.10.2. Relationship between outer membrane permeability and efflux pumps

Interaction between the outer membrane permeability and efflux pumps is also of a multiplicative nature. Decrease or loss of porins results in reduced antibiotic uptake, leading to resistance. However, in the presence of drug efflux, the resistance is amplified multiplicatively. This effect has been shown in *P. aeruginosa* (Li *et al*, 2000b), where either inactivation of the MexAB-OprM pumps or permeabilisation of

the outer membrane alone resulted in a very drastic, but similar decrease in the antibiotic resistance of this organism.

1.10.3. Relationship between antibacterial-inactivating enzymes and efflux pumps

Relationship between enzymes that degrade antibiotics and efflux pumps is additive in nature as both these mechanisms work in parallel to lower the antibiotic concentration. In *E. coli*, it was shown that strains lacking either the AcrAB pump or the β-lactamase enzyme had similar MIC values for β-lactams (Mazzariol *et al*, 2000a).

1.10.4. Interaction between antibacterial target alterations and efflux pumps

This interaction has been very well documented in case of quinolone resistance. DNA gyrase and topoisomerase are the target enzymes for this group of antibiotics. Mutations in the target genes (*gyrA/B* and *parC/E*) have been reported in various organisms that overexpress efflux pumps. A study in *E. coli* showed that about 90% of the clinical isolates resistant to ciprofloxacin overexpressed the AcrAB pump, in addition to the presence of target mutations (Mazzariol *et al*, 2000b). In *P. aeruginosa*, it has also been shown that strains that overexpress an efflux pump and also have mutations in DNA gyrase are more resistant to quinolones than those carrying only one of these resistance mechanisms (Lomovskaya *et al*, 1999; Nakajima *et al*, 2002)

1.11. Potential methods for inhibition of efflux pumps in bacteria

Studies have shown that inactivating efflux pumps in different organisms makes them highly susceptible to a variety of antibiotics. As such, this makes efflux

pumps potentially very effective antibacterial targets. There are various theoretical approaches being employed to find efflux pump inhibitors (EPIs) and they are summarized as follows:

1. to identify an inhibitor against a specific pump known to be responsible for efflux of a specific agent or class
2. to identify a "broad spectrum" pump inhibitor that affects multiple related or unrelated pumps and/or multiple antibiotics
3. to identify an inhibitor that counters efflux by compensatory changes in influx of an antibacterial agent
4. to identify a non-target based inhibitor that perturbs the bacterial cell wall/membrane, thus rendering efflux less efficient.
5. to modify a single antibacterial agent to make it a poor substrate for efflux pumps

Inhibition of Tet pumps has been studied for many years (Nelson *et al*, 1993). The most potent inhibitor found so far, 13-cyclopentylthio-5-OH tetracycline (13-CPTC), a derivative of doxycycline that is a competitive inhibitor of the TetB protein. The combination of 13-CPTC with doxycycline had either a synergistic or an additive effect (depending on the Gram-type), reducing the MIC values of doxycycline by four- to ten-fold (Nelson & Levy, 1999). Some natural compounds like ginsenosides (from gensin) have also been identified as potential inhibitors of the TekK or TetC pump (Nelson, 2002).

Microcide Pharmaceuticals has reported EPIs that potentiated the activity of levofloxacin by as much as 64-fold in MexAB-expressing strains of *P. aeruginosa*,

while not affecting the levofloxacin MIC in strains in which the MexAB pump was inactivated (Lomovskaya & Watkins, 2001a). These EPIs led to an increase in the accumulation of the antibiotic inside the cell without disrupting the proton-gradient. The first compound reported by Microcide was MC-207,110, a low molecular weight dipeptide amide (phenylalanylarginyl-β-naphthylamide). This compound lacks antibacterial activity of its own but, at a concentration of 10 μg/ml, potentiates the activity of levofloxacin by 8-fold. (Lomovskaya *et al*, 2001). Further chemical modifications of MC-207,110 led to other peptides that showed potentiation activity for *P. aeruginosa* strains producing MexAB-OprM, MexCD-OprJ, and MexEF-OprN pumps, and *E. coli* strains producing AcrAB-TolC pumps. EPIs of this category are specific for RND pumps only, and do not inhibit any other pumps.

These EPIs have been tested in animal models of *P. aeruginosa* infection (Griffith *et al*, 2000) and have been confirmed to potentiate the activity of levofloxacin. It was found that infection of animal models with strains resistant to levofloxacin (MIC values of up to 64 μg/ml), levofloxacin was effective when used in combination with the EPI, while, when used alone, it was ineffective. These studies clearly indicate the usefulness of EPIs as combinational agents along with antibiotics. However, the need to combine EPIs with antibiotics of similar pharmacokinetic characteristics makes their clinical use very complicated.

1.12. Hypothesis and Objectives

Hypothesis: Proton gradient-dependent efflux of fluoroquinolones by the means of RND family pumps contributes to the resistance of *S. marcescens* to this group of antibiotics.

Specific Objectives:

1. To identify proton gradient-dependent efflux of fluoroquinolones as a resistance mechanism in *S. marcescens*.
2. To identify, clone, and sequence the RND pump-encoding gene(s) of *S. marcescens.*
3. To study the structure and function of *S. marcescens* RND pump(s).
4. To assess the effect of clinical settings on the expression of *S. marcescens* RND pump-encoding gene(s) by studying the regulation of pump gene(s).

Two

Materials and Methods

It is common sense to take a method and try it.
If it fails, admit it frankly and try another.
But above all, try something
-F. D. Roosevelt

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. *Serratia marcescens* UOC-67 ATCC 13880 was the source of the *sdeAB* and *sdeCDE* operons. All *S. marcescens* strains were maintained on T-soy medium (Difco) at 28°C or 37°C. All *E. coli* strains were maintained on Luria-Bertani (LB) medium (Difco) at 37°C. Freezer stocks of bacterial cultures were prepared by addition of dimethyl sulfoxide (DMSO) to a final concentration of 7% (v/v) to the mid-log phase cultures. Transformed bacterial cultures were maintained in the presence of appropriate antibiotics. Ampicillin (100 μg/ml), kanamycin (40 μg/ml), streptomycin (40 μg/ml), and tetracycline (5 μg/ml) were added to *E. coli* strains transformed with plasmids or cosmids carrying Ap^r, Km^r, Sm^r, and Tc^r markers.

Mutant strains of *S. marcescens*, UOC-67WL, UOC-67WLN, and UOC-67WLO were derived by serially passaging the wild-type strain UOC-67 in T-soy broth medium supplemented with ciprofloxacin, norfloxacin, or ofloxacin, respectively. For generation of mutant strains, UOC-67 was first grown in T-soy broth containing 0.2 μg/ml of the appropriate antibiotic and then subcultured in broth supplemented with 2-fold increments of the appropriate antibiotic up to 16 μg/ml with overnight incubation at 37°C with shaking.

2.2. Antibiotic susceptibility assay

Susceptibilities of the bacterial strains to different antibiotics were tested using the two-fold dilution method with an inoculum of 10 cfu (BSAC, 1991).

Table 2.1. Bacterial strains used in this study

Strain	Characteristic	Source/Reference
S. marcescens UOC-67	Type strain	ATCC 13880
S. marcescens T-849	Clinical isolate	D. Hoban, Health Sciences Centre, University of Manitoba
S. marcescens T-850	Clinical isolate	D. Hoban
S. marcescens T-851	Clinical isolate	D. Hoban
S. marcescens T-852	Clinical isolate	D. Hoban
S. marcescens T-853	Clinical isolate	D. Hoban
S. marcescens T-854	Clinical isolate	D. Hoban
S. marcescens T-855	Clinical isolate	D. Hoban
S. marcescens T-856	Clinical isolate	D. Hoban
S. marcescens T-857	Clinical isolate	D. Hoban
S. marcescens T-858	Clinical isolate	D. Hoban
S. marcescens T-859	Clinical isolate	D. Hoban
S. marcescens T-860	Clinical isolate	D. Hoban
S. marcescens T-861	Clinical isolate	D. Hoban
S. marcescens UOC-67WL	Fluoroquinolone resistant mutant derived from serial passaging of *S. marcescens* UOC-67 on media supplemented with ciprofloxacin	This study
S. marcescens UOC-67WLN	Fluoroquinolone resistant mutant derived from serial passaging of *S. marcescens* UOC-67 on media supplemented with norfloxacin	This study

S. marcescens UOC-67WLO	Fluoroquinolone resistant mutant derived from serial passaging of *S. marcescens* UOC-67 on media supplemented with ofloxacin	This study
E. coli NM522	*SupE thiΔ (lac-proAB) hsd5* F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Promega
E. coli EZ cells	*F'::Tn10(Tc^r) proA*$^+$*B*$^+$ *lacI*^{q}*ZΔM15 recA1 lac glnV44 thi-1 gyrA96 relA1*	Qiagen
E. coli HS832	*acrAB::kan*	Sulavik *et al*, 2001
E. coli AG102MB	*marR1 acrB::kan*	Elkins & Nikaido, 2002
E. coli HNCE1a	*marR1 acrB::kan ΔacrD*	Elkins & Nikaido, 2002
E. coli BL923	*recA*$^-$ *ΔtolC*	Thanabalu *et al*, 1998
E. coli LBB1136	Δ*acrA*, *tolC*::Tn10	J. Fralick, Texas Tech. University Health Sciences Centre
E. coli LBB1135	Δ*acrA*	J. Fralick
E. coli LB1175	*tolC*::Tn10	J. Fralick
E. coli VCS257	*supE44, supF58, hsdS3* (r^-_B, m^-_B), *dapD8, lacY1, glnV44,* Δ*(gal-uvrB)47, tyrT58, gyrA29,* Δ*thyA57*	Stratagene
E. coli MT616	MT607 (*pro-82 thi-l hsdR17 supE44*):pRK600	Finan *et al*, 1986
E. coli CC118 λ-pir	*E. coli* CC118 lysogenized with *pir* transducing λ phage	T. Charles, University of Waterloo

Table 2.2. Plasmids/Cosmids used in this study

Plasmid/Cosmid	Characteristics	Source/Reference
pKS (-)	ColE1 replicon, Ap^r, *lacZ'*	Stratagene
pUC18	ColE1 replicon, Ap^r, *lacZ'*	Invitrogen
pDrive	Phage F1 origin, Ap^r, Km^r, *lacZ'*	Qiagen
pRK7813	Cosmid vector, IncP replicon, Tc^r	Jones & Gutterson, 1987
pUC4-kixx	ColE1 replicon, *lacZ'*, Ap^r, Km^r cassette within a 1.4 kb *Sma*I fragment	Pharmacia
pRK600	ColE1 replicon with RK2 transfer gene, Cm^r	Finan *et al*, 1986
pKNG101	Suicide vector, *pir⁻ oriR6K mobRK2 sacB* Sm^r	Kaniga *et al*, 1991
pRKO37	pRK7813 containing *sdeAB* genes within 21 kb *S. marcescens* genomic insert	This study
pUCAB	pUC18 containing *S. marcescens sdeAB* genes within a 5 kb *Eco*RV fragment from pRKO37	This study
pClone63	pDrive with 630 bp PCR product from within *sdeB*	This study
pKS17	pKS(-) with 1.7 kb PCR product from within *sdeB*	This study
pKS17:Km	pKS17 with 1.4 kb *Sma*1 Km^r cassette cloned in 1.7 kb PCR product from *sdeB* gene	This study
pSO421	pKNG101 containing 2.1 kb insert (*sdeB*:Km^r) from pKS17:Km	This study
pRKH10	pRK7813 containing *sdeCDE* genes within 15 kb *S. marcescens* genomic insert	This study
pRKC51	pRK7813 containing *sdeCDE* genes within 40 kb *S. marcescens* genomic insert	This study

pUCCDE	pUC18 containing *S. marcescens* *sdeCDE* genes within a 12 kb *Eco*RI/*Kpn*I fragment	This study
pUCCD	pUC18 containing *sdeCD* genes within a 5 kb *Nco*I-digested fragment from pRKC51	This study
pClone17	pDrive with 1.7 kb PCR product from within *sdeD*	This study
pK423	423 bp *Eco*RI/*Pst*I fragment of *sdeD* cloned in pKS	This study
pK1000	1 kb fragment from 1.7 kb PCR product of *sdeD* with ~700 bp deleted from the middle	This study
pKNG1000	1 kb deletion fragment from pK1000 cloned in pKNG101	This study
pUCHF	*hasF* gene cloned in pUC18	This study
pUCR1	~750 bp upstream region of *sdeAB* gene containing *sdeR* cloned in pUC18	This study

Results were reported as the MIC, the minimum concentration of antibiotic that inhibited visible growth determined by absence of turbidity in the broth after 18 hrs of incubation (without shaking) at 37°C. Susceptibilities of various strains to SDS and ethidium bromide were tested by the same method.

2.3. Ethidium bromide (EtBr) accumulation assay

Accumulation of ethidium bromide by all thirteen clinical isolates, the mutant UOC-67WL, and the wild-type strain UOC-67 was measured using a modification of a previously-described method (Neyfakh *et al*, 1991). Cells were grown overnight in T-soy broth, harvested, and resuspended to an $A_{600} = 0.2$ in 50 mM sodium phosphate buffer, pH 7.0. Ethidium bromide was added to a final concentration of 2 μg/ml. Fluorescence of the samples was measured every 30 s for a period of 10 min at excitation and emission wavelengths of 530 nm and 600 nm, respectively, using a Shimadzu RF-1501 spectrofluorophotometer. Proton gradient uncoupler carbonyl cyanide m-chlorophenylhydrozone (CCCP) (100mM in DMSO) was added to a final concentration of 100 μM after 5 min. Cells equilibrated with 50 mM sodium phosphate buffer without ethidium bromide were used as blanks.

2.4. Fluoroquinolone accumulation assays

Accumulation of ciprofloxacin, ofloxacin, and norfloxacin was performed following a method previously described by Mortimer and Piddock (Mortimer & Piddock, 1991) with some modifications. Cells were grown to late log phase at 37°C in T-soy broth, harvested by centrifugation, washed once with 50 mM sodium phosphate buffer (pH 7.0), resuspended in the same buffer to an $A_{600} = 20.0$, and equilibrated for 10 min at 37°C. The antibiotic to be tested was added to a final

concentration of 10 μg/ml, and 0.5 ml of culture was removed every 30 s for a period of 10 min, and immediately diluted in 1 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.0). Carbonyl cyanide m-chlorophenylhydrozone (CCCP) was added to a final concentration of 100 μM 5 min after the addition of antibiotic. The samples were centrifuged at 19,600 x g for 10 min. The pellet was washed with 1 ml of 50 mM sodium phosphate buffer, resuspended in 1 ml of 0.1 M glycine hydrochloride (pH 3.0), and incubated for at least 15 h at room temperature. The samples were then centrifuged at 19,600 x g for 10 min. Fluorescence of the supernatant was measured using a Shimadzu RF-1501 spectrofluorometer as follows: ciprofloxacin at excitation and emission wavelengths of 279 nm and 447 nm; norfloxacin at 281 nm and 440 nm; and ofloxacin at 292 nm and 496 nm, respectively. The concentration of the antibiotics in the supernatant was calculated using a standard curve for the respective antibiotic (concentration ranging from 100-1000 ng) in 0.1 M glycine hydrochloride, pH 3.0. The results were expressed as nanogram of antibiotic incorporated per milligram (dry weight) of bacteria.

2.5. DNA isolation

Plasmid and genomic DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA fragment purification, and DNA ligation were all performed by standard techniques (Ausubel, 1989). A rapid plasmid isolation method described by Kado and Liu (Kado & Liu, 1981) was used for the screening of potential transformants, with some modifications. Bacterial colonies were scraped with a sterile toothpick and resuspended thoroughly in a microfuge tube containing 40 μl of STE buffer (100 mM NaCl, 20 mM Tris-Cl, pH 7.5, 10 mM EDTA). Twenty

microlitres of Phenol:Chloroform:Isoamyl alcohol::25:24:1 was added and vortexed for 1 min to emulsify the solution. The top layer was collected and microfuged for 2 min. The supernatant was placed in a microfuge tube containing 1 µl of RNase (1 mg/ml). Samples were loaded on 0.8% agarose gel and the rate of migration was compared with that of vector without the insert.

2.6. Preparation of competent cells and transformation

Competent cells were prepared using the $CaCl_2$ treatment method (Ausubel, 1989). Log phase cultures (2.5 to 3 hours incubation at 37°C) were harvested by centrifugation at 1000X g for 5 min at 4°C. The cells were resuspended in 1/10 of the original culture volume of ice-cold 0.1 M $CaCl_2$, and incubated on ice for 30 min. Cells were centrifuged again for 5 min at 1000 x g at 4°C and resuspended in 1/25 volume of ice cold 0.1 M $CaCl_2$. Glycerol was added to a final concentration of 23%, cells were aliquoted, and stored at -70°C. Occasionally, competent cells were also prepared on a small scale by spinning down 5 ml of log phase culture, and resuspending the cells in 500 µl of ice cold 0.1 M $CaCl_2$. Cells were incubated on ice for 30 minutes and transformed immediately.

For transformation, 100 µl of competent cells were mixed with 10 ng to 1µg DNA in a 10 µl volume, and kept on ice for 30 min. Cells were then heat shocked for 3 min at 42°C and cooled on ice for another 5 min. One ml of LB broth was added and cells were incubated for 50 min for transformation with Ap^r plasmids or 90 min for plasmids carrying Sm^r, Km^r, or Tc^r markers before plating on LB-agar supplemented with appropriate antibiotics.

2.7. Triparental mating

Triparental mating of *E. coli* strains was performed using the method described by Goldberg and Ohman (Goldberg & Ohman, 1984). Briefly, 0.1 ml of overnight cultures of the donor, recipient and helper strains, incubated at 37°C, 42°C, and 28°C respectively, and mixed in 2 ml of L-broth. The mixture was filtered using a 0.45 μm Nalgene filter unit, the filter was then removed and placed (cell side up) on an LB-agar plate. The plate was incubated overnight at 28°C. The following day, bacteria from the filter were resuspended in 2-5 ml of saline (0.85% NaCl) and the suspension was diluted to 1:100 in saline. One hundred μl of undiluted and 1:100 dilution suspension was spread plated on selective plates containing appropriate antibiotics and the plates were incubated at 37°C until distinct colonies appeared. *E. coli* MT616 (Finan *et al*, 1986) was used as the helper strain.

2.8. Southern blotting

Detection of nucleic acid by Southern blotting was performed using the non-radioactive digoxigenin (DIG) DNA labeling and detection kit (Roche Diagnostics, Mannheim, Germany). Restriction fragment and PCR product probes were random-primed labeled with DIG-11-dUTP provided with the DIG labeling kit using manufacturer's instructions.

Plasmid, cosmid, or genomic DNA was resolved on 0.8 % agarose gel, and washed twice in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 15 min each, followed by two washes for 15 min each in neutralization buffer (1.5 M NaCl, 1 M Tris, pH 8.0). DNA was transferred onto Hybond-N nylon membrane (Amersham) overnight using 20X SSC buffer (3 M NaCl, 300 mM sodium citrate, pH 7.0). DNA

was fixed on the membrane by cross-linking under UV light on a transilluminator for 3 min. DNA-bound membranes were incubated for 1 hr in 15 ml of pre-hybridization solution [5X SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 1% (w/v) blocking reagent] at 58°C. Subsequently, the DNA probe was denatured at 100 °C for 10 min and added to 10 ml of standard hybridization buffer [5X SSC, 0.1% (w/v) N-laurylsarcosine and 1% (w/v) blocking reagent (Roche, Canada)] to a final concentration of 5 to 25 ng/ml. The hybridization reaction was performed overnight at 64°C in a hybridization oven with constant agitation. After hybridization was complete, two low-stringency washes (2X SSC, 0.1 % SDS) were performed at room temperature for 5 min each, and two high-stringency washes (0.1X SSC, 0.1% SDS) were performed at 68°C.

For chemiluminescent detection following the post-hybridization washes, membranes were equilibrated in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min and then washed in the blocking solution [1% (w/v) blocking reagent (Roche, Canada) in maleic acid buffer] for 30 min with gentle agitation. The membrane was subsequently incubated in a solution of 1:10,000 dilution of Anti-DIG-alkaline phosphatase Fab fragments (Roche, Canada) in blocking solution for 30 min. The membrane was then washed twice in washing buffer [0.3% (w/v) Tween® 20 in maleic acid buffer A] for 15 min each, followed by 2 min wash in the detection buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl). Membranes were then coated with 250 µl of a 1:100 dilution of CDP-*Star*™ (Roche, Canada) in the detection buffer and placed between two acetate sheets. The membranes were then exposed to

Kodak X-Omat X-ray film (East Kodak Co., Rochester, New York) for 10 min to 1 hr to detect the chemiluminescent reaction.

Following detection, membranes were stripped of probes by rinsing in water for 2 min followed by two 15 min washes in the probe stripping solution (0.2 M NaOH, 0.1 % SDS). Membranes were then washed 2 times in 2X SSC and stored at 4 °C in 2X SSC until ready for another detection.

2.9. PCR and DNA sequencing

PRC primers used in this study are listed in the Table 2.3. PCR for identification of RND pump genes was carried out by using two primers, ACRB1236 and ACRB2954 (Gibco BRL) to amplify a ~1.7 Kb region from the genome of *S. marcescens* UOC-67.

Sequences of primers were derived from two signature sequences of RND proteins, as described by Tseng and coworkers (Tseng *et al*, 1999). These two PCR products were cloned in the pDrive vector using the Qiagen PCR cloning kit following the manufacturer's instructions.

PCR of QRDR regions of the *gyrA* and *parC* genes was performed using primers GYRAF and GYRAR; and PARCF and PARCR, respectively. Template DNA for the QRDR regions was prepared by boiling an overnight culture of the appropriate strain of *S. marcescens* for 10 min, and spinning the samples for 2 min at 19,600X g. Two µl of the supernatant was used as the template for a 50 µl PCR reaction.

Table 2.3. PCR primers used in this study

Name	Sequence (5'→ 3')	Target
ACRB1236 ACRB2954	GTGGATGACGCCATCGTTGTG GGTCATCAGGATCGGACGTAA	1.7 kb region between conserved motifs of RND pump proteins
EACRB1236 BACRB2954	GgaattcCGTGGATGACGCAATCGTTGTG GAagatctTCGCGACAGGCCTCCAGCGCCG	1.7 kb region within *sdeB*, with *Eco*RI and BglII restriction sites engineered in the 5` end (shown as underlined bases in lower case)
GYRAF GYRAR	CCCGTAAAGCGTGCTTTGTG CTTCGGTATAACGCATCGCC	QRDR (~350 bp) of *S. marcescens gyrA*
PARCF PARCR	GACTCATGACGGTGTAGAGCG ACGAACTCGAGCAGGTCATCG	QRDR (~500 bp) of *S. marcescens parC*
YEGF SDECR	TTGATCCGCCGTCCAGCCGAAC TCACCTGCATCAGGACTTCCTC	*sdeC* of *S. marcescens sdeCDE*
SDEDF YEGR	GAGGAAGTCCTGATGCAGGTGA CGCCACCGAGGACGCCATGGTT	*sdeD* of *S. marcescens sdeCDE*
SDEF1 SDEA	CCAGACAGCGCTTACTTATCGG GTCAGAAGTCTCGGCTGGGAGC	*sdeA* of *S. marcescens sdeAB*
SDEB SDER1	GCTCCCAGCCGAGACTTCTGAC GTCGGGCAATGCCCGACCTTT	*sdeB* of *S. marcescens sdeAB*
TOCF TOLCR	CCGCAGACTCTGCTAGAATCGGCA GCTGTGAGGCGGTGGGCAGAAGTG	*S. marcescens tolC*
MARE	CGgaattcATGCCTGCGTTCGGCGGAAG	750 bp region upstream of *sdeAB*

MARB	CGggatccCACTGCCCAACCGATCAG	
HISE HISB	TGgaattcATGTGCATCGGGCAGAGGGT CGggatccGCA**GTGGTGGTGGTG**GTGGT-GCGTGCATCTCACCG	*sdeR* with engineered *Eco*RI and *Bam*HI sites (shown as underlined bases in lower case), and His codons added as overhangs are shown as bold bases, while His codons already present the 3' terminal of the target sequence are underlined.

DNA sequencing was carried out at the automated sequencing facility of the National Research Council/Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada.

2.10. Cloning of PCR products

Two strategies were used to clone PCR products into cloning vectors, when the Qiagen PCR cloning kit was not used. The first strategy involved designing primers containing restriction enzyme sites in the 5` end as mismatched sequences. After amplification of the target DNA sequence, the PCR product was purified using the Ultra Clean DNA purification kit (BioCan, Canada) following manufacturer's instructions and then digested using the appropriate restriction enzyme. The purified PCR product was then cloned in cloning vector digested with the same set of restriction enzymes. The second strategy involved purifying the PCR product from the agarose gel as described above, and then treating 5-10 μg of purified DNA with 5-10 units of T4 DNA polymerase supplemented with 100 μM dNTPs and 1X T4 DNA polymerase reaction buffer (New England Biolabs) for 30 min at 12°C. Reaction was stopped by heating at the reaction mix at 75°C for 20 min. The PCR product was then cloned in a cloning vector using blunt-end ligation.

2.11. Preparation of RNA

RNA was prepared as described by Ausubel *et. al.* (Ausubel, 1989) with slight modifications. A 10 ml overnight bacterial culture was harvested by centrifugation at 12,000X g, and resuspended in 10 ml of protoplast buffer (15 mM Tris-HCl, pH 8.0, 0.45 M sucrose, 8 mM EDTA) and 50 μl of 50 mg/ml lysozyme, followed by incubation on ice for 10 min. Protoplasts were collected by centrifuging for 5 min in

an SS-34 rotor at 5,900X g and resuspended in 0.5 ml of Gram-negative lysing buffer [10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM sodium citrate, 1.5% (w/v) SDS]. After 5 min incubation on ice, 250 µl of saturated NaCl was added and the sample was centrifuged at high speed. Phenol:Chloroform:Isoamyl alcohol::25:24:1 was used to precipitate protein contaminants and RNA was precipitated using ice-cold absolute ethanol at -60°C for 2 hours. Precipitated RNA was washed once with 70% ethanol and the pellet was resuspended in 100 µl of diethylpyrocarbonate (DEPC) treated water. Ten microlitres of RNase Out (Invitrogen) were added to the resuspended RNA sample and stored at -70°C.

All solutions used in the preparation of RNA samples were prepared in DEPC- treated water. DEPC treatment was performed by adding 0.2 ml of DEPC to 100 ml of water followed by vigorous shaking. The solution was left overnight at room temperature and then autoclaved to inactivate the remaining DEPC.

