# The Role of OmpF and OmpC Outer Membrane Porins and RND Efflux Pumps in Antibiotic Resistance of *Serratia marcescens*

by

**Sanela Begic**

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

**Doctor of Philosophy**

Department of Microbiology

University of Manitoba

Winnipeg, Manitoba, Canada

# The Role of OmpF and OmpC Outer Membrane Porins and RND Efflux Pumps in Antibiotic Resistance of *Serratia marcescens*

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**Thesis Abstract**

*Serratia marcescens* is a Gram-negative enterobacterium that has become an important opportunistic pathogen, largely due to its high degree of natural antibiotic resistance. One factor contributing to this natural antibiotic resistance is reduced outer membrane permeability, which is controlled in part by OmpC and OmpF porin proteins.

To investigate the direct role of these porins in the diffusion of antibiotics across the outer membrane, an *ompF/ompC* porin-deficient strain of *S. marcescens* was created. Considerable similarity between the *S. marcescens* porins and those from other members of *Enterobacteriaceae* was detected by sequence alignment, with the exception of a change in a conserved region of the third external loop (L3) of the *S. marcescens* OmpC protein. *S. marcescens* OmpC has an aspartic acid, not glycine in position 112; methionine instead of aspartic acid in position 114 and glutamine in position 124, while in *S. marcescens* OmpF, this is a glycine. To investigate the role of amino acid positions 112, 114 and 124 and how the observed changes within OmpC porin may play a part in pore permeability, two OmpC sites have been altered to the *Enterobacteriaceae* consensus (D112G and M114D) through site-directed mutagenesis. Also, Q124G in OmpC and G124Q in OmpF and double mutants of these amino acid residues were constructed. Antibiotic accumulation assays and MICs of the strains harboring the mutated porins were performed, while liposome swelling experiments were performed on purified porins. Results demonstrated that the amino acid at position 114 is not responsible for either antibiotic size or ionic selection, the amino acid at position 112 is responsible for size selection only, and position 124 is involved in both, size and ionic selection.

Another factor contributing to natural antibiotic resistance is active efflux. Our group previously identified two Resistance-Nodulation-Cell Division (RND) efflux pump complexes; SdeAB and SdeCDE, and a TolC-like outer membrane protein (HasF). Additionally, a regulatory protein-encoding gene in the upstream region of the *sdeAB* was identified (*sdeR*), and found to be 40% homologous to MarA, an *E. coli* transcriptional regulator. To provide conclusive evidence as to the role of these components in *S. marcescens*, *sdeB*, *sdeD*, *hasF* and *sdeR* deletion mutants were constructed. The *sdeB*, *hasF* and *sdeR* knockout strains were consistently more susceptible to antibiotics than the parent strains, with the *sdeB/hasF* double knockout strain showing the highest susceptibility. A marked increase in fluoroquinolone (e.g. ciprofloxacin) accumulation was observed for strains deficient in either the *sdeB* or *hasF* genes when compared to the parental strains, with the highest ciprofloxacin accumulation observed for the *sdeB/hasF* double knockout. Due to the comparable susceptibility of the *sdeB* and the *hasF* individual knockouts, we concluded that *S. marcescens* HasF is the sole outer membrane component of the SdeAB pump and confirmed that SdeAB is the major efflux pump. In addition, minimum inhibitory concentration (MIC) data for *sdeR*-deficient and overexpressing strains indicate that SdeR is an activator of *sdeAB* and acts to enhance the overall multidrug resistance of *S. marcescens*. *sdeCDE* knockout strains showed no change in antibiotic susceptibility in comparison to the parental strains for any of the substrates with the exception of novobiocin. In addition, novobiocin was the only antibiotic to be accumulated by *sdeCDE* deficient strains. Our results demonstrate that SdeCDE is a highly specific pump belonging to the Resistance-Nodulation-Cell Division family.

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**List of Abbreviations**

| | |
|---|---|
| Å | Angstrom |
| ABC | ATP-Binding Cassette |
| Amp | Ampicillin |
| ATCC | American Type Culture Collection |
| ATP | Adenosine 5'-triphosphate |
| bp | base pairs |
| Car | Carbenicillin |
| CCCP | Carbonyl Cyanide m-Chlorophenylhydrazone |
| Cef | Cephalothin |
| Cfc | Cephaloridine |
| Chl | Chloramphenicol |
| Cip | Ciprofloxacin |
| Cla acid | Clavulanic acid |
| Da | Dalton |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DNP | dinitrophenyl |
| EDTA | Ethylenediamine-tetra-acetic acid |
| ESBL | Extended spectrum β–lactamases |
| EtBr | Ethidium Bromide |
| g | gram(s) |
| HTH | Helix-turn-helix |
| IHF | Integration host factor |
| kb | kilobases |
| kDa | kiloDalton |
| Km | Kanamycin |
| L | Litre |
| M | Molar |
| 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) |
| ml | millilitre |
| mM | millimolar |
| Nal | Nalidixic acid |
| ng | nanogram |
| Nor | Norfloxacin |
| Nov | Novobiocin |

| | |
|---|---|
| OBS | OmpR binding sites |
| Ofl | Ofloxacin |
| OMF | Outer membrane factor |
| ONPG | ortho-nitrophenyl-b-D-galactopyranoside |
| ORF | Open reading frame |
| PAGE | Polyacrylamide gel electrophoresis |
| PBP | Penicillin binding protein |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| QRDR | Quinolone resistance determining region |
| RNA | Ribonucleic acid |
| RND | Resistance-nodulation-cell division |
| SDS | Sodium dodecyl sulfate |
| SMR | Small multidrug resistance |
| Str | Streptomycin |
| TMS | Transmembrane spanner |
| v/v | volume/volume |
| w/v | weight/volume |
| xg | Relative centrifugal force represented by 'x' multiplication of '*g*' Earth's gravitational force |
| µg | microgram |
| µl | microlitre |
| µmole | micromole |

## CHAPTER 1 Literature Review

### 1.1 Introduction to *Serratia marcescens*

*Serratia marcescens* is a Gram-negative facultative anaerobic rod-shaped bacterium belonging to the *Enterobacteriaceae* family (Grimont and Grimont 1984). It is motile by peritrichous flagella and found in soils, water and on plant surfaces. It is now recognized as an opportunistic nosocomial human pathogen responsible for urinary tract infections, pneumonia, and other respiratory infections, meningitis, endocarditis, and septicemia (Hejazi and Falkiner 1997). Bacteraemia, as a result of *S. marcescens* infections, occurs in surgical patients or those in intensive care units, while endocarditis is more common in intravenous drug users (Brouqui and Raoult 2001).

Historically, *S. marcescens* has often been erroneously identified due to a common misconception that it always produces a red pigment (prodigiosin) (Franczek at al. 1986); however, most clinical isolates do not produce this pigment (Holmes and Gross 1990). During the 1960's, the incidence of *S. marcescens* infections increased making it the second most common cause of nosocomial infections after *Escherichia coli*. This is due in part to *S. marcescens* being able to adhere to medical devices, such as catheters (Saito et al. 1989). 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 and 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 study reported that neonatal lower respiratory tract

infections were caused most often by *S. marcescens* (Alberts et al. 2001). Although *S. marcescens* is not a part of the normal conjuctival flora, its capability to survive in contact lens solutions leads to a variety of ocular infections, including purulent conjunctivitis, keratitis, corneal ulcers and endophthalmitis (Atlee et al. 1970; Duffey 1995; Johnson et al. 1992).

For purposes of my work, I have selected to use the ATCC 13880 *S. marcescens* wild type strain from the American Type Culture Collection, also designated as UOC-67. This strain was selected for our work before wild type strain Db11 was sequenced by the Sanger Institute (http://www.sanger.ac.uk/Projects/S_marcescens/). UOC-67 was initially isolated from pond water and has several additional designations, such as CDC 813-60, NCIB 9155 and NCTC 10211. I had selected it initially for easier comparisons to other *S. marcescens* literature that had used this particular wild type strain in their research and because at the time, it was unclear which strain would be sequenced. Likewise, in Chapters 4 and 5, I had selected to use clinical isolate designated as T-861 received from the lab of Dr. Darryl Hoban (University of Manitoba, Department of Medical Microbiology) rather than other clinical isolates, because T-861 showed the highest resistance to fluoroquinolones within our experimental parameters.

**1.2 Antibiotic resistance in *S. marcescens***

*S. marcescens* is recognized for its high antibiotic resistance (Kumar and Worobec 2003; Fujimaki et al. 1989). In the 1960's, reports indicated that gentamicin was the only effective antibiotic therapy for many *S. marcescens* infections. However, by the 1980's, the frequency of the infections declined and successful treatment of the infections improved with the introduction of newer antibiotics, such as cephalosporins

and imipenem (Saito et al. 1989). More recently, *S. marcescens* strains have been identified with the capability to resist many of these newer agents and it is feared that most of the infections will soon not be treatable (Hejazi and Falkiner 1997). Most strains of *S. marcescens* seem to be intrinsically resistant to ampicillin, cefuroxime, and tetracycline, just to name a few agents (Lambert and O'Grady 1992). Quinolone resistance was reported almost immediately after these drugs came into use (Fujimaki et al. 1989).

Generally, the basis of the antibiotic resistance is multifactorial, involving different mechanisms for each group of antibiotics. For example, resistance to β–lactam antibiotics is achieved by outer membrane permeability mediated by porins, β-lactamases found in the periplasm which degrade these drugs, and inner membrane penicillin-binding proteins, which are the target for these antibiotics. Alterations in any of these components can result in antibiotic resistance. Likewise, resistance to quinolones can be a result of mutations in DNA gyrase, the target enzyme for this group of antibiotics, and/or over-expression of multi-drug resistance efflux pumps. This thesis describes the analysis of two of these resistance mechanisms: a) decreased outer membrane permeability; and b) resistance-nodulation-cell division (RND) pump mediated active efflux.

### 1.2.1 β–lactam Resistance

#### 1.2.1.1 β-lactamases

Penicillin was the first β–lactam to be used clinically for treatment of *S. marcescens* infections. β-lactams function by preventing cell wall synthesis, specifically, by inhibiting transpeptidation, the last step of peptidoglycan maturation (Lambert 1992).

The targets of the β–lactam are transpeptidase and carboxypeptidase enzymes, commonly called penicillin binding proteins (PBPs). Resistance to these agents is caused in part by β–lactamases, enzymes that cleave the β–lactam ring of the antibiotic molecule. Various strains of *S. marcescens* are 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. Metallo-β-lactamases contain two zinc atoms at the active site and are distinguished by EDTA-mediated inhibitions. *S. marcescens* metallo-β-lactamases are encoded by plasmids (Ito et al. 1995). However, chromosomally encoded serine-based β–lactamases, SME-1 and SME-2, have also been reported in some strains of *S. marcescens* (Queenan et al. 2000). These enzymes are known to confer resistance to both the traditional β–lactamases and cephalosporins. ESBLs have been found in different species of members of the family *Enterobacteriaceae, P. aeruginosa, Haemophilus influenzae*, and *Neisseria gonorrhoeae* as well as in *S. marcescens* (Bradford 2001). This group of β–lactamases is characterized by inhibition by clavulanic acid (Bush et al. 1995).

**1.2.1.2 PBP-mediated resistance**

This is a less common mechanism of β–lactam inactivation (Spratt 1994), which may partially be due to the necessity to reduce β–lactam affinity without a major reduction in substrate affinity. This mechanism is important in many Gram-positive staphylococcal (Song et al. 1987) and streptococcal (Dowson et al. 1989) organisms and in some Gram-negative bacterial species of *Neisseria* (Spratt et al. 1989) and *Haemophilus* (Clairoux et al. 1992). Generally, single amino acid mutations do not result in resistance. An altered PBP is thought to result from homologous recombination events

between different PBPs (Spratt 1994). Since there are multiple PBP targets for β–lactams, it is harder to achieve resistance through this mechanism.

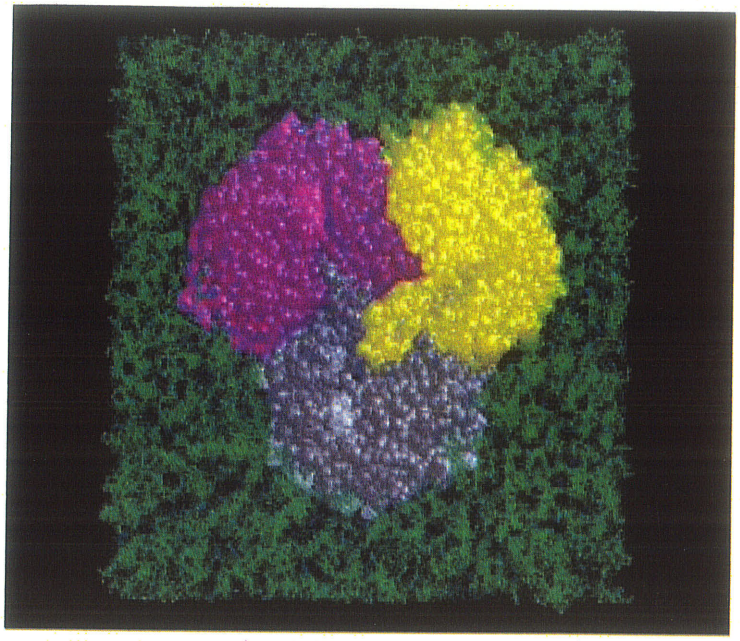
### 1.2.1.3 Outer membrane permeability and porins

Penicillins and cephalosporins are the most commonly used classes of β–lactam antibiotics against *S. marcescens* infections. For these agents 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 amongst others (Nikaido and Nakae 1979) into and out of the cell's periplasm. 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 DNA or amino acid homologies, most have 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 zig-zags 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) revealed a conserved 16-strand anti-parallel β–barrel structure for each monomer (Cowan et al. 1992), implicating the role of a long loop, or eyelet region, which extends into the porin channel directing the channel size and ion selectivity (Cowan et al. 1992).

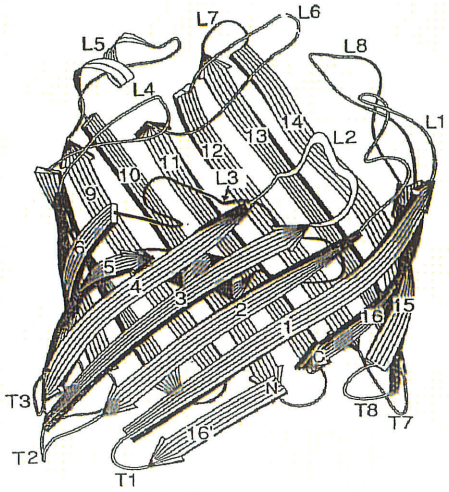
Porins are classified as either non-specific or specific. Non-specific porins act as general diffusion pores, while specific porins possess binding sites for specific solutes (Hancock 1991; Nikaido 1992). In general, it is usually the non-specific porins that are involved in the passage of β–lactam antibiotics (*E. coli* OmpF/C (Zimmermann 1980);

**Figure 1.1.** Three-dimensional structure of the OmpF porin of *E. coli* (Neuwald et al. 1995). (a) The OmpF trimer seen from "above". Porin occurs as a trimer, a protein made up of three identical units. These units are colored differently in the picture. The green spheres are the head groups of the lipids, the thin green wires their tails. (b) Three-dimensional figure of the *E.coli* OmpF porin. Beta-strands are designated by thick arrows while loops are shown as thin strands. The extracellular side of the porin would be at the top of the figure. The third external loop is designated L3 and can be seen folding down into the pore lumen.

a)



b)


L7
L6
L5
L8
L4
L1
14
13
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10
L3
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6
5
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3
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1
16
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N
T3
T8
T7
16'
T2
T1

OprF of *P. aeruginosa* (Angus et al. 1982)) and changes in porin copy number, size, selectivity, or function that can alter the rate of diffusion of hydrophilic β–lactam antibiotics (Nikaido and 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 different 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 and 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 also been identified in many other Gram-negative organisms (Hancock 1986), including *S. marcescens* (Hutsul 1996).

### 1.2.2 Aminoglycoside resistance

Gentamicin, tobramycin, amikacin and streptromycin are some of the common aminoglycosides used to treat bacterial infections. These agents 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

ribosome binding. Resistance to aminoglycosides is widespread, with more than 50 aminoglycoside-modifying enzymes identified to date (Fluit et al. 2001). These 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 grouped 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).

**1.2.3 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 A2B2 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 Regions (QRDR), while the GyrB subunit is responsible for ATP hydrolysis and capturing of the DNA strand (Heddle and Maxwell 2002). Resistance to quinolones generally arises from mutations in the QRDR of *gyrA*

(Yoshida et al.1990). 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 found in *S. marcescens*, displaying the greatest diversity of mutations in the *gyrA* gene (Fujimaki et al. 1989). Three mutations in *gyrA* have been identified: 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 which is discussed in detail in Section 1.6.

**1.3 *E. coli* porins**

In 1976, a 36.5 kDa outer membrane protein was identified to be responsible for the non-specific permeability of outer membranes in *E. coli* B (Nakae 1976). It was decided that it be called a porin, due to its apparent pore-forming property.

Porins form water-filled open channels that span the outer membrane of Gram-negative bacteria. As mentioned previously, porins may be classified as non-specific or specific and distinguished into trimeric and monomeric groups. Most porins form trimers to form a functioning unit which form such a strong association that it requires a heating at temperatures above 80°C for 10 minutes in SDS for dissociation to occur (Benz 1988).

Porins have been identified in many Gram-negative bacteria including: *Salmonella typhi* (Puente et al 1989), *P. aeruginosa* (Nikaido et al 1991), *H. influenzae* (Burns and Smith 1987), *Neisseria* (Barlow et al. 1989), *Bordetella pertussis* (Li et al. 1991), and *Pasteurella multocida* (Chevalier et al. 1993).

The porin originally described by Nakae (1976) in *E. coli* B is now known as the OmpF porin. A second homologous porin known as OmpC is also produced by *E. coli* K-

12 strains (Mizuno et al. 1983). These two types of porins are present in high copy numbers and are major constituents of the outer membrane. The total number of OmpF and OmpC porins in the *E.coli* outer membrane remains approximately constant and are reciprocally regulated such that as the number of one porin type increases, the number of the other porin decreased (Csonka 1989). These porins are similar in amino acid sequence and structure and both function as cation selective pores (Mizuno et al. 1983). The molecular weight exclusion limit of OmpF, OmpC and related porins of this type is about 600 Da (Decad and Nikaido 1976; Basle et al. 2006).

A third general diffusion porin, PhoE, can be produced by *E. coli*. PhoE is also similar in sequence, size and structure to the OmpF/C belonging to the same family of porins (Jeanteur et al. 1991); however it is anion selective and is produced as part of the *pho* regulon under phosphate-limited conditions (Tommassen et al. 1987). Although its expression is regulated in response to phosphate it does not have a phosphate binding site (Bauer et al. 1988) and will allow selective passage of any anion within its exclusion limit.

LamB, ScrY and Tsx are examples of specific porins in *E. coli*, and OmpA is an example of a monomeric porin. LamB, also known as maltoporin, is expressed in response to maltose and facilitates the diffusion of maltose and maltodextrins (Charbit et al. 1994). Tsx is a nucleoside specific channel (Fsihi et al. 1993). ScrY is a plasmid encoded sucrose specific porin (Schulein et al. 1991). All three of these specific channels also exhibit some general diffusion activity. The OmpA channel forms a pore similar in diameter to the OmpF/C porins, however, it has a much lower permeability rate. This is believed to be due to the possibility of open and closed states (Sugawara and Nikaido

1994). It is estimated that only 2-3% of OmpA channels are open at a given time. The contribution of OmpA to *E. coli's* membrane permeability is thought to be minimal since most diffusion would occur through the OmpF and OmpC porins (Sugawara and Nikaido 1994).

The crystallized structure of the *E. coli* OmpF porin (Cowan et al. 1992) revealed that each porin barrel is formed by 16 anti-parallel β-strands. The barrel of the *E.coli* porins is a pseudocyclic structure since there is a non-covalent interaction between the N and C termini. Short turns are formed on the periplasmic side, while longer turns form on the external side. The third external loop is important as it functions to constrict the pore size by folding inward. The pore is constricted at about half the height of the barrel by the third loop and once past this constriction zone, it opens up. The diameter of the channel at the constriction zone measures 7 x 11 Å. The design of the channel is advantageous because the restriction allows for size selection with less effect on diffusion rate by limiting it to only a small portion. Within the porin barrel area are a number of charged residues that also function to constrict the pore lumen. Positive groups on one side of the wall attract negative groups on the other to pull out side chains into the pore lumen (Nikaido 1994).

Although OmpF porins exist as a trimer, the crystallization data showed that each monomer has one pore, therefore there are three distinct pores per functional porin (Cowan et al. 1992). Trimer association is strong because R groups of monomeric units interdigitate with the other two monomers. Also, the second loop reaches over to "grasp" the neighbouring subunit at the site where the third loop is depressed as it reaches down into the channel. There are no hydrophobic stretches like in the inner membrane proteins

(alpha-helix). This may be related to the method of membrane protein translocation since hydrophobic proteins would be predicted to stop in the inner membrane (Singer 1990). Instead, the amphipathic beta-strand structure is characteristic of porins. Mostly hydrophilic amino acid groups face inward (water-filled channels) and hydrophobic groups face out (lipid bilayer) (Cowan et al. 1992). However, outer surface amino acids involved in intermolecular associations to form the trimer need not be hydrophobic.

Various *E. coli* mutants with altered porins have been informative in determining amino acid residues involved in porin function. Single site mutants and large deletions have been characterized that can be classified according to effect on function in proper folding, outer membrane insertion and/or stability of the porin, or in determining pore characteristics. For instance, the C-terminal phenylalanine present not only in porins, but practically all outer membrane proteins sequenced, has been shown to be important in outer membrane assembly/insertion (Struyve et al. 1991).

The third loop, by folding down into the pore, is important in determining size and ion selectivity of respective porins. A number of the various *E. coli* porin mutants have had alterations involving the third loop. For example, the anion selectivity of the PhoE porin was shown to be primarily due to a lysine residue within the third loop not present in OmpF or OmpC (Bauer et al. 1989). An important feature of the third loop is the motif PEFGG, which is believed to be important in turn formation at this site in the loop and appears to be conserved across enterobacterial porins. The Glycine-144 to aspartic acid mutation characterized by Fourel et al (1993) occurred in the PEFGG motif, changing it to PEFDG. As expected, this affected porin stability and pore size (Jeanteur et al. 1994).

An interesting phenomenon displayed by the classical porins is known as voltage gating. Application of voltage above a threshold value appeared to reversibly close channels inserted in a planar lipid bilayer (Morgan et al. 1990; Schindler and Rosenbusch 1978; Xu et al 1986). Other groups have found that the channels close infrequently even without the application of a potential across the bilayer (Benz et al. 1978). It is debated as to what significance this observation has *in vivo*. The only potential across the outer membrane that is known is the Donnan potential which was shown not to trigger channel closing or opening (Sen et al. 1988). This aspect of porin character has yet to be fully discovered.

Two pore sizes have been shown to occur in each of the *E. coli* OmpF and OmpC porins in response to external pH. Smaller and larger channels are recorded at lower and higher pH, respectively (Todt et al. 1992). The switch occurs around neutral pH and a histidine was suspected as chemical mutagenesis of the only histidine in OmpF and OmpC, H-21, resulted in a higher proportion of larger channels regardless of pH (Todt et al. 1992). This suspected a role for histidine-21 in the pH-induced switch in channel size, possibly through an interaction with the third loop in the pore. However, crystallographic data demonstrated that the H-21 residue was present on the external surface interacting with another monomer unit (Cowan et al. 1992), making it unclear as to how it would be able to function in regulating pore size.

### 1.4 *Serratia marcescens* porins

Two porins, OmpC and OmpF, have been identified in UOC-67 *S. marcescens* strain (Hutsul and Worobec 1994; Hutsul and Worobec 1997). OmpC has a molecular weight of 40 kDa while OmpF is a 41 kDa protein. The two structural genes have been

cloned and sequenced. Similarly, others have reported two major porins, namely Omp1 and Omp2, which are osmoregulated in a similar way to the *E. coli* OmpF and OmpC (Sanchez et al. 1997) and a smaller Omp3 porin at 39 kDa (Ruiz et al. 2003). Based on the size similarity, osmoregulation and sequence similarities, *S. marcescens* OmpF is therefore also referred to as Omp1 and OmpC as Omp2. Although, the OmpC porin of *S. marcescens*, at an amino acid level, shows an approximately 60% homology to other enterobacterial porins, a notable difference was found in the eyelet region, which instead of the conserved PEFGG(D) motif, has the sequence PEFDGM. With this in mind, in an earlier study, D112 and M114 were selected for individual alteration to glycine (G) and aspartic acid (D), respectively, to reflect the enterobacterial consensus sequence. Individual and double mutations of these sites were transformed into the *E. coli* porin deficient strain, BZB1107 (Hutsul 1996). The relative permeability of *S. marcescens* wild-type OmpF and OmpC and the mutant *S. marcescens* OmpC/OmpF porins to cephaloridine, cephalothin, cefotaxime and glucose were tested using the liposome swelling assay following the protocol described in Nikaido *et al.* 1991. Liposome swelling assays demonstrated that the *S. marcescens* OmpF and the OmpC with the 112 residue change both allowed more rapid diffusion of uncharged solutes than the wild type OmpC porin. This mutation appeared to increase the permeability of OmpC to that detected for enterobacterial porins having the consensus sequence. Similar results have been reported for *Enterobacter aerogenes* (De et al. 2001; Bornet et al., 2004) 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 ionic selection. No change in the permeability

of the porin resulted from the change of methionine to aspartate at position 114 (Hutsul 1996). The production of the double mutation $D^{112}G$ and $M^{114}D$ has shown that the simultaneous presence of both mutations has a combined effect on ion selectivity resembling that of the wild-type OmpC (Famobio 2005).

### 1.5 Regulation of the *E. coli* OmpF and OmpC porins

#### 1.5.1 Introduction

*E. coli* OmpF and OmpC porins are regulated in response to external osmolarity and temperature (Csonka 1989). The total number of the two types of porins remains constant for the most part while the ratios between the two vary. With increasing growth temperature and medium osmolarity, the membrane levels of OmpC increases while OmpF decreases and vice versa. The significance of this response is thought to be related to the two environments that *E. coli* may find itself in; the animal host and the external environment. In the nutrient rich, but hostile environments of the animal gut, the less permeable porin, OmpC, predominates. However, where nutrients are scarce and the environment is less hostile, OmpF will be the major porin.

In addition to osmolarity and temperature, other conditions such as, oxidative stress (Greenberg et al. 1991), pH (Thomas and Booth 1992) and the presence of salicylate (Rosner et al. 1991), tetracycline and chloramphenicol (Cohen et al. 1988), have been shown to regulate the levels of the OmpF and/or OmpC porins.

Two major mechanisms involved in the porin regulation have been characterized in *E. coli.* These include the OmpR/EnvZ system and the *micF* system.

### 1.5.2 The EnvZ/OmpR system

The *ompB* locus is postulated to be involved in the regulation of the structural genes, *ompF* and *ompC*. This was supported by studies using *lacZ-ompC/F* fusions and *ompB* mutants (Hall and Silhavy 1981). Further characterization of this locus demonstrated two gene products, *ompR* and *envZ* (Hall and Silhavy 1981). From results of complementation experiments, it was suggested that the OmpR protein was a transcriptional activator of the two porin genes while EnvZ encoded an envelope protein responsible for "sensing" the environmental osmolarity. The EnvZ/OmpR system is a two component histidine kinase regulatory system (Hall and Silhavy 1981). The system functions by modulating the activity of a transcriptional regulator protein through phoshorylation and dephosphorylation. In this system, EnvZ is the modulator and OmpR the transcriptional regulator.

The EnvZ transmembrane sensor has two membrane spanning segments. The N-terminus begins in the cytoplasm, spans the membrane once leaving the majority of the amino-half of the protein in the periplasm before spanning the membrane one more time. The C-terminal end is in the cytoplasm and possesses both phosphatase and kinase activities. The way in which the EnvZ histidine kinase exactly 'senses' the osmolarity is still relatively unknown as the sensing domain has not been fully identified (Roberts et al. 1994).

The second function of EnvZ is the modulation of OmpR activity. In the presence of ATP, the EnvZ autophosphorylates at its Histidine-243 (His-243) residue (Roberts et al. 1994). The phosphate group is transferred to OmpR (the Asp-55 site on N-terminus) which activates it for transcriptional activation (Aiba et al. 1989). The phosphorylation of

OmpR facilitates its binding to the operator regions of the *ompF* and *ompC* genes and activates transcription of each. EnvZ will also remove the phosphate group from OmpR through its phosphatase activity. The level of OmpR-P is then achieved through the modulation of the kinase and phosphatase activities of EnvZ (Russo and Silhavy 1991).

Six OmpR molecules bind to the -100 to -40 region of *ompF* at three consecutive 20 bp binding units, referred to as F1, F2 and F3, with varying affinity (Harlocker et al. 1995). The F1 unit has the strongest affinity and most resembles the consensus sequence (Fig. 1.2a) (Harlocker et al. 1995). Binding of OmpR to the subsequent sites requires prior binding of OmpR to the first of the 20 bp units. OmpR bound to all three sites stabilize each other (Harlocker et al. 1995). It has been suggested that cooperative binding ensures that OmpR binds to high affinity sites before low affinity sites. The ability of OmpR to bind to sites in a hierarchical manner allows differential expression in different conditions (Huang et al. 1997). At low levels of OmpR-P, the higher affinity sites will be occupied allowing transcription of *ompF* (Fig. 1.2b). As the levels of OmpR-P rise, the lower affinity sites of *ompC* will be bound and *ompC* will be activated. However, the levels of OmpF are lower at high osmolarity (high OmpR-P), therefore this mechanism does not account for the repression of the *ompF* gene. The two additional OmpR-binding sites (OBS) found upstream at -380 to -360 are essential for the negative expression of OmpF (Huang et al. 1994). OmpR will bind these lower affinity sites and repress *ompF* transcription. Binding at these sites requires prior binding at the downstream sequences which indicates interaction between the OmpR molecules. This interaction is accomplished because the DNA can bend at poly-T sites causing a loop to form between the OmpR complexes. This bending of the DNA is facilitated by the *E. coli*

integration host factor protein which upon binding DNA causes a change in DNA conformation (Ramani et al. 1992). The exact mechanism of repression through this interaction is not fully understood.

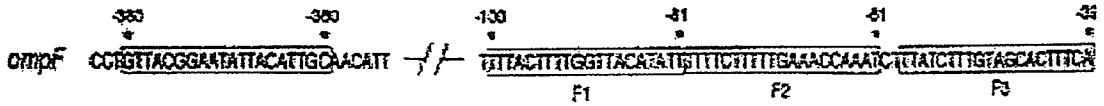
### 1.5.3 *micF*

A 93-nucleotide RNA called *micF* was first discovered when a cloned fragment of DNA upstream of *ompC* inhibited the production of OmpF (Mizuno et al. 1984). Further analysis demonstrated a short RNA transcript encoded immediately upstream of *ompC* that is transcribed in the opposite direction. The transcript was found to be 70% complementary to the 5' end of *ompF* mRNA and proposed to hybridize with the *ompF* transcript and inhibit translation (Fig. 1.2c) (Mizuno et al. 1994; Chen et al. 2004). *micF* was shown to decrease the levels of *ompF* mRNA when expressed in high copy number (Mizuno et al. 1984) by destabilizing the *ompF* transcript by allowing accessibility of certain ribonucleases (Schmidt and Delihas 1995).

*micF* appears to be under the control of the *ompB* locus via the same OmpR binding sites as *ompC* (Mizuno et al. 1984). It has been shown that it plays a major role even in osmoregulation but only at low-to-intermediate levels of osmolarity as the OmpR/EnvZ system plays such a strong influence here. *micF* levels also increase in response to salicylate (Rosner et al. 1991), chloramphenicol, tetracycline, ethanol, oxidative stress and increased temperature (Andersen et al. 1989) and thereby this results in reduced OmpF levels in the membrane.

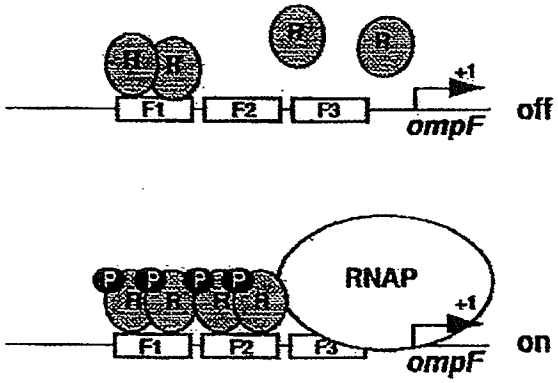
**Figure 1.2.** (a, b) Model of the OmpR-binding sites within the *E. coli ompF* gene and (c, d) proposed formation of MicC-*ompC* and MicF-*ompF* duplexes. a) F1 unit within *ompF* most strongly resembles the consensus sequence (Harlocker et al. 1995); b) Although occupancy of the F2 site may occur in the absence of OmpR phosphorylation, this occupancy does not seem to be sufficient to active *ompF* transcription. In contrast, phosphorylation of OmpR results in increased occupancy of the F2 site due to the stimulation of cooperative interactions. Therefore, increased occupancy of the F2 site is responsible for the observed activation of *ompF* transcription (Huang et al. 1997); c) Base-pairing between wild-type *ompF* mRNA and wild-type MicF RNA of the *E. coli* genome (Chen et al. 2004); d) Base-pairing between wild-type *ompC* mRNA and wild-type MicC RNA of the *E. coli* genome (Chen et al. 2004). Ribosome-binding sites (RBS) and start codons for *ompC* and *ompF* are underlined.

a)




Consensus TTTACTTTTTGNNACATNNN

b)




c) **wt *ompF* mRNA**

RBS start codon

5′.....AUAAAAAAAACCAUGAGGGUAAUAAAUAAUGAUGAAGCGCAAUAU.....

UUUACUUACUGCCAUUAUUUAUUACUACUUCG–5′ **wt MicF RNA**

d) **wt *ompC* mRNA**

RBS start codon

5′.....CAUAAAAAAGCAAAUAAAGGCAUAUAACAGAGGGUUAAUAACAUGAAAGUUAAA.....

UUGUCUUU UAUUUU UUAUUUCCGUAUAUUG–5′ **wt MicC RNA**

### 1.5.4 *micC*

A 109-nucleotide RNA called *micC* was recently identified which contributes to the regulation of synthesis of OmpC (Chen et al. 2004). The *E. coli micC* gene is located between the *ompN* porin gene and a gene of unknown function (*ydbK*) (Chen et al. 2004). MicC inhibits *ompC* expression at the post-transcriptional level by an antisense mechanism that involves the formation of base pairs between 22 nucleotides at the 5' end of MicC and nucleotides just before the ribosome binding site of the *ompC* mRNA (Fig. 1.2d) (Chen et al. 2004). Parallels can be drawn between between the 109-nucleotide MicC RNA and the 93-nucleotide MicF RNA. Both repress the expression of porins by base pairing near the ribosome binding site, essentially blocking translation. Both are also encoded opposite other porin genes in *E. coli*.

MicC and MicF RNAs are generally expressed under different environmental conditions. MicF levels are elevated under most conditions in which MicC levels are low, while MicC levels are highest under those conditions in which MicF levels are low. These observations are in agreement with the previous finding that OmpF and OmpC show reciprocal expression under many different conditions (Csonka 1989). When examined in sequence, the base pairing between the MicC and MicF RNAs and their targets is more extensive than that observed for most of the *E. coli* small RNAs that act by base pairing. The *ompF* mRNA-MicF RNA duplex has been enzymatically and chemically characterized, and the region of base pairing was found to encompass 24 nucleotides (Chen et al. 2004). The *ompC* mRNA-MicC RNA duplex, on the other hand, involves 16 contiguous nucleotides just before the *ompC* ribosome binding site in the upstream direction and an additional 6 nucleotides further upstream. The research to

determine the advantage of antisense regulation for controlling the synthesis of outer membrane proteins is on-going.

**1.6 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. In this type of resistance, there is no alteration or degradation of the antibiotic. The phenomenon of MDR was first described by Levy and colleagues in 1980 (McMurry et al. 1980) as being responsible for tetracycline resistance in *E. coli*. The MDR phenotype is associated with over-expression of transporters that pump out (efflux) a broad range of structurally unrelated compounds from the cells in an energy-dependent manner. The resistance of bacterial cells to antibiotics is often a complex phenomenon involving the combination of reduced influx and increased efflux across the cell envelope. Analysis of various available genome sequences has shown that known and putative drug efflux transporters constitute about 6-18% of the genome of all the organisms sequenced to date (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 presently classified into five families (Fig. 1.3): a) Major Facilitator Superfamily (MFS) (Marger and Saier 1993); b) ATP Binding Cassette (ABC) superfamily (van Veen and Konings 1998); c) Small

Multidrug Resistance (SMR) family (Paulsen et al. 1996); d) Resistance-Nodulation-Cell-Division (RND) family (Saier et al. 1994) and e) Multidrug And Toxic Compound Extrusion (MATE) family (Brown et al. 1999).

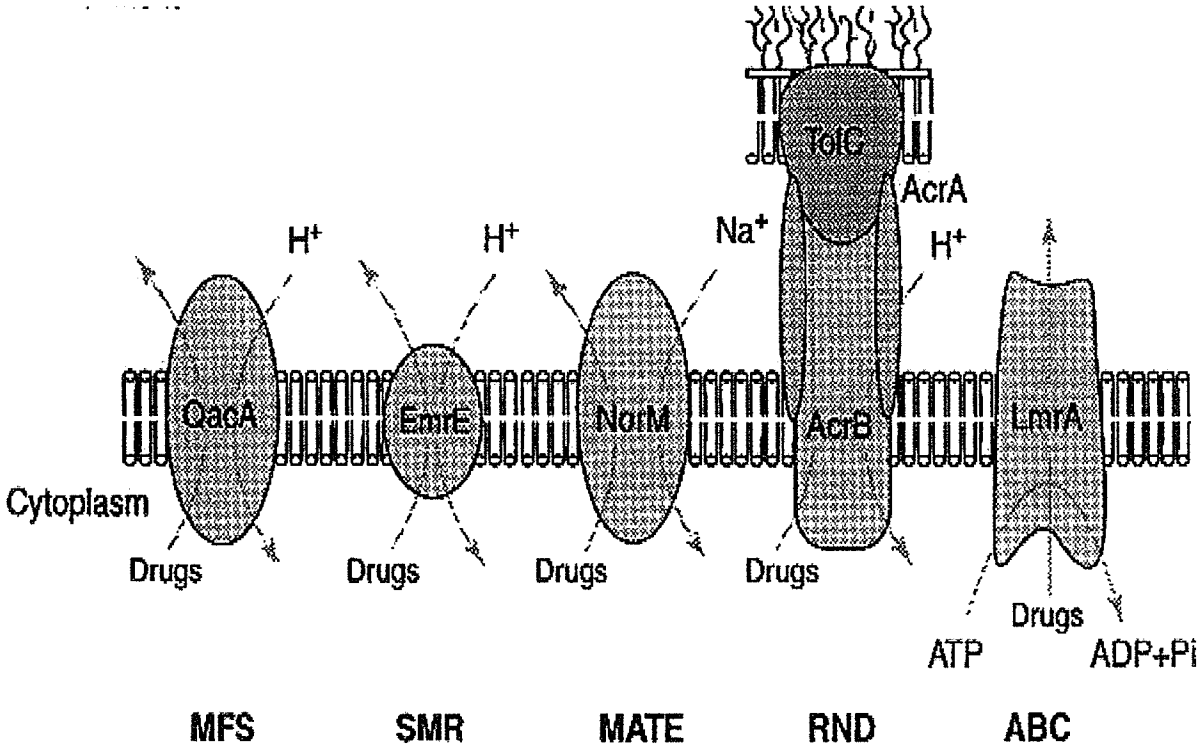
### 1.6.1 Major Facilitator Superfamily (MFS)

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 proton-motive force. Six families of the MFS are known to export drugs, two of which are prokaryotie-specific, two are eukaryotie-specific, and two are ubiquitous (Saier and Paulsen 2001). These transporters usually function as single-component pumps such as NorA pump of *Staphylococcus aureus* (Yoshida et al. 1990), but in Gram-negative bacteria they function with the membrane fusion protein (MFP), and outer membrane protein (OMP) components, an example being the EmrAB-TolC pump of *E. coli* (Lomovskaya and Lewis 1992). Drug pumps belonging to this superfamily usually belong to families consisting of 12- or 14-transmembrane spanners (TMS) (Pao et al. 1998).

### 1.6.2 ATP-Binding Cassette (ABC) Superfamily

ABC transporters contain 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 and 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

**Figure 1.3.** Diagrammatic representation of multidrug efflux pumps belonging to 5 different families with their representatives (Paulsen 2003).


TolC
AcrA
H+
H+
Na+
H+
QacA
EmrE
NorM
AcrB
LmrA
Cytoplasm
Drugs
Drugs
Drugs
Drugs
Drugs
ATP
ADP+Pi
MFS
SMR
MATE
RND
ABC

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.

### 1.6.3 Small Multidrug Resistance (SMR) Family

Transporters belonging to the SMR family contain only about 110 amino acid residues and 4 TMSs. These use the proton-motive force as an energy source and function as tetramers (Ma and Chang 2004). Some of the well-characterized pumps of this family include the Smr pumps of Staphylococcus aureus (Grinius et al. 1992) and EmrE pump of E. coli (Schuldiner et al. 1997). These bind a vast range of substrates, with a preference for cationic, lipophilic antibiotics.

### 1.6.4 Multidrug and Toxic Compound Extrusion (MATE) Family

In spite of similar membrane topology, these proteins show no sequence homology to members of MFS. These use Na+ gradient as the energy source to pump out cationic dyes and fluoroquinolones (Morita et al. 1998). Examples of proteins belonging to this family include NorM of *Vibrio parahaemolyticus* and YdhE of *E. coli* (Morita et al. 1998).

### 1.6.5 Resistance-Nodulation-Cell Division (RND) Superfamily

Proteins belonging to the RND superfamily were initially thought to be present only in bacteria, however, they have now been reported in eukaryotes and archaea (Tseng et al. 1999). These are all, except for one, chromosomally-encoded (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 consist of 12 TMSs and function by forming complexes with a MFP and OMP in Gram-negative organisms. There are 2 large periplasmic loops between TMSs 1 and 2 and TMSs 7 and 8 (Fujihira et al. 2002). N-terminal halves of RND family proteins are homologous to the C-terminal halves, and therefore proteins are believed to have risen from an intragenic tandem duplication event that occurred in the primordial system before the divergence of the family members.

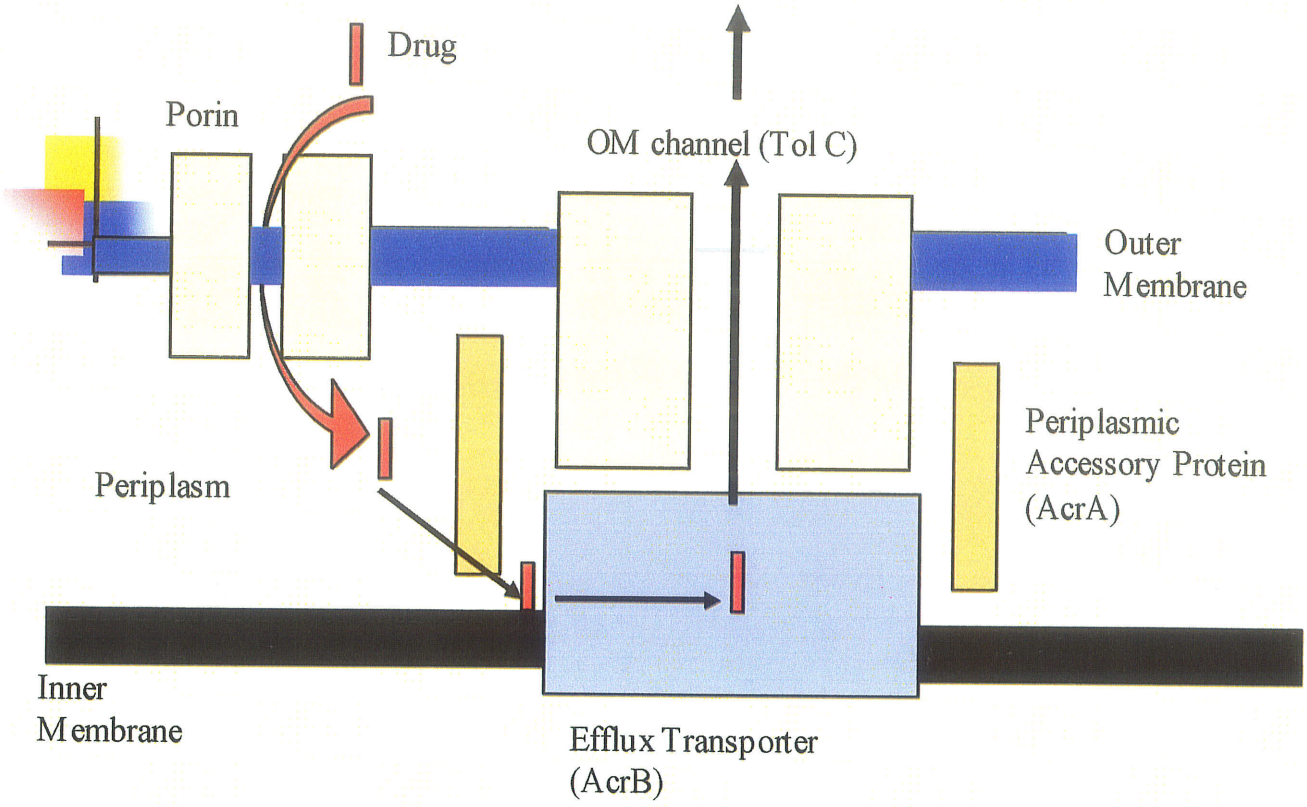
Three major criteria (energy source, phylogenetic relationship, and substrate specificity) were used to divide the RND superfamily into seven families: Heavy Metal Efflux (HME) family (Gram negative bacteria), Hydrophobe/Amphiphile Efflux-1 (HAE-1) family (Gram negative bacteria), Nodulation Factor Exporter (NFE) family (Gram negative bacteria), SecDF family (Gram negative and positive bacteria and archaea), HAE-2 family (Gram positive bacteria), Eukaryotic Sterol Transporter (EST) family, and the HAE-3 family (archaea and spirochetes). All seven members of this superfamily function via substrate:proton antiport (Nies and Silver 1995). Functionally uncharacterized RND proteins that fall outside of these seven families have also been identified. Some of the eukaryotic homologues function as enzymes and receptors instead of, or in addition to, transporters. The sizes and topological patterns of members from all seven families are strikingly similar. These proteins have 12 transmembrane α–helical spanners (TMSs) and large hydrophilic extracytoplasmic domains between TMSs 1 and 2 and TMS 7 and 8 (Fujihira et al. 2002). It is believed that the origin of these proteins is from an internal gene duplication event.

### 1.6.5.1 RND pumps in *E. coli*

Analysis of the *E. coli* genome has revealed the presence of 7 putative and known RND transporters (Nishino and Yamaguchi 2001). The AcrAB-TolC system has been identified as the predominant drug efflux pump of this organism, and in addition to being the best-characterized RND pump to date, it also has a broad substrate specificity. These pumps form a three-component system, consisting of an inner membrane component, a periplasmic component, and an outer membrane component.

The AcrAB efflux system is composed of AcrB, an RND transporter, and the periplasmic accessory protein, AcrA (Fig. 1.4). The mode of interaction between the components remains largely unclear. The cooperation between AcrB, AcrA, and TolC is believed to allow the direct efflux of the antibiotic molecules into the medium rather than the periplasm. In 2000, the high resolution crystalline structure of TolC was reported (Rosenberg et al. 2000) revealing that unlike the conventional porin-like proteins, which are completely embedded in the outer membrane, a TolC trimer, in addition to an outer-membrane-spanning domain, has a long periplasmic α–helical barrel domain, composed of 12 α–helices. A similar structure has also been proposed for OprM, the outer membrane component of the MexAB-OprM pump of *P. aeruginosa* (Poole et al. 1993) and for CmeC, the outer membrane component of the CmeABC pump of *Campylobacter jejuni* (Gibreel et al. 2007). Recent studies have shown that AcrA exists as a trimer and that these trimers may be cross-linked to AcrB (Zgurskaya and Nikaido 2000). However, efforts to cross-link either of the proteins to TolC were unsuccessful. Based on these studies, possible modes of interaction between AcrB, AcrA, and TolC have been suggested and will be described later. However, there is no experimental evidence to

**Figure 1.4.** AcrAB-TolC RND pump of *E. coli* in conjunction with outer membrane permeability.


Drug
Porin
OM channel (Tol C)
Outer
Membrane
Periplasmic
Accessory Protein
(AcrA)
Periplasm
Inner
Membrane
Efflux Transporter
(AcrB)

establish an actual mechanism for the interaction between the different components of AcrAB-TolC pump of *E. coli* or any other RND efflux pump.

### 1.7 Structure of RND efflux pumps

In Gram-negative bacteria, RND efflux pumps form a three-component complex transversing both the inner and outer membranes (Lomovskaya and Watkins 2001). As mentioned, the tripartite system 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 cooperation of the three-component membrane-traversing structure facilitates direct passage of the substrate into the medium. All three components of RND efflux pumps can either be one gene cluster (Mex AB-OprM of *P.aeruginosa* (Poole et al. 1993)) or in other cases, including the AcrAB pump of *E. coli* (Ma et al. 1993), the gene encoding the outer membrane component is not present in the gene cluster encoding these pumps.

#### 1.7.1 Inner membrane pump component

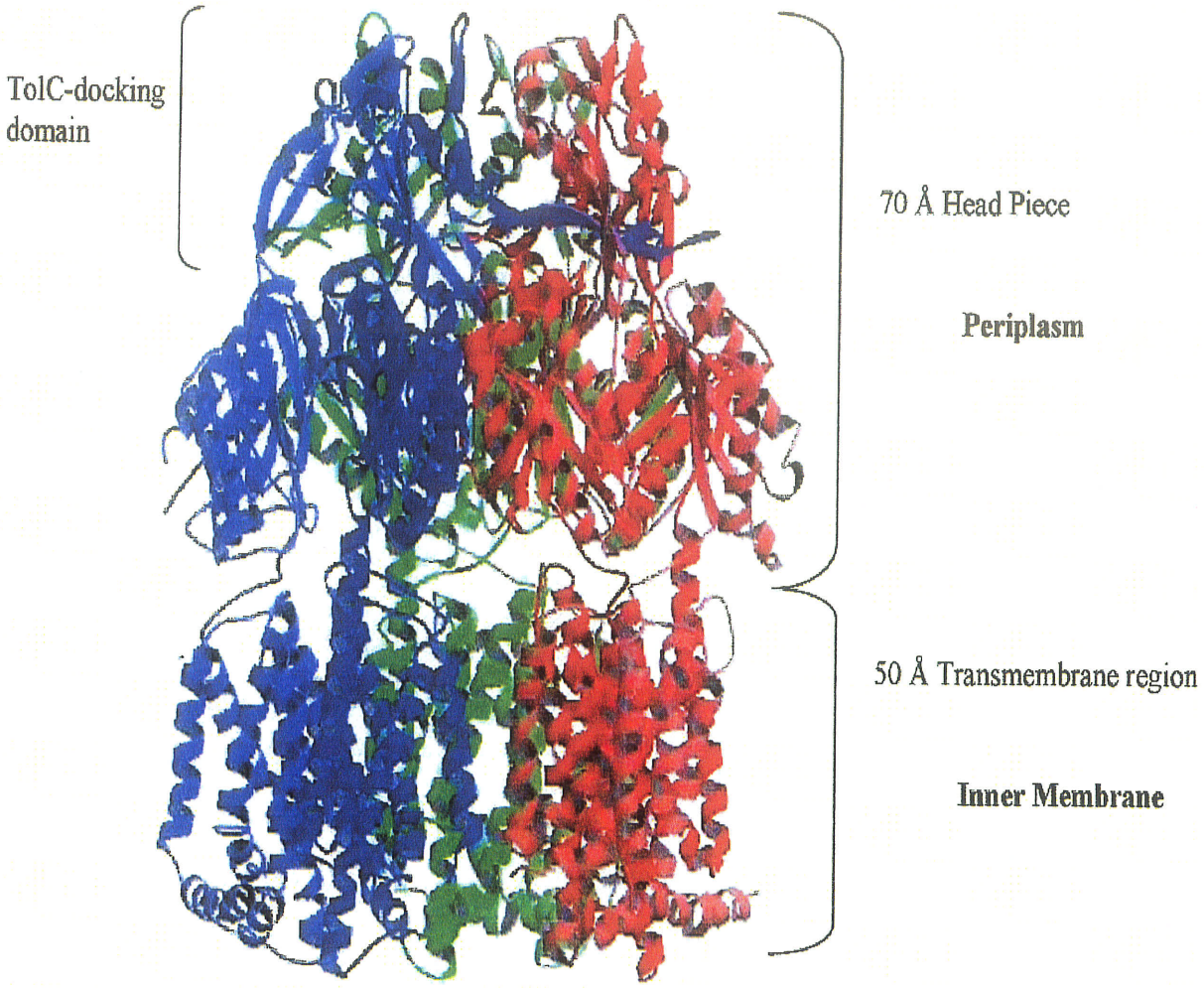
The inner membrane component of RND efflux pumps is responsible for the identification of the molecule to be effluxed (Zgurskaya and Nikaido 1999). Two of the best-studied RND transporters, AcrB of *E. coli* and MexB of *P. aeruginosa*, pump out lipophilic and amphiphilic compounds (Zgurskaya and Nikaido 1999) and can recognize a variety of substrates. 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 and play a role in substrate specificity (Fujihira et al. 2002).

The crystal structure of the inner membrane component AcrB at 3.5 Å resolution demonstrated that AcrB is arranged as a homotrimer forming a jelly fish like structure (Murakami et al. 2002) (Fig. 1.5). Each trimer is composed of a 50 Å thick transmembrane 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 the two large periplasmic loops, with the loops from one promoter interacting with loops of the other two promoters 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 3 vestibules leading into the periplasm. This structure suggests that the substrates translocated from the cell interior and from 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 homology among RND transporters.

The crystal structure of AcrB also revealed that multiple molecules of ligands bind simultaneously to the extremely large central 5000 cubic Å cavity, primarily by hydrophobic, aromatic stacking and van der Waals interactions (Murakami et al. 2002). Different substrates are 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 monomer. There are no negatively charged amino acid residues near the substrate binding

**Figure 1.5.** Three-dimensional structure of the AcrB trimer at 3.5 Å (Murakami et al. 2002).


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

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 membrane bilayer and protein side-chains.

### 1.7.2 Periplasmic pump component

The periplasmic proteins of the RND multidrug complex belong to the membrane fusion protein (MFP) family. These proteins are common in Gram-negative transport systems and have not been identified in Gram-positive bacteria, archaea or eukaryotes. 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 and Nikaido 1999), thus facilitating the transfer of substrates across the periplasm.

AcrA of the *E. coli* AcrAB pump, is a highly asymmetric molecule approximately 20 nm in length (Zgurskaya and Nikaido 1999). 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 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. The presence of the β–domain on the C-terminal end has led to speculations that this end is inserted in the outer membrane in a porin-like fashion although the crystal structure of *P. aeruginosa* MexA has not shown this arrangement (Higgins et al. 2004). Instead, the structure by Higgins et al. (2004) has shown 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).

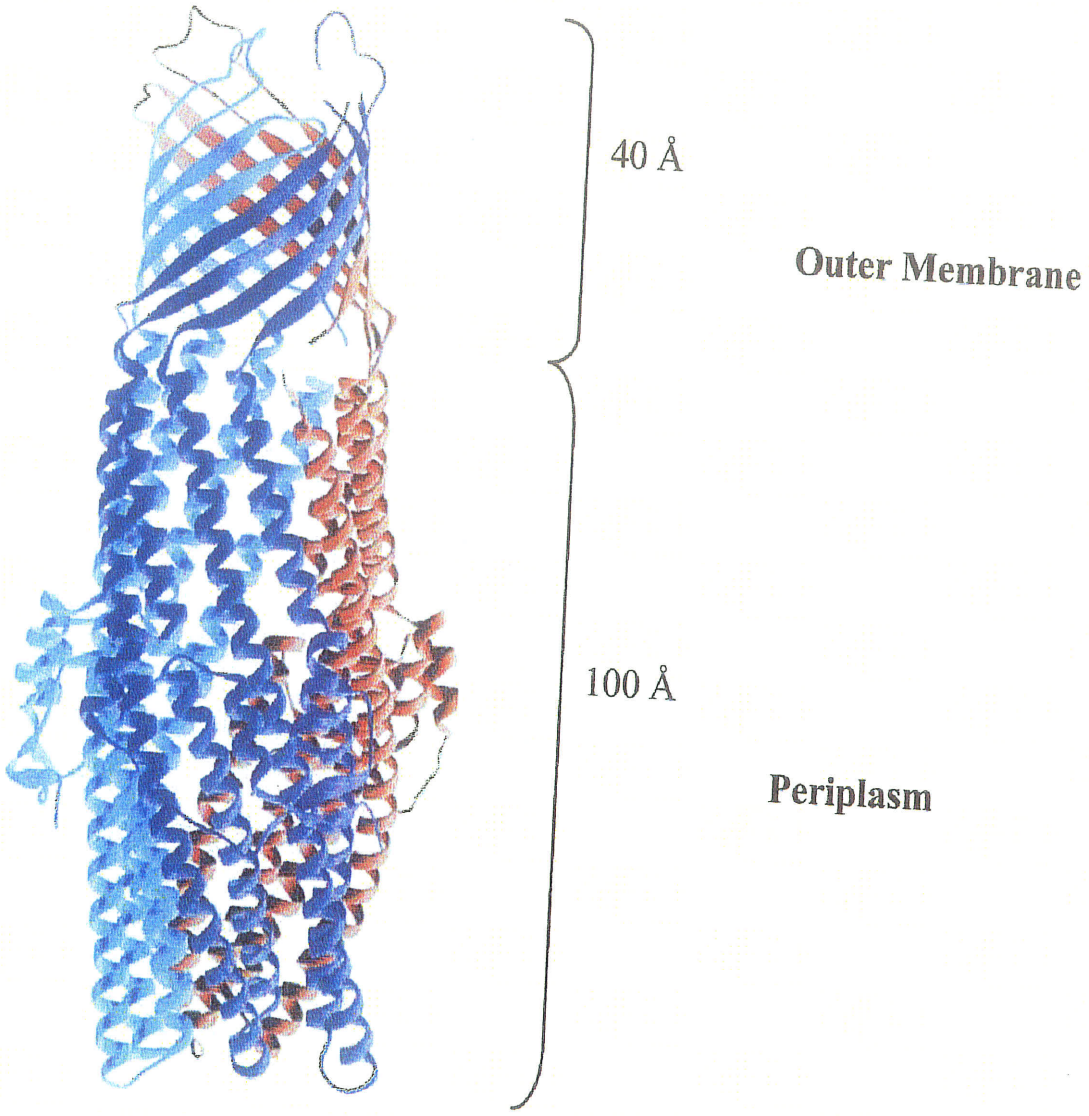
### 1.7.3 Outer membrane components

Outer membrane components serve 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 and Nikaido 1999). For example, TolC, the outer membrane component of the AcrAB pump is believed to work with all efflux systems in *E. coli* that require an OMF.

TolC is a trimeric outer membrane protein. The crystal structure of the protein reveals the presence of an alpha–helical barrel which forms an approximately 100 Å long tunnel, through the periplasm (Koronakis et al. 2000). This barrel is anchored by a 12-stranded outer membrane β–barrel (Fig. 1.6). TolC has 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 alpha-helical barrel (Wong et al. 2001). 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 β–helical barrel being 100 Å, and the outer membrane β–barrel 40 Å. The alpha-barrel consists of 12 alpha-helices packed in an antiparallel arrangement to form a hollow cylinder. Koronakis and coworkers proposed that the alpha-helical barrel of the TolC protein is virtually

**Figure 1.6.** 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).


40 Å
Outer Membrane
100 Å
Periplasm

uniform in diameter near the β–barrel, however, the long helices are tapered at the proximal end. 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. 2002). 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.

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, 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 identify at the amino acid level.

**1.8 Natural function of RND pumps**

It was believed that these pumps were developed by Gram-negative pathogens as a defense mechanism to counter the increasing antibiotic concentrations in their environment (Webber and Piddock 2003). However, through phylogenetic studies, the presence of RND pump homologs is noticed in Gram-positive bacteria, archae, and eukaryotes. It has been established that proteins belonging to RND pump family are part

of an ancient family of proteins with representation in all major kingdoms (Tseng et al. 1999) whose primary function is to act in a buffering capacity.

The AcrAB pump of *E. coli* was found to have the highest affinity for bile salts. The natural habitat for *E. coli* is the enteric tract (rich in bile salts). 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).

**1.9 Mechanism of action of RND efflux pumps**

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

Substrate (in) + nH+(out) $\longrightarrow$ 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 proton motive force is coupled to the export process in these pumps. However, what is known is that these transporters are very versatile as far as substrate recognition is concerned, having the ability to export hydrophobic cations, neutral, zwitteronic, and negatively charged compounds. Different substrates seem to 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 cubic Å. A common feature of all of these substrates seems to be large hydrophobic domains which ensures the partition of substrate molecules into the phospholipid bilayer, favoring the notion that substrate binding occurs in the membrane environment. This was confirmed by the AcrB crystal structure (Murakami et al. 2002).

There are 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 and Nikaido 1999). Substrates are collected in the central cavity and then actively transported through the pore in TolC. To facilitate a tighter interaction between the outer and inner membrane components, the periplasmic component simply brings these two membranes together in closer contact.

Based on the crystal structures of TolC and AcrB in *E. coli*, a model of interaction has been predicted (Zgurskaya and Nikaido 1999). The alpha-helical tail of TolC 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 towards the extracellular side. The interaction is facilitated by AcrA, which is, via its β–barrel, embedded into the outer membrane. This interaction results in a conformational change in the AcrB protein, which in turn causes folding back of the AcrA protein upon itself to bring the two membranes closer together. This results in the interaction of the alpha-helical tail of TolC with the funnel shaped headpiece of AcrB, which may serve as a signal for the opening of the TolC channel. As the diameter of the top of the AcrB headpiece (40 Å) is almost equal to the diameter of the helical TolC tail, these two proteins might form a continuous channel once TolC is brought together with AcrB. Futhermore, there is a ring of aspartate residues present in the channel opening of the TolC protein on the periplasmic side that plays a role in the cation binding of the protein (Andersen et al. 2002). This further suggests that, once TolC

interacts with the AcrB, the substrate is somehow translocated from the cavity of AcrB to TolC channel, and then TolC guides the substrate out of the cell.

### 1.10 Regulation of RND pump gene expression

Overexpression of the AcrAB pump is found to be toxic for *E. coli* (Ma et al. 1993). Therefore, it is important to have an elaborate mechanism for regulation of the expression of many of these RND pumps in place. The majority of these transporter genes are controlled by transcriptional regulatory proteins which consist of repressors and activators of the target gene, and function at local and/or global levels.

#### 1.10.1 Local regulators

Local regulators, mostly repressors, are known to control the expression of many multidrug pumps. One example of a repressor is the *acrR* of *E. coli*, transcribed divergently from *acrAB* genes, encoded a repressor of the TetR repressor family, as it possesses a helix-turn-helix (HTH) DNA binding domain (Tikhonova and Zgurskaya 2004). AcrR represses 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.