2.12. Northern blotting

Five to ten µg of RNA samples were treated with 5-10 units of DNase (Bio/Can Canada), and diluted to concentrations of 1µg/µl. One µl of each sample was spotted onto nitrocellulose membrane (Hybond NX, Amersham Biosciences). RNA probes for detection were constructed using the PCR products cloned in the pDrive vector (pClone17 and pClone63) using the T7 RNA polymerase and DIG-UTP. Northern dot blots were performed using the DIG labeling kit (Roche Biochemicals, Canada) following manufacturer's instructions. The density of individual spots was measured using FluorChem 2.01 software (Alpha Innotech Corp.).

2.13. Construction and screening of genomic library of *S. marcescens* UOC-67

A cosmid library of *S. marcescens* UOC-67 was constructed in the low-copy number cosmid vector pRK7813 (Jones & Gutterson, 1987). Chromosomal DNA was isolated from *S. marcescens* using the method described by Hancock (Hancock, 2000) and partially digested with *Eco*RI. Digested DNA was size-fractionated on a continuous sucrose gradient [10-40% (v/v)] by spinning the samples at 25,000 rpm in a SW-27 rotor. Inserts ranging from 9- to 23-kb were collected, ethanol-precipitated, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) to a total of 100 µl. The cosmid vector pRK7813 was digested with *Eco*RI and treated with sheep intestinal alkaline phosphatase (USB Corp., USA) to prevent self-ligation. Ligation of vector to genomic inserts was performed with insert:vector ratio of 1:1 and 1:2 using T4 DNA ligase (NEB) at a total DNA concentration of 1µg/µl. The ligation reactions were incubated at the room temperature overnight. The ligated product was packaged with Gigapack II Gold packaging extract (Stratagene) following the manufacturer's instructions. One-four µl of ligated DNA (0.1-1.0 µg) was added to the packaging extract and mixed by pipetting. The tube was incubated at room temperature for 2 hrs. Five hundred µl of SM buffer [100 mM NaCl; 8 mM $MgSO_4.7H_2O$; 50 mM Tris-HCl, pH 8.0, 0.005% (w/v) gelatin] was added to the tube, followed by 20 µl of chloroform. The tube was spun briefly to sediment the debris and the supernatant containing phage was stored at 4°C until ready for titration. *E. coli* VCS257 was used as the host strain for the titration and amplification of the library. One hundred µl of 1:10 dilution of the library was added to 200 µl of OD_{600} = 0.5 of *E. coli* VCS257 cultured in LB medium supplemented with 0.2% (w/v) maltose

and 10 mM $MgSO_4$, and incubated at room temperature for 30 min without shaking. Eight hundred µl of LB broth was added to the mixture followed by another incubation at 37°C, shaking the tube gently by inversion every 15 min to allow for expression of the resistance gene. Cells were pelleted by spinning briefly and resuspended in 200 µl of LB broth, and spread on LB-tetracyline plates and incubated overnight at 37°C.

To screen the cosmid clones, colonies were inoculated in pools of 10 in LB broth supplemented with 10 µg/ml of tetracycline and incubated overnight at 37°C with shaking. Cosmids were prepared using the method described above for the isolation of plasmids, and screened by Southern blotting using the DIG-labeled DNA probes constructed from pClone63 and pClone17. Positive cosmid pools were checked again by preparing individual cosmid clones and screening by Southern detection. Approximately 1200 colonies were screened by this method. Freezer stock cosmid bank was prepared in DMSO by freezing the cosmid clones in pools of 10 clones.

2.14. SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Lugtenberg and coworkers (Lugtenberg *et al*, 1975) at a polyacrylamide concentration of 12% (w/v). Protein samples were solubilized with a buffer containing 12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 2% (v/v) glycerol, 0.0125% bromophenol blue, and 1% (v/v) β-mercaptoethanol. Samples were heated at 90-100°C for 3-5 min prior to loading. Electrophoresis was conducted at 100-200 V with a 3% acrylamide stacking gel over

the separating gel. Prestained broad range molecular weight marker (New England Biolabs) was used for the determination of molecular weights. Protein bands were stained for 1 hour using Coomassie Blue staining solution [30% (v/v) ethanol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie Blue R-250]. Destaining of gels was performed using destaining solution [15% (v/v) methanol and 7% (v/v) glacial acetic acid] for 1 hr and then overnight with fresh destaining solution.

2.15. Whole cell lysis

Whole cell lysis was performed for rapid examination of gene expression (Hitchcock & Brown, 1983). A 1.5 ml aliquot of an overnight culture of the bacterial strain was centrifuged at 12,000X g for 1 min and cells were resuspended in 100 µl of cell lysis buffer [2% (w/v) SDS, 4% (w/v) dithiothreitol (DTT), 10% (v/v) glycerol and 1 M Tris-HCl, pH 6.8]. The resulting cell lysate was quantified using the Ultrospec 4000 UV/visible spectrophotometer. Protein samples (50-100 µg) were boiled for 3-5 minutes and resolved on 12% polyacrylamide gel.

2.16. Cell envelope preparation

Cell envelope proteins were isolated by using the method described by Lugtenberg and colleagues (Lugtenberg *et al*, 1975) from a 50 ml overnight bacterial culture. Cells were harvested by centrifugation at 5,000X g for 10 min in a Sorvall centrifuge, and resuspended in 20 ml of 50 mM Tris-HCl, 2 mM EDTA, pH 8.5. Cells were lysed by passage through a French press twice at 18,000 psi. Unlysed cells and debris was separated by centrifugation at 1,200X g for 10 min. The supernatant was centrifuged at 100,000X g for 1 hr at 4°C.

2.17. Separation of outer and inner membranes

Separation of inner and outer membranes was performed using the method described by Hancock and Nikaido (Hancock & Nikaido, 1978) with slight modifications. An overnight bacterial culture was subcultured in 2 L of LB broth, supplemented with appropriate antibiotic, and grown to an OD_{600} = 0.8-1.0. Cells were harvested by spinning at 7000 rpm in a Sorvall-SS34 rotor for 10 min, and resuspended in 50 mM Tris and 2 mM EDTA, pH 8.0. Cells were then lysed by passing twice through a French Press pressure cell at 15,000 psi. Cell debris was removed by centrifuging the samples at 3000 rpm for 10 min, at 4°C.

Membranes were collected by a two-stage (9 ml 70% sucrose and 12 ml 18% sucrose) density gradient centrifugation by layering 10 ml of samples on top of the sucrose gradient and spinning at 23,000 rpm for 2 hrs. at 4°C in a SW27 rotor. Membranes were collected from the band at the junction of 18% and 70% steps and 2 ml of sample was loaded on a 4-step sucrose gradient (3 ml 70%, 8 ml 64%, 8 ml 58%, and 8 ml 52% sucrose). The samples were then centrifuged overnight at 23,000 rpm at 4°C. The inner (upper band) and the outer membranes (lower band) were collected and diluted with at least 2 volumes of distilled H_2O to dilute the sucrose to below 20%. Samples were centrifuged once again at 47,000 rpm in a 60Ti rotor for 1 hr at 4°C. Resulting pellet was resuspended in approximately 0.5 ml of 50 mM Tris, pH 8.0, and resolved on an 8% or 13% SDS-PAGE gel.

2.18. Western Immunoblotting

Following SDS-PAGE described above, protein samples were transferred overnight to the Immobilon-P nitrocellulose membrane (Millipore) using a Bio-Rad

Trans-Blot Cell containing glycine transfer buffer [150 mM glycine, 20% (v/v) methanol, 20 mM Tris-HCl, pH 8.3] at 10 mA constant current as described by Burnette (Burnette, 1981). Immunodetection of proteins was conducted as described by Towbin and coworkers (Towbin *et al*, 1979). The blotted membrane was incubated at room temperature in phosphate buffered saline (PBS) buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 16.6 mM K_2HPO_4, 5.4 mM KCl, pH 7.4) containing 1% skim milk for 1 hour, rinsed with PBS without the skim milk, and then immunostained for 2 hours using PBS-diluted rabbit anti-AcrA antibody (1:10,000) (H. Nikaido, University of California, Berkeley, CA, USA). A secondary affinity isolated antibody, goat-anti-rabbit IgG conjugated to horseradish peroxidase (1:1000) (Sigma), was added to the membrane and incubated for 2 hours. Detection of protein bands was performed using the colorimetric method in the presence of 50 ml PBS containing 0.0125 (v/v) of hydrogen peroxide (Fisher Scientific) and 10 ml of 0.3% (w/v) 4-chloro-1-naphthol (Sigma).

2.19. Computer Analysis of DNA and amino acid sequences sequences

Computer analysis of DNA and von-Heijne transmembrane plots for RND proteins were performed using the OMIGA 2.0 software of Oxford Moleculars. PSIPRED protein structure prediction server (www.psipred.net) was used to predict amino acid residues forming transmembrane helices (McGuffin *et al*, 2000). Phylogenetic trees were constructed using the online server of Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

2.20. Three-dimensional structure predictions

Three-dimensional structure predictions were performed using the Conserved Domain Database (CDD) software of NCBI.

THREE

Establishment of Proton Gradient-Dependent Efflux in *Serratia marcescens*

Just don't give up trying to do what you really want to do.
-Ella Fitzgerald

3.1. Abstract

Thirteen clinical isolates of *S. marcescens* were tested for their susceptibilities to a variety of fluoroquinolones, with the ATCC strain UOC-67 used as a control. Two clinical isolates, T-860 and T-861, displayed significantly higher resistances than all other strains to nalidixic acid, ciprofloxacin, norfloxacin, and ofloxacin.

In order to determine if proton gradient-dependent efflux could be contributing to fluoroquinolone resistance, ethidium bromide accumulation assays were carried out for all strains. Similar results were obtained for all strains, with a rapid increase in levels of accumulated ethidium bromide upon the addition of the proton gradient uncoupler CCCP, indicating that proton gradient-dependent efflux mechanism was active in all of the strains.

To further investigate the extent of proton gradient-dependent efflux, strains with MIC values of $\geq$2 μg/ml for fluoroquinolone antibiotics were subjected to fluoroquinolone accumulation assays. Of the strains tested, the clinical isolate T-861 tested positive for increased accumulation of all fluoroquinolones tested (ciprofloxacin, norfloxacin, and ofloxacin) upon CCCP addition, indicating that only this isolate is capable of proton-dependent efflux of these drugs.

This study was followed by Western immunoblot experiments using the anti-AcrA antibody, for *E. coli* AcrA protein of AcrAB efflux pump. Results indicated that clinical isolate T-861 was expressing an AcrA-like protein.

Laboratory derived fluoroquinolone-resistant mutant strains were isolated by serial passaging of the wild-type strain UOC-67 in media supplemented with

ciprofloxacin, norfloxacin, or ofloxacin respectively. These strains were capable of effluxing one or more of fluoroquinolones tested.

Mutations in genes encoding the target enzymes for fluoroquinolones, DNA gyrase and topoisomerase, are responsible for fluoroquinolone resistance of different organisms. With this in mind, *S. marcescens* strains resistant to fluoroquinolones were tested for the presence of target mutations in the genes encoding DNA gyrase and topoisomerase. An S83→R mutation was found to be present in the *gyrA* sequence of fluoroquinolone resistant strains.

In combination, these results established proton gradient-dependent efflux of fluoroquinolones to be a mechanism of resistance in *S. marcescens,* and indicated that active efflux, along with target mutations, contribute to the fluoroquinolone resistance of this organism.

3.2. Antibiotic susceptibilities of *S. marcescens* strains

Thirteen clinical isolates of *S. marcescens* (Table 2.1) were tested for their susceptibilities to different antimicrobials, namely, ciprofloxacin, norfloxacin, ofloxacin, nalidixic acid, ampicillin, kanamycin, and ethidium bromide. The wild-type strain, UOC-67, was used as the control. Results of MIC experiments for the clinical isolates and UOC-67 are summarized in Table 3.1. All strains tested were highly resistant to ampicillin, exhibiting MICs of ≥16 μg/ml. Interestingly, UOC-67 had the highest MIC for ampicillin. All strains had an MIC of ≥1 μg/ml for kanamycin except for

Table 3.1. Minimum Inhibitory Concentration values (μg ml^{-1}) of *S. marcescens* strains

Strain	Cip	Nor	Ofx	Nal	Ap	Km	Cb	EtBr
T-849	0.5	0.25	0.25	16	32	2	32	128
T-850	0.125	0.5	0.25	16	64	1	4	256
T-851	0.5	0.25	0.5	4	125	2	8	256
T-852	0.5	0.125	1	16	64	4	16	512
T-853	1	2	1	8	16	4	2	512
T-854	0.25	1	0.5	8	16	2	4	512
T-855	0.025	0.5	0.5	8	64	4	16	512
T-856	2	4	2	32	16	1	4	1024
T-857	1	1	0.125	2	32	2	4	256
T-858	0.064	0.5	0.125	2	32	2	4	512
T-859	0.064	0.25	0.125	2	64	2	32	256
T-860	4	8	4	512	64	2	32	256
T-861	4	32	4	512	64	0.5	16	256
UOC-67	0.032	0.125	0.5	4	250	2	8	256

Cip, ciprofloxacin, Nor, norfloxacin; Ofx, ofloxacin; Nal, nalidixic acid; Ap, ampicillin; Km, kanamycin; Cb, carbenicillin; EtBr, ethidium bromide.

T-861, where the MIC was determined to be only 0.5 μg/ml. The clinical isolate T-853 was most susceptible to carbenicillin with an MIC of 2 μg/ml while all other strains tested had MICs of ≥4 μg/ml. All strains were highly resistant to ethidium bromide with MIC of ≥128 μg/ml.

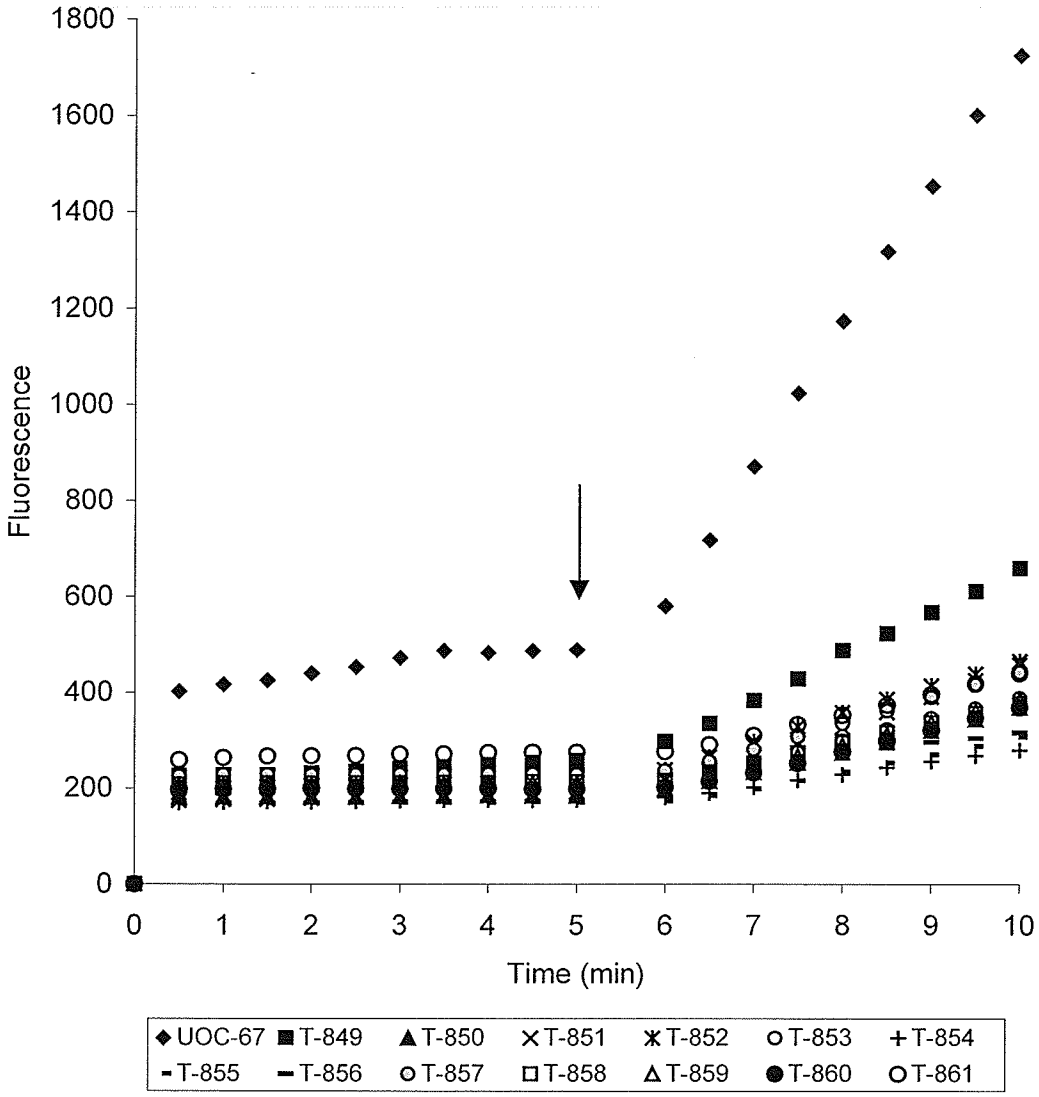
With respect to quinolones, all strains exhibited very high resistance to nalidixic acid with an MIC of ≥2 μg/ml, with the clinical isolates T-860 and T-861 being most resistant with MICs of 512 μg/ml each. T-860 and T-861 were most resistant to the three fluoroquinolones tested (ciprofloxacin, norfloxacin, and ofloxacin), with MIC values ranging from 4 to 32 μg/ml.

3.3. Ethidium bromide accumulation assays

Ethidium bromide accumulation assays were performed to ascertain the presence of a proton-gradient efflux mechanism. Results of assays using all strains are shown in Fig. 3.1. All strains, including the wild-type UOC-67, maintained low constant intracellular levels of ethidium bromide but upon addition of CCCP, 5 min after the addition of ethidium bromide, they all showed a rapid increase in the accumulation of ethidium bromide. Interestingly, the wild-type strain UOC-67 accumulated more ethidium bromide than any of the clinical isolates before the addition of CCCP, and even after the addition of CCCP, the amount of ethidium bromide accumulated by UOC-67 was the highest when compared to all clinical isolates.

Fig. 3.1. Ethidium bromide accumulation by UOC-67 and 13 clinical isolates of *S. marcescens*. Accumulation of ethidium bromide was monitored for 10 min and CCCP was added 5 min after incubation (shown by the arrow). Fluorescence of ethidium bromide was measured at 530 (excitation) and 600 (emission) nm. Data presented is a representative of that obtained from three independent assays performed on three independent cultures.

Fig. 3.1.


1800
1600
1400
1200
1000
800
600
400
200
0
Fluorescence
0
1
2
3
4
5
6
7
8
9
10
Time (min)
UOC-67
T-849
T-850
T-851
T-852
T-853
T-854
T-855
T-856
T-857
T-858
T-859
T-860
T-861

3.4. Fluoroquinolone accumulation assays

Strains that exhibited an MIC of ≥2 μg/ml for any of the three fluoroquinolones were considered resistant and tested for the accumulation of that particular fluoroquinolone. The clinical isolate, T-853 with an MIC of 2 μg/ml for norfloxacin, was tested for norfloxacin accumulation. It accumulated norfloxacin in a linear fashion with time and there was no effect of addition of CCCP, proving that this strain does not efflux the antibiotic (Fig. 3.2a). T-856 had an MIC of 2 μg/ml for both ciprofloxacin and ofloxacin and that of 4 μg/ml for norfloxacin. This strain was not found to efflux any of the three antibiotics in a proton gradient-dependent manner, as it accumulated the antibiotics with time with no effect of CCCP addition (Fig. 3.2b).

The clinical isolate T-860 had an MIC of 4 μg/ml for ciprofloxacin and ofloxacin, and 8 μg/ml for norfloxacin. Interestingly, it was also not found to efflux any of the antibiotic tested inspite of high MICs (Fig. 3.2c). T-861 was another clinical isolate that exhibited high MIC values for all three fluoroquinolones with values of 4 μg/ml, 32 μg/ml, and 4 μg/ml for ciprofloxacin, norfloxacin, and ofloxacin, respectively. This strain showed low constant intracellular levels of all three antibiotics but once CCCP was added there was a rapid accumulation of all three antibiotics suggesting that T-861 was effluxing all three fluoroquinolones (Fig. 3.2d).

The wild-type strain UOC-67 was not found to efflux any of the three fluoroquinolones (Fig. 3.2e).

Fig. 3.2. Fluoroquinolone accumulation by clinical isolates T-853, T-856, T-860, T-861, and the wild-type UOC-67. T-853 (a) was measured for the accumulation of norfloxacin (Nor); and T-856 (b), T-860 (c), T-861 (d), and UOC-67 (e) were tested for accumulation of norfloxacin (Nor), ciprofloxacin (Cip), and ofloxacin (Ofx). CCCP was added 5 min after addition of the antibiotic (shown by the arrow). Results are expressed as ng of antibiotic accumulated per mg (dry weight) of cells. Data presented is a representative of that obtained from three independent assays performed on three independent cultures.

Fig. 3.2a. T-853

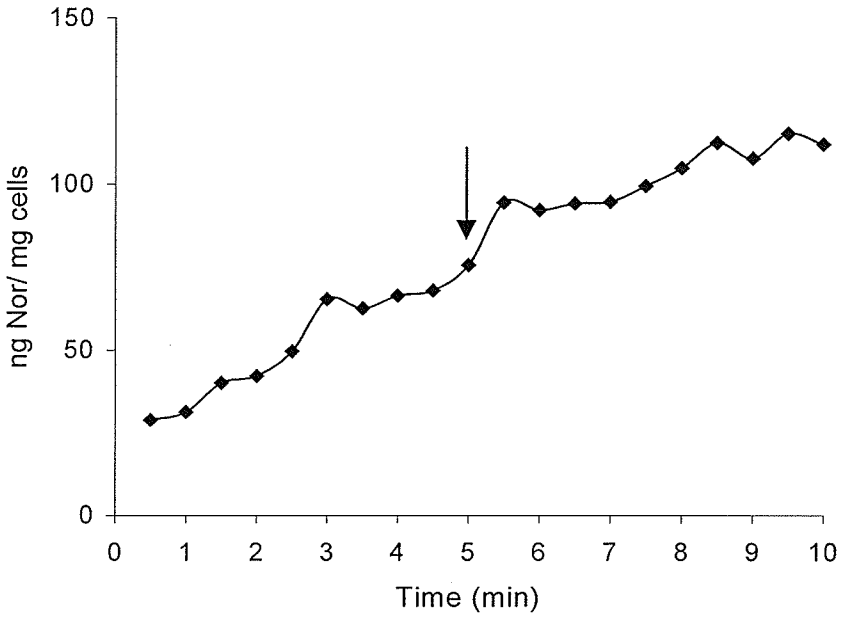


Fig. 3.2b. T-856

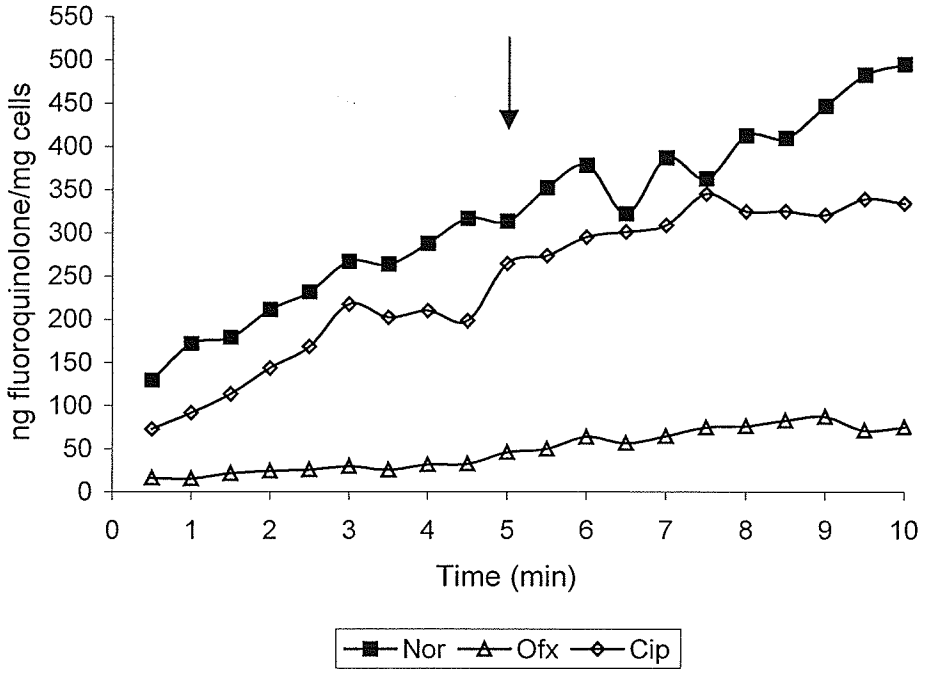

ng fluoroquinolone/mg cells
550
500
450
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
Time (min)
Nor
Ofx
Cip

Fig. 3.2c. T-860

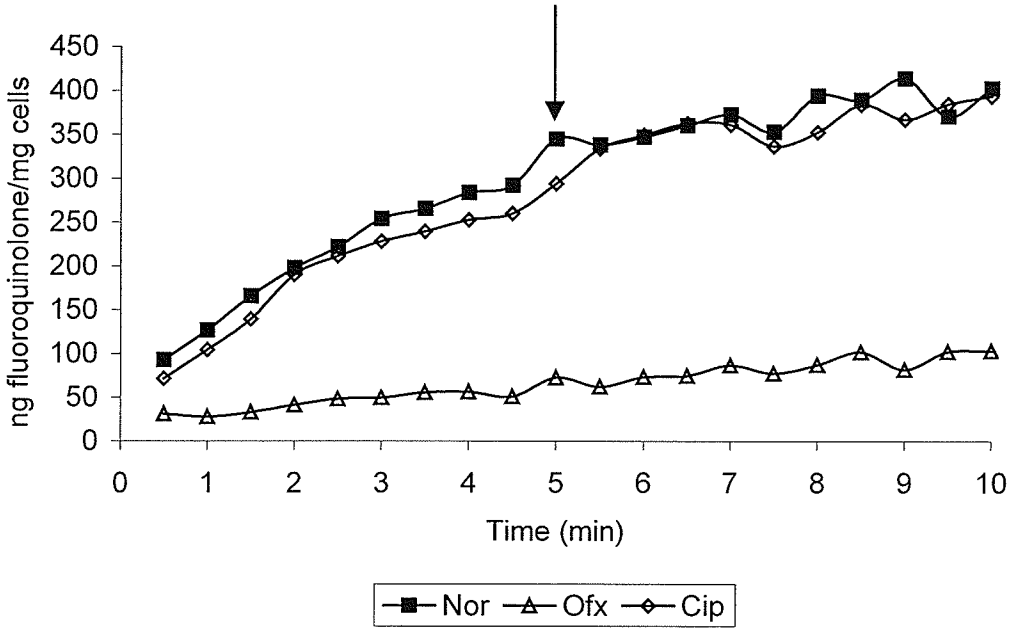

ng fluoroquinolone/mg cells
450
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
Time (min)
Nor
Ofx
Cip