#### 1.10.2 Two-component regulatory systems

Some efflux pumps are known to be regulated by two-component systems consisting of a sensor kinase and a response regulator. These systems are found extensively in bacteria and allow them to react to changes in their environment. In response to the environmental stimuli, the sensor kinase phosphorylates the response regulator, which in turn activates or represses the target gene (Stock et al. 2000). An

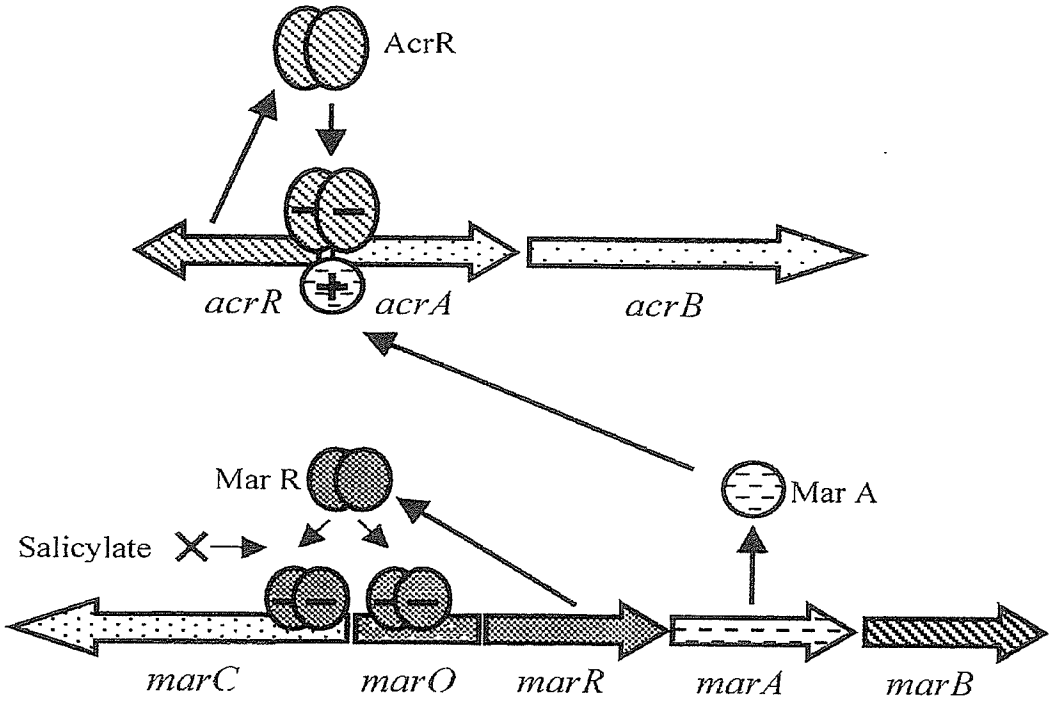
example of this type of system that regulates an RND pump is the BaeSR system for the MdtABC pump of *E. coli* (Baranova and Nikaido 2002; Nagakubo et al. 2002).

### 1.10.3 Global regulators

The role of global regulators in the expression of efflux pumps has been best-studied for the *E. coli* AcrAB-TolC system (Fig.1.7). 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 and 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 and 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 constitutively regulate the expression of over 60 *E. coli* genes (Barbosa and Levy 2000). MarA also activates the transcription of *micF*, to produce an antisense RNA that downregulates the expression of *ompF* (Delihas and Forst 2001). A combination of *ompF* reduction and overexpression of *acrAB-tolC*, results in a highly effective mechanism by which MarA can coordinate a response to the presence of a variety of structurally unrelated hydrophobic and hydrophilic antibiotics.

**Figure 1.7.** Schematic representation of the regulation of the *acrAB* locus by AcrR and MarA. AcrR represses (-) transcription of the *acrRAB* genes, while MarA upregulates (+) the expression of the *acrAB* genes. Expression of the *marRAB* operon is repressed by MarR, encoded by *marR*. 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 and Brennan 2002).

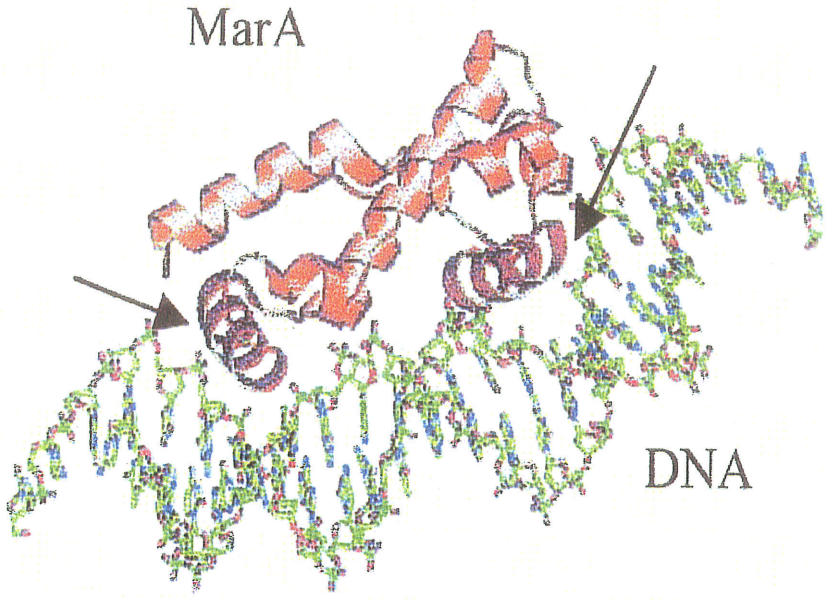

AcrR
acrR
acrA
acrB
Mar R
Mar A
Salicylate
marC
marO
marR
marA
marB

The crystal structure of MarA bound to the *marRAB* operon has been solved (Fig 1.8) (Rhee et al. 1998). MarA binds to the DNA as a monomer and possesses two separate helix-turn-helix (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. A typical HTH motif is capable of recognizing only 6 bp, the presence of two 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 and Rosner 1995). Site II, approximately 80% homologous to site I is not required for repression since a site II-deleted mutant (*marO133*) was repressed in trans by wild-type MarR (Martin and Rosner 1995). The absence of site II did not prevent MarR from complexing with the site I of *marO133*. MarR-DNA interactions can be inhibited by several anionic compounds, including salicylate (Alekshun and Levy 1999) through still an unknown mechanism.

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

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


MarA
DNA

the transcription of *acrAB*. Also, SdiA, an *E. coli* protein that is homologous to the receptor of acyl homoserine lactone quorum-sensing signal, positively regulates the AcrAB expression (Rahmati et al. 2002).

### 1.11 Efflux pumps in *Serratia marcescens*

Our group (Kumar and Worobec 2002) was the first to identify *S. marcescens* genes homologous to known efflux pumps by using PCR primers designed for two conserved motifs within the RND protein (Kumar 2004). Two different RND pump complex-encoding operons were identified, *sdeAB* and *sdeCDE*. *sdeAB* encodes a MFP (membrane fusion protein) and an RND pump, with the predicted molecular weight of 42.8 kDa for SdeA, and 112.5 kDa for SdeB. *sdeCDE* encodes a MFP and two different RND pumps with predicted molecular weights of SdeC, SdeD and SdeE being 47.8 kDa, 111.5 kDa and 110 kDa, respectively.

Since no outer membrane component gene was found linked to either of the loci, the then incomplete database of the *S. marcescens* genome sequencing project (http://www.sanger.ac.uk/Projects/S_marcescens/Sma.art) was searched for the presence of a *tolC*-like gene. One such gene (*hasF*) was identified, having the deduced amino acid sequence approximately 80% homologous to the *E. coli* TolC with a predicted molecular weight of 54 kDa (Kumar 2004).

An additional RND pump, SdeXY, was found to confer norfloxacin and tetracycline resistance and appears to be a close homologue of the *E. coli* AcrAB-TolC pump (Chen et al. 2003).

## 1.12 Synergy between efflux pumps and other resistance mechanisms

Widespread use of antimicrobial agents has given rise to a variety of resistance mechanisms in bacteria. To summarize, these mechanisms are:

1. Alteration of 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 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.

### 1.12.1 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 the single-component pumps that pump substrate into the periplasm, or the multicomponent pumps that pump substrate into the external medium), the net effect is additive. If there are two different kinds of pumps (a single component and a multi-component type) working together, the effect is multifactorial. This model also explains how overexpression of the single-component pumps, such as the Tet pump, can result in extremely high

resistance in Gram-negative bacteria. As these pumps are only able to pump out antibiotics into the periplasm, there is always a possibility of the antibiotic diffusing back into the cytoplasm. However, multi-component pumps that are expressed constitutively can perhaps synergize with Tet pumps and make the pumping more effective.

### 1.12.2 Relationship between outer membrane permeability and efflux pumps

Interaction between the outer membrane permeability and efflux pumps also has the multiplicative effect. An example of this is a study by Mallea et al. 1998 where simultaneous changes in envelope permeability (porin deficiency) and efflux mechanism were clearly evident in two clinical isolates of *Enterobacter aerogenes* strains, leading to very high cumulative antibiotic resistance.

### 1.12.3 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. 2000).

### 1.12.4 Interaction between antimicrobial target alterations and efflux pumps

This interaction has been very well documented in cases of quinolone resistance. DNA gyrase and topoisomerase are 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. 2000). In *P. aeruginosa*, it has 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).

### 1.13 Thesis Objectives

As the literature review has suggested, both outer membrane permeability and RND efflux pumps contribute to the overall antibiotic resistance of *S. marcescens*, as well as *Salmonella* and *Campylobacter*. My specific objectives to further investigate this became:

1. To prove that outer membrane permeability affects antibiotic resistance in *Serratia marcescens*
    a. by showing that natural factors such as temperature, pH, salicylate and osmotic pressuse affect *ompF* and *ompC* expression
    b. by showing that conditions that affect *ompF* and *ompC* expression affect antibiotic resistance
    c. by showing that mutations affecting pore size affect antibiotic resistance
2. To prove that efflux affects antibiotic resistance in *Serratia marcescens*
    a. by showing that SdeAB functions as a multidrug RND efflux pump
    b. by showing that SdeCDE functions as a minor RND efflux pump with limited substrate specificity

## CHAPTER 2 Site-directed mutagenesis studies to probe the role of specific residues in the external loop (L3) of OmpF and OmpC porins in susceptibility of *Serratia marcescens* to antibiotics

The material presented herein resulted in the publication Begic, S., and Worobec, E. A. 2007a. Site-directed mutagenesis studies to probe the role of specific residues in the external loop (L3) of OmpF and OmpC porins in susceptibility of *Serratia marcescens* to antibiotics. Can. J. Microbiol. **53:** 710-719. I planned and completed the majority of experiments outlined in this chapter with assistance from summer students Craig Harlos and Kiet Do, with special suggestions regarding liposome swelling assays made by Dr. Jo-Anne Hutsul (whom I also thank for the pM8BH12, 13(+) and pEX1.8JH constructs, primer design and initial site-directed mutagenesis studies on sites 112 and 114), and valuable input regarding fluoroquinolone uptake assays made by Dr. Ayush Kumar. I thank O. Famobio for the P8BK and P8BAK *ompC* constructs. I wrote the manuscript with full guidance and feedback from my advisor and important suggestions were made by Dr. T. de Kievit.

### 2.1 Abstract

*Serratia marcescens* is a nosocomial bacterium with natural resistance to a broad spectrum of antibiotics, making treatment challenging. One factor contributing to this natural antibiotic resistance is reduced outer membrane permeability, controlled in part by OmpF and OmpC porin proteins. To investigate the direct role of these porins in the diffusion of antibiotics across the outer membrane, we have created an *ompF/ompC* porin-deficient strain of *S. marcescens*. A considerable similarity between the *S. marcescens* porins and those from other members of *Enterobacteriaceae* was detected by

sequence alignment, with the exception of a change in a conserved region of the third external loop (L3) of the *S. marcescens* OmpC protein. *S. marcescens* OmpC has an aspartic acid not glycine in position 112; methionine instead of aspartic acid in position 114 and glutamine in position 124, while in *S. marcescens* OmpF, this is a glycine. To investigate the role of amino acid positions 112, 114 and 124 and how the observed changes within OmpC porin may play a part in pore permeability, 2 OmpC sites have been altered back to the *Enterobacteriaceae* consensus (D112G and M114D) through site-directed mutagenesis. Also, Q124G in OmpC and G124Q in OmpF and double mutants of these amino acid residues were constructed. Antibiotic accumulation assays and MICs of the strains harboring the mutated porins were performed, while liposome swelling experiments were performed on purified porins. Our results demonstrate that the amino acid at position 114 is not responsible for either antibiotic size or ionic selection, the amino acid at position 112 is responsible for size selection only, and position 124 is involved in both, size and ionic selection.

**2.2 Introduction**

*Serratia marcescens* is a Gram negative enteric bacterium which has become an important opportunistic pathogen associated with a number of life-threatening diseases and nosocomial infections, such as urinary tract infections, respiratory tract infections, meningitis, septicaemia and wound infections (Hejazi and Falkiner 1997). This organism possesses a high intrinsic resistance to a variety of antibiotics, including aminoglycosides, β-lactams, first- and second-generation cephalosporins, and quinolones (Kumar and Worobec 2003; Fujimaki et al. 1989) making treatment very difficult. The basis of this antibiotic resistance is multifactorial, involving different mechanisms for

each group of antibiotics. For example, resistance to β–lactam antibiotics is achieved by outer membrane impermeability mediated by porins (Gutmann et al. 1984), β-lactamases found in the periplasm which degrade these drugs (Sanders and Sanders 1992), and altered inner membrane penicillin-binding proteins, which are the target for these antibiotics (Gunkel et al. 1991). Likewise, resistance to quinolones can be a result of mutations in DNA gyrase (Fujimaki et al. 1989), the target enzyme for this group of antibiotics, and/or over-expression of multi-drug resistance efflux pumps (Ma et al. 1992).

The multifactorial nature of antibiotic resistance was demonstrated in a study using *Enterobacter aerogenes*, a related nosocomial pathogen. Investigators reported a simultaneous change in membrane permeability due to porin deficiency concomitant with the expression of an efflux mechanism which renders this bacterium resistant to both β–lactam and fluoroquinolone antibiotics (Mallea et al. 1998). One of our objectives was to study the role of outer membrane permeability in the β–lactam resistance of *S. marcescens.*

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 behaves like a molecular sieve to allow the passage of small hydrophilic molecules such as nutrients, waste products and β–lactam antibiotics (Nikaido 1994) into and out of the cell. The degree of permeability of this membrane depends on the presence of pore-forming porin proteins, which are membrane-spanning molecules that form water-filled channels (Nikaido 1994). Channel size and hydrophilicity are determined by an eyelet loop of the polypeptide chain which extends into the pore

(Nikaido 1994). Non-specific porins are typically involved in the passage of β–lactam and other families of antibiotics [e.g. *E. coli* OmpF/C (Nakae 1976; Mizuno et al. 1983) or OprF of *P. aeruginosa* (Woodruff and Hancock 1989)].

Two major non-specific porins, OmpF and OmpC, have been identified in *S. marcescens* (Hutsul and Worobec 1994; Hutsul and Worobec 1997). Although the role of OmpF and OmpC in the antibiotic resistance of *E. coli* (Mortimer and Piddock 1993) and other members of *Enterobacteriaceae*, such as *Enterobacter aerogenes* and *Klebsiella pneumoniae* has been studied extensively (Gutmann et al. 1984), little is known about *S. marcescens* porins. Changes in porin copy number, size, selectivity, or function can alter the rate of diffusion of hydrophilic β–lactam and other antibiotics through the porins (Mortimer and Piddock 1993; Gutmann et al. 1984). One of the aims of this study was to construct *ompF* and *ompC* deficient mutants, as well as a double-deficient mutant, in order to study the role of these proteins in *S. marcescens* antibiotic resistance.

The enterobacterial non-specific diffusion porins are a homologous group of proteins with a reported average homology of 79% at the amino acid level (Jeanteur et al. 1991). Sequence analysis has confirmed that *S. marcescens* porins are closely conserved with those of *E. coli* (Hutsul and Worobec 1994; Hutsul and Worobec 1997). The three-dimensional crystallographic resolution of the *E. coli* OmpF porin revealed a trimeric structure, consisting of monomeric β–barrels composed of 16 anti-parallel β–strands that form the pore (Cowan et al. 1992). One large loop, L3, folds into the channel, and through its charge and bulkiness, defines a constriction zone which governs pore activity (Fig. 2.1). Considerable similarity between the *S. marcescens* porins and those from the

*Enterobacteriaceae* has been observed with the noted exception of a change in a conserved region of the L3 of the *S. marcescens* UOC-67 OmpC (Fig. 2.1). At the tip of L3 is a turn formed by the PEFX112G motif trend in all enterobacterial porins (Jeanteur et al. 1991) with X112= glycine (G). The G-G sequence is thought to be conserved because of unique torsion angles it forms which aid in the turn formation of the PEFXG region (Cowan et al. 1992). Although the *S. marcescens* OmpF L3 motif is conserved with that of other enterobacterial porins, we have found a discrepancy in the OmpC sequence where X at amino acid position 112 is an aspartic acid (D) (according to OmpC numbering), not a glycine (Hutsul and Worobec 1994) (Fig. 2.2). Aspartic acid is also turn promoting (Paul and Rosenbusch 1985) therefore D112 in *S. marcescens* OmpC is not expected to disrupt the structure or function of the overall motif, however, due to its bulky side chain, we expect the aspartic acid to protrude into the pore lumen leading to a reduced permeability through the porin as was seen for *E. coli* OmpF porin (Jeanteur et al. 1994). In enterobacterial porins, the PEFXG motif is followed by an aspartic acid, however again the *S. marcescens* OmpC porin differs from the rest of the group as the motif is followed by the hydrophobic residues, methionine (M114). These changes may reflect a divergence this porin has taken from other OmpC porins, indicating a possible difference in the pore characteristic of the *S. marcescens* porin. In addition, in *S. marcescens* OmpC, amino acid position 124 (according to OmpC numbering) is occupied by a glutamine (Q) residue (Fig. 2.2), and in *S. marcescens* OmpF, a glycine. We predict that the glutamine side chain within this strain of *S. marcescens* OmpC may extend into the pore channel contributing to size constriction by conferring a smaller channel in OmpC in comparison to OmpF which has a glycine.

**Figure 2.1.** Loop 3 *S. marcescens* UOC-67 OmpC porin built by threading the sequence of the protein into the co-ordinates of the solved *E. coli* OmpC crystal structure using PHYRE (Protein Homology/analogY Recognition Engine). Key amino acids in positions 112 and 124 are labeled.

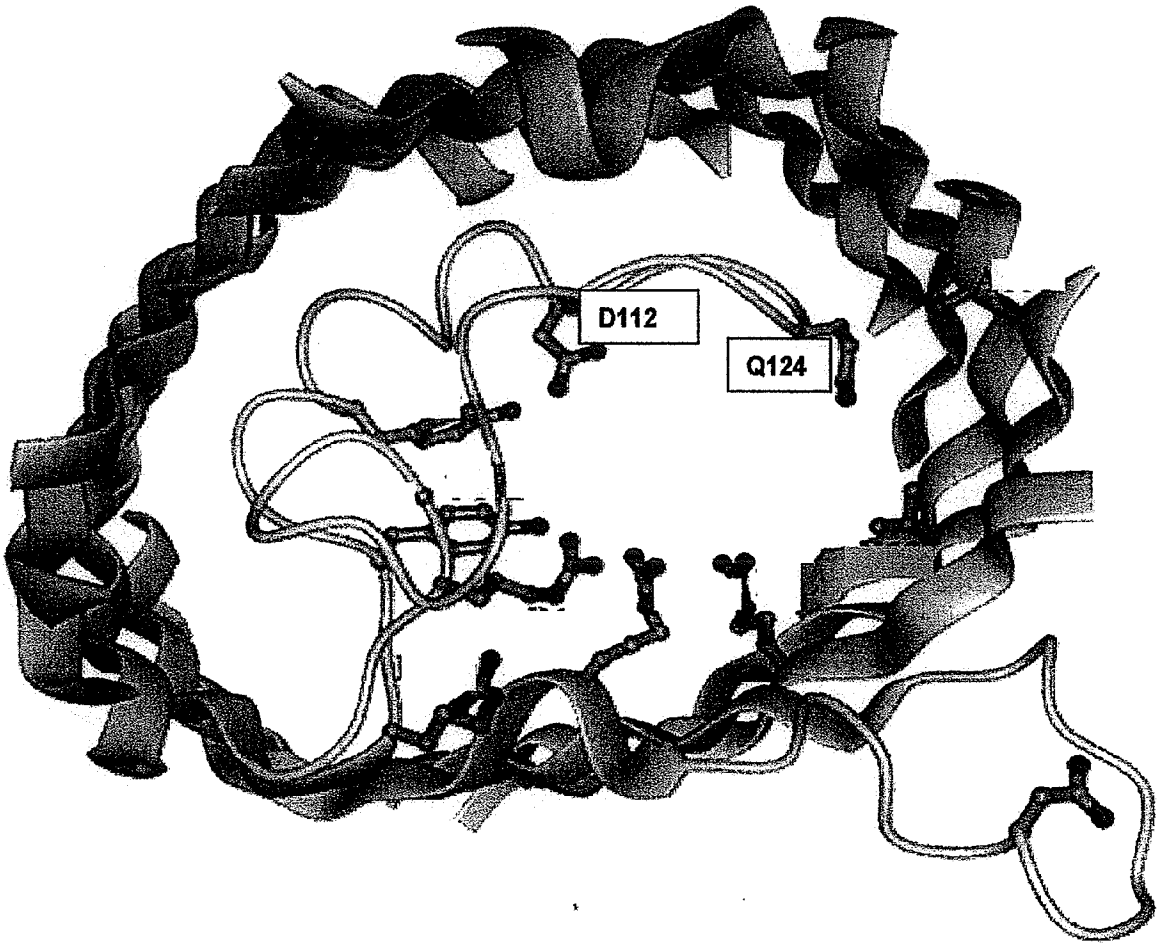

D112
Q124

**Figure 2.2.** Amino acid sequence comparison of the *S. marcescens* UOC-67 OmpC and OmpF L3 to the *Enterobacteriaceae* OmpC and OmpF consensus sequence (according to *S. marcescens* OmpC numbering) (Hutsul, 1996). Residues 112, 114 and 124 are designated at the top of the sequences. Differences between the *Enterobacteriaceaea* OmpC porin consensus sequence and *S. marcescens* OmpC sequence appear in bold.

| | 112 114 124 |
|---|---|
| *Enterobacteriaceae* OmpC consensus: | PEF**GG**D**D**TYG-SDNFMQQRG |
| *Enterobacteriaceae* OmpF consensus: | PEFGGDTAY-SDDFFVGRV |
| *S. marcescens* UOC -67 OmpC sequence: | PEF**D**G**M**TYG-ADQFMFQRS |
| *S. marcescens* UOC-67 OmpF sequence: | PEFGGDTYTYSDNFMTGRT |

To investigate the role of amino acid positions 112 and 114 and how the observed changes within the *S. marcescens* UOC-67 OmpC porin may play a part in pore permeability, two OmpC residue sites were changed to the consensus (D112G and M114D). Amino acid 124 in both *S. marcescens* porins was altered (Q124G in *S. marcescens* OmpC and G124Q in *S. marcescens* OmpF) in order to evaluate the porin permeability and investigate the effect of the glutamine side-chain within OmpC. An OmpC double mutant preserving the entire motif, PEFGGD, was also investigated. Additionally, two other L3 OmpC double mutants (D112G with Q124G; and M114D with Q124G) were produced in order to examine the OmpC porin permeability and investigate the roles these amino acids play in function.

## 2.3 Materials and Methods

### 2.3.1 Bacterial strains, plasmids and site-directed mutagenesis

All strains and plasmids used in this study are outlined in Table 2.1. All bacterial strains were grown in Luria-Bertani agar (LB), Trypticase Soy Agar (TSA) and Tryptone Soy Broth (TSB) [BD Diagnostics Systems]. The ampicillin resistant strains (containing pUC18, pKS(+), pUC18:9F, P8BK(+), P8BAK and all pEX1.8 constructs) were grown on LB plates and in LB broth containing 100 μg/mL ampicillin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The kanamycin resistant strains (with pKIXX, pUC18:9F:Kan$^r$) were grown on LB plates and in LB broth containing 25 μg/mL kanamycin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The streptomycin resistant strains (with pKNGFKan$^r$, pKNGBAK, pKNGCGm$^r$) were grown on LB plates and in LB broth containing 50 μg/mL streptomycin [Sigma-Aldrich Chemical Co. (St. Louis, MO.)].

**Table 2.1.** Bacterial strains and plasmids used in the study.

| Strains | Characteristic | Source/Reference |
|---|---|---|
| UOC-67 | Wild type *S. marcescens*, ATCC 13880 | American Type Culture Collection |
| MT616 | *E. coli*, MT607 (*pro-82 thi-I hsdR17 supE44*):pRK600 | Finan *et al.*, 1986 |
| CC118 | *E. coli, araD 139Δ(ara, leu) 7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB argEam recA1* | Dr. C. Manoil, University of Washington, Seattle, USA |
| SM100 | OmpF, OmpC porin-deficient *S. marcescens* | This study |
| SM200 | OmpC porin-deficient *S. marcescens*, wt OmpF (*ompC* deletionally mutated) | This study |
| SM300 | OmpF porin-deficient *S. marcescens*, wt OmpC | This study |
| SM400 | OmpC porin-deficient *S. marcescens*, wt OmpF (*ompC* insertionally mutated) | This study |
| SM600 | SM100 with wild type OmpF and OmpC | This study |
| SM700 | SM100 with wild type OmpF | This study |
| SM800 | SM100 with wild type OmpC | This study |
| SM1 | *S. marcescens ompC*, D112G, transformed into SM100 | Hutsul, 1996 |
| SM2 | *S. marcescens ompC*, M114D, transformed into SM100 | Hutsul, 1996 |
| SM3 | *S. marcescens ompC*, Q124G, transformed into SM100 | This study |
| SM4 | *S. marcescens ompC*, D112G/M114D, transformed into SM100 | Famobio, 2005 |
| SM5 | *S. marcescens ompC*, D112G/Q124G, transformed into SM100 | This study |
| SM6 | *S. marcescens ompC*, M114D/Q124G, transformed into SM100 | This study |
| SM7 | *S. marcescens ompF*, G124Q, transformed into SM100 | This study |
| Plasmids | | |

| | | |
|---|---|---|
| pKS (+) | ColE1 replicon, $Amp^r$, *lacZ'* | Stratagene |
| pM8BH12(+) | Wild-type *PstI/KpnI* fragment that can be replaced by the mutant *PstI/KpnI* fragment within wt *ompC*, pKS vector background | Hutsul, 1996. |
| pM8BH13(+) | Wild-type *Hin*DIII/*Hin*DIII fragment that can be replaced by the mutant *Hin*DIII/*Hin*DIII fragment within wt *ompF*, pKS vector background | Hutsul, 1996. |
| P8BK(+) | pKS(+) with 1.1-kb *BamHI/KpnI* fragment, *ompC* | Famobio, 2005 |
| P8BAK | P8BK(+) missing 400 bp AatII in the *BamHI/KpnI* fragment, *ompC* | Famobio, 2005 |
| pKS:ompC:$Gm^r$ | P8BK with a 900-bp $Gm^r$ insertion in the *Bam*HI/*Kpn*I flanked *ompC* fragment | This study |
| pUC18 | ColE1 replicon, $Amp^r$, *lacZ'* | Stratagene |
| pUC18:9F | pUC18 with 0.9-kb *Hin*DIII *ompF* fragment | This study |
| pUC18:9F:$Kan^r$ | pUC18:9F with a 1.4-kb $Kan^r$ insertion in the *Hin*DIII *ompF* fragment | This study |
| pEX1.8 | ColE1 replicon, $Amp^r$/$Car^r$, carries $lacI^Q$ and a $P_{tac}$ promoter | Pearson *et al.*, 1997 |
| pEX1.8JH | a pEX1.8 plasmid with a *Bam*HI site upstream of the P*tac* promoter removed by Erase-a-base system | Hutsul 1996, unpublished. |
| pEX1.8JH1 | a pEX1.8 plasmid with *Eco*RI, *Sma*I and *Bam*HI sites within MCS removed by Erase-a-base system | Hutsul 1996, unpublished. |
| pEXF | pEX1.8 with 1.1-kb *Eco*RI/*Sma*I *ompF* fragment | This study |
| pEXFE/S | pEX1.8JH with 1.1-kb *Eco*RI/*Sma*I *ompF* fragment | This study |
| pEXC | pEX1.8 with 1.1-kb *Eco*RI/*Hin*DIII *ompC* fragment | This study |
| pEXCH/H | pEX1.8JH1 with 1.1-kb *Hin*DIII*Hin*DIII *ompC* fragment | This study |
| pEXFC | pEX1.8JH with 1.1-kb *Eco*RI/*Sma*I *ompF* fragment and 1.1-kb *Hin*DIII/*Hin*DIII *ompC* fragment | This study |
| pKNG101 | Suicide vector, *pir- oriR6K mobRK2 sacB* $Str^r$ | Kaniga *et al.*, 1991 |

| | | |
|---|---|---|
| pKNGBAK | pKNG101 with an approximate 0.8-kb *ompC* deletion fragment | This study |
| pKNGCGm$^r$ | pKNG101 with 2.0-kb *ompC* insertion fragment | This study |
| pKNGFKan$^r$ | pKNG101 with 2.3-kb *ompF* insertion fragment | This study |
| pKIXX | 1.4-kb Kan$^r$ cassette flanked by *Sma*I | Pharmacia PL Biochemicals |
| pUCGM | 900-bp Gm$^r$ cassette flanked by *Pst*I | Schweizer *et al.*, 1993 |

Note:
Abbreviations: Kan, kanamycin; Amp, ampicillin; Str, streptomycin; Car, carbenicillin; Gm, gentamicin.

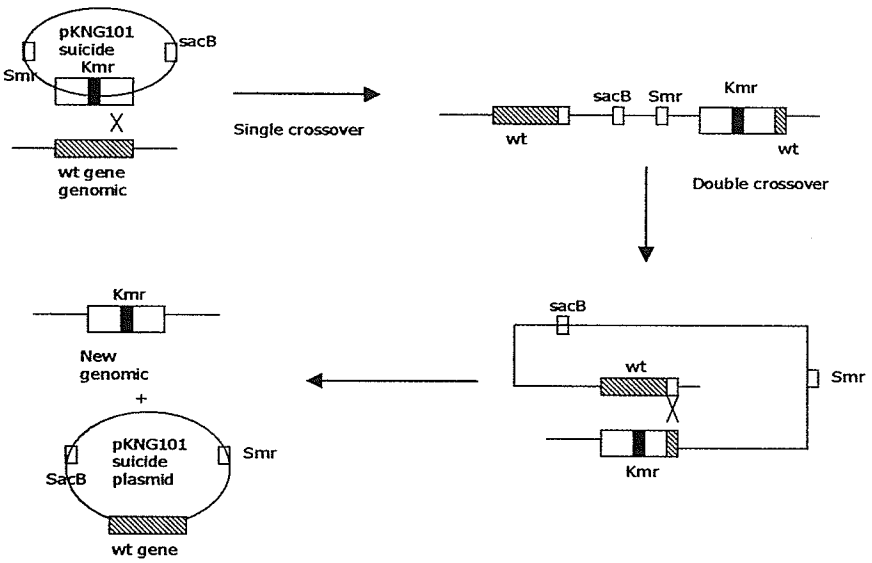
The gentamicin resistant strains (with pUCGM, pKS:ompC:Gm$^r$) were grown on LB plates and in LB broth containing 20 μg/mL gentamicin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. SM200 and SM300 strains were grown on LB plates and in LB broth containing 100 μg/mL ampicillin and 50 μg/mL streptomycin; SM600, SM700 and SM800 on 100 μg/mL ampicillin; SM400 containing 20 μg/mL gentamicin and 50 μg/mL streptomycin and SM100 containing 25 μg/mL kanamycin and 20 μg/mL gentamicin.

### 2.3.2 Construction of *S. marcescens* mutant strains

For the schematic representation of the two-step generalized protocol used to make insertional or deletional mutations, please refer to Figure 2.3. Primers used throughout this study are listed in Table 2.2. Two methods were employed to create the *ompC* knockout strains. Firstly, two unique *Aat*II restriction sites within p8BK(+) separated by approximately 400 bases, were identified to be a feasible target for creating a deletion mutation in the *ompC* coding region (Figure 2.4a). p8BK(+) was digested with *Aat*II, and religated to release a 0.4-kb fragment to result in p8BAK. After transformation of p8BAK into *S. marcescens* UOC-67 for propagation, the *ompC* deletion mutation was confirmed by restriction digestion with *Kpn*I/*Bam*HI, PCR of p8BAK with OmpCfor/rev primers and sequencing of the mutated *ompC* gene and vector flanking regions. p8BAK was digested with *Kpn*I to remove the 0.8-kb mutated *ompC*, ends were made flush with T4 DNA polymerase and then digested with *Bam*HI. The 0.8-kb mutated *ompC* fragment was ligated into *Sma*I/*Bam*HI sites of pKNG101 replacement vector resulting in pKNGBAK which was transformed into *E. coli* CC118 λpir for stability.

**Figure 2.3.** Schematic representation of the two-step homologous recombination process for a) insertional and b) deletional gene mutations. Gene knockouts were created in Chapter 2 (for *ompF* and *ompC),* Chapter 4 (*sdeR, hasF* and *sdeB*) and Chapter 5 (*sdeD*) with this approach.

a)


pKNG101
suicide
Kmr
sacB
Smr
wt gene
genomic
Single crossover
sacB
Smr
Kmr
wt
wt
Double crossover
Kmr
New
genomic
+
pKNG101
suicide
plasmid
SacB
Smr
wt gene
sacB
wt
Smr
Kmr

b)

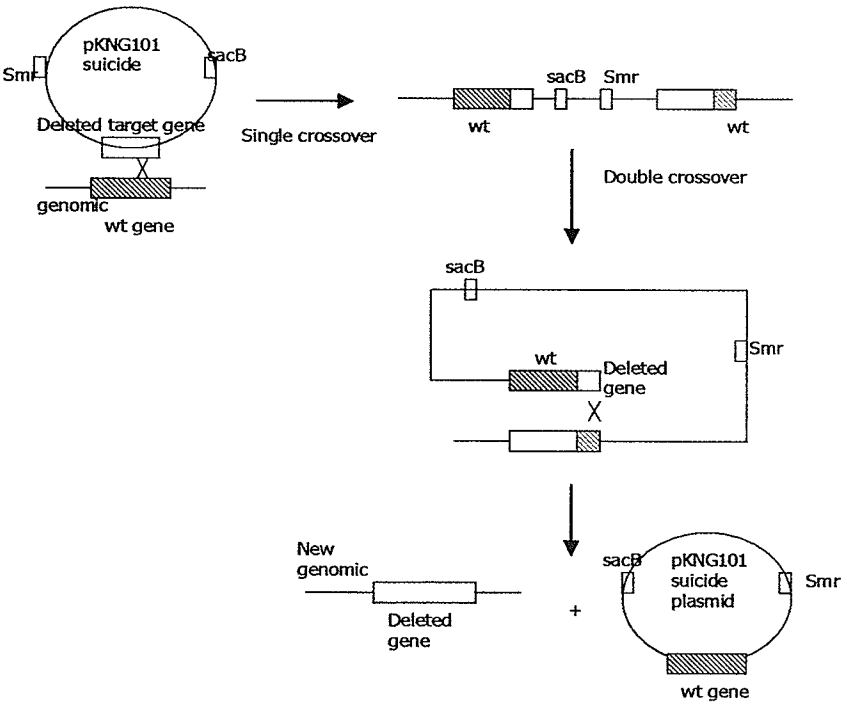

pKNG101
suicide
Smr
sacB
Deleted target gene
genomic
wt gene
Single crossover
sacB
Smr
wt
wt
Double crossover
sacB
wt
Deleted
gene
Smr
New
genomic
Deleted
gene
+
sacB
pKNG101
suicide
plasmid
Smr
wt gene

**Table 2.2.** Primers used in the study. All restriction sites are underlined.

| Name | Sequence (5' to 3') | Description | PCR Tm |
|---|---|---|---|
| OmpCf (forward) | CTAGGTACCCAACAAAGACGGTAAT | Amplification of a 1.1-kb sequence within *ompC* (*Kpn*I site engineered) | 61°C |
| OmpCr (reverse) | CGGGGATCCCAACCACGTTGTCGGT | Same as above (*Bam*HI site engineered) | |
| OmpCfor (forward) | GACGGTAATAAACTGGATTTGTA | Amplification of a 1.1-kb sequence within *ompC* (for screening transconjugants) | 54°C |
| OmpCrev (reverse) | GGTTTACAGGCAAAAAAAACGCG | Same as above | |
| OmpFfor (forward) | TTTATACGATCACACGTTTTTTAAAC | Amplification of a 0.9-kb sequence within *ompF* (for screening transconjugants) | 57°C |
| OmpFrev (reverse) | TAAGTAGAGGAAATGCCCCAGC | Same as above | |
| SacBf (forward) | AAGAAAATGCCAATAGGATATC | Analysis of *ompF* and *ompC* transconjugant after *S. marcescens-E. coli* conjugations, 1.0 kb | 56°C |
| SacBr (reverse) | TTCTATCCTAAAAGACCAAACAC | Same as above | |
| OmpFfor, OmpFrev, OmpCfor, OmpCrev | Above | Multiplex reaction using both sets of primers (OmpFfor/rev and OmpCfor/rev) for detection of | 56°C |

| | | | |
|---|---|---|---|
| | | double mutants | |
| Kmf (forward) | TAGAAAAACTCATCGAGCATC | Amplification of $Km^r$ cassette (650 bp) | 51°C |
| Kmr (reverse) | CGCCAGAGTTGTTTCTGAAA | Same as above | |
| OmpFKmf (forward) | TTCTTGCAGTGGTAATCCCG | Amplification of the 650 bp $Km^r$ cassette and some of *ompF* within *ompF* transconjugant (0.8 kb product) | 52.5°C |
| OmpFKmr (reverse) | CGCCAGAGTTGTTTCTGAAA | Same as above | |
| Gmf (forward) | GTCGACTCTAGAGGATCCCCG | Amplification of $Gm^r$ cassette (650 bp) | 56°C |
| Gmr (reverse) | ACGTAGATCACATAAGCA | Same as above | |
| OmpCGmf (forward) | GACGGTAATAAACTGGATTTGTA | Amplification of the 650 bp $Gm^r$ cassette and some of *ompC* within *ompC* transconjugant (1.1 kb product) | 54.5°C |
| OmpCGmr (reverse) | ACGTAGATCACATAAGCA | Same as above | |
| Kmfor (forward) | ATACCGGTTAGAAAAACTCATCG | Amplification of $Km^r$ cassette (1.4 kb) for insertion into the *ompF* gene (*Pin*AI site engineered) | 55 °C |
| Kmrev (reverse) | GCACCGGTGTCGATTTTTGTGATG | Same as above | |
| pEXFf (forward) | CAATGGAATTCAGGGTAATAATGAT | Amplification of a 1.1-kb sequence of wt *ompF* for complementation (*Eco*RI site engineered) | 55.5°C |
| pEXFr (reverse) | CATCCCGGGAGGTGGAGATGTTTT | Same as above | |

| | | | |
|---|---|---|---|
| (reverse) | | (*Sma*I site engineered) | |
| pEXCf (forward) | ACGGAATTCAGGATAATAACGATGAAA | Amplification of a 1.1-kb sequence of wt *ompC* for complementation (*Eco*RI site engineered) | 58°C |
| pEXCr (reverse) | CACAAGCTTCCAGGTCGACGAATTTGACC | Same as above (*Hin*DIII site engineered) | |
| pEXFCf1 (forward) | CAATGGAATTCAGGGTAATAATGAT | Amplification of a 1.1-kb of wt *ompF* for double complementation (*Eco*RI site engineered) | 56.5°C |
| pEXFCr1 (reverse) | CATCCCGGGAGGTGGAGATGTTTT | Same as above (*Sma*I site engineered) | |
| pEXFCf2 (forward) | TTCAAGCTTTGGATTAATATAACCCT | Amplification of a 1.1-kb sequence of wt *ompC* for double complementation (*Hin*DIII site engineered) | 60.5°C |
| pEXFCr2 (reverse) | CACAAGCTTCCAGGTCGACGAATT | Same as above (*Hin*DIII site engineered) | |
| D112G | CCTGAGTTCGGTGGTATGACCTAC | Site directed mutagenesis within PEFDGM motif of *ompC* – from I. Famobio | 60°C |
| M114D | CCTGAGTTCGACGGTGACACCTAC | Site directed mutagenesis within PEFDGM motif of *ompC* – from I. Famobio | 61°C |
| D112G/M114D | CCTGAGTTCGGTGGTGACACCTAC | Site directed mutagenesis within PEFDGM motif of *ompC* – | 62°C |

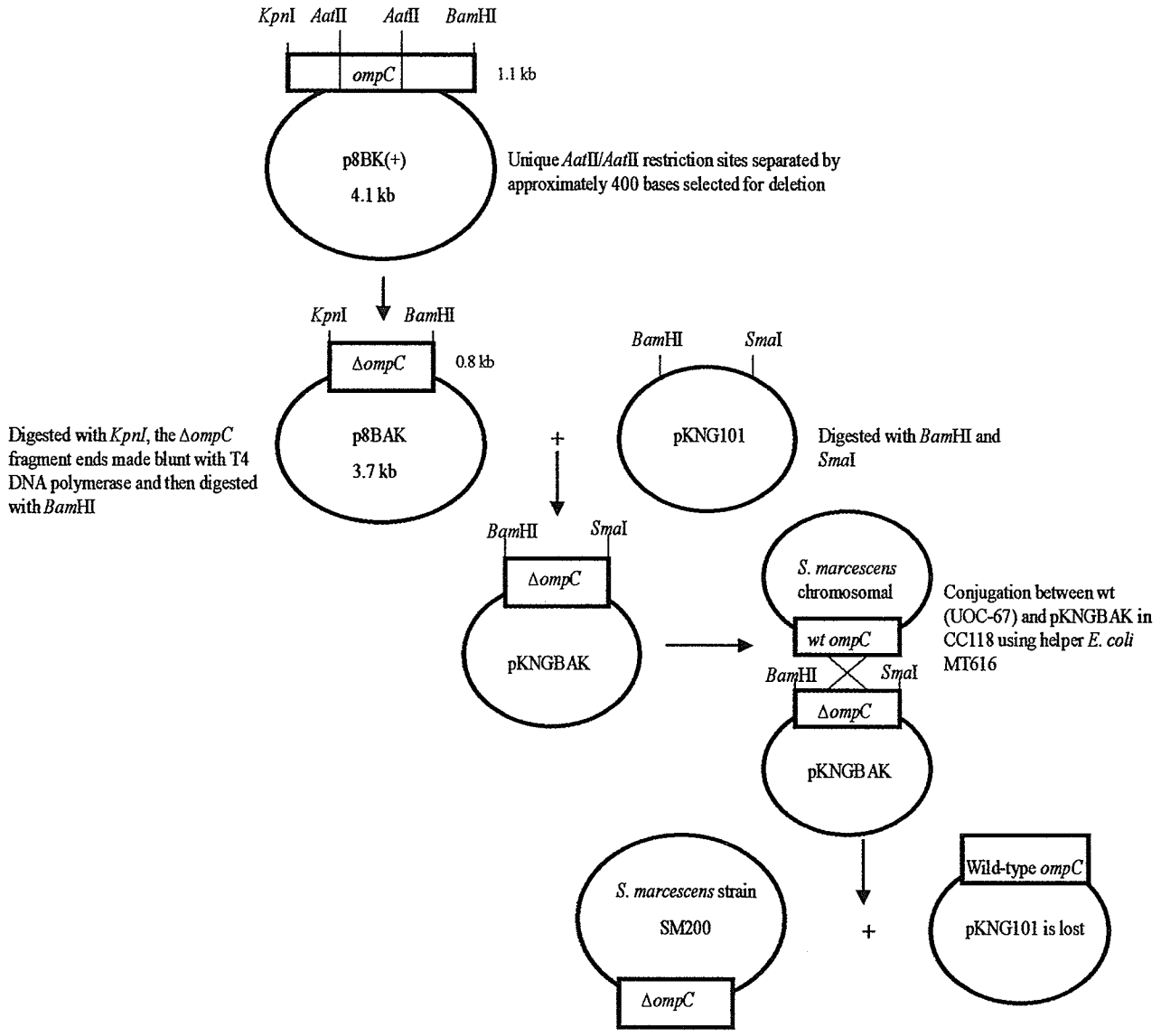
| | | | |
|---|---|---|---|
| | | from I. Famobio | |
| Q124G | TTCATGTTCGGGCGTTCCAGC | Site directed mutagenesis of *ompC* | 55°C |
| D112G/Q124G | CCTGAGTTCGGTGGTATGACCTAC | Site directed mutagenesis of *ompC* | 60°C |
| M114D/Q124G | CCTGAGTTCGACGGTGACACCTAC | Site directed mutagenesis of *ompC* | 61°C |
| G124Q | TTCATGACCCAGCGTACCAACGGC | Site directed mutagenesis of *ompF* | 58.5°C |

**Figure 2.4.** Construction of *S. marcescens ompC* mutant strains.

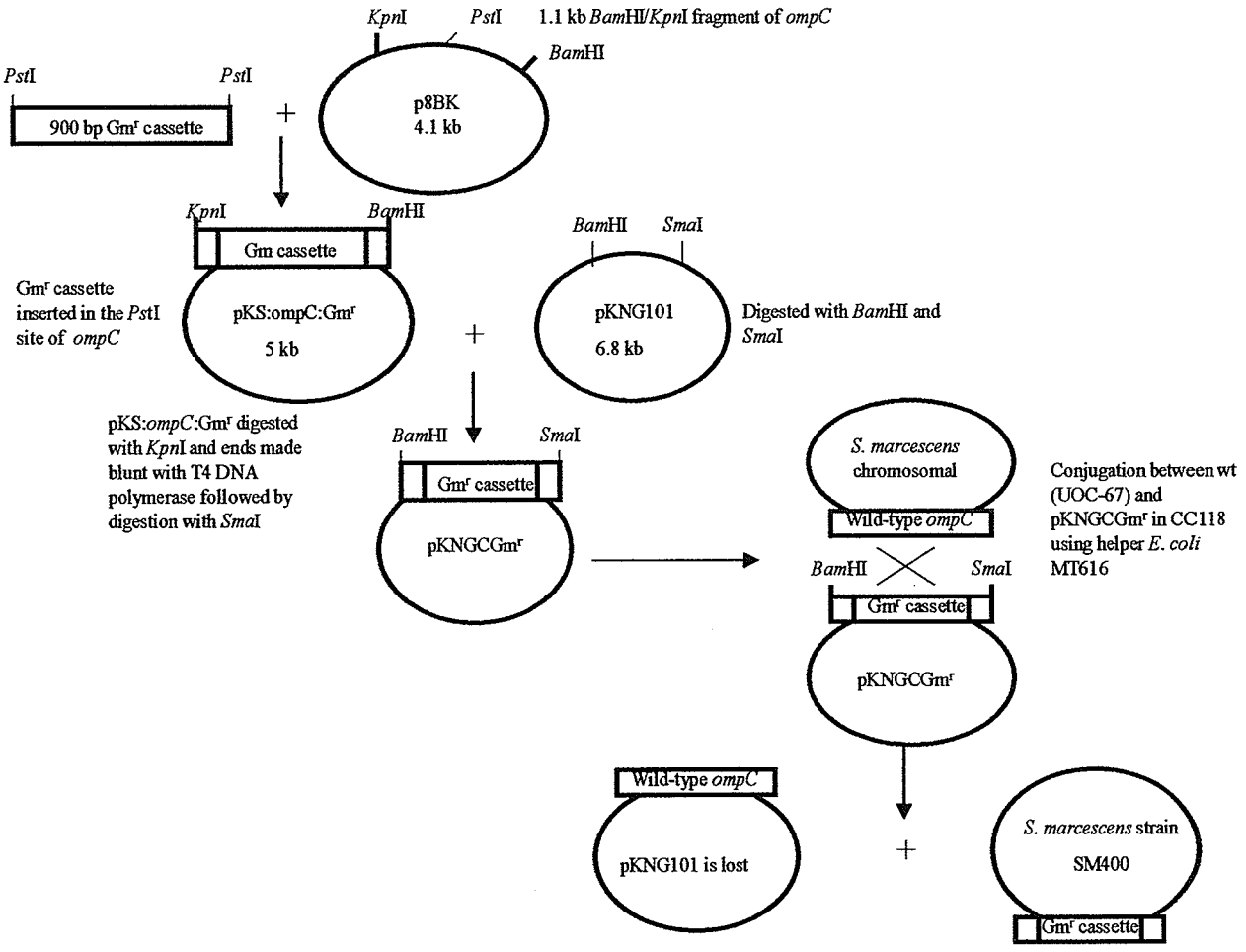
a) Deletion: Two unique *Aat*II restriction sites within p8BK(+) separated by approximately 400 bases were identified to be a feasible target for creating a deletion mutation in the *ompC* coding region. The mutated 0.8-kb *ompC* fragment was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118 for stability.

b) Insertion: A 900-bp $Gm^r$ cassette was inserted into a unique *Pst*I restriction site within the *Kpn*I/*Bam*HI flanked *ompC* fragment (p8BK). The disrupted *ompC* fragment was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118. Conjugation between the *E. coli* strain harboring each of the disrupted *S. marcescens ompC* genes individually and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986).

a)


KpnI AatII AatII BamHI
ompC
1.1 kb
p8BK(+)
4.1 kb
Unique AatII/AatII restriction sites separated by approximately 400 bases selected for deletion
KpnI BamHI
ΔompC
0.8 kb
p8BAK
3.7 kb
Digested with KpnI, the ΔompC fragment ends made blunt with T4 DNA polymerase and then digested with BamHI
BamHI SmaI
pKNG101
Digested with BamHI and SmaI
BamHI SmaI
ΔompC
pKNGBAK
S. marcescens chromosomal
wt ompC
BamHI SmaI
ΔompC
pKNGBAK
Conjugation between wt (UOC-67) and pKNGBAK in CC118 using helper E. coli MT616
S. marcescens strain
SM200
ΔompC
Wild-type ompC
pKNG101 is lost

b)


KpnI
PstI
1.1 kb BamHI/KpnI fragment of ompC
BamHI
PstI
PstI
900 bp Gmr cassette
p8BK
4.1 kb
KpnI
BamHI
Gm cassette
Gmr cassette inserted in the PstI site of ompC
pKS:ompC:Gmr
5 kb
BamHI
SmaI
pKNG101
6.8 kb
Digested with BamHI and SmaI
pKS:ompC:Gmr digested with KpnI and ends made blunt with T4 DNA polymerase followed by digestion with SmaI
BamHI
SmaI
Gmr cassette
pKNGCGmr
S. marcescens chromosomal
Wild-type ompC
BamHI
SmaI
Gmr cassette
pKNGCGmr
Conjugation between wt (UOC-67) and pKNGCGmr in CC118 using helper E. coli MT616
Wild-type ompC
pKNG101 is lost
S. marcescens strain SM400
Gmr cassette

pKNG101 is a suicide vector that contains a conditional origin of replication (*oriR6K*), the *strAB* genes encoding streptomycin phosphotransferase ($Str^r$), an origin of transfer (*mobRK2*), the *sacB* gene mediating sucrose sensitivity, and a multiple cloning site with a few restriction sites (Kaniga et al. 1991).

Introduction of the manipulated gene (*ompC*) into the cell on an unstable plasmid such as pKNG101, which can only replicate in the presence of λpir protein, results in homologous recombination of the altered gene with the wild type chromosomal copy of the gene when mated with the UOC-67 wild type strain. Firstly, a single homologous recombination event leads to the integration of pKNGBAK into the chromosome. In this case, the transconjugants, which are selected for streptomycin resistance, are sensitive to sucrose due to the synthesis of levans (toxic compounds the formation of which is catalyzed by levanosucrase encoded by the *sacB* gene). For the second homologous recombination event to occur, a single colony from the streptomycin plates is grown in a rich medium deprived of this antibiotic, promoting the occurrence of a second cross-over that replaces the wild type allele with the mutant one, followed by an excision of the plasmid-borne *sacB* from the chromosome (Schweizer 1992; Schweizer and Hoang 1995). Such *ompC* mutant strains are selected by their ability to grow on TSA (tryptic soy broth) containing varying concentrations of sucrose (sucrose resistance). Therefore, the primary integration of the plasmid into the host chromosome results in gene duplication, whereas the second recombination event results in resolving the co-integrate by replacing the original gene (Figure 2.3).

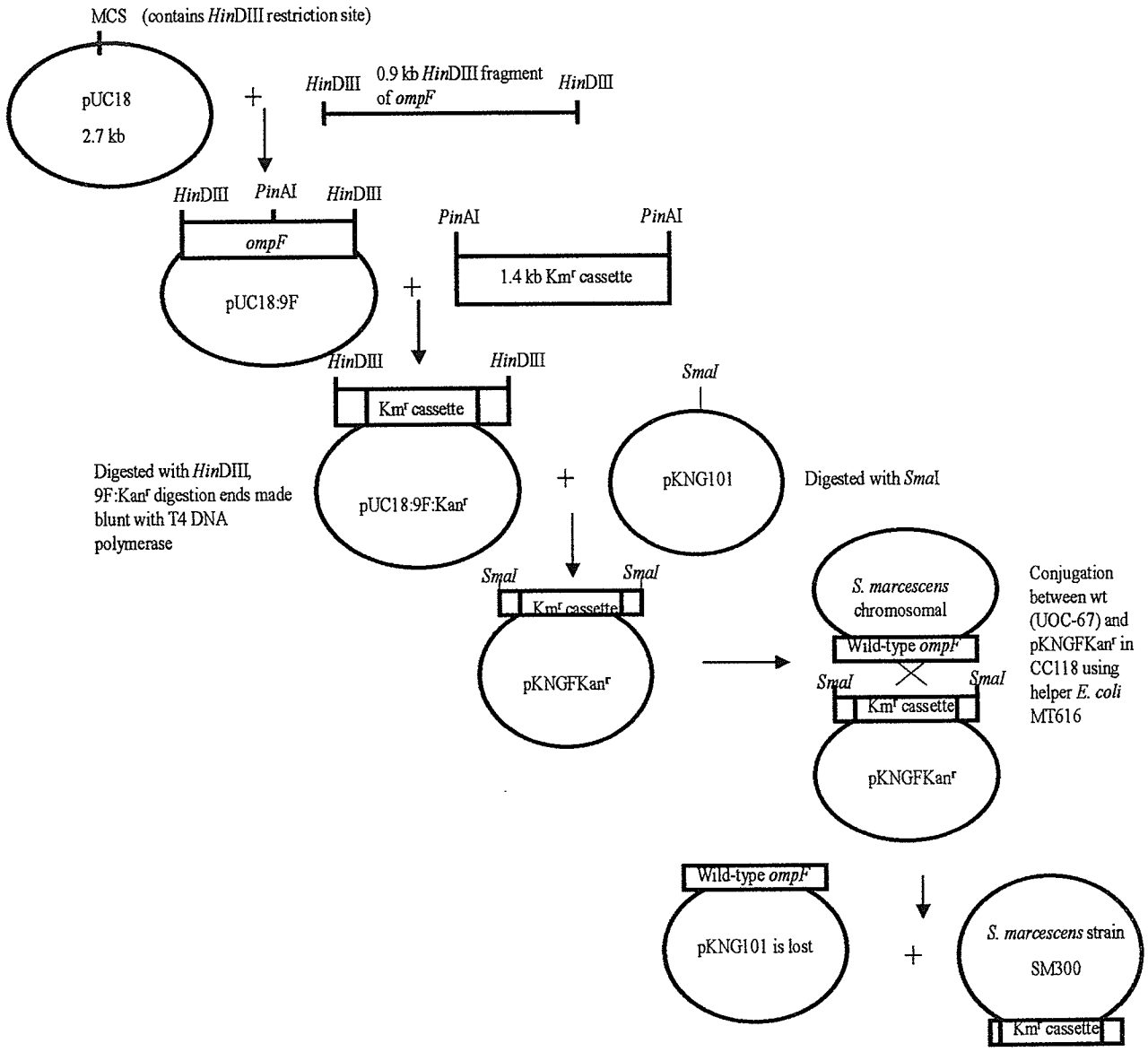
Secondly, in order to create an *ompC* mutant that I could use in the construction of the double porin deficient mutant, it was important to disrupt the *ompC* insertionally,

rather than deletionally to allow for antibiotic selection. A 900-bp *Pst*I-flanked $Gm^r$ cassette was inserted into a unique *Pst*I restriction site within the *Kpn*I/*Bam*HI flanked *ompC* fragment of P8BK resulting in pKS:ompC:$Gm^r$ (Figure 2.4b). After transformation of pKS:ompC:$Gm^r$ into *S. marcescens* UOC-67, the *ompC* insertion mutation was confirmed by restriction digestion with *Kpn*I/*Bam*HI, PCR of pKS:ompC:$Gm^r$ with OmpCfor/rev primers and sequencing of the mutated gene. pKS:ompC:$Gm^r$ was digested with *Kpn*I to remove the 2-kb *ompC* insertion, ends were made flush with T4 DNA polymerase and digested with *Bam*HI. The 2-kb insertionally inactivated *ompC* gene was ligated into pKNG101 replacement vector resulting in pKNGCG$m^r$ which was transformed into *E. coli* CC118 λpir in the similar fashion as above.

The *ompF* gene was cloned as a 0.9-kb *Hin*DIII fragment into a high copy number plasmid vector, pUC18 to construct pUC18:9F. A 1.4-kb *Pin*AI-flanked kanamycin resistance cassette was inserted into a unique *Pin*AI restriction site within this fragment (Figure 2.5) resulting in pUC18:9F:$Kan^r$. After transformation of pUC18:9F:$Kan^r$ into *S. marcescens* UOC-67, the *ompF* insertion mutation was confirmed by restriction digestion with *Hin*DIII, PCR of pUC18:9F:$Kan^r$ with OmpFfor/rev primers and sequencing of the mutated gene. pUC18:9F:$Kan^r$ was digested with *Hin*DIII to remove the 2.3-kb insertionally inactivated *ompF* gene and ends were made flush with T4 DNA polymerase. The *ompF* fragment was then ligated into the *Sma*I cut pKNG101 replacement vector resulting in pKNGFK$an^r$ which was transformed into *E. coli* CC118 in a similar fashion as for *ompC*.

**Figure 2.5.** Construction of *S. marcescens ompF* mutant strain.

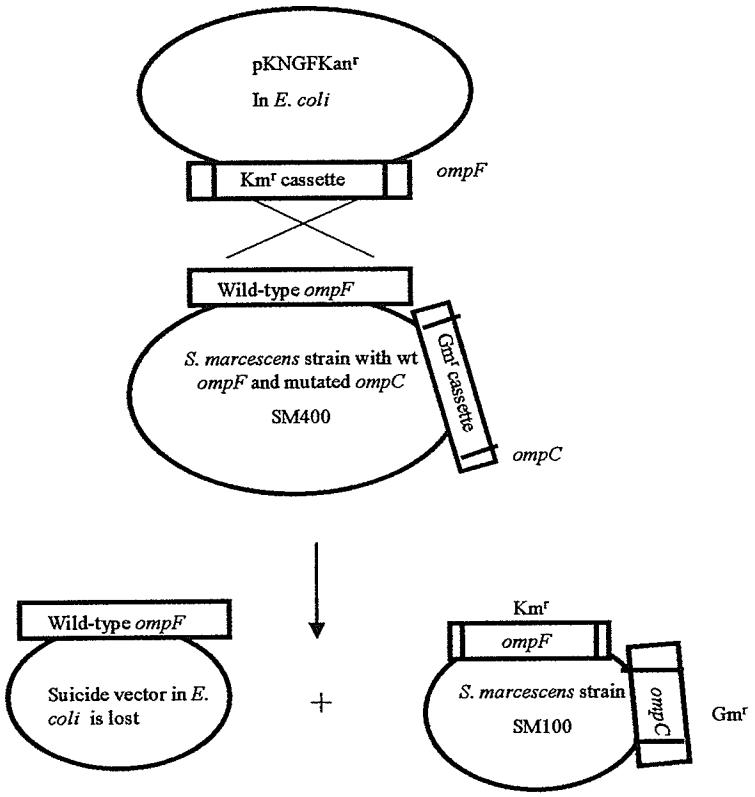
A 1.4 kb $Km^r$ cassette was inserted into a unique *Pin*AI restriction site within the *Hin*DIII flanked *ompF* fragment (pUC18:9F). The insertionally disrupted *ompF* gene was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118. Conjugation between the *E. coli* strain harboring the disrupted *S. marcescens ompF* gene and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986).


MCS (contains HinDIII restriction site)
pUC18
2.7 kb
HinDIII
0.9 kb HinDIII fragment of ompF
HinDIII
HinDIII
PinAI
HinDIII
ompF
pUC18:9F
PinAI
PinAI
1.4 kb Kmr cassette
HinDIII
HinDIII
Kmr cassette
pUC18:9F:Kanr
SmaI
pKNG101
Digested with HinDIII, 9F:Kanr digestion ends made blunt with T4 DNA polymerase
Digested with SmaI
SmaI
SmaI
Kmr cassette
pKNGFKanr
S. marcescens chromosomal
Wild-type ompF
SmaI
SmaI
Kmr cassette
pKNGFKanr
Conjugation between wt (UOC-67) and pKNGFKanr in CC118 using helper E. coli MT616
Wild-type ompF
pKNG101 is lost
S. marcescens strain SM300
Kmr cassette

Conjugation between the *E. coli* CC118 strain harboring each of the disrupted *S. marcescens ompF* and *ompC* genes (both insertional and deletional *ompC* mutations) individually and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986) to create SM300, SM400 (insertional mutation) and SM200 (deletional mutation) respectively. To create the double porin deficient mutant strain (SM100), pKNGFKan$^r$ was mobilized from *E. coli* CC118 into the *S. marcescens ompC* mutant strain (SM400) via conjugation (Figure 2.6). Analysis of transconjugants was based on sucrose resistance due to the absence of synthesis of lethal levano compounds, catalyzed by levanosucrase, the product of the *sacB* gene.

A multiplex PCR was done with optimized conditions that used both *ompF* and *ompC* primers. These optimized conditions included first optimizing the PCR conditions for each primer pair separately (OmpFfor/rev; OmpCfor/rev), then using equimolar concentrations of each primer set (0.2 μM of each primer) along with the 1X Qiagen PCR Buffer, and the HotStarTaq DNA polymerase in order to minimize primer dimers. Insertional and deletional mutations were confirmed by sequence analysis of the gel isolated correct sized PCR-product fragment (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada). The template DNA for PCR amplifications was the genomic DNA obtained from the putative *S. marcescens ompC* and *ompF* mutant strains.

**Figure 2.6.** Constructional approach to creating *S. marcescens* double mutant strain SM100 (*ompF/C* double mutant). The knockout construct in *E. coli* (pKNGFKan$^r$) with a single insertional mutation in one gene of interest (*ompF*) was mobilized from *E. coli* CC118 into the *S. marcescens ompC* mutant (SM400) via conjugation. To select for double mutants, both kanamycin and gentamicin selection pressure was used.


pKNGFKan$^r$
In *E. coli*
Km$^r$ cassette
*ompF*
Wild-type *ompF*
*S. marcescens* strain with wt *ompF* and mutated *ompC*
SM400
Gm$^r$ cassette
*ompC*
Wild-type *ompF*
Suicide vector in *E. coli* is lost
+
Km$^r$
*ompF*
*S. marcescens* strain
SM100
*ompC*
Gm$^r$

### 2.3.3 Complementation of porin mutants

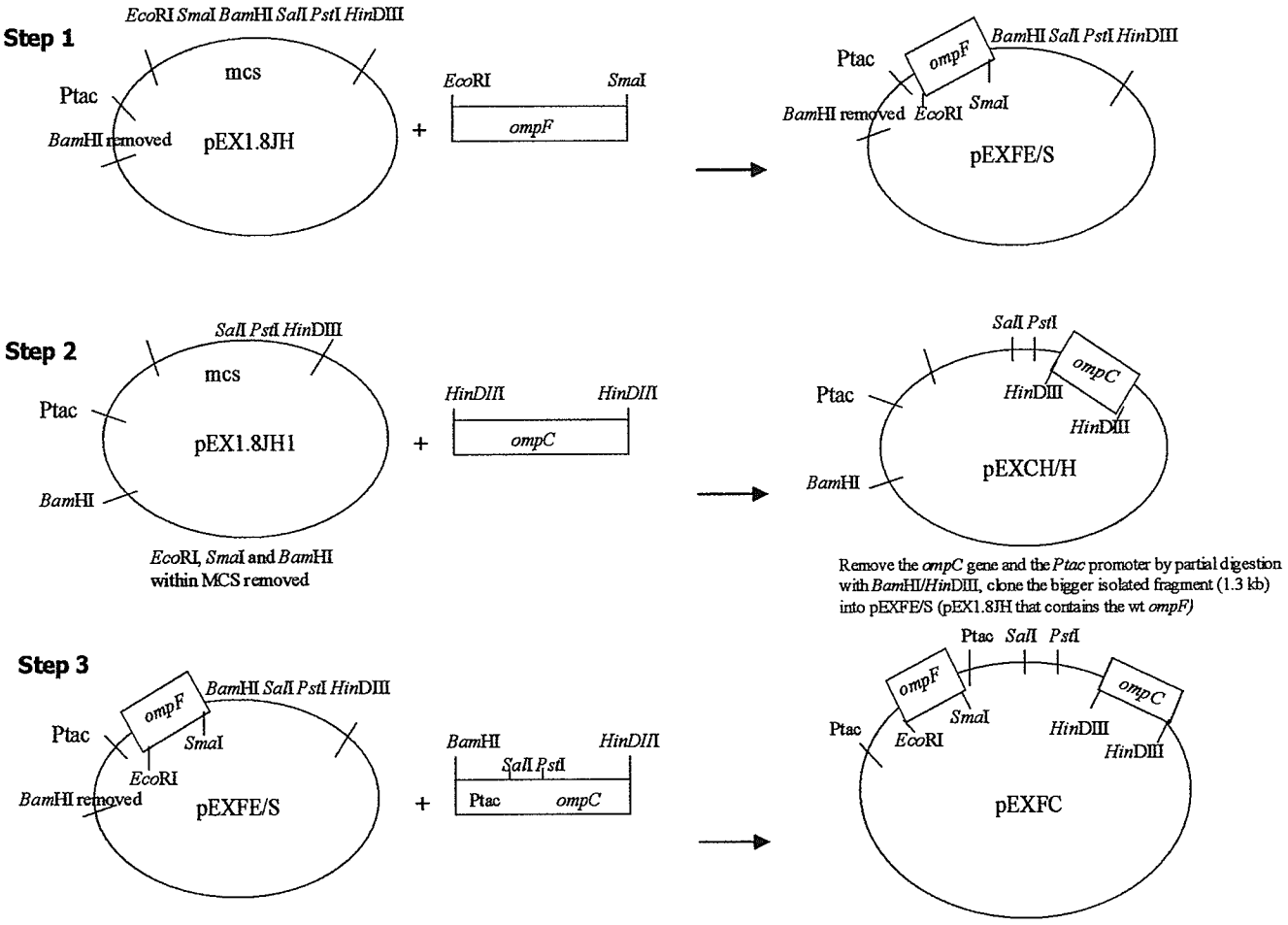
Complementation was carried out by introducing *ompF* and *ompC* into SM100 (OmpF/C porin-deficient strain). For this, *ompF* and *ompC* wild type porin genes were cloned into the pEX1.8 high copy number expression vector, which carries the $P_{tac}$ promoter for the expression of these genes. The coding sequences of *ompF* and *ompC* genes were PCR amplified from genomic DNA (UOC-67). Primers can be found in Table 2.2. The primers for *ompF* and *ompC* PCR reactions contained additional engineered *Eco*RI/*Sma*I sites and *Eco*RI/*Hin*DIII sites, respectively. The products from PCR amplifications individually (1.1-kb for *ompF* and 1.1-kb *ompC*) were digested with *Eco*RI/*Sma*I for *ompF* and *Eco*RI/*Hin*DIII for *ompC* and cloned into the *Eco*RI/*Sma*I and *Eco*RI/*Hin*DIII sites of pEX1.8 to create pEXF and pEXC, respectively. To create pEXFC, the primers for the *ompF* PCR reaction contained additional engineered *Eco*RI and *Sma*I sites. The *ompF* product (1.1-kb) from this PCR amplification was digested with *Eco*RI/*Sma*I and cloned into the *Eco*RI/*Sma*I sites of pEX1.8JH (a pEX1.8 with a *Bam*HI site in front of the P*tac* promoter removed by Erase-a-base system) to construct pEXFE/S (Figure 2.7 Step 1). The primers for the *ompC* PCR reaction contained additional engineered *Hin*DIII sites. The *ompC* product (1.1-kb) from this PCR amplification was digested with *Hin*DIII and cloned into the *Hin*DIII site of pEX1.8JH1 (a pEX1.8 with *Eco*RI, *Sma*I and *Bam*HI sites within the MCS removed) to construct pEXCH/H (Figure 2.7 Step 2). The *ompC* gene and the P*tac* promoter were then excised from pEXCH/H by a *Bam*HI and *Hin*DIII partial digestion. This fragment was then inserted into the *Bam*HI and *Hin*DIII sites within the pEXFE/S MCS to construct pEXFC (Figure 2.7 Step 3).

**Figure 2.7.** Diagrammatic representation of pEXFC construction.

Step 1.The primers for the *ompF* PCR reaction contained an additional engineered *Eco*RI and *Sma*I sites (pEXFCf1/r1). The *ompF* product from this PCR amplification was digested with *Eco*RI/*Sma*I and cloned into the *Eco*RI/*Sma*I sites of pEX1.8JH (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI site in front of the Ptac promoter removed by Erase-a-base system) to construct pEXFE/S.

Step 2. The primers for the *ompC* PCR reaction contained additional engineered *Hin*DIII sites (pEXFCf2/r2). The *ompC* product from this PCR amplification was digested with *Hin*DIII and cloned into the *Hin*DIII site of pEX1.8JH1 (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI, *Eco*RI and *Sma*I sites within MCS removed) to construct pEXCH/H.

Step 3. The *ompC* gene (including the Ptac promoter) was then excised from the pEXCH/H vector by *Bam*HI and *Hin*DIII partial digestion and inserted into the *Bam*HI and *Hin*DIII sites within the pEXFE/S to construct pEXFC.


Step 1
EcoRI SmaI BamHI SalI PstI HinDIII
mcs
Ptac
BamHI removed
pEX1.8JH
EcoRI
SmaI
ompF
Ptac
ompF
BamHI SalI PstI HinDIII
BamHI removed
EcoRI
SmaI
pEXFE/S
Step 2
SalI PstI HinDIII
mcs
Ptac
pEX1.8JH1
BamHI
EcoRI, SmaI and BamHI
within MCS removed
HinDIII
HinDIII
ompC
SalI PstI
Ptac
ompC
HinDIII
HinDIII
BamHI
pEXCH/H
Remove the ompC gene and the Ptac promoter by partial digestion
with BamHI/HinDIII, clone the bigger isolated fragment (1.3 kb)
into pEXFE/S (pEX1.8JH that contains the wt ompF)
Step 3
ompF
BamHI SalI PstI HinDIII
Ptac
SmaI
EcoRI
BamHI removed
pEXFE/S
BamHI
HinDIII
SalI PstI
Ptac
ompC
Ptac SalI PstI
ompF
ompC
Ptac
SmaI
EcoRI
HinDIII
HinDIII
pEXFC

To construct strain SM600, electroporation of pEXFC into SM100 directly was optimized by using a 40 μL log phase cell suspension (after resuspending the cells in cold 10% glycerol and freezing them in aliquots) and 10 μL of DNA in a low ionic strength buffer (TE). Electroporation was done at 1.45 volts in short 1 second intervals for 3 seconds. 1 ml of SOC media (20g/1L Bacto Tryptone, 5g/1L Bacto Yeast Extract, 5M NaCl, 1M KCl, 1M $MgCl_2$, 1M $MgSO_4$, 1M glucose) was immediately added to these cells and left to incubate at 37°C for one hour without shaking and then plated out. SOC media contains twice the amount of Bacto Tryptone in comparison to LB media, which provides more protein precursors for rapid repair of cell walls damaged by the electroporation process, and maintains isotonicity to prevent cell death by osmotic rupture (Electroporation-Competent cells, Stratagene, modified protocol under catalog number 200123). SM700 was constructed by transferring the pEXF into SM100 and SM800 was constructed by transferring the pEXC into SM100 in the same fashion described for pEXFC. Selection of transconjugants occurred by incorporation of ampicillin into the growth medium. Transformants and knockout strains were verified by SDS-PAGE protein analysis. Proteins were expressed under the control of the $P_{tac}$ promoter and induced by the addition of varying concentrations (0.5-5 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] to the growth medium.

### 2.3.4 Site-directed mutagenesis of *ompF* and *ompC* genes

Jo-Anne Hutsul initiated work on *ompC* sites 112 and 114 that I have completed. D112G, M114D and Q124G mutants (Table 2.2) were constructed by cloning a 0.5-kb *PstI*/*KpnI* *ompC* fragment into pKS(+) and introducing the amino acid change through

PCR (Molecular Biology: Current Innovations and Future Trends; Thiolas et al. 2004). The 0.5-kb fragment with each individual mutation was introduced into pM8BH12(+). Recombinant D112G/M114D was constructed by cloning this 0.5 kb *PstI/KpnI ompC* fragment that includes the M114D mutation into pKS(+), using PCR to introduce position 112 alteration and introducing this fragment into pM8BH12(+). Recombinants D112G/Q124G and M114D/Q124G were constructed by cloning the 0.5 kb *PstI/KpnI ompC* fragment that includes the Q124G mutation into pKS(+), using PCR to introduce position 112 and 114 alterations respectively, and introducing these fragments into pM8BH12(+). The G124Q mutant was created by cloning a 0.9 kb *Hin*DIII/*Hin*DIII *ompF* fragment into pKS(+), introducing the amino acid change through PCR, and then introducing the 0.9 kb fragment containing the G124Q mutation into pM8BH13(+). All mutated *ompF* and *ompC* fragments were then transferred into SM100 (*ompF/ompC* deficient strain) via electroporation described above using SOC. Amplification reactions were performed in a total volume of 50 μL containing 10 mM dATP, 10 mM dCTP, 10 mM dGTP and 10 mM dTTP, 40 pmol/μL primer, 20 ng template, and 1 U *Pfx* Polymerase in 1X PCRx Enhancer solution (Invitrogen). For double-site recombinants, genomic DNA obtained from the single recombinant was used as a PCR template to introduce the base change in the second site. The reaction mixtures were subjected to amplification in Techne Genius programmed for 35 cycles of 1 min at 95°C, 1 min at 52°C, and 5 min at 72°C. Amplification products were electrophoresed in 0.8% agarose gels in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.2), stained with ethidium bromide and inspected by UV light transillumination (FluorChem™ 8900; Alpha Innotech, San Leandro, California). All modifications were confirmed via

sequence analysis of each specific fragment (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada).

### 2.3.5 Porin Purification and Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Cell envelope preparations of SM100, SM300, SM400, SM600, SM700 and SM800 were prepared (Lugtenberg et al. 1975) from a 50 ml overnight bacterial culture. Cells were harvested by centrifugation at 5,000 xg 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 1,200 xg for 10 min. The supernatant was centrifuged at 100,000 xg for 1 hour at 4°C. Porins were isolated as described by Malouin et al. 1990. First, the SDS-insoluble fraction was obtained by solubilizing the envelopes with 2% (w/v) SDS, at 32°C for 30 min, and centrifuging at 100,000 xg for 30 min. The supernatant was discarded and the pellet was resuspended in 50 mM Tris-HCl, pH 8.0. After solubilization at 37°C, the samples were centrifuged at 100,000 xg for 1 hour. Porins were solubilized from the pellet with 50 mM Tris-HCl, pH 7.7, 1% (w/v) SDS, 5 mM EDTA, and 0.4 M NaCl for 1 hour at 37°C. Cell envelopes and porins were analyzed via SDS-PAGE. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) of cell envelopes of *ompF* and *ompC* knockout mutant and complemented strains was carried out as described by Lugtenberg et al. 1975, using 12% (w/v) polyacrylamide gels. Prior to loading, the samples were heated at 95°C for 5 min in 12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 0.0125% bromophenol blue, 2% (v/v) glycerol and 1% (v/v) β–mercaptoethanol. In order to separate porin samples, 4 M urea

was added to the SDS-PAGE system. For complemented strains SM600, SM700 and SM800, 0.5, 1, or 5 mM IPTG was added to the growth medium for porin expression. 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. Proteins were stained for 20-30 min using Coomassie Blue staining solution (Sigma, St. Louis, MO) in 30% (v/v) isopropanol/10% (v/v) acetic acid. The gels were destained with 20% (v/v) methanol/7.5% (v/v) acetic acid for 20-30 min and then overnight with fresh destain solution. Stained SDS-PAGE gels were wrapped in cellophane (Bio-Rad) and air dried.

**2.3.6 Antibiotic susceptibility tests**

Susceptibility of SM100, SM200, SM300, SM400, SM600 and SM1-SM7 to ampicillin (0.008 - 64 mg/L), carbenicillin (0.008 - 64 mg/L), norfloxacin (0.001 - 32 mg/L), ciprofloxacin (0.001 - 32 mg/L), ofloxacin (0.001 - 32 mg/L), cephaloridine (0.001 - 16 mg/L), and cephalothin (0.001 - 16 mg/L) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was tested using the minimum inhibitory concentration (MIC) two-fold broth dilution method (National Committee for Clinical Laboratory Standards protocol with modifications). For β–lactams, ampicillin and carbenicillin susceptibilities were tested in the presence and absence of clavulanic acid, an agent that affects the ability of β–lactamases to hydrolyze these antibiotics. Complemented strains SM600, SM700 and SM800 were tested in the presence of 1 mM IPTG, an agent that induces expression of genes cloned under *tac* promoter. Overnight cultures in Mueller Hinton (MH) broth were diluted 1000-fold in fresh broth, grown at 37°C until $OD_{600}$=0.5-0.9 and 5 μL of the bacterial suspension was inoculated in MH containing serial dilutions of each antibiotic.

Results were reported as MIC, the concentration of antibiotic that inhibited visible growth determined by absence of turbidity in MH after 18 hours of shaking incubation at 37°C.

**2.3.7 Fluoroquinolone accumulation**

The accumulation of ciprofloxacin by SM100, SM200, SM300, SM600 and SM1-SM7 was measured using the method of Mortimer and Piddock, 1991. Cultures in 3L batches were grown until $OD_{600}$ reaches 0.5-0.7. Cells were harvested by centrifugation at 4000 x *g* for 15 min at room temperature, resuspended to 30 ml in phosphate buffered saline (PBS) pH 7.5 and washed two times. For complemented strain SM600, accumulation was performed in presence of 1 mM IPTG. Ciprofloxacin was added to a final concentration of 10 μg/mL and 0.5 ml of culture was removed in 30 sec intervals for a period of 9 minutes and immediately diluted in 1 ml of cold PBS. Next, a rapid centrifugation (13,000 x *g* at 4°C for 5 min) was carried out, followed by a wash in cold PBS, suspension in 1 mL of HCl-glycine 0.1 M pH 3.0 to lyse the cells, and overnight incubation at room temperature. This suspension was centrifuged at 11,000 x *g* for 5 min to remove cellular debris and antibiotic concentration measured in a RF-1501 Shimadzu spectrofluorometer. The fluorescence of ciprofloxacin was measured at 279 nm excitation wavelength and 447 nm emission wavelength. Antibiotic concentration was calculated using a standard curve for the antibiotic (concentration ranging from 100 to 1000 ng) in 0.1 M HCl-glycine 0.1 M pH 3.0. The results were expressed as nanograms of antibiotic incorporated per milligram of dry mass of bacteria.

### 2.3.8 Liposome swelling assays

Liposome swelling assays were carried out as previously described (Nikaido and Rosenberg 1983). Phosphatidylcholine (6.2 μmole) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] and dicetylphosphate (0.2 μmole) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] were placed in a test tube and allowed to dry using mild heat. Dried lipid film was suspended in 200 μL (equivalent to 10 μg of porin) of aqueous suspension of each isolated porin. Protein concentration was determined through optical density measurement ($A_{280} - A_{260}$ = 1 mg/mL). Lipid was dispersed first by shaking, and then by sonication using a Fisher Sonic Dismembrator Model 300 for three 15-second bursts at 35%. Liposomes were then dried in a Speed-Vac under medium heat and stored overnight in the dark in a vacuum dessicator. Next, 400 μL of 12 mM stachyose, 4 mM Na-NAD (pH 6.0), and 1 mM imidazole-NAD (pH 6.0) were added to the dried liposomes, and left undisturbed at room temperature for 2 hours. Resuspension was completed by gently shaking with the hand. The suspension was then filtered through a 8 μM Millipore membrane filter to remove large aggregates. To measure liposome swelling, an isotonic concentration of each solute (glucose (18 mM), maltose (18 mM), cephaloridine (18 mM), cephalothin (9 mM) and ampicillin (9 mM)) was prepared in 1 mM Na-NAD (pH 6.0), and 1 mM imidazole-NAD (pH 6.0). The liposome suspension (20 μL) was added to a cuvette containing 600 μL of solute, mixed quickly and liposome swelling was measured following optical density change at 400 nm every 10 s over a period of 90 s.

## 2.4 Results

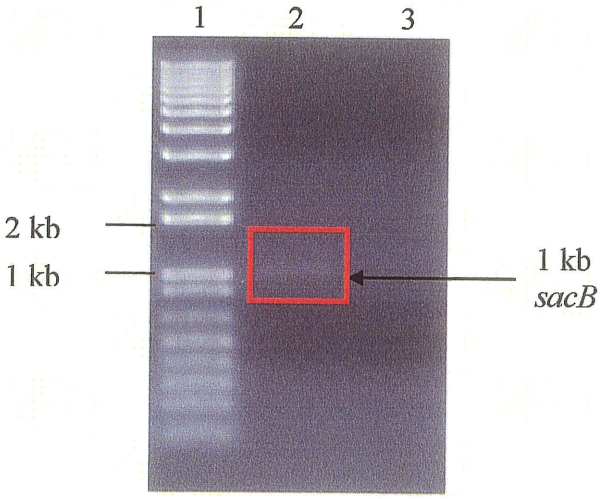
### 2.4.1 Construction of *S. marcescens* mutant strains

PCR amplification of *S. marcescens ompF* insertion mutant genomic DNA using OmpFfor/rev primers resulted in two products, a 2.3-kb band representing the *ompF* insertion fragment and the 0.9-kb wild type *ompF* gene after the single crossover event (Figure 2.8), and only the 2.3-kb insertion fragment after the double crossover event (Figure 2.9). A 0.85-kb band using the OmpFKmf/r kanamycin$^r$ cassette primers (Figure 2.10) was also found. PCR amplification of *S. marcescens ompC* deletion mutant genomic DNA using OmpCfor/rev primers illustrates two products, a 0.8-kb band representing the *ompC* deletion fragment and a 1.1-kb wild type *ompC* gene after the single crossover event (Figure 2.11), and only the 0.8-kb deletion fragment after the double crossover event (Figure 2.12). PCR amplification of *S. marcescens ompC* insertion mutant genomic DNA using OmpCfor/rev primers denote two products, a 2.0-kb *ompC* insertion band and a 1.1-kb wild type *ompC* gene band after the single crossover event (Figure 2.13), and only the 2.0-kb insertion fragment after after the double crossover event (Figure 2.14). Additionally, a 1.1-kb band was found after PCR amplification using OmpCGmf/r gentamicin cassette primers with the *ompC* insertion mutant DNA (Figure 2.15). PCR products obtained from genomic DNA from putative *S. marcescens ompF* and *ompC* double mutants illustrate a 2.3-kb insertion band for *ompF* and a 2.0-kb insertion band for *ompC* using OmpFfor/rev and OmpCfor/rev multiplex primers (Figure 2.16).

**Figure 2.8.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompF* mutants after the single crossover event.

a) Lane 1, 1 kb ladder; Lane 2, *sacB* (SacBf/r) product; Lane 3, ddH2O. Molecular weight of product is 1 kb. b) Lane 1 and Lane 2, *ompF* (OmpFfor/rev) products (2 insertion mutants shown). Important bands indicated by the arrows; bands shown are around 0.9-kb and 2.3-kb. 3 μL of samples was loaded in each lane.

a)




b)

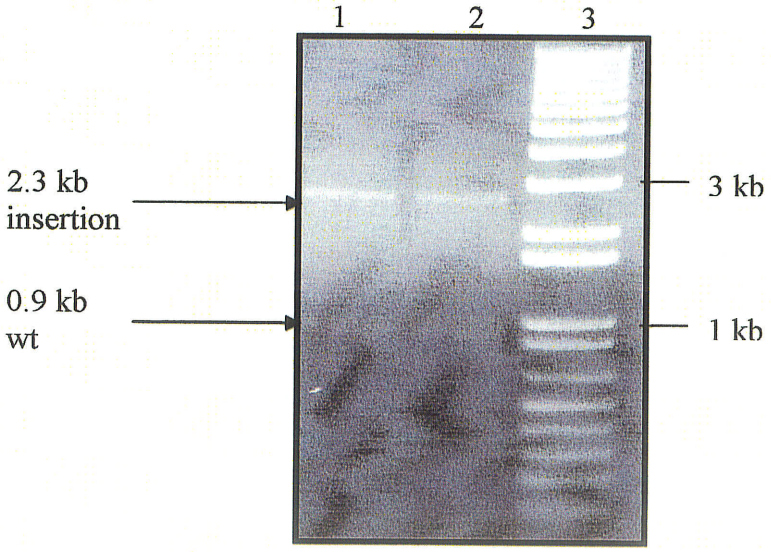


**Figure 2.9.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompF* mutants after sucrose selection.

Lane 1,1 kb ladder; Lane 2, *sacB* product (SacBf/r; no band visible); Lane 3, *ompF* (OmpFfor/rev) product (2.3 kb band boxed in red); Lane 4, ddH2O. 3 μL of all samples was loaded in each lane.

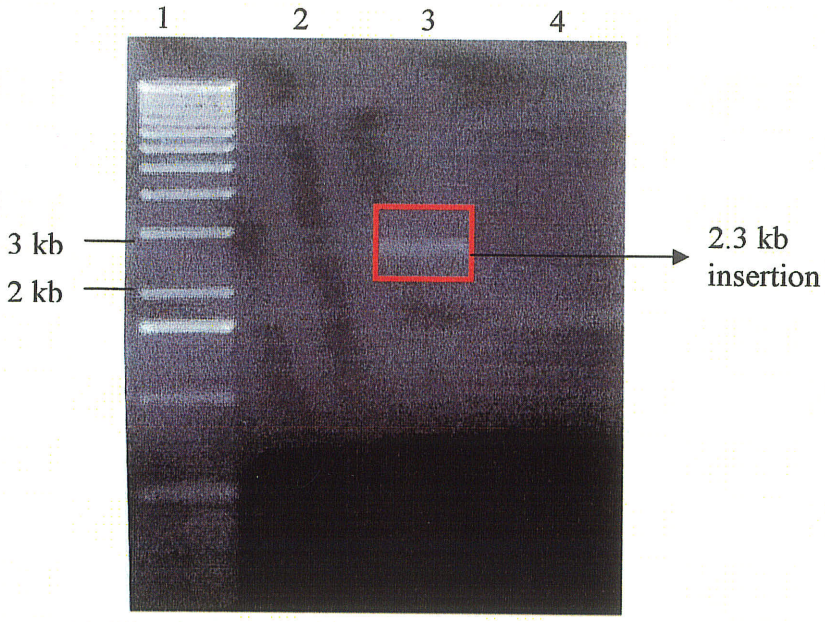
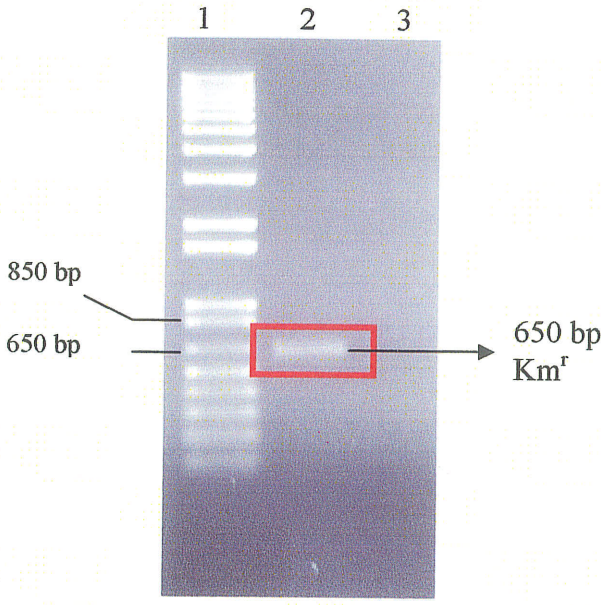

1
2
3
4
3 kb
2 kb
2.3 kb
insertion

**Figure 2.10.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompF* mutants using kanamycin$^r$ cassette primers.

a) Lane 1,1 kb(+) ladder; Lane 2, product using kan$^r$ primers from within the cassette (650 bp amplified); Lane 3, ddH2O

b) Lane 1, 1 kb ladder; Lane 2, larger fragment amplified (OmpFKmf/r, some of *ompF* (about 200 bp) and some of the cassette (650 bp) yielding an approximate 850 bp product (boxed). There is a shift between this band and the 650 bp band in a) indicating the presence of the cassette; Lane 3, 1 kb ladder; and Lane 4, ddH2O. 3 μL of all samples loaded in each lane.

a)


1
2
3
850 bp
650 bp
650 bp
Km^r

b)

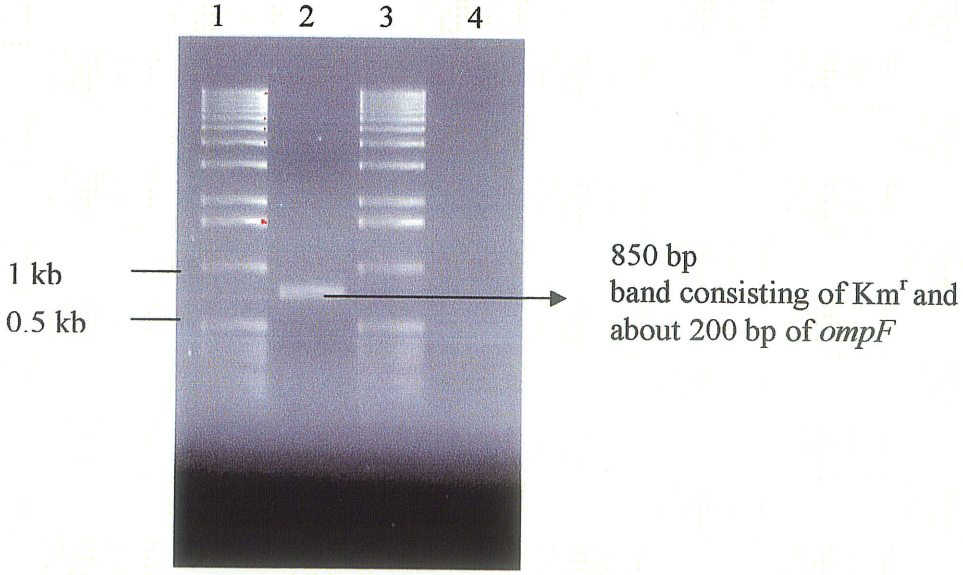
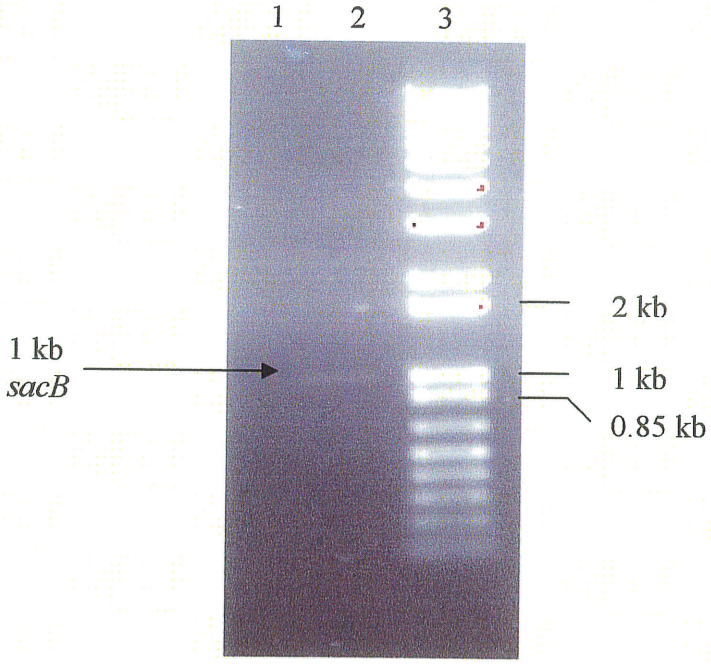

1
2
3
4
1 kb
0.5 kb
850 bp
band consisting of Km^r and
about 200 bp of ompF

**Figure 2.11.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompC* mutants after the single crossover event. This picture illustrates the *ompC* deletion mutant.

a) Lane 1, ddH2O; Lane 2, *sacB* (SacBf/r) product; Lane 3, 1 kb(+) ladder. Molecular weight of product is 1.0 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *ompC* product (OmpCfor/rev); Lane 3, ddH2O. Important bands indicated by the arrows; bands around 1.1-kb and 0.8-kb. 5 μL of samples loaded in each lane.

a)


1
2
3
2 kb
1 kb
1 kb
sacB
0.85 kb

b)

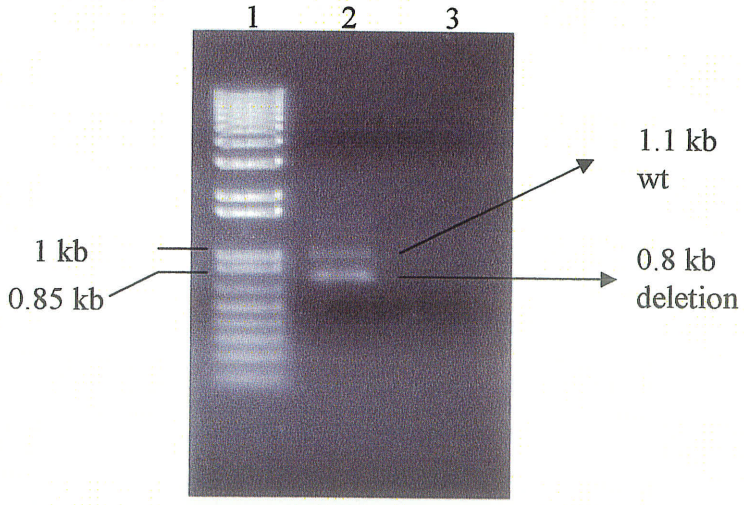

1
2
3
1.1 kb
wt
1 kb
0.85 kb
0.8 kb
deletion

**Figure 2.12.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompC* mutants after sucrose selection.

This picture illustrates the *ompC* deletion mutant.

Lane 1,1 kb(+) ladder; Lane 2, *ompC* (OmpCfor/rev) product (0.8-kb band boxed in red); Lane 3, *sacB* product (SacBf/r; no band visible); Lane 4, ddH2O. 3 μL of samples loaded in each lane.

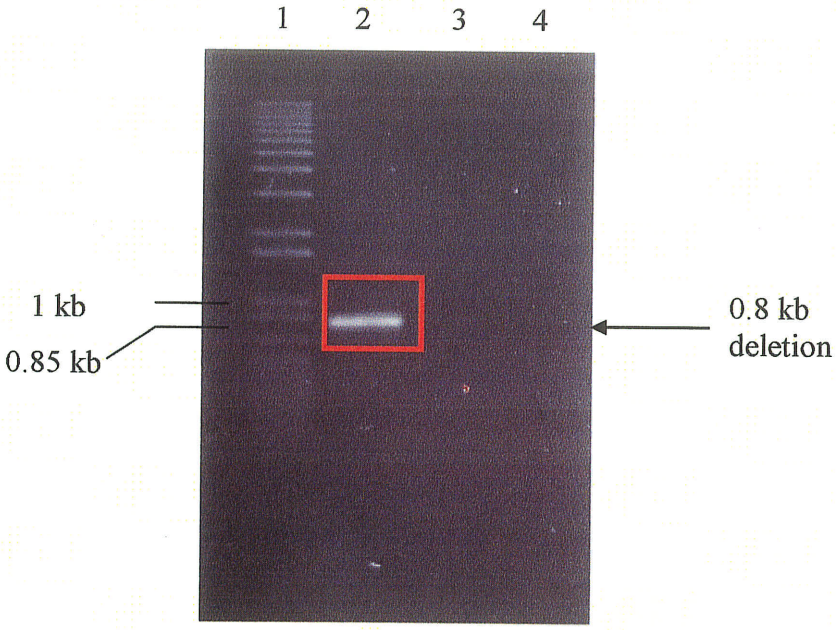
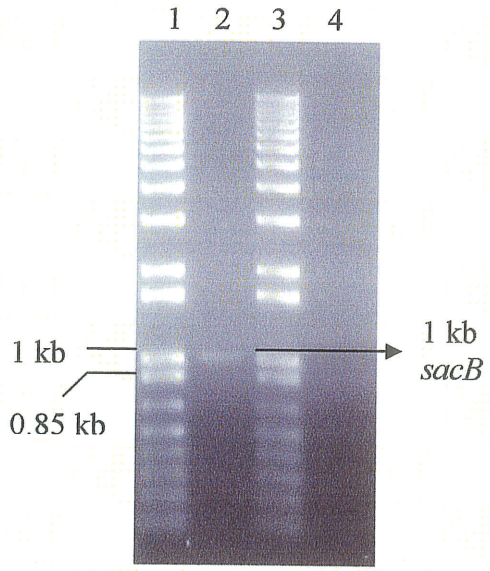

1
2
3
4
1 kb
0.85 kb
0.8 kb
deletion

**Figure 2.13.** PCR products obtained from genomic DNA from putative *S. marcescens ompC* mutants after the single crossover event.

This picture denotes the *ompC* insertion mutant used in preparation of the double *ompF/ompC* knockout.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* (SacBf/r) product; Lane 3, 1 kb ladder; Lane 4, ddH2O. Molecular weight of product is 1 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *ompC* (OmpCfor/rev) product; Lane 3, ddH2O. Important bands indicated by arrows; bands shown are around 1.1-kb and 2.0-kb. 7 μL of samples loaded in each lane.

a)


1 2 3 4
1 kb
0.85 kb
1 kb
*sacB*

b)

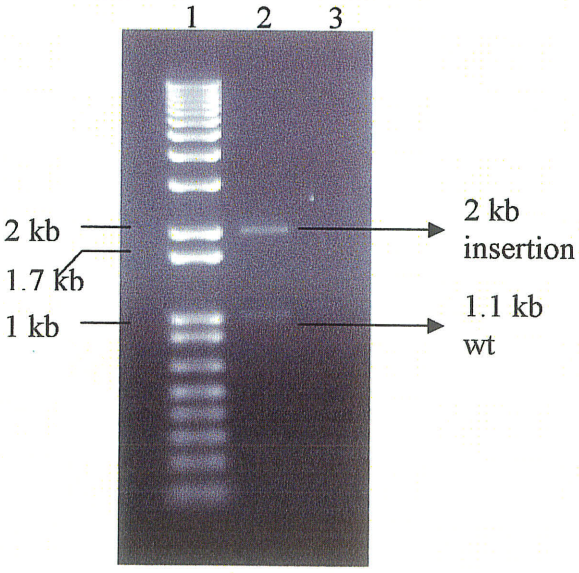

1 2 3
2 kb
1.7 kb
1 kb
2 kb
insertion
1.1 kb
wt

**Figure 2.14.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompC* mutants after sucrose selection.

This picture illustrates the *ompC* insertion mutant used in preparation of the double *ompF/ompC* knockout.

Lane 1,1 kb(+) ladder; Lane 2, *ompC* (OmpCfor/rev) product (2.0 kb band boxed in red); Lane 3, *sacB* product (SacBf/r; no band visible); Lane 4, ddH2O. 5 μL of samples loaded in each lane.

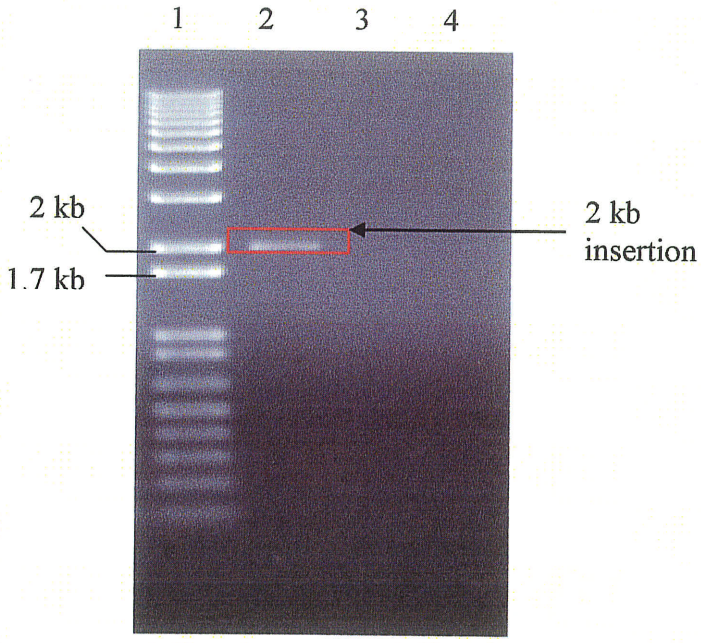
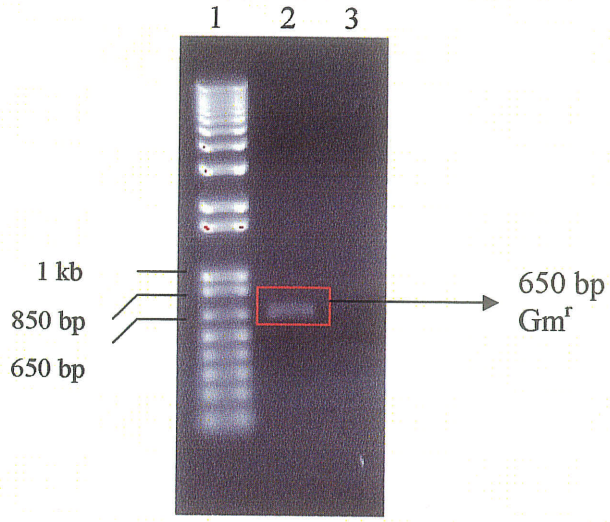

1
2
3
4
2 kb
1.7 kb
2 kb
insertion

**Figure 2.15.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompC* mutants using gentamicin$^r$ cassette primers.

a) Lane 1,1 kb(+) ladder; Lane 2, product using Gm$^r$ primers from within the cassette (650 bp amplified); Lane 3, ddH2O

b) Lane 1, 1 kb ladder; Lane 2, larger fragment amplified (OmpCGmf/r, some of *ompC* (about 450 bp) and some of the cassette (650 bp) yielding an approximate 1.1 kb product (boxed). There is a shift in this lane compared to Lane 2 of a) indicating the presence of the cassette. Lane 3, ddH2O. 4 μL of samples loaded in each lane.

a)


1
2
3
1 kb
850 bp
650 bp
650 bp
Gm^r

b)

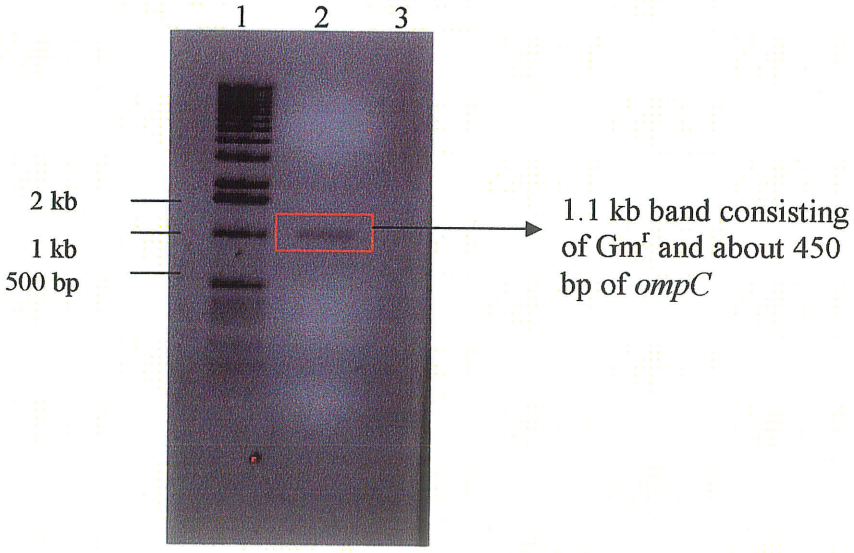
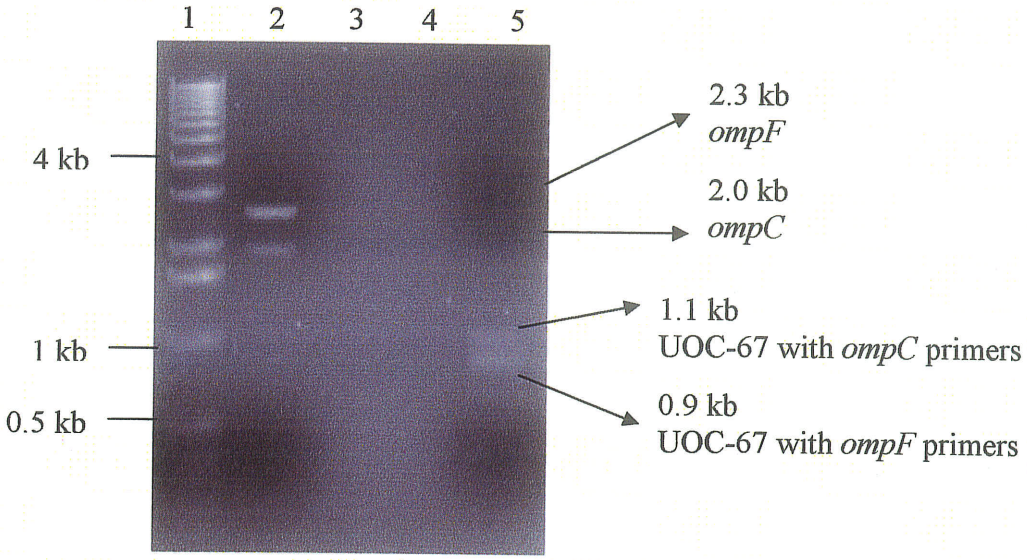

1
2
3
2 kb
1 kb
500 bp
1.1 kb band consisting of Gm^r and about 450 bp of ompC

**Figure 2.16.** PCR products obtained from genomic DNA from putative *S. marcescens* *ompF* and *ompC* double mutants after sucrose selection.

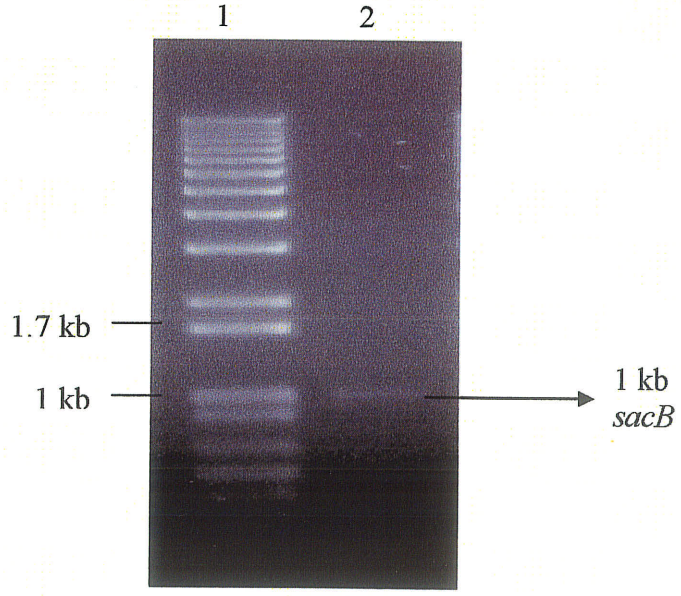
a) Lane 1, 1 kb ladder; Lane 2, *ompF/C* product (OmpFfor/rev and OmpCfor/rev primers); Lane 3, ddH2O; Lane 4, *sacB* product; Lane 5, wt UOC-67 control (OmpFfor/rev and OmpCfor/rev primers). Molecular weights of PCR products are 2.3 kb for *ompF* and 2.0 for *ompC*, respectively. Molecular weights of PCR products for UOC-67 are represented by 1.1 kb for *ompC* primers and 0.9 kb for *ompF* primers, respectively. No band is obtained using *sacB* primers. 7 μL of samples loaded in each lane.

b) After initial selection on kanamycin and gentamicin (no sucrose), *sacB* product (SacBf/r) were used on the mutant for positive control as indicated by the presence of a 1.0-kb in Lane 2. Lane 1, 1 kb(+) ladder.

a)


1
2
3
4
5
4 kb
1 kb
0.5 kb
2.3 kb
ompF
2.0 kb
ompC
1.1 kb
UOC-67 with ompC primers
0.9 kb
UOC-67 with ompF primers

b)


1
2
1.7 kb
1 kb
1 kb
sacB

### 2.4.2 Complementation of porin mutants

Figure 2.17 shows a 7.4 vector band corresponding to pEX1.8 and the 1.1-kb band corresponding to the *ompF* (Figure 2.17a) and *ompC* (Figure 2.17b) genes. The primers for the PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites for the *ompC* (pEXCf/r primers) and additional engineered *Eco*RI and *Sma*I sites for the *ompF* reaction (pEXFf/r primers). Figure 2.18 shows the stepwise digestion of pEXFC where the first digestion was performed with *Eco*RI/*Sma*I (Step 1) to release the wild type *ompF* gene, indicated by the 1.1-kb band, followed by a second digestion performed with *Hin*DIII (Step 2) resulting in the wild type *ompC* gene release (1.1-kb).

SDS-PAGE analysis of cell envelope and purified porins from *S. marcescens* SM100 and SM600 in Figures 2.19 and 2.20 confirm absence of porin proteins expressed in SM100 (Figure 2.19c and 2.20c) and an over-expression of proteins in varying concentrations of IPTG for SM600 (Figure 2.19a, 2.19b, 2.19c, 2.20c).

Additionally, please refer to the appendix for confirmation sequences throughout.

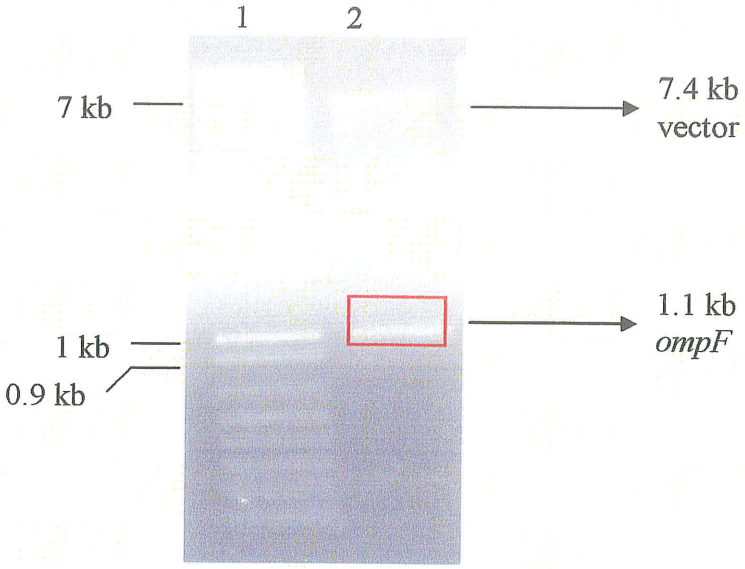
### 2.4.3 Site-directed mutagenesis of *ompF* and *ompC* genes

Agarose gel electrophoresis of the *S. marcescens ompC* and *ompF* fragments used for site-directed mutagenesis at position 124 within Loop 3 is shown in Figure 2.21. pM8BH12(+) digestions with *Pst*I/*Kpn*I results in a 0.5-kb *ompC* fragment (Figure 2.21a); whereas pM8BH13(+) digestions with *Hin*DIII results in a 0.9-kb *ompF* mutated fragment (Figure 2.21b).

**Figure 2.17.** Restriction digestion products of a) pEXF (*Eco*RI/*Sma*I) and b) pEXC (*Eco*RI/*Hin*DIII) constructs. The primers for *ompF* PCR reaction contained additional engineered *Eco*RI and *Sma*I sites (pEXFf/r). The primers for *ompC* PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites (pEXCf/r). The products from PCR amplifications individually (1.1-kb for *ompF* and 1.1-kb for *ompC*) were digested with *Eco*RI/*Sma*I for *ompF* and *Eco*RI/*Hin*DIII for *ompC* and cloned into the *EcoR*I/*Sma*I and *Eco*RI/*Hin*DIII sites of pEX1.8, respectively.

Lane 1, 1 kb ladder; Lane 2 a) vector band (7.4 kb) is the upper band (indicated by an arrow) and the insert *ompF* wild type gene is the lower band; Lane 2 b) vector band (7.4 kb) and the insert *ompC* wild type gene

a)


1
2
7 kb
7.4 kb
vector
1 kb
0.9 kb
1.1 kb
ompF

b)

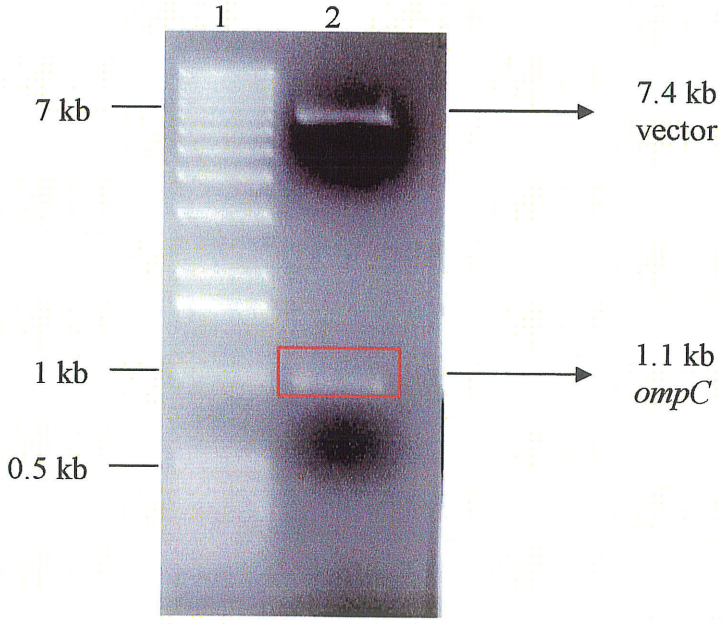
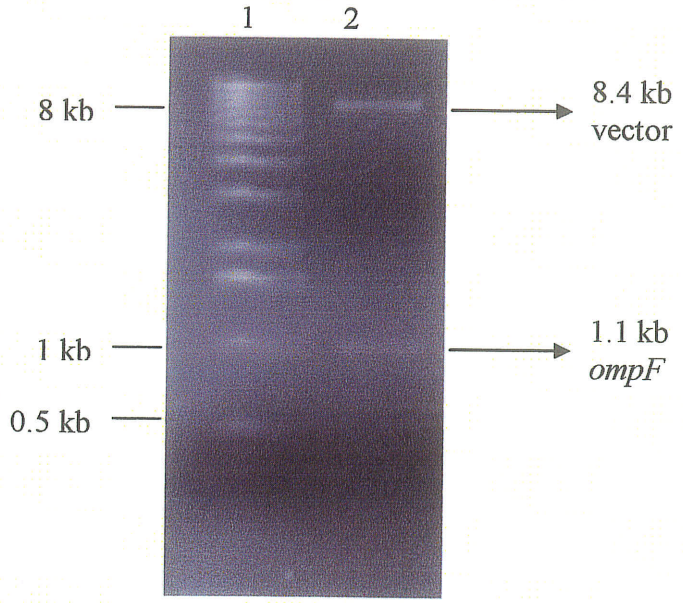

1
2
7 kb
7.4 kb
vector
1 kb
0.5 kb
1.1 kb
ompC

**Figure 2.18.** Restriction digestion products of pEXFC. To test for the presence of both wild type genes in pEX1.8JH, a stepwise digestion was done where first digestion was performed with *Eco*RI/*Sma*I (Step 1) to release the *ompF* gene (1.1 kb, indicated by arrow in Lane 2). The remaining 8.4 kb vector contains the gel purified wild type *ompC*. A second digestion was performed with *Hin*DIII (Step 2) resulting in the wild type *ompC* gene (1.1 kb, indicated by arrow in Lane 2), and the approximate 7.4 kb vector band. 1 kb ladder (3 μL) is shown in Lane 1 of both Step 1 and Step 2.

Step 1


1
2
8 kb
1 kb
0.5 kb
8.4 kb
vector
1.1 kb
ompF

Step 2

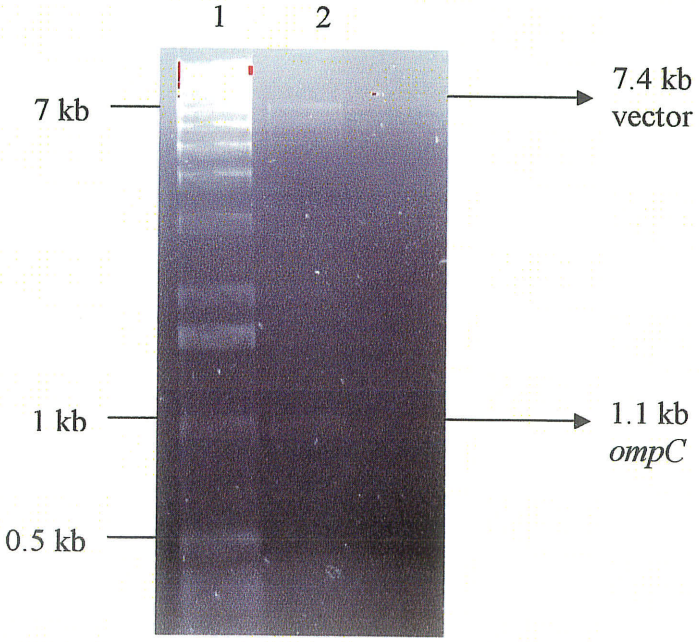

1
2
7 kb
1 kb
0.5 kb
7.4 kb
vector
1.1 kb
ompC

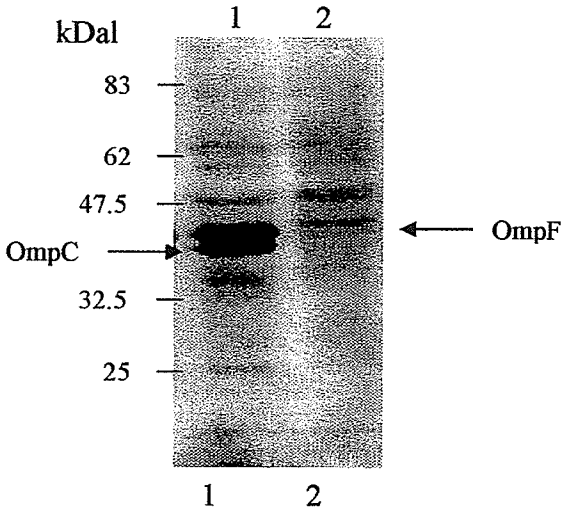
**Figure 2.19.** SDS-PAGE of cell envelopes and purified porins from *S. marcescens* porin knockout (SM100) and porin complemented strains (SM600 and SM700).

a) Lane 1, cell envelope preparations of SM600 (OmpF/C porin complemented strain) induced by 5 mM IPTG; and Lane 2, SM700 (OmpF complement) induced by 1 mM IPTG. Molecular weights of markers are indicated along the side of the gel. 4M urea was added to the gel for resolution.

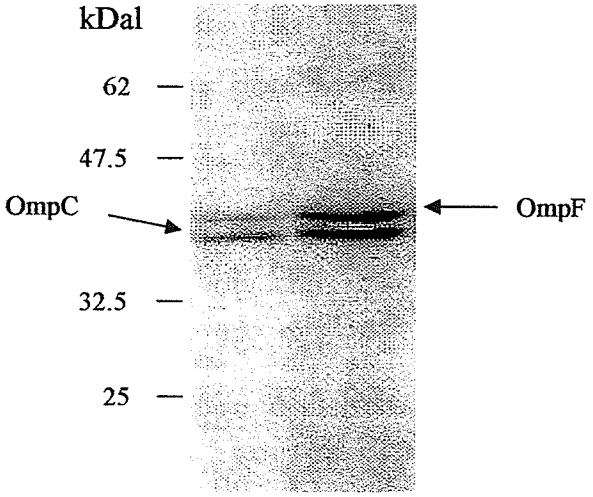
b) Purified porins isolated from the above envelope (SM600). Lane 1, wt UOC-67 (approximately 2 μg/lane loaded); and Lane 2, SM600 induced by 1 mM IPTG. 4M urea was added to the gel for resolution. OmpF is the top band (around 41 kDa), OmpC the bottom (40 kDa).

c) Lane 1, wt UOC-67; Lane 2, cell envelope preparations of SM600 induced by 5 mM IPTG and Lane 3, SM100 (OmpF/C deficient). 4M urea was added to the gel for resolution. 50 μg of cell envelope preparation was used.

a)


kDal
1
2
83
62
47.5
OmpC
32.5
25
OmpF

b)


1
2
kDal
62
47.5
OmpC
32.5
25
OmpF

c)

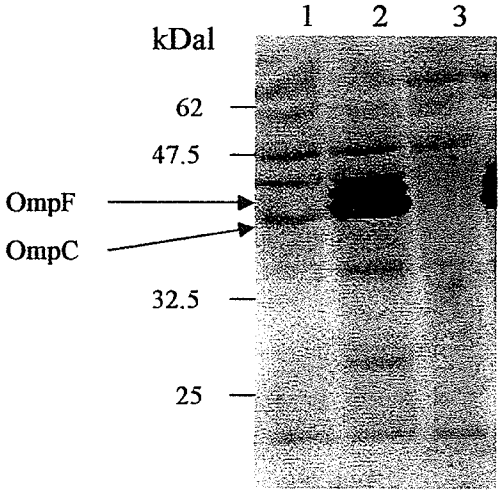

1
2
3
kDal
62
47.5
OmpF
OmpC
32.5
25

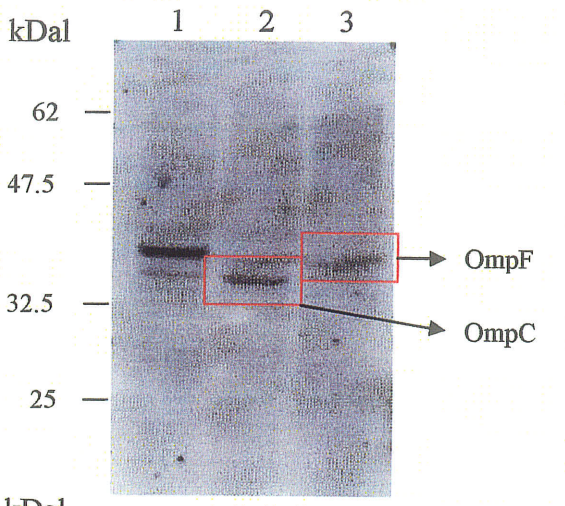
**Figure 2.20.** SDS-PAGE of cell envelopes from *S. marcescens* porin knockout (SM100, SM300 and SM400) and porin complemented strains (SM600, SM700 and SM800).

a) Lane 1, cell envelope preparations of SM600 (OmpF/C porin complemented strain) induced by 1 mM IPTG; Lane 2, SM300 (wt OmpC/OmpF knockout); and Lane 3, SM400 (wt OmpF/OmpC knockout). Molecular weights of markers are indicated along the side of the gel. 4M urea was added to the gel for resolution.