Fig. 3.2d. T-861

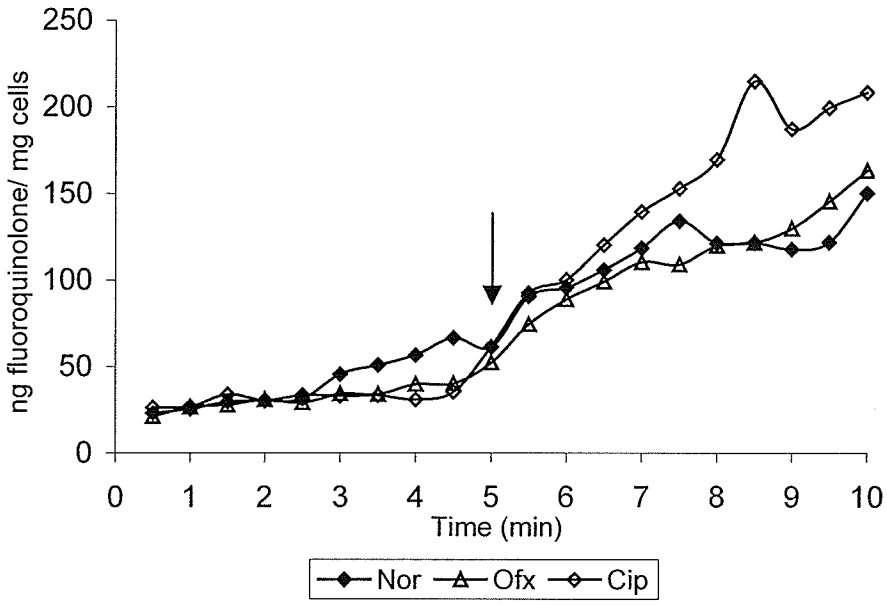
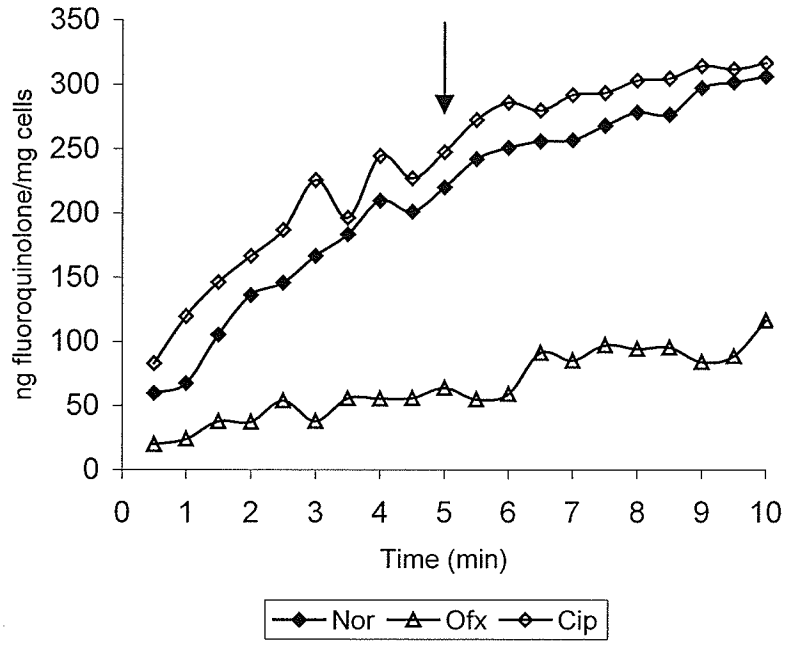

250
200
150
100
50
0
ng fluoroquinolone/ mg cells
0
1
2
3
4
5
6
7
8
9
10
Time (min)
Nor
Ofx
Cip

Fig. 3.2e. UOC-67



3.5. Western Immunoblot

The Western immunoblot from the SDS-PAGE urea gel of the whole cell lysate of UOC-67 (wild-type), and T-861 (the clinical isolate capable of effluxing fluoroquinolones) showed a positive reaction with antibodies for AcrA protein of *E. coli* AcrAB pump (Fig. 3.3). Two protein bands were visualized, with the intensity of one of the bands higher for T-861 than that of UOC-67, suggesting the presence of two different, yet related, RND pump proteins.

3.6. Isolation and characterization of mutant strains of *S. marcescens*

Isolation of three different mutant strains was performed by serial passaging UOC-67 in media supplemented with ciprofloxacin, norfloxacin, or ofloxacin. Strains were named UOC-67WL, UOC-67WLN, and UOC-67WLO, respectively. All three mutant strains were tested for their susceptibilities to ciprofloxacin, norfloxacin, ofloxacin, and nalidixic acid. The results of antibiotic susceptibility testing are summarized in Table 3.2. All mutant strains showed at least a 16-fold increase in their resistance to all four antibiotics when compared to the parent strain.

Results from MIC assays for the mutant strains showed that growth of the wild-type strain UOC-67 in media supplemented with different fluoroquinolones can result in a drastic increase in its resistance to these antibiotics. To determine if energy-dependent efflux was playing a role in the increased flouroquinolone resistance of the mutant strains, fluoroquinolone accumulation assays were performed for UOC-67WL, UOC-67WLN, and UOC-67WLO. UOC-67WL, the mutant strain isolated from ciprofloxacin-supplemented media, showed a drastic increase in the accumulation of ciprofloxacin and norfloxacin upon addition of CCCP, and to some

Table 3.2. Minimum Inhibitory Concentration values (μg ml^{-1}) and relative resistance for quinolones for mutant strains of *S. marcescens*

Strain	Cip		Nor		Ofx		Nal	
	MIC	**RR[a]**	**MIC**	**RR**	**MIC**	**RR**	**MIC**	**RR**
UOC-67WL	32	1024	64	512	32	64	128	32
UOC-67WLN	8	256	32	256	16	32	>1024	>256
UOC-67WLO	4	128	16	128	8	16	256	64

[a] relative resistance, the number denotes the MIC of the mutant strain divided by the MIC of the parent strain, UOC-67.

UOC-67WL, isolated from ciprofloxacin-supplemented media

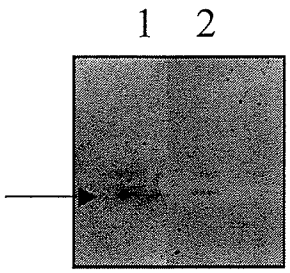
UOC-67WLN, isolated from norfloxacin-supplemented media

UOC-67WLO, isolated from ofloxacin-supplemented media

Cip, ciprofloxacin; Nor, norfloxacin; Ofx, ofloxacin; Nal, nalidixic acid

Fig. 3.3. Immunoblot analysis of AcrA-like protein(s) from T-861 (1) and UOC-67 (2). Protein (150 μg) from the whole cell lysate of each strain was resolved on SDS-PAGE and immunodetection was performed using anti-AcrA antibody (1:4000). The arrow points to the protein-band (~42 kDa) being over expressed in T-861.

Fig. 3.3.



extent, ofloxacin, suggesting that this strain was effluxing both ciprofloxacin and norfloxacin, and also ofloxacin to some extent (Fig 3.4a). UOC-67WLN, the mutant strain isolated from media supplemented with norfloxacin, also showed similar accumulation patterns as UOC-67WL, with efflux of ciprofloxacin and norfloxacin and also to some extent ofloxacin (Fig. 3.4b). UOC-67WLO, the strain isolated from ofloxacin-supplemented media, did not show any signs of efflux of ciprofloxacin or norfloxacin (Fig. 3.4c).

There was some increase in the accumulation of ofloxacin, suggesting that UOC-67WLO was, probably, able to efflux very small amounts of this antibiotic.

3.7. Target mutation analysis

Sequencing of the Quinolone Resistance Determining Regions (QRDRs) of the *gyrA* and *parC* genes was performed for the clinical isolates T-856, T-860 and T-861, and the mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO, along with the wild-type UOC-67. The QRDR of *gyrA* showed a Ser83→Arg mutation in T-860, T-861, UOC-67WL, and UOC-67WLN (Fig 3.5a). No mutations were seen in the QRDR region of *parC* gene in any of the strains (Fig. 3.5b).

3.8. n-hexane tolerance assay

Resistance to organic solvents is an indication of active efflux. With this in mind, the n-hexane tolerance assay was performed for the clinical isolate T-861, and mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO. UOC-67 was used as the control strain. While *S. marcescens* T-861, UOC-67WL, UOC-67WLN, and UOC-67WLO were found to be resistant to n-hexane, UOC-67 was found to be susceptible (Fig. 3.6).

Fig. 3.4. Fluoroquinolone accumulation by mutant strains of *S. marcescens*. UOC-67WL (a), UOC-67WLN (b), and UOC-67WLO (c) are the mutant strains of *S. marcescens* derived from UOC-67 selected on media supplemented by ciprofloxacin, norfloxacin, and ofloxacin, respectively. CCCP was added 5 min after addition of the antibiotic (shown by the arrow) and results were expressed as nanogram of antibiotic accumulated per mg (dry weight) of cells. Data presented are a representative of those obtained from three independent assays performed on three independent cultures.

Fig. 3.4a. UOC-67WL

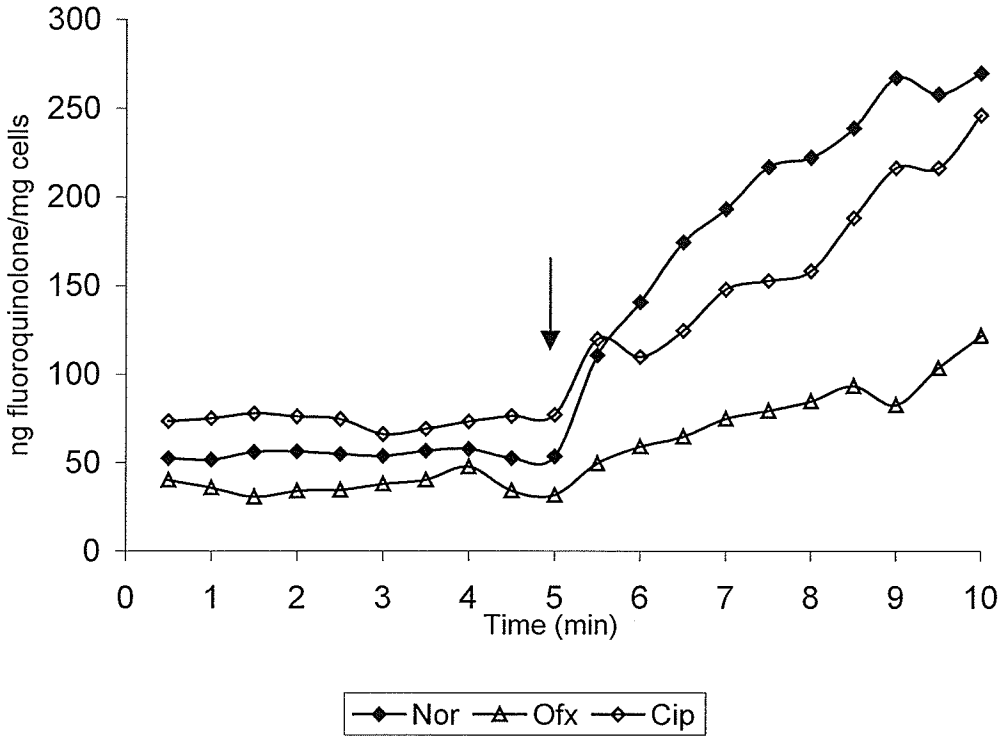


Fig. 3.4b. UOC-67WLN

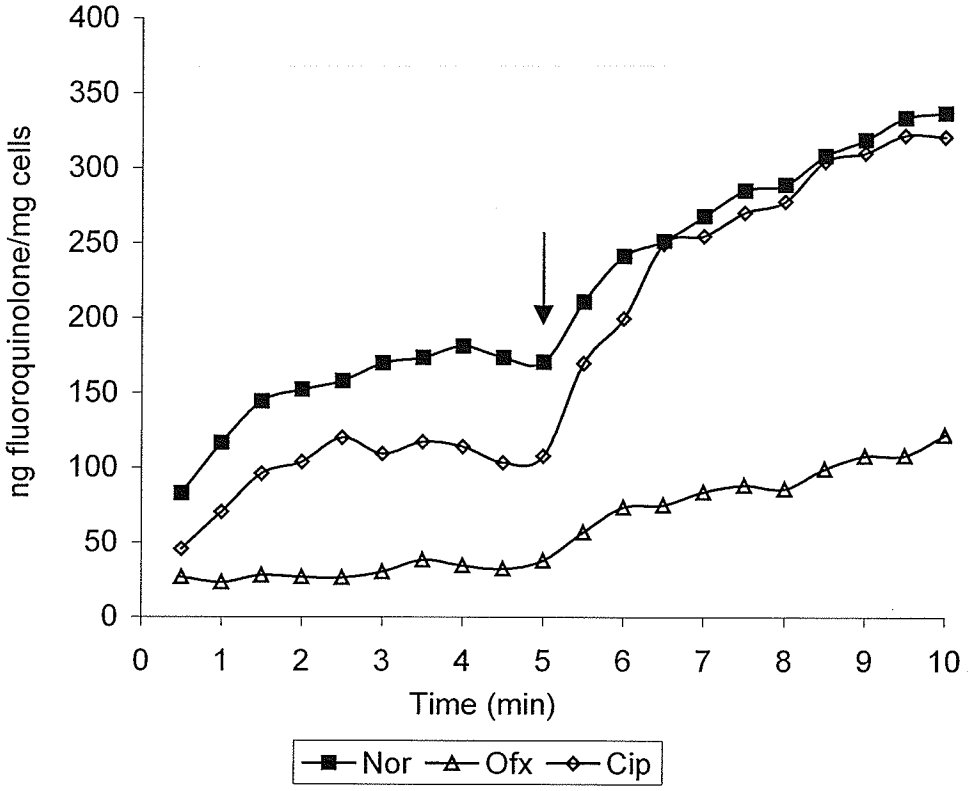


Fig. 3.4c. UOC-67WLO

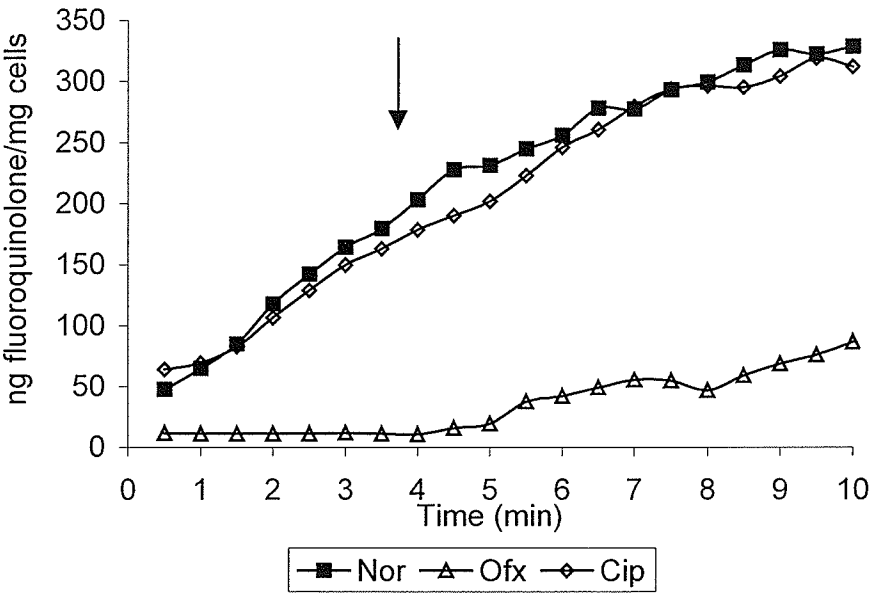


Fig. 3.5. Alignment of deduced amino acid residues of the QRDRs of *gyrA* (a) and *parC* (b) genes of *S. marcescens* strains. UOC-67 is the wild-type, T-856, T-860, and T-861 are clinical isolates, and UOC-67WL, UOC-67WLN, and UOC-67WLO are mutant strains isolated by serial passaging of UOC-67 in media supplemented with ciprofloxacin, norfloxacin, or ofloxacin, respectively. Amino residues indicated in bold letters (in shaded boxes) indicate the known point mutation sites responsible for fluoroquinolone resistance in *E. coli*. Amino acid residues altered are underlined.

Fig. 3.5a

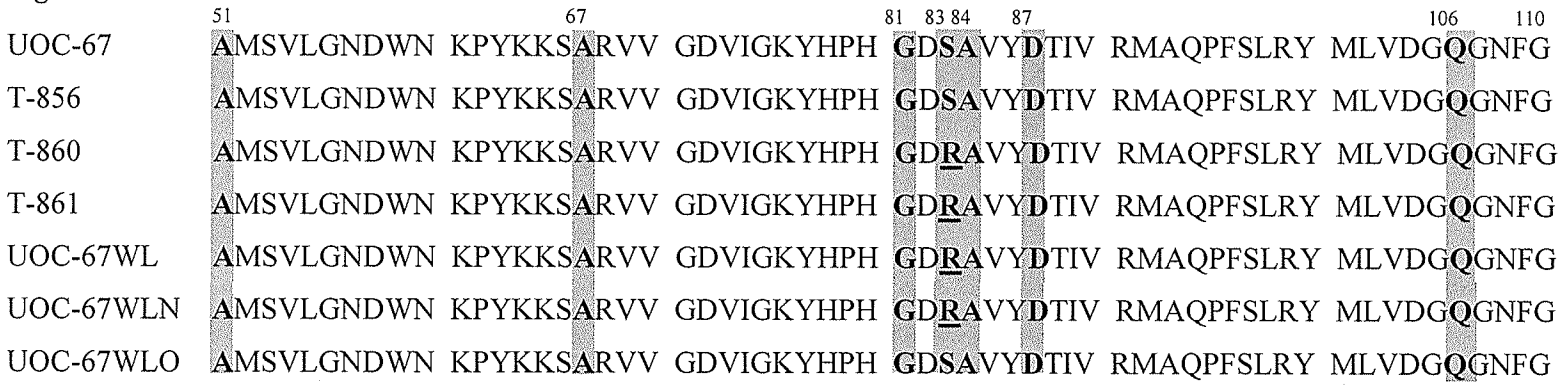
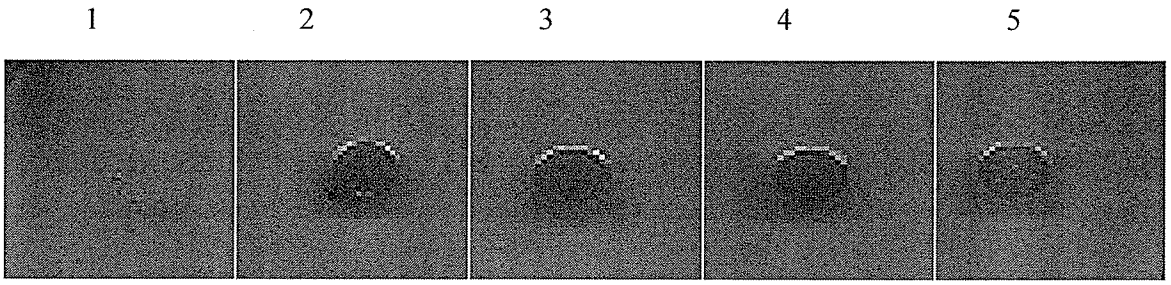


Fig. 3.5b

Fig. 3.6. Organic solvent (n-hexane) tolerance of various *S. marcescens* strains: UOC-67 (1), T-861 (2), UOC-67WL (3), UOC-67WLN (4), and UOC-67WLO (5). Five microliters of equilibrated mid-logarithmic-phase culture (10^8 cfu/ml) of each strain was spotted on LB-agar and allowed to dry. The plate was flooded with n-hexane to a depth of 2-3 mm, sealed, and incubated at 37°C for 16-20 hrs. Growth at spots indicates resistance to n-hexane.

Fig. 3.6.



3.9. Discussion

Screening of *S. marcescens* clinical isolates for resistance to fluoroquinolones resulted in the identification of 4 isolates with MICs of ≥ 2 μg/μl for one or more fluoroquinolones. Ethidium bromide accumulation assays were performed to screen the clinical isolates for the presence of proton gradient-dependent efflux. Ethidium bromide, a substrate for efflux pumps (Paulsen *et al*, 1996a), can be used to examine for the presence of an efflux mechanism in an organism by measuring the fluorescence of the cells. The fluorescence of ethidium bromide increases dramatically once it is incorporated into cellular DNA. As a result, if ethidium bromide is a substrate for an efflux pump that is inhibited, the intracellular fluorescence should increase as more ethidium bromide enters the cells. Efflux pumps that utilize the proton gradient as the source energy can be inhibited by an uncoupler, such as CCCP. The fluorescence of ethidium bromide before the addition of CCCP for all 13 clinical isolates was similar, whereas the wild-type strain showed a higher fluorescence before the CCCP was added (Fig. 3.1). This may be explained by a lower efflux rate or higher influx rate of ethidium bromide in UOC-67, as compared to the clinical isolates. This hypothesis is further supported by the fact that greater accumulation of ethidium bromide was achieved when studying the wild-type strain. The ethidium bromide studies point strongly to the presence of proton gradient-dependent efflux in *S. marcescens*.

Fluoroquionolone accumulation assays were performed for 4 clinical isolates that demonstrated high resistance to these antibiotics: T-853, T-856, T-860, and T-861, in order to screen them for efflux of fluoroquinolones and to determine if there is a role of proton gradient-dependent efflux in the fluoroquinolone resistance of these isolates.

Three of the clinical isolates, T-853 (Fig. 3.2a), T-856 (Fig. 3.2b), and T-860 (Fig. 3.2c) did not show any increase in the accumulation of relevant antibiotic upon the addition of CCCP. Moreover, the accumulation patterns of these strains were found to be very similar to that of the wild-type strain, UOC-67 (Fig. 3.2e). T-861, however, showed increased accumulation of all three fluoroquinolones tested (Fig. 3.2d) upon addition of CCCP suggesting that once CCCP disrupted the proton gradient, more fluoroquinonolone accumulated inside the cells as a result of inhibition of efflux. Comparison of accumulation curves of T-861 and UOC-67 for these drugs revealed that while the wild-type strain continued to accumulate the drug before the addition of CCCP, T-861 maintained constant low intracellular levels of the drug until CCCP was added. This was strong evidence that T-861 was capable of effluxing ciprofloxacin, norfloxacin, and ofloxacin in an energy dependent manner.

Western immunoblot detection was performed on UOC-67 and the fluoroquinolone-effluxing clinical isolate, T-861 using *E. coli* anti-AcrA antibody. Following blot development, 2 bands were observed, representing proteins of a size similar to that of MFPs, with one of the proteins being overproduced in T-861 (Fig. 3.3). These results suggest that the clinical isolate T-861 overexpresses an RND pump MFP-like protein.

Three different fluoroquinolone resistant mutant strains of *S. marcescens* were isolated by the serial passaging of UOC-67 in media supplemented with ciprofloxacin, norfloxacin, or ofloxacin, respectively, in an attempt to isolate pump-overexpressing strains. When tested for their susceptibilities for fluoroquinolones, all strains showed a marked increase in the resistance to these antibiotics (Table 3.2). UOC-67WL and UOC-

67WLN were effluxing both ciprofloxacin and norfloxacin and, to some extent ofloxacin (Figs. 3.4a and 3.4b). In fact, the accumulation pattern was very similar to that seen for the clinical isolate T-861 (Fig. 3.2d). UOC-67WLO, however, did not exhibit any efflux of ciprofloxacin and norfloxacin, although, it did appear to efflux ofloxacin to some extent (Fig. 3.4c). Accumulation data for the 3 mutant strains suggest that UOC-67WL and UOC-67WLN were overexpressing proton gradient-dependent pumps capable of pumping out norfloxacin and ciprofloxacin, and perhaps ofloxacin. These results further indicate that the presence of ciprofloxacin and norfloxacin results in up-regulation of either the same pump or two different pumps in *S. marcescens*. As far as UOC-67WLO is concerned, it appears that ofloxacin was not able to up-regulate any proton gradient-dependent pump. However, there is a definite decrease in the uptake of ofloxacin in this strain, as it showed very low intracellular amounts of the antibiotic before as well as after the addition of CCCP, suggesting that the major mechanism of resistance to ofloxacin is reduced uptake.

All clinical isolates and the mutant strains were screened for the presence of the target gene mutations. It is well known that in *E. coli* mutations in the QRDR of *gyrA* and *parC* and energy-dependent efflux, either together or independently, are responsible for resistance to fluoroquinolones. In *E. coli*, *gyrA* mutations in codons 67, 81, 82, 83, 84, 87, and 106 have been documented to be responsible for the development of quinolone resistance (Cambau *et al*, 1993; Conrad *et al*, 1996; Everett *et al*, 1996; Ruiz, 2003; Tavio *et al*, 1999; Troung *et al*, 1997; Vila *et al*, 1994). Recently, alterations in position 51, a codon outside the QRDR, have also been reported to confer resistance (Friedman *et al*, 2001). In *E. coli*, the presence of a single mutation in any of the above

codons can confer a high degree of resistance to nalidixic acid, however, for higher resistance to fluoroquinolones, additional mutation(s) in *gyrA* and/or in another target, such as *parC*, is required (Ruiz, 2003). In the *parC* gene of *E. coli*, the most common mutations are observed in codons 78, 80, and 84 (Heisig, 1996) and, as such, this enzyme is now recognized as a secondary target in *E. coli* for quinolone antibiotics.

In *S. marcescens*, much diversity has been documented in *gyrA* mutations, with mutations present in codons 81, 83, and 87 (Weigel *et al*, 1998). However, no double mutations have been reported in the *gyrA* QRDR (Kim *et al*, 1998; Weigel *et al*, 1998). Findings from the *gyrA* QRDR analysis in this study are consistent with this, as only a Ser83→Arg (AGC→AGA) mutation was found in T-861, UOC-67WL, and UOC-67WLN (Fig. 3.5a). There were no other mutations present in the *gyrA* QRDR in spite of the fact that these strains exhibited high MIC values for all fluoroquinolones tested. Interestingly, there was no mutation present in the QRDR of the *parC* gene for any of these strains (Fig. 3.5b).