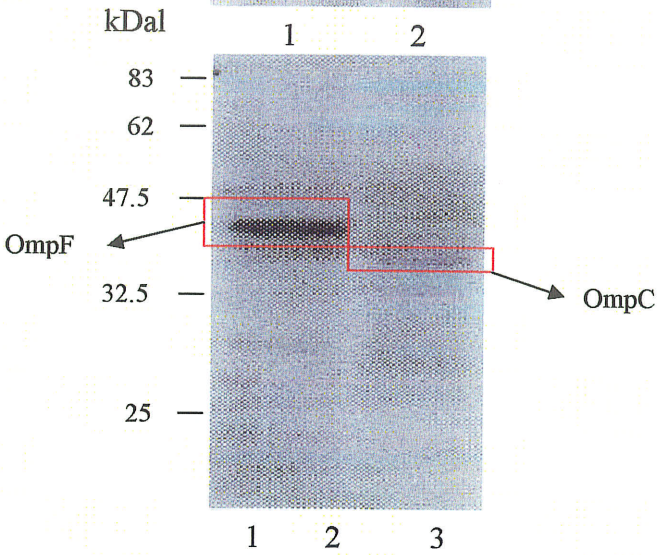
b) Lane 1, cell envelope preparations of SM700 (wt OmpF) induced by 1 mM IPTG; and Lane 2, SM800 (wt OmpC) induced by 0.5 mM IPTG. 4M urea was added to the gel for resolution.

c) Lane 1, cell envelope preparations (in absence of urea) of SM100 (OmpF/C deficient); Lane 2, SM600 (OmpF/C porin complemented strain) induced by 1 mM IPTG; and Lane 3, wt UOC-67. OmpF and OmpC are indicated by one band as no urea was added here and they appear approximately the same size. 50 μg of cell envelope preparation was used.

a)


kDal
1
2
3
62
47.5
32.5
25
OmpF
OmpC

b)


kDal
1
2
83
62
47.5
OmpF
32.5
25
OmpC

c)

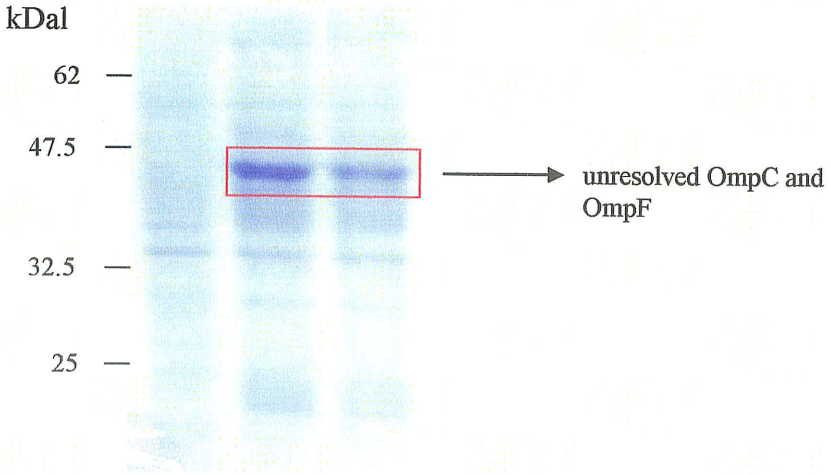
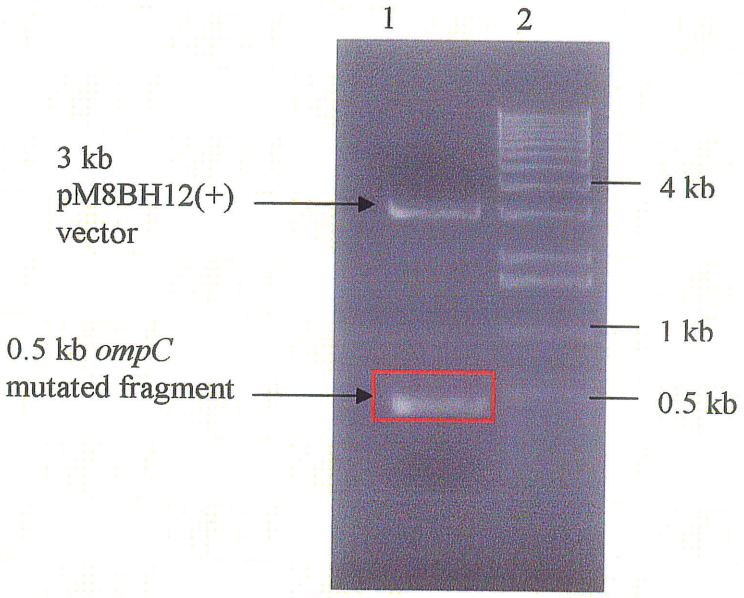

1
2
3
kDal
62
47.5
32.5
25
unresolved OmpC and
OmpF

**Figure 2.21.** Restriction digestions products of the *S. marcescens* a) *ompC* and b) *ompF* fragments used for site-directed mutagenesis at position 124 within Loop 3.

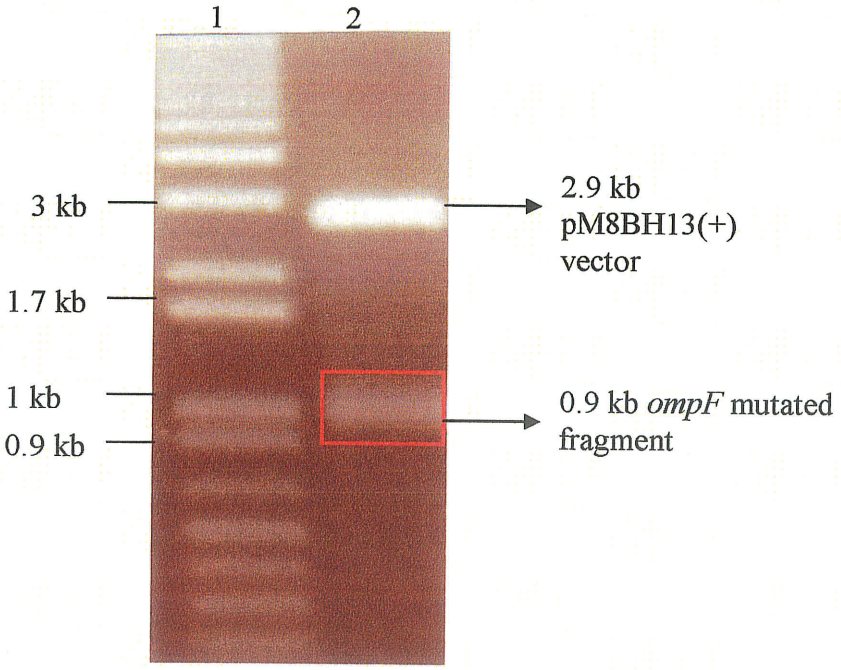
a) *ompC* was digested with *Pst*I and *Kpn*I. Lane 1, 0.5-kb *ompC* (boxed in red) insert band and 3-kb vector pM8BH12(+) band; Lane 2, 3 μL 1 kb ladder.

b) *ompF* was digested with *Hin*DIII. Lane 1, 3 μL 1 kb(+) ladder; Lane 2, 0.9-kb *ompF* (boxed in red) insert band and 2.9-kb vector pM8BH13(+) band.

a)


1
2
3 kb
pM8BH12(+)
vector
4 kb
1 kb
0.5 kb *ompC*
mutated fragment
0.5 kb

b)


1
2
3 kb
1.7 kb
1 kb
0.9 kb
2.9 kb
pM8BH13(+)
vector
0.9 kb *ompF* mutated
fragment

### 2.4.4 Antibiotic susceptibility tests

The double knockout strain (SM100), showed reproducible (n = 3) decreases in susceptibilities to all antibiotics (Table 2.3). For strains containing the wild type *ompF* (SM200, SM400), the wild type *ompC* (SM300) and both wild type porin genes (SM600), the susceptibilities to all antibiotics increased (Table 2.3) with respect to SM100. MIC values for SM600 were identical to those for wild type UOC-67 for all antibiotics tested. For SM700 (wt OmpF) and SM800 (wt OmpC) susceptibility to all antibiotics increased in comparison to each respective knockout strain (SM300 and SM400 respectively), however susceptibility was not as high as when both porin genes are present (SM600). For SM100, the addition of clavulanic acid increased antibiotic susceptibility by 64-fold, suggesting that this agent has prevented the ability of β–lactamases to hydrolyze ampicillin and carbenicillin.

For negatively-charged antibiotics, such as ampicillin, carbenicillin and cephalothin, the MIC values for the recombinants with D112G and M114D OmpC remained identical to that of the strain expressing only wild type OmpC (Table 2.3). However, susceptibility increased for the mutant with OmpC Q124G over wild type, and decreased for the OmpF G124Q mutant when compared to the strain expressing only wild type OmpF. The D112G/M114D OmpC MIC remained unchanged when compared to that of the wild type, whereas the susceptibility for D112G/Q124G and M114D/Q124G increased (Table 2.3). When clavulanic acid was used, susceptibility for all mutants was increased by at least eight-fold (Table 2.3).

**Table 2.3.** The susceptibilities of wild type and various mutant *S. marcescens* strains to a range of antibiotics.

| MIC (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | Nor | Ofx | Cip | Amp (+ cla acid) | Car (+ cla acid) | Cfc | Cef |
| UOC-67 (wild type) | 1 | 1 | 1 | 1 (1) | 1 (1) | 1 | 1 |
| SM100 (*ompF/ompC* deficient) | 2048 | 2048 | 2048 | 4096 (64) | 4096 (64) | 2048 | 2048 |
| SM200 (*ompC* deficient, wild type *ompF*) | 4 | 4 | 4 | 4 (2) | 4 (2) | 4 | 4 |
| SM400 (*ompC* deficient, wild type *ompF*) | 8 | 4 | 4 | 8 (2) | 4 (2) | 2 | 8 |
| SM300 (*ompF* deficient, wild type *ompC*) | 16 | 16 | 32 | 32 (16) | 32 (16) | 16 | 8 |
| SM600 (*ompF/ompC* wild type) | 1 | 1 | 1 | 1 (1) | 1 (1) | 1 | 1 |
| SM700 (*ompC* deficient, wild type *ompF*) | 4 | 2 | 2 | 4 (2) | 4 (2) | 4 | 2 |
| SM800 (*ompF* deficient, wild type *ompC*) | 4 | 2 | 2 | 4 (2) | 4 (2) | 2 | 4 |
| SM1 (OmpC D112G) | 4 | 4 | 8 | 32 (4) | 32 (4) | 2 | 8 |
| SM2 (OmpC M114D) | 16 | 16 | 32 | 32 (4) | 32 (4) | 16 | 8 |
| SM3 (OmpC Q124G) | 4 | 4 | 8 | 8 (1) | 8 (1) | 2 | 2 |
| SM4 (OmpC D112G/M114D) | 4 | 4 | 8 | 32 (4) | 32 (4) | 2 | 8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SM5 (OmpC D112G/Q124G) | 0.25 | 0.25 | 0.5 | 8 (1) | 8 (1) | 0.125 | 2 |
| SM6 (OmpC M114D/Q124G) | 4 | 4 | 8 | 8 (1) | 8 (1) | 2 | 2 |
| SM7 (OmpF G124Q) | 16 | 16 | 32 | 256 (32) | 256 (32) | 16 | 128 |

Note:
For complemented strain SM600, SM700 and SM800, MIC values reported are in the presence of 1 mM IPTG.
Nor, norfloxacin; Ofx, ofloxacin; Cip, ciprofloxacin; Amp, ampicillin; Car, carbenicillin; Cfc, cephaloridine; Cef, cephalothin; Cla acid, clavulanic acid. Results are representative of 3 trials.

For uncharged antibiotics, such as norfloxacin, ciprofloxacin, ofloxacin and cephaloridine, there was at least a four-fold increase in susceptibility for both, the OmpC D112G mutant, and the Q124G mutant for each of these antibiotics. Unlike what was observed for charged antibiotics, only the MIC value for M114D remained identical to that of the wild type OmpC (Table 2.3). The OmpF G124Q mutant showed a decrease in susceptibility when compared to the wild type OmpF. The susceptibility for OmpC double mutants M114D/Q124G and D112G/M114D increased in each case for all uncharged antibiotics, and the susceptibility of D112G/Q124G increased drastically when compared to the wild type OmpC (Table 2.3).

**2.4.5 Fluoroquinolone accumulation**

The loss of both porins (SM100) resulted in a dramatic reduction in fluoroquinolone accumulation as compared to SM600 (OmpF/OmpC porin complemented strain) (Figure 2.22). Loss of OmpF alone (SM300) and loss of OmpC alone (SM200 and SM400) resulted in an increase in ciprofloxacin accumulation rate as compared to SM100. The accumulation rate of ciprofloxacin (Figure 2.22) by SM600 was restored to that of UOC-67.

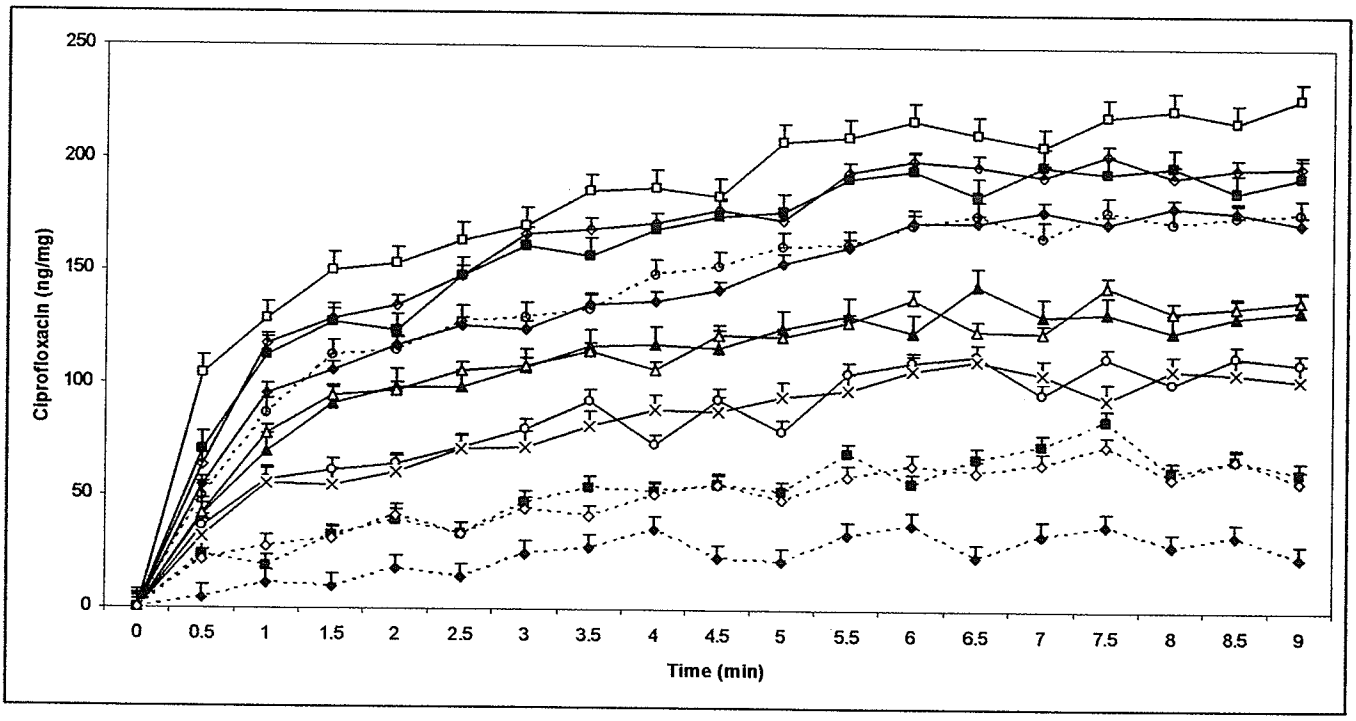
The results of ciprofloxacin accumulation for the loop 3 mutants are also found in Figure 2.22. Both SM3 (OmpC Q124G) and SM1 (OmpC D112G) had a higher accumulation than SM300 (wild type OmpC). Accumulation was comparable for SM2 (M114D) and SM300 for ciprofloxacin (Figure 2.22a). SM5 (D112G/Q124G) had higher accumulation compared to SM300 (Figure 2.22a). The rate of SM5 (D112G/Q124G) uptake surpassed that of even the two wild type OmpF strains (SM200 and SM400) (Figure 2.22a). The SM6 (M114D/Q124G) double mutant and the SM4

**Figure 2.22.** Accumulation of ciprofloxacin by a) wild type and *ompC* mutant *S. marcescens* strains (site directed mutants and knockouts) and b) wild type and *ompF* mutant *S. marcescens* strains (site directed mutant and knockouts). Results are graphed by the EXCEL system.

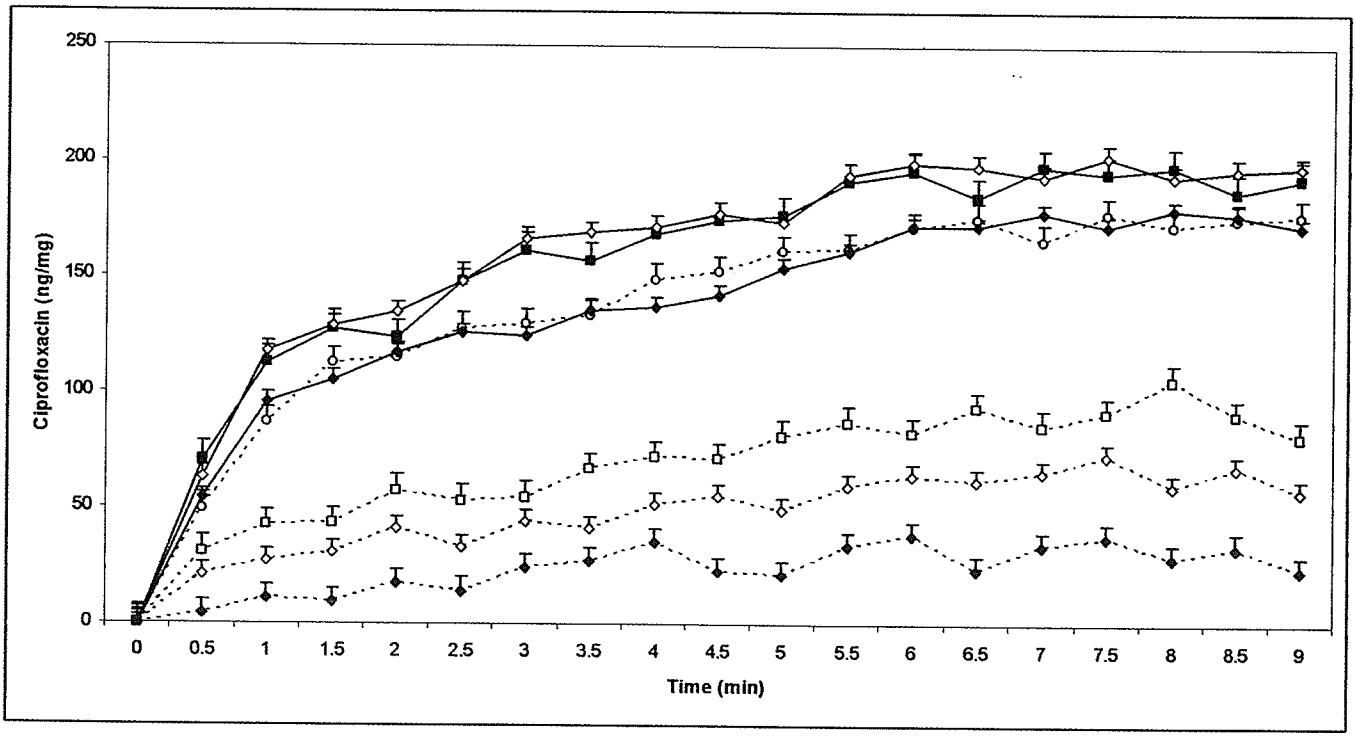
Strains for Panel a) are depicted as follows: UOC-67 (wild type *S. marcescens* - ■, solid line); SM100 (*ompF/ompC* deficient - ♦, broken line); SM200 (*ompC* deficient, wild type *ompF* - ♦, solid line); SM300 (*ompF* deficient, wild type *ompC* - ◊, broken line), SM400 (*ompC* deficient, wild type *ompF* - ○, broken line); SM600 (*ompF/ompC* wild type - ◊, solid line); SM1 (OmpC D112G - ○, solid line); SM2 (OmpC M114D - ■, broken line); SM3 (OmpC Q124G - ▲, solid line); SM4 (OmpC D112G/M114D - x, solid line); SM5 (OmpC D112G/Q124G - □, solid line) and SM6 (OmpC M114D/Q124G - Δ, solid line).

Strains for Panel b) are depicted as follows: UOC-67 (wild type *S. marcescens* - ■, solid line); SM100 (*ompF/ompC* deficient - ♦, broken line); SM200 (*ompC* deficient, wild type *ompF* - ♦, solid line); SM300 (*ompF* deficient, wild type *ompC* - ◊, broken line), SM400 (*ompC* deficient, wild type *ompF* - ○, broken line); SM600 (*ompF/ompC* wild type - ◊, solid line) and SM7 (OmpF G124Q - □, broken line). All trials performed in triplicate. For complemented strain SM600, accumulation performed in presence of 1 mM IPTG.

a)


Ciprofloxacin (ng/mg)
250
200
150
100
50
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9
Time (min)

b)


Ciprofloxacin (ng/mg)
250
200
150
100
50
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9
Time (min)

(D112G/M114D) double mutant had a higher accumulation than SM300 (Figure 2.22). The SM7 (G124Q) OmpF mutant strain accumulated less ciprofloxacin (Figure 2.22b) than SM200 and SM400. OmpF/OmpC deficient strain (SM100) still accumulated the smallest amount of antibiotic.

Table 2.4 shows the initial rate of ciprofloxacin uptake after 30 sec and 1 min intervals and confirms our overall accumulation data in that, UOC-67 and SM600 display a similar initial diffusion rate into the cell, SM7 (G124Q) OmpF mutant strain accumulated less ciprofloxacin initially than the wt OmpF strains, SM200 and SM400 and SM5 (D112G/Q124G) strain had a statistically much higher initial accumulation rate than SM300 (wt OmpC). SM5 had a slightly higher initial diffusion rate than both UOC-67 and SM600. SM100 however had the lowest initial diffusion rate than any of the strains tested. All strains had a higher initial ciprofloxacin uptake at the 30 sec time interval which then slowed down gradually past 1 minute.

It was difficult to make reproducible quantitative estimations from these assays mainly due to variations between culture batches, especially for norfloxacin, one of the fluoroquinolones used in my initial studies. I experienced much less difficulty with ciprofloxacin, which is why all accumulation data reported is that of ciprofloxacin.

**2.4.6 Liposome swelling assays**

To compare the relative permeation rates, the sugar (maltose) and antibiotic (cephaloridine, ampicillin and cephalothin)-diffusion values were normalized to that of glucose (Bredin et al. 2002).

When compared to the wild type OmpC, OmpC D112G allowed for a faster diffusion rate for both, maltose and cephaloridine, while the diffusion rate of OmpC

**Table 2.4.** Initial ciprofloxacin uptake (initial diffusion into cells for the rate per minute taken after the first 30 second time interval and the 1 minute interval) for the wild type and mutant *S. marcescens* strains.

| Ciprofloxacin uptake rate of diffusion (ng/mg/min) | | |
|---|---|---|
| | Initial rate at 30 seconds | Rate at 1 minute |
| UOC-67 wt | 140.64 (5.71) | 112.21 (10.23) |
| SM100 *ompF/ompC* deficient | 9.10 (0.51) | 11.21 (7.04) |
| SM200 *ompC*-deficient | 108.88 (3.11) | 95.68 (7.04) |
| SM300 *ompF*-deficient | 42.26 (3.44) | 27.54 (6.07) |
| SM400 *ompC*-deficient | 98.88 (7.41) | 86.55 (6.33) |
| SM600 wt *ompF/ompC* | 126.86 (6.21) | 117.32 (5.21) |
| SM1 D112G | 73.08 (3.11) | 57.32 (5.78) |
| SM2 M114D | 47.10 (3.94) | 18.54 (7.33) |
| SM3 Q124G | 82.12 (9.16) | 69.77 (7.01) |
| SM4 D112G/M114D | 64.22 (3.45) | 55.67 (7.01) |
| SM5 D112G/Q124G | 208.84 (10.23) | 128.43 (9.71) |
| SM6 M114D/Q124G | 84.66 (4.42) | 77.50 (3.71) |
| SM7 G124Q | 62.22 (6.89) | 42.33 (9.14) |

Note: Standard deviation is indicated in brackets.
For complemented strain SM600, accumulation performed in presence of 1 mM IPTG.

The results were expressed as nanograms of ciprofloxacin per milligram (dry weight) of cells.

Y = Fluorescence

W = Dry weight (mg)

Z = ng antibiotic accumulated

= Y/slope of the standard curve for given antibiotic

ng/mg of cells = Z/W

Mortimer and Piddock, 1991.

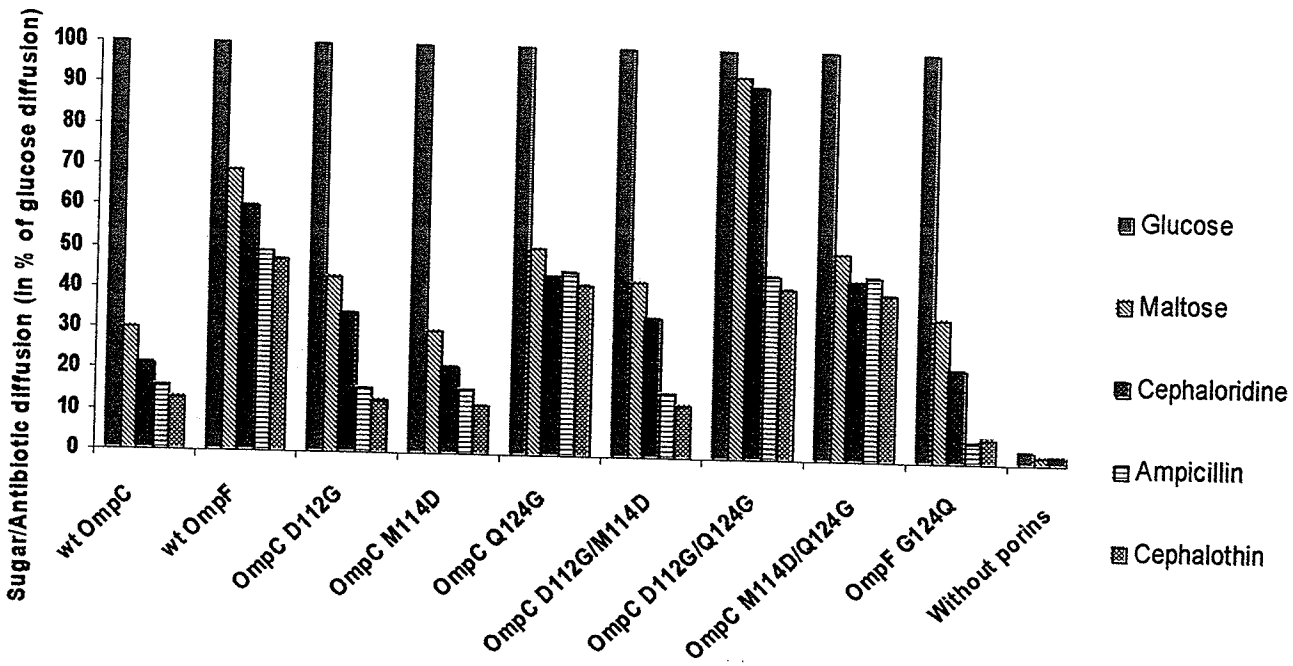
M114D remained unchanged (Figure 2.23). An increase in the diffusion rate for OmpC Q124G was observed when compared to wild type OmpC for both maltose and cephaloridine whereas there was a decrease in the diffusion rate for OmpF G124Q when compared to wild type OmpF (Figure 2.23a). The rate of diffusion for OmpC M114D/Q124G double mutant was similar to that of OmpC Q124G, whereas, the rate of OmpC D112G/M114D mutant was the same as for OmpC D112G. However, the diffusion rate of OmpC D112G/Q124G double mutant was higher than for wild type OmpC for both maltose and cephaloridine (Figure 2.23a).

For charged antibiotics, such as ampicillin and cephalothin, somewhat different results were observed. When compared to wild type OmpC, neither OmpC D112G nor OmpC M114D demonstrated a change in the permeability rate to either ampicillin or cephalothin (Figure 2.23a). This was also the case for OmpC D112G/M114D double mutant. We observed an increase in the diffusion rate for the OmpC Q124G recombinant when compared to wild type OmpC for both antibiotics, whereas there was a decrease in the diffusion rate for the OmpF G124Q recombinant when compared to wild type OmpF (Figure 2.23a). The rate of diffusion for both, the OmpC M114D/Q124G double mutant as well as for the OmpC D112G/Q124G mutant was almost identical to that of OmpC Q124G (Figure 2.23a).

**Figure 2.23.** Sugar and antibiotic-diffusion properties of *S. marcescens* wild-type OmpF, OmpC and site-directed mutated porins. For liposome-swelling assays, 20 μL aliquots of porin-containing proteoliposomes were diluted in 0.6 mL of an isotonic solution of glucose, maltose, cephaloridine, cephalothin and ampicillin. Results reported are a mean of three independent experiments.

(a) Diffusion properties of maltose (344 Da); cephaloridine (415.6 Da); cephalothin (418.4 Da) and ampicillin (371. 39 Da). Mean diffusion rates obtained for glucose (180.16 Da), presented for each porin with standard deviation (SD) in (b), was used to normalize diffusion of the other substrates.

a)




b)

| Porin Type | Mean diffusion rate for glucose | SD |
|---|---|---|
| wt OmpF | 0.527 | +/- 0.004 |
| Q124G | 0.484 | +/- 0.004 |
| D112G | 0.475 | +/- 0.002 |
| G124Q | 0.429 | +/- 0.002 |
| M114D | 0.403 | +/- 0.003 |
| wt OmpC | 0.406 | +/- 0.006 |
| D112G/M114D | 0.478 | +/- 0.002 |
| D112G/Q124G | 0.581 | +/- 0.002 |
| M114D/Q124G | 0.484 | +/- 0.003 |
| Liposome without porins | 0.024 | +/- 0.002 |

## 2.5 Discussion

Across the antibiotic spectra tested, the *ompF/ompC* deficient strain (SM100) exhibited the highest resistance, followed by the *ompF* deficient strain (SM300) and the *ompC* deficient strains (SM200, SM400). Our results are comparable to those reported by Mortimer and Piddock 1993, where the susceptibilities of an *E. coli ompF* deficient strain to a range of antibiotics were reduced by approximately two to four-fold as compared to the *ompC* deficient strain, with the lowest susceptibility displayed by the *E. coli ompF/ompC* deficient strain. In addition, the presence of clavulanic acid resulted in increased susceptibility of SM200/SM400 and SM300 to ampicillin and carbenicillin suggesting that there is a combination of antibiotic resistance mechanisms at work and that neither porin is solely involved in antibiotic uptake. However, complementing SM100 with both wild type *ompF* and *ompC* genes (SM600) resulted in MIC values identical to wild type UOC-67, confirming that both proteins are important and are working in concert to maximize antibiotic uptake.

My MIC values vary from those reported by Kumar and Worobec (2005a) for the same strain. This difference is due to the MIC protocols used. In Kumar and Worobec (2005a), for example, the MIC procedure does not include shaking. The British Society for Antimicrobial Chemotherapy protocol often results in values quite different than the values produced by the NCCLS method which I used (Jones et al. 2004).

Data from the antibiotic accumulation studies confirmed that OmpF plays a bigger role than OmpC in facilitating the diffusion of fluoroquinolones into the periplasm, since the reduction in accumulation was more extreme for SM300 than SM200 and SM400. In the absence of both OmpF and OmpC (SM100), the

accumulation of fluoroquinolones is reduced by up to 70%. Interestingly, SM100, which is missing OmpF and OmpC, was not completely void of fluoroquinolone accumulation. Sanchez et al. 1997 reported a third, smaller porin, called Omp3 that may be used for antibiotic accumulation in addition to OmpF and OmpC, perhaps explaining why SM100 still accumulated minimal amounts of fluoroquinolones. Alternatively, there is a possibility of a different, non-porin, accumulation pathway(s) such as self-promoted uptake or via a carrier protein (Mortimer and Piddock 1993).

The *S. marcescens* OmpC porin sequence also diverges from the enterobacterial Loop3 PEFGGD consensus motif with methionine replacing aspartate at position 114. OmpC with substitutions of an aspartate to glycine at position 112, and glutamine to glycine at position 124 allowed for a more rapid diffusion of maltose, cephaloridine and fluoroquinolones than the wild type protein. Replacing bulkier, charged residues with the uncharged, smaller glycine residues allowed for a faster diffusion of the uncharged maltose and cephaloridine and reduced the constriction of the pore to allow for less restricted antibiotic uptake than the wild type OmpC. This is consistent with an *Enterobacter aerogenes* study (Thiolas et al. 2004) where a glycine to aspartate substitution at position 112 of the Omp36 osmoporin led to a restricted antibiotic uptake resulting in a twelve-fold increase in resistance.

The OmpC double substitution D112G/Q124G allowed for a synergistic effect when uncharged antibiotics were tested, as reflected in faster permeability and higher uptake than wild type OmpC. However, when charged antibiotics were tested, the two OmpC substitutions at positions 112 and 124 no longer had the same effect. The Q124G change resulted in higher susceptibility and faster diffusion rate to anionic antibiotics, as

compared to the wild type OmpC, while the D112G change resulted in same diffusion rate and antibiotic susceptibility as wild type OmpC. In addition, no synergistic effect was seen for the double substitution D112G/Q124G when charged antibiotics were tested with diffusion rates and susceptibility values being identical to those observed for Q124G alone. Based on these combined results, we suggest that the amino acid at position 112 plays an important role in pore constriction, but not ionic selection. Our results agree with those reported by Jeanteur et al. (1994) for *E. coli* OmpF, where through crystallization studies, it was determined that the bulkier aspartate side chain protrudes into the pore lumen, thus constricting the pore, while the amino acid in position 124 is important for both size and ionic selection. We thus predict that the glutamine 124 side chain within *S. marcescens* OmpC extends into the pore channel contributing to size constriction by conferring a smaller channel in OmpC in comparison to OmpF which has a glycine at the same position.

One may predict that having methionine instead of aspartate at position 114 may affect porin function by affecting the loop conformation, however, there was no difference between M114D porin and wild type OmpC observed from liposome swelling experiments, susceptibility profiles and accumulation experiments. In order to confidently determine if any effect M114D may have is not masked by the other divergence this porin has from the consensus, we constructed double mutants; D112G/M114D and M114D/Q124G. All experimental results for these mutants were the same as for either D112G or Q124G alone, substantiating our initial findings.

To investigate the role of glycine at position 124 of *S. marcescens* OmpF, G124Q mutant was constructed, in order to mimic OmpC. We observed a much higher resistance

profile for G124Q as opposed to the wild type OmpF, concomitant with lower accumulation of ciprofloxacin suggesting that the glutamine at this position is at least partially responsible for cationic selectivity of *S. marcescens* OmpF porin. Therefore, due to the bulkiness of this glutamine side chain and the steric hindrance it provides, we suggest that glutamine may extend into the pore channel conferring a smaller channel in OmpC porins in comparison to OmpF which has a glycine. The failure of the β–lactamase inhibitor clavulanic acid to fully restore β–lactam susceptibility, as observed for G124Q, also suggests a drastically altered channel in this strain since the glutamine residue would affect the stability of the porin conformation by altering the folding of the porin, thus preventing clavulanic acid entry (Mallea et al. 1998).

In conclusion, we have established that two notable changes in the OmpC (glutamine (124) and aspartate (112)) contribute to size constriction by conferring a smaller channel in OmpC in comparison to OmpF, while methionine 114 has no effect. Any change which results in the loss of outer membrane permeability leads to a significant reduction in the concentration of accumulated antibiotic, resulting in an increase in the overall antibiotic resistance.

## CHAPTER 3 Fluoroquinolone resistance of *Serratia marcescens* UOC-67: sucrose, salicylate, temperature, and pH induction of phenotypic resistance

Parts of the material presented herein resulted in the publication Begic, S., and Worobec, E. A. 2007b. Fluoroquinolone resistance of *Serratia marcescens*: sucrose, salicylate, temperature, and pH induction of phenotypic resistance. Can. J. Microbiol. **53:** 1239-1245. I completed the majority of experiments outlined in this chapter, with assistance from summer research assistant Peter Sytnik and Kiet Do for antibiotic accumulation trials and MIC tests. I wrote the manuscript with full guidance and support from my advisor and valuable suggestions were made by Dr. T. de Kievit and Dr. R. Sparling. Original studies which resulted in the manuscript cited above used solely norfloxacin to study the susceptibility and internal antibiotic accumulation in *S. marcescens*, whereas this chapter concentrates on ciprofloxacin.

### 3.1 Abstract

*Serratia marcescens* is a nosocomial agent with a natural resistance to a broad spectrum of antibiotics, making the treatment of its infections very challenging. This study examines the influence of salicylate, sucrose, temperature and pH variability on membrane permeability and susceptibility of *S. marcescens* to ciprofloxacin (hydrophilic fluoroquinolone) and nalidixic acid (hydrophobic quinolone). Resistance of wild-type *S. marcescens* UOC-67 (ATCC 13880) to ciprofloxacin and nalidixic acid was assessed by Minimal Inhibitory Concentration assays (MIC) after growth in the presence of varying concentrations of sucrose, salicylate, different temperatures and pH values. Ciprofloxacin and nalidixic acid accumulation was determined in the absence and presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore which collapses proton-

motive-force (PMF), and Phe-Arg-β-naphthylamide (PAβN), an efflux pump inhibitor. Accumulation of ciprofloxacin decreased when *S. marcescens* was grown in high concentrations of salicylate (8 mM) and sucrose (10% w/v), high temperature (42°C) and pH 6, and was restored in the presence of CCCP, due to the collapse of proton gradient-dependent efflux in *S. marcescens* UOC-67. Although nalidixic acid accumulation was observed, it was not affected by salicylate, sucrose, pH or temperature changes. In the absence of PAβN, and either in the presence or absence of CCCP, a plateau is reached in the nalidixic acid accumulation for all environmental conditions. With the addition of 20 mg/L PAβN nalidixic acid accumulation is restored for all environmental conditions, suggesting that this quinolone is recognized by a yet to be identified *S. marcescens* pump which does not use the PMF as its energy source.

**3.2 Introduction**

*Serratia marcescens* is a Gram negative enteric bacterium which has become an important opportunistic pathogen associated with a number of life-threatening diseases and nosocomial infections, such as urinary tract infections, respiratory tract infections, meningitis, septicaemia and wound infections (Hejazi and Falkiner 1997). Antibiotic resistance of *S. marcescens* is in part due to a reduction in expression of certain outer membrane porins which decreases outer membrane permeability (Hutsul and Worobec 1997). Our group has characterized two *S. marcescens* non-selective porins, OmpC and OmpF (Hutsul and Worobec 1997). In strain UOC-67, OmpF is believed to have a slightly larger pore diameter resulting in a faster rate of diffusion of substrates through OmpF than through OmpC (Hutsul and Worobec 1997). Additionally, efflux systems

also account for phenotypic multi-drug resistance (MDR) in some strains of *S. marcescens* (Kumar and Worobec 2005a).

We have previously published a report on the effect of osmotic stress, salicylate, temperature and pH on regulation of *S. marcescens* porin gene expression (Begic and Worobec 2006). Lower growth temperature (28°C), pH 8 and 0% sucrose and salicylate induce the expression from the *ompF* porin gene, whereas the induction of *ompC* expression is stimulated by a higher growth temperature (42°C), pH 6 and high concentrations of sucrose (10% w/v) and salicylate (8 mM). Due to suppression of OmpF synthesis at these conditions, we predict that salicylate, sucrose, high temperature and acidic pH will increase the resistance of *S. marcescens* UOC-67 to several antibiotics, including ampicillin, tetracycline, chloramphenicol, cephalosporins and quinolones in much the same way as has been observed with *Escherichia coli* (Foulds et al. 1989; Rosner 1985). Here we determine the effect of sucrose, salicylate, temperature and pH on the permeability and susceptibility of *S. marcescens* to ciprofloxacin (hydrophilic fluoroquinolone) and nalidixic acid (hydrophobic quinolone) in order to confirm that an increase in resistance is a result of decreased production of OmpF. The effect of this reduction in permeability on efflux is also examined.

## 3.3 Materials and Methods

### 3.3.1 Bacterial strains

Wild type *S. marcescens* UOC-67 (American Type Culture Collection 13880) was used throughout this study. This strain was maintained on Trypticase Soy Agar (TSA) slants and cultured in Tryptone Soy Broth (TSB; tryptone, soytone, sodium chloride) [BD Diagnostics Systems].

### 3.3.2 Antibiotic susceptibility testing

Salicylate and sucrose were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo.). Susceptibility of UOC-67 to ciprofloxacin and nalidixic acid [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was tested using a modified version of Clinical and Laboratory Standards Institute protocol using the minimum inhibitory concentration (MIC) two-fold broth dilution method in TSB medium (Berlanga and Vinas 2000) instead of MH (Mueller-Hinton). In this study, where I have used an additional environmental factor in the MIC assay, I had difficulty performing the assay in the presence of the traditional MH broth (meat infusion, casein hydrolysate, starch) because I noticed the turbidity was difficult to distinguish. Other groups have also used TSB when testing environmental conditions such as salicylate (Berlanga and Vinas 2000). Overnight cultures in TSB were diluted 1000-fold in fresh broth, grown at 37°C until $OD_{600}$=0.5-0.9 and 5 µl of the bacterial suspension was inoculated in TSB containing serial dilutions of each antibiotic and the respective environmental factor. For pH experiments, external pH was manipulated by adjusting the TSB with 1 M HCl (pH 6) or 1 M NaOH (pH 8). TSB media metabolites are not affected by pH changes used in this study (Freitag et al. 1997). Results were reported as MIC, the concentration of antibiotic that inhibited visible growth determined by absence of turbidity in TSB after 18 hours of shaking at 37°C. For temperature studies, cultures were incubated at 28°C, 37°C and 42°C.

### 3.3.3 Fluoroquinolone accumulation

The accumulation of ciprofloxacin and nalidixic acid was measured using the method of Mortimer and Piddock, 1991. Cultures in 10L batches were grown in varying concentrations of sucrose (5%, 8%, 10%), salicylate (1 mM, 5 mM, 8 mM), temperature

(28°C, 37°C, 42°C) and pH (6, 7, 8) until the $OD_{600}$ = 0.5-0.7. Cells were harvested by centrifugation at 4000 xg for 15 minutes at room temperature, resuspended to 100 ml in phosphate buffered saline (PBS) pH 7.5 and washed two times. Nalidixic acid or ciprofloxacin was added to a final concentration of 10 μg/ml, and 0.5 ml of culture was removed in one minute intervals for a period of 10 minutes and immediately diluted in 1 ml of cold PBS. Next, a rapid centrifugation (13,000 xg at 4°C for 5 min) was carried out, followed by a wash in cold PBS, suspension in 1 ml of HCl-glycine 0.1 M pH 3.0 to lyse the cells, and overnight incubation at room temperature. This suspension was centrifuged at 11,000 xg for 5 minutes to remove cellular debris and antibiotic concentration was measured using a RF-1501 Shimadzu spectrofluorometer. The fluorescence of antibiotics was measured as follows: ciprofloxacin at 279 nm excitation wavelength and 447 nm emission wavelength; nalidixic acid at 330 nm excitation wavelength and 417 nm emission wavelength. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was added from an ethanol stock solution (70%) that was further diluted in 10 mM NaOH to a final concentration of 100 μM, 5 minutes after the addition of antibiotic. To test for nalidixic acid accumulation, Phe-Arg-β-naphthylamide (PAβN) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was added to a final concentration of 20 mg/L 5 minutes after the addition of nalidixic acid accumulation.

Antibiotic concentration was calculated using a standard curve for each antibiotic (concentration ranging from 100 to 1000 ng) in 0.1 M HCl-glycine 0.1 M pH 3.0. The result is expressed as nanograms of antibiotic incorporated per milligram (dry weight) of bacteria. All data was graphed by the EXCEL computer system.

For accumulation of ciprofloxacin by UOC-67 in the presence of 70% ethanol and absence of CCCP, the above procedure was followed, with exception that 1L starting culture was used as opposed to 10L and cells were resuspended to 10 ml in phosphate buffered saline (PBS) pH 7.5. 100 µl of ethanol was added after 5 minutes of antibiotic incubation as this volume is equivalent to the amount of ethanol in CCCP assays. T=5 min was chosen as CCCP is added 5 minutes after the addition of ciprofloxacin in assays where CCCP is used.

## 3.4 Results

### 3.4.1 Antibiotic susceptibility testing

Susceptibility to ciprofloxacin was affected by the therapeutic concentration of salicylate (1 mM) (Berlanga and Vinas 2000), and continued to decrease with increasing concentrations of salicylate. Susceptibility to ciprofloxacin did not change in the presence of 5% sucrose, however, the addition of 8% sucrose and 10% sucrose caused two-fold and four-fold decreases in susceptibility, respectively. In response to growth at 28°C as compared to 37°C, susceptibility increased eight-fold and decreased at 42°C two-fold. In response to growth at pH 6 as compared to pH 7, susceptibility decreased eight-fold, and increased at pH 8 by two-fold. Susceptibility to nalidixic acid was not affected by any concentrations of salicylate and sucrose used, or in conjunction with varying growth temperature or pH values (Table 3.1).

### 3.4.2 Fluoroquinolone accumulation

A significant decrease in the accumulation of ciprofloxacin was detected at all salicylate concentrations, with the accumulation at 8 mM salicylate being approximately half that determined after growth in the absence of salicylate (Figure 3.1a). After 5

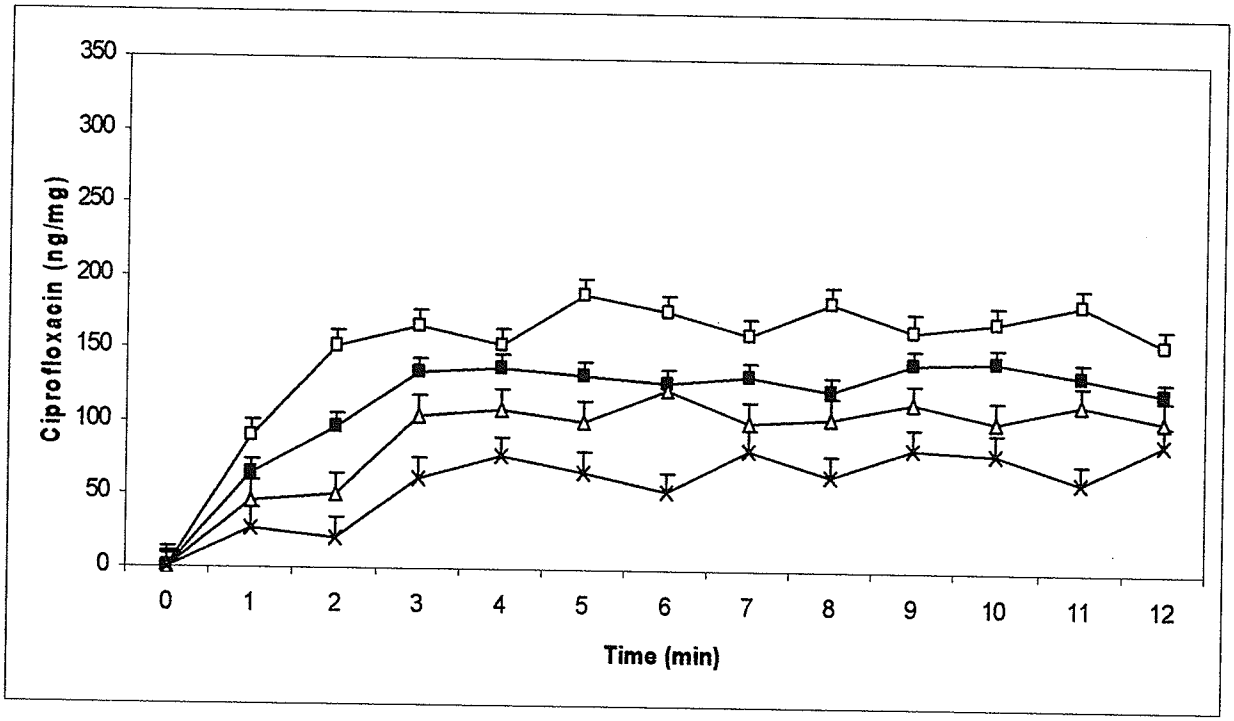
**Table 3.1.** Ciprofloxacin (Cip) and nalidixic acid (Nal) MICs (μg/ml) for *S. marcescens* grown at different concentrations of salicylate, sucrose, and at different temperatures and pH.

| **Antimicrobial agent** | **MIC (μg/ml)** |
|---|---|
| Cip | 16 |
| Cip + 1 mM salicylate | 64 |
| Cip + 5 mM salicylate | 128 |
| Cip + 8 mM salicylate | 256 |
| Nal | 512 |
| Nal + 1 mM salicylate | 512 |
| Nal + 5 mM salicylate | 512 |
| Nal + 8 mM salicylate | 512 |
| Cip + 5% sucrose | 16 |
| Cip + 8% sucrose | 32 |
| Cip + 10% sucrose | 64 |
| Nal + 5% sucrose | 512 |
| Nal + 8% sucrose | 512 |
| Nal + 10% sucrose | 512 |
| Cip + 28°C | 2 |
| Cip + 37°C | 16 |
| Cip + 42°C | 32 |
| Nal + 28°C | 512 |
| Nal + 37°C | 512 |
| Nal + 42°C | 512 |
| Cip + pH 6 | 128 |
| Cip + pH 7 | 16 |
| Cip + pH 8 | 8 |
| Nal + pH 6 | 512 |
| Nal + pH 7 | 512 |
| Nal + pH 8 | 512 |

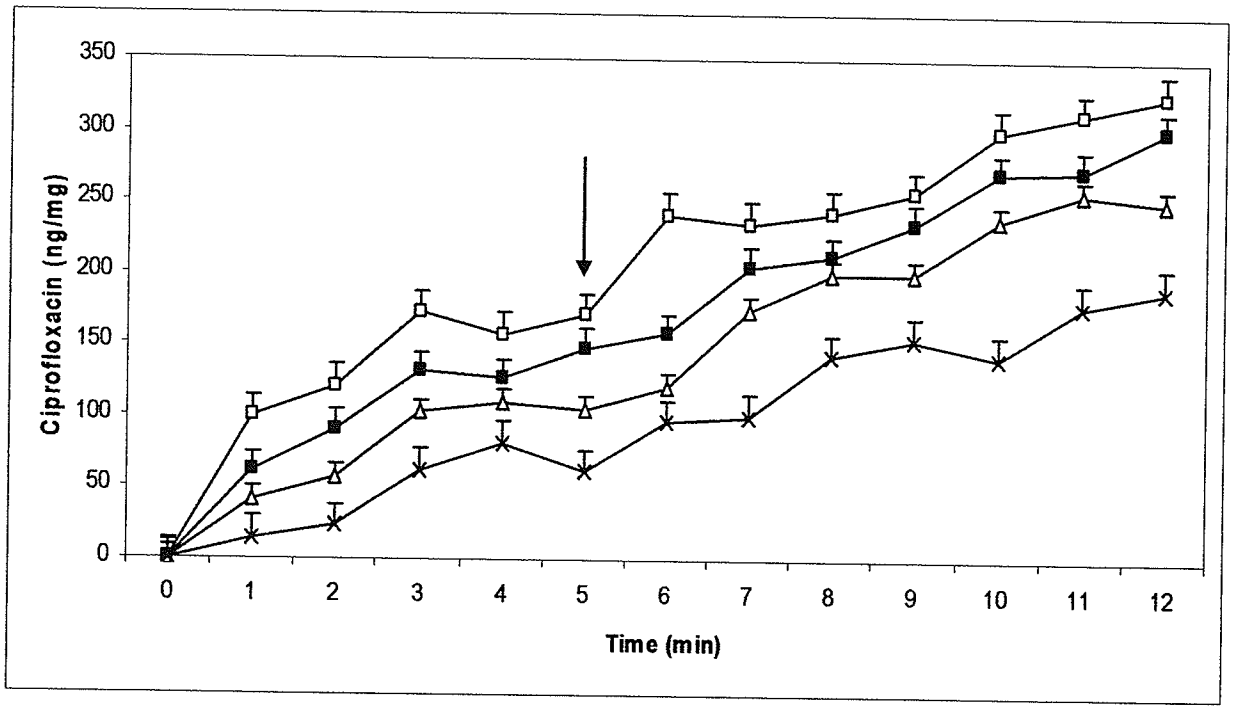
All trials were performed in triplicate with identical results.

**Figure 3.1.** Effect of salicylate on ciprofloxacin and nalidixic acid accumulation by *S. marcescens* UOC-67. a) Accumulation of ciprofloxacin (10 μg/ml external concentration) without CCCP and b) with CCCP. c) Accumulation of nalidixic acid (10 μg/ml external concentration) without CCCP and d) with CCCP. Varying concentrations of salicylate are depicted as: no salicylate (□), 1 mM salicylate (■), 5 mm salicylate (Δ), and 8 mm salicylate (x). The addition of CCCP is indicated by the arrow. All trials were performed in triplicate.

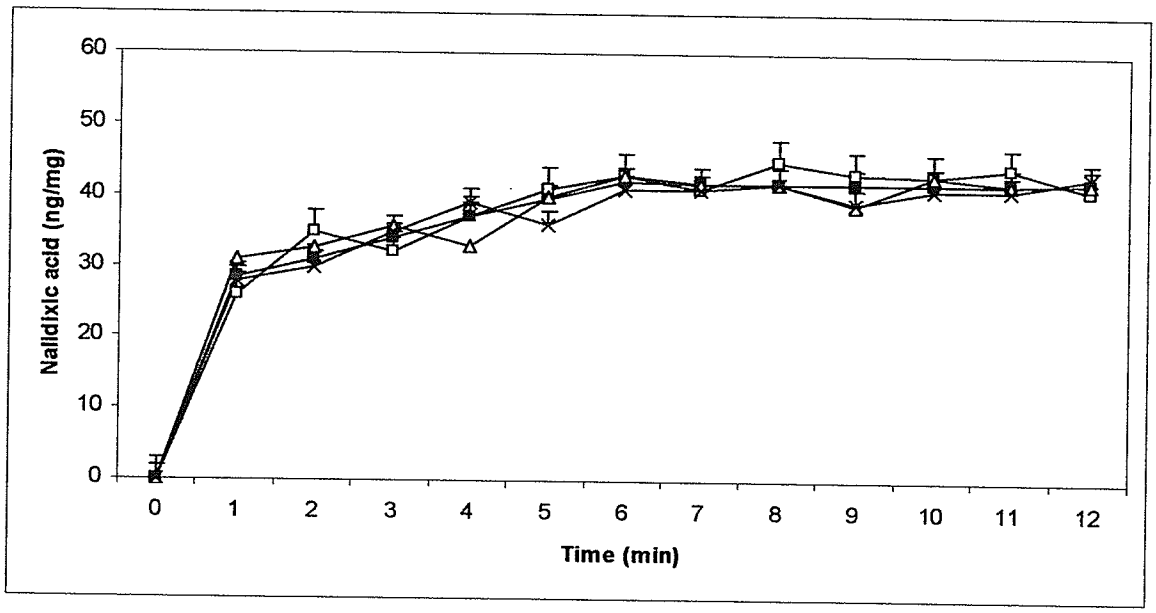
a)


Ciprofloxacin (ng/mg)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

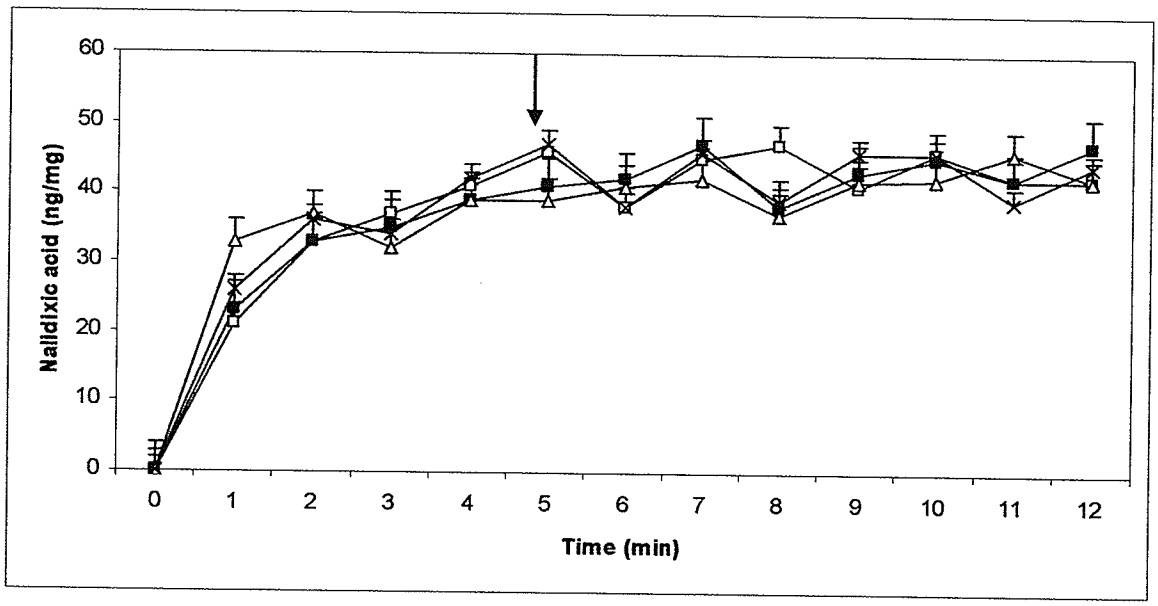
b)


Ciprofloxacin (ng/mg)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

minutes of antibiotic accumulation, a plateau was reached. Upon addition of proton motive force inhibitor CCCP at T = 5 min, the accumulation of ciprofloxacin was restored (Figure 3.1b). Salicylate in the growth media had no effect on nalidixic acid accumulation in the presence or absence of CCCP (Figure 3.1 c and d).

No significant effect on ciprofloxacin accumulation was detected with 5% sucrose, whereas the accumulation decreased by approximately 50% with the addition of 8% sucrose, and >70% with the addition of 10% sucrose (Figure 3.2a). CCCP restored the accumulation of ciprofloxacin (Figure 3.2b). Sucrose had no effect on nalidixic acid accumulation in the presence or absence of CCCP (Figure 3.2 c and d).

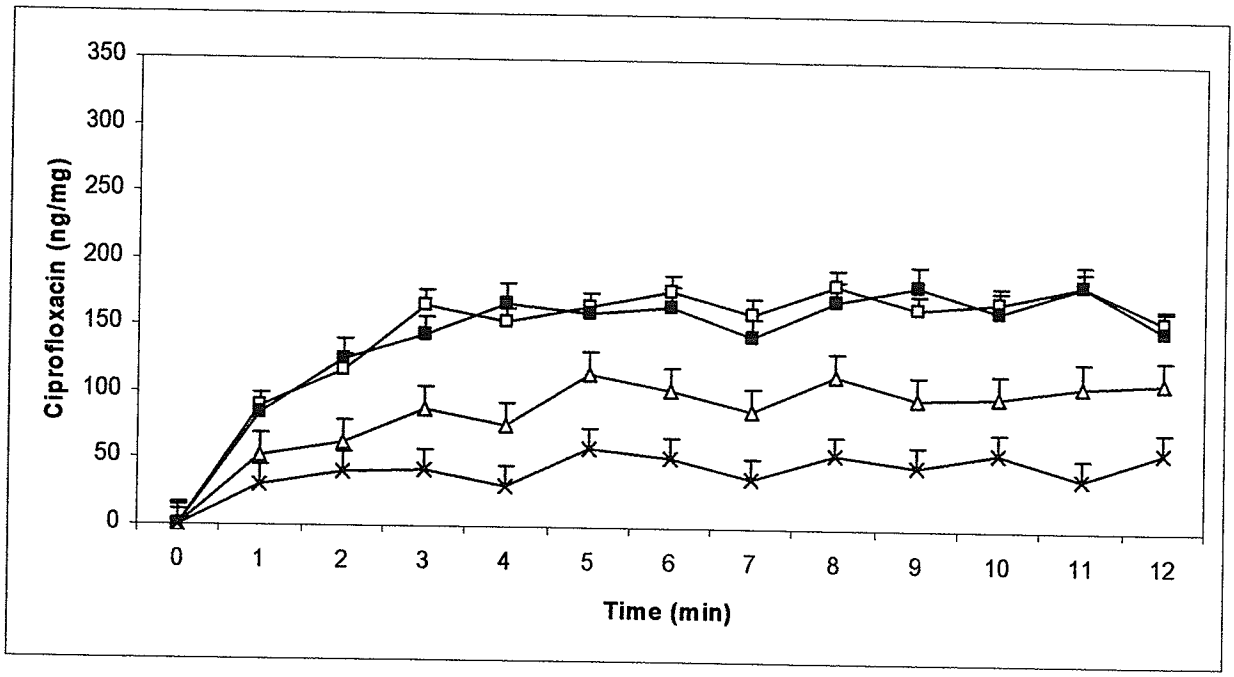
An increase in ciprofloxacin uptake was detected at 28°C, and a decrease in uptake was detected at 42°C, as compared to 37°C (Figure 3.3a). CCCP addition resulted in restoring the accumulation for all three temperatures (Figure 3.3b). Varying temperature had no significant effect on nalidixic acid accumulation in the presence or absence of CCCP (Figure 3.3 c and d).

As compared to pH 7, an increase in ciprofloxacin uptake was observed at pH 8 and decreasing the pH to 6 resulted in a concomitant decrease in ciprofloxacin uptake (Figure 3.4a). CCCP restored fluoroquinolone accumulation at all three pH values (Figure 3.4b). Varying pH values had no significant effect on nalidixic acid accumulation in absence or presence of CCCP (Figure 3.4 c and d).

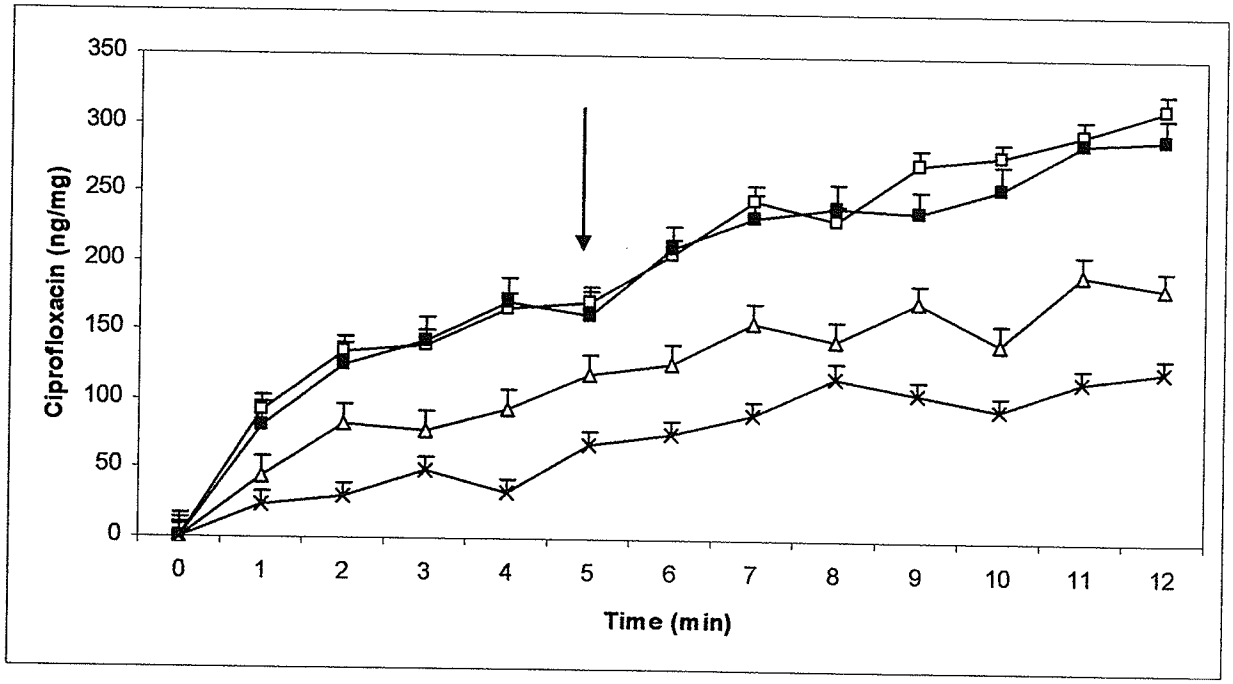
PAβN, an efflux pump competitive inhibitor (Lomovskaya et al. 2001), restored nalidixic acid accumulation at T = 5 min thus allowing for an overall higher accumulation of this antibiotic when UOC-67 was grown in media containing all test concentrations of salicylate and sucrose, as well as in the presence of varying temperatures and pH values

**Figure 3.2.** Effect of sucrose on ciprofloxacin and nalidixic acid accumulation by *S. marcescens* UOC-67. a) Accumulation of ciprofloxacin (10 μg/ml external concentration) without CCCP and b) with CCCP. c) Accumulation of nalidixic acid (10 μg/ml external concentration) without CCCP and d) with CCCP. Varying concentrations of sucrose are depicted as: no sucrose (□), 5% sucrose (■), 8% sucrose (Δ), and 10% sucrose (x). The addition of CCCP is indicated by the arrow. All trials were performed in triplicate.