UOC-67WLO was found to be unique in that, while exhibiting high MIC values ($\geq$ 4 μg/ml) for all antibiotics tested, there were no mutations in either the *gyrA* or the *parC* gene. Among the strains with the Ser-83 mutation, the MIC values varied up to 16-fold for different antibiotics. UOC-67WLO had similar MIC values for ciprofloxacin, norfloxacin, and ofloxacin. In spite of the comparable MIC values, UOC-67WLO appears to be lacking mutations that could confer resistance to quinolones. From this data it can be inferred that a Ser-83 mutation in the *gyrA* QRDR is likely to be the most important point mutation responsible for fluoroquinolone resistance of *S. marcescens*. It is interesting that the serial passaging of UOC-67 in ciprofloxacin as well as norfloxacin-

supplemented media resulted in the same mutation as seen in the highly resistant clinical isolate, T-861.

Although there were no mutations present in the QRDR of the *parC* gene, a Thr-59→Asn mutation was found in T-861 and UOC-67WL (Fig. 3.5b). The significance of this mutation is unclear, as it was not found in any of the other strains tested that were resistant to fluoroquinolones, and is not documented for any species elsewhere. However, as this mutation was present in UOC-67WL, it is likely a result of the ciprofloxacin pressure imposed on UOC-67 and thus may be contributing to fluoroquinolone resistance.

Hexane tolerance was assayed for T-861 and the 3 mutant strains, and indicated that all are resistant to hexane, in contrast to the wild-type UOC-67 (Fig. 3.6). As active efflux is the major mechanism of resistance to organic solvents, the data for hexane tolerance suggests that all of the above strains are over-expressing one or more efflux pumps.

In summary, the above data demonstrate that active efflux of fluoroquinolones, along with target mutation(s), is established as a resistance mechanism in *S. marcescens*.

FOUR

Molecular Characterization of the *Serratia marcescens* SdeAB Multidrug Efflux pump

When it is dark enough, you can see the stars.
-Charles A. Beard

4.1. Abstract

The partial sequence of a *mexF*-like RND pump gene was identified in *S. marcescens* by PCR, using primers for two conserved motifs of RND proteins. To clone, sequence, and characterize the complete gene sequence, a genomic bank of *S. marcescens* was created and screened by Southern blot analysis. One cosmid clone tested positive, and a 5 kb *Eco*RV fragment from the cosmid clone was subcloned into the cloning vector pUC18.

Sequencing of the subclone revealed the presence of an MFP- and a RND pump-encoding gene in an operon. The locus was named *sdeAB* (serratia drug efflux). Expression of the *sdeB* gene was measured in fluoroquinolone-resistant clinical isolate and laboratory-derived mutant strains by Northern blot analysis, and was found to be over-expressed in two of the three mutant strains.

The *sdeAB* locus, cloned in pUC18, was introduced in an AcrB-deficient strain of *E. coli*, and susceptibility to various antimicrobials was measured. Results show that the SdeAB pump of *S. marcescens* is a multidrug efflux pump that can be upregulated by norfloxacin and ciprofloxacin, and is responsible for resistance to fluoroquinolones, detergent, and organic solvents.

4.2. Identification of the *sdeAB* locus

PCR of the wild-type *S. marcescens* UOC-67 genomic DNA using primers for conserved motifs for RND pumps resulted in a 1.7 kb product. Sequencing of this PCR product revealed a very high degree of DNA homology (~60%) with the *mexF* gene of *P. aeruginosa* and subsequent amino acid prediction revealed the presence of conserved motifs found in RND pump proteins. RND protein signature sequences were also

identified (Fig. 4.1). This PCR product was named *sdeB* (*Serratia* Drug Efflux) and cloned in the cloning vector pBluescript KS(-). A DIG-labeled probe was synthesized using the cloned PCR product.

4.3. Cloning of the *sdeAB* locus

A genomic bank of *S. marcescens* UOC-67 was constructed in the cosmid pRK7813 and screened for the *sdeAB* operon using the DIG-labeled probe constructed from the cloned 1.7 kb *sdeB* fragment cloned in pKS(-). One cosmid clone, pRKO37, was identified to be harboring the *sdeB* gene (Fig. 4.2) within a 21 kb insert. A 5 kb *Eco*RV fragment was subcloned into the cloning vector pUC18 to construct the plasmid pUCAB, and sequenced. Sequencing of this 5 kb *Eco*RV cosmid-derived fragment and its further analysis using the OMIGA 2.0 software revealed the presence of 2 open reading frames (ORFs) of 1185 bp and 3140 bp, respectively (Fig. 4.3a). Based on the sequence similarity, the 1185 bp ORF was identified as a gene encoding the putative MFP, while the 3140 bp ORF is homologous to RND efflux pump-encoding genes. This locus was named *sdeAB*, with *sdeA* being the gene for the MFP, and *sdeB* for the RND pump gene (Fig. 4.3b). The sequence of this operon has been submitted to GenBank under the Accession No. AY168756.

Transmembrane helices in the SdeB protein were calculated using OMIGA 2.0 software, and results revealed the presence of 12 transmembrane helices with 2 long loops between helices 1 and 2, and 6 and 7 (Fig. 4.4), a characteristic of RND proteins.

Phylogenetic analysis of SdeB with RND pump proteins from *E. coli* and *P. aeruginosa* esbalished that it is closely related to the MexF protein to *P. aeruginosa* insead of any of the *E. coli* pumps (Fig. 4.5).

Fig. 4.1. Alignment predicted amino acid sequence of the 1.7 kb *S. marcescens sdeB* fragment and known RND efflux pumps of *E. coli* (AcrB, AcrF, and MdtC) and *P. aeruginosa* (MexB, MexD, MexY, and MexF). Alignment was performed using OMIGA 2.0 software. Amino acid residues shaded in gray represent the conserved amino acid residues in RND family pumps. Numbers indicate positions of amino acids in each protein.

Fig. 4.1

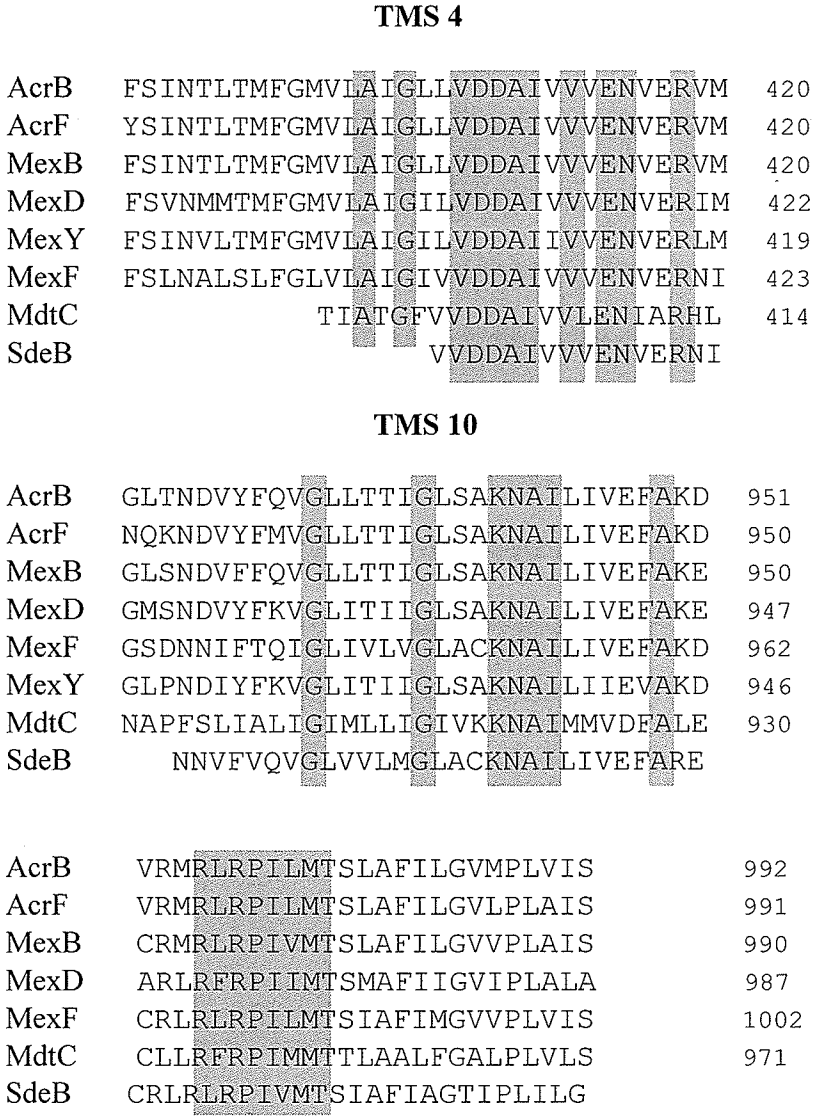

TMS 4
AcrB FSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
AcrF YSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
MexB FSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
MexD FSVNMMTMFGMVLAIGILVDDAIVVVENVERIM 422
MexY FSINVLTMFGMVLAIGILVDDAIIVVENVERLM 419
MexF FSLNALSLFGLVLAIGIVVDDAIVVVENVERNI 423
MdtC TIATGFVVDDAIVVLENIARHL 414
SdeB VVDDAIVVVENVERNI
TMS 10
AcrB GLTNDVYFQVGLLTTIGLSAKNAILIVEFAKD 951
AcrF NQKNDVYFMVGLLTTIGLSAKNAILIVEFAKD 950
MexB GLSNDVFFQVGLLTTIGLSAKNAILIVEFAKE 950
MexD GMSNDVYFKVGLITIIGLSAKNAILIVEFAKE 947
MexF GSDNNIFTQIGLIVLVGLACKNAILIVEFAKD 962
MexY GLPNDIYFKVGLITIIGLSAKNAILIIEVAKD 946
MdtC NAPFSLIALIGIMLLIGIVKKNAIMMVDFALE 930
SdeB NNVFVQVGLVVLMGLACKNAILIVEFARE
AcrB VRMRLRPILMTSLAFILGVMPLVIS 992
AcrF VRMRLRPILMTSLAFILGVLPLAIS 991
MexB CRMRLRPIVMTSLAFILGVVPLAIS 990
MexD ARLRFRPIIMTSMAFIIGVIPLALA 987
MexF CRLRLRPILMTSIAFIMGVVPLVIS 1002
MdtC CLLRFRPIMMTTLAALFGALPLVLS 971
SdeB CRLRLRPIVMTSIAFIAGTIPLILG

Fig. 4.2. Southern blot detection of the *sdeB* gene in the *S. marcescens* cosmid clone pRKO37 after restriction-enzyme digestion. Lane 1, *Eco*RI; Lane 2, *Sma*I; and Lane 3, *Xba*I. Numbers indicate the size of *Hind*III-digested λ-DNA molecular weight markers.

Fig. 4.2.

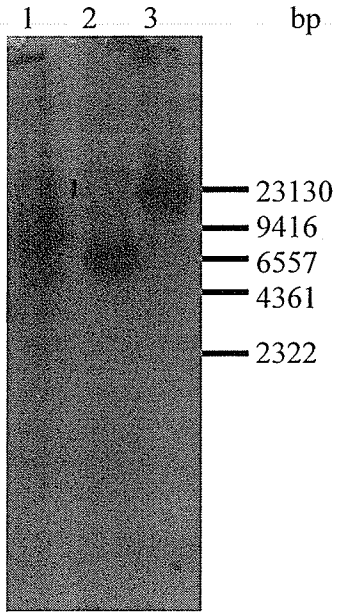


Fig. 4.3a. Nucleotide sequence of the *sdeAB* locus of *S. marcescens* UOC-67. Initiation codons for *sdeA* and *sdeB* are shown in bold. Shaded nucleotides represent *sdeA* and *sdeB* open reading frames.

Fig. 4.3a.

sdeA

```
   1 atggcaaatc aaccaaactc atcgttcaac gccggcggcc gcacgcgcgc cgtcgctctg
  61 ggccagcggc tctccggggt ggcgctgttg gccgccctgc tcgccgggtg tgacaacagc
 121 gtcgcgcaca atgccccgcc gccgccgccg gtggtcagcg ccgccagcgt ggtggtcaag
 181 ccgatcagcc agtgggatgc gttcaatggc cgggtcgagg cggtgcagag cgtgcaactg
 241 cgcccacggg tctctggtta catcgagcgg gtcaactaca ccgaaggcga cgaggtgaag
 301 aaaggccagg tgctgttcac catcgacgat cgcacctacc gcgccgcgcg tgaacaggcg
 361 caggccgagc tggtgcgggc gcgcaatcag gccgctctgg cgcgcagcga atcctcgcgc
 421 accgagaaac tgatcggcac tcaggccatc tcgcaagagg tgtgggaaca gcgccgttcg
 481 tccgccgcgc aggcgcaaag caacgtgctg gcggcgcagg cccagctcga tatggcgcag
 541 cttaatctcg actttacccg cgtcaccgcc ccgatcgacg ggcgcgccag ccgcgcgatg
 601 atcaccgccg gcaacctggt caccgccggc gacagcgcca gcgtgctgac gacgctggtg
 661 tcgctcgaca aggtctacgt ctatttcgac gtggatgaag ccaccttcct gcgttatcag
 721 cagcaaggcc ggcacgatgc ccgcctgccg gtgaaagtcg gcctggtggg cgaagacggc
 781 actccgcatc aagggctggt ggacttcact gataaccaac tgaacgccgg gaccggcacc
 841 atccgcatgc gcgccctgct cgacaaccgc gatcgccgct tcacgccggg gctgttcgct
 901 cgggtacaga tgcccggcag cgccgagttc aacgccatgc tgatcgacga caaagcggtg
 961 atgaccgatc agaaccgcaa gttcgtttac atcgtcgata aagacggcaa ggcgcagcgc
1021 cgcgacatcg acgtgggccg gatggccgaa ggattgcgca ttgtgcaaaa aggcttggca
1081 aacggcgatc gggtgatcgt cgacggcatg cagaaagtgt ttatgccggg catgccggtc
1141 gacgcgaaaa gcgtcgccat gaccactacc gcttccgcac ttaactaagc tcccagccga
                                    sdeB
1201 gacttctgac ccatggactt ttcccgtttt ttcatcgacc ggccgatctt tgccgcggtg
1261 ctgtcgatac tgatttttgt cgccggggtg atcgccatcc cgctgctgcc gatcagcgag
1321 tacccggacg tggtgccgcc cagcgtgcag gtgcgcgcag aatacccggg cgccaacccg
1381 aaagagatcg ctgaaacggt ggccacgccg ctggaagaag cgatcaacgg cgtcgaaaac
1441 atgatgtaca tgaaatcggt cgccggttcc gacggcgtgc tggtgaccac cgtcaccttc
1501 cgccccggca ccgatcccga tcaggcgcag gtgcaggtgc aaaaccgcgt ggcgcaggcc
1561 gaagcgcgcc tgccggaaga cgtgcgccgc cagggcatca ctacccagaa acagtctccg
1621 gcgctgacgc tggtggtgca tctggtgtcg ccttccggca agtacgactc gctgtacctg
1681 cgcaactacg ccaccctgaa ggtcaaggat gaactggctc gcctgccggg cgtcggccag
1741 gtgcagatct tcggcgccgg tgaatacgcg atgcgcatct ggctcgatcc gaacaaggtc
1801 gccgcgcgcg ggctgaccgc ctccgacgtg gtgagcgcca tgcaagagca aaacgtgcag
1861 gtctccgccg gccagctcgg cgcggagccg atgccgacgc gcagcgacta tctgctgtcg
1921 atcaacgccc agggccgcct gcaaaccgaa gaagagttcg gcaatatcat cctcaaaagc
1981 ggcgataacg gcgagatcgt gcggctgcgc gacgtggcgc gcatcgagat gggttccggc
2041 agctacgcgc tgcgcgccca gctcaataac aaggatgcgg tcggcatcgg catcttccag
2101 tcgccgggcg ccaacgccat cgagctgtcg gacgcggtgc gcggcaagat ggccgagctg
```

2161 gcgacccgct tcccggatgg catgagctgg aaatctccgt acgatccgac ggttttcgtg
2221 cgcgattcga tccgcgcggt ggtggatacc ctgctggaag cggtgatcct ggtggtgctg
2281 gtggttattc tgttcctgca gacctggcgc gcctcgatca ttccgctgtt ggcggtgccg
2341 atctccgtgg tcggcacctt cgccgcgctg tatctgctgg gcttctcgct caacaccctc
2401 agtctgttcg ggctggtgct ggcgatcggc atcgtggtgg atgacgccat cgtggtggtg
2461 gagaacgtcg agcgcaacat cgaagagggg ctgtcgccgc tggcggcggc gcaccaggcg
2521 atgcgcgagg tctccggccc gatcatcgcc atcgccgtgg tgctgtgcgc ggtgttcgta
2581 ccgatggcgt tcctttccgg cgtcaccggc cagttctaca agcagttcgc cgtcaccatc
2641 gccatctcga cggtgatctc cgccatcaac tcgctgaacc tgtcgccggc gctggcggcg
2701 cgcttgctga aaccgcacgg cgcgccgaaa gatatgccgt cgcgcctgat cgacaggctg
2761 ttcggttggc tgttccggcc gttcaaccgc ttcttcgcca gcggttcgca acgctaccag
2821 cgcggcgtct cccgcgtgct gggccggcgc ggcgcggtgt tcatcgtcta tctgctgctg
2881 ctggccgccg ccggggtgat gtttaagacc gtgcccggcg gctttatccc gacgcaggac
2941 aagctgtatc tgattggcgg cgtgaagatg ccggaaggcg cctcgctgga gcgcaccgat
3001 gcggtgatcc gcaagatgag cgccatcggt atgagcgtgg acggcgtcac cgatgcggtg
3061 gccttccccg gcctgaacgc gctgcagttc accaatacgc cgaataccgg cacggtgttc
3121 ttcgcgctgg agtcattgag cacccgtacc cgcaccgcgg cgcagatcaa cgccgagatc
3181 aacgcgcgca tttcgcagat ccaggaaggt ttcgccttct cgatcatgcc gccgccgatc
3241 ctcggcatcg gccagggttc cggctattcg ctgtacgtgc aggatcgcgg cgggctgggt
3301 tacggcgcgc tgcagaccgc gatcaacacg atgtccggcg ccattatgca aacgccaggc
3361 atggggttcc cgatctcctc ttatcaggcc aacgtgggcc aacgtgacgc caaaatcgat
3421 cgcgacaagg ccaaggccca gggggtgccg ctcaatgcgc tgttcagcac gctgcagacc
3481 tacctcggtt cgtcttatat caacgacttc aaccgctatg gccgcacctg gaaggtgatg
3541 gcgcaggccg acgcccagtt ccgcgacagc gtggaagaca tcgccaacct gcgtacccgc
3601 aacgacaagg gcgaaatggt gccgatcggc agcatggtca gcataggcac cacttacggc
3661 ccggatccgg tgatccgtta taacggcttc ccggcggcgg atctgatcgg cgacgccgat
3721 ccgcgcgtgc tctcctctac ccaggcgatg ggtgcgctga cgcagatggc cgataagctg
3781 ttgccgaacg gcatgaacat ccagtggacc gatctgagct atcagcaatc gacccagggc
3841 aacgccgcgc tggtggtctt cccggtggca gtgctgctgg cgttcctcgc gctggcggcg
3901 ctgtatgaaa gctggacgct gccgctggcg gtgatcctga tcgtgccgat gaccatgctg
3961 tcggcgctgt tcggcgtgtg gctgaccggc ggcgacaaca acgtgttcgt gcaggtcggg
4021 ctggtcgttc tgatggggct ggcctgcaag aacgccattc tgatcgtcga gttcgcccgc
4081 gagctggaaa tgcagggcaa aggcatcgtc gaggcggcgc tggaggcctg tcgcctgcgg
4141 ttgcgcccga tcgtgatgac ctccatcgcc ttcatcgccg gcaccatccc gctgatcctc
4201 ggccacggcg ccggcgccga ggtgcgcggc gtcaccggca tcacggtgtt ctccggcatg
4261 ctgggggtca ccctgttcgg gctgttcctg acgccggtgt tctacgtcac cctgcggcgc
4321 ctggtggcgc gcaaagcgca gccgcagacg gcgtaa

Fig. 4.3b. Predicted amino acid sequences of *S. marcescens* SdeA and SdeB proteins. Sequences were derived using OMIGA 2.0 software. Conserved amino acid residues from MFP (Dinh *et al*, 1994) and RND (Tseng *et al*, 1999) families are shown in bold and are underlined. Transmembrane spanner predictions for SdeB were performed using the PSIPRED protein structure prediction server (McGuffin *et al*, 2000) and are shown as overlined residues.

Fig. 4.3b.

SdeA

```
MANQPNSSFN AGGRTRAVAL GQRLSGVALL AALLAGCDNS VAHNAPPPPP VVSAASVVVK  60
PISQWDAFNG RVEAVQSVQL RPRVSGYIER VNYTEGDEVK KGQVLFTIDD RTYRAAREQA  120
QAELVRARNQ AALARSESSR TEKLIGTQAI SQEVWEQRRS SAAQAQSNVL AAQAQLDMAQ  180
LNLDFTRVTA PIDGRASRAM ITAGNLVTAG DSASVLTTLV SLDKVYVYFD VDEATFLRYQ  240
QQGRHDARLP VKVGLVGEDG TPHQGLVDFT DNQLNAGTGT IRMRALLDNR DRRFTPGLFA  300
RVQMPGSAEF NAMLIDDKAV MTDQNRKFVY IVDKDGKAQR RDIDVGRMAE GLRIVQKGLA  360
NGDRVIVDGM QKVFMPGMPV DAKSVAMTTT ASALN                              395
```

SdeB

```
MDFSRFFIDR PIFAAVLSIL IFVAGVIAIP LLPISEYPDV VPPSVQVRAE YPGANPKEIA  60
ETVATPLEEA INGVENMMYM KSVAGSDGVL VTTVTFRPGT DPDQAQVQVQ NRVAQAEARL  120
PEDVRRQGIT TQKQSPALTL VVHLVSPSGK YDSLYLRNYA TLKVKDELAR LPGVGQVQIF  180
GAGEYAMRIW LDPNKVAARG LTASDVVSAM QEQNVQVSAG QLGAEPMPTR SDYLLSINAQ  240
GRLQTEEEFG NIILKSGDNG EIVRLRDVAR IEMGSGSYAL RAQLNNKDAV GIGIFQSPGA  300
NAIELSDAVR GKMAELATRF PDGMSWKSPY DPTVFVRDSI RAVVDTLLEA VILVVLVVIL  360
FLQTWRASII PLLAVPISVV GTFAALYLLG FSLNTLSLFG LVLAIGIVVD DAIVVVENVE  420
RNIEEGLSPL AAAHQAMREV SGPIIAIAVV LCAVFVPMAF LSGVTGQFYK QFAVTIAIST  480
VISAINSLNL SPALAARLLK PHGAPKDMPS RLIDRLFGWL FRPFNRFFAS GSQRYQRGVS  540
RVLGRRGAVF IVYLLLLAAA GVMFKTVPGG FIPTQDKLYL IGGVKMPEGA SLERTDAVIR  600
KMSAIGMSVD GVTDAVAFPG LNALQFTNTP NTGTVFFALE SLSTRTRTAA QINAEINARI  660
SQIQEGFAFS IMPPPILGIG QGSGYSLYVQ DRGGLGYGAL QTAINTMSGA IMQTPGMGFP  720
ISSYQANVGQ RDAKIDRDKA KAQGVPLNAL FSTLQTYLGS SYINDFNRYG RTWKVMAQAD  780
AQFRDSVEDI ANLRTRNDKG EMVPIGSMVS IGTTYGPDPV IRYNGFPAAD LIGDADPRVL  840
SSTQAMGALT QMADKLLPNG MNIQWTDLSY QQSTQGNAAL VVFPVAVLLA FLALAALYES  900
WTLPLAVILI VPMTMLSALF GVWLTGGDNN VFVQVGLVVL MGLACKNAIL IVEFARELEM  960
QGKGIVEAAL EACRLRLRPI VMTSIAFIAG TIPLILGHGA GAEVRGVTGI TVFSGMLGVT  1020
LFGLFLTPVF YVTLRRLVAR KAQPQTAAA                                     1049
```

Fig. 4.4. von Heijne transmembrane helices in *S. marcescens* SdeB. Transmembrane helices were plotted using OMIGA 2.0 software, plotting hydrophobicity as positive and hydrophilicity as negative. Each predicted transmembrane helix is numbered on the plot.

Fig. 4.4.

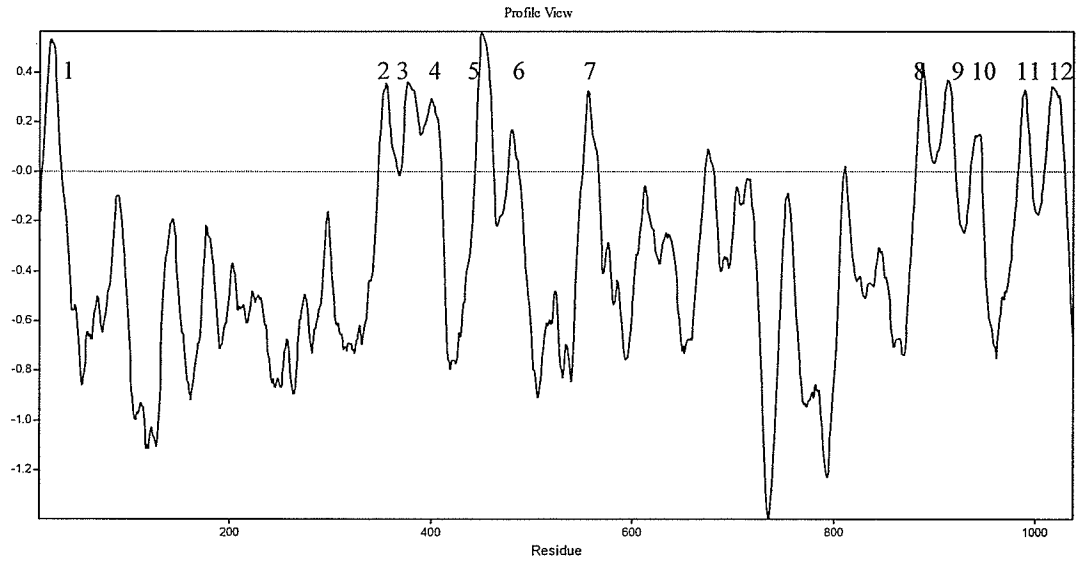
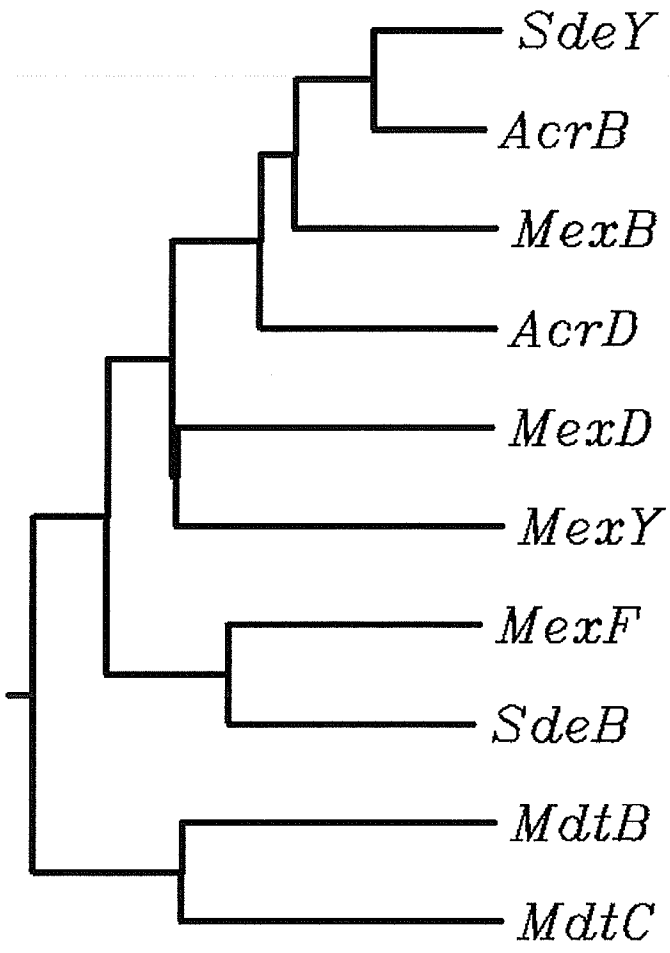

Profile View
1
2 3 4
5
6
7
8
9 10
11 12
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
200
400
600
800
1000
Residue

Fig. 4.5. Rooted distance tree based on alignement of SdeB protein of *S. marcescens* with RND pump proteins of *E. coli* and *P. aeruginosa*. The phylogenetic tree for SdeB and different RND pumps of *E. coli* (AcrB, AcrD, MdtC, and MdtD), *P. aeruginosa* (MexB, MexD, MexF, and MexY), and SdeY of *S. marcescens* (Chen *et al*, 2003) was prepared using the online server of Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

Fig. 4.5.



4.4. Comparison of expression levels of *sdeB* in the clinical isolate and mutant *S. marcescens* strains

Expression of *sdeB* was measured in strains that were found to efflux fluoroquinolones namely, the clinical isolate T-861, and the mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO. The wild-type strain UOC-67 was used as the control. Northern blotting results revealed over-expression of the *sdeB* gene in mutant strains UOC-67WL (2-fold) and UOC-67WLN (3-fold), as compared to wild-type UOC-67 (Fig. 4.6). The clinical isolate T-861 was also found to over-express *sdeB* to some extent (1.5-fold). No difference was seen in the expression levels of *sdeB* in UOC-67 and UOC-67WLO.

4.5. Three-dimensional structure prediction of SdeB protein

The amino acid sequence of *sdeAB* gene products was deduced using OMIGA 2.0 software. The SdeA protein was found to consist of 395 amino acid residues with a predicted molecular weight of 42.8 kDa, while SdeB consists of 1047 amino acid residues with a predicted molecular weight of 112.5 kDa. Three-dimensional structure prediction of the SdeB protein was performed using the CDD software of NCBI, revealing a very similar structure to that of the AcrB protein of *E. coli* (Fig. 4.7).

4.6. Antibiotic susceptibility of *E. coli* AG102MB expressing the SdeAB pump

The plasmid pUCAB containing *sdeAB* genes was transformed into the AcrB-deficient strain of *E. coli*, AG102MB, and the antibiotic resistance profile compared with the parent strain without the plasmid. The results are summarized in the Table 4.1.

Fig. 4.6. Northern blot analysis of various *S. marcescens* strains for expression of *sdeB* gene. Expression levels of *sdeB* was measured in *S. marcescens* UOC-67 (1), T-861 (2), UOC-67WL (3), UOC-67WLN (4), and UOC-67WLO (5). Total RNA (1μg) was spotted and hybridization was performed using DIG-labeled mRNA probe for *sdeB* gene. Intensity of each spot was measured using FluorChem 2.0.

Fig. 4.6.

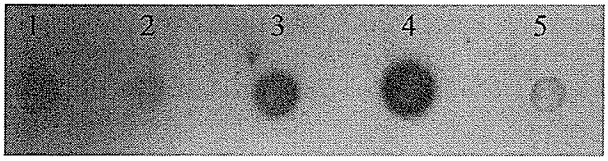


Fig. 4.7. Model of the three-dimensional structure of the *S. marcescens* SdeB protein using CDD software, revealing similiarity to *E. coli* AcrB. Residues shown in red are similar/identical to those in AcrB, while those shown in pink are different from AcrB.

Fig. 4.7.

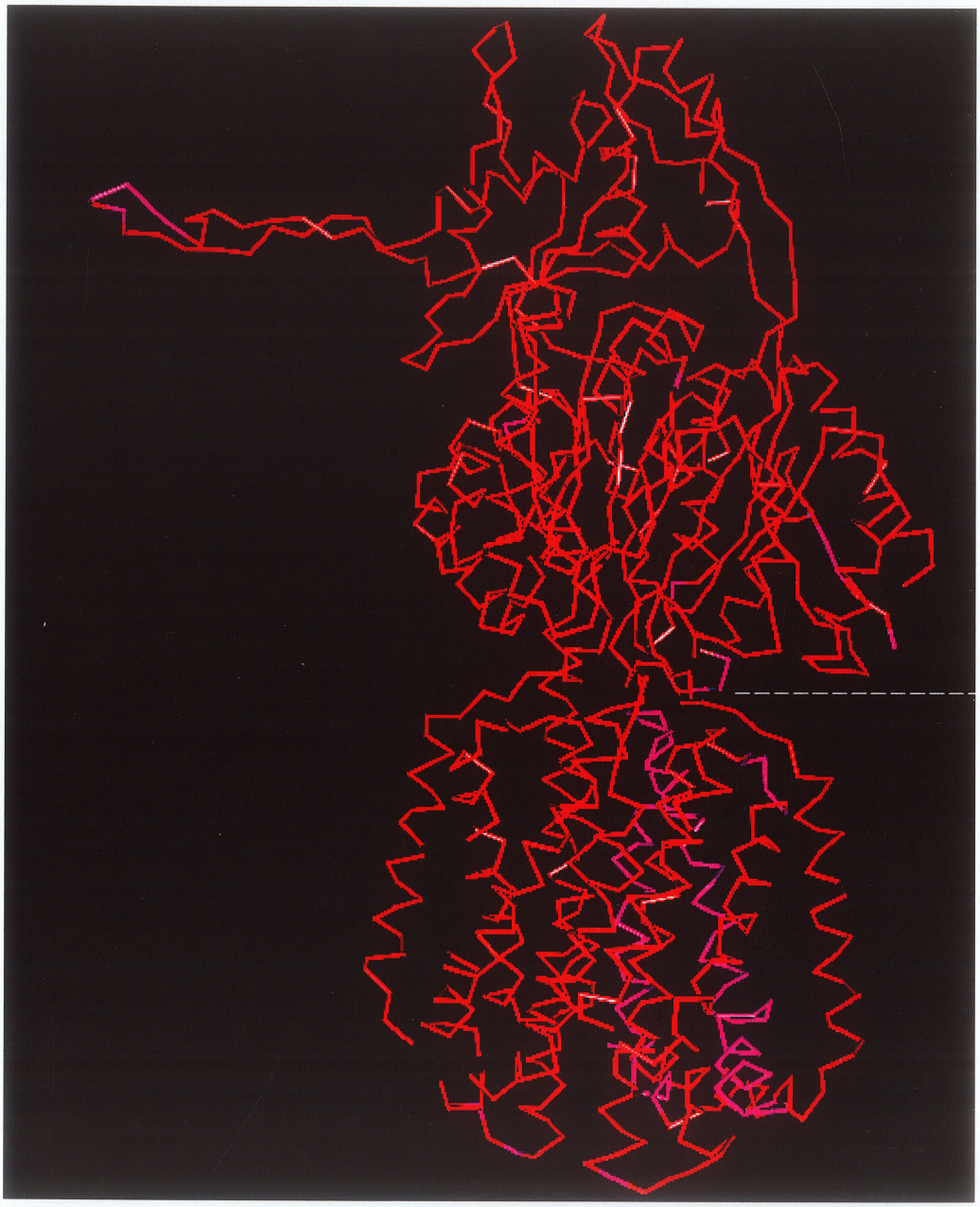


Table 4.1. Antibiotic susceptibility of *E. coli* AG102MB expressing the *S. marcescens* SdeAB efflux pump.

	Relative MIC[a]						
Strain	**Cip**	**Nor**	**Ofx**	**Chl**	**Nov**	**SDS**	**EtBr**
AG102MB	1	1	1	1	1	1	1
AG102MB/pUC18	1	1	1	1	1	1	1
AG102MB/pUCAB	>64	16	16	>64	1	8	64

Cip, Ciprofloxacin; Nor, norfloxacin; Ofx, ofloxacin; Chl, chloramphenicol; Nov, novobiocin; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide.

pUCAB contains the *sdeAB* locus as cloned in the cloning vector pUC18.

[a]relative MIC is the MIC value obtained for the test strain divided by the MIC value of *E. coli* AG102MB

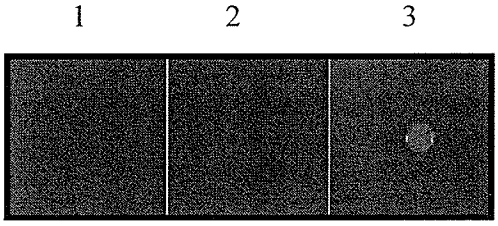
Introduction of the *sdeAB* genes to *E. coli* AG102MB resulted in a >64-fold increase in resistance to ciprofloxacin and chloramphenicol, and a 16-fold increase in resistance to norfloxacin and ofloxacin. Resistance to SDS increased by 8-fold and that to ethidium bromide increased by 64-fold. These results prove that SdeAB pump of *S. marcescens* is responsible for efflux of ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol, SDS, and ethidium bromide.

4.7. n-hexane tolerance of *E. coli* AG102MB expressing SdeAB pump

E. coli AG102MB was found to be resistant to n-hexane only when it was supplemented with pUCAB containing the *S. marcescens sdeAB* locus. The host strain was unable to grow on n-hexane plates by itself or when supplemented with the cloning vector pUC18 (Fig. 4.8).

Fig. 4.8. Organic solvent (n-hexane) tolerance of various *E. coli* AG102MB strains. *E. coli* AG102MB (1) and *E. coli* AG102MB expressing *S. marcescens* SdeAB (3) efflux pump were tested for tolerance to n-hexane. *E. coli* AG102MB:pUC18 (2) was used as control. *E. coli* AG102MB is the AcrB deletion strain (Elkins & Nikaido, 2002), *E. coli* AG102MB:pUCAB is expressing the *S. marcescens* SdeAB efflux pump. Five microliters of equilibrated (0.5 MacFarland standard) mid-logarithmic-phase culture (10^8 cfu/ml) of each strain was spotted on LB-agar and allowed to dry. The plate was flooded with n-hexane to a depth of 2-3 mm, sealed, and incubated at 37°C for 16-20 hrs. Growth at spots indicates resistance to n-hexane.

Fig. 4.8.



4.8. Discussion

Once active efflux was identified as a resistance mechanism in *S. marcescens*, the next aim was to identify the efflux pump(s) in this organism. Since RND pumps are most prevalent in Gram-negative organisms, primers were designed for conserved motifs in RND proteins to identify such proteins. PCR from the *S. marcescens* UOC-67 genome resulted in a product similar (~60%) to the *mexF* gene of *P. aeruginosa*. Following this, a cosmid genomic bank of *S. marcescens* UOC-67 was constructed in an effort to identify and clone the entire operon containing genes for the MFP component, RND pump, as well as the outer membrane component, if present. Screening of the cosmid bank was performed using the 1.7 kb *mexF*-like fragment as a probe.

One cosmid clone containing the *mexF*-like fragment was identified. Subcloning and subsequent sequencing of the complete locus revealed the presence of MFP- and RND pump-encoding genes, based on the presence of conserved amino acid residues (Fig. 4.3b). There was no gene found for the outer-membrane component. This operon was named *sdeAB*, with *sdeA* being the gene for the MFP, and *sdeB* the gene for the RND pump. Three-dimensional structure prediction and transmembrane spanner plots further established *sdeB* as a gene encoding an RND pump (Figs. 4.4 and 4.7). Phylogenetic analysis of SdeB with different RND pumps from *E. coli* and *P. aeruginosa* revealed that SdeB was indeed more closely related to MexF of *P. aeruginosa* than any of the *E. coli* pumps (Fig. 4.5).

Northern blot analysis was performed to compare the transcript levels of *sdeB* gene in different isolates of *S. marcescens* found to be effluxing fluoroquinolones. Results showed UOC-67WL and UOC-67WLN were over-expressing *sdeB* when

compared to the wild-type strain, UOC-67 (Fig. 4.6). Incidently, these two strains had very similar patterns of accumulation for fluoroquinolones, with efflux of ciprofloxacin and norfloxacin, and to some extent ofloxacin. UOC-67WL and UOC-67WLN were isolated by serial passaging of UOC-67 in media supplemented with ciprofloxacin and norfloxacin, respectively. This suggests that the presence of either ciprofloxacin or norfloxacin can lead to up-regulation of *sdeB*. UOC-67WLO, which was isolated from media supplemented with ofloxacin, had expression levels of *sdeB* were similar to that for the wild-type UOC-67, indicating that ofloxacin is not capable of up-regulating this particular pump. MIC data for UOC-67WL and UOC-67WLN, however suggests that, even though ofloxacin cannot up-regulate the SdeB pump, it is definitely a substrate for this pump (Table 4.2). There was only a minimal increase in the expression of *sdeB* in the clinical isolate T-861 in comparison to UOC-67. However, the accumulation data of T-861 was very similar to that of UOC-67WL and UOC-67WLN (Figs. 4.2e, 4.3a, and 4.3b), suggesting that there might be an additional pump in this isolate that could be effluxing fluoroquinolones in a proton gradient-dependent manner.

An AcrB-deficient mutant, *E. coli* AG102MB, was complemented with *sdeAB* to study the function of SdeAB pump. Upon introduction of the SdeAB pump, *E. coli* AG102MB showed a remarkable increase in the resistance to ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol, SDS, ethidium bromide, and n-hexane (Table 4.1 and Fig. 4.8). This data also further establishes the fact that ofloxacin is a substrate for SdeAB pump as suggested above.

In summary, results presented in this chapter identify SdeAB as an efflux pump in *S. marcescens* with a very broad substrate specificity, being able to pump out a variety of

substrates including fluoroquinolones, chloramphenicol, detergents, dyes, and organic solvents.

FIVE

Molecular Characterization of the *Serratia marcescens* SdeCDE RND Efflux Pump

Vision: the art of seeing things invisible.
-Jonathan Swift

5.1. Abstract

The partial sequence of a *yegO*-like RND pump gene was identified in *S. marcescens* by PCR, using primers for two conserved motifs of RND proteins. To clone, sequence, and characterize the complete gene sequence, the genomic bank of *S. marcescens* was screened by Southern blot analysis. A ~5 kb *Nco*I fragment from the cosmid clone was subcloned in pUC18 cloning vector and sequenced.

Sequencing of the subclone revealed the presence of an MFP- and a RND pump-encoding gene in an operon. The locus was named *sdeCD*. This clone was introduced in the AcrB-deficient strain of *E. coli* and susceptibility to various antimicrobials was measured. No change was observed in the susceptibility of the *E. coli* strain upon introduction of the *sdeCD* locus. Further analysis of the region downstream of the *sdeD* gene revealed presence of another RND pump gene and called *sdeE*. A 12 kb *Eco*RI/*Kpn*I fragment from the cosmid clone was subcloned in pUC18 and the entire *sdeCDE* locus introduced in the *E. coli* AcrB-mutant. However, there was still no change in the antimicrobial susceptibility of the *E. coli* strain

Northern blot analysis showed minimal expression of the *sdeD* gene in *S. marcescens* strains that were resistant to fluoroquinolone. Results indicate that the *sdeCDE* locus is a multimeric RND pump-encoding locus; however, it is not responsible for resistance to fluoroquinolones, detergent, and organic solvents and that fluoroquinolones cannot upregulate the expression of this locus.

5.2. Identification of the *sdeCDE* locus

Close analysis of the 1.7 kb PCR product described in the previous section revealed that there was another product besides the *sdeB* gene fragment. Sequencing of

this PCR product showed that it was homologous (~60%) to the *mdtB* gene of *E. coli* and contained RND protein signature sequences (Fig. 5.1). This partial gene fragment was named as *sdeD* and cloned into the cloning vector pDrive and a DIG-labeled probe synthesized.

5.3. Cloning of *sdeCDE* locus

The genomic bank of *S. marcescens* was screened for the presence of *sdeD* gene using the DIG-labeled probe described above. Two different cosmid clones were identified, pRKC51 (containing a ~40 kb genomic insert) (Fig. 5.2), and pRKH10 (containing a 15 kb insert) that harbored the *sdeD* gene. A 5 kb *Nco*I pRKC51-derived fragment was cloned in the vector pUC18 to construct the plasmid pUCCD. Sequencing of the insert revealed the presence of 2 ORFs of 1350 bp and 3108 bp, respectively. The 1350 bp ORF, named *sdeC*, was identified as an MFP encoding gene, and the 3108 bp ORF, named *sdeD*, was identified as a RND-pump encoding gene. However, analysis of the partial sequence downstream of the *sdeD* gene revealed possible presence of another ORF homologous to the RND pump-encoding genes. To clone this entire locus with all 3 ORFs, a 12 kb *Eco*RI/*Kpn*I fragment was subcloned from the cosmid clone pRKC51 into pUC18 to construct the plasmid pUCCDE. Sequencing of the third ORF downstream of the *sdeD* gene did indeed reveal the presence of another RND pump-encoding gene of 3063 bp which was named *sdeE* (Fig. 5.3a). The sequence of the entire *sdeCDE* locus has been submitted to GenBank under the Accession No. AY168757.

Fig. 5.1. Alignment of the predicted amino acid sequence of portions of the 1.7-kb *S. marcescens sdeD* fragment and known RND efflux pumps of *E. coli* (AcrB, AcrF, and MdtC) and *P. aeruginosa* (MexB, MexD, MexY, and MexF). Alignment was performed using OMIGA 2.0 software. Amino acid residues shaded in gray represent the conserved amino acid residues in RND family pumps.

Fig. 5.1.

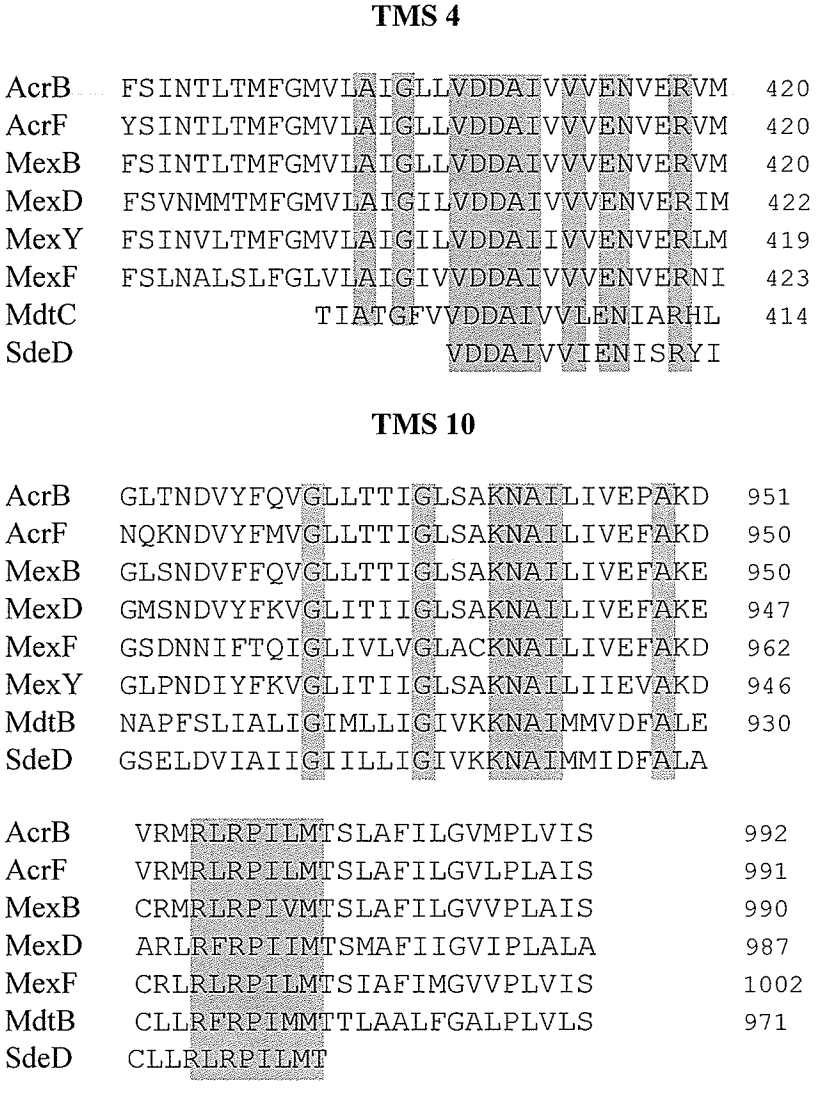

TMS 4
AcrB FSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
AcrF YSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
MexB FSINTLTMFGMVLAIGLLVDDAIVVVENVERVM 420
MexD FSVNMMTMFGMVLAIGILVDDAIVVVENVERIM 422
MexY FSINVLTMFGMVLAIGILVDDAIIVVENVERLM 419
MexF FSLNALSLFGLVLAIGIVVDDAIVVVENVERNI 423
MdtC TIATGFVVDDAIVVLENIARHL 414
SdeD VDDAIVVIENISRYI
TMS 10
AcrB GLTNDVYFQVGLLTTIGLSAKNAILIVEPAKD 951
AcrF NQKNDVYFMVGLLTTIGLSAKNAILIVEFAKD 950
MexB GLSNDVFFQVGLLTTIGLSAKNAILIVEFAKE 950
MexD GMSNDVYFKVGLITIIGLSAKNAILIVEFAKE 947
MexF GSDNNIFTQIGLIVLVGLACKNAILIVEFAKD 962
MexY GLPNDIYFKVGLITIIGLSAKNAILIIEVAKD 946
MdtB NAPFSLIALIGIMLLIGIVKKNAIMMVDFALE 930
SdeD GSELDVIAIIGIILLIGIVKKNAIMMIDFALA
AcrB VRMRLRPILMTSLAFILGVMPLVIS 992
AcrF VRMRLRPILMTSLAFILGVLPLAIS 991
MexB CRMRLRPIVMTSLAFILGVVPLAIS 990
MexD ARLRFRPIIMTSMAFIIGVIPLALA 987
MexF CRLRLRPILMTSIAFIMGVVPLVIS 1002
MdtB CLLRFRPIMMTTLAALFGALPLVLS 971
SdeD CLLRLRPILMT

Fig. 5.2. Southern blot detection of the *S. marcescens sdeD* gene in the cosmid clone pRKC51 after restriction-enzyme digestion, Lane 1, *Bam*HI; Lane 2, *Eco*RI; Lane 3, *Eco*RV; Lane 4, *Hind*III; Lane 5, *Kpn*I; Lane 6, *Sma*I. Numbers indicate molecular weights of *Hind*III-digested λ-DNA fragments.

Fig. 5.2.

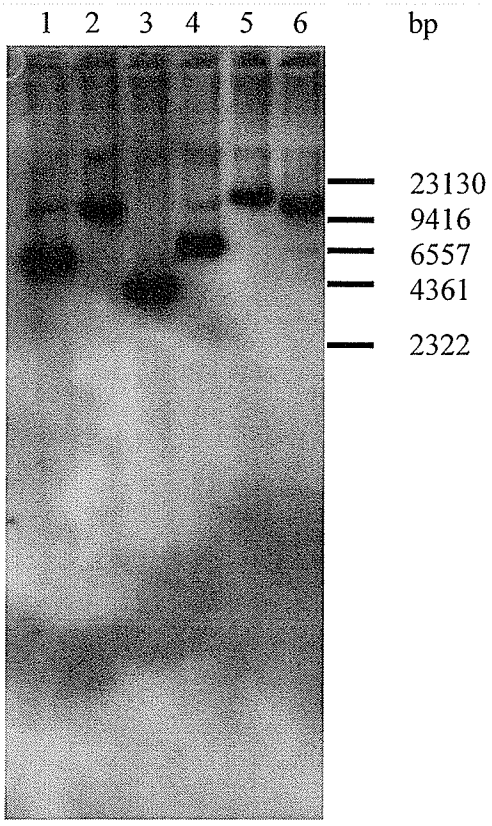


Fig. 5.3a. Nucleotide sequence of the *sdeCDE* locus of *S. marcescens*. Initiation codons for *sdeC*, *sdeD*, and *sdeE* are shown in bold. Shaded nucleotides represent *sdeC, sdeD,* and *sdeE* open reading frames. No obvious -10, -35, or ribosome binding sites were found in the upstream region of *sdeCDE* locus.