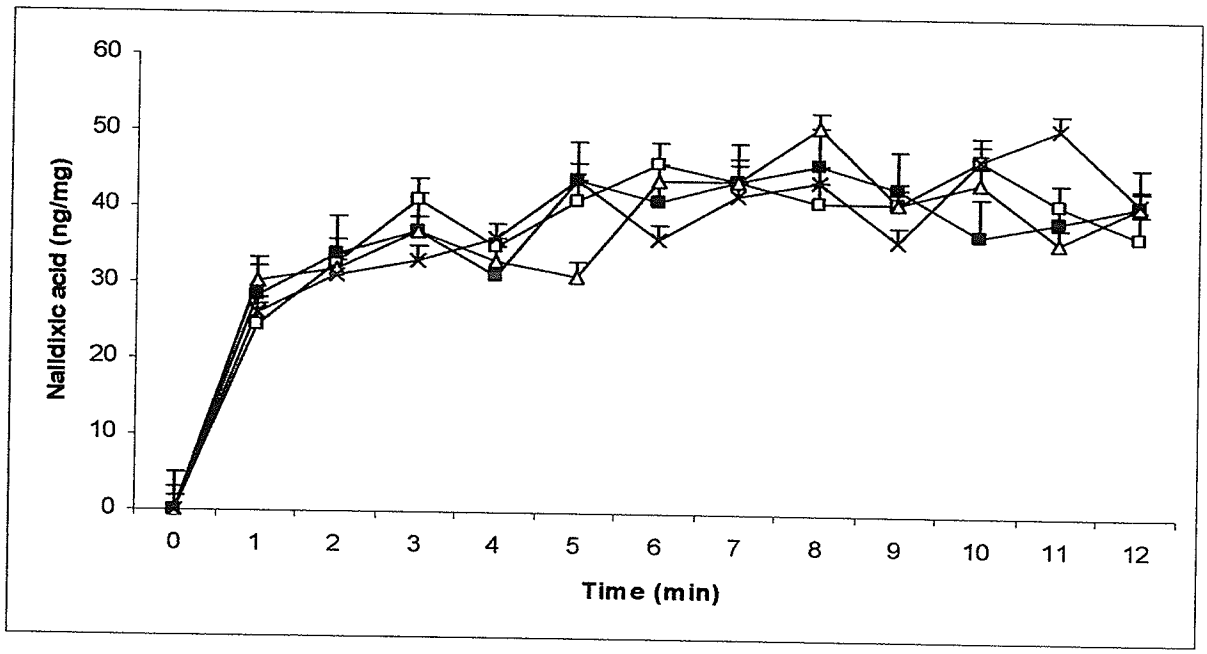
a)


Ciprofloxacin (ng/mg)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

b)


Ciprofloxacin (ng/mg)
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)

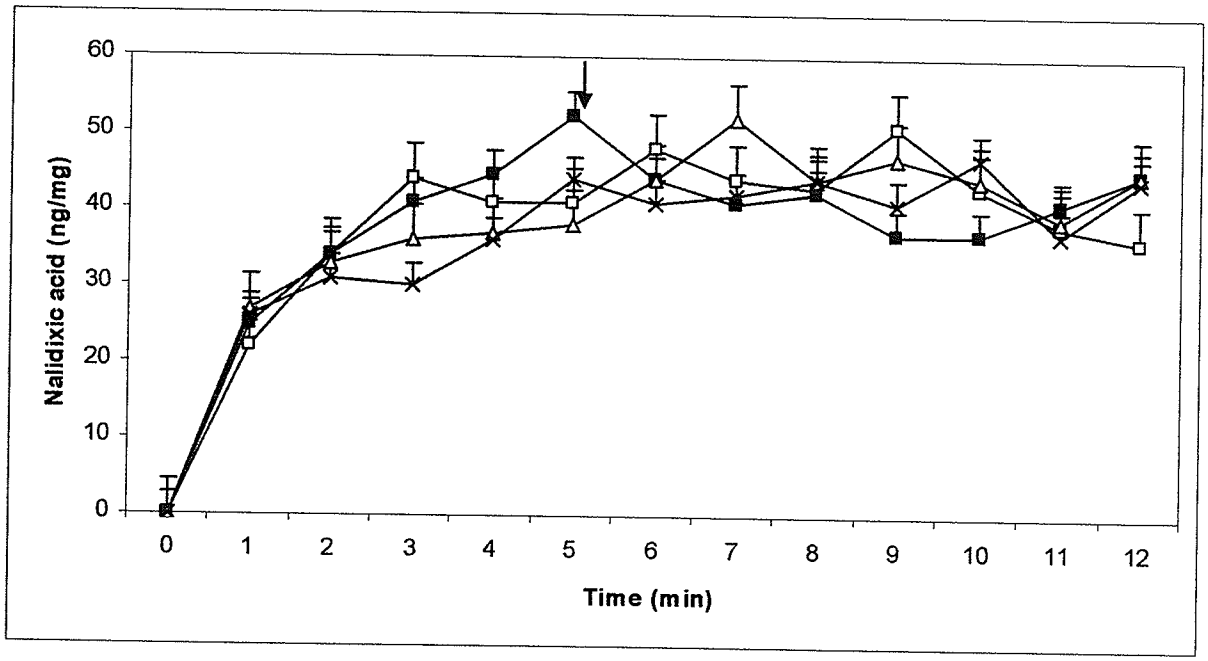
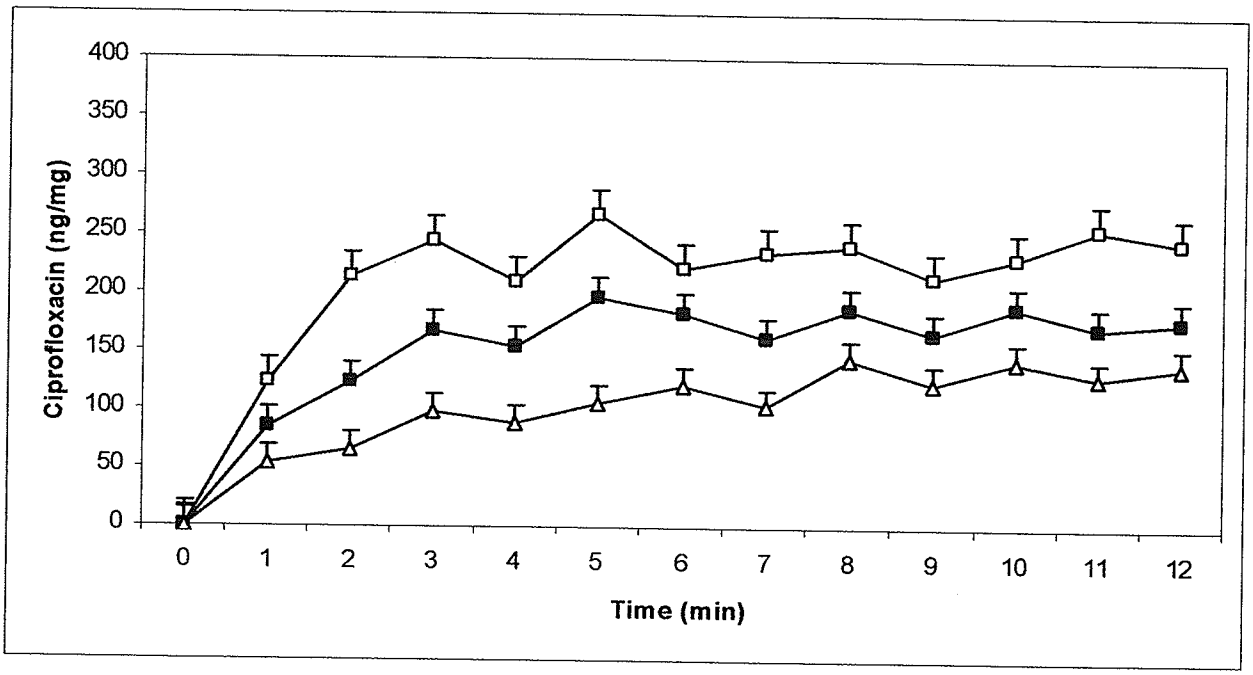
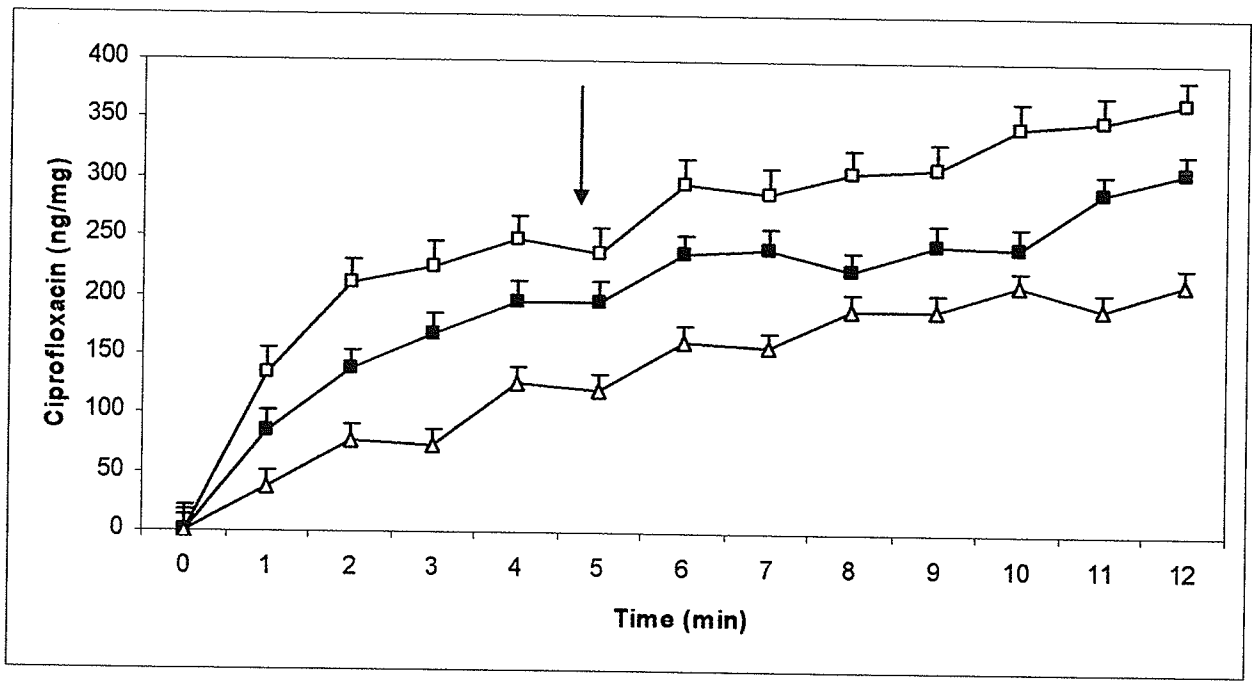

Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

**Figure 3.3.** Effect of temperature on ciprofloxacin and nalidixic acid accumulation by *S. marcescens* UOC-67. a) Accumulation of ciprofloxacin (10 μg/ml external concentration) without CCCP and b) with CCCP. c) Accumulation of nalidixic acid (10 μg/ml external concentration) without CCCP and d) with CCCP. Varying temperatures are depicted as: 28°C (□), 37°C (■), and 42°C (Δ). The addition of CCCP is indicated by the arrow. All trials were performed in triplicate.

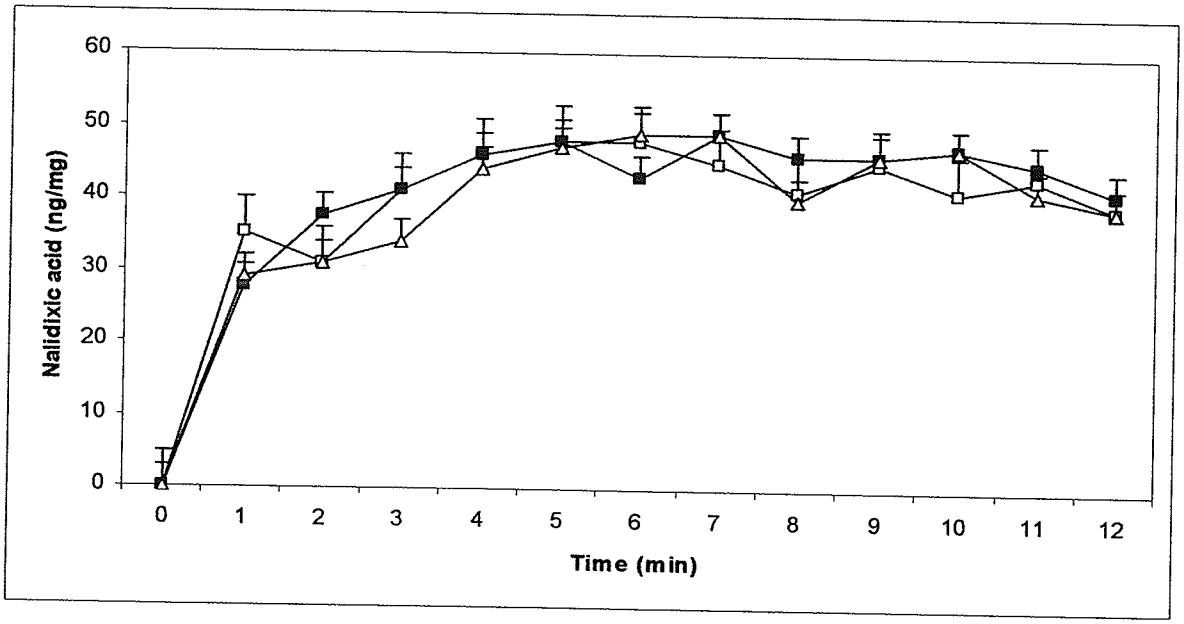
a)


Ciprofloxacin (ng/mg)
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

b)


Ciprofloxacin (ng/mg)
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)

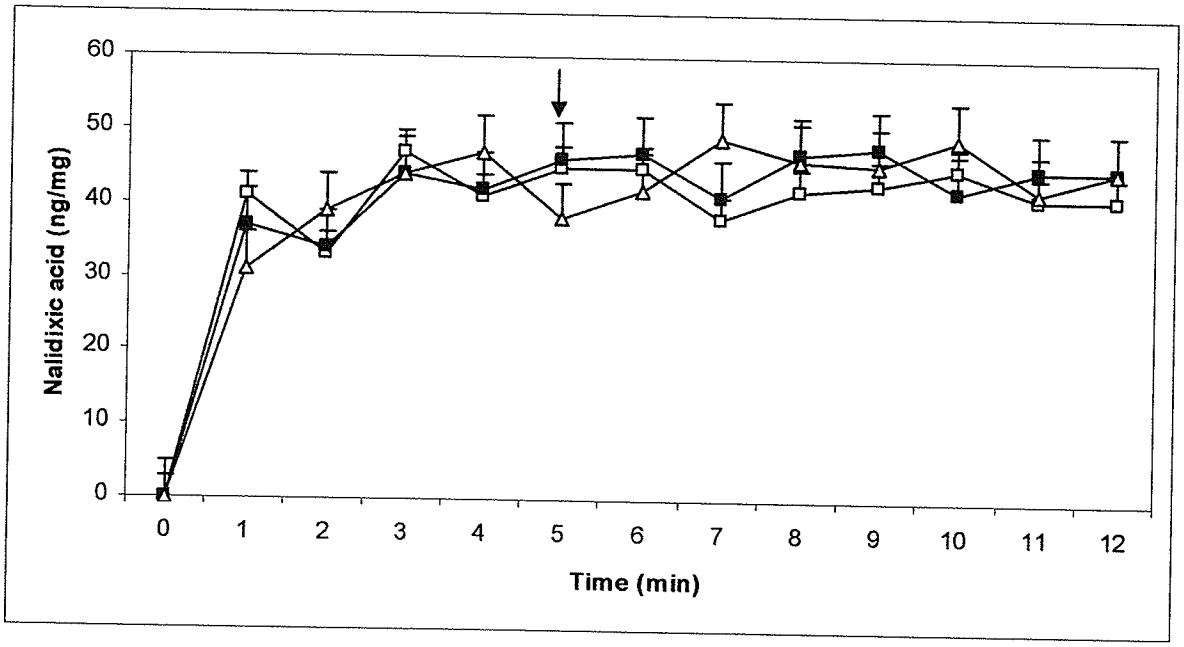
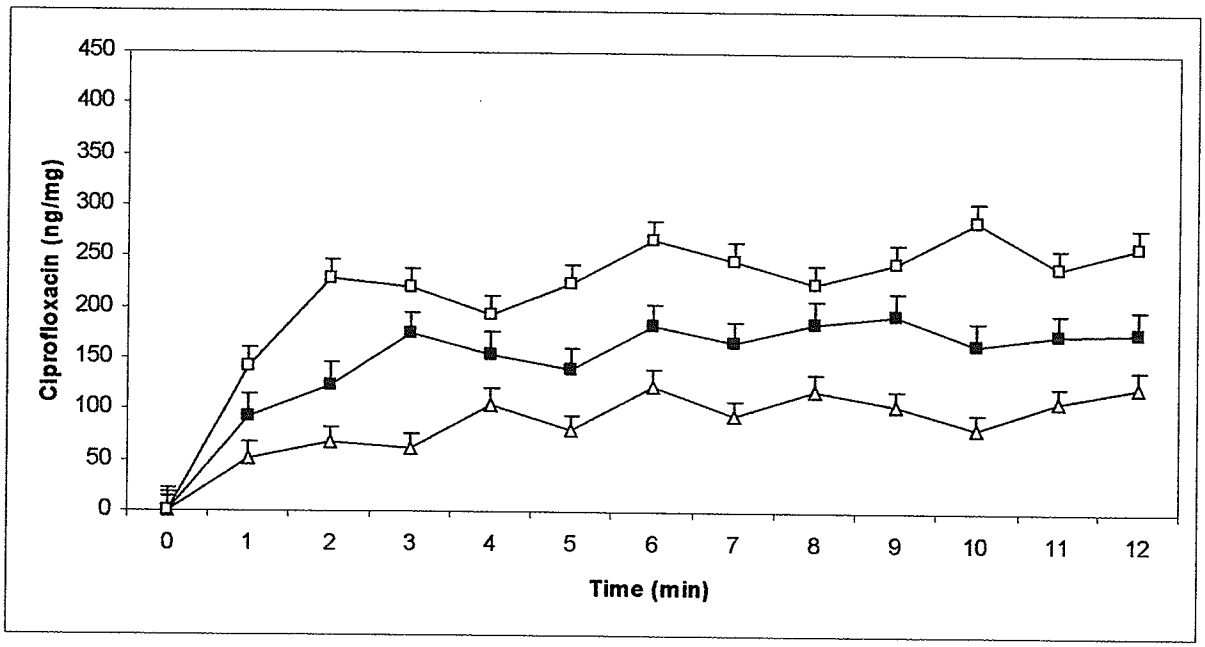
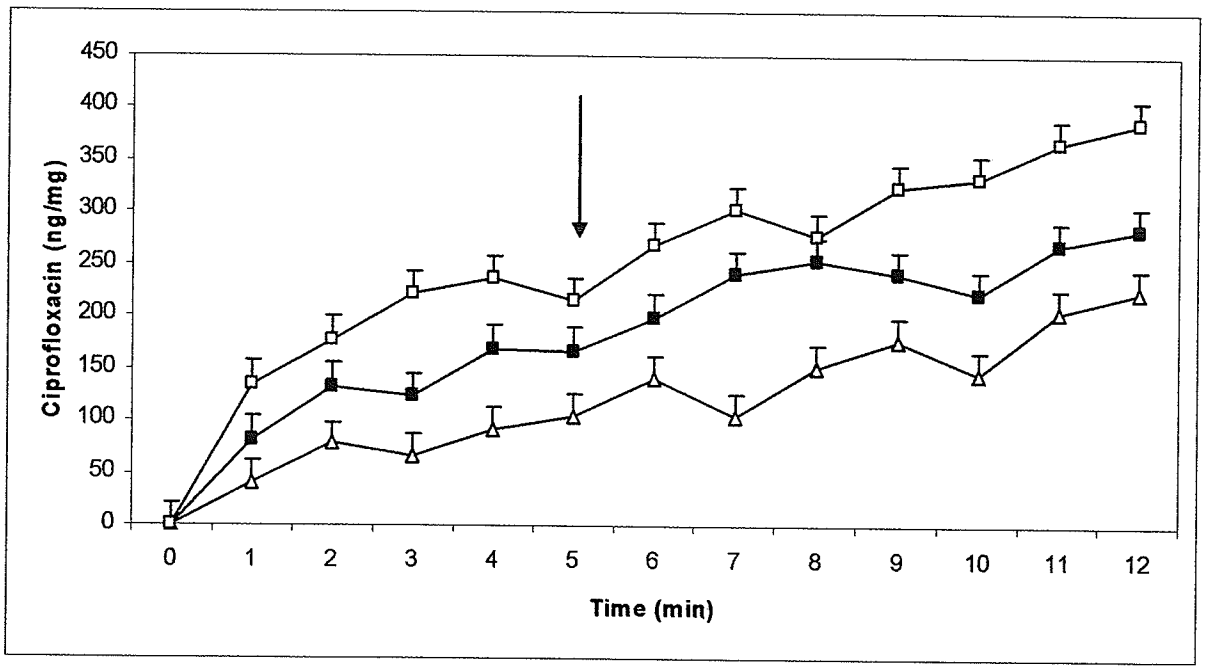

Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

**Figure 3.4.** Effect of pH on ciprofloxacin and nalidixic acid accumulation by *S. marcescens* UOC-67. a) Accumulation of ciprofloxacin (10 μg/ml external concentration) without CCCP and b) with CCCP. c) Accumulation of nalidixic acid (10 μg/ml external concentration) without CCCP and d) with CCCP. Varying pH values are depicted as: pH 8 (□), pH 7 (■), and pH 6 (Δ). The addition of CCCP is indicated by the arrow. All trials were performed in triplicate.

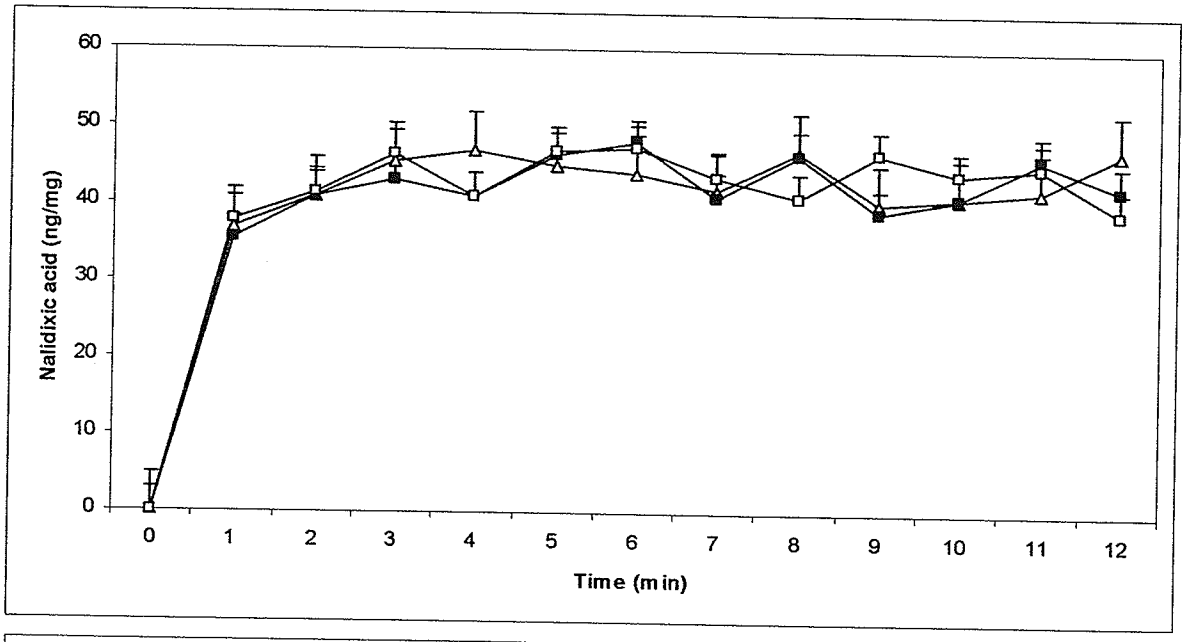
a)


Ciprofloxacin (ng/mg)
450
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

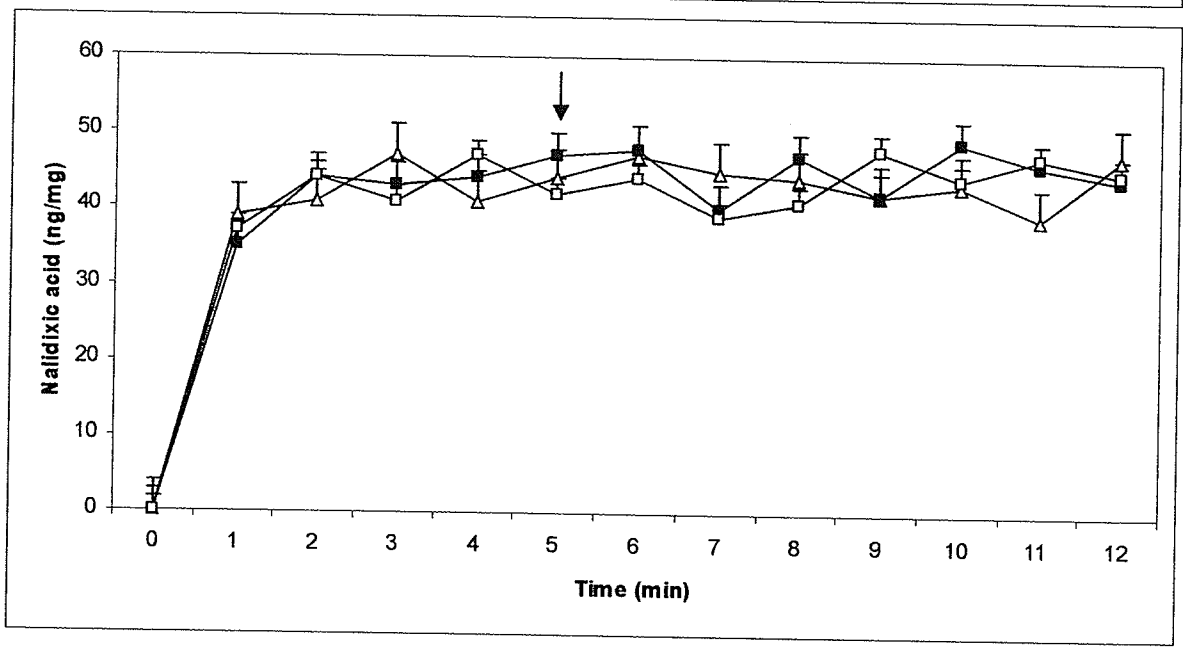
b)


Ciprofloxacin (ng/mg)
450
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)


Nalidixic acid (ng/mg)
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

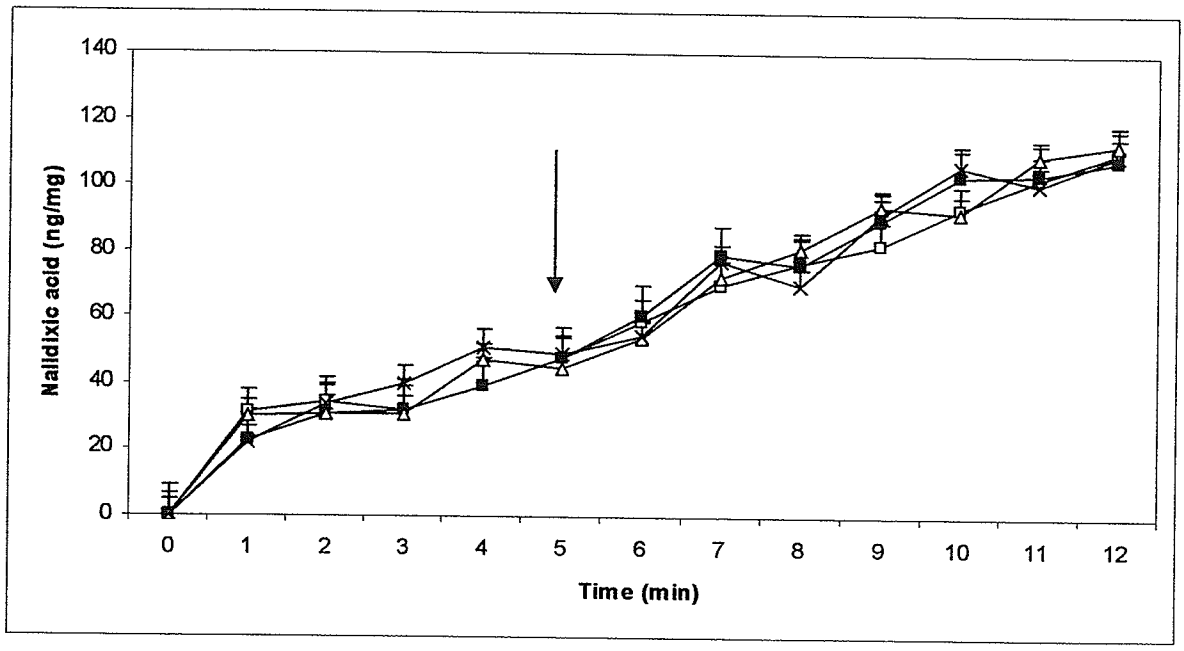
(Figure 3.5 a-d). This is in sharp contrast to the plateau reached in the absence of PAβN, where the accumulation of nalidixic acid for all environmental conditions remained unchanged after T = 5 at approximately 40-45 ng/mg (Figure 3.1-4 c and d). PAβN had no effect on ciprofloxacin accumulation (Figure 3.6 a-d).

Ciprofloxacin accumulation by UOC-67 conducted in the presence of ethanol alone (no CCCP) was also studied (Figure 3.7). Here we see that ethanol alone added from a 70% stock solution does not have an effect on ciprofloxacin accumulation within our experimental parameters as similar results were obtained in presence and absence of 70% ethanol.

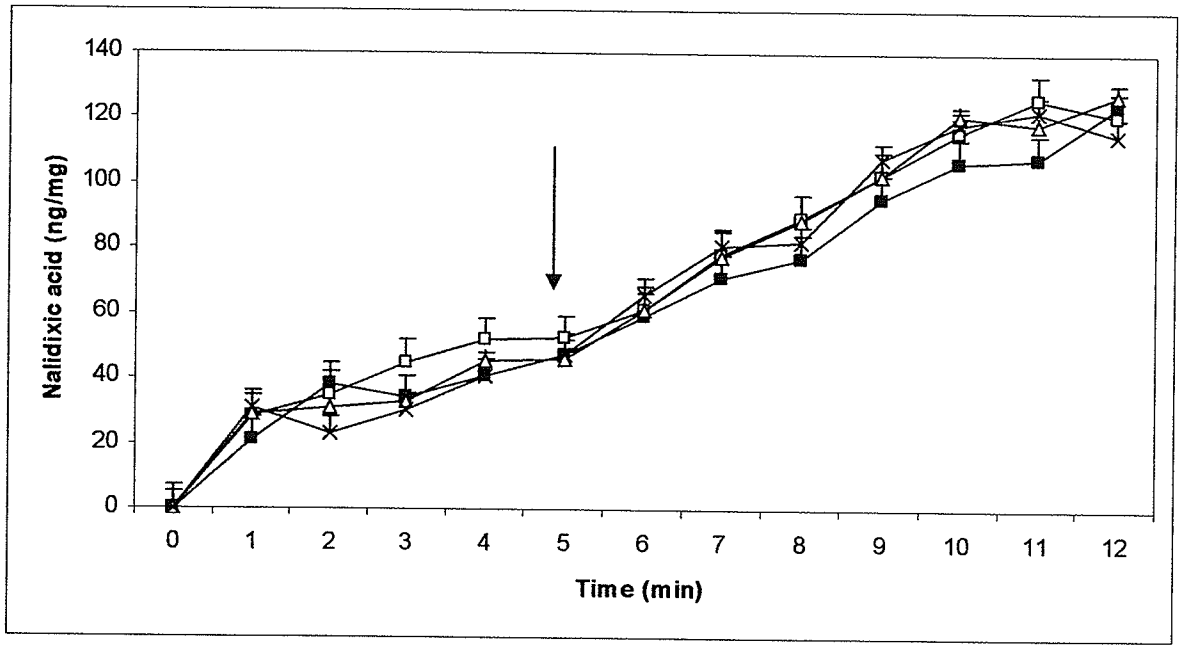
In my assays, I have added CCCP 5 minutes after the addition of antibiotic as it is at this time interval that the antibiotic seems to find its binding site on the efflux transporter (Figure 3.1a) and the internal antibiotic accumulation plateaus. I have also tried several assays where CCCP was added to minute 10 of the experiment, well after the efflux pump has started to expel the drug out of the cell (Figure 3.8). Again, CCCP de-energizes the pump and the antibiotic accumulation continues whereas in its absence accumulation more or less plateaus off. The trends in Figure 3.8b where CCCP was added 10 minutes into the experiment and Figure 3.1b where CCCP was added 5 minutes into the experiment, are the same, however there is a slight uptake difference at the important 5 minute mark between the two assays. In Figure 3.1b, at 5 minutes, 0 mM salicylate added, the antibiotic concentration is approximately 160 ng/ml, whereas in Figure 3.8b, at 5 minutes, 0 mM salicylate added, the antibiotic concentration is slightly higher at approximately 180 ng/mg. This difference is negligible however and in both

**Figure 3.5.** Effect of PAβN on nalidixic acid accumulation by *S. marcescens* UOC-67. Accumulation of nalidixic acid (10 μg/ml external concentration) with PAβN in the presence of: a) Varying concentrations of salicylate: no salicylate (□), 1 mM salicylate (■), 5 mm salicylate (Δ), and 8 mm salicylate (x); b) varying concentrations of sucrose: no sucrose (□), 5% sucrose (■), 8% sucrose (Δ), and 10% sucrose (x); c) varying temperatures: 28°C (□), 37°C (■), and 42°C (Δ); and d) varying pH: pH 8 (□), pH 7 (■), and pH 6 (Δ). The addition of PAβN is indicated by the arrow. All trials were performed in triplicate. For nalidixic acid accumulations in the absence of PAβN, please refer to Figures 3.1c, 3.2c, 3.3c and 3.4c.

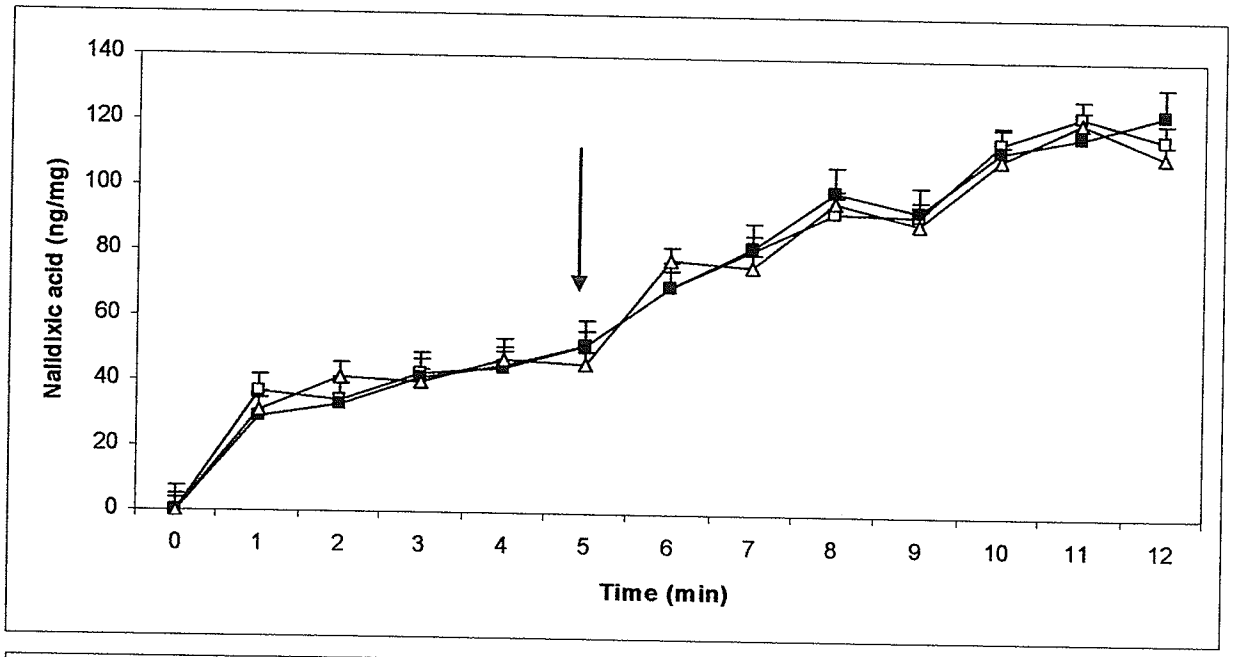
a)


Nalidixic acid (ng/mg)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

b)


Nalidixic acid (ng/mg)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Nalidixic acid (ng/mg)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)

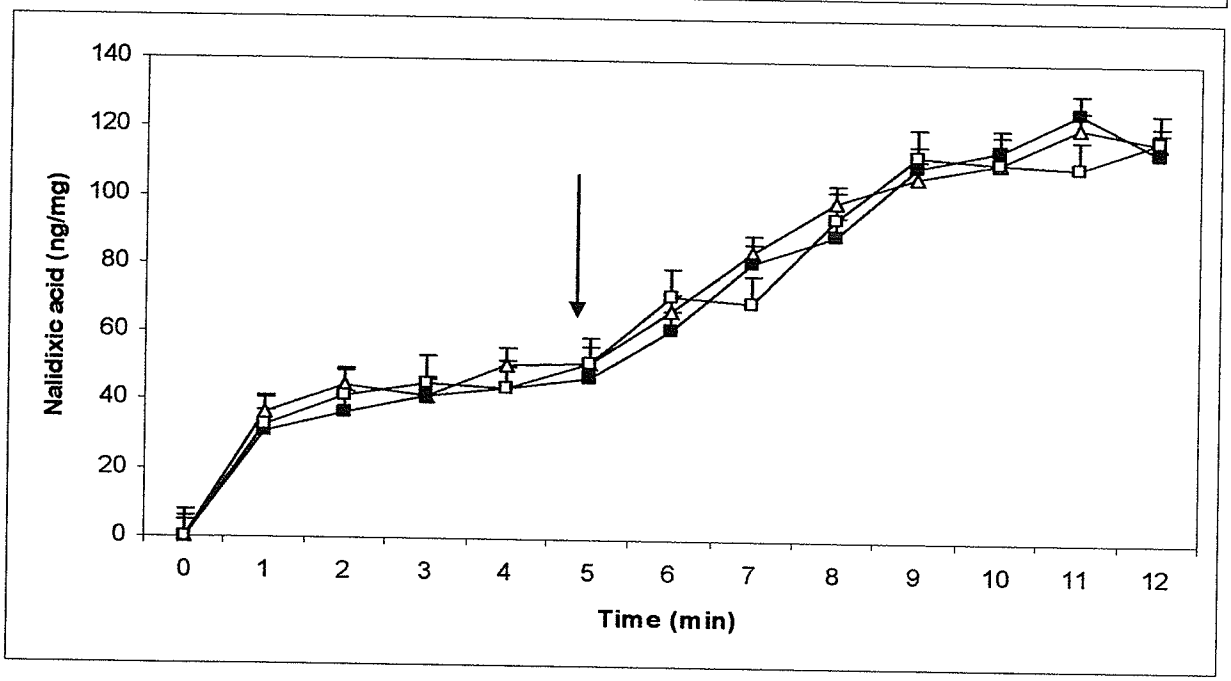
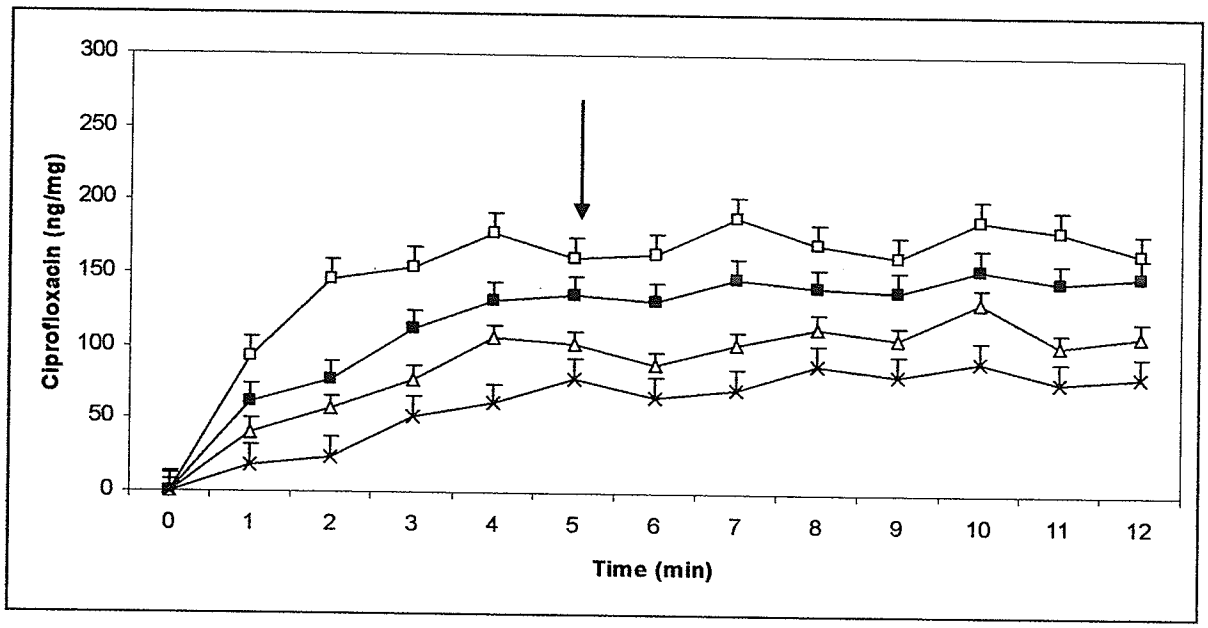
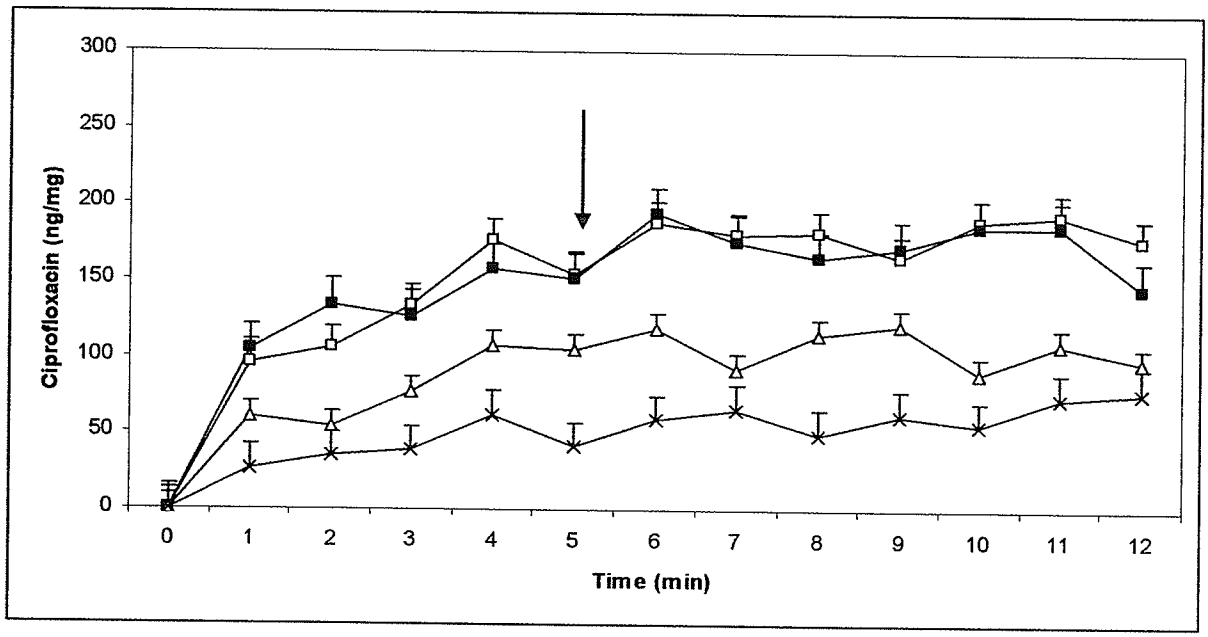

Nalidixic acid (ng/mg)
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

**Figure 3.6.** Effect of PAβN on ciprofloxacin accumulation by *S. marcescens* UOC-67. Accumulation of ciprofloxacin (10 μg/ml external concentration) with PAβN in the presence of: a) Varying concentrations of salicylate: no salicylate (□), 1 mM salicylate (■), 5 mm salicylate (Δ), and 8 mm salicylate (x); b) varying concentrations of sucrose: no sucrose (□), 5% sucrose (■), 8% sucrose (Δ), and 10% sucrose (x); c) varying temperatures: 28°C (□), 37°C (■), and 42°C (Δ); and d) varying pH: pH 8 (□), pH 7 (■), and pH 6 (Δ). The addition of PAβN is indicated by the arrow. All trials were performed in triplicate.

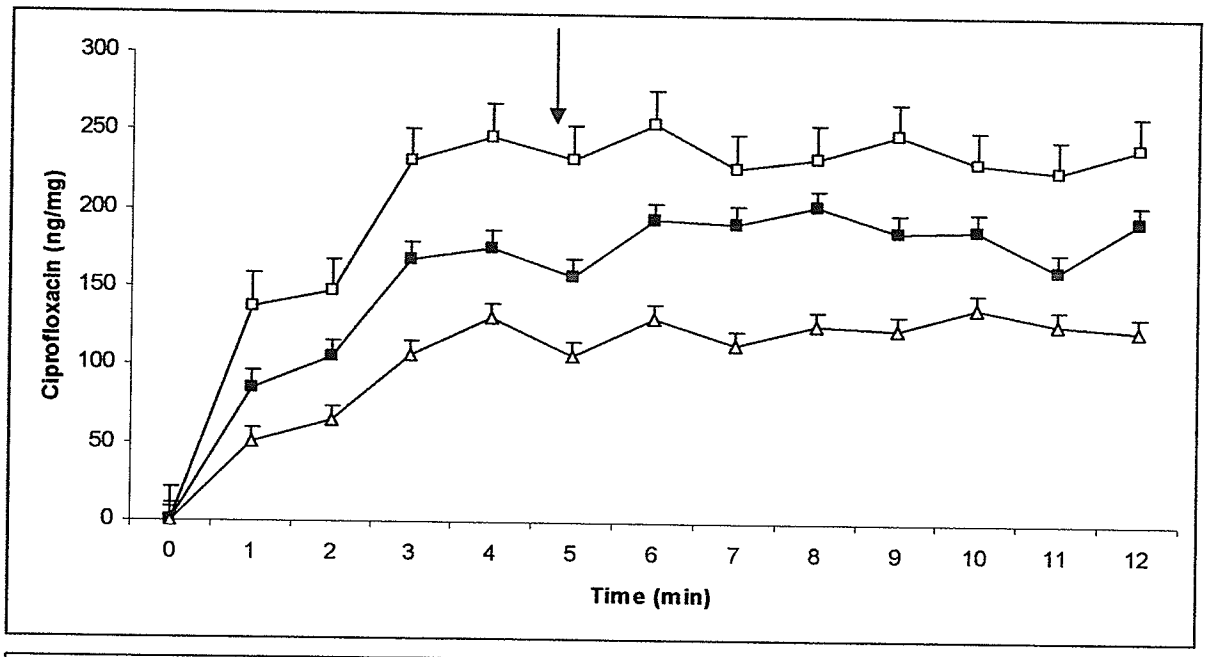
a)


Ciprofloxacin (ng/mg)
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

b)


Ciprofloxacin (ng/mg)
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

c)


Ciprofloxacin (ng/mg)
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

d)

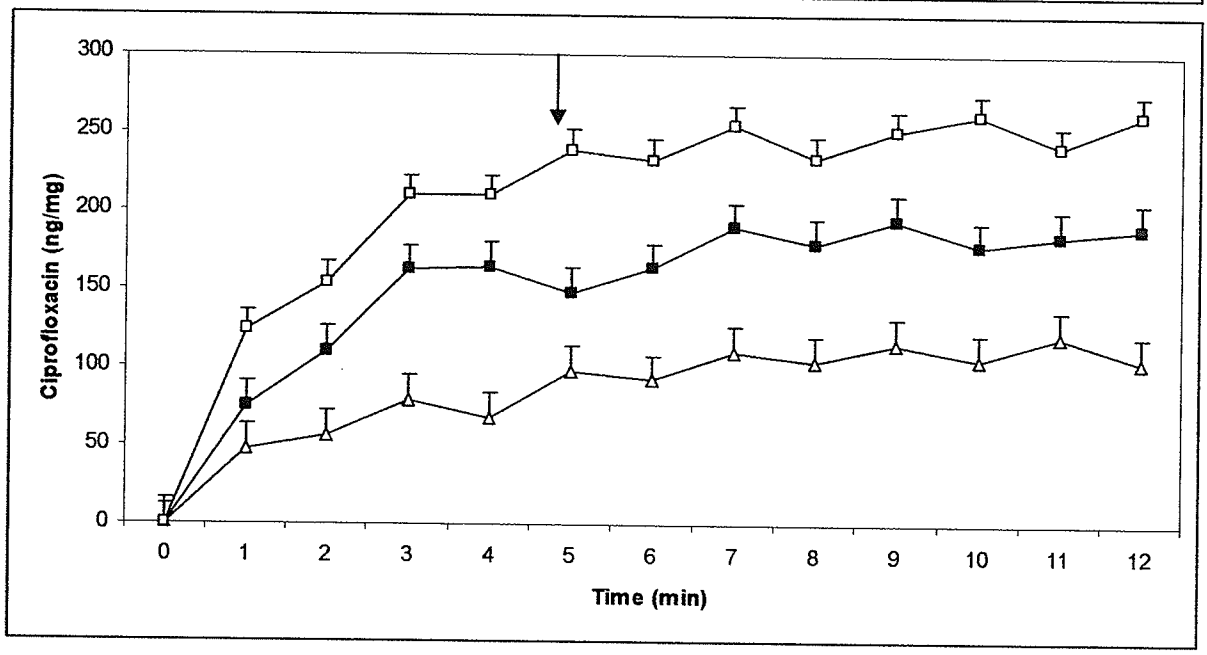

Ciprofloxacin (ng/mg)
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time (min)

**Figure 3.7.** Accumulation of ciprofloxacin by *S. marcescens* UOC-67 in the presence of 70% ethanol (no CCCP). Fluoroquinolone accumulation assay was prepared as described in 3.3.3. 100 µl of 70% ethanol was added after 5 minutes of antibiotic incubation. This is equivalent to the amount of ethanol in CCCP preparations. Accumulation in absence of ethanol (□) and accumulation in presence of ethanol is designated by (■). Trials performed in triplicate.

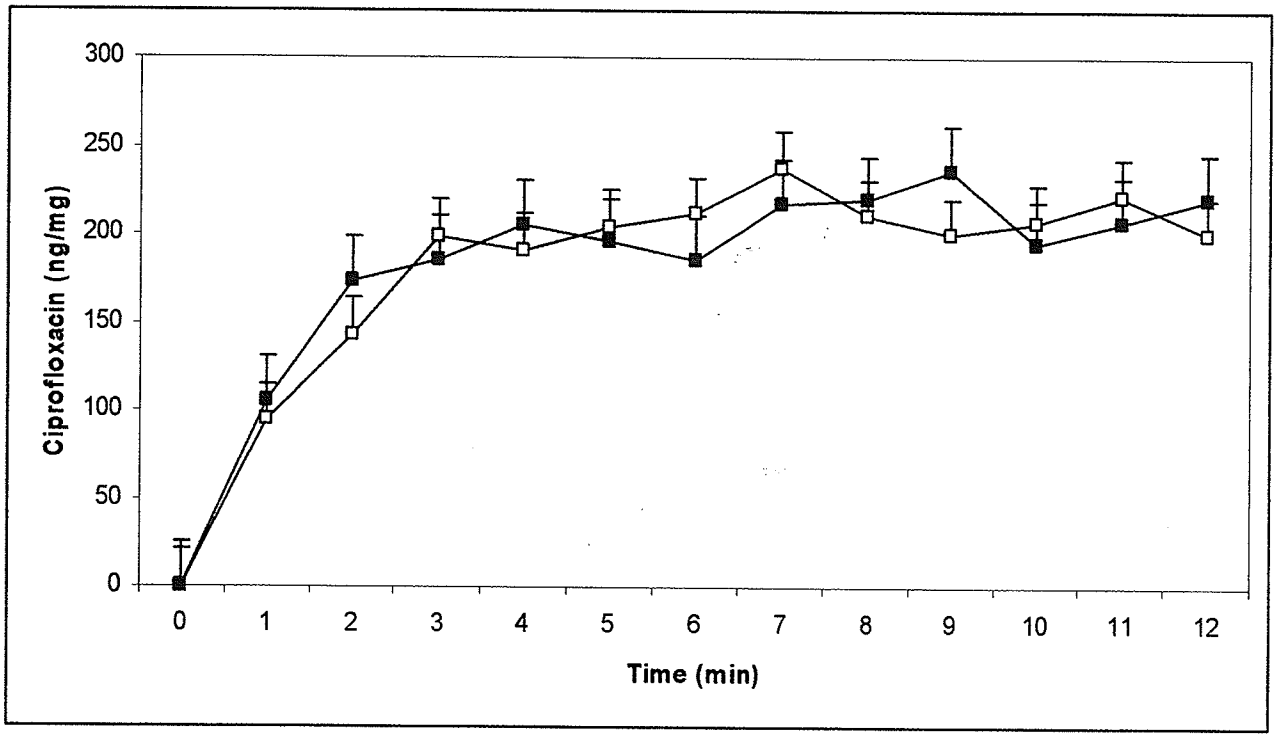
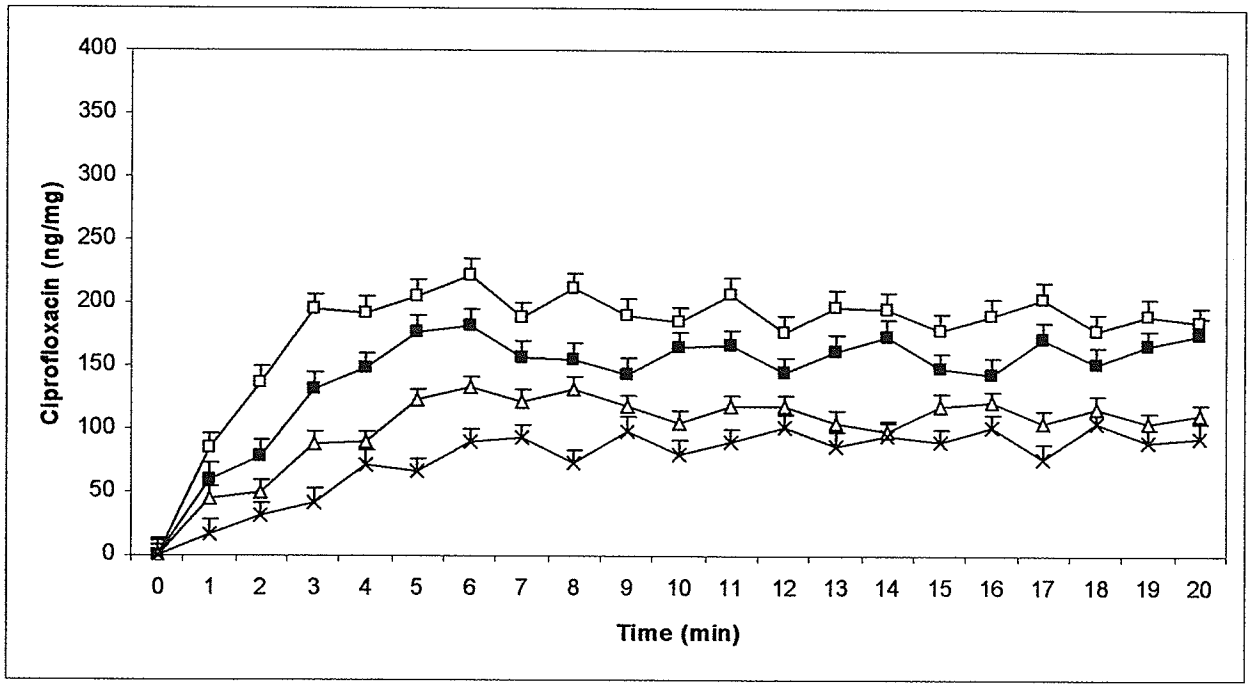
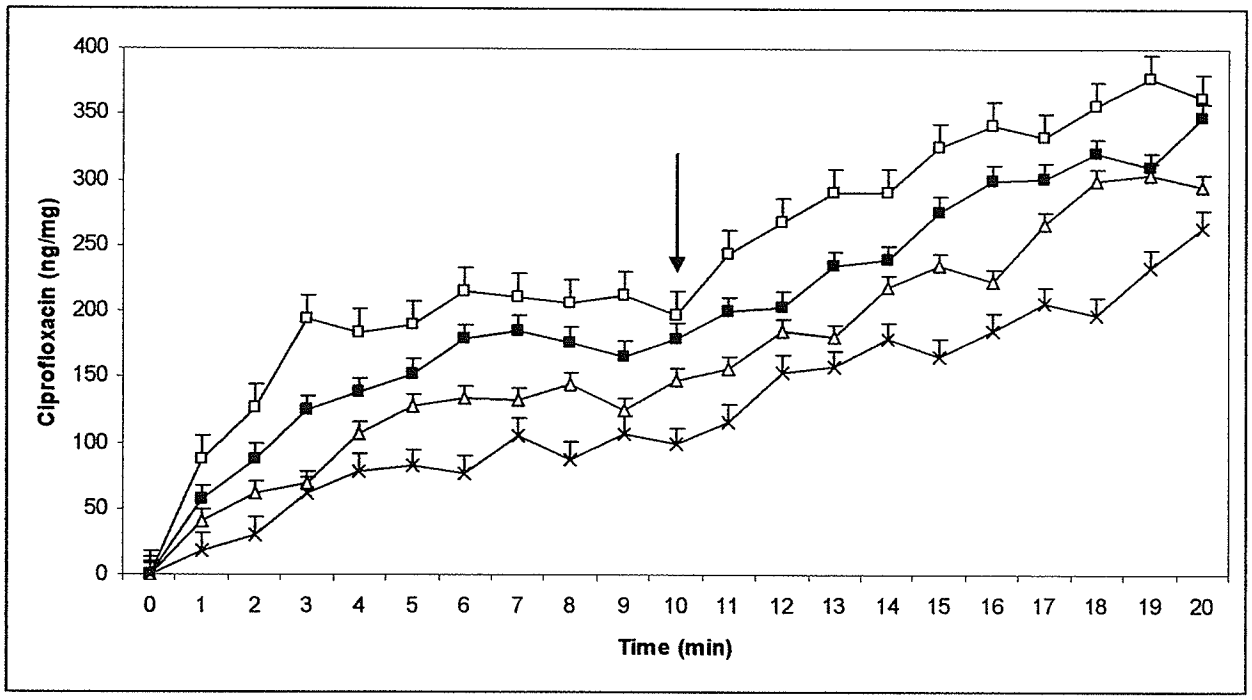

Ciprofloxacin (ng/mg)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (min)

**Figure 3.8.** Effect of salicylate on ciprofloxacin accumulation (10 μg/ml external concentration) by *S. marcescens* UOC-67 in the a) absence of CCCP and b) presence of CCCP added at minute 10 of the assay. Varying concentrations of salicylate are depicted as: no salicylate (□), 1 mM salicylate (■), 5 mm salicylate (Δ), and 8 mm salicylate (x). The addition of CCCP is indicated by the arrow. All trials were performed in triplicate. For ciprofloxacin accumulation in the presence of CCCP added at min=5 of the assay, please refer to Figure 3.1b.

a)


Ciprofloxacin (ng/mg)
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (min)

b)


Ciprofloxacin (ng/mg)
400
350
300
250
200
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (min)

cases, accumulation continues in the presence of CCCP added at either minute 5 or minute 10 of the accumulation assay.

**3.5 Discussion**

In an earlier study (Begic and Worobec 2006), we found that *S. marcescens* UOC-67 growing at a higher temperature, acidic pH or in the presence of high concentrations of sucrose and salicylate, demonstrated a decreased expression from *ompF* and an increase from *ompC*. This differential porin expression influenced the permeability of this bacterium to different antibiotics. In this study, reduced OmpF expression resulted in decreased intracellular ciprofloxacin accumulation and overall lower ciprofloxacin susceptibility.

Based on MIC results, *S. marcescens* UOC-67 exhibited increased resistance to ciprofloxacin after growth in high concentrations of sucrose, salicylate, acidic pH and high temperature. Accordingly, accumulation assays for a hydrophilic versus a hydrophobic antibiotic were tested. Due to the ordering of water molecules within the porin eyelet channel and the presence of acidic and basic amino acid residues on opposite sides of the channel wall, hydrophobic molecules, such as nalidixic acid, are probably not able to cross the outer membrane via porins as is the case for hydrophilic molecules (Mortimer and Piddock 1991; Nikaido et al. 1983; Nikaido 2001; Berlanga et al. 2000). Instead, nalidixic acid might be able to enter the *S. marcescens* periplasm through the lipid bilayer via passive diffusion (Piddock et al. 1991; Vaara 1992; Berlanga et al. 2000). This would account for the fact that we did not observe a change in the accumulation pattern of nalidixic acid under any varying conditions such as sucrose, salicylate, pH or temperature.

Addition of CCCP did not have any effect on overall nalidixic acid accumulation suggesting that this inhibitor is unable to affect a nalidixic acid efflux pump or that there is no efflux of nalidixic acid. However, when PAβN was utilized as the efflux pump inhibitor instead of CCCP, overall nalidixic acid accumulation under all conditions increased suggesting that this quinolone is indeed recognized by a pump. We did not observe a change in accumulation of nalidixic acid under different concentrations of salicylate, sucrose and at different temperatures and pH values in assays performed using PAβN, demonstrating that changes in *ompF* and *ompC* expression did not affect nalidixic acid uptake.

MIC values in this thesis differ from those reported by Kumar and Worobec (2005a) where the ciprofloxacin MIC for UOC-67 was cited as 0.032 μg/ml in comparison to my value of 16 μg/ml (Table 3.1). This discrepancy is likely due to different MIC protocols and media being used in each case. In Kumar and Worobec 2005a, cultures were grown in MH broth without shaking while I cultured the organisms in TSB broth with shaking, similar to Berlanga and Vinas (2000) who reported an MIC value of 60 μg/ml for ciprofloxacin for a *S. marcescens* NIMA wild type strain. In addition, shaking has been known to play a role in gene regulation such that many proteins can be differentially expressed under standing and shaking conditions, including Acr efflux proteins (Florczyk et al 2001). This, in addition to MIC culture media, may account for the discrepancy in MIC values.

Kumar and Worobec (2005a) report similar accumulation curves for ciprofloxacin, however no CCCP effect was observed for UOC-67 (UOC-67 continued to accumulate antibiotics in a steady fashion before and after the uncoupler addition). I

report here that CCCP does however have an effect on the accumulation of ciprofloxacin. It was also reported (Kumar and Worobec 2005a) that a strain generated by serial passaging of UOC-67 exclusively in ciprofloxacin-supplemented medium (UOC-WL) was in fact affected by CCCP, which is at odds with the reported concept that CCCP has no effect on ciprofloxacin accumulation of UOC-67 alone. It is very difficult to make quantitative estimates from accumulation assays. One thing to note in terms of the CCCP studies is that in accumulation assays, CCCP is prepared in an ethanol solution which may have a physiological effect. In our studies in this chapter only, CCCP was solubilized in ethanol (70%) and then further diluted in 10 mM NaOH to dilute the amount of ethanol in the medium. Studies have shown that with CCCP prepared in this fashion, ethanol has no significant effect on bacterial viability (Lambert and Le Pecq 1984). Additionally, Figure 3.7 shows that 70% ethanol alone does not have an effect on ciprofloxacin accumulation within our experimental parameters.

There are several additional problems with CCCP, one of which is that CCCP may be excreted out of the cell using efflux pumps (Yin et al. 2006), specifically with the aid of the accessory protein (such as perhaps *S. marcescens* SdeA or SdeC). This may explain why a relatively high concentration of CCCP (100 μM) is required to see an effect in accumulation assays. The second problem may be that CCCP has cytotoxic effects on quinolone sensitive strains as was the case in *E. coli* (Cho et al. 2001). Here the authors only required 10 μM CCCP to inhibit ofloxacin accumulation in quinolone-susceptible strains. In this same study, 100 μM CCCP was used to collapse the efflux pump in quinolone-resistant strains and prevent ofloxacin from being excreted out of the cell (Cho et al. 2001).

CCCP is a protonophore that is intercepted by the efflux transporter on the inner membrane as it crosses towards the cytoplasm. In the absence of drug efflux, CCCP diffuses across the inner membrane from the periplasmic space in the protonated form, disrupting the pH differential as it moves into the cytoplasm (Yin et al. 2006). The molecule next releases its proton to become a lipophilic soluble anion that rapidly diffuses back into the periplasm. The possibility remains that CCCP may bind to the inner membrane transporter (eg. with *S. marcescens* SdeB or SdeD/E) through hydrophobic interactions on the inner leaflet side acting as a competitive inhibitor. However, it is speculated that proton translocation and drug transport may occur at different locations, in which case there would be no competition for the same transporter binding site and the drug would be effluxed (Yin et al. 2006).

To further discuss results of this study, PAβN had no effect on accumulation of ciprofloxacin in *S. marcescens* (Figure 3.6). It has been suggested that the magnitude of the effect of PAβN is strongly dependent on the nature of a particular substrate, indicating that different antibiotics may have different binding sites on the pump and that inhibition by PAβN is binding site specific (Lomovskaya et al. 2001; Mamelli et al. 2003). According to this interpretation, ciprofloxacin rather than PAβN may be a stronger competitor for the inner membrane transporter and thus *S. marcescens* efflux pumps can still extrude ciprofloxacin even when exposed to PAβN (Lomovskaya et al. 2001; Mamelli et al. 2003). Similar results have been reported for *Campylobacter* (Mamelli et al. 2003) and *Pseudomonas aeruginosa* (Lomovskaya et al. 2001).

In terms of the overall ciprofloxacin uptake pattern, in the presence and absence of CCCP, our results agree with those reported for *Klebsiella pneumoniae* (Domenico et

al. 1990; Sawai et al. 1987) and *E. coli* strains (Cohen et al. 1993). In addition, our results are similar to ciprofloxacin accumulation values reported by Berlanga and Vinas, 2000, where these authors tested the effect of 1 mM salicylate on the *S. marcescens* outer membrane permeability. In terms of the overall nalidixic acid uptake pattern in the presence of PAβN, our results suggest that *S. marcescens* produces an efflux pump that is able to discharge nalidixic acid as easily as ciprofloxacin. A similar result has been reported for *E. coli* isolates (Saenz et al. 2004).

Our results agree with those reported for *E. coli* and *P. aeruginosa* strains where acid pH and a higher temperature (>40°C) were found to decrease the overall accumulation of several fluoroquinolones (Piddock et al. 1991). It is important to keep in mind, that acidic pH in addition to decreasing the expression of *E. coli ompF*, also alters the overall fluoroquinolone electric charge from the zwitterionic form (which favours accumulation) to a negative charge which would result in fewer zwitterions, hence less fluoroquinolone accumulation (Piddock et al. 1991). Therefore, lower ciprofloxacin accumulation at pH 6, is due to both a decrease in *ompF* expression and the effect of acidic pH on the antibiotic itself.

It must be recognized that resistance caused by reduced OmpF expression has been shown to require the presence and often enhanced expression of efflux systems that actively pump out drugs from the cytoplasm (Hooper 2001). The simultaneous decrease in porin production and an increase in efflux activity is very common in gram-negative bacteria and is how bacteria become hyper-resistant to antimicrobials (Poole 2001). However, while the CCCP and PAβN results support the existence of an efflux pump, this does not necessarily indicate the pump's level of expression. Notably, the

equilibrium levels of antibiotics in the cell are controlled by the rate of influx and rate of efflux. Reduced OmpF expression would suggest the strain to be less permeable than a susceptible strain, which could lead to higher resistance at a constant level of pump expression (Szabo et al. 2006). Therefore, in recognition that reduced outer membrane permeability works in concert with expression of multidrug efflux pumps, we must also acknowledge that reduced porin expression does not necessarily lead to pump overexpression (Szabo et al. 2006).

In conclusion, salicylate, sucrose, increased temperature and an acidic pH reduces the uptake (via porins) and availability of ciprofloxacin, leading to an increased resistance to this antibiotic. The same conditions had no effect on nalidixic acid susceptibility. Similar conclusions regarding the effect of salicylate on ciprofloxacin in *S. marcescens* NIMA strain have been reached by Berlanga and Vinas (2000).

# CHAPTER 4 The role of *Serratia marcescens* SdeAB multidrug efflux pump and TolC-homolog in fluoroquinolone resistance studied via gene knockout mutagenesis

Parts of the material presented herein resulted in the publication Begic, S., and Worobec, E. A. 2008a. The role of *Serratia marcescens* SdeAB multidrug efflux pump and TolC-homolog in fluoroquinolone resistance studied via gene knockout mutagenesis. Microbiology. **154:** 454-461. I planned and completed the majority of experiments outlined in this chapter, with *tolC* cloning assistance from Honours Project students Annie Ducas and Craig Harlos and lab assistant Leif Larsen. I wrote the manuscript with full guidance and feedback from my advisor and important suggestions were made by Dr. T. de Kievit.

## 4.1 Abstract

*Serratia marcescens* is a prominent opportunistic nosocomial pathogen resistant to several classes of antibiotics. The major mechanism for fluoroquinolone resistance in various Gram-negative pathogens is active efflux. Our group previously identified SdeAB, a Resistance-Nodulation-Cell Division (RND) efflux pump complex, and a TolC-like outer membrane protein (HasF), which together mediate energy-dependent fluoroquinolone efflux. Additionally, a regulatory protein-encoding gene in the upstream region of the *sdeAB* was identified (*sdeR*), and found to be 40% homologous to MarA, an *E. coli* transcriptional regulator. To provide conclusive evidence as to the role of these components in *S. marcescens, sdeB, hasF* and *sdeR* deletion mutants were constructed. Suicide vectors were created and introduced via tri-parental mating into *S. marcescens* UOC-67 (wild type) and, for *sdeB* and *hasF*, T-861 (clinical isolate). We have analyzed these genetically altered strains using Minimal Inhibitory Concentration assays (MIC) for

a wide range of compounds (fluoroquinolones, SDS, novobiocin, ethidium bromide and chloramphenicol). Intracellular accumulation of a variety of fluoroquinolones was measured fluorospectroscopically. The *sdeB*, *hasF* and *sdeR* knockout strains were consistently more susceptible to antibiotics than the parent strains, with the *sdeB/hasF* double knockout strain showing the highest susceptibility. A marked increase in fluoroquinolone (ciprofloxacin) accumulation was observed for strains deficient in either the *sdeB* or *hasF* genes when compared to the parental strains, with the highest ciprofloxacin accumulation observed for the *sdeB/hasF* double knockout. Antibiotic accumulation assays for the *sdeB* knockout mutant strains performed in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore that is a proton motive force inhibitor, demonstrated that SdeAB mediated efflux is proton motive force dependent. Due to the comparable susceptibility of the *sdeB* and the *hasF* individual knockouts, we conclude that *S. marcescens* HasF is the sole outer membrane component of the SdeAB pump. In addition, MIC data for *sdeR*-deficient and overexpressing strains confirm that SdeR is an activator of *sdeAB* and acts to enhance the overall multidrug resistance of *S. marcescens*.

**4.2 Introduction**

*Serratia marcescens* is a prominent opportunistic pathogen, responsible for serious infections in immunocompromised individuals due primarily to its high intrinsic antibiotic resistance. It has been shown to be resistant to all major classes of antibiotics used, including fluoroquinolones, making the treatment of infections very difficult (Lambert and O'Grady 1992; Aucken and Pitt 1998; Fujimaki et al. 1989). The major mechanism for fluoroquinolone resistance in various Gram negative organisms, including

*S. marcescens*, is the active efflux of the antibiotic molecule mediated by 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 (e.g. *Escherichia coli* TolC) to form a tripartite system (Li and Nikaido 2004, and references therein).

The Worobec lab has established active efflux as a resistance mechanism in *S. marcescens*, and presented molecular characterization of two different efflux pumps (Kumar and Worobec 2005a). Of these, they have demonstrated that the SdeAB RND pump is a multidrug efflux pump with a wide range of substrates, having a high degree of homology to the AcrAB pump of *E. coli*. The *sdeAB* operon (GenBank accession no. AY168756) consists of the *sdeA* gene encoding for a periplasmic fusion protein and the *sdeB* gene encoding for the transporter of the RND pump on the inner membrane. We have also reported on an outer-membrane component called HasF (TolC-homolog; GenBank accession no. X98513) involved in energy-dependent efflux of antimicrobial agents (Kumar and Worobec 2005b). Computer-generated analysis of the *S. marcescens* HasF revealed a very similar structure to that of *E. coli* TolC, having the channel-tunnel structure characteristic of outer membrane components of RND efflux pumps. No other *tolC* homolog was found upon searching the *S. marcescens* Db11 genome (Kumar and Worobec 2005b; http://www.sanger.ac.uk/Projects/S_marcescens/).

Most of the efflux pumps characterized to date have a regulatory protein encoding gene in the upstream from the pump encoding genes (Alekshun and Levy 1997; Barbosa and Levy 2000; Hachler et al. 1991). Upstream from the *sdeAB* locus is *sdeR* (GenBank accession no. AY623133), a 405 bp ORF which is transcribed in the opposite direction to

*sdeAB* (Kumar 2004). At the amino acid level, SdeR is 40% homologous to the MarA protein of *E. coli*, a transcriptional activator of the AcrAB-TolC drug efflux pump (Alekshun and Levy 1997; Barbosa and Levy 2000; Hachler et al. 1991). Amino acid prediction and 3-dimensional structural prediction also showed similarity to the MarA protein of *E. coli* with high degree of conservation of the DNA binding helices.

In this study, we address the importance of the SdeAB pump and the TolC-like protein in fluoroquinolone resistance by constructing *hasF* and *sdeB* gene knockouts. Additionally, through knockout mutagenesis, we demonstrate the importance of SdeR in the regulation of the expression of the SdeAB efflux pump system.

### 4.3 Materials and Methods

#### 4.3.1 Bacterial strains and plasmids

All strains and plasmids used in this study are outlined in Table 4.1. All bacterial strains were grown in Luria-Bertani agar (LB), Trypticase Soy Agar (TSA) and Tryptone Soy Broth (TSB) [BD Diagnostics Systems]. The ampicillin resistant strains (containing pKS(+), pKS:SdeB, pKS:HasF, pKS:ΔHasF, pKS:SdeR and all pEX1.8 constructs) were grown on LB plates and in LB broth containing 100 μg/mL ampicillin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The kanamycin resistant strains (with pKIXX, pKS:SdeR:$Km^r$, pKS:SdeB:$Km^r$) were grown on LB plates and in LB broth containing 25 μg/mL kanamycin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The streptomycin resistant strains (with pKNG101, pKNGsdeB, pKNGhasF, pKNGsdeR, pKNGhasFGm$^r$) were grown on LB plates and in LB broth containing 50 μg/mL streptomycin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The gentamicin resistant

**Table 4.1.** Bacterial strains and plasmids used in the study.

| Strains | Characteristic | Source/Reference |
|---|---|---|
| UOC-67 | Wild type *S. marcescens*, ATCC 13880 | American Type Culture Collection |
| MT616 | *E. coli*, MT607 (*pro-82 thi-I hsdR17 supE44*):pRK600 | Finan *et al.* 1986 |
| CC118 | *E. coli, araD 139Δ(ara, leu) 7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB argEam recA1* | Dr. C. Manoil, University of Washington, Seattle, USA |
| T-861 | Clinical isolate of *S. marcescens* | D. Hoban, Health Science Centre, Winnipeg |
| SDEAB1 | *sdeB*-deficient *S. marcescens* UOC-67 | This study |
| SDEAB2 | *sdeB*-deficient *S. marcescens* T-861 | This study |
| HASF100 | *hasF*-deficient *S. marcescens* UOC-67 (*hasF* deletionally mutated) | This study |
| HASF200 | *hasF*-deficient *S. marcescens* T-861 (*hasF* deletionally mutated) | This study |
| HASF300 | *hasF*-deficient *S. marcescens* UOC-67 (*hasF* insertionally mutated) | This study |
| SDEAB3/HASF300 | *sdeB/hasF*-deficient *S. marcescens* | This study |
| SDER1 | *sdeR*-deficient *S. marcescens* UOC-67 | This study |
| SDER2 | SDER1/pEXR | This study |
| SM3000 | SDEAB1 with pEXS | This study |
| SM2000 | HASF300 with pEXH | This study |
| SM1000 | SDEAB3/HASF300 with pEXSH | This study |
| SDER3 | UOC-67/pEXR | This study |
| SDER4 | UOC-67/pEX1.8 | This study |
| Plasmids | | |
| pKS (+) | ColE1 replicon, $Amp^r$, *lacZ'* | Stratagene |
| pKS:SdeB | pKS with 1.7-kb *Eco*RI/*Eco*RI *sdeB* fragment | This study |

| | | |
|---|---|---|
| pKS:SdeB:Km$^r$ | pKS:SdeB with a 1.4-kb Kan$^r$ insertion in *sdeB* | This study |
| pKS:HasF | pKS with a 1.5-kb *Eco*RI/*Eco*RI *hasF* fragment | This study |
| pKS:ΔHasF | pKS:HasF with *Hin*CII/*Kpn*I 700-bp deleted | This study |
| pKS:hasF:Gm$^r$ | pKS:HasF with a 900-bp Gm$^r$ insertion in the *Eco*RI/*Eco*RI flanked *hasF* fragment | This study |
| pKS:SdeR | pKS with 400-base *Bam*HI/*Eco*RI *sdeR* fragment | This study |
| pKS:SdeR:Km$^r$ | pKS:SdeR with a 1.4-kb Kan$^r$ insertion in *sdeR* | This study |
| pKIXX | 1.4-kb Kan$^r$ cassette flanked by *Pst*I for *sdeB* insertion and *Eco*RV for *sdeR* insertion | Pharmacia PL Biochemicals |
| pUCGM | 900-bp Gm$^r$ cassette flanked by *Pst*I | Schweizer *et al.*, 1993 |
| pEX1.8 | CoE1 replicon, Amp$^r$-Car$^r$, carries *lacI*$^Q$ and a $P_{tac}$ promoter | Pearson *et al.*,. 1997 |
| pEX1.8JH | a pEX1.8 plasmid with a *Bam*HI site upstream of the P*tac* promoter removed by Erase-a-base system | Hutsul 1996, unpublished |
| pEX1.8JH1 | a pEX1.8 plasmid with *Eco*RI, *Sma*I and *Bam*HI sites within MCS removed by Erase-a-base system | Hutsul 1996, unpublished. |
| pEXSH | pEX1.8JH with 3.1-kb *Eco*RI/*Eco*RI *sdeB* fragment and 1.5-kb *Hin*DIII/*Hin*DIII *hasF* fragment | This study |
| pEXH | pEX1.8 with wild type 1.5-bp *Eco*RI/*Hin*DIII *hasF* fragment | This study |
| pEXHH/H | pEX1.8JH1 with wild type 1.5-bp *Hin*DIII/*Hin*DIII *hasF* fragment | This study |
| pEXS | pEX1.8 with wild type 3.1-kb *Eco*RI/*Hin*DIII *sdeB* fragment | This study |
| pEXSE/E | pEX1.8JH with wild type 3.1-kb *Eco*RI/*Eco*RI *sdeB* fragment | This study |

| | | |
|---|---|---|
| pEXR | pEX1.8 with 405-bp *Eco*RI/*Hin*DIII *sdeR* fragment | This study |
| pKNG101 | Suicide vector, *pir- oriR6K mobRK2 sacB* $Str^r$ | Kaniga *et al.*, 1991 |
| pKNGsdeB | pKNG101 with 3.1-kb insertionally-disrupted *sdeB* fragment | This study |
| pKNGhasF | pKNG101 with 800-bp deleted *hasF* | This study |
| pKNGhasFGm$^r$ | pKNG101 with 2.4-kb *hasF* insertion fragment | This study |
| pKNGsdeR | pKNG101 with 1.8-kb insertionally-disrupted *sdeR* fragment | This study |

Abbreviations: Kan, kanamycin; Amp, ampicillin; Str, streptomycin; Car, carbenicillin; Gm, gentamicin.

strains (with pUCGM, pKS:hasF:Gm$^r$) were grown on LB plates and in LB broth containing 20 μg/mL gentamicin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. SDEAB1, SDEAB2, HASF100, HASF200 and SDER1 strains were grown on LB plates and in LB broth containing 100 μg/mL ampicillin and 50 μg/mL streptomycin, HASF300 was grown on media containing 20 μg/mL gentamicin and 50 μg/mL streptomycin and SDEAB3/HASF300 was grown on media containing 25 μg/mL kanamycin and 20 μg/mL gentamicin.UOC-67, MT616, CC118 and T-861 were grown in absence of any antibiotic.

### 4.3.2 Construction of *S. marcescens hasF, sdeB* and *sdeR*-deficient strains

Primers used throughout this study are listed in Table 4.2. Two methods were employed to create the *hasF* knockout. Firstly, unique *Hin*CII/*Kpn*I restriction sites within pKS:HasF (Kumar and Worobec 2005b), separated by approximately 700 bases, were identified to be a feasible target for creating a deletion mutation in the *hasF* coding region (Figure 4.1a). pKS:HasF was digested with *Hin*CII/*Kpn*I, and religated to release a 0.8-kb fragment to result in pKS:ΔHasF. After transformation of pKS:ΔHasF into *S. marcescens* UOC-67, the *hasF* deletion mutation was confirmed by restriction digestion with *Eco*RI, PCR of pKS:ΔHasF with HasF1for/rev primers and sequencing of the mutated gene and vector flanking regions. pKS:ΔHasF was digested with *Eco*RI to remove the 0.8-kb mutated *hasF* fragment, the ends of the fragment were made flush with T4 DNA polymerase and subsequently digested with *Sma*I in order to be inserted into the *Sma*I site of pKNG101. The mutated *hasF* fragment was then ligated into the pKNG101 replacement vector resulting in pKNGhasF which was then transformed into *E. coli* CC118 λpir for stability. Please see Section 2.3.2 for a detailed description. pKNG101 is a suicide vector that contains a conditional origin of replication (*oriR6K*), the *strAB* genes

**Table 4.2.** Primers used in the study. All restriction sites are underlined.

| Name | Sequence (5' to 3') | Description | PCR Tm |
|---|---|---|---|
| HasFfor (forward) | AGGAATTCGTCTACAAACAGGC | Amplification of a 1.5-kb sequence within *hasF* (*Eco*RI site engineered) | 55°C |
| HasFrev (reverse) | CGAATTCGGCGGGCAGAAGC | Same as above (*Eco*RI site engineered) | |
| HasF1for (forward) | ACAGGCCAGGGAAAGTAA | Amplification of a 1.5-kb sequence within *hasF* (for screening transconjugants) | 49°C |
| HasF1rev (reverse) | ATCGGACAAAAAAAAACGG | Same as above | |
| SdeBfor (forward) | TGAATTCTCCCGTTTTTTCATCGA | Amplification of a 1.7-kb sequence within *sdeB* (*Eco*RI site engineered) | 57°C |
| SdeBrev (reverse) | AGAATTCAAGCCGCCGGGCAC | Same as above (*Eco*RI site engineered) | |
| SdeB1for (forward) | GGCCGATCTTTGCCGCGGTG | Amplification of a 1.7-kb sequence within *sdeB* (for screening transconjugants) | 57°C |
| SdeB1rev (reverse) | AGCACGCGGGAGACGCCG | Same as above | |
| SdeRfor (forward) | ATGAATTCGTGCATCGGGCAGAGGG | Amplification of a 0.4-kb sequence within *sdeR* (*Eco*RI site engineered) | 59°C |
| SdeRrev (reverse) | TCGGATCCAGTGGTGCGTGCATCT | Same as above (*Bam*HI site engineered) | |
| SdeRf (forward) | GGCAGAGGGTGACCCTGCCGA | Amplification of a 0.4-kb sequence within *sdeR* (for screening transconjugants) | 57°C |
| SdeRr (reverse) | GCTGGCTGCGCCAGCGCGCG | Same as above | |
| SdeRfor1 (forward) | ATGAATTCATGTGCATCGGGCAG | Amplification of a 0.4-kb sequence within *sdeR* for overexpression/complementation (*Eco*RI site engineered) | 57°C |
| SdeRrev1 (reverse) | CGAAGCTTTCAGTGGTGCGTGCA | Same as above (*Hin*DIII site engineered) | |

| | | | |
|---|---|---|---|
| SacBf (forward) | AAGAAAATGCCAATAGGATATC | Analysis of *sdeB, hasF and sdeR* transconjugant after *S. marcescens-E. coli* conjugations, 1.0 kb | 56°C |
| SacBr (reverse) | TTCTATCCTAAAAGACCAAACAC | Same as above | |
| HasF1for/rev and SdeB1for/rev | Above | Multiplex reaction using both sets of primers (HasF1for/rev and SdeB1for/rev) for detection of double mutants | 53°C |
| Kmf (forward) | TAGAAAAACTCATCGAGCATC | Amplification of $Km^r$ cassette (650 bp) | 51°C |
| Kmr (reverse) | CGCCAGAGTTGTTTCTGAAA | Same as above | |
| SdeBKmf (forward) | GGCCGATCTTTGCCGCGGTG | Amplification of the 650 bp $Km^r$ cassette and some of *sdeB* within *sdeB* transconjugant (1.7 kb product) | 58°C |
| SdeBKmr (reverse) | CGCCAGAGTTGTTTCTGAAA | Same as above | |
| Gmf (forward) | GTCGACTCTAGAGGATCCCCG | Amplification of $Gm^r$ cassette (650 bp) | 56°C |
| Gmr (reverse) | ACGTAGATCACATAAGCA | Same as above | |
| HasFGmf (forward) | ACAGGCCAGGGAAAGTAA | Amplification of the 650 bp $Gm^r$ cassette and some of *hasF* within *hasF* transconjugant (0.9 kb product) | 54°C |
| HasFGmr (reverse) | ACGTAGATCACATAAGCA | Same as above | |
| SdeRKmf (forward) | GGCAGAGGGTGACCCTGCCGA | Amplification of the 650 bp $Km^r$ cassette and some of *sdeR* within *sdeR* transconjugant (0.8 kb product) | 53°C |
| SdeRKmr (reverse) | CGCCAGAGTTGTTTCTGAAA | Same as above | |
| Kmfor1 (forward) | ATGATATCTAGAAAAACTCATCG | Amplification of $Km^r$ cassette (1.4 kb) for insertion into the *sdeR* gene (*Eco*RV site engineered) | 54°C |
| Kmrev1 (reverse) | GCGATATCGTCGATTTTTGTGATG | Same as above | |
| pEXSfor (forward) | CCGAATTCGAGACTTCTGACCCAT | Amplification of a 3.1-kb sequence of wt *sdeB* for | 55°C |

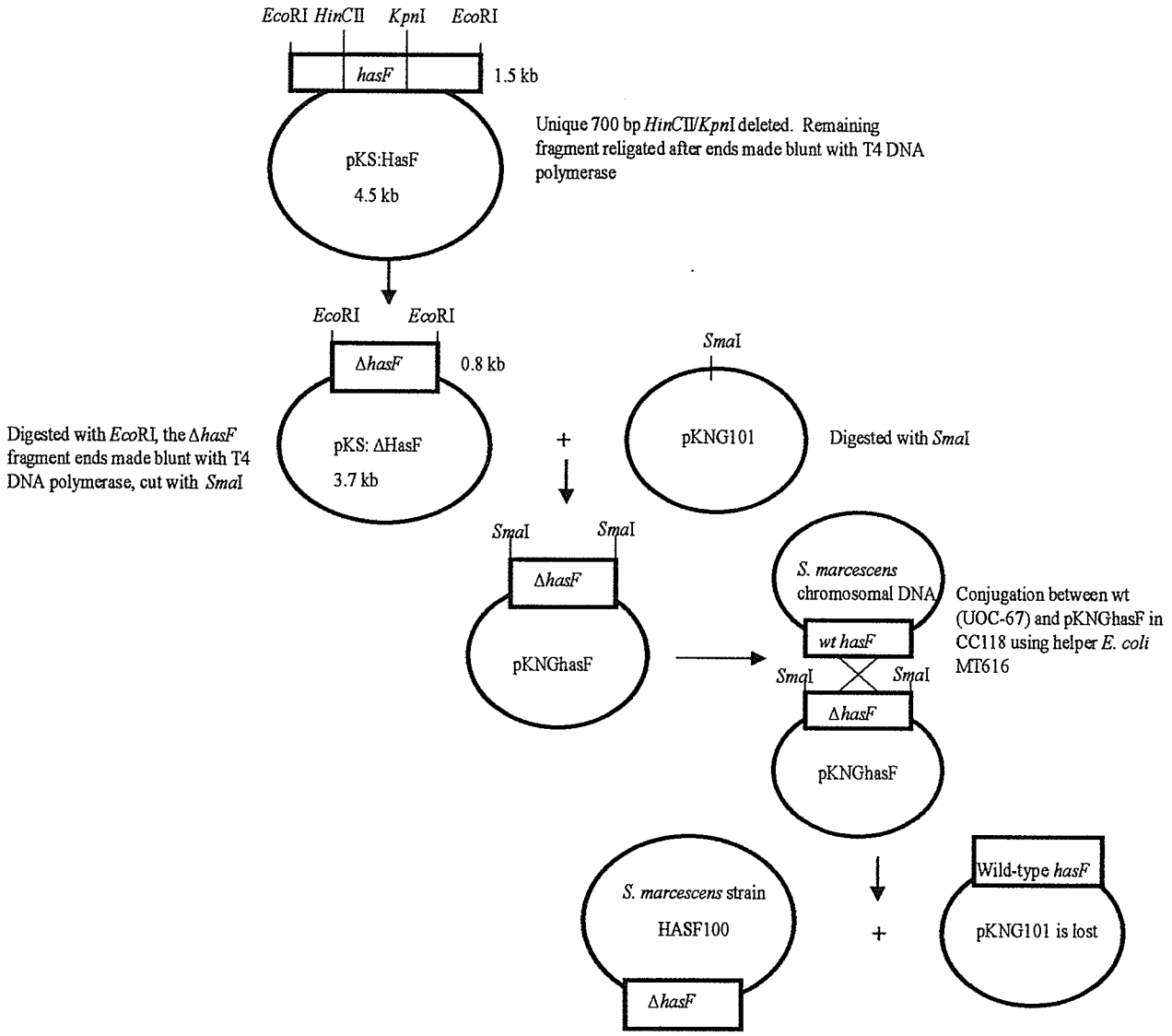
| | | | |
|---|---|---|---|
| | | complementation (*Eco*RI site engineered) | |
| pEXSrev (reverse) | GCAAGCTTAGGGTGACGTAG | Same as above (*Hin*DIII site engineered) | |
| pEXHfor (forward) | CTGAATTCGCACAACAAGGAATGC | Amplification of a 1.5-kb sequence of wt *hasF* for complementation (*Eco*RI site engineered) | 57°C |
| pEXHrev (reverse) | CGAAGCTTAGTTGCGGAATGGGT | Same as above (*Hin*DIII site engineered) | |
| pEXSHf1 (forward) | CCGAATTCGAGACTTCTGACCCAT | Amplification of a 3.1-kb of wt *sdeB* for double complementation (*Eco*RI site engineered) | 55°C |
| pEXSHr1 (reverse) | GCGAATTCAGGGTGACGTAG | Same as above (*Eco*RI site engineered) | |
| pEXSHf2 (forward) | CTAAGCTTGCACAACAAGGAATGC | Amplification of a 1.5-kb sequence of wt *hasF* for double complementation (*Hin*DII site engineered) | 59.5°C |
| pEXSHr2 (reverse) | CGAAGCTTAGTTGCGGAATGGGT | Same as above (*Hin*DIII site engineered) | |

**Figure 4.1.** Construction of *S. marcescens hasF* mutant strains.

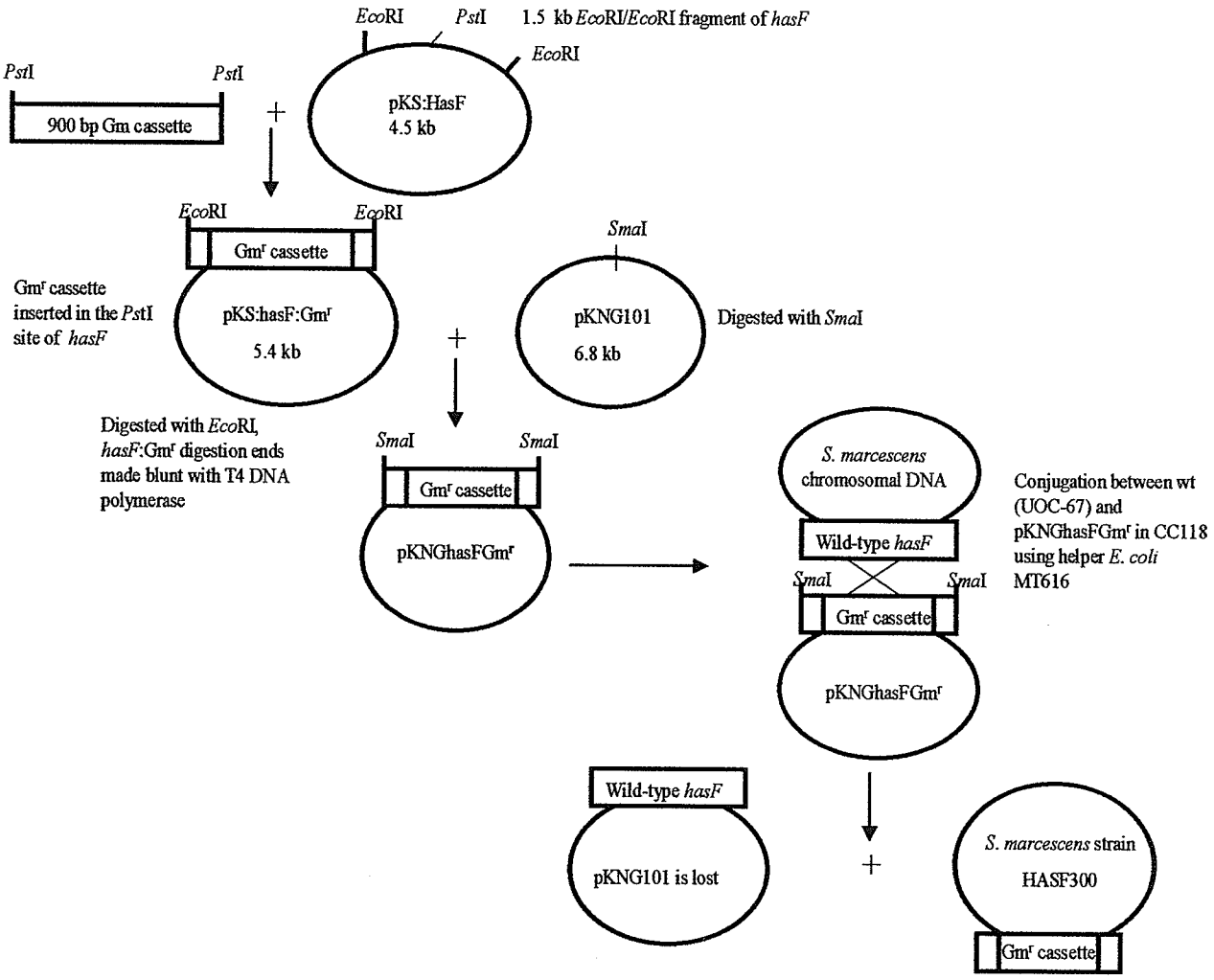
a) Deletion: Unique *Hin*CII/*Kpn*I restriction sites within pKS:HasF separated by approximately 700 bases were excised. The mutated *hasF* fragment (0.8-kb *hasF* fragment) was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118 for stability.

b) Insertion: A 900-bp $Gm^r$ cassette was inserted into a unique *Pst*I restriction site within the *Eco*RI/*Eco*RI flanked *hasF* fragment (pKS:HasF). The disrupted *hasF* fragment was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118. Conjugation between the *E. coli* strain harboring each of the disrupted *S. marcescens hasF* gene and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986) to create HASF300.

a)


EcoRI HinCII KpnI EcoRI
hasF
1.5 kb
pKS:HasF
4.5 kb
Unique 700 bp HinCII/KpnI deleted. Remaining fragment religated after ends made blunt with T4 DNA polymerase
EcoRI EcoRI
ΔhasF
0.8 kb
pKS: ΔHasF
3.7 kb
Digested with EcoRI, the ΔhasF fragment ends made blunt with T4 DNA polymerase, cut with SmaI
SmaI
pKNG101
Digested with SmaI
+
SmaI SmaI
ΔhasF
pKNGhasF
S. marcescens chromosomal DNA
wt hasF
SmaI SmaI
ΔhasF
pKNGhasF
Conjugation between wt (UOC-67) and pKNGhasF in CC118 using helper E. coli MT616
S. marcescens strain
HASF100
ΔhasF
+
Wild-type hasF
pKNG101 is lost

b)


EcoRI
PstI
1.5 kb EcoRI/EcoRI fragment of hasF
EcoRI
PstI
PstI
900 bp Gm cassette
pKS:HasF
4.5 kb
EcoRI
EcoRI
Gmr cassette
Gmr cassette inserted in the PstI site of hasF
pKS:hasF:Gmr
5.4 kb
SmaI
pKNG101
6.8 kb
Digested with SmaI
Digested with EcoRI, hasF:Gmr digestion ends made blunt with T4 DNA polymerase
SmaI
SmaI
Gmr cassette
pKNGhasFGmr
S. marcescens chromosomal DNA
Wild-type hasF
SmaI
SmaI
Gmr cassette
pKNGhasFGmr
Conjugation between wt (UOC-67) and pKNGhasFGmr in CC118 using helper E. coli MT616
Wild-type hasF
pKNG101 is lost
S. marcescens strain HASF300
Gmr cassette

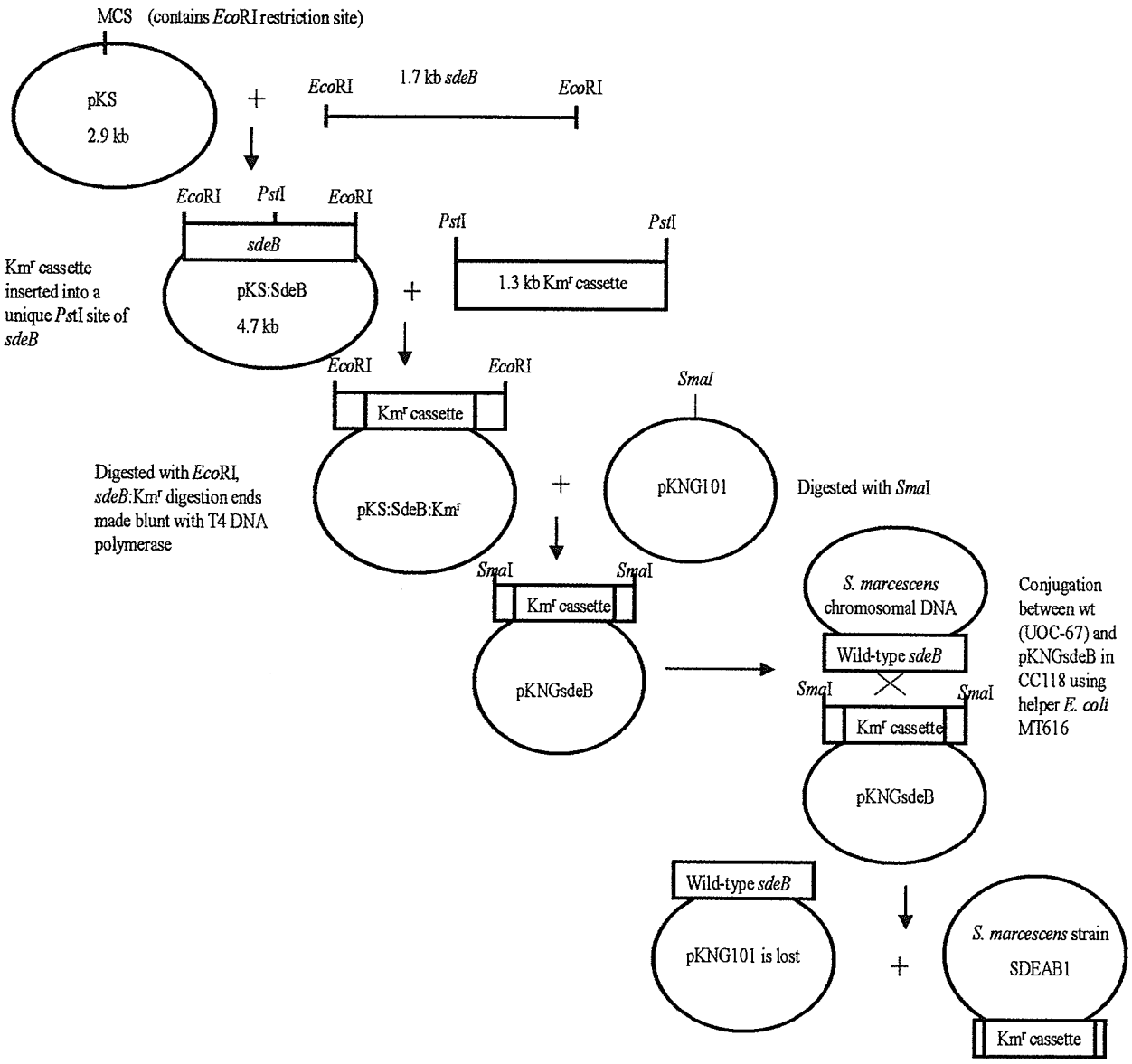
encoding streptomycin phosphotransferase ($Str^r$), an origin of transfer (*mobRK2*), the *sacB* gene mediating sucrose sensitivity, and multiple restriction sites (Kaniga et al.1991).

In order to create a *hasF* mutant that I could use in the construction of the *hasF/sdeB* double deficient mutant, it was important to disrupt the *hasF* insertionally rather than deletionally to allow for double antibiotic selection. A 900-bp *Pst*I-flanked $Gm^r$ cassette was inserted into a unique *Pst*I restriction site within the *Eco*RI/*Eco*RI flanked *hasF* fragment of pKS:HasF resulting in pKS:hasF:$Gm^r$ (Figure 4.1 b). After transformation of pKS:hasF:$Gm^r$ into *S. marcescens* UOC-67 for propagation, the *hasF* insertion mutation was confirmed by restriction digestion with *Eco*RI, PCR of pKS:hasF:$Gm^r$ with HasF1for/rev primers and sequencing of the mutated gene. pKS:hasF:$Gm^r$ was digested with *Eco*RI to remove the 2.4-kb *hasF* insertion and the ends of the fragment were made flush with T4 DNA polymerase. The insertionally-inactivated *hasF* gene was then ligated into the pKNG101 replacement vector resulting in pKNGhasF$Gm^r$ which was then transformed into *E. coli* CC118 in the similar fashion as above.

The *sdeB* gene (Kumar and Worobec 2005a) was cloned as a 1.7-kb *Eco*RI/*Eco*RI PCR-amplified (SdeBfor/SdeBrev) fragment into pKS to result in pKS:SdeB, which was transformed into UOC-67 (Figure 4.2). A kanamycin resistance cassette was inserted into a unique *Pst*I restriction site within the *sdeB* fragment resulting in the pKS:SdeB:$Km^r$. After transformation of pKS:SdeB:$Km^r$ into *S. marcescens* UOC-67, the *sdeB* insertion mutation was confirmed by restriction digestion with *Eco*RI, PCR of pKS:SdeB:$Km^r$ with

**Figure 4.2.** Construction of *S. marcescens sdeB* mutant strain. The *sdeB* gene was cloned as a 1.7-kb *Eco*RI/*Eco*RI PCR fragment into pKS then disrupted by the insertion of a kanamycin resistance cassette into a unique *Pst*I restriction site. The disrupted *sdeB* fragment was ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118.

Conjugation between the *E. coli* strain harboring the disrupted gene and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 to create SDEAB1 (Finan et al. 1986).


MCS (contains EcoRI restriction site)
pKS
2.9 kb
EcoRI
1.7 kb sdeB
EcoRI
EcoRI
PstI
EcoRI
sdeB
pKS:SdeB
4.7 kb
PstI
PstI
1.3 kb Kmr cassette
Kmr cassette inserted into a unique PstI site of sdeB
EcoRI
EcoRI
Kmr cassette
pKS:SdeB:Kmr
SmaI
pKNG101
Digested with EcoRI, sdeB:Kmr digestion ends made blunt with T4 DNA polymerase
Digested with SmaI
SmaI
SmaI
Kmr cassette
pKNGsdeB
S. marcescens chromosomal DNA
Wild-type sdeB
SmaI
SmaI
Kmr cassette
pKNGsdeB
Conjugation between wt (UOC-67) and pKNGsdeB in CC118 using helper E. coli MT616
Wild-type sdeB
pKNG101 is lost
S. marcescens strain SDEAB1
Kmr cassette

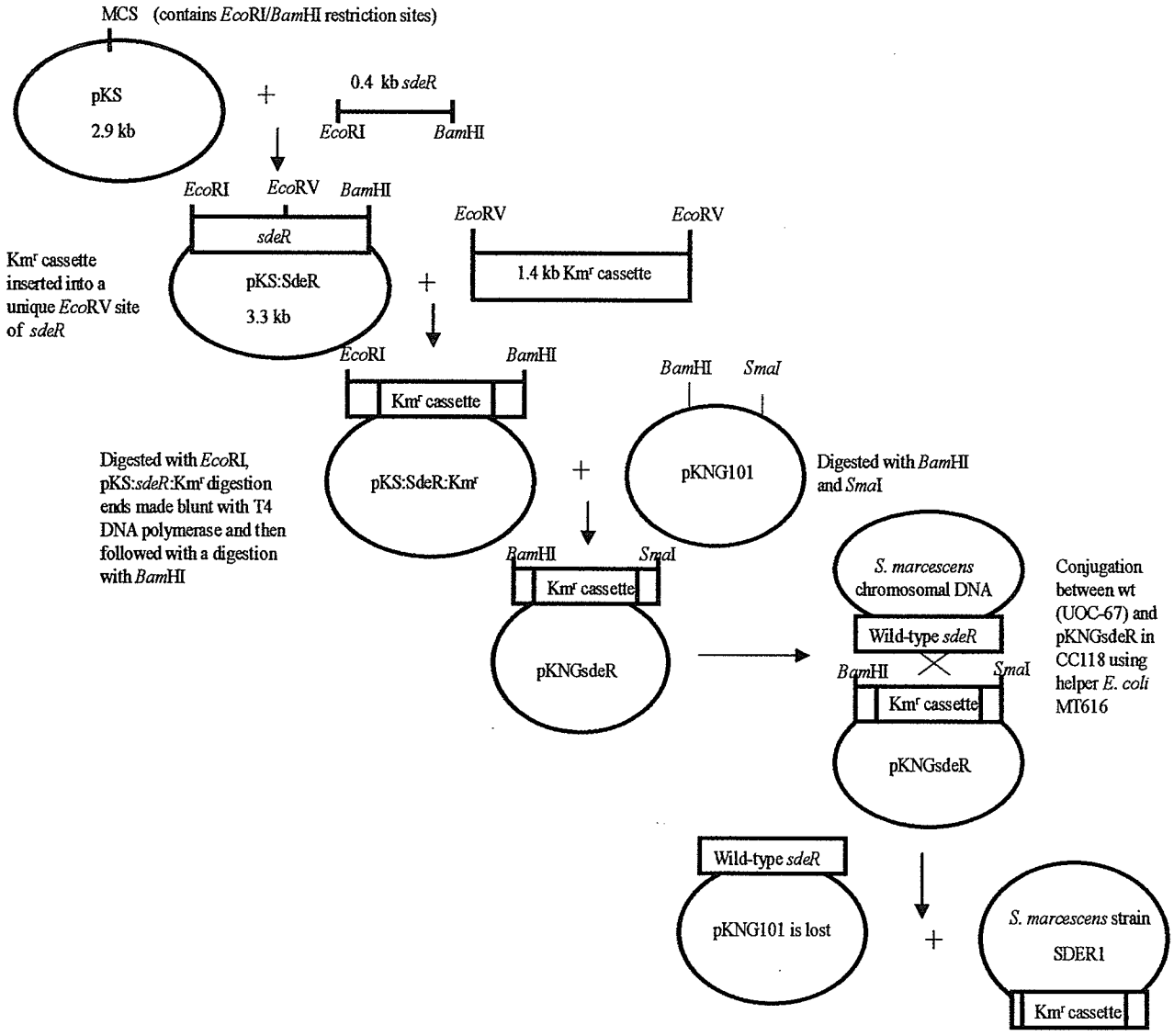
SdeBfor/SdeBrev primers and sequencing of the mutated gene. The pKS:SdeB:Km$^r$ was digested with *Eco*RI to remove the 3-kb *sdeB* insertion fragment, the ends of the fragment were made flush with T4 DNA polymerase (Figure 4.2). The disrupted *sdeB* fragment was ligated into the pKNG101 replacement vector to create pKNGsdeB which was transformed into *E. coli* CC118.

The *sdeR* gene was cloned as a 0.4-kb *Bam*HI/*Eco*RI PCR-amplified (SdeRfor/rev) fragment into pKS yielding pKS:SdeR. A kanamycin resistance cassette was inserted into a unique *Eco*RV restriction site within the pKS:SdeR fragment resulting in pKS:SdeR:Km$^r$. After transformation of pKS:SdeR:Km$^r$ into *S. marcescens* UOC-67, the *sdeR* insertion mutation was confirmed by restriction digestion with *Eco*RI/*Bam*HI, PCR of pKS:SdeR:Km$^r$ with SdeRfor/rev primers and sequencing of the mutated gene. The pKS:SdeR:Km$^r$ was digested with *Eco*RI and the ends were made flush with T4 DNA polymerase (Figure 4.3). The disrupted *sdeR* fragments was ligated into the pKNG101 replacement vector to create pKNGsdeR and transformed into *E. coli* CC118.

Conjugation between the *E. coli* strain harboring each of the disrupted *S. marcescens sdeB*, *hasF* (both insertional and deletional mutations) and *sdeR* genes and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986) to create SDEAB1, HASF100 (deletional mutation), HASF300 (insertional mutation) and SDER1, respectively. Conjugation was also carried out between the *E. coli* strain harboring each of the disrupted *S. marcescens sdeB* and *hasF* genes and the *S. marcescens* clinical isolate strain (T-861), using *E. coli* helper strain MT616 (Finan et al. 1986) for comparison purposes. For the double deficient mutant

**Figure 4.3.** Construction of *S. marcescens sdeR* mutant strain. The *sdeR* gene was cloned as a 0.4-kb *Bam*HI/*Eco*RI PCR fragment into pKS and disrupted by the insertion of a kanamycin resistance cassette into a unique *Eco*RV restriction site. The disrupted *sdeR* fragment was ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118.

Conjugation between the *E. coli* strain harboring the disrupted gene and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 to create SDER1 (Finan et al. 1986).

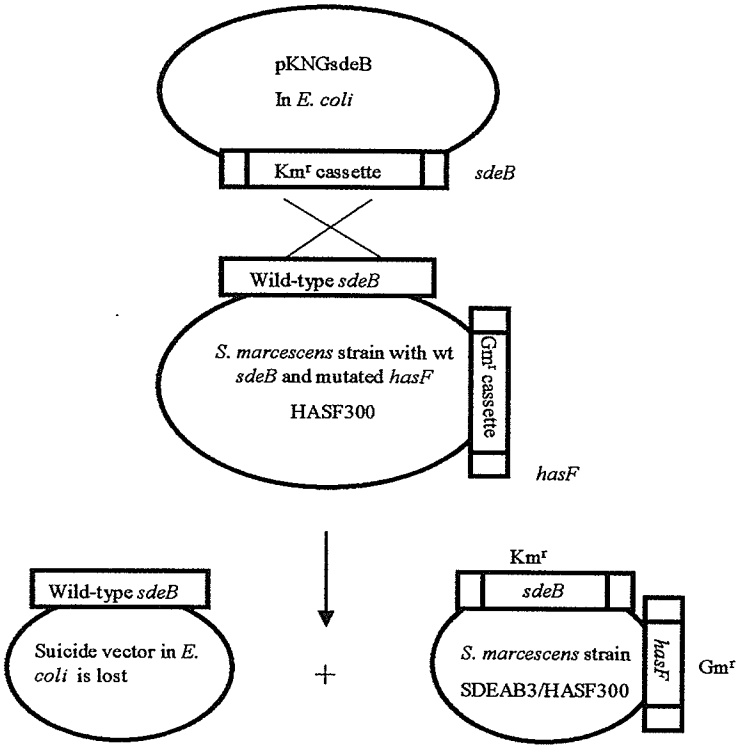

MCS (contains EcoRI/BamHI restriction sites)
pKS
2.9 kb
0.4 kb sdeR
EcoRI
BamHI
EcoRI
EcoRV
BamHI
sdeR
pKS:SdeR
3.3 kb
EcoRV
EcoRV
1.4 kb Kmr cassette
Kmr cassette inserted into a unique EcoRV site of sdeR
EcoRI
BamHI
Kmr cassette
pKS:SdeR:Kmr
BamHI
SmaI
pKNG101
Digested with EcoRI, pKS:sdeR:Kmr digestion ends made blunt with T4 DNA polymerase and then followed with a digestion with BamHI
Digested with BamHI and SmaI
BamHI
SmaI
Kmr cassette
pKNGsdeR
S. marcescens chromosomal DNA
Wild-type sdeR
BamHI
SmaI
Kmr cassette
pKNGsdeR
Conjugation between wt (UOC-67) and pKNGsdeR in CC118 using helper E. coli MT616
Wild-type sdeR
pKNG101 is lost
S. marcescens strain SDER1
Kmr cassette

(SDEAB3/HASF300), pKNGsdeB was mobilized from *E. coli* CC118 into the *S. marcescens hasF* mutant strain (HASF300) via conjugation (Figure 4.4). Analysis of transconjugants was based on sucrose resistance due to the absence of synthesis of lethal levano compounds, catalyzed by levanosucrase, the product of the *sacB* gene (See section 2.3.2 for full description). A multiplex PCR was done with optimized conditions that used both *sdeB* and *hasF* primers. These optimized conditions included first optimizing the PCR conditions for each primer pair separately (HasF1for/rev; SdeB1for/rev), then using equimolar concentrations of each primer set (0.2 µM of each primer) along with the 1X Qiagen PCR Buffer, and the HotStarTäq DNA polymerase in order to minimize primer dimers. Insertional and deletional mutations were confirmed by sequence analysis of the gel isolated correct sized PCR-product fragment (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada). The template DNA for PCR amplifications was the genomic DNA obtained from the putative *S. marcescens hasF, sdeB* and *sdeR* mutant strains.

### 4.3.3 Complementation of *sdeB, hasF* and *sdeR*-deficient strains

For complementation purposes, the expression vector pEX1.8 was used for cloning a 1.5-kb wild type *Eco*RI/*Hin*DIII *hasF* fragment (pEXH), a 3.1-kb wild type *Eco*RI/*Hin*DIII *sdeB* fragment (pEXS) and a 0.4-kb wild type *Eco*RI/*Hin*DIII *sdeR* fragment (pEXR). The coding sequences of the *hasF, sdeB* and *sdeR* genes were PCR amplified from genomic DNA (UOC-67). Primers can be found in Table 4.2. The primers for each PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites. The products from PCR amplifications (1.5-kb for *hasF;* 3.1-kb for *sdeB* and 0.4- kb *sdeR*) were digested with *Eco*RI/*Hin*DIII and cloned into the *Eco*RI/*Hin*DIII sites of pEX1.8 to

**Figure 4.4.** Constructional approach to creating *S. marcescens* double mutant strain SDEAB3/HASF300 (*sdeB/hasF* double mutant). The knockout construct in *E. coli* (pKNGsdeB) with a single insertional mutation in one gene of interest (*sdeB*) was mobilized from *E. coli* CC118 into the *S. marcescens hasF* mutant (HASF300) via conjugation. To select for double mutants, both kanamycin and gentamicin selection pressure was used.


pKNGsdeB
In E. coli
Km^r cassette
sdeB
Wild-type sdeB
S. marcescens strain with wt sdeB and mutated hasF
HASF300
Gm^r cassette
hasF
Wild-type sdeB
Suicide vector in E. coli is lost
+
Km^r
sdeB
S. marcescens strain
SDEAB3/HASF300
hasF
Gm^r

create pEXH; pEXS and pEXR, respectively.

To create pEXSH, the primers for the *sdeB* PCR reaction contained additional engineered *Eco*RI sites. The *sdeB* product (3.1-kb) from this PCR amplification was digested with *Eco*RI and cloned into the *Eco*RI site of pEX1.8JH (a pEX1.8 with a *Bam*HI site in front of the P*tac* promoter removed by Erase-a-base system) to construct pEXSE/E (Figure 4.5 Step 1). The primers for the *hasF* PCR reaction contained additional engineered *Hin*DIII sites. The *hasF* product (1.5-kb) from this PCR amplification was digested with *Hin*DIII and cloned into the *Hin*DIII site of pEX1.8JH1 (a pEX1.8 with *Eco*RI, *Sma*I and *Bam*HI sites within the MCS removed) to construct pEXHH/H (Figure 4.5 Step 2). The *hasF* gene and the P*tac* promoter were excised from pEXHH/H by a *Bam*HI and *Hin*DIII partial digestion. This fragment was then inserted into the *Bam*HI and *Hin*DIII sites within the pEXSE/E MCS to construct pEXSH (Figure 4.5 Step 3).

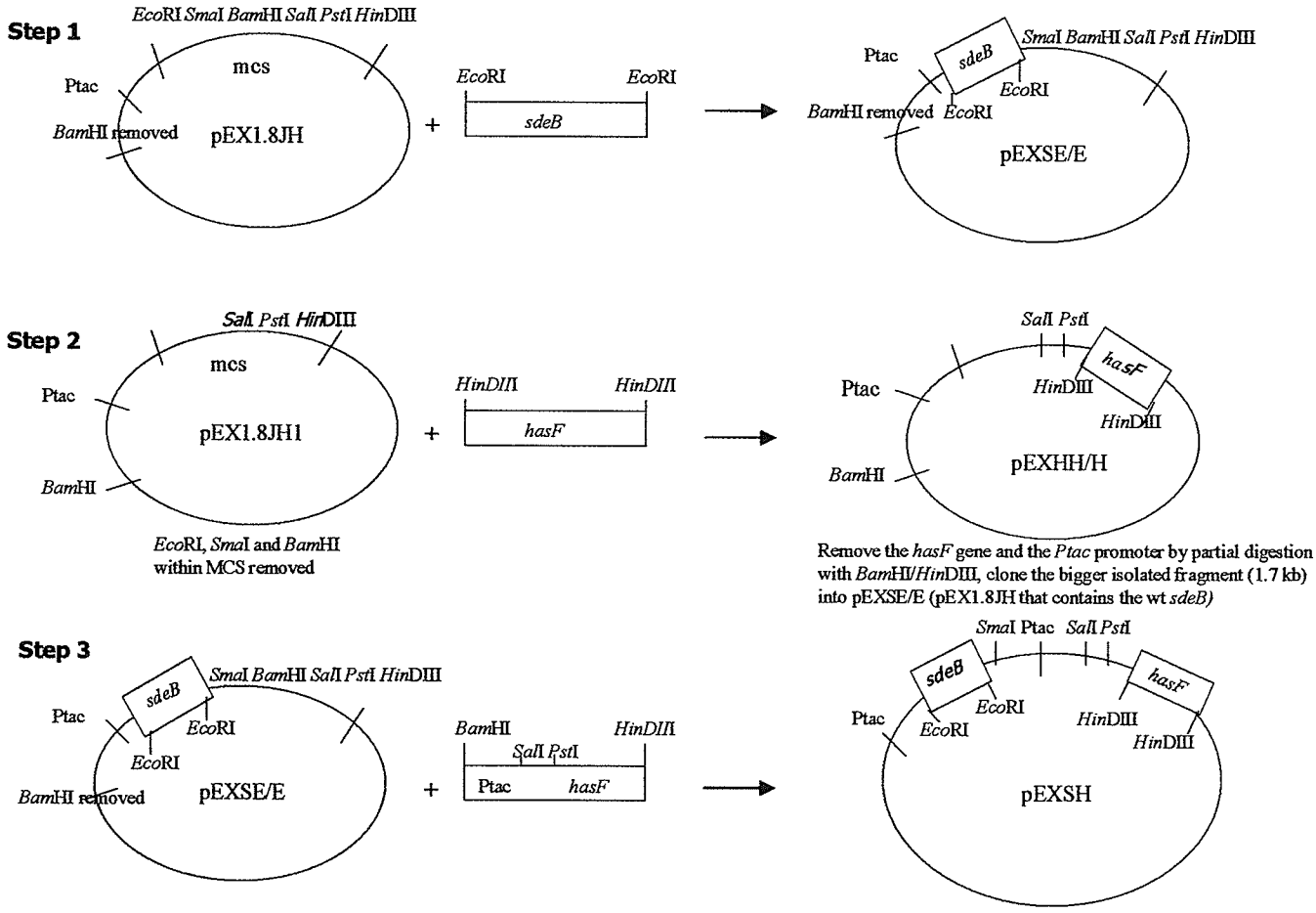
To construct strain SM1000, electroporation of pEXSH into SDEAB3/HASF300 (*sdeB/hasF* deficient strain) was optimized by using a 40 μL cell suspension (after resuspending the cells in cold 10% glycerol and freezing them in aliquots) and 10 μL of DNA in a low ionic strength buffer (TE). Electroporation was done at 1.45 volts in short 1 second intervals for about 3 seconds. 1 ml of SOC media (Bacto Tryptone, Bacto Yeast Extract, 5M NaCl, 1M KCl, 1M $MgCl_2$, 1M $MgSO_4$, 1M glucose) was immediately added to these cells and left to incubate at 37°C for about an hour without shaking and then plated out. SOC media contains twice the amount of Bacto Tryptone in comparison to LB media, which provides more protein precursors for rapid repair of cell walls damaged by the electroporation process, and maintains isotonicity to prevent cell death

**Figure 4.5.** Diagrammatic representation of pEXSH construction.

Step 1. The primers for the *sdeB* PCR reaction contained additional engineered *Eco*RI sites (pEXSHf1/r1). The *sdeB* product from this PCR amplification was digested with *Eco*RI and cloned into the *Eco*RI site of pEX1.8JH (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI site in front of the Ptac promoter removed by Erase-a-base system) to construct pEXSE/E.

Step 2. The primers for the *hasF* PCR reaction contained additional engineered *Hin*DIII sites (pEXSHf2/r2). The *hasF* product from this PCR amplification was digested with *Hin*DIII and cloned into the *Hin*DIII sites of pEX1.8JH1 (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI, *Eco*RI and *Sma*I sites within MCS removed) to construct pEXHH/H.

Step 3. The *hasF* gene (including the Ptac promoter) was then excised from the pEXHH/H vector by *Bam*HI and *Hin*DIII partial digestion and inserted into the *Bam*HI and *Hin*DIII sites within the pEXSE/E MCS to construct pEXSH.


Step 1
EcoRI SmaI BamHI SalI PstI HinDIII
mcs
Ptac
BamHI removed
pEX1.8JH
EcoRI
EcoRI
sdeB
Ptac
sdeB
SmaI BamHI SalI PstI HinDIII
EcoRI
BamHI removed EcoRI
pEXSE/E
Step 2
SalI PstI HinDIII
mcs
Ptac
pEX1.8JH1
BamHI
EcoRI, SmaI and BamHI
within MCS removed
HinDIII
HinDIII
hasF
SalI PstI
Ptac
hasF
HinDIII
HinDIII
BamHI
pEXHH/H
Remove the hasF gene and the Ptac promoter by partial digestion
with BamHI/HinDIII, clone the bigger isolated fragment (1.7 kb)
into pEXSE/E (pEX1.8JH that contains the wt sdeB)
Step 3
sdeB
SmaI BamHI SalI PstI HinDIII
Ptac
EcoRI
EcoRI
BamHI removed
pEXSE/E
BamHI
HinDIII
SalI PstI
Ptac
hasF
SmaI Ptac SalI PstI
sdeB
hasF
Ptac
EcoRI
EcoRI
HinDIII
HinDIII
pEXSH

by osmotic rupture (Electroporation-Competent cells, Stratagene, modified protocol under catalog number 200123). SM2000 was constructed by transferring the pEXH into HASF300 and SM3000 was constructed by transferring the pEXS into SDEAB1 in the same fashion described for pEXSH. SDER2 was constructed by transferring the pEXR into SDER1. Selection of transconjugants occurred by incorporation of ampicillin into the growth medium. Transformants were verified by SDS-PAGE protein analysis. The *sdeB* and the *hasF* genes were expressed under the control of the $P_{tac}$ promoter and induced by the addition of varying concentrations (0.5-5 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] to the growth medium.

**4.3.4 *sdeR* over-expression**

A 0.4-kb *Eco*RI/*Hin*DIII *sdeR* PCR fragment (SdeRfor1/rev1 including the entire open reading frame and ribosome binding site) was cloned into the *Eco*RI/*Hin*DIII site of pEX1.8 (pEXR) and transformed into UOC-67 to generate SDER3. pEX1.8 alone was also transformed into UOC-67 to generate SDER4 to use as a control. Cloning was confirmed via restriction digestion of pEXR with *Eco*RI/*Hin*DIII .

**4.3.5 Cell envelope preparation, Whole cell lysis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis**

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 5,000 xg 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 1,200 x *g* for 10 min. The supernatant was centrifuged at 100,000 x *g* for 1 hour at 4°C.

Whole cell lysis was performed for rapid examination of gene expression. A 1.5 ml aliquot of an overnight culture of the bacterial strain was centrifuged at 12,000 xg for 1 min and cells were resuspended in 100 µl of cell lysis buffer (2% (w/v) SDS, 4% (w/v) DTT, 10% (v/v) glycerol and 1 M Tris-HCl, pH 6.8). The resulting cell lysate was quantified using the Ultraspec 4000 UV/visible spectrophotometer. Samples (50-100 µg) were boiled for 5 minutes and resolved on a 12% SDS-PAGE gel.