Fig. 5.3a.

```
1    agttgtttct tgctcatcac aaaggctcca tgggttaaat gatttgcctg ttaattatta
61   gcgccaaata caggaaatcg ttaaaacgta ttggctataa gccggaataa atcacttata
121  aacatagcac gcgcaaatca cccacttatt aagacaatgc tgacggttcc tccatatttc
                    sdeC
181  cttcattttt atgccgtctt cgctggctaa actagtatcc tgtaaggagt gtcatttttg
241  tggcaatgag tgcccgcgcc cctgcctttt ggagagaatt ttcaccacca tgaatgcaaa
301  accccaacgt cgttccctgc tgctgcgcct gctggttgcg gcgattgcga tcatcgtcgc
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481  gatgtcgccg gtgcaagcgg cgaccgccac gcaacagacg gtgccacgct atctttccgg
541  cttgggcacc gccaccgccg ccaataccgt gaccgtcacc agccgggtcg atggccaact
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661  cgatccccgg ccattccagg ttcagttgac ccaggctcag gggcagttgg caaaagatca
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841  gatcaaggcc gatcagggcg cggtcgacag cgccaaactg cagatcacct acagccgcat
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1381 ccggcaggtg gtgatcagcg ccggcctcaa cgccggcgat cgggtggtga ccgacggcat
1441 cgatcgcctg accgaaggca tgcaggtgga agtgctcgcg ccgagcgaag cgccggcggc
                                                    sdeD
1501 ggccaacgcc aagcgcgaac ctgacgttca gaggaagtcc tgatgcaggt gatgcctccc
1561 aacgccggcg gcggtccgtc ccgcctgttt attctgcgcc cggtcgccac cactctgctg
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1741 gtggtgacct ccgccattac cgcgccgctg gaacgccagt tcggccagat gtccggcctg
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```

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6781 acgatcgcga aaccatggcg cgcctcggca tttcggtgtc cgacgccaac aacctgttga
6841 acaacgcctt cggccagcga cagatttcga ccatctatca accgctcaac caatacaaag
6901 tcgtgatgga agtggcgccg ccctacactc aggacgtgag ctcgttggac aaaatgttca
6961 tcatcaacaa cgaaggcaag gcgataccgc tctcctactt cgccagttgg cgaccggcca
7021 acgcgccgct gtcggtaaac catcaggggc tgtccgccgc ctcgaccatc tcctttaacc
7081 tgccggacgg cggcagcctg tcggacgcca ccgcggcggt agagcgcacc atgactcagc
7141 tcggcgtgcc gtccacggtg cgcggcgcgt tcgccggcac cgcccaggta ttccaggaca
7201 ccctgaagtc gcagctcatc ctgatcctgg cagcgatcgc cacggtgtat atcgtgctcg
7261 gcgtgctgta tgagagctat atccatccgc tgaccatcct ttccacgctg ccttccgccg
7321 gcgtgggggc gctgctggcg ctcgagctgt tcggcgcacc gttcagcctg atcgcgctga
7381 tcggcatcat gctgttgatc ggtatcgtga agaaaaatgc catcatgatg gtcgacttcg
7441 cgctcgaagc gcagcgcaat ggcggcatca gcgcccgcga ggcgattttc caggccagcc
7501 tgctgcgttt ccggccgatc atgatgacca cgctggcggc gttgttcggc gcgctgccgc
7561 tggtgctgac ccgcggcgac ggcgcggagc tgcgccagcc gctcggcatc accatcgccg
7621 gcggcctggt gatgagccag ctgctgacgc tgtacaccac cccggtggtc taccttttact
7681 tcgaccggct gcaggcgaag ttccgccgca acaagcaact ggccccactg ccccattaat
7741 ggccgaccga tgctgatgaa acacaccgcc acggtacgct ggcaactctg gatagtggca
7801 ttcggcttct ttatgcagac gctggatacc actatcgtca acactgccct
```

Fig. 5.3b. Predicted amino acid sequences of *S. marcescens* SdeC, SdeD, and SdeE proteins. Sequences were derived using OMIGA 2.0 software. Conserved amino acid residues from MFP (Dinh *et al*, 1994) and RND (Tseng *et al*, 1999) families are shown in bold underlined letters. Transmembrane spanner predictions for SdeD and SdeD were performed using the PSIPRED protein structure prediction server (McGuffin *et al*, 2000) and are shown as overlined residues.

Fig. 5.3b

SdeC

```
MPSSLAKLVS CKECHFCGNE CPRPCLLERI FTTMNAKPQR RSLLLRLLVA AIAIIVAVLA   60
WRHFSAAQPT AGTTPGAHQA AGKTGAGRAA GGRRGAPMSP VQAATATQQT VPRYLSGLGT  120
ATAANTVTVT SRVDGQLMAI HFTEGQQVKA GDLLAEIDPR PFQVQLTQAQ GQLAKDQATL  180
ANARRDLARY QQLVKTNLVS RQELDTQASL VQQTEGAIKA DQGAVDSAKL QITYSRITAP  240
IDGRVGLKLV DVGNYVTSGS TTGLVVITQT HPIDVVFTLP EGNIADLLKA QKAGPVSVEA  300
WDRTNQNKLT TGSLLSLDNQ IDTATGTIKL KARFANEDDA LFPNQFVNAR LQVDTLHDAV  360
VIPTAALQMG NEGNFVWTLG EDNKVSKHRV TAGVQDSRQV VISAGLNAGD RVVTDGIDRL  420
TEGMQVEVLA PSEAPAAANA KREPDVQRKS                                   450
```

SdeD

```
MPPNAGGGPS RLFILRPVAT TLLMVAILLA GIIGYRALPV SALPEVDYPT IQVVTLYPGA   60
SPDVVTSAI TAPLERQFGQ MSGLKQMASQ SAGGASVVTL QFQLALPLDV AEQEVQAAINA  120
ATNLLPNDL PYPPIYSKVN PADPPILTLA VTSTAMPMTQ VEDMVETRVA QKISQVTGVGL  180
VTLAGGQRPA VRVKLNAAAV AAYGLDSETI RAAISNANVN SAKGSLDGPT RSVTLSANDQ  240
MKSADDYRQL IVAYQNGAAI RLQDIATIEQ GAENTRLAAW ANQQQAIVLN IQRQPGVNVI  300
TTADSIREML PTLIKSLPKS VDVKVLTDRT TTIRASVSDV QFELLLAVAL VVMVIYVFLR  360
NVPATIIPSV AVPLSLVGTF AAMYFLRFSI NNLTLMALTI ATGFVVDDAI VVIENISRYI  420
EKGEKPLDAA LKGAGEIGFT TISLTFSLVA VLIPLLFMGD IVGRLFREFA VTLAVAILIS  480
AVVSLTLTPM MCARMLSHES LRKQNRFSAA SERFFERVIA RYGRWLKTVL NHPWLTLGVA  540
VGTLALTVLL YLLIPKGFFP VQDNGIIQGT LEALQSVSFS NMAERQQQVA AQILKDPAVE  600
SLTSFVCVDG SNATLNSGRL QINLKPLSER SDRIPAIISR LQQQTAQFPG VKLYLQPVQD  660
LTIDTQVSRT QYQFTLQAMS LDDLSLWVPQ LMDELKQTPQ LADVTSDWQD RGLVAYVNVD  720
RDSASRLGVT MSDADNALYN AFGQRLISTI YTQANQYRVV LEHDVSATPG LAALNEIRLS  780
GNDGAVVPLS AIAKIEERFG LPPRRHHEHF PSATVSFNVA DGYSLGEAVD AVTLAEKNLN  840
MPRDITTQFQ GATLAFQAAL GSTLWLILAA VVAMYIVLGV LYESFIHPVT ILSTLPTAGV  900
GALLALMMAG SELDVIAIIG IILLIGIVKK NAIMMIDFAL AAEREQGLSP RDAICQACLL  960
RLRPILMTTL AALLGALPLM LSTGVGAELR HPLGVCMVGG LIMSQILTLF TTPVIYLLFD 1020
KLARNTHRQL DTQELPPP                                               1038
```

SdeE

```
MFIHRPVATT LLTLAIAISG AIGFRLLPVS PLPQVDFPVI SISASLPGAS PETMASSVAT   60
PLERALGRIA GVNEMTSMSS LGSTRVILQF DLDRDINGAA RDVQAAINAA QSLLPTGMPS  120
RPSYRKVNPS DAPIMILTLT SDTYSQGQLY DFASTQLAQK ISQTEGVGDV SVGGSSLPAV  180
RVELNPSALF NQGVSLDSVR QAIANANVRR PQGAVENPQQ RWQIQANDAL KTADAYRPLI  240
IHYNNGSAVR LADVAEVKDS VQDVRNAGMT DAKPAIILAI SRAPDANIIE TVDRIRAELP  300
ALQENIPASI QLNVAQDRSP TIRASLAEVE QSLAIAIGLV ILVVFIFLRS GRATLIPAVA  360
VPVSLIGSFA AMYLCGFSLN NLSLMALTIA TGFVVDDAIV VLENISRHVE AGMKPINAAL  420
LGAREVGFTV LSMSVSLVAV FIPLLLMEGL PGRLFREFAV TLSVSIGLSL IVSLTLTPMM  480
CAYLLRHQPA RSQRRARGFG KMLLALQQGY GRSLNWVLGH SRWVLAVFLA TVALNVWLYI  540
SIPKTFFPEQ DTGRLMGFIQ ADQSISFQAM RGKLEDFMKI VREDPDVENV TGFTGGSRTN  600
SGSMFISLKP LSVRSDDAQK VIARLRARLA KEPGASLFLM AVQDIRVGGR QANASYQYTL  660
LADDLAALRE WEPKIRTALA ALPELADVNS DQQDKGSEMD LVYDRETMAR LGISVSDANN  720
LLNNAFGQRQ ISTIYQPLNQ YKVVMEVAPP YTQDVSSLDK MFIINNEGKA IPLSYFASWR  780
PANAPLSVNH QGLSAASTIS FNLPDGGSLS DATAAVERTM TQLGVPSTVR GAFAGTAQVF  840
QDTLKSQLIL ILAAIATVYI VLGVLYESYI HPLTILSTLP SAGVGALLAL ELFGAPFSLI  900
ALIGIMLLIG IVKKNAIMMV DFALEAQRNG GISAREAIFQ ASLLRFRPIM MTTLAALFGA  960
LPLVLTRGDG AELRQPLGIT IAGGLVMSQL LTLYTTPVVY LYFDRLQAKF RRNKQLAPLP 1020
HHH                                                               1023
```

Transmembrane helices were calculated for SdeD and SdeE proteins using OMIGA 2.0 software and the results showed a similar topology as RND proteins (Fig. 5.4).

Phylogenetic analysis of SdeD and SdeE protein revealed that they are very closely related to MdtC and MdtD proteins of *E. coli* (Fig. 5.5).

5.4. Measurement of expression levels of *sdeD* in clinical isolate and mutant strains of *S. marcescens*

Expression of *sdeD* was measured in the clinical isolate T-861, and the mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO, with the wild-type UOC-67 used as control. Results from Northern blotting showed an identical low level expression of the *sdeD* gene in all the strains tested (Fig. 5.6).

5.5. Three-dimensional structure prediction of SdeD and SdeE proteins

The amino acid sequence of *sdeCDE* gene products revealed that SdeC was a 450 amino acid residues long with a predicted molecular weight of 47.8 kDa, SdeD consisted of 1036 amino acid residues with a predicted molecular weight of 111.5 kDa, while SdeE had a predicted molecular weight of 110 kDa with 1021 amino acids. Three-dimensional structures were predicted for both SdeD and SdeE proteins using CDD software and both proteins showed a structure similar to that of AcrB protein of *E. coli* (Fig. 5.7).

5.6. Antibiotic susceptibility of *E. coli* AG102MB expressing the SdeCDE pump

E. coli AG102MB was supplemented with plasmids pUCCD (expressing *sdeCD* genes) and pUCCDE (expressing *sdeCDE* genes) and antibiotic resistance profile compared with the non-supplemented parent strain. The results are summarized in the Table 5.1. There was no increase in the resistance to any of the antimicrobial agent tested upon introduction of pUCCD or pUCCDE plasmids, suggesting that SdeCDE pump was not effluxing any these agents.

Tolerance to hexane was also tested for *E. coli* AG102MB supplemented with pUCCD and pUCCDE. Neither of the two plasmids increased tolerance of the *E. coli* strain to n-hexane.

Fig. 5.4. von Heijne transmembrane helices in (a) *S. marcescens* SdeD and (b) *S. marcescens* SdeE proteins. Transmembrane helices were plotted using OMIGA 2.0 software. Hydrophobicity is indicated by positive numbers while hydrophilicity is indicated by negative numbers. Each predicted transmembrane helix is represented by numbers of the plot.

Fig. 5.4(a). SdeD

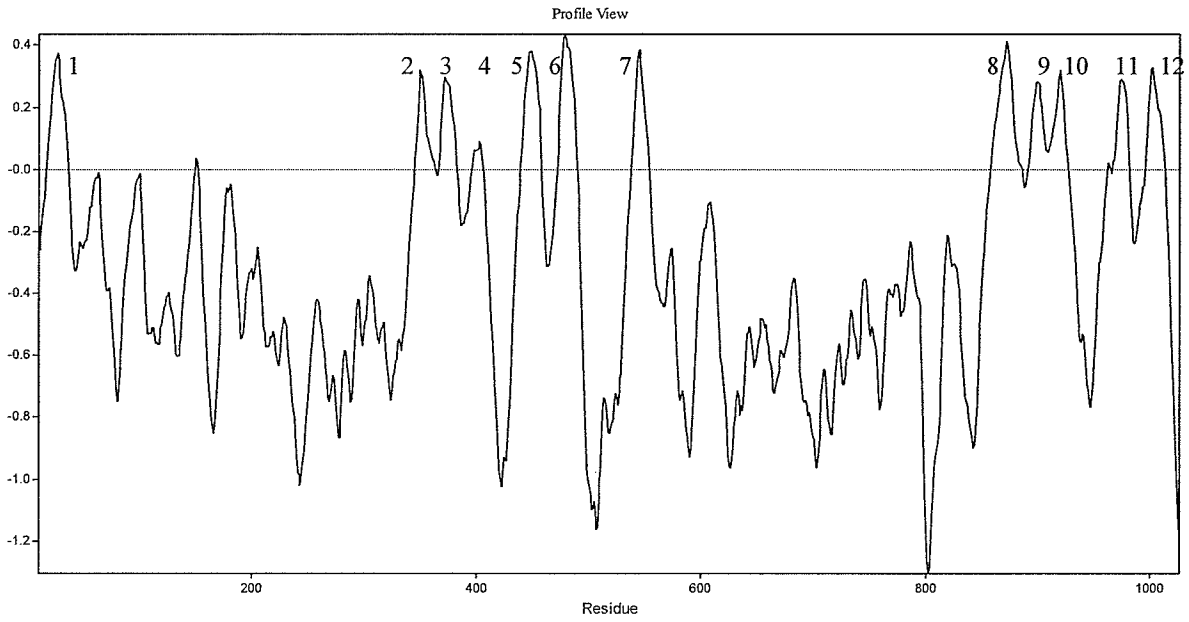

Profile View
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
1
2
3
4
5
6
7
8
9
10
11
12
200
400
600
800
1000
Residue

Fig. 5.4(b). SdeE

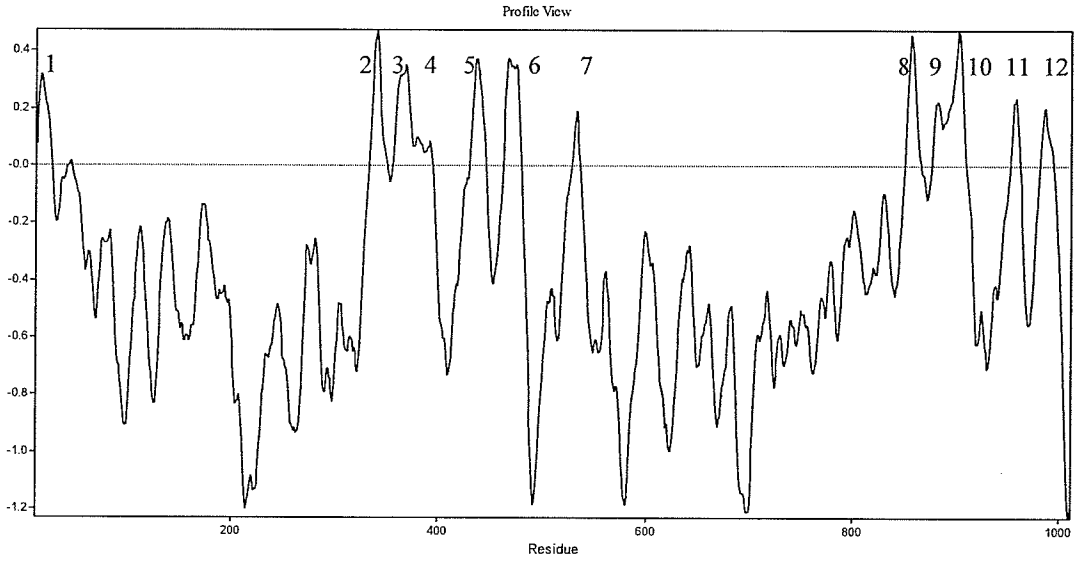

Profile View
1
2
3
4
5
6
7
8
9
10
11
12
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
200
400
600
800
1000
Residue

Fig. 5.5. Unrooted distance tree based on alignments of SdeD and SdeE proteins of *S. marcescens* with RND pump proteins of *E. coli* and *P. aeruginosa*. The phylogenetic tree for SdeB and different RND pumps of *E. coli* (AcrB, AcrD, MdtC, and MdtD), *P. aeruginosa* (MexB, MexD, MexF, and MexY), and SdeY of *S. marcescens* (Chen *et al*, 2003) was constructed using the online server of Kyoto University Bioinformatics Center (http://clustalw.genome.jp).

Fig. 5.5

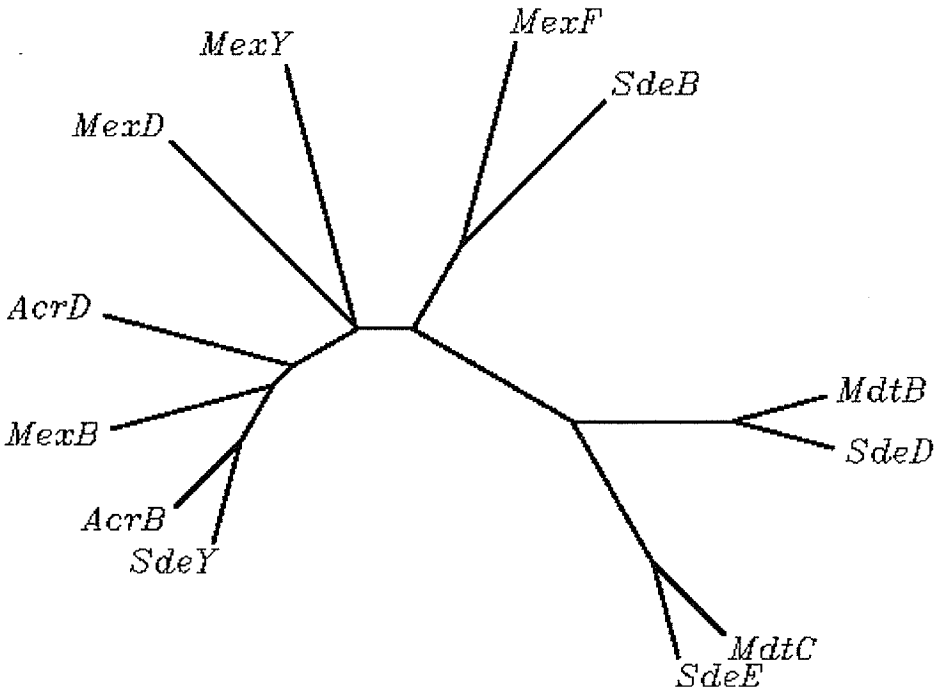


Fig. 5.6. Northern blot analysis of various *S. marcescens* strains for expression of *sdeD*. Expression levels of *sdeD* was measured in *S. marcescens* UOC-67 (1), T-861 (2), UOC-67WL (3), UOC-67WLN (4), and UOC-67WLO (5). Total RNA (1μg) was spotted and hybridization was performed using DIG-labeled mRNA probe for *sdeD* gene. Intensity of spots was measured using FluorChem 2.0.

Fig. 5.6.

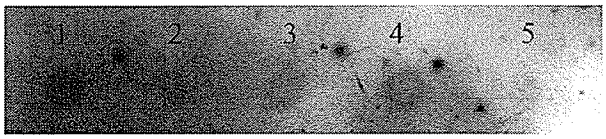


Fig. 5.7. Three-dimensional structure prediction of *S. marcescens* SdeD (a) and SdeE (b) proteins using CDD software, revealing similarity to *E. coli* AcrB. Residues shown in red are similar/identical to *E. coli* AcrB while those shown in pink are different from AcrB.

Fig. 5.7. (a) SdeD

Fig. 5.7. (b) SdeE

Table 5.1. Antibiotic susceptibility of *E. coli* AG102MB expressing the *S. marcescens* SdeCDE efflux pump

	Relative MIC[a]						
Strain	**Cip**	**Nor**	**Ofx**	**Chl**	**Nov**	**SDS**	**EtBr**
AG102MB	1	1	1	1	1	1	1
AG102MB/pUC18	1	1	1	1	1	1	1
AG102MB/pUCCD	1	1	1	1	1	1	1
AG102MB/pUCCDE	1	1	1	1	1	1	1

Cip, Ciprofloxacin; Nor, norfloxacin; Ofx, ofloxacin; Chl, chloramphenicol; Nov, novobiocin; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide.

PUCCD contains the *sdeCD* locus as cloned in the cloning vector pUC18

pUCCDE contains the *sdeCDE* locus as cloned in the cloning vector pUC18

[a]relative MIC is the MIC value obtained for the test strain divided by the MIC value of *E. coli* AG102MB

5.7. Discussion

In addition to the *sdeB* gene fragment, PCR from the genomic DNA of *S. marcescens* UOC-67 resulted in a second product of 1.7 kb. Sequencing of this PCR product revealed its high homology (~60%) to the *mdtB* gene of the *E. coli* MdtABC system, a multicomponent RND system of *E. coli* that contains two different RND pump proteins instead of one. This PCR product was cloned, and a DIG-labeled probe constructed. The cosmid bank of *S. marcescens* UOC-67 was screened, and two different clones, pRKC51 and pRKH10, were found to be carrying the *mdtB*-like gene. PRKC51, containing a larger insert, was selected for further analysis. Subcloning and subsequent sequencing revealed the presence of MFP- and RND pump-encoding genes, based on the presence of conserved amino acid residues (Fig. 5.3b). This locus was named *sdeCD*. Further examination of the predicted amino acid residues from *sdeD* using the von-Heijne transmembrane helices plot (Fig. 5.4a) and 3-dimensional structure predictions (Fig. 5.7a) established SdeD as an RND pump protein.

Expression levels of *sdeD* were examined in *S. marcescens* UOC-67, T-861, UOC-67WL, UOC-67WLN, and UOC-67WLO. There was no apparent difference in the expression of *sdeD* in any of the strains, with all strains showing minimal expression of the gene (Fig. 5.6). Data from Northern blot analysis showed that none of the fluoroquinolone resistant strains were over-expressing *sdeD*, and that none of the fluoroquinolones tested (ciprofloxacin, norfloxacin, and ofloxacin) up-regulated the expression of this gene in *S. marcescens* mutants, UOC-67WL, UOC-67WLN, or UOC-67WLO.

To further analyze the gene products of the *sdeCD* locus, a plasmid carrying it was introduced into the *acrB*-deletion mutant *E. coli* AG102MB. No change in the

susceptibility of the host strain to various antimicrobials was observed as a result (Table 5.1), suggesting that either the gene products of *sdeCD* do not recognize fluoroquinolones, detergents, or dyes as their substrates, or that the pump is simply not functional.

The MdtABC system is unique in that, in addition to genes encoding the MFP and the RND pump proteins, it has an extra RND pump-encoding gene, *mdtC* (Baranova & Nikaido, 2002; Nagakubo *et al*, 2002). Both RND proteins have been shown to be required for the activity of the pump complex. Since introduction of *sdeCD* in *E. coli* AG102MB did not give any indication that the pump might be functioning, the region downstream of *sdeD* was examined for the presence of another RND pump-encoding gene that might be required for the functioning of this pump. Analysis of few hundred nucleotides present downstream of *sdeD* in pUCCD vector did in fact reveal the presence of an additional RND pump gene. A ~12-kb fragment was subcloned from pRKC51 into pUC18 to clone this second RND pump gene along with *sdeCD*. Sequencing of this third gene and subsequent analysis revealed that it was indeed an RND pump-encoding gene, based on the presence of sequence homologies and the presence of signature sequences (Fig. 5.3b). This gene has been named *sdeE*. It was further confirmed to be a RND pump-encoding gene by von-Heijne transmembrane helices plot (Fig. 5.4b) and 3-dimensional structure predictions (Fig. 5.7b). Phylogenetic analysis of SdeD and SdeE proteins with RND proteins of *E. coli* and *P. aeruginosa* also revealed that both these proteins were very closely related to MdtB and MdtC proteins of *E. coli*.

The entire *sdeCDE* operon was introduced into *E. coli* AG102MB by triparental mating, and susceptibility of the host strain was tested for fluoroquinolones,

chloramphenicol, SDS, and ethidium bromide in order to examine if there was any change in the resistance profile when both RND pump genes were present in *E. coli* AG102MB (Table 5.1). However, the data showed that SdeCDE pump was not effluxing any of the agents tested. Finally, susceptibility to novobiocin was tested for *E. coli* AG102MB supplemented with *sdeCDE*, as the MdtABC system has been shown to efflux novobiocin (Nagakubo *et al*, 2002). The SdeCDE pump was found not to efflux this drug either.

From the data above, it appears that *sdeCDE* could be encoding a multicomponent RND pump of *S. marcescens*, the first of its kind identified in this organism. However it not clear what compounds constitute the substrates for this pump, or even if the pump is active.

SIX

Molecular Characterization of a TolC Homolog of *Serratia marcescens*

The search for truth is more precious than its possession
-Albert Einstein

6.1. Abstract

A *tolC*-like gene was identified in *S. marcescens* upon scanning the incomplete database of the *S. marcescens* genome sequencing project (http://www.sanger.ac.uk/Projects/S_marcescens/). The gene was identified and amplified using PCR and cloned in the pUC18 vector. Sequencing revealed about 80% amino acid similarity with the *E. coli tolC*.

Introduction of the *S. marcescens tolC* gene in an *E. coli* strain deficient in TolC resulted in an increase in resistance to SDS and ethidium bromide. To confirm if the increase in resistance to ethidium bromide was the result of proton gradient-dependent efflux, ethidium bromide accumulation assays were performed, with results proving the involvement of *S. marcescens* TolC in proton gradient-dependent efflux.

6.2. Identification, cloning, and sequencing of TolC homolog of *S. marcescens*

The incomplete database of the *S. marcescens* genome sequencing project at the University of Cambridge (http://www.sanger.ac.uk/Projects/S_marcescens/) was screened for the presence of a *tolC*-like gene using the *E. coli tolC* sequence as the reference. Primers were designed with *Eco*R1 and *Bam*HI restriction sites engineered in their 5'-ends and PCR product was cloned in the pUC18 vector to construct the plasmid pUCHF and the sequence analyzed (Fig. 6.1). The sequence has been submitted to GenBank under the Accession No. AY631047.

6.3. Three-dimensional structure prediction of *S. marcescens* TolC protein

The amino acid sequence of the ORF was deduced (Fig. 6.2a) and was found to be ~80% similar to the TolC protein of *E. coli* (Fig. 6.2b). The amino acid sequence of *S. marcescens* TolC revealed that it contained 505 amino acid residues with a predicted

molecular weight of 54 kDa. The predicted 3-dimensional structure of *S. marcescens* TolC (Fig. 6.3) showed a structure very similar to that of *E. coli* TolC with presence of the β-barrel pore and α-helical tail.