SDS-PAGE of cell lysates from *S. marcescens sdeB* knockout mutant and complemented strains and cell envelopes from *S. marcescens hasF* and *sdeR* knockout mutant and complemented strains was also performed using the method described by Lugtenberg (Lugtenberg et al. 1975) using 12% (w/v) polyacrylamide gels. 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. For complemented strains SM2000, SM1000 and SDER2, 0.5, 1, 3 or 4 mM IPTG was added to the growth medium for gene expression. Samples were heated at 95°C for 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. Bands were stained for up to 1 hour using Coomassie Blue staining solution (Sigma, St. Louis, MO) in 30% (v/v) isopropanol/10% (v/v) acetic acid. The gels were destained with 20% (v/v) methanol/7.5% (v/v) acetic acid for 30 min and then overnight with fresh destain solution.

### 4.3.6 Antibiotic susceptibility tests

Susceptibility of SDEAB1, SDEAB2, HASF100, HASF200, HASF300, SDEAB3/HASF300, SM1000, SM2000, SM3000, SDER1, SDER2, SDER3 and SDER4 to norfloxacin (0.001-32 mg/L), ciprofloxacin (0.001-32 mg/L), ofloxacin (0.001-32 mg/L), chloramphenicol (0.002-16 mg/L), novobiocin (0.001-16 mg/L), sodium dodecyl sulphate (SDS) (0.001-8 mg/L) and ethidium bromide (0.002-16 mg/L) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was tested using the minimum inhibitory concentration (MIC) two-fold MH broth dilution method (National Committee for Clinical and Laboratory Standards protocol with modifications). Complemented strains SM3000, SM2000, SM1000, SDER2 and SDER3 were tested in the presence of 1 and 4 mM IPTG, an agent that induced genes cloned under the control of the *tac* promoter. Overnight cultures in MH broth were diluted 1000-fold in fresh broth, grown at 37°C until $OD_{600}$=0.5-0.9 and 5 µL of the bacterial suspension was inoculated in MH containing serial dilutions of each antibiotic. Results were reported as MIC, the concentration of antibiotic that inhibited visible growth determined by absence of turbidity in MH after 18 hours of shaking at 37°C.

### 4.3.7 Fluoroquinolone accumulation

The accumulation of ciprofloxacin by SDEAB1, SDEAB2, HASF200, HASF300, SDEAB3/HASF300, SM1000, SM2000 and SM3000 was measured using the method of Mortimer and Piddock 1991. Cultures in 3L batches were grown until $OD_{600}$ reaches 0.5-0.7. Cells were harvested by centrifugation at 4000 x *g* for 15 min at room temperature, resuspended to 30 ml in phosphate buffered saline (PBS) pH 7.5 and washed two times. Pellets were suspended in 1/10 volume of PBS. For complemented strains SM1000,

SM2000 and SM3000, accumulation was performed in the presence of 1 mM IPTG. Ciprofloxacin was added to a final concentration of 10 μg/mL and 0.5 ml of culture was removed in 30 sec intervals for a period of 12 minutes and immediately diluted in 1 ml of cold PBS. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was added to a final concentration of 100 μM after 5 minutes of antibiotic incubation. After incubation, a rapid centrifugation (13,000 x *g* at 4°C for 1 min) was carried out, followed by a wash in cold PBS, suspension in 1 mL of HCl-glycine 0.1 M pH 3.0 to lyse the cells, and overnight incubation at room temperature. This suspension was centrifuged at 11,000 x *g* for 5 min to remove cellular debris and antibiotic concentration measured in a RF-1501 Shimadzu spectrofluorometer. The fluorescence of ciprofloxacin was measured at 279 nm excitation wavelength and 447 nm emission wavelength. Ciprofloxacin concentration was calculated using a standard curve (concentration ranging from 100 to 1000 ng) in 0.1 M HCl-glycine 0.1 M pH 3.0. The results were expressed as nanograms of ciprofloxacin incorporated per milligram (dry weight) of bacteria.

An extended ciprofloxacin accumulation assay was also performed where antibiotic uptake was measured for 30 minutes (1 min intervals) for UOC-67 and T-861. All other assay conditions remained the same as above.

## 4.4 Results

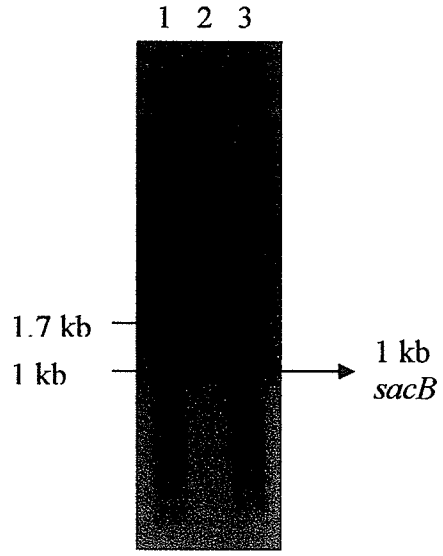
### 4.4.1 Construction of *S. marcescens* mutant strains

PCR amplification of *S. marcescens hasF* deletion mutant genomic DNA using HasF1for/rev primers resulted in two products, a 0.8-kb band representing the *hasF* deletion fragment and the 1.5-kb wild type *hasF* gene after the single crossover event (Figure 4.6), and only the 0.8-kb deletion fragment after the double crossover event (Figure 4.7). PCR of *S. marcescens hasF* insertion mutant genomic DNA using HasF1for/rev primers resulted in two products, a 2.4-kb insertion fragment and a 1.5-kb wild type *hasF* gene after the single crossover event (Figure 4.8), and only the 2.4-kb insertion fragment after the double crossover event (Figure 4.9). Additionally, a 0.9-kb band was depicted when HasFGmf/r gentamicin cassette primers were used to examine the *hasF* insertion mutant (Figure 4.10). PCR amplification of *S. marcescens sdeB* genomic DNA using SdeB1for/rev primers resulted in two products, a 3.0-kb fragment representing the *sdeB* insertion fragment and the 1.7-kb wild type *sdeB* gene after the first crossover event (Figure 4.11), and only the 3.0-kb insertion fragment after the second crossover event (Figure 4.12). A 1.7-kb fragment using the SdeBKmf/r kanamycin$^r$ cassette primers (Figure 4.13) is seen. PCR of *S. marcescens sdeR* genomic DNA using SdeRf/r primers resulted in two products, a 1.8-kb fragment representing the *sdeR* insertion fragment and the 0.4-kb wild type *sdeR* gene after the single crossover (Figure 4.14), and only the 1.8-kb insertion fragment after the double crossover (Figure 4.15). A 0.8-kb fragment was found (Figure 4.16) when using the SdeRKmf/r kanamycin$^r$ cassette primers. PCR products obtained from genomic DNA from putative *S. marcescens sdeB* and *hasF* double mutants illustrate a 3.0-kb insertion band for *sdeB* and a 2.4-kb insertion

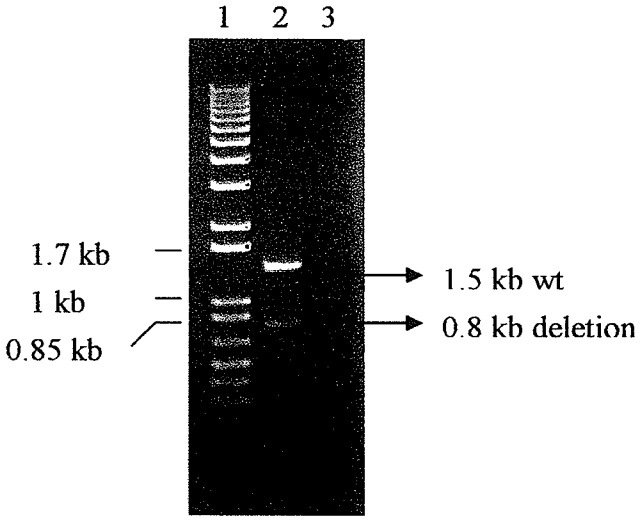
**Figure 4.6.** PCR products obtained from genomic DNA from putative *S. marcescens* *hasF* mutants after the single crossover event. This picture illustrates the *hasF* deletion mutant.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* (SacBf/r) PCR product; Lane 3, 1 kb(+) ladder. Molecular weight of product is 1 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *hasF* PCR product (HasF1for/rev); Lane 3, ddH2O. Important bands indicated by the arrows; bands around 1.5-kb and 0.8-kb. 4 μL of samples were loaded in each lane.

a)


1 2 3
1.7 kb
1 kb
1 kb
*sacB*

b)


1 2 3
1.7 kb
1 kb
0.85 kb
1.5 kb wt
0.8 kb deletion

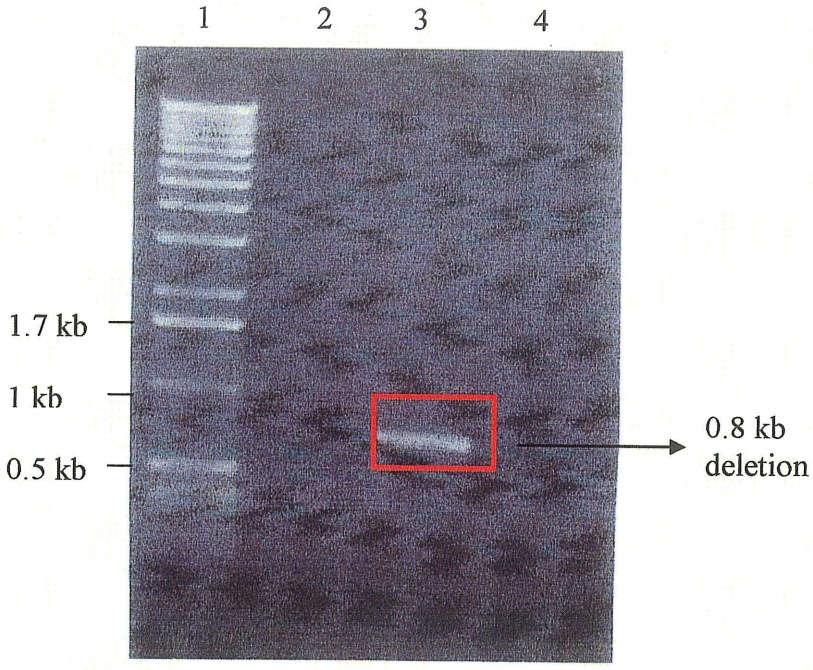
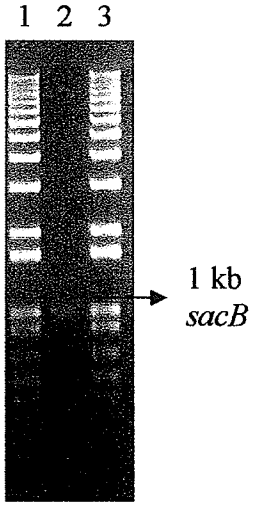

1
2
3
4
1.7 kb
1 kb
0.5 kb
0.8 kb
deletion

**Figure 4.8.** PCR products obtained from genomic DNA from putative *S. marcescens* *hasF* mutants after the single crossover event. This picture illustrates the *hasF* insertion mutant used in preparation of the double *sdeB/hasF* knockout.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* (SacBf/r) product; Lane 3, 1 kb(+) ladder. Molecular weight of product is 1 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *hasF* (HasF1for/rev) product; Lane 3, ddH2O. Important bands indicated by arrows; bands shown are around 1.5-kb and 2.4-kb. 6 μL of samples were loaded in each lane.

a)




b)

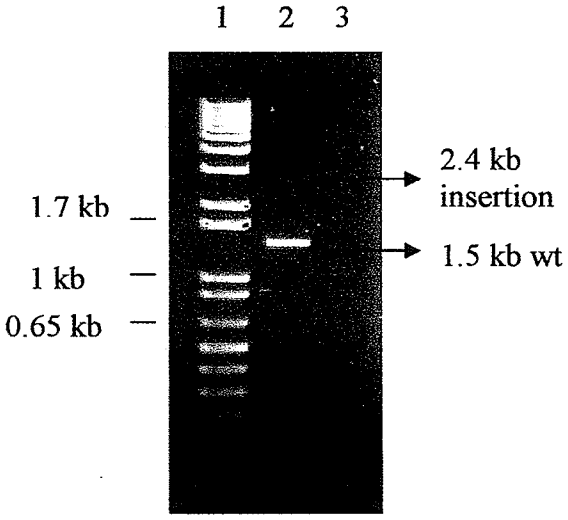


**Figure 4.9.** PCR products obtained from genomic DNA from putative *S. marcescens* *hasF* mutants after sucrose selection. This picture illustrates the *hasF* insertion mutant used in preparation of the double *sdeB/hasF* knockout.

Lane 1, *sacB* product (SacBf/r; no band visible); Lane 2, ddH2O; Lane 3, *hasF* (HasF1for/rev) product (2.4 kb band indicated); Lane 4, 1 kb(+) ladder. 3 μL of samples were loaded in each lane.

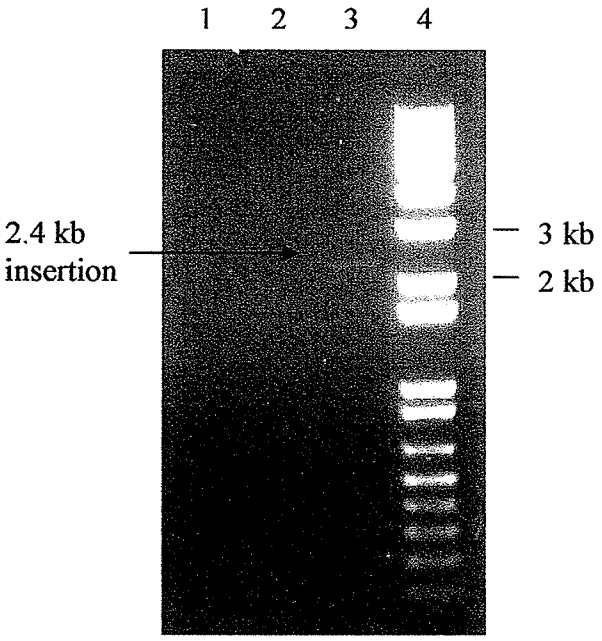
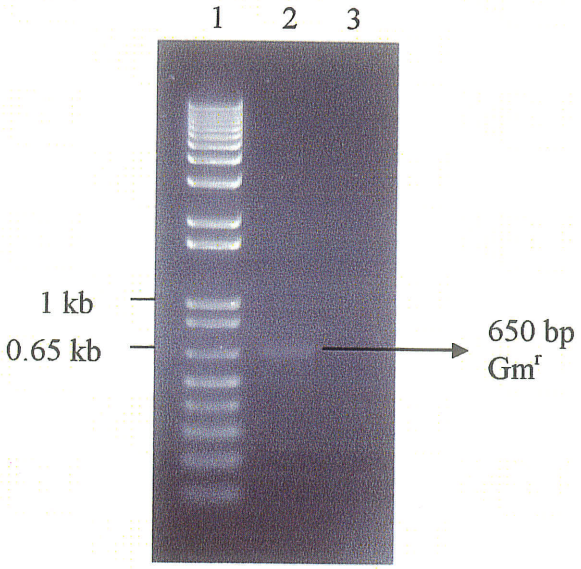

1
2
3
4
2.4 kb
insertion
3 kb
2 kb

**Figure 4.10.** PCR products obtained from genomic DNA from putative *S. marcescens* *hasF* mutants using gentamicin$^r$ cassette primers.

c) Lane 1,1 kb(+) ladder; Lane 2, product using Gm$^r$ primers from within the cassette (650 bp amplified); Lane 3, ddH2O.

d) Lane 1, 1 kb ladder; Lane 2, larger fragment amplified (HasFGmf/r, some of *hasF* (about 300 bp) and some of the cassette (650 bp) yielding an approximate 0.9 kb product (boxed). There is a shift in this lane compared to Lane 2 of a) indicating the presence of the cassette. Lane 3, ddH2O. 3 μL of samples were loaded in each lane.

a)


1
2
3
1 kb
0.65 kb
650 bp
Gm^r

b)

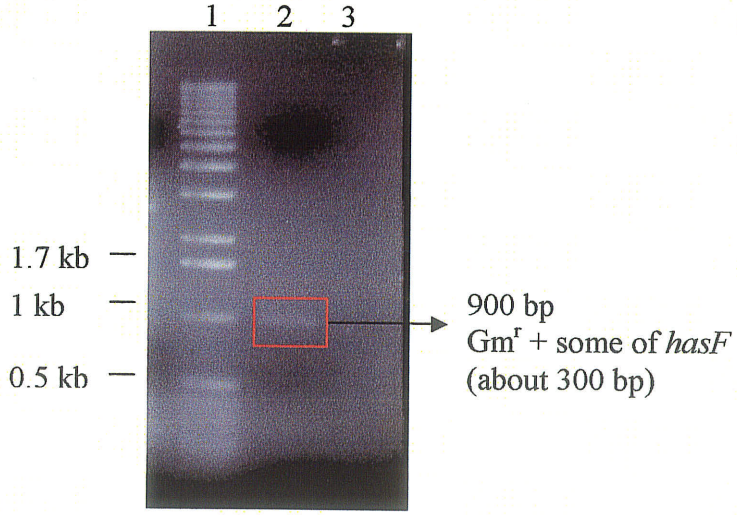
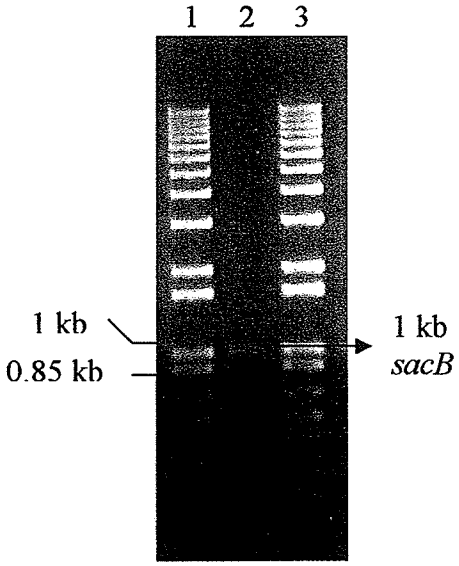

1
2
3
1.7 kb
1 kb
0.5 kb
900 bp
Gm^r + some of hasF
(about 300 bp)

**Figure 4.11.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeB* mutants after the single crossover event.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* (SacBf/r) product; Lane 3, 1 kb(+) ladder. Molecular weight of product is 1.0 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *sdeB* product (SdeB1for/rev); Lane 3, ddH2O. Important bands indicated by the arrows; bands around 1.7-kb and 3.0-kb. 5 μL of samples were loaded in each lane.

a)




b)

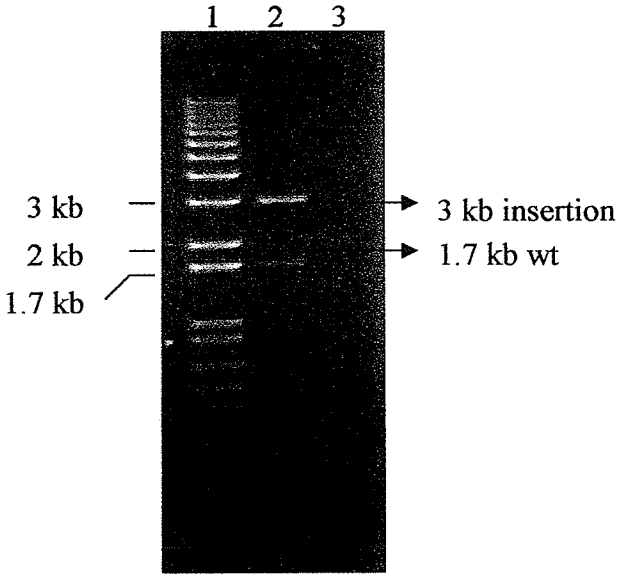


**Figure 4.12.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeB* mutants after sucrose selection.

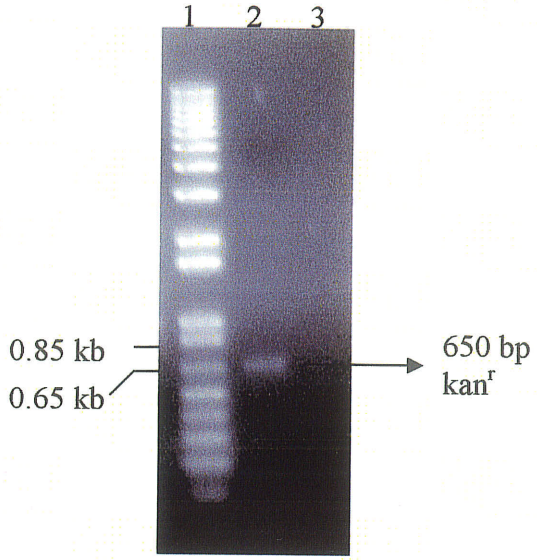
Lane 1,1 kb ladder; Lane 2, ddH2O; Lane 3, *sdeB* (SdeB1for/rev) product (3.0 kb band indicated); Lane 4, *sacB* product (SacBf/r; no band visible). 10 μL of samples were loaded in each lane.

**Figure 4.13.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeB* mutants using kanamycin$^r$ cassette primers.

c) Lane 1,1 kb(+) ladder; Lane 2, product using kan$^r$ primers from within the cassette (650 bp amplified); Lane 3, ddH2O.

d) Lane 1, 1 kb ladder; Lane 2, larger fragment amplified (SdeBKmf/r, some of *sdeB* (about 1 kb) and some of the cassette (650 bp) yielding an approximate 1.7 kb product (boxed); Lane 3, ddH2O. There is a shift between the band in Lane 2 and the 650 bp band in a) indicating the presence of the cassette. 6 μL of samples were loaded in each lane.

a)


1
2
3
0.85 kb
0.65 kb
650 bp
kan^r

b)

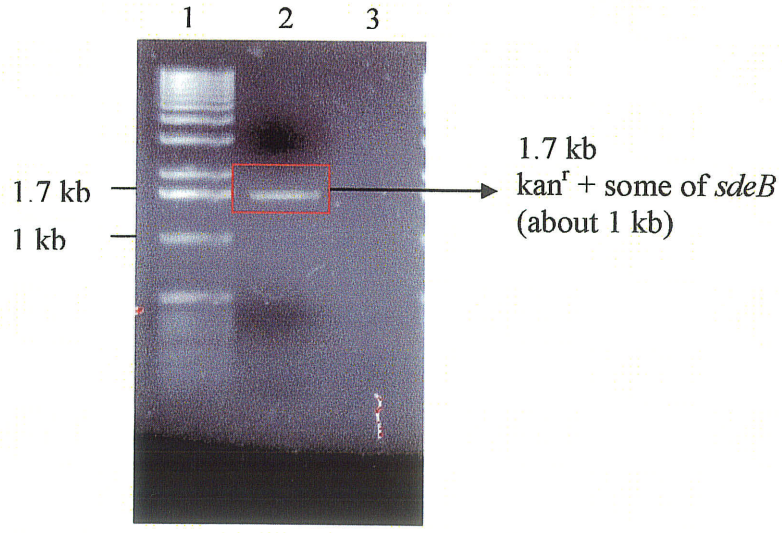
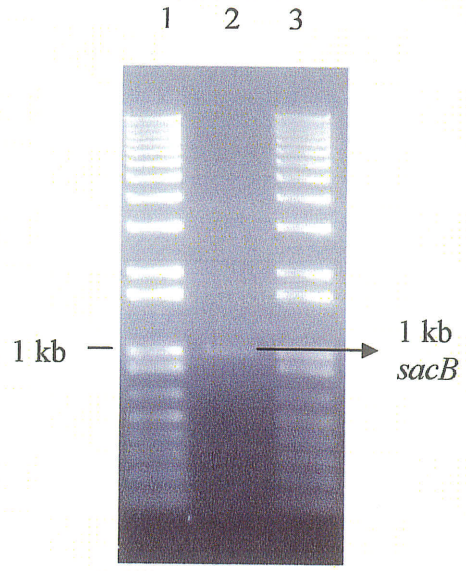

1
2
3
1.7 kb
1 kb
1.7 kb
kan^r + some of sdeB
(about 1 kb)

**Figure 4.14.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeR* mutants after the single crossover event.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* (SacBf/r) product; Lane 3, 1 kb(+) ladder. Molecular weight of product is 1 kb. b) Lane 1, 1 kb(+) ladder; Lane 2, *sdeR* (SdeRf/r) product; Lane 3, ddH2O. Important bands indicated by the arrows; bands shown are around 0.4-kb and 1.8-kb. 8 μL of samples were loaded in each lane.

a)




b)

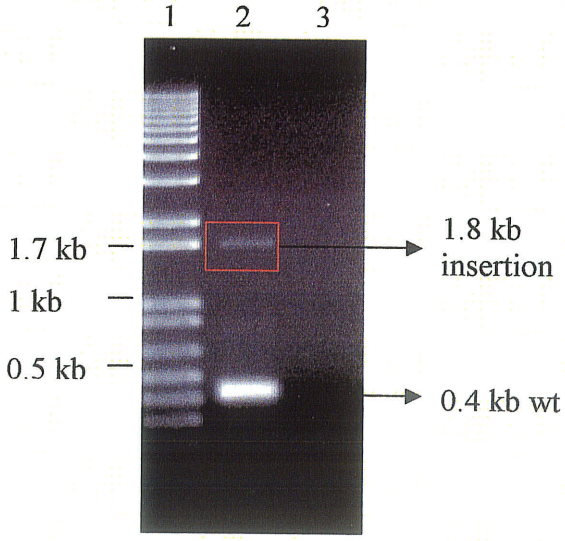


**Figure 4.15.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeR* mutants after sucrose selection.

Lane 1,1 kb ladder; Lane 2, *sdeR* (SdeRf/r) product (1.8 kb band indicated by arrow); Lane 3, ddH2O; Lane 4, *sacB* product (SacBf/r; no band visible); Lane 5, wt UOC-67 control (SdeRf/r primers); 0.4 kb band indicated by arrow. 7 μL of samples were loaded in each lane.

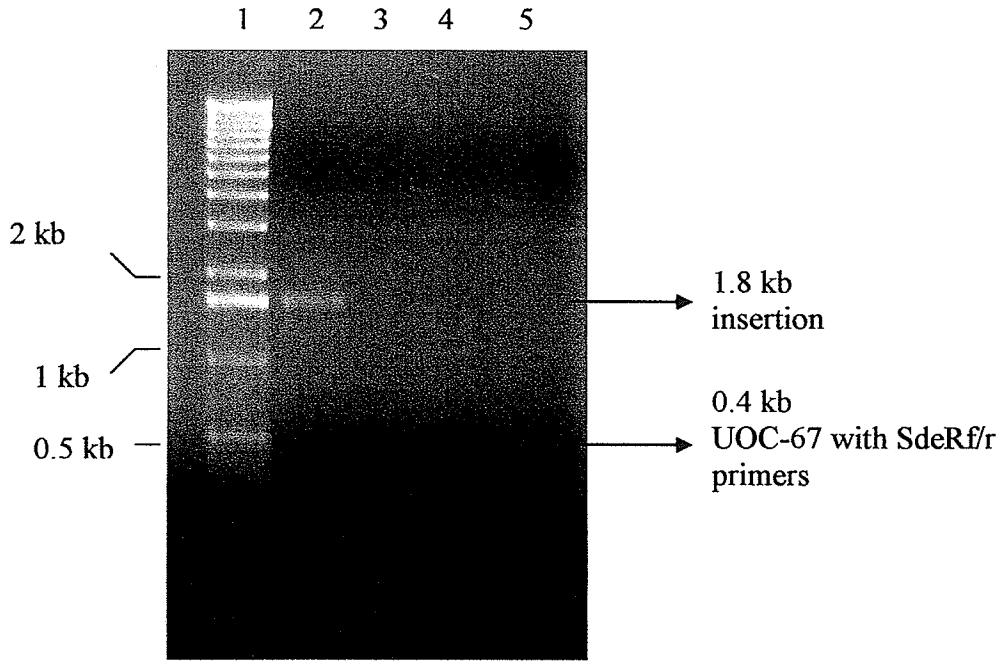
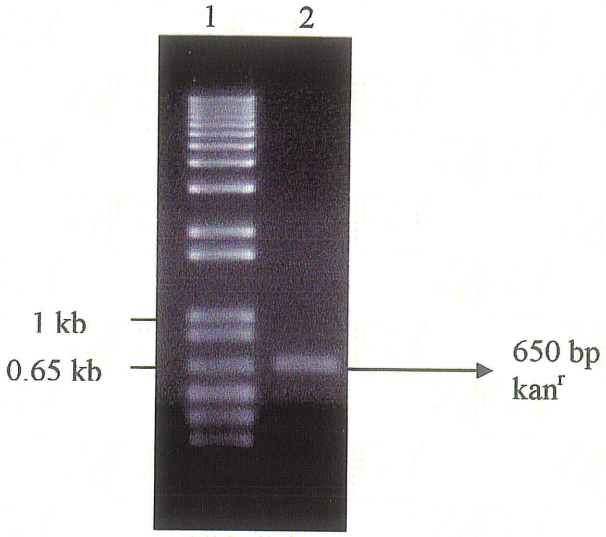

1 2 3 4 5
2 kb
1 kb
0.5 kb
1.8 kb
insertion
0.4 kb
UOC-67 with SdeRf/r
primers

**Figure 4.16.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeR* mutants using kanamycin$^r$ cassette primers.

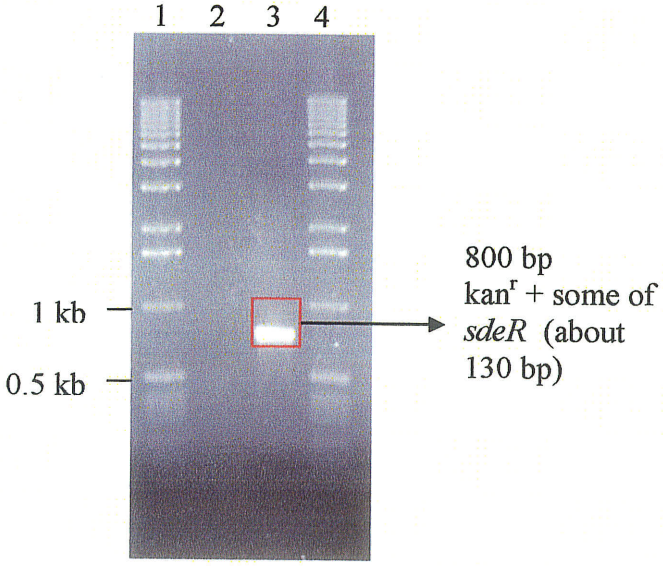
a) Lane 1,1 kb(+) ladder; Lane 2, product using kan$^r$ primers from within the cassette (650 bp amplified); Lane 3, ddH2O

b) Lane 1, 1 kb ladder; Lane 2, ddH2O; Lane 3, larger fragment amplified (SdeRKmf/r, some of *sdeR* (about 130 bp) and some of the cassette (650 bp) yielding an approximate 0.8 kb product (boxed) and Lane 4, 1 kb ladder. There is a shift between this band and the 650 bp band in a) indicating the presence of the cassette. 8 μL of samples were loaded in each lane.

a)


1
2
1 kb
0.65 kb
650 bp
kan^r

b)


1
2
3
4
1 kb
0.5 kb
800 bp
kan^r + some of
sdeR (about
130 bp)

band for *hasF* using HasF1for/rev and SdeB1for/rev multiplex primers (Figure 4.17).

### 4.4.2 Complementation of *sdeB, hasF* and *sdeR*-deficient strains

Figure 4.18a shows a 7.4 kb vector band corresponding to pEX1.8 and the entire 3.1-kb *sdeB* wild type gene fragment. The primers for the PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites (pEXSfor/rev). Figure 4.18b depicts a 7.4 kb vector band corresponding to pEX1.8 and the entire 1.5-kb *hasF* wild type gene fragment. The primers for the PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites (pEXHfor/rev). Lastly, Figure 4.18c depicts a 7.4 kb vector band corresponding to pEX1.8 and the entire 0.4-kb *sdeR* wild type gene fragment. The primers for the PCR reaction contained additional engineered *Eco*RI and *Hin*DIII sites (SdeRfor1/rev1). Figure 4.19 shows the *Eco*RI/*Hin*DIII digestion of pEXSH resulting in the release of the wild type *sdeB* (3.1-kb) and wild type *hasF* (1.5-kb).

SDS-PAGE analysis of cell envelope preparations of HASF300 and SM2000 in Figure 4.20a confirms HasF absence and HasF over-expression (induced with IPTG), respectively. SDS-PAGE analysis of whole cell lysates of SDEAB3/HASF300 and SM1000 in Figure 4.20b confirms HasF and SdeB absence and HasF/SdeB over-expression (induced by IPTG), respectively. SDS-PAGE analysis of SDER1 and SDER2 in Figure 4.21 confirms SdeR absence and SdeR over-expression (induced by IPTG), respectively.

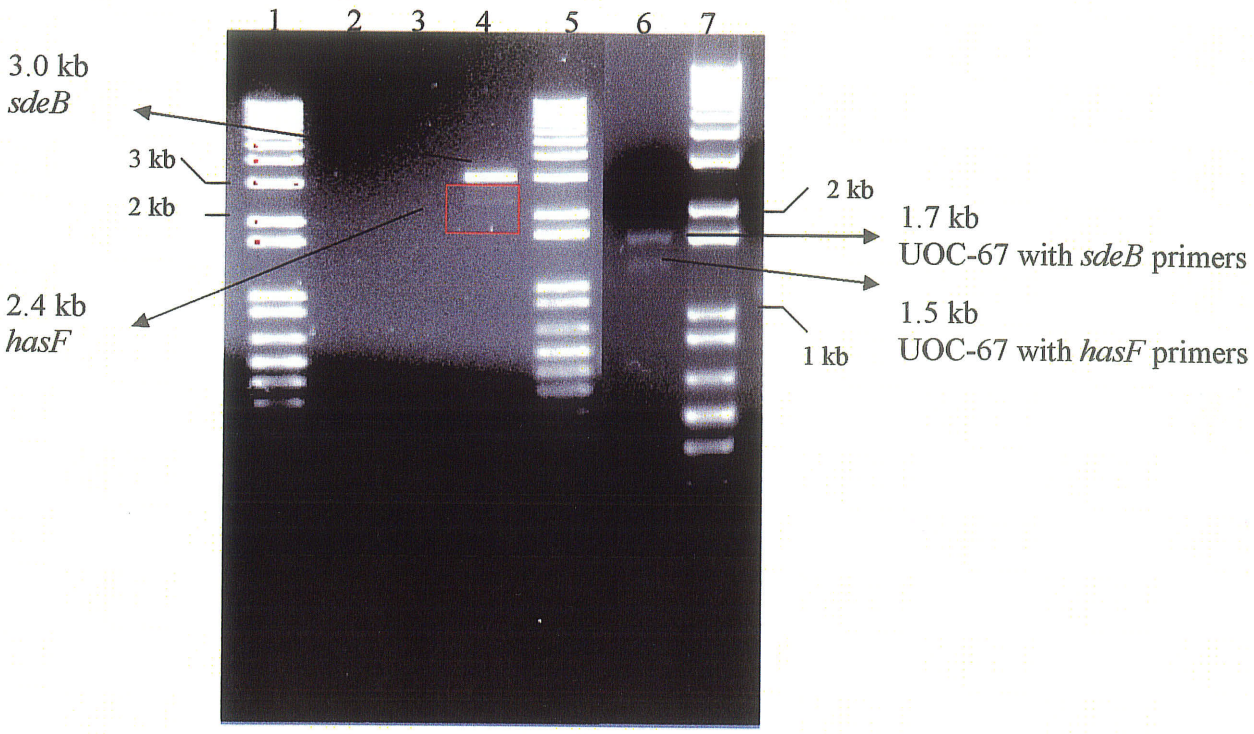
Additionally, please refer to the appendix for confirmation sequences throughout.

**Figure 4.17.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeB* and *hasF* double mutants after sucrose selection.

a) Lane 1, 1 kb(+) ladder; Lane 2, *sacB* product; Lane 3, ddH2O; Lane 4, *hasF/sdeB* product (HasF1for/rev and SdeB1for/rev primers); Lane 5, 1 kb(+) ladder; Lane 6, wt UOC-67 control (HasF1for/rev and SdeB1for/rev primers); Lane 7, 1 kb(+) ladder. Molecular weights of PCR products are represented by 2.4 kb for *hasF* and 3.0 for *sdeB*, respectively. Molecular weights of PCR products for UOC-67 are represented by 1.7 kb for *sdeB* primers and 1.5 kb for *hasF* primers, respectively. No band is obtained using *sacB* primers. 10 μL of samples were loaded in each lane.

b) After initial selection on kanamycin and gentamicin (no sucrose), *sacB* primers (SacBf/r) were used on the mutant for positive control as indicated by the presence of a 1.0-kb in Lane 2. Lane 1 and 3, 1 kb(+) ladder.

a)


1 2 3 4 5 6 7
3.0 kb
sdeB
3 kb
2 kb
2.4 kb
hasF
2 kb
1.7 kb
UOC-67 with sdeB primers
1.5 kb
UOC-67 with hasF primers
1 kb

b)

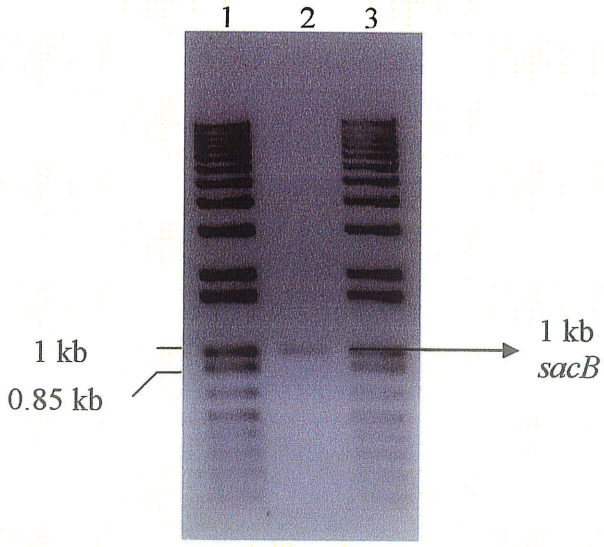
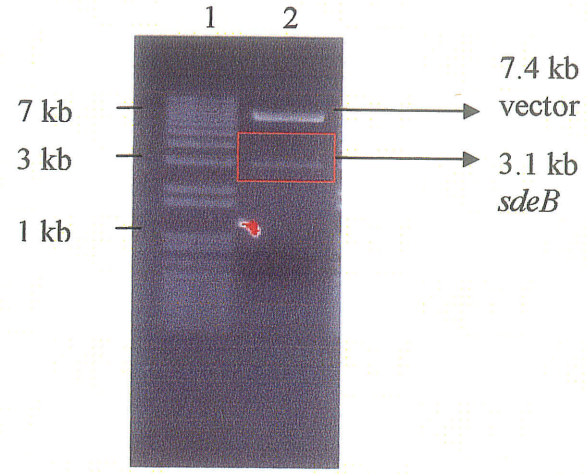

1 2 3
1 kb
0.85 kb
1 kb
sacB

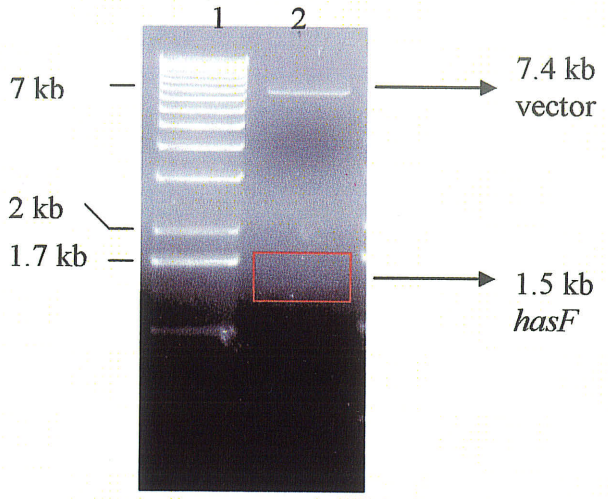
**Figure 4.18.** Restriction digestion products of a) pEXS; b) pEXH and c) pEXR constructs (*Eco*RI/*Hin*DIII digestion). The primers for each PCR reaction contained an additional engineered *Eco*RI and *Hin*DIII sites (pEXSfor/rev for a; pEXHfor/rev for b; SdeRfor1/rev1 for c). The products from PCR amplifications individually (1.5-kb for *hasF;* 3.1-kb for *sdeB* and 0.4-kb for *sdeR*) were digested with *Eco*RI/*Hin*DIII and cloned into the *Eco*RI/*Hin*DIII sites of pEX1.8.

Lane 1, 1 kb(+) ladder; Lane 2 a) vector band (7.4 kb) is the upper band (indicated by an arrow) and the insert *sdeB* wild type band is the lower band; Lane 2 b) vector band (7.4 kb) and the insert *hasF* wild type band; Lane 2 c) vector band (7.4 kb) and the insert *sdeR* wild type band.

a)


1
2
7 kb
3 kb
1 kb
7.4 kb
vector
3.1 kb
sdeB

b)


1
2
7 kb
2 kb
1.7 kb
7.4 kb
vector
1.5 kb
hasF

c)

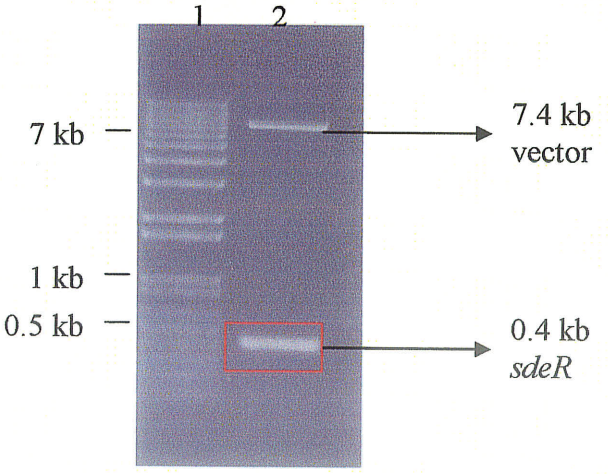

1
2
7 kb
1 kb
0.5 kb
7.4 kb
vector
0.4 kb
sdeR

**Figure 4.19.** Restriction digestion products of pEXSH (*Eco*RI/*Hin*DIII digestion). The vector band (7.4 kb) is the upper band and the insert wild type bands are the lower bands (indicated by red box) in Lane 2. 1 kb ladder (3 μL) is shown in Lanes 1 and 3.

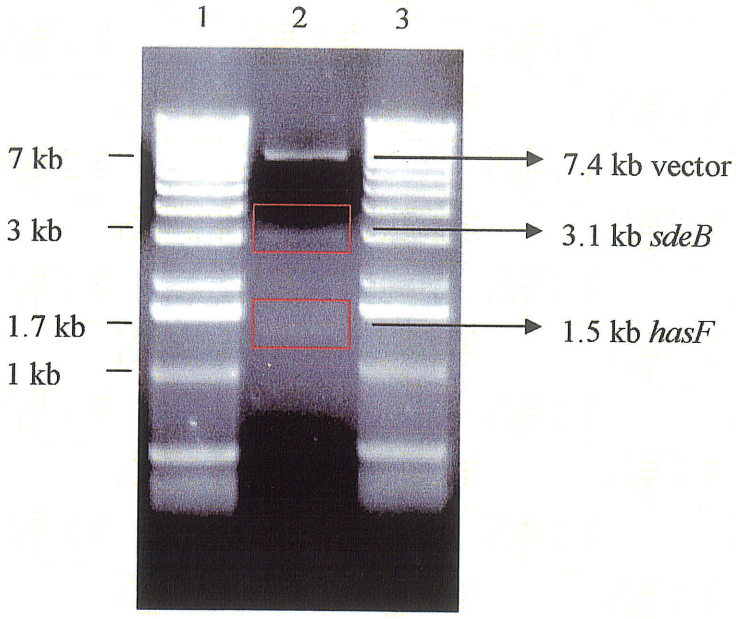
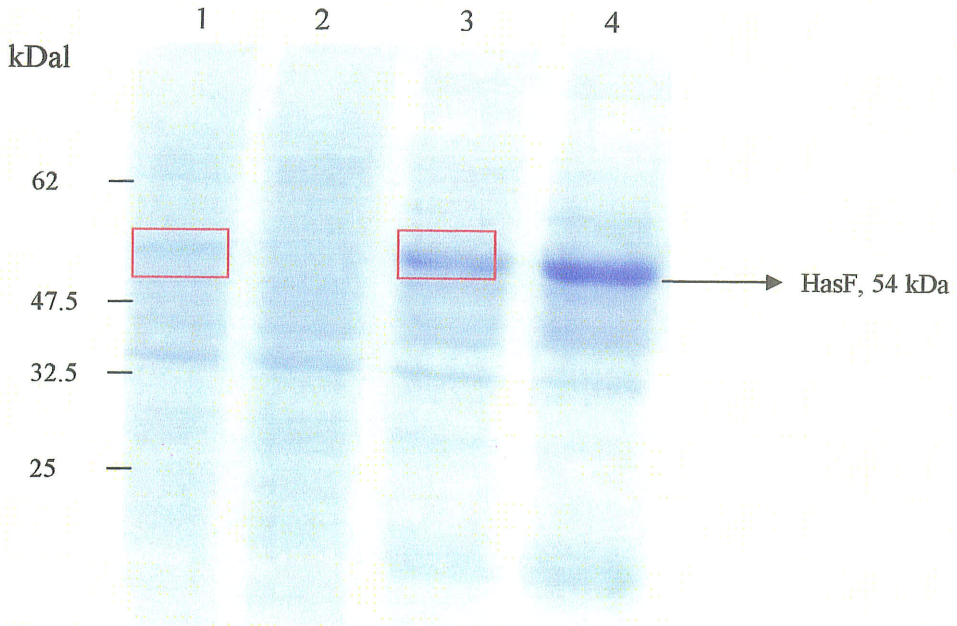

1
2
3
7 kb
3 kb
1.7 kb
1 kb
7.4 kb vector
3.1 kb sdeB
1.5 kb hasF

**Figure 4.20.** SDS-PAGE of cell envelope preparations and whole cell lysates from *S. marcescens* HasF and SdeB knockout mutants and complemented strains.

a) Lane 1, cell envelope preparations of UOC-67; Lane 2, HasF knockout (HASF300); Lane 3, HasF complemented *S. marcescens* (SM2000) induced by 3 mM IPTG; and Lane 4, HasF complemented (SM2000) induced by 4 mM IPTG. Molecular weights of markers are indicated along the side of the gel. Molecular weight of HasF is 54 kDa.

b) Lane 1, whole cell lysates of UOC-67; Lane 2, double *sdeB/hasF* complement (SM1000) induced by 0.5 mM IPTG; Lane 3, double *sdeB/hasF* complement (SM1000) induced by 1 mM IPTG; Lane 4, *sdeB/hasF* double knockout mutant (SDEAB3/HASF300); and Lane 5, UOC-67. Molecular weight of HasF is 54 kDa and SdeB is 112 kDa. 50 μg of cell envelope preparation and 70 μg of cell lysates were used.

a)


1
2
3
4
kDal
62
47.5
32.5
25
HasF, 54 kDa

b)

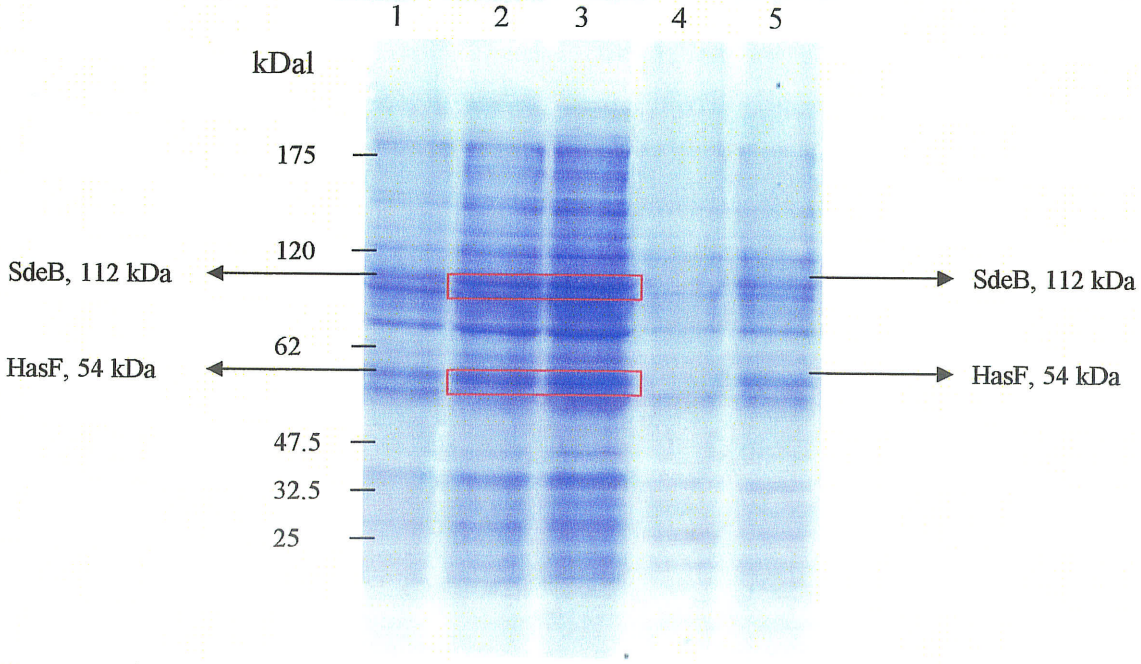
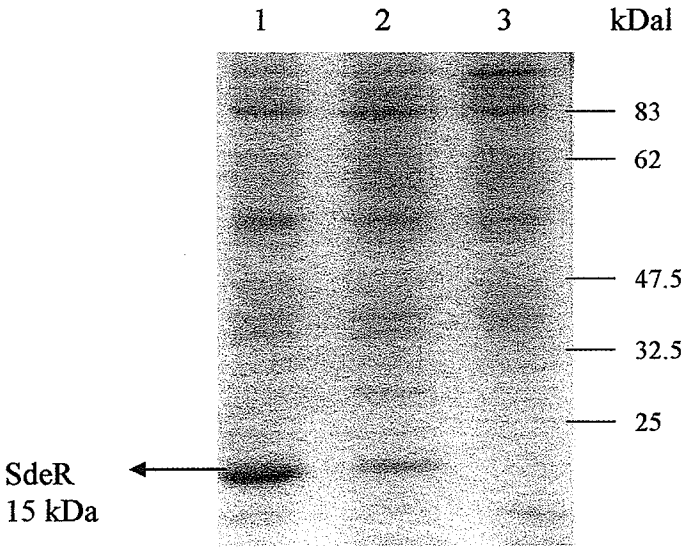

1
2
3
4
5
kDal
175
120
62
47.5
32.5
25
SdeB, 112 kDa
HasF, 54 kDa
SdeB, 112 kDa
HasF, 54 kDa

**Figure 4.21.** SDS-PAGE of cell envelope preparations of SdeR complement (SDER2) and SdeR knockout (SDER1) strains of *S. marcescens*.

Lane 1, SdeR complement (SDER2) induced by 4 mM IPTG; Lane 2, UOC-67; and Lane 3, SdeR knockout (SDER1). Molecular weights of markers are indicated along the side of the gel. Molecular weight of SdeR is 15 kDa. 50 μg of cell envelope preparation was used.


1
2
3
kDal
83
62
47.5
32.5
25
SdeR
15 kDa

### 4.4.3 Antibiotic susceptibility tests

The most dramatic increase in antibiotic susceptibility was found with the double knockout strain (SDEAB3/HASF300) in comparison to wild type UOC-67 (Table 4.3). For SDEAB1 and HASF100/HASF300 susceptibility to all compounds tested increased, in comparison to UOC-67 and for SDEAB2, and HASF200 in comparison to T-861. MIC values for HASF100 were identical to those of SDEAB1 for all compounds tested, except for chloramphenicol where there is a slight difference. A similar trend was found for HASF200 and SDEAB2. MIC values for SM1000, the *hasF/sdeB* complemented strain, showed a dramatic increase in antibiotic resistance when induced by 4 mM IPTG in the growth medium. Restoration of resistance to wild type levels was seen for SM1000 when induced by 1 mM IPTG. SM2000 (*hasF* complemented strain) and SM3000 (*sdeB* complemented strain) under the induction of 1 mM IPTG were both identical to those of wild-type UOC-67 for all antibiotics tested, suggesting that both SdeB and HasF are equally important for *S. marcescens* multidrug resistance.

The *sdeR* overexpressing strain (SDER3), studied under 1 mM and 4 mM IPTG induction, was considerably more resistant than either UOC-67 or SDER4 (UOC-67/pEX1.8) (Table 4.3) for all compounds tested, while SDER1 (*sdeR*-deficient) was more susceptible to all compounds. Complementation of SDER1 with *sdeR* (SDER2) resulted in an increase in resistance when induced by 4 mM IPTG in the growth medium. Restoration of resistance to wild type levels was seen for SDER2 when induced by 1 mM IPTG.

**Table 4.3.** The susceptibilities of wild type, clinical isolate and various *sdeB* and *hasF* mutant *S. marcescens* strains to a range of antibiotics.

Abbreviations: Nor, norfloxacin; Ofx, ofloxacin; Cip, ciprofloxacin; Chl, chloramphenicol; Nov, novobiocin; SDS, sodium dodecyl sulphate; EtBr, ethidium bromide. Results are representative of three trials.

| | MIC (μg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | Cip | Nor | Ofl | Chl | Nov | SDS | EtBr |
| UOC-67 (wild type) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |
| T-861 (clinical isolate) | 128 | 128 | 128 | 256 | 256 | 4 | 8 |
| SDEAB1 (*sdeB*-deficient in UOC-67) | 0.125 | 0.125 | 0.125 | 4 | 16 | 0.25 | 0.25 |
| SDEAB2 (*sdeB*-deficient in T-861) | 0.008 | 0.008 | 0.008 | 16 | 32 | 0.25 | 0.25 |
| HASF100 (*hasF*-deficient in UOC-67, deletionally disrupted) | 0.125 | 0.125 | 0.125 | 8 | 16 | 0.25 | 0.25 |
| HASF200 (*hasF*-deficient in T-861) | 0.008 | 0.008 | 0.008 | 32 | 32 | 0.25 | 0.25 |
| HASF300 (*hasF*-deficient in UOC-67, insertionally disrupted) | 0.125 | 0.125 | 0.125 | 4 | 4 | 0.25 | 0.25 |
| SDEAB3/HASF300 (*sdeB/hasF*-deficient in UOC-67) | 0.002 | 0.002 | 0.002 | 0.004 | 0.016 | 0.004 | 0.004 |
| SM3000 (*sdeB* complemented SDEAB1, induced by 1 mM IPTG) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |
| SM2000 (*hasF* complemented HASF300, induced by 1 mM IPTG) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |
| SM1000 (*sdeB*, *hasF* complemented SDEAB3/HASF300, induced by 1 mM IPTG) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |
| SM1000 (*sdeB*, *hasF* complemented SDEAB3/HASF300, induced by 4 mM IPTG) | 128 | 128 | 128 | 256 | 512 | 64 | 64 |
| SDER1 (*sdeR*-deficient UOC-67) | 0.25 | 0.25 | 0.25 | 8 | 8 | 0.25 | 0.25 |
| SDER2 (*sdeR* complemented SDER1, induced by 1 mM IPTG) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |
| SDER2 (*sdeR* complemented SDER1, induced by 4 mM IPTG) | 64 | 64 | 64 | 256 | 128 | 128 | 64 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SDER3 (*sdeR* over-expressing UOC-67, induced by 1 mM IPTG) | 16 | 8 | 16 | 32 | 64 | 16 | 16 |
| SDER3 (*sdeR* over-expressing UOC-67, induced by 4 mM IPTG) | 256 | 256 | 128 | 256 | 512 | 64 | 64 |
| SDER4 (UOC-67 with vector) | 1 | 1 | 1 | 32 | 64 | 1 | 1 |

Note:
For complemented strains, MIC values reported are in the presence of either 1 or 4 mM IPTG.

### 4.4.4 Fluoroquinolone accumulation

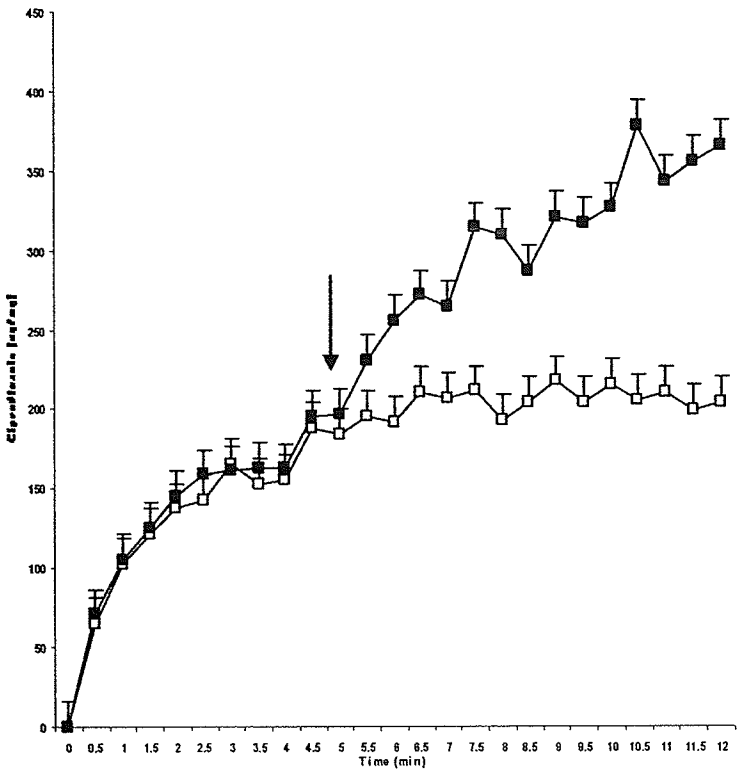
The loss of the functioning efflux pump SdeAB (SDEAB1 and SDEAB2) resulted in a significant increase in ciprofloxacin accumulation as compared to UOC-67 (Figure 4.22a and 4.22b) and T-861 parental strains, respectively (Figure 4.22d and 4.22e). The addition of CCCP, a proton motive force inhibitor, increased the accumulation of UOC-67 (Figure 4.22a) and T-861 (Figure 4.22d); however it had no effect upon ciprofloxacin accumulation in the knockout mutant strains (Figure 4.22b and 4.22e). Ciprofloxacin accumulation for the SM3000 (*sdeB* complemented) strain was very similar to that of the UOC-67 in the absence and presence of CCCP (Figure 4.22c).

The loss of the functioning *hasF* gene (HASF300 and HASF200) also resulted in a notable increase in ciprofloxacin accumulation as compared to UOC-67 (Figure 4.23a and 4.23b) and T-861 parental strains, respectively (Figure 4.23d and 4.23e). As expected, the addition of CCCP increased the accumulation by UOC-67 (Figure 4.23a) and T-861 (Figure 4.23d); however it had no effect upon ciprofloxacin accumulation in the HASF300 (Figure 4.23b) and HASF200 knockout mutant strains (Figure 4.23e). Ciprofloxacin accumulation for the SM2000 (*hasF* complemented) strain was very similar to that of the UOC-67 in the absence and presence of CCCP (Figure 4.23c).

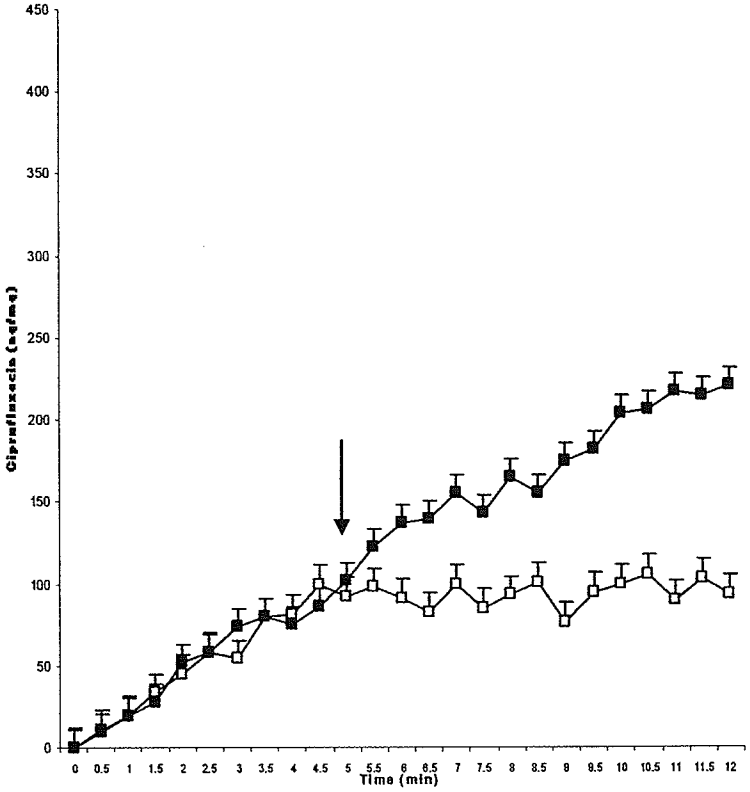
Much like the accumulation of SDEAB1 and HASF300, the loss of both *hasF* and *sdeB* (SDEAB3/HASF300) resulted in an increased ciprofloxacin accumulation (Figure 4.24c). Addition of CCCP resulted in a slightly further increase in ciprofloxacin accumulation for the double knockout strain, but not significant (SDEAB3/HASF300) (Figure 4.24c). SM1000 (*sdeB/hasF* complemented) in the absence of CCCP showed the lowest accumulation, lower than that of either SDEAB1 or HASF300 alone suggesting

**Figure 4.22.** Accumulation of ciprofloxacin by a) wild type UOC-67 b) mutant *sdeB* UOC-67 c) complemented *sdeB* UOC-67 d) clinical isolate T-861 and e) mutant *sdeB S. marcescens* T-861 in the absence and presence of CCCP. Results were graphed by the EXCEL computer program. Various strains are depicted as follows: a) UOC-67 in presence of CCCP (■); UOC-67 in absence of CCCP (□); b) SDEAB1 in presence of CCCP (▲); SDEAB1 in absence of CCCP (Δ); c) SM3000 in presence of CCCP (●); SM3000 in absence of CCCP (○); d) T-861 in presence of CCCP (■); T-861 in absence of CCCP (□) and e) SDEAB2 in presence of CCCP (▲); SDEAB2 in absence of CCCP (Δ). Proton motive force uncoupler CCCP (100 μM) was added 5 minutes after incubation (shown by the arrow). All trials were performed in triplicate. For results regarding addition of CCCP after 10 minutes of incubation (for UOC-67), please refer to Figure 3.8 in Chapter 3. For complemented strain SM3000, accumulation performed in presence of 1 mM IPTG.

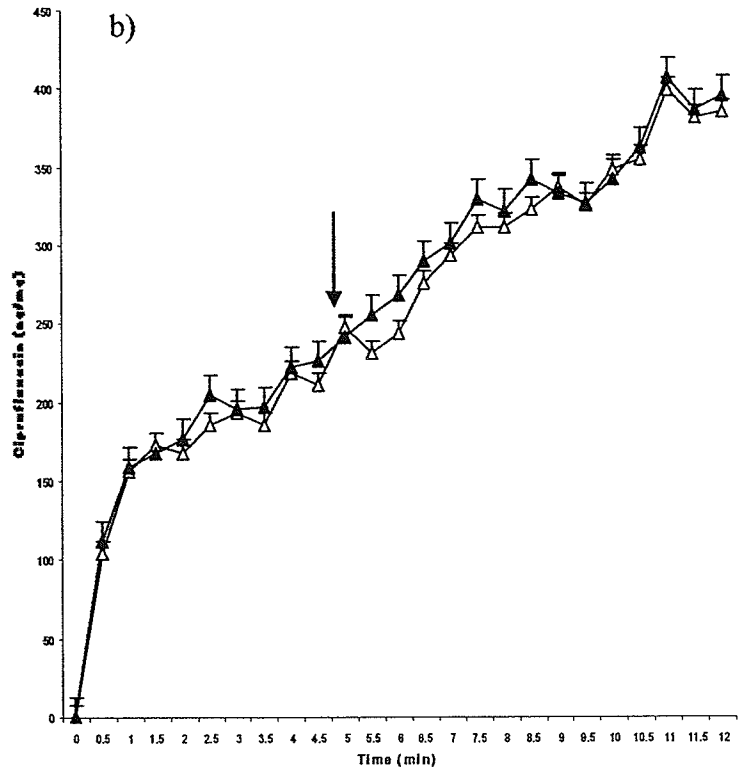
a)



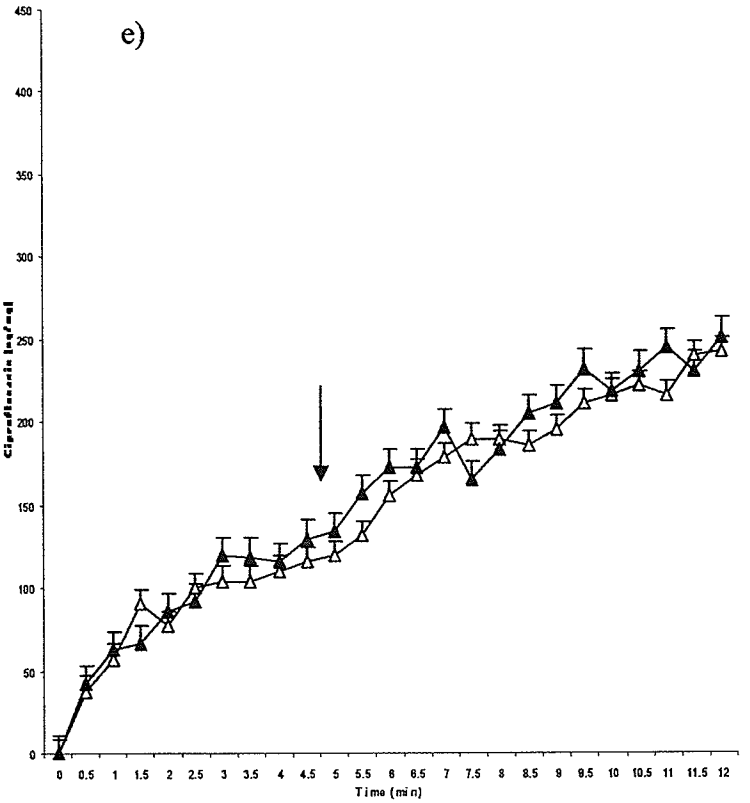

d)




b)




e)



c)

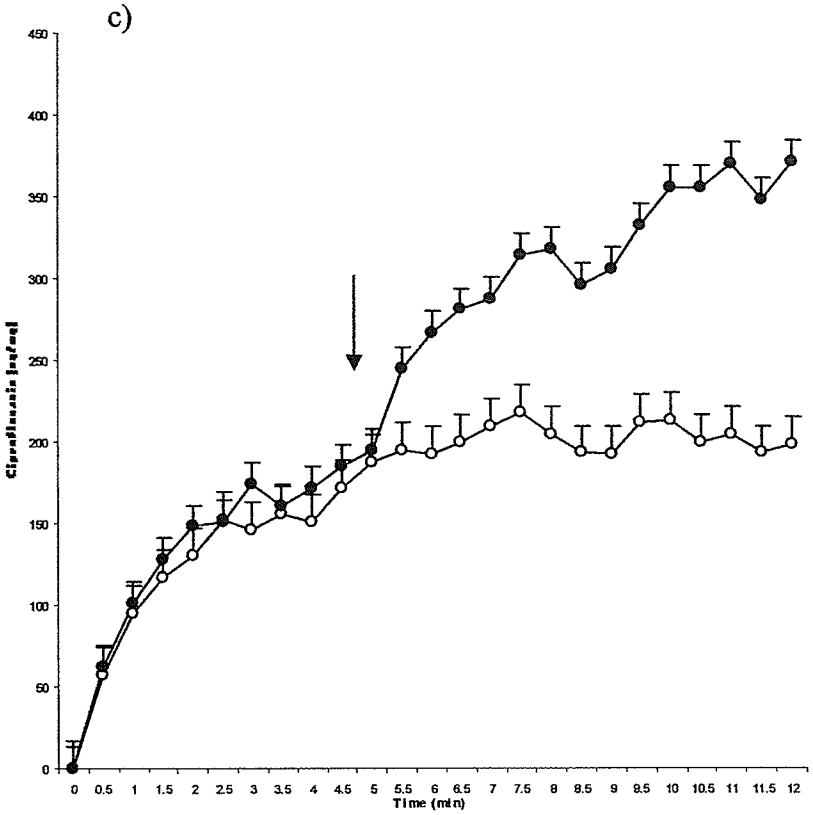
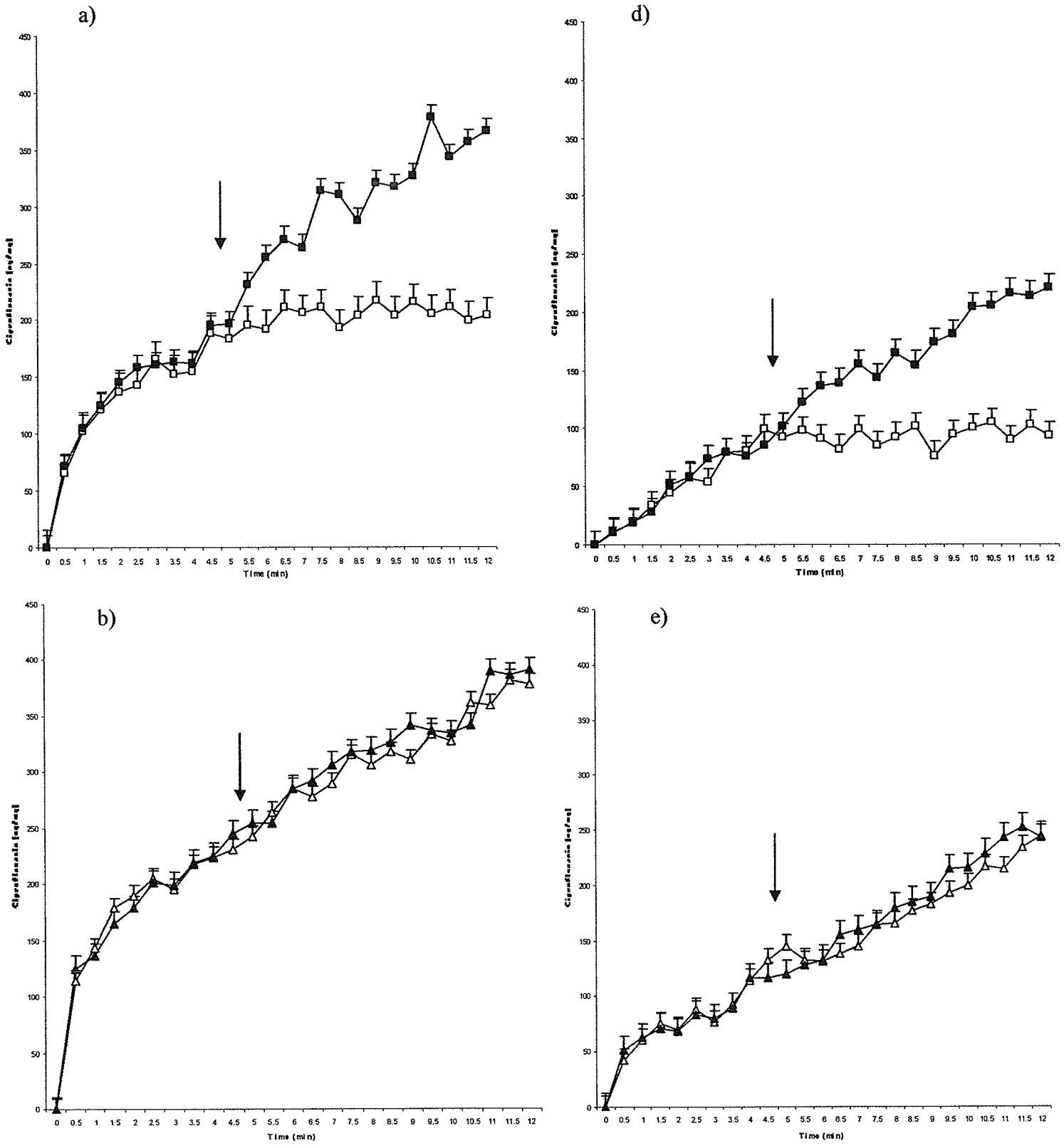
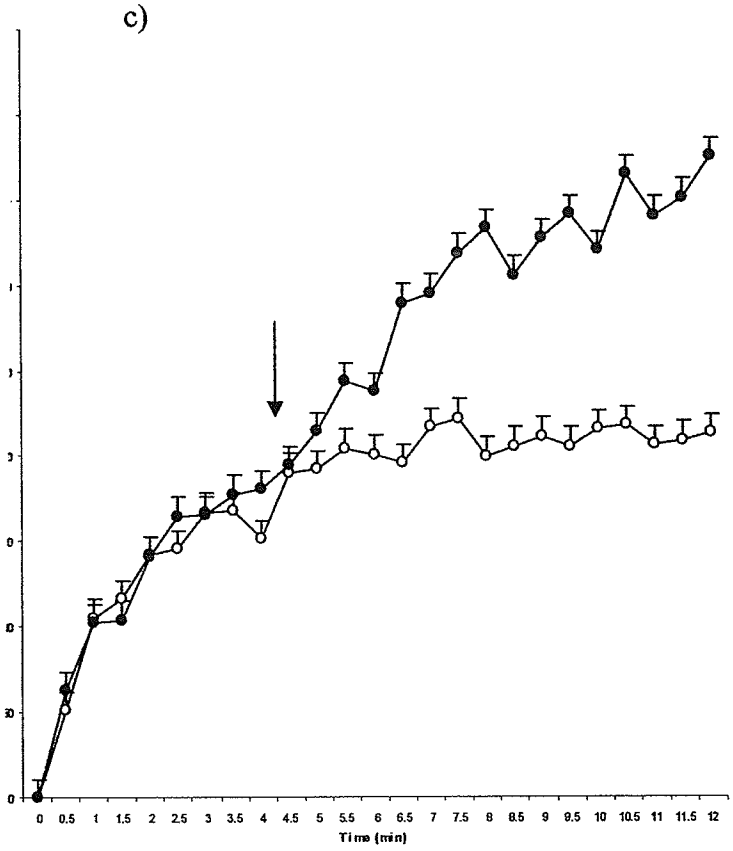

450
400
350
300
250
200
150
100
50
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5 11 11.5 12
Time (min)

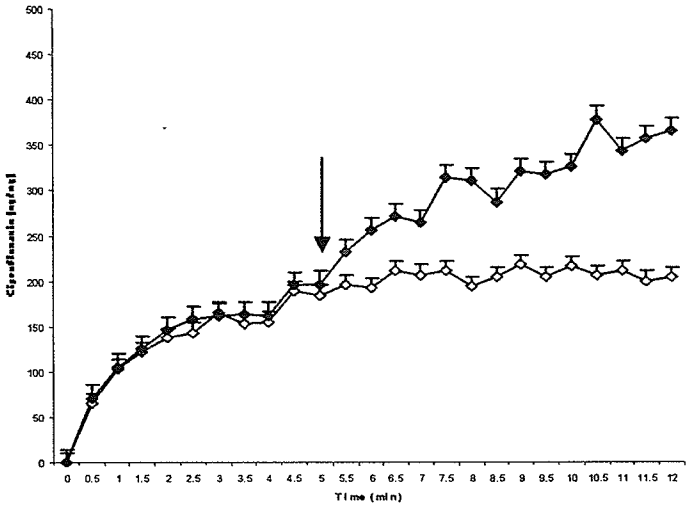
**Figure 4.23**. Accumulation of ciprofloxacin by a) wild type UOC-67 b) mutant *hasF* UOC-67 c) complemented *hasF* UOC-67 d) clinical isolate T-861 and e) mutant *hasF S. marcescens* T-861 in the absence and presence of CCCP. Results were graphed by the EXCEL computer program. Various strains are depicted as follows: a) UOC-67 in presence of CCCP (■); UOC-67 in absence of CCCP (□); b) HASF300 in presence of CCCP (▲); HASF300 in absence of CCCP (Δ); c) SM2000 in presence of CCCP (●); SM2000 in absence of CCCP (○); d) T-861 in presence of CCCP (■); T-861 in absence of CCCP (□) and e) HASF200 in presence of CCCP (▲); HASF200 in absence of CCCP (Δ). Proton motive force uncoupler CCCP (100 μM) was added 5 minutes after incubation (shown by the arrow). All trials were performed in triplicate. For complemented strain SM2000, accumulation performed in presence of 1 mM IPTG.


a)
d)
b)
e)
Time (min)

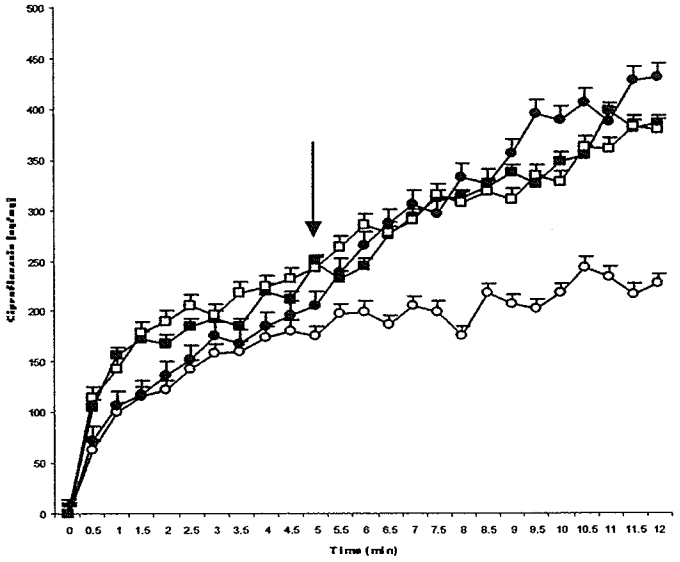
c)



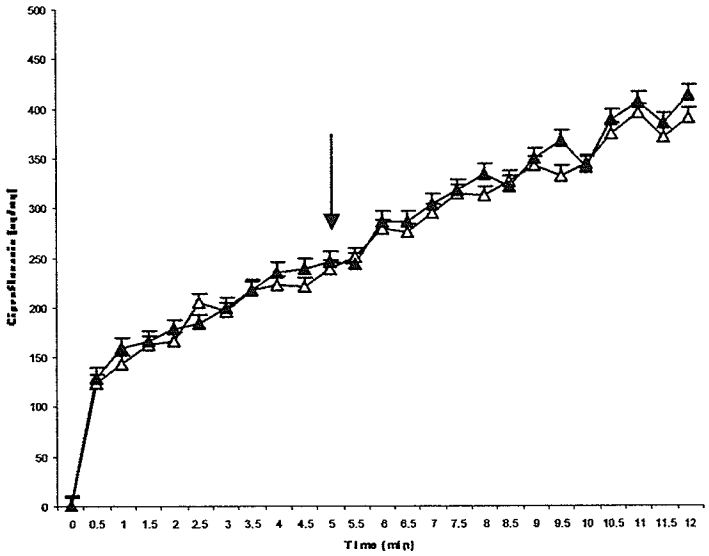
a)




b)




c)



that the *sdeB* and *hasF* have a synergistic effect (Figure 4.24b). After the addition of CCCP, at equilibrium SM1000 showed a significant increase in accumulation (Figure 4.24b). Ciprofloxacin accumulation for the SM1000 strain was very similar to that of the UOC-67 (Figure 4.24a).

Table 4.4 shows the initial rate of ciprofloxacin uptake for the 30 sec and 1 min time intervals and confirms our overall accumulation data in that, SDEAB1 and SDEAB2 display a higher initial diffusion rate into the cell than UOC-67 and T-861, respectively. The table permits a mathematical illustration of what is seen in the figures. Also, complemented strains SM1000, 2000 and 3000 show similar initial accumulation to UOC-67. Additionally, SDEAB3/HASF300 has a higher initial diffusion rate than UOC-67 and also reached the highest equilibrium concentration similar to that in the presence of CCCP.

For these studies, antibiotic uptake was measured for 12 minutes in 30 sec intervals. Additionally, Figure 4.25 shows an extended ciprofloxacin accumulation assay for wild type UOC-67 strain and the T-861 clinical strain where uptake measurements were extended to 30 minutes (in 1 min intervals). For the T-861 strain, accumulation past minute 20 decreases gradually due to the efflux pump activity, while in the presence of CCCP, accumulation continues past 30 minutes. For UOC-67, CCCP clearly also has the same effect, however the accumulation did not decrease as slowly and gradually as for T-861 in the absence of CCCP, and showed more of a plateau. Additionally, for UOC-67, accumulation did not increase as rapidly in the presence of CCCP as for T-861. This reflects T-861 being more resistant to quinolones presumably due to increased pump production and as such would efflux more antibiotic than a more susceptible strain such

**Table 4.4.** Initial ciprofloxacin uptake (initial diffusion into cells for the rate per minute taken after the first 30 second time interval and the 1 minute interval) for the wild type and mutant *S. marcescens* strains.

| Ciprofloxacin uptake rate of diffusion (ng/mg/min) | | |
|---|---|---|
| | Initial rate at 30 seconds | Rate at 1 minute |
| wt UOC-67 | 130.64 (11.41) | 103.21 (15.32) |
| SDEAB1 | 208.44 (10.11) | 156.43 (11.37) |
| SM3000 | 114.46 (15.41) | 94.75 (10.53) |
| HASF300 | 229.50 (12.71) | 143.33 (11.29) |
| SM2000 | 102.24 (11.43) | 105.43 (10.68) |
| SDEAB3/HASF300 | 248.64 (12.36) | 143.75 (14.07) |
| SM1000 | 124.22 (6.46) | 99.58 (10.99) |
| T-861 | 22.46 (2.04) | 18.94 (5.24) |
| SDEAB2 | 75.10 (9.66) | 57.07 (7.17) |
| HASF200 | 84.64 (6.48) | 61.05 (8.02) |

Note: Standard deviation is indicated in brackets. For complemented strains SM3000, SM2000 and SM1000 accumulation performed in presence of 1 mM IPTG.

The results were expressed as nanograms of ciprofloxacin per milligram (dry weight) of cells.

Y = Fluorescence

W = Dry weight (mg)

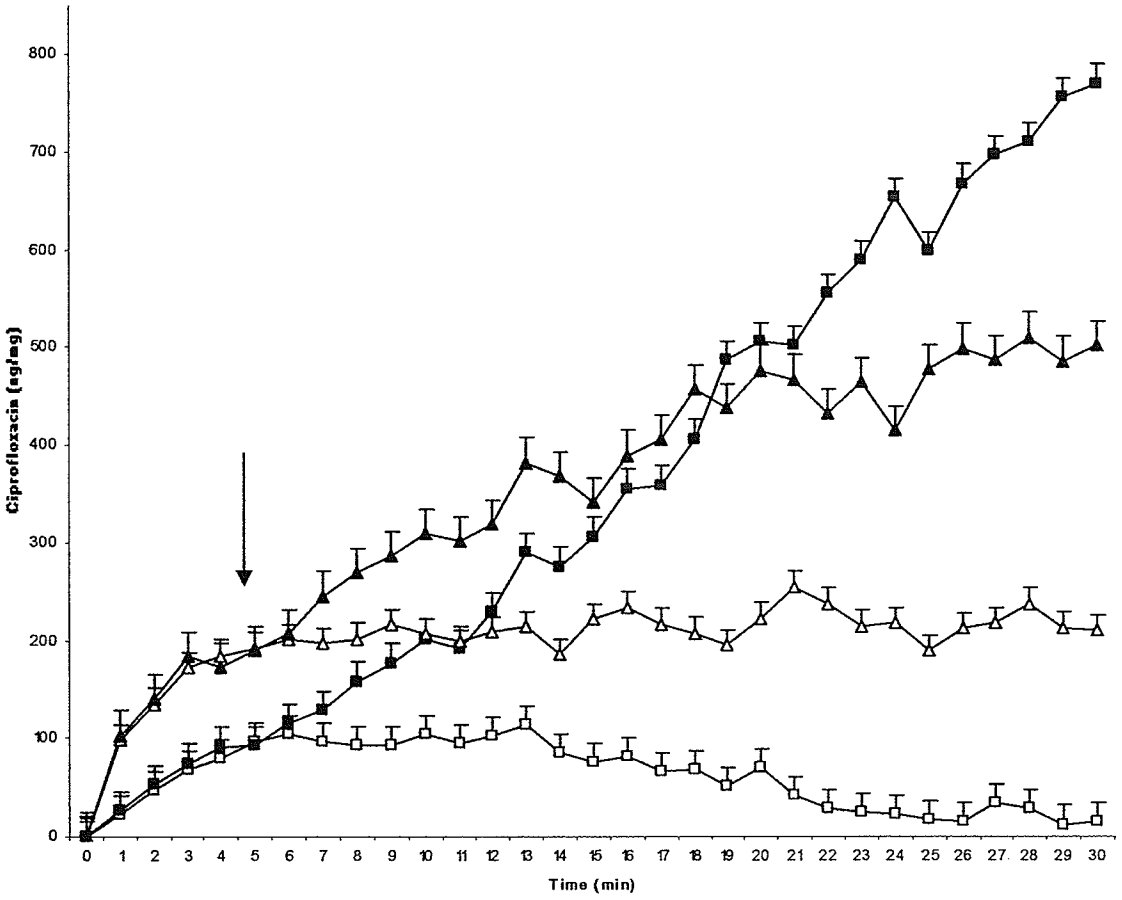
Z = ng antibiotic accumulated

= Y/slope of the standard curve for given antibiotic

ng/mg of cells = Z/W

Mortimer and Piddock, 1991.

**Figure 4.25.** Accumulation of ciprofloxacin by UOC-67 and T-861 over a period of 30 minutes. Results were graphed by the EXCEL computer program. Various strains are depicted as follows: UOC-67, no CCCP (Δ); T-861, no CCCP (□); UOC-67 in presence of CCCP (▲); T-861 in presence of CCCP (■). Proton motive force uncoupler CCCP (100 μM) was added 5 minutes after incubation (shown by the arrow). All trials were performed in triplicate.


Ciprofloxacin (ng/mg)
800
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Time (min)

as UOC-67. This also suggests that passive diffusion is faster in this strain, suggesting porins have bigger channels in T-861.

**4.5 Discussion**

Three different RND pumps have been identified in a variety of *S. marcescens*; SdeAB, SdeCDE (Kumar and Worobec 2005a) and SdeXY (Chen et al. 2003). SdeCDE appears to be a homolog of the *E. coli* MdtABC system (Kumar and Worobec 2005a). Preliminary results thus far demonstrate that SdeCDE is a very selective pump and provides very limited drug resistance, with novobiocin being its only substrate (data not shown). Novobiocin is an inhibitor of DNA gyrase that targets the GyrB subunit (Maxwell and Lawson 2003). Similar results have been reported for MdtABC of *E. coli* (Nagakubo et al. 2002). SdeXY, confers norfloxacin and tetracycline resistance, and appears to be a close homolog of the *E. coli* AcrAB-TolC pump (Chen et al. 2003). Our study demonstrates that *S. marcescens* SdeAB is the major pump, functioning with broad substrate specificity.

*sdeB* and *hasF* knockouts resulted in an increase in susceptibility for all compounds tested, demonstrating that both *hasF* and *sdeB* mutants were hyper-susceptible to a range of antibiotics, dyes and detergents. This finding is consistent with Buckley et al. 2006, where the authors reported a similar antibiotic susceptibility trend for *acrB* and *tolC* knockouts in *Salmonella enterica*, and by Ruzin et al. 2007, where the same finding was reported for the *adeB* knockout strain (inner membrane transporter of the AdeABC multidrug efflux pump homologous to AcrAB of *E. coli*). Hyper-susceptibility to a variety of compounds was also reported for *acrAB* deletion mutants in *E. coli* (Ma et al. 1995), a pump to which *S. marcescens* SdeAB is closely related.

Similarly, in *Helicobacter pylori*, knockout mutant strains in which TolC homologs were inactivated, displayed higher susceptibility to antibiotics and ethidium bromide (van Amsterdam et al. 2005), similar to the *hasF* knockout strains. Also, in *Brucella suis*, mutations in the *bepC* encoding a TolC homolog, increased the antimicrobial susceptibility, contributing to the intrinsic resistance/susceptibility phenotype (Posadas et al. 2007). Corresponding mutant strains complemented with *sdeB* (SM3000) or *hasF* (SM2000), resulted in a restoration of resistance to wild type levels, demonstrating the overall importance of both of these components. MIC values of the two strains used, vary from those reported by Kumar and Worobec, 2005a however a different MIC procedure was used. This, consistent with what is discussed in Jones et al. 2004 where MIC values from two different methods were compared for the same strains with different results, likely explains why my values are different.

The *sdeR* mutation resulted in a decrease in resistance which was restored to wild type levels upon complementation in 1 mM IPTG growth medium, and resulted in increased resistance in 4 mM IPTG growth medium. Overexpression of *sdeR* significantly increased resistance further. These results demonstrate the role of SdeR as an activator of SdeAB expression. Similar results were found in *Enterobacter aerogenes* where the RamA activator was shown to induce the expression of the efflux pump and act as an activator (Chollet et al. 2004). SdeR is 40% homologous to the MarA protein of *E. coli*, a transcriptional activator of the AcrAB-TolC drug efflux pump and the amino acid sequence of the two DNA binding motifs essential to the regulatory function of MarA are well conserved in SdeR. In addition to suggesting that SdeR and MarA may recognize the

same set of operator sequences, we have detected the presence of a putative marbox in the *sdeR* promoter which is well conserved according to the consensus (Kumar 2004).

Strains in the study were examined spectrofluorimetrically for efflux of ciprofloxacin before and after the addition of the uncoupler CCCP. Knockout mutant strains (SDEAB1, SDEAB2, HASF300, HASF200, SDEAB3/HASF300) that do not efflux antibiotics are not affected by the addition of CCCP. When the HasF outer membrane protein (HASF300, HASF200) or both HasF and the pump (SDEAB3/HASF300) are no longer produced, mutant strains continue to accumulate antibiotics in a steady fashion before and after the addition of the uncoupler (Figure 4.22, 4.23 and 4.24). Whereas, antibiotic accumulation of the wild type strain, UOC-67 and the clinical T-861 isolate was low, but increased after the addition of CCCP as the proton gradient-dependent efflux collapsed (Figure 4.22, 4.23 and 4.24). This is a similar finding to that reported by Pumbwe and Piddock 2002 where the *cmeB* gene encoding a *Campylobacter jejuni* multidrug efflux pump was knocked out and the results of mutant strains displayed a higher ciprofloxacin accumulation in comparison to that of the wild type *C. jejuni*. For strains SM3000 and SM2000 complemented with *sdeB* and *hasF* genes, respectively, the accumulation trend was similar to that of UOC-67 where the levels increased after the CCCP collapsed the gradient. This suggests that both components, SdeB and HasF are important in the drug efflux as the accumulation levels for each are very similar to that of UOC-67. For the *sdeB/hasF* double-complemented strain SM1000, in the absence of CCCP, accumulation levels were lower than those of the corresponding uncomplemented mutants SDEAB1 or HASF300, suggesting that most of the compound is effluxed out of the cell through the restored outer membrane channel.

With the addition of CCCP, SM1000 accumulation levels increased to that of SDEAB3/HASF300 suggesting the collapse of the gradient did restore the accumulation once again (Figure 4.24).

There are differences in the ciprofloxacin accumulation of the clinical isolate T-861 (Figure 4.22) as compared to values reported by Kumar and Worobec 2002. In Kumar and Worobec 2002, the T-861 ciprofloxacin accumulation with CCCP is higher, reaching approximately 200 ng/mg at minute 10, whereas in Figure 4.22d, at the same time interval in the presence of CCCP, only approximately 170 ng/mg is reached. There may be several reasons for this difference including human error or due to a physiological change in the strain over time. In addition to the efflux pump mechanism, for instance, T-861, could have changed to use another method of antibiotic resistance, such as reduced outer membrane permeability, which may account for the overall lower accumulation levels as compared to Kumar and Worobec 2002. My focus, however, was not necessarily on the ciprofloxacin accumulation differences between T-861 or UOC-67, but rather on the differences between these strains and their respective knockout strains. In this respect, I was consistently able to make qualitative interpretations regarding the presence or absence of proton gradient-dependent efflux, rather than solely focusing on the antibiotic accumulation of the clinical isolate T-861.

We hypothesize that there is at least another uptake system responsible for the uptake kinetics after the SdeAB efflux pump has been knocked out. Some early literature (Chen et al. 2003), describes another close homologue of the AcrAB-TolC pump in *E. coli,* in addition to the SdeAB major RND pump described here, named the SdeXY pump. Early studies show that this pump may be responsible for norfloxacin uptake, and

though work on other fluoroquinolones was not exhaustive, a pump with homology to AcrAB-TolC such as SdeXY would most probably confer resistance to other antibiotics as well, including ciprofloxacin used in my studies. This may be responsible for the trend we see in Figures 4.22 and 4.23 where there is fast initial uptake followed by continuous slower uptake.

Additionally, our early studies show that the SdeCDE pump does not efflux ciprofloxacin (Chapter 5). However the SdeXY pump has been reported to efflux fluoroquinolones (Chen et al. 2003), which could account for the minor change in ciprofloxacin accumulation for the knockout mutant strains in the absence and presence of CCCP. Ciprofloxacin accumulation for the *sdeB* and *hasF* knockout mutant strains (SDEAB1, HASF300) was very similar (Figure 4.24) and both had a comparable susceptibility profile (Table 4.3), suggesting that *S. marcescens* HasF is the only outer membrane component of efflux pumps in this organism.

In conclusion, using knockout mutagenesis, we have established the importance of the *S. marcescens* SdeAB multidrug efflux pump as the primary RND pump responsible for increasing *S. marcescens* resistance to a range of compounds. Additionally, we have established the role of HasF in contributing to the intrinsic resistance of *S. marcescens* to a variety of substances and the role of SdeR as an activator of the SdeAB efflux pump.

## CHAPTER 5 Characterization of the *Serratia marcescens* SdeCDE multidrug efflux pump studied via gene knockout mutagenesis

Parts of the material presented herein resulted in the publication Begic, S., and Worobec, E. A. 2008b. Characterization of the *Serratia marcescens* SdeCDE multidrug efflux pump studied via gene knockout mutagenesis. Canadian Journal of Microbiology. **54:** 411-416. I completed all of the experiments in this chapter with cloning assistance from Honours Project student Annie Ducas and summer assistant Craig Harlos. I wrote the manuscript with full guidance and support from my advisor.

### 5.1 Abstract

*Serratia marcescens* is an important nosocomial agent having high antibiotic resistance. A major mechanism for *S. marcescens* antibiotic resistance is active efflux. In order to ascertain the substrate specificity of the *S. marcescens* UOC-67 SdeCDE efflux pump, we constructed pump gene deletion mutants. *sdeCDE* knockout strains showed no change in antibiotic susceptibility in comparison to the parental strains for any of the substrates with the exception of novobiocin. In addition, novobiocin was the only antibiotic to be accumulated by *sdeCDE* deficient strains. Based on the substrates used in our study, we conclude that SdeCDE is a Resistance-Nodulation-Cell Division family pump with limited substrate specificity.

### 5.2 Introduction

*Serratia marcescens* is a nosocomial pathogen that is often reported as the causative agent of septicemia, meningitis, endocarditis, and wound infections. High intrinsic resistance to a variety of antibiotics makes the treatment of *S. marcescens* infections difficult. *S. marcescens* has been found to be resistant to β-lactams,

aminoglycosides and quinolones. *S. marcescens* resistance to quinolones was reported shortly after the initial clinical use (Fujimaki et al. 1989).

The major mechanism for fluoroquinolone resistance in Gram negative organisms (Ma et al. 1995; Poole et al. 1993; Giraud et al. 2000) is the active efflux of the antibiotic molecule mediated by efflux pumps belonging to the Resistance-Nodulation-Cell Division (RND) family. Bacteria pump out antibiotic molecules against a concentration gradient in an energy-dependent manner. RND pumps (inner membrane transporters) use the proton gradient as their energy source and work in conjunction with a membrane fusion protein (MFP), and an outer membrane protein (e.g. *Escherichia coli* TolC; *S. marcescens* HasF (http://www.sanger.ac.uk/Projects/*S. marcescens*/)) to form a tripartite system (Kumar and Worobec 2005a; Li and Nikaido 2004, and references therein). These three components form a continuous channel that aids in the efflux of antimicrobials directly out of the cell.

We have previously reported the importance of the *S. marcescens* SdeAB RND pump which functions with broad substrate specificity (Begic and Worobec 2007; Kumar and Worobec 2005b). We have also reported another RND pump, SdeCDE, consisting of a MFP (*sdeC*) and two different RND pump transporters (*sdeD* and *sdeE*), both of which are required for the activity of the pump complex (Kumar and Worobec 2005b). At the DNA level, this pump has a 60% homology to *E. coli mdtABC,* which also contains two RND transporter-encoding genes, *mdtB* and *mdtC* (Baranova and Nikaido 2002). In *E. coli*, MdtABC is expressed at a minimal level, using bile salts and novobiocin as the only known substrates (Nagakubo et al. 2002). In this study, we address the role of the SdeCDE pump in *S. marcescens* resistance via construction of pump deficient strains.

This is only the third RND *S. marcescens* pump to be characterized, the others being SdeAB (Begic and Worobec 2007; Kumar and Worobec 2005b), and SdeXY, which confers norfloxacin and tetracycline resistance and is a close homologue of the *E. coli* AcrAB-TolC pump (Chen et al. 2003).

**5.3 Materials and Methods**

Strains and plasmids used in this study are outlined in Table 5.1. All bacterial strains were grown on Luria-Bertani agar (LB), Trypticase Soy Agar (TSA) and in Tryptone Soy Broth (TSB) [BD Diagnostics Systems]. Ampicillin resistant strains (containing pDrive, pClone1.7 and all pEX1.8 constructs) were grown on LB agar and in LB broth containing 100 μg/mL ampicillin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. The gentamicin resistant strains (with pUCGM, pClone1.7:$Gm^r$) were grown on LB plates and in LB broth containing 20 μg/mL gentamicin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. Streptomycin resistant strains (with pKNG101, pKNGsdeD) were grown on LB agar and in LB broth containing 50 μg/mL streptomycin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)]. SDEAB1 was grown on LB agar and in LB broth containing 100 μg/mL ampicillin and 50 μg/mL streptomycin, SDECDE1 and SDECDE2 on media containing 20 μg/mL gentamicin and 50 μg/mL streptomycin and SDECDE3 on media containing 25 μg/mL kanamycin and 20 μg/mL gentamicin. UOC-67, MT616 and CC118 were grown in absence of any antibiotic.

**Table 5.1.** Bacterial strains and plasmids used in the study.

| Strains | Characteristic | Source/Reference |
|---|---|---|
| UOC-67 | Wild type *S. marcescens*, ATCC 13880 | American Type Culture Collection |
| MT616 | *E. coli*, MT607 (*pro-82 thi-I hsdR17 supE44*):pRK600 | Finan et al.1986 |
| CC118 | *E. coli, araD 139Δ(ara, leu) 7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB argEam recA1* | Dr. C. Manoil, University of Washington, Seattle, USA |
| T-861 | Clinical isolate of *S. marcescens* | D. Hoban, Health Science Centre, Winnipeg, MB, Canada |
| SDEAB1 | *sdeB*-deficient *S. marcescens* UOC-67 | Begic and Worobec 2007, Chapter 4 |
| SDECDE1 | *sdeD*-deficient *S. marcescens* UOC-67 | This study |
| SDECDE2 | *sdeD*-deficient *S. marcescens* T-861 | This study |
| SDECDE3 | *sdeB*-deficient *S. marcescens* SDECDE1 (*sdeB/sdeD* deficient *S. marcescens*) | This study |
| SDECDE4 | SDECDE1 with pEXD | This study |
| SDECDE5 | SDECDE3 with pEXD | This study |
| SDECDE6 | SDECDE3 with pEXS | This study |
| SDECDE7 | SDECDE3 with pEXSD | This study |
| Plasmids | | |
| pDrive | Phage F1 origin, $Amp^r$, $Kan^r$, *lacZ'* | Qiagen |
| pClone1.7 | pDrive with 1.7-kb *Eco*RI/*Eco*RI *sdeD* fragment | This study |
| pClone1.7:$Gm^r$ | pClone1.7 with a 0.9-kb $Gm^r$ insertion in the *Sal*I *sdeD* fragment | This study |
| pKNG101 | Suicide vector, *pir- oriR6K mobRK2 sacB* $Str^r$ | Kaniga *et al.*, 1991 |
| pKNGsdeD | pKNG101 with 2.6-kb insertionally-disrupted *sdeD* fragment | This study |
| pUCGM | 900-bp $Gm^r$ cassette flanked by *Sal*I | Schweizer *et al.*, 1993 |
| pEX1.8 | CoE1 replicon, $Amp^r$-$Car^r$, carries $lacI^Q$ and a $P_{tac}$ promoter | Pearson *et al.*, 1997 |
| pEX1.8JH | a pEX1.8 plasmid with a *Bam*HI site in front of the P*tac* promoter removed by Erase-a-base system | Hutsul, unpublished. |

| | | |
|---|---|---|
| pEX1.8JH1 | a pEX1.8 plasmid with *Eco*RI, *Sma*I and *Bam*HI sites within MCS removed by Erase-a-base system | Hutsul 1996, unpublished. |
| pEXS | pEX1.8 with wild type 3.1-kb *Eco*RI/*Hin*DIII *sdeB* fragment | Chapter 4 |
| pEXSE/E | pEX1.8JH with wild type *Eco*RI/*Eco*RI 3.1-kb *sdeB* fragment | Chapter 4 |
| pEXD | pEX1.8 with wild type *Eco*RI/*Pst*I 4.9-kb *sdeDE* fragment | This study |
| pEXDP/P | pEX1.8JH1 with wild type *Pst*I/*Pst*I 4.9-kb *sdeDE* fragment | This study |
| pEXSD | pEX1.8JH with 3.1-kb *Eco*RI/*Eco*RI *sdeB* fragment and 4.9-kb *Pst*I/*Pst*I *sdeDE* fragment | This study |

Note: Abbreviations: Amp, ampicillin; Str, streptomycin; Car, carbenicillin; Gm, gentamicin.

Primers used throughout this study are listed in Table 5.2. As shown in Figure 5.1, the *sdeD* gene (Kumar and Worobec 2005a; GenBank accession no. AY168757) was cloned as a 1.7-kb *Eco*RI/*Eco*RI fragment into pDrive to result in pClone1.7, and transformed into UOC-67. The 1.7-kb *sdeD* gene fragment was disrupted by the insertion of a gentamicin resistance cassette into a unique *Sal*I restriction site to yield pClone1.7:Gm$^r$. After transformation of pClone1.7:Gm$^r$ into *S. marcescens* UOC-67 for propagation, the *sdeD* insertion mutation was confirmed by restriction digestion with *Eco*RI, PCR of pClone1.7:Gm$^r$ with SdeD1f/r primers and sequencing of the mutated gene. pClone1.7:Gm$^r$ was digested with *Eco*RI to remove the 2.6-kb *sdeD* insertion and the ends of the fragment were made flush with T4 DNA polymerase. The *sdeD* insertion mutant was then ligated into the pKNG101 replacement vector to construct pKNGsdeD which was transformed into *E. coli* CC118 (See Section 2.3.2 for a detailed description). pKNG101 is a suicide vector that contains a conditional origin of replication (*oriR6K*), the *strAB* genes encoding streptomycin phosphotransferase (Str$^r$), an origin of transfer (*mobRK2*), the *sacB* gene mediating sucrose sensitivity, and multiple restriction sites (Kaniga et al. 1991). Conjugation between the *E. coli* strain harboring the disrupted *S. marcescens sdeD* gene and wild type *S. marcescens*, as well as the *S. marcescens* clinical isolate strain (T-861) was carried out using *E. coli* helper strain MT616 (Finan et al. 1986) to construct SDECDE1 and SDECDE2, respectively.

For the double deficient mutant (SDECDE3, deficient in both SdeAB and SdeCDE pump functions), pKNGsdeB (Begic and Worobec 2008a, Chapter 4) was mobilized from *E. coli* CC118 into the *S. marcescens sdeD* mutant strain (SDECDE1) via

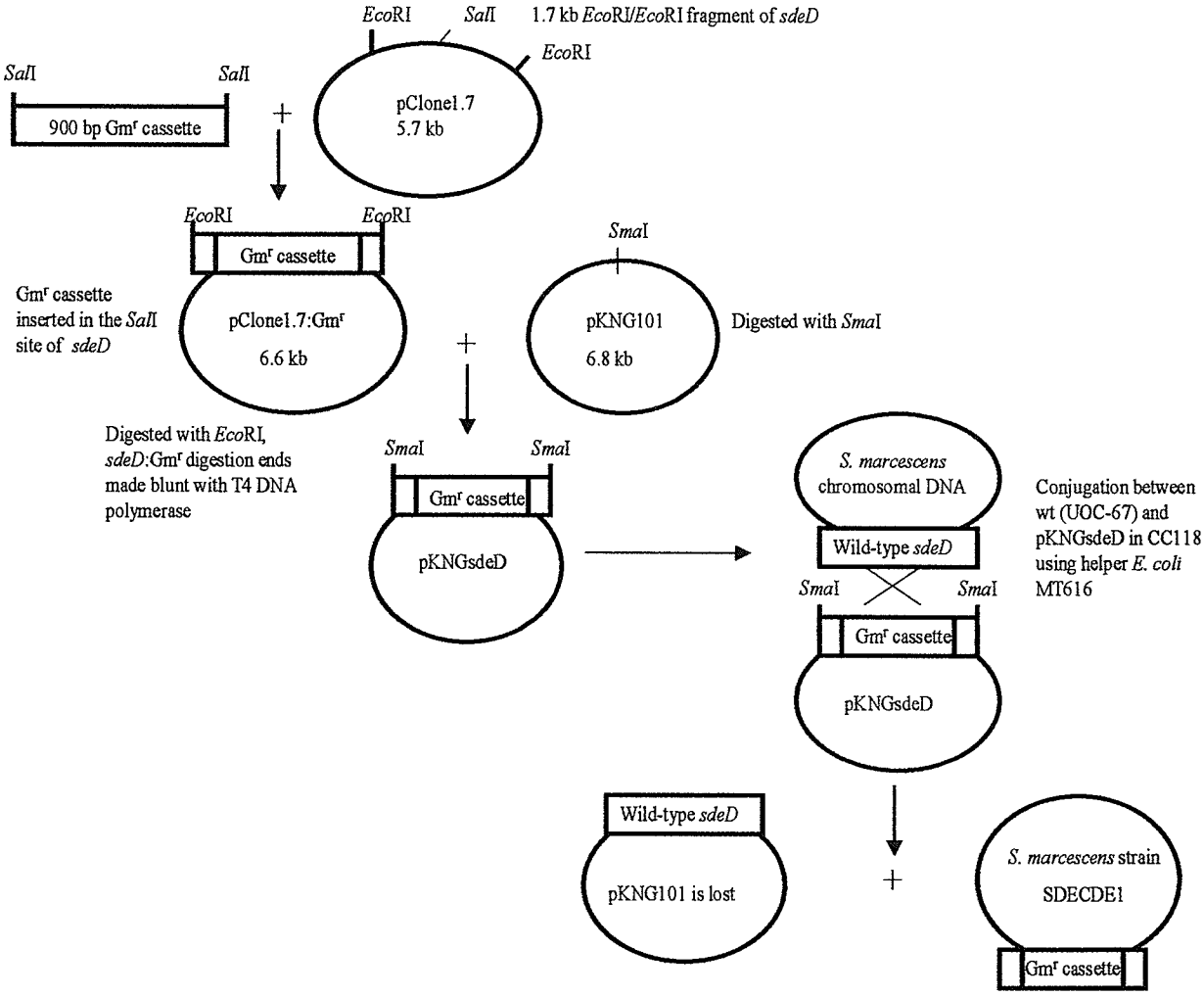
**Table 5.2.** Primers used in this study. All restriction sites are underlined.

| Name | Sequence (5' to 3') | Description | PCR Tm |
|---|---|---|---|
| SdeDfor (forward) | TCGGAATTCACGCCGCGCTGAAAG | Amplification of a 1.7-kb sequence within *sdeB* (*Eco*RI site engineered) | 61°C |
| SdeDrev (reverse) | TACGAATTCAGACGCCGAGCGGAT | Same as above (*Eco*RI site engineered) | |
| SdeD1f (forward) | GCCGGCGAGATCGGCTTCACCA | Amplification of a 1.7-kb sequence within *sdeD* (for screening transconjugants) | 59°C |
| SdeD1r (reverse) | CGCCGACGCCGGTGCTCAGC | Same as above | |
| SdeB1for (forward) | GGCCGATCTTTGCCGCGGTG | Amplification of a 1.7-kb sequence within *sdeB* (for screening transconjugants) | 57°C |
| SdeB1rev (reverse) | AGCACGCGGGAGACGCCG | Same as above | |
| SacBf (forward) | AAGAAAATGCCAATAGGATATC | Analysis of *sdeD* transconjugant after *S. marcescens-E. coli* conjugations, 1.0 kb | 56°C |
| SacBr (reverse) | TTCTATCCTAAAAGACCAAACAC | Same as above | |
| SdeD1f/r and SdeB1for/rev | Above | Multiplex reaction using both sets of primers (SdeD1f/r and SdeB1for/rev) for detection of double mutants | 58.5°C |
| Gmf (forward) | ATCCCCGGGTACCGAGCTC | Amplification of $Gm^r$ cassette (650 bp) | 54°C |

| | | | |
|---|---|---|---|
| Gmr (reverse) | CATAAGCACCAAGCGCGTTGGC | Same as above | |
| SdeDGmf (forward) | GCCGGCGAGATCGGCTTCACCA | Amplification of the 650 bp $Gm^r$ cassette and some of *sdeD* within *sdeD* transconjugant (1.1 kb product) | 58°C |
| SdeDGmr (reverse) | CATAAGCACCAAGCGCGTTGGC | Same as above | |
| pEXDfor (forward) | CAGAATTCCCGGCTTCGTGGTGGATG A | Amplification of an approximately 4.9-kb sequence of wt *sdeDE* for complementation (*Eco*RI site engineered) | 62°C |
| pEXDrev (reverse) | GTCTGCAGGGTGTACAGCGTCAG | Same as above (*Pst*I site engineered) | |
| pEXSDfor1 (forward) | CCGAATTCGAGACTTCTGACCCAT | Amplification of a 3.1-kb of wt *sdeB* for double complementation (*Eco*RI site engineered) | 55°C |
| pEXSDrev1 (reverse) | GCGAATTCAGGGTGACGTAG | Same as above (*Eco*RI site engineered) | |
| pEXSDfor2 | CACTGCAGCCGGCTTCGTGGTGGATG A | Amplification of a 4.9-kb sequence of wt *sdeDE* for double complementation (*Pst*I site engineered) | 62°C |
| pEXSDrev2 | GTCTGCAGGGTGTACAGCGTCAG | Same as above (*Pst*I site engineered) | |

**Figure 5.1.** Construction of *S. marcescens sdeD* mutant strain. A 900-bp $Gm^r$ cassette was inserted into a unique *Sal*I restriction site within the *Eco*RI/*Eco*RI flanked *sdeD* fragment (pClone1.7). The disrupted *sdeD* fragment was then ligated into the pKNG101 replacement vector and transformed into *E. coli* CC118.

Conjugation between the *E. coli* strain harboring the disrupted *S. marcescens sdeD* gene and the wild type *S. marcescens* strain, was carried out using *E. coli* helper strain MT616 (Finan et al. 1986) to create SDECDE1.


EcoRI
SalI
1.7 kb EcoRI/EcoRI fragment of sdeD
EcoRI
SalI
SalI
900 bp Gmr cassette
pClone1.7
5.7 kb
EcoRI
EcoRI
Gmr cassette
Gmr cassette inserted in the SalI site of sdeD
pClone1.7:Gmr
6.6 kb
SmaI
pKNG101
6.8 kb
Digested with SmaI
Digested with EcoRI, sdeD:Gmr digestion ends made blunt with T4 DNA polymerase
SmaI
SmaI
Gmr cassette
pKNGsdeD
S. marcescens chromosomal DNA
Wild-type sdeD
SmaI
SmaI
Gmr cassette
pKNGsdeD
Conjugation between wt (UOC-67) and pKNGsdeD in CC118 using helper E. coli MT616
Wild-type sdeD
pKNG101 is lost
S. marcescens strain SDECDE1
Gmr cassette

conjugation (Figure 5.2). Analysis of transconjugants was based on sucrose resistance due to the absence of synthesis of lethal levano compounds, catalyzed by levanosucrase, the product of the *sacB* gene. A multiplex PCR was done with optimized conditions that used both *sdeB* and *sdeD* primers. These optimized conditions included first optimizing the PCR conditions for each primer pair separately (SdeD1f/r; SdeB1for/rev), then using equimolar concentrations of each primer set (0.2 μM of each primer) along with the 1X Qiagen PCR Buffer, and the HotStarTaq DNA polymerase in order to minimize primer dimers. Insertional mutations were confirmed by sequence analysis of the gel isolated correct sized PCR-product fragment (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada). The template DNA for PCR amplifications was the genomic DNA obtained from the putative *S. marcescens sdeD* mutant strains.

For complementation purposes, the expression vector pEX1.8 was used for cloning a 4.9-kb wild type *Eco*RI/*Pst*I *sdeDE* fragment (also contains *sdeE* as *sdeD* and *sdeE* genes are arranged as an operon) (pEXD). To complement both *sdeB* and *sdeD* simultaneously (pEXSD), primers (Table 4.2) for the *sdeB* PCR reaction contained additional engineered *Eco*RI sites (Chapter 4). The *sdeB* product (3.1 kb) from this PCR amplification was digested with *Eco*RI and cloned into the *Eco*RI site of pEX1.8JH (a pEX1.8 with a *Bam*HI site in front of the P*tac* promoter removed by Erase-a-base system) to construct pEXSE/E (Figure 5.3 Step 1). The primers for the *sdeDE* PCR reaction contained additional engineered *Pst*I sites. The *sdeDE* product (4.9 kb) from this PCR amplification was digested with *Pst*I and cloned into the *Pst*I sites of pEX1.8JH1 (a pEX1.8 with *Eco*RI, *Sma*I and *Bam*HI sites within the MCS removed) to construct

**Figure 5.2.** Constructional approach to creating *S. marcescens* double mutant strain SDECDE3 used in the study (*sdeB/sdeD* double mutant). The knockout construct in *E. coli* (pKNGsdeB, Chapter 4) with a single insertional mutation in one gene of interest (*sdeB*) was mobilized from *E. coli* CC118 into the *S. marcescens sdeD* mutant (SDECDE1) via conjugation. To select for double mutants, both kanamycin and gentamicin selection pressure was used.

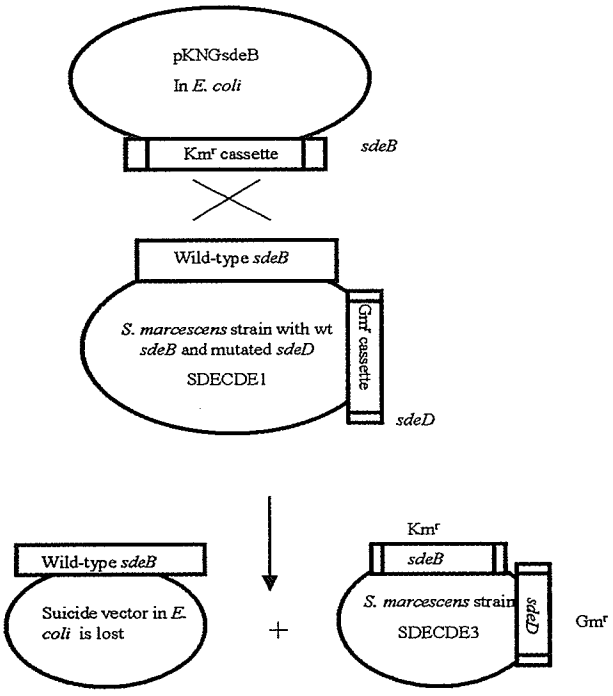
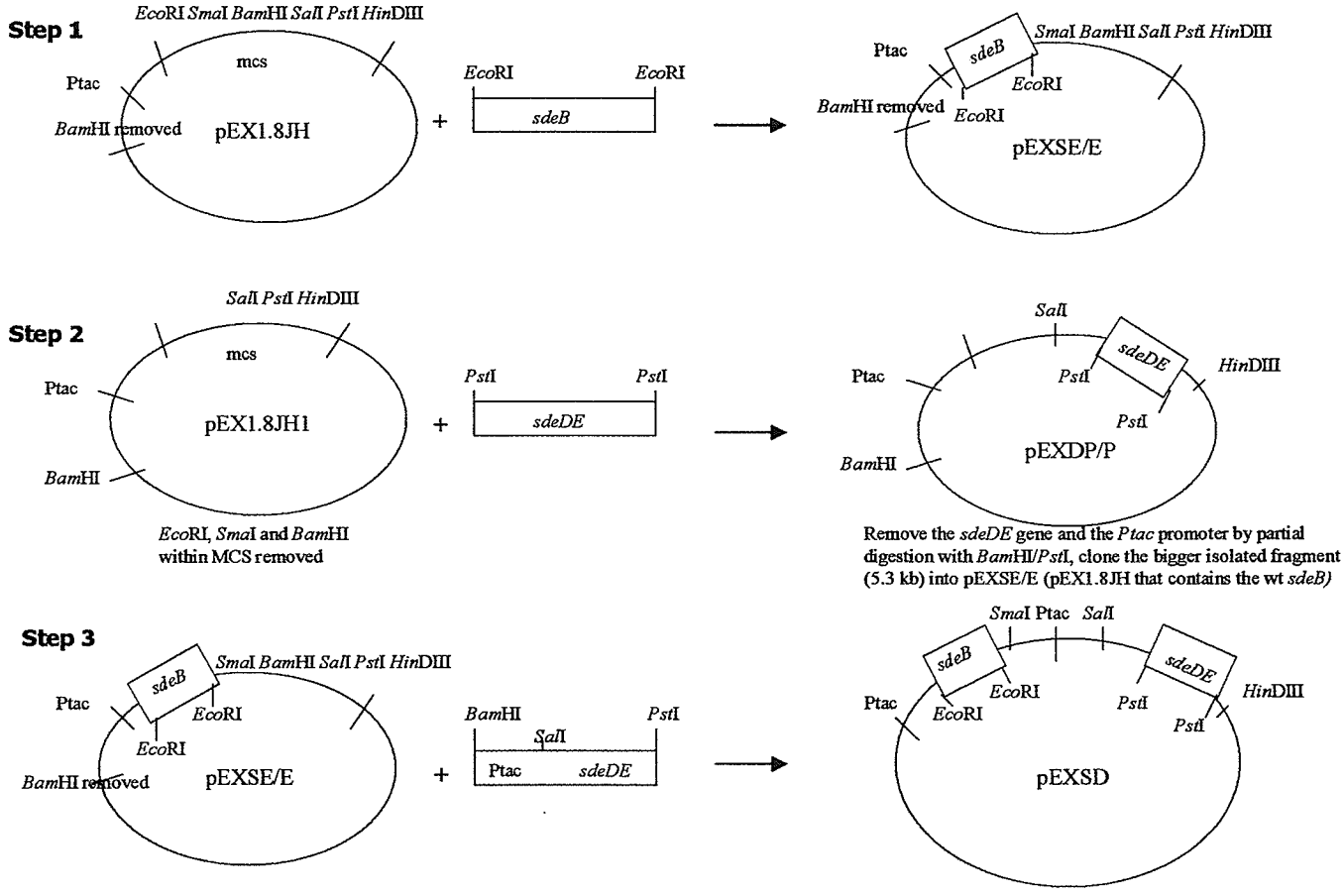

pKNGsdeB
In E. coli
Kmr cassette
sdeB
Wild-type sdeB
S. marcescens strain with wt sdeB and mutated sdeD
SDECDE1
Gmr cassette
sdeD
Wild-type sdeB
Suicide vector in E. coli is lost
+
Kmr
sdeB
S. marcescens strain
SDECDE3
sdeD
Gmr

**Figure 5.3.** Diagrammatic representation of pEXSD construction.

Step 1. The primers for the *sdeB* PCR reaction contained additional engineered *Eco*RI sites (pEXSDfor1/rev1). The *sdeB* product from this PCR amplification was digested with *Eco*RI and cloned into the *Eco*RI site of pEX1.8JH (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI site in front of the Ptac promoter removed by Erase-a-base system) to construct pEXSE/E.

Step 2. The primers for the *sdeDE* PCR reaction contained additional engineered *Pst*I sites (pEXSDfor2/rev2). The *sdeDE* product from this PCR amplification was digested with *Pst*I and cloned into the *Pst*I site of pEX1.8JH1 (a pEX1.8 plasmid provided by Dr. Jo-Anne Hutsul with a *Bam*HI, *Eco*RI and *Sma*I sites within MCS removed) to construct pEXDP/P.

Step 3. The *sdeDE* genes (including the Ptac promoter) were then excised from the pEXDP/P vector by *Bam*HI and *Pst*I partial digestion and inserted into the *Bam*HI and *Pst*I sites within the pEXSE/E MCS to construct pEXSD.


Step 1
EcoRI SmaI BamHI SalI PstI HinDIII
mcs
Ptac
BamHI removed
pEX1.8JH
EcoRI
EcoRI
sdeB
sdeB
SmaI BamHI SalI PstI HinDIII
Ptac
EcoRI
BamHI removed
EcoRI
pEXSE/E
Step 2
SalI PstI HinDIII
mcs
Ptac
pEX1.8JH1
BamHI
EcoRI, SmaI and BamHI
within MCS removed
PstI
PstI
sdeDE
SalI
sdeDE
HinDIII
Ptac
PstI
PstI
BamHI
pEXDP/P
Remove the sdeDE gene and the Ptac promoter by partial
digestion with BamHI/PstI, clone the bigger isolated fragment
(5.3 kb) into pEXSE/E (pEX1.8JH that contains the wt sdeB)
Step 3
sdeB
SmaI BamHI SalI PstI HinDIII
Ptac
EcoRI
EcoRI
BamHI removed
pEXSE/E
BamHI
PstI
SalI
Ptac
sdeDE
SmaI Ptac SalI
sdeB
sdeDE
Ptac
EcoRI
EcoRI
PstI
HinDIII
PstI
pEXSD

pEXDP/P (Figure 5.3 Step 2). The *sdeDE* genes (including the P*tac* promoter) were then excised from pEXDP/P by a *Bam*HI and *Pst*I partial digestion. This fragment was then inserted into the *Bam*HI and *Pst*I sites within the pEXSE/E MCS to construct pEXSD (Figure 5.3 Step 3).

To construct strain SDECDE4, electroporation of pEXD into SDECDE1 (*sdeD* deficient strain) was optimized by using a 40 μL cell suspension and 10 μL of DNA in a low ionic strength buffer (TE). Electroporation was done at 1.45 volts in short 1 second intervals for about 3 seconds. 1 ml of SOC media (20g/1L Bacto Tryptone, 5g/1L Bacto Yeast Extract, 5M NaCl, 1M KCl, 1M $MgCl_2$, 1M $MgSO_4$, 1M glucose) was immediately added to these cells and left to incubate at 37°C for about an hour without shaking and then plated out. SOC media contains twice the amount of Bacto Tryptone in comparison to LB media, which provides more protein precursors in Bacto Tryptone for rapid repair of cell walls damaged by the electroporation process and maintains isotonicity to prevent cell death by osmotic rupture (Electroporation-Competent cells, Stratagene, modified protocol under catalog number 200123). SDECDE5 was constructed by transferring the pEXD into SDECDE3, SDECDE6 by transferring the pEXS into SDECDE3 and SDECDE7 by transferring pEXSD into SDECDE3 in the same fashion described above. Selected transformants were verified by SDS-PAGE protein analysis. The *sdeD* and *sdeB* genes were expressed under the control of the $P_{tac}$ promoter and induced by the addition of varying concentrations (0.5-5 mM) of isopropyl-β-D-thiogalactopyranoside (IPTG) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] to the growth medium.

Whole cell lysis was performed for rapid examination of gene expression. A 1.5 ml aliquot of an overnight culture of the bacterial strain was centrifuged at 12,000 x *g* for

1 min and cells were resuspended in 100 µl of cell lysis buffer (2% (w/v) SDS, 4% (w/v) DTT, 10% (v/v) glycerol and 1 M Tris-HCl, pH 6.8). The resulting cell lysate was quantified using the Ultraspec 4000 UV/visible spectrophotometer. Samples (50-100 µg) were boiled for 5 minutes and resolved on a 12% SDS-PAGE gel. Because whole cell lysates represent overall protein expression, it is often difficult to distinguish absence/presence of certain bands in a given area on the SDS-PAGE gel.

SDS-PAGE of whole cell lysates from *S. marcescens sdeB* and *sdeD* knockout mutant and complemented strains was performed using the method described by Lugtenberg (Lugtenberg et al. 1975) using 12% (w/v) polyacrylamide gels. 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. For complemented strains SDECDE4, SDECDE5, SDECDE6 and SDECDE7, 0.5 or 1 mM IPTG was added to the growth medium for gene expression. Samples were heated at 95°C for 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. Bands were stained for up to 1 hour using Coomassie Blue staining solution (Sigma, St. Louis, MO) in 30% (v/v) isopropanol/10% (v/v) acetic acid. The gels were destained with 20% (v/v) methanol/7.5% (v/v) acetic acid for 30 min and then overnight with fresh destain solution.

Susceptibility of SDECDE1 through SDECDE7 to norfloxacin (0.001-32 mg/L), ciprofloxacin (0.001-32 mg/L), ofloxacin (0.001-32 mg/L), chloramphenicol (0.002-16 mg/L), novobiocin (0.001-16 mg/L), sodium dodecyl sulphate (SDS) (0.001-8 mg/L),

ethidium bromide (0.002-16 mg/L), deoxycholic acid sodium salt (0.008-4 mg/L) and cholic acid sodium salt (0.008-4 mg/L) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was tested using the minimum inhibitory concentration (MIC) two-fold broth dilution method (National Committee for Clinical and Laboratory Standards with modifications). Complemented strains SDECDE4, SDECDE5, SDECDE6 and SDECDE7 were tested in the presence of 1 mM IPTG, an agent that induced genes cloned under control of the *tac* promoter. Overnight cultures in MH