6.4. Antibiotic susceptibility of *E. coli* BL923 expressing *S. marcescens* TolC

E. coli BL923, a TolC-deficient strain, was transformed with pUCHF and expression of the *S. marcescens* TolC protein confirmed by resolution of outer membrane proteins by SDS-PAGE (Fig. 6.4). Antibiotic resistance profiles of *E. coli* BL923 with and without pUCHF were compared. Results are summarized in the Table 6.1. Expression of the *S. marcescens tolC* in *E. coli* BL923 resulted in a 64-fold increase in the resistance to ethidium bromide and a >32-fold increase in the resistance to SDS, although there was no change in the resistance to any other antimicrobial tested.

Fig. 6.1. Nucleotide sequence of the *S. marcescens tolC*. The initiation codon is shown in bold. Shaded nucleotides represent *S. marcescens tolC* open reading frame. Target sequences for TOCF and TOLCR primers are underlined.

Fig. 6.1.

1 ccgcagactc tgctagaatc ggcaactatt tcgtctatcg tcagcgctaa catagcaata
61 ttgctgcaca acaaggaatg caaatgaaga aactgctccc ccttcttatc ggactgagcc
121 tgggcggctt cagtgcaatg agccaggcag agaacctgct gcaggtctac aaacaggcca
181 gggaaagtaa cccggatctg cgcaaaagtg ctgctgaccg cgacgccgca ttcgaaaaga
241 tcaatgaagc gcgcagcccg ctgttgccgc agctcggttt aaccgcaggt tacgattaca
301 ccaacggtta tcgtgacagc aatggcgtta acagcaacgt gaccagcggt tcgctggcat
361 tgacgcaaac cctcttcgac atgtcgaaat ggcgccagct gacgctgcag gaaaagaccg
421 ccggcatctc cgacgtcacc ttccagaccg cagagcagtc gctgattctc aacactgcca
481 ccgcttactt caacgtgctg aaagcgatcg acacgctgtc ctatacccag gcgcagaaag
541 acgcggtcta ccgcacgctg gatcaaacca cccaacgctt caacgtgggc ctggtggcga
601 tcaccgacgt gcagaacgcc cgttcgaact atgacaccgt gctggcggcc gaagtctccg
661 cccgcaacga tctggataac gcgctggaaa cgctgcgcca ggtgaccggg gccttctacc
721 cggagctggc ctcgttgaat accgatcgct tcagtaccca gcgcccggaa gcggtgaaca
781 acctgctgaa agaagcggaa gcgcgcaacc tgagcctgct gtccgcccgt ctgagccagg
841 atctggcgcg tgagcaaatc cgcgcagccc aaactggcta tatgccgacg attgacgtca
901 gcgcgtcgac cggcatcagc aataccaaat acaacggctc caacaccggc ggcgcgaatg
961 ccgcacgcta cagcgactcc gatgcgggcc aaaataaggt cggcatcagc ttcaacctgc
1021 cgctatacag cggcggcgcg accaactcgc aggtcaaaca agcgcagtac ggcttcgtcg
1081 gcgccagtga gcagttggaa agctcccacc gcagcgtggt gcagaccgtg cgttcgtcgt
1141 tcaacaacgt taacgcctcg atcagcagca tcaacgccta caaacaggcg gtaatctccg
1201 cgcagagctc cctggacgcg atggaagccg gttatcaggt cggtacccgc accatcgtcg
1261 atgtgctgga cgccaccacc accctgtata acgccaaacg tcaactgtcc gacgcgcgtt
1321 atacctatct gatcaaccag ctgaacatca agtcggcgct cggcacgctg aaccagaacg
1381 atctgctgct gctgaatggc gcgttgggca aaccggtgtc gaccgcgcct gacgccgtcg
1441 caccgcaaaa ccgcgcgcag gacgcctatg cggacggtta tcaggacaat gcgccgatgc
1501 aacaaaccgc ggcgccggca ccggcggcca ctcgcgtatc cgcgccggcc gttaccacca
1561 gccaaccggc tcgcaacagc ggcaacccat tccgtaactg atgcgcgcac gttgataaac
1621 acggggtccg gcctgcgccg cgccccgttt tttttgcccc gatttcccca cttctgccca
1681 ccgcctcaca gc

Fig. 6.2a. Amino acid sequence of the *S. marcescens* TolC, as derived using OMIGA 2.0.

Fig. 6.2a

```
MKKLLPLLIG LSLGGFSAMS QAENLLQVYK QARESNPDLR KSAADRDAAF EKINEARSPL  60
LPQLGLTAGY DYTNGYRDSN GVNSNVTSGS LALTQTLFDM SKWRQLTLQE KTAGISDVTF  120
QTAEQQLILN TATAYFNVLK AIDTLSYTQA QKDAVYRTLD QTTQRFTWGL VAITDVQNAR  180
SNYDTVLAAE VSARNDLDNA LETLRQVTGA FYPELASLNT DRFSTQRPEA VNNLLKEAEA  240
RNLSLLSARL SQDLAREQIR AAQTGYMPTI DVSASTGISN TKYNGSNTGG ANAARYSDSD  300
AGQNKVGISF NLPLYSGGAT NSQVKQAQYG FVGASEQLES SHRSVVQTVR SSFNNVNVSI  360
SSINAYKQAV ISAQSSLDAM EAGYQVGTRT IVDVLDATTT LYNAKRQLSD ARYTYLINQL  420
NIKSALGTLN QNDLLLLNGA LGKPVSTAPD AVAPQNRAQD AYADGYQDNA PMQQTAAPAP  480
AATRASAPAV TTSQPARNSG NPFRN                                        505
```

Fig. 6.2b. Amino acid alignment of *S. marcescens* TolC and *E. coli* TolC. Alignment was performed using OMIGA 2.0 software. Identical amino acids are indicated by dot (.) and gaps are indicated by dash (-).

Fig. 6.2b.

```
          1                                                  50
EcTolC    MKKLLPILIG LSLSGFSSLS QAENLMQVYQ QARLSNPELR KSAADRDAAF
SmTolC    ......L... ...G..AM. .....L...K ...E...D.. ..........

          51                                                 100
EcTolC    EKINEARSPL LPQLGLGADY TYSNGYRDAN GINSNATSAS LQLTQSIFDM
SmTolC    .......... ......T.G. D.T.....S. .V...V..G. .A...TL...

          101                                                150
EcTolC    SKWRALTLQE KAAGIQDVTY QTDQQTLILN TATAYFNVLN AIDVLSYTQA
SmTolC    ....Q..... .T...S...F ..AE.Q.... .........K ...T......

          151                                                200
EcTolC    QKEAIYRQLD QTTQRFNVGL VAITDVQKAR AQYDTVLANE VTARNNLDNA
SmTolC    ..D.V..T.. ......TW.. .......N.. SN......A. .S...D....

          201                                                250
EcTolC    VEHVRQITGN YYPGTAALNV ENFKTDKPQP VNALLKEAEK RNLSLLQARL
SmTolC    L.TL..V..A F..EL.S..T DR.S.QR.EA ..N.L....A ......S...

          251                                                300
EcTolC    SQDLAREKFA RRRMVTYRLW DLTASTGFLT PLIAVRKP-- CAAVPYDDSN
SmTolC    .......QIR AAQTGYMPTI .VS....ISN TKYNGSNTGG AN.AR.S..D

          301                                                350
EcTolC    MGQNKVGLSF SLPIYQGGMV NSQVTQAQYN FVGAASTWKV PIVASCQRA.
SmTolC    A......I.. N..LYS..AT ....K....G ....SEQLES SHRSVV.TVR

          351                                                400
EcTolC    FCFSNINASI SSINRYTQAA VSAQSSLDAM EAGYSVGTRT IVDVLDATTS
SmTolC    SS.N.V.V.. ....A.K..V I......... ....Q..... .........T

          401                                                450
EcTolC    CT-AQARAGN PRYN...... NIKSALGTLN EQDLLALNNA LSKPISTNPE
SmTolC    LYN.KRQLSD A..T...... .......... QN...L..G. .G..V..A.D

          451                                                500
EcTolC    NVAPQTPEQN AIADGYAPDS -------PAP VVQQTSARTT TS-----NGH
SmTolC    A....NRA.D .Y....QDNA PMQQTAA... AATRA..PAV .TSQPAR.SG

          501
EcTolC    NPFRN
SmTolC    .....
```

Fig. 6.3. Three-dimensional structure prediction of *S. marcescens* TolC using CDD software. The structure shows similarity with that of *E. coli* TolC. Residues shown in red are similar/identical to the *E. coli* TolC, while those shown in pink are different from TolC protein of *E. coli*.

Fig. 6.3.

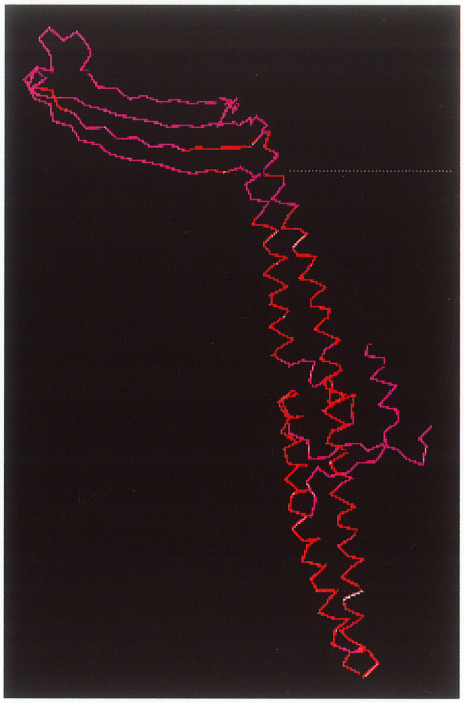


Fig. 6.4. Expression of *S. marcescens* TolC (indicated by the arrow) in *E. coli* TolC-deficient strain BL923. Fifty μg of cell envelope preparation was resolved on 12% SDS-PAGE. Lane 1, *E. coli* BL923; Lane 2, *E. coli* BL923:pUC18; Lane 3, *E. coli* BL923:pUCHF. Numbers indicate the weight of molecular weight markers.

Fig. 6.4.

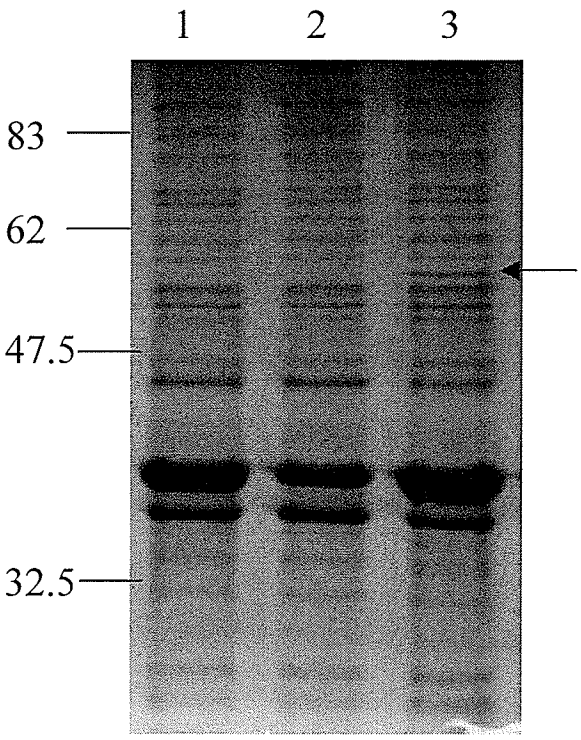


Table 6.1. Antibiotic susceptibility of *E. coli* BL923 expressing the *S. marcescens* TolC

Relative MICs[a]						
Strain	**Cip**	**Nor**	**Ofx**	**Chl**	**SDS**	**EtBr**
E. coli BL923	1	1	1	1	1	1
E. coli BL923 /pUC18	1	1	1	1	1	1
E. coli BL923/pUCHF	1	1	1	1	>32	64

Cip, Ciprofloxacin; Nor, norfloxacin; Ofx, ofloxacin; Chl, chloramphenicol; SDS, sodium dodecyl sulfate; EtBr, ethidium bromide.

pUCHF contains the *S. marcescens tolC* cloned in the cloning vector pUC18.

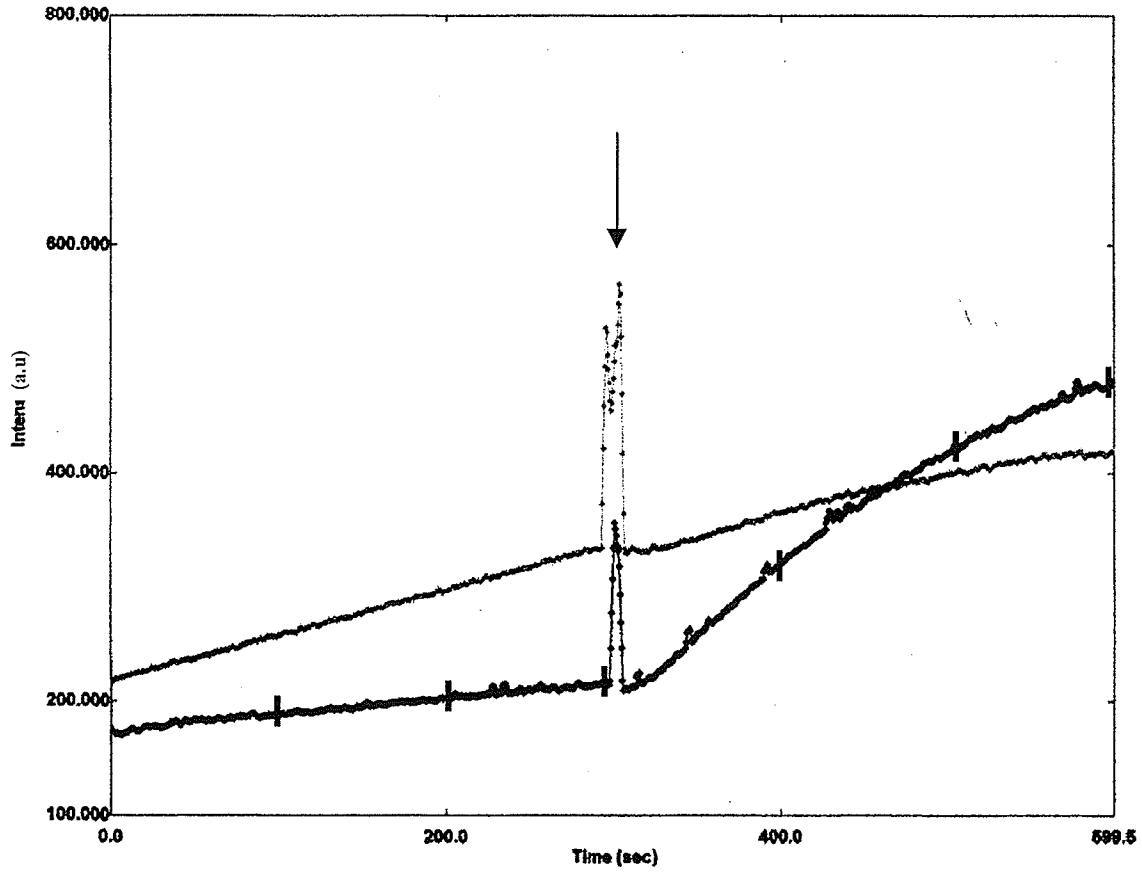
[a]relative MIC is the MIC value obtained for the test strain divided by the MIC value of *E. coli* BL923

6.5. Ethidium bromide accumulation by *E. coli* BL923 expressing *S. marcescens* TolC

Accumulation of ethidium bromide was monitored in for *E. coli* BL923 strains expressing the *S. marcescens* TolC. It was observed that *E. coli* BL923 showed a steady increase in the intracellular ethidium bromide and addition of CCCP did not cause any increase in the amount of ethidium bromide inside cells. However, when supplemented with the *S. marcescens tolC* gene, cells maintained a constant level of intracellular ethidium bromide until CCCP was added, after which the intracellular amounts of ethidium bromide increased dramatically. This indicates that *E. coli* BL923 did not efflux ethidium bromide but when supplemented with *S. marcescens tolC*, the bacterium was able to efflux ethidium bromide in proton gradient-dependent fashion.

Fig. 6.5. Accumulation of ethidium bromide by *E. coli* BL923 (—) and *E. coli* BL923/pUCHF (++) in real time. Accumulation was monitored for a total of 10 min, and CCCP was added 5 min after incubation (shown by the arrow). Fluorescence of ethidium bromide was measured at 530 (excitation) and 600 (emission) nm (a.u., arbitrary unit)

Fig. 6.5.


800.000
600.000
400.000
200.000
100.000
Intens (a.u)
0.0
200.0
400.0
599.5
Time (sec)

6.6. Discussion

Since no outer membrane component-encoding gene was present in the operons encoding the SdeAB and SdeCDE pumps, the incomplete database of *S. marcescens* genomic sequencing project (http://www.sanger.ac.uk/Projects/S_marcescens/) was searched for the presence of an *E. coli tolC*-like gene. One such gene was identified. This gene was amplified using PCR primers and cloned in the cloning vector pUC18 to construct the plasmid pUCHF. Sequencing of this *S. marcescens tolC*-homolog and subsequent amino acid sequence determination revealed about 80% amino acid homology (Fig. 6.2b) to the *E. coli* counterpart with very similar 3-dimensional structures (Fig. 6.3). It was found that this gene was previously reported as the outer membrane component of an ABC-iron transport system in *S. marcescens* (Binet & Wandersman, 1996), although the authors had not investigated its possible role in proton gradient-dependent efflux.

Functional analysis of *S. marcescens tolC* was carried out in an *E. coli tolC*-deficient strain BL923. Introduction of the *S. marcescens tolC* gene resulted in up to 64-fold increase in resistance to SDS and ethidium bromide (Table 6.1). There was no change in the susceptibility to fluoroquinolones or chloramphenicol indicating that the pumps present in *E. coli* BL923 were not effluxing these antibiotics. To find out whether the resistance conferred by the *S. marcescens tolC*-homolog was a result of active efflux, ethidium bromide accumulation assays were performed. It was observed that *E. coli* BL923 accumulated ethidium bromide with time, and that there was no effect of addition of the inhibitor CCCP. However, when *E. coli* BL923 was complemented with *S. marcescens tolC*-homolog, it showed low constant levels of intracellular ethidium bromide but upon addition of CCCP, there was exponential increase with time in the

intracellular levels of the compound (Fig. 6.5). Ethidium bromide accumulation established the role of *S. marcescens tolC* in active efflux.

Since there was no other homolog of *E. coli tolC* was found in the genome search of *S. marcescens*, it is likely that the gene product of *S. marcescens tolC* is the only efflux pump associated outer membrane component present in this organism. This is similar to *E. coli*, where TolC is so far the only functional outer membrane component known to be acting in conjunction with different efflux pumps.

SEVEN

Characterization of *sdeR*, a Gene Encoding the Putative Regulator of the *Serratia marcescens* SdeAB Pump

Motivation is what gets you started.
Habit is what keeps you going.
-Unknown

7.1. Introduction

Most of the efflux pumps characterized to date have a regulatory protein-encoding gene in the upstream region of the pump encoding genes. With this in mind, upstream region of the *sdeAB* locus was analyzed for the presence of a gene encoding regulatory protein. A 405-bp ORF (*sdeR*) was identified, which was transcribed in the opposite direction of *sdeAB*. The deduced amino acid sequence was found to be about 40 % similar to the MarA protein of *E. coli*, a transcriptional activator of AraC/XylS family. The intragenic region between the *sdeR* and *sdeA* was analyzed for *S. marcescens* strains resistant to fluoroquinolones, in an effort to identify a possible binding site(s) for SdeR.

7.2. Identification, cloning, and sequencing of *sdeR*

A ~700-bp region upstream of the *sdeAB* locus was sequenced and analyzed from the cosmid clone pRK037 and primers were designed to amplify the region. The product was cloned in the pUC18 vector to construct the plasmid pUCR1. The cloned product was then sequenced and analyzed for ORFs (Fig. 7.1). One ORF of 405 bp was identified having about 50% DNA homology to the *marA* gene of *E. coli*, and named *sdeR*. The sequence of the *sdeR* gene was submitted to the GenBank under the Accession No. AY623133 (Fig. 7.2).

7.3. Three-dimensional structure prediction of SdeR

The amino acid sequence of SdeR was deduced using OMIGA 2.0 software (Fig. 7.3), and the predicted molecular weight was found to be ~15 kDa. The amino acid sequence of SdeR was found to be ~40% similar to that of the MarA protein of *E. coli* (Fig. 7.4). Expression of SdeR from the plasmid pUCR1 was monitored in *E. coli* NM522 cells using SDS-PAGE (Fig. 7.5). The 3-dimensional structure of SdeR was

predicted using the CDD software of NCBI. The structure was found to be very similar to that of the MarA, with the presence of putative DNA binding helices (Fig. 7.6)

7.4. Alignment of the predicted amino acid sequence of SdeR from different *S. marcescens* strains

The 405 bp *sdeR* gene was amplified from the *S. marcescens* clinical isolate, T-861, and mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO. PCR products from all 4 strains were sequenced and amino acid sequences were deduced. The predicted amino acid sequences were aligned with that of *S. marcescens* UOC-67 SdeR using OMIGA 2.0 and all four sequences were found to be identical to that of UOC-67.

7.5. Analysis of the intergenic region between *sdeA* and *sdeR* from different strains of *S. marcescens*

The intergenic region between *sdeA* and *sdeR* was amplified from *S. marcescens* T-861, and the mutant strains UOC-67WL, UOC-67WLN, and UOC-67WLO. PCR products were sequenced and aligned with the intergenic region between *sdeA* and *sdeR* of *S. marcescens* UOC-67 (Fig.7.7). Although no obvious -10 or ribosome binding sites were found, 2 putative -35 sites were found. T-861 and UOC-67WL had 9 mutations within the putative SdeR binding site [determined based on similarity to 'marbox' sequences from *E. coli* (Alekshun & Levy, 1997)] when compared to the UOC-67 sequence. There were also some mutations found in these two strains outside the putative binding site. Interestingly, T-861 and UOC-67WL had identical base substitutions. Surprisingly, there was no change in the intergenic region of UOC-67WLN, the strain overexpressing *sdeB*.

Fig. 7.1. Analysis of the ~700-bp upstream region of *sdeAB* for open reading frames. ORF search was performed using OMIGA 2.0 software. Dots represent initiation codons, while arrow heads represent the stop codons. The *sdeR* ORF is shown with the solid arrow while other potential ORFs are represented with broken arrows.

Fig. 7.1.

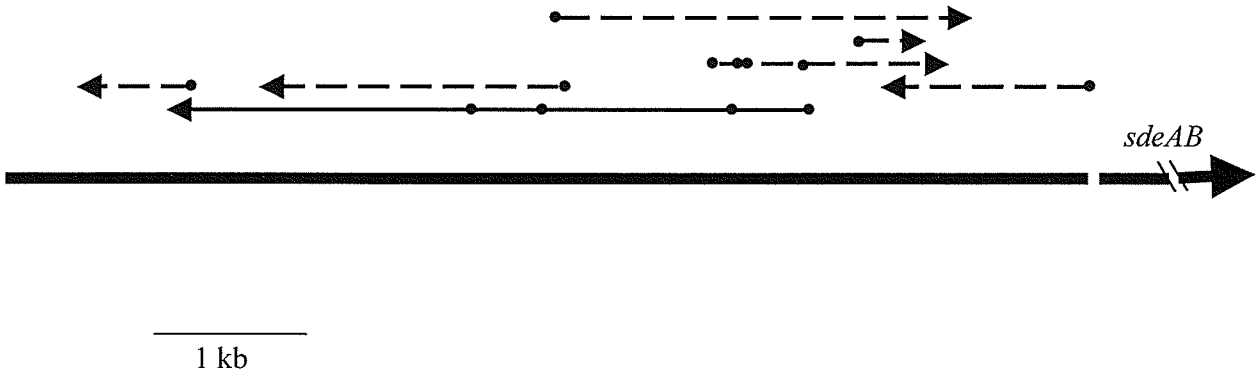


Fig. 7.2. The nucleotide sequence of the *sdeR* gene from UOC-67. The initiation codon is shown in bold.

Fig. 7.2.

```
1   atgtgcatcg ggcagagggt gaccctgccg aatgatggga gcatctgcat gaacactacg
61  ggctttatta ccgatttgaa ggcgtggatc gacaacaatc tggaagagaa actggatatc
121 aacaccgtgg cggaccgcgc gggctattcc aaatggcatc tgcagcgcat gttcaaacgc
181 cagaccggct acgcgctggg ggagtatatc cgcatgcaaa aactgaaagt gtcggcggag
241 cgcctggcca acagcggcga gcctatcgtc agcgtggcga tctcgctcgg cttcgactca
301 cagcagtcgt tcaaccgcag cttcaaacgc cagttcggcc aaacgccggg cgactggcgc
361 cgcgcgctgg cgcagccagc gtcggtgaga tgcacgcacc ac
```

Fig. 7.3. Amino acid sequence of SdeR protein, deduced using OMIGA 2.0 software.

Fig. 7.3.

```
MCIGQRVTLP NDGSICMNTT GFITDLKAWI DNNLEEKLDI NTVADRAGYS KWHLQRMFKR  60
QTGYALGEYI RMQKLKVSAE RLANSGEPIV SVAISLGFDS QQSFNRSFKR QFGQTPGDWR  120
RALAQPASVR CTHH                                                    134
```

Fig. 7.4. Amino acid alignment of MarA and SdeR. Mismatches and gaps are shown as (x), while shaded (x) represent amino acid with similar chemical properties. DNA binding helices of MarA are shown by bars.

Fig. 7.4.

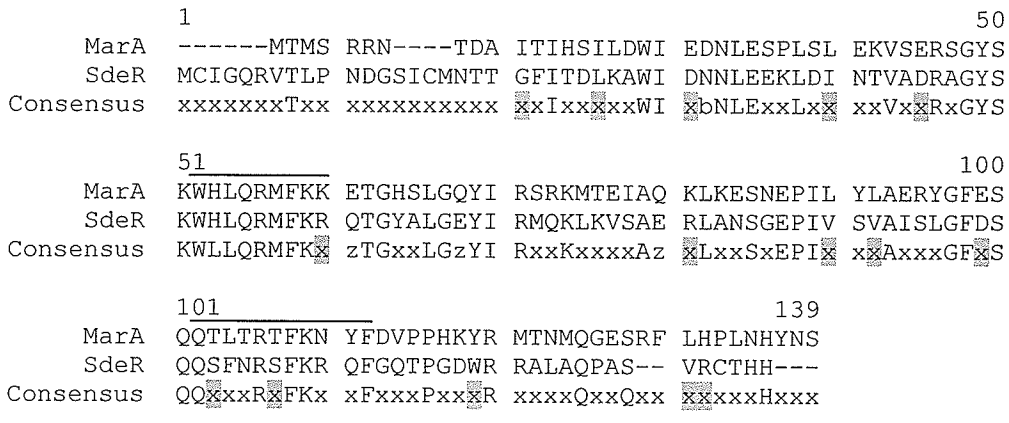

1 50
MarA ------MTMS RRN----TDA ITIHSILDWI EDNLESPLSL EKVSERSGYS
SdeR MCIGQRVTLP NDGSICMNTT GFITDLKAWI DNNLEEKLDI NTVADRAGYS
Consensus xxxxxxxTxx xxxxxxxxxx xxIxxxxxWI xbNLExxLxx xxVxxRxGYS
51 100
MarA KWHLQRMFKK ETGHSLGQYI RSRKMTEIAQ KLKESNEPIL YLAERYGFES
SdeR KWHLQRMFKR QTGYALGEYI RMQKLKVSAE RLANSGEPIV SVAISLGFDS
Consensus KWLLQRMFKx zTGxxLGzYI RxxKxxxxAz xLxxSxEPIx xxAxxxGFxS
101 139
MarA QQTLTRTFKN YFDVPPHKYR MTNMQGESRF LHPLNHYNS
SdeR QQSFNRSFKR QFGQTPGDWR RALAQPAS-- VRCTHH---
Consensus QQxxxxRxFKx xFxxxPxxxR xxxxQxxQxx xxxxxxHxxx

Fig. 7.5. Expression of SdeR (indicated by the arrow) in *E. coli* NM522. Fifty μg of cell envelope preparation was resolved on 15% SDS-PAGE. Lane 1, *E. coli* NM522; Lane 2, *E. coli* NM522:pUC18; Lane 3, *E. coli* NM522:pUCR1; Lane 4, Molecular weight markers. Numbers indicate the weight of molecular weight markers in kDa.

Fig. 7.5.

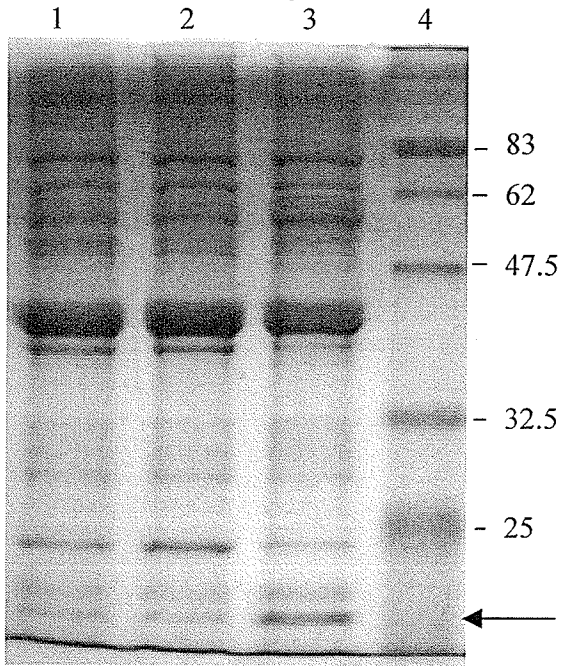


Fig. 7.6. Three-dimensional structure prediction of SdeR using CDD software. The structure shows similarity with that of the MarA protein of the AraC/XylS family of transcriptional activators. Residues shown in red are similar/identical to the MarA while those shown in pink are different from MarA protein. Barrels indicate the helices in the structure. Predicted DNA binding helices are indicated by arrows.

Fig. 7.6.

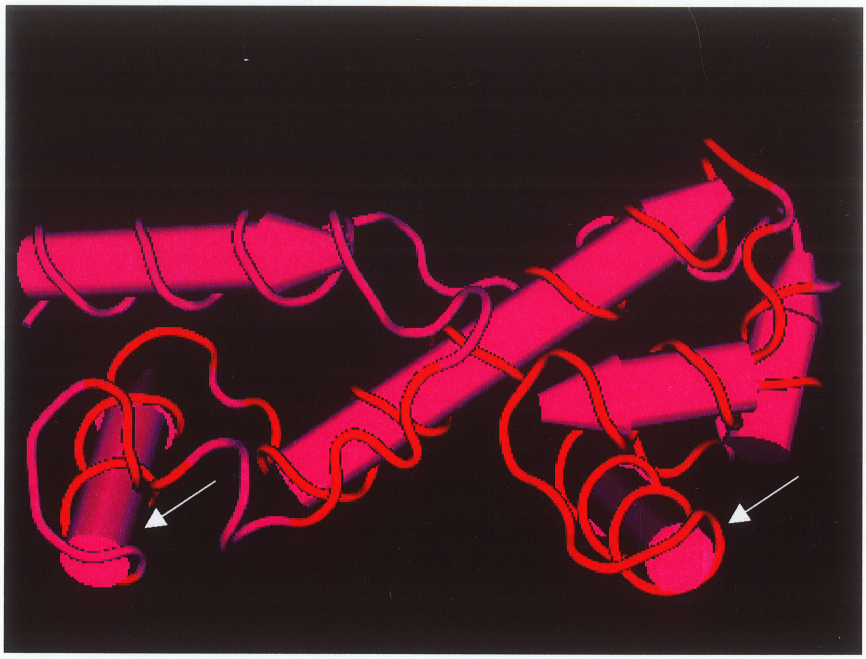
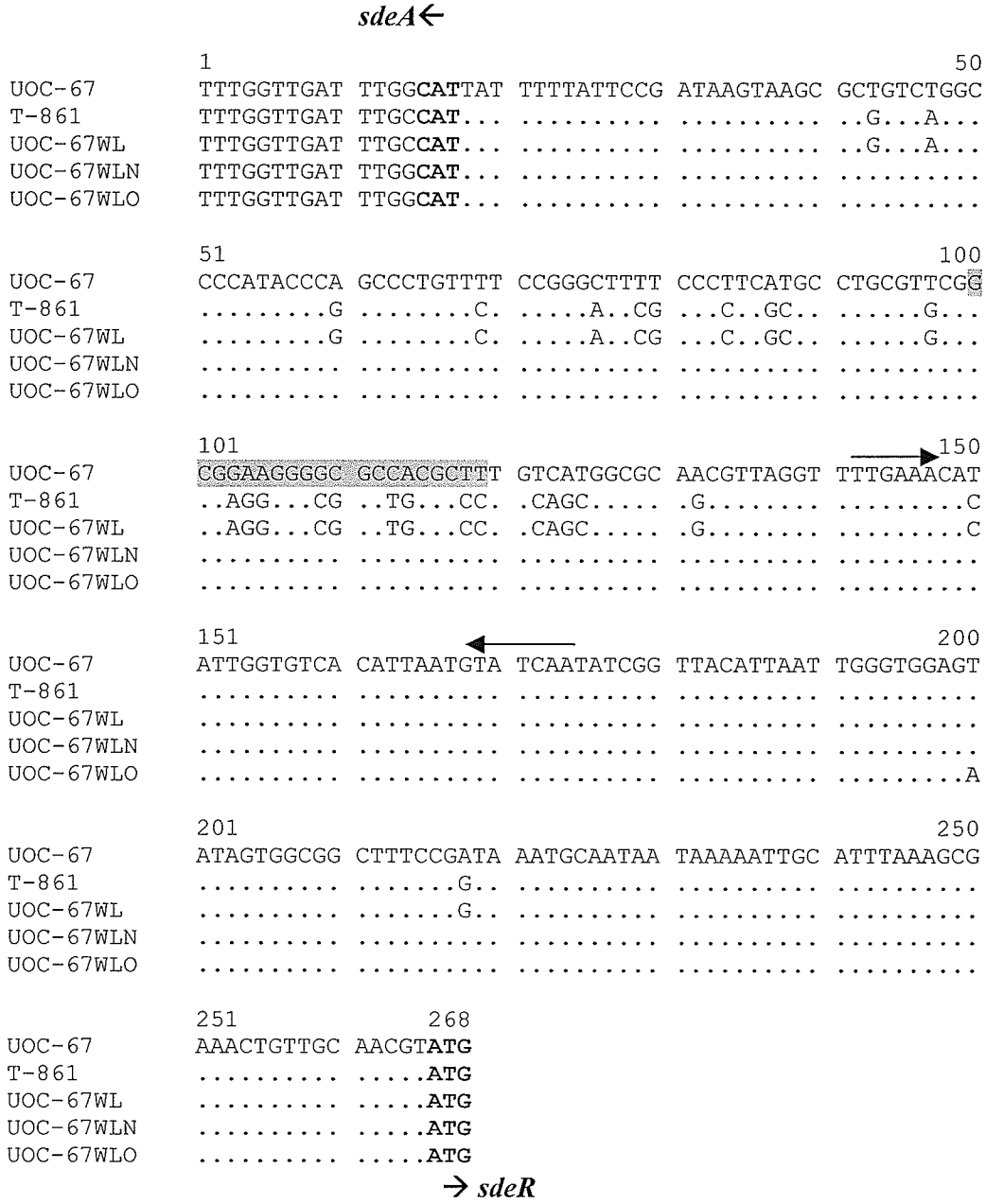


Fig. 7.7. Alignment of the intergenic region of *sdeA* and *sdeR* from different *S. marcescens* strains. Identical bases are shown by dots (.). A putative binding site for SdeR is highlighted. Initiation codons for *sdeA* and *sdeR* genes are shown in bold, arrows indicate the direction of transcription. Two putative -35 sites are shown by arrows.

Fig. 7.7.


sdeA←
1 50
UOC-67 TTTGGTTGAT TTGGCATTAT TTTTATTCCG ATAAGTAAGC GCTGTCTGGC
T-861 TTTGGTTGAT TTGCCAT... ....G...A...
UOC-67WL TTTGGTTGAT TTGCCAT... ....G...A...
UOC-67WLN TTTGGTTGAT TTGGCAT... ....
UOC-67WLO TTTGGTTGAT TTGGCAT... ....
51 100
UOC-67 CCCATACCCA GCCCTGTTTT CCGGGCTTTT CCCTTCATGC CTGCGTTCGG
T-861 .....G ....A..CG ...C..GC.. .....G...
UOC-67WL .....G ....A..CG ...C..GC.. .....G...
UOC-67WLN ....
UOC-67WLO ....
101 150
UOC-67 CGGAAGGGGC GCCACGCTTT GTCATGGCGC AACGTTAGGT TTTGAAACAT
T-861 ..AGG...CG ..TG...CC. .CAGC..... .G..... C
UOC-67WL ..AGG...CG ..TG...CC. .CAGC..... .G..... C
UOC-67WLN ....
UOC-67WLO ....
151 200
UOC-67 ATTGGTGTCA CATTAATGTA TCAATATCGG TTACATTAAT TGGGTGGAGT
T-861 ....
UOC-67WL ....
UOC-67WLN ....
UOC-67WLO ....A
201 250
UOC-67 ATAGTGGCGG CTTTCCGATA AATGCAATAA TAAAAATTGC ATTTAAAGCG
T-861 ....G.. ....
UOC-67WL ....G.. ....
UOC-67WLN ....
UOC-67WLO ....
251 268
UOC-67 AAACTGTTGC AACGTATG
T-861 ....ATG
UOC-67WL ....ATG
UOC-67WLN ....ATG
UOC-67WLO ....ATG
→ *sdeR*

7.6. Discussion

Since *sdeAB* exhibited a differential expression in various strains of *S. marcescens*, it was decided to analyze its upstream region in order to find a possible regulatory protein-encoding gene. A 405-bp ORF was identified that is divergently transcribed. Sequencing of this ORF revealed a ~50% DNA similarity with the *marA* gene of *E. coli*. Amino acid prediction and subsequent 3-dimensional structural prediction also showed similarity with the MarA protein of *E. coli* with a high degree of conservation of the DNA binding helices (Fig. 7.6). MarA is an AraC/XylS family global regulator and is known to transcriptionally regulate about 60 different genes in *E. coli* (Barbosa & Levy, 2000) by binding to a site (marbox) overlapping or upstream of the -35 signal. Activation of the *marA* regulon renders the cell resistant to various antibiotics, superoxides, and organic solvents. The MarA protein consists of 2 DNA binding domains which were found to be highly conserved in the sequence of SdeR (Fig. 7.4).

Sequence of SdeR from different strains of *S. marcescens* was analyzed in order to find any base change that could affect its binding to the DNA resulting in differential expression of *sdeAB* in these strains. However, no differences were found in the *sdeR* sequence in any of the strains. Since MarA is known to bind to promoter regions of different genes, including its own, the intergenic *sdeA-sdeR* region, that contains both the *sdeAB* and *sdeR* promoters, was examined from different *S. marcescens* strains to find any possible mutation that could be linked to the overexpression of *sdeAB* in UOC-67WL and UOC-67WLN (Fig. 7.7). UOC-67WL did show a significant number of sequence alterations in the putative SdeR binding

site as well as outside this box, but no change was observed in the *sdeA-sdeR* intergenic region of UOC-67WLN. However, even though the clinical isolate T-861 exhibited only a minimal overexpression of *sdeAB*, its *sdeA-sdeR* intergenic region had the same mutations as seen for UOC-67WL. Further experimentation is required to establish the role of SdeR in the expression of *sdeAB*. As for UOC-67WLN, since neither the *sdeR* nor *sdeA-sdeR* intergenic region had any mutations, it appears that there is at least one more regulatory protein involved. This could be similar to what is seen for the AcrAB-TolC pump of *E. coli*, where the expression of *acrAB* and *tolC* genes is not only under the control of a local repressor, AcrR, but also a global regulator MarA (Grkovic *et al*, 2001).

EIGHT

Conclusions

A journey of thousand miles begins
with a single step.
-Confucius

Prior to this work, it was not known if active efflux is a mechanism of antibiotic resistance in *S. marcescens*. The objective of this thesis was to identify active efflux as a resistance mechanism in this organism, and to characterize the efflux pump(s) responsible for the MDR phenotype of *S. marcescens*.

Objective 1: To identify proton gradient-dependent efflux of fluoroquinolones as a resistance mechanism in *S. marcescens*

Clinical isolates of *S. marcescens* were screened for resistance to fluoroquinolones, and the presence of energy-dependent efflux of fluoroquinolones by these strains was examined using ethidium bromide accumulation assays (Chapter 3). Once clinical isolate was found to exhibit proton gradient-dependent efflux of fluoroquinolones. Lab-derived mutants, capable of effluxing fluoroquinolones, were isolated by culturing the wild-type (found to be a non-fluoroquinolone effluxing strain) in the presence of fluoroquinolones in the culture media. This data establishes that clinical isolates of *S. marcescens* utilize active efflux as a resistance mechanism to fluoroquinolones, and that exposure to this group of antibiotics can lead to conversion of an efflux-negative strain to an efflux-positive strain.

Fluoroquiolone resistant strains were also analyzed for target gene mutations, with the results indicating that both active efflux and target gene mutations are responsible for the fluoroquinolone resistant phenotype of *S. marcescens*.

One intriguing outcome from this section is that exposure to one type of fluoroquinolone antibiotic can lead to resistance of *S. marcescens* to a wide variety of

antimicrobials, as evident from the antimicrobial resistance profile of the lab-derived mutant strains.

Objective 2: To identify, clone, and sequence the RND pump-encoding gene(s) of *S. marcescens*

To identify, clone, and sequence the RND pump-encoding gene(s) of *S. marcescens*, PCR primers were designed for two conserved motifs (approximately 600 amino acid residues apart) within the RND protein, and the PCR reaction was performed on the *S. marcescens* wild-type DNA (Chapters 4 and 5). The reaction resulted in two different products, with sequence homology studies revealing that both of these products were likely to be a part of RND pump-encoding genes. Using the two PCR products as DNA probes, a genomic library of *S. marcescens* was screened for the complete gene sequences. Two different RND pump complex-encoding operons were identified, subcloned, and sequenced. One operon was found to be encoding an MFP and RND pump and was named *sdeAB*, with the predicted molecular weight of SdeA 42.8 kDa, and that of SdeB 112.5 kDa. Phylogenetic analysis revealed that SdeB is more closely related to the MexF protein of *P. aeruginosa* than to any of the *E. coli* pumps. The other operon identified was found to encode an MFP and two different RND pumps and was named *sdeCDE* with predicted molecular weights of SdeC, SdeD, and SdeE 47.8 kDa, 111.5 kDa, and 110 kDa, respectively. Phylogenetic analysis demonstrated that SdeD and SdeE are very closely related to MdtB and MdtC proteins of *E. coli* and thus the SdeCDE is most likely a homolog of MdtABC system of *E. coli*.

Since no outer membrane component gene was found linked to either of the loci, the incomplete database of the *S. marcescens* genome sequencing project (Sanger Institute) was searched for the presence of a *tolC*-like gene. Once such gene was identified, primers were designed to PCR amplify the gene from *S. marcescens*, and the gene was cloned and sequenced (Chapter 6). The deduced amino acid sequence from the gene was found to be approximately 80% homologous to the *E. coli tolC* with a predicted molecular weight of 54 kDa.

Objective 3: To study the structure and function of *S. marcescens* RND pump(s)

Computer-aided structural analysis of the *S. marcescens* RND pumps was performed (Chapters 4 and 5). Transmembrane helices in SdeB, SdeD, and SdeE proteins were predicted using the PSIPRED server and von Heijne plots. Results showed a typical RND protein topology for all three RND pump proteins with the presence of 12 transmembrane helices with characteristically large periplasmic loops between helices 1 and 2, and 7 and 8.

Three-dimensional modeling of all three *S. marcescens* RND pump proteins was performed by using the CDD software of NCBI, with the structures showing a very high degree of homology to that of AcrB of *E. coli*.

Functional analysis of SdeAB and SdeCDE pumps was performed in an *acrB*-deletion mutant strain of *E. coli,* AG102MB (Chapters 4 and 5). It was observed that, upon introduction of the *sdeAB* locus, *E. coli* AG102MB demonstrated increased resistance not only to fluoroquinolones, but also to chloramphenicol, SDS, ethidium

bromide, and hexane. This established SdeAB as a multidrug efflux pump of *S. marcescens*.

Introduction of the *sdeCDE* locus to *E. coli* AG102MB, however, failed to confer resistance to any of the compounds tested, suggesting that either the SdeCDE pump does not efflux compounds tested or not expressed .

Structural analysis of the *S. marcescens* TolC, revealed a very similar structure to that of *E. coli* TolC (Chapter 6). To functionally characterize the *S. marcescens* TolC, it was introduced in a *tolC*-deletion mutant of *E. coli*, BL923. This resulted in an increase in resistance of *E. coli* BL923 to SDS and ethidium bromide. To examine whether the increase in resistance to ethidium bromide was a function of active efflux, ethidium bromide accumulation assays were performed. Data obtained established the role of *S. marcescens* TolC in proton gradient-dependent efflux. Since no other *tolC* homolog was found in the search of *S. marcescens* genome, *S. marcescens* TolC is most likely the outer membrane component of all functional RND pumps of this organism.

Objective 4: To assess the effect of clinical settings on the expression of *S. marcescens* RND pump-encoding gene(s) by studying the regulation of pump gene(s) expression

To assess the effect of clinical settings, i.e. the presence of antibiotics in the growth environment, on the emergence of MDR phenotype, Northern blot analysis was performed to measure the expression of *sdeAB* and *sdeCDE* in the fluoroquinolone-effluxing clinical isolate and lab-derived mutants of *S. marcescens*.

Results demonstrated that SdeAB was being over-expressed in two of the three mutant strains and also to some extent in the clinical isolate (Chapter 4). The data established that one or more RND pump-encoding genes are over-expressed if cells are exposed to antibiotics in their environment. While no over-expression of *sdeCDE* was observed in any of the strains tested, its over-expression in response to some other antimicrobial agent cannot be ruled out.

Since the *sdeAB* locus was found to be over-expressed in response to the presence of fluoroquinolones in the bacterial environment, the region upstream of this locus was examined for the presence of a regulatory gene. One such gene was identified, cloned, and sequenced (Chapter 7). The gene product was found to be approximately 40% similar to the MarA protein of *E. coli* that belongs to the AraC/XylS family of transcriptional activators, and was named SdeR. The sequence of *sdeR* was analyzed from strains of *S. marcescens* over-expressing *sdeAB*, but no changes were found. The intergenic region between *sdeA* and *sdeR* was also analyzed from different strains of *S. marcescens*. The clinical isolate T-861, and the mutant strain UOC-67WL have 27 different base pair changes within this region, 9 of which were within the putative SdeR binding region. However, UOC-67WLN, the other mutant overexpressing *sdeAB*, did not have of any mutations in this region, suggesting presence of an alternate regulatory protein, as seen in AcrAB-TolC pump of *E. coli*, expression of which is under the control of not only a local repressor AcrR but also a global regulator MarA (Grkovic *et al*, 2001).

In summary, work done in this thesis identifies two different RND proteins, SdeAB and SdeCDE in *S. marcescens*. SdeAB pump is characterized as a multidrug efflux pump. A TolC-like outer membrane component was also identified and characterized. Furthermore, a putative *E. coli* MarA-like regulator was also reported. Results obtained in this work will contribute to a better understanding of the multidrug resistance phenomenon in *S. marcescens*.

NINE

Future Studies

Future depends on what
you do in present.
-Mahatma Gandhi

This thesis describes the characterization of two different RND pumps in *S. marcescens*, SdeAB and SdeCDE along with a TolC-like outer membrane protein. A putative regulator of efflux pumps, SdeR, was also identified. This work has provided the groundwork needed to further characterize the role of efflux in the overall antibiotic resistance of *S. marcescens*.

9.1. Construction of *S. marcescens sdeAB* and *sdeCDE* knock-out strains

Deletion mutants of *S. marcescens* for SdeAB and SdeCDE pumps should be constructed using the technique of homologous recombination. My attempts to construct the deletion mutants were not successful because of various problems with the excision of suicide vector after its co-integration in the *S. marcescens* genome. Suicide vectors to knock out both of these pumps were created during the course of this work and are listed in Table 2.2. These vectors can be introduced in *S. marcescens* strains either by tri-parental mating or electroporation and a suitable culture media can be used to promote excision. The construction of SdeAB pump knock-out mutants would provide conclusive evidence as to the importance of this pump in *S. marcescens*. In order to meet this objective, SdeAB knock-outs should be constructed in at least one of the *sdeAB*-overexpressing mutant and the clinical isolate T-861, as it would be then possible to determine the effect of the knock out on the fluoroquinolone resistant strains of *S. marcescens*. These experiments may also provide evidence for a different kind of active pump in the clinical isolate, as suggested by fluoroquinolone accumulation assays and the Northern blot experiments. SdeCDE knock-out mutants should be constructed in order to provide a means of determining the potential substrates for this pump in *S. marcescens*.

9.2. Characterization of the SdeCDE pump

The SdeCDE pump is a multicomponent RND efflux pump. Its counterpart in *E. coli*, the MdtABC pump, has been shown to require both RND pumps (MdtB and MdtC) to be active. However, SdeCDE pump was not found to efflux any of the compounds tested in this work, suggesting that the pump may be inactive in the strains tested. Analysis of few hundred bases downstream of *sdeE* revealed that there may be a MFS pump-encoding gene present. Such a gene is present downstream of *mdtABC* locus as well, however it is not required for the functioning of pump. It will be interesting to investigate the role of MFS protein in the functioning of SdeCDE pump. Cosmid clones of *S. marcescens,* that contain the *sdeCDE* locus, can be screened for the presence of complete MFS-encoding gene sequence. If these attempts are not successful then the cosmid bank of *S. marcescens* can be screened for the MFS-encoding gene.

To understand the functioning of the SdeCDE pump, it is necessary to overexpress this pump as it seems to be expressed at a minimal level in *S. marcescens*. This can be done by growing *S. marcescens* UOC-67 in the presence of bile salts, as bile salts have been shown to be a substrate for MdtABC pump, it might result in the overexpression of *sdeCDE*. Expression can then be monitored by Northern blot. Once the pump is overexpressed, substrates for this pump can be determined by doing MIC experiments. The MdtABC system has been shown to be controlled by an adjacently encoded two-component system, and thus presence of a similar gene should be investigated close to the *sdeCDE* locus as well in order to understand the regulatory mechanism for this pump.

Once the substrates for this pump are determined, site-specific mutants can be created in order to understand the mechanism of functioning of multicomponent RND pumps. Candidates for site-directed mutagenesis can selected by the doing an alignment search between, MdtB, MdtC, SdeD, and SdeE, residues that are conserved between these four proteins but not in other RND pumps can be selected for these experiments. Results from such experiments will not only help in characterization of SdeCDE pump in *S. marcescens* but also other multicomponent pumps from other organisms.

9.3. Construction of the *S. marcescens tolC*-deletion mutant

Construction of *S. marcescens tolC*-deletion mutant will aid in further characterization of the gene product, and will also help in determining if it is the only outer membrane component of this organism, as suggested by the *S. marcescens* genome sequencing project. Deletion mutants should be constructed using the homologous recombination technique. Once the knock-outs are constructed, susceptibility of the strain to different antibiotics, particularly fluoroquinolones, can be measured by MICs and compared with that of strains with *sdeAB* knock-out strains. A comparable susceptibility of these two different knock-out strains will demonstrate whether or not *S. marcescens* TolC is the only outer membrane component of efflux pumps in this organism. Accumulation of ethidium bromide in real time can also be measured for the *S. marcescens tolC*-deletion and the parent strain. Complete abolition of efflux would indicate that it is the only outer-membrane component in *S. marcescens*.

9.4. Characterization of SdeR

Identification of SdeR is an important step in understanding the regulation of SdeAB pump. Since I was able to isolate *sdeAB* over-expressing mutants, these strains can be used to study the role of SdeR in the expression of *sdeAB*. Data from sequence analysis shows that it is most likely a transcriptional activator of *sdeAB*. A putative binding site of the protein was also identified, however this must be experimentally confirmed by nuclease protection assays. A His-tag clone of *sdeR* was constructed during the course of this work and it should be used to purify the SdeR protein, to be used for the nuclease protection assay. These assays will help identify the binding site for SdeR within the intergenic region of *sdeA* and *sdeR*. Although the sequence analysis revealed presence of only one putative binding site, it is highly likely that there are two binding sites present, one each for *sdeA* and *sdeR* genes. These sites can be determined accurately by using nuclease protection assays.

The plasmid pUCR1, containing *sdeR*, can be introduced in *S. marcescens* UOC-67 and its susceptibility to antibiotics measured and compared to that antibiotic susceptibility profile of *S. marcescens* UOC-67 without pUCR1. If UOC-67 confers an MDR phenotype upon over-expression of *sdeR*, as a result of introduction of the gene on a multicopy vector, the hypothesis that SdeR acts as an MDR activator can be confirmed. Further, Northern blot experiments should be performed for *sdeAB* from UOC-67 in absence and presence of pUCR1, this would confirm if SdeR is an activator of the SdeAB pump.

Once SdeR is confirmed to be an activator of MDR systems in *S. marcescens*, expression of porin genes should also be monitored upon over-expression of *sdeR* in order to confirm the possible global nature of this protein.

TEN

Bibliography

It is good to have an end to journey toward,
but, it is the journey that matters in the end
-Unknown

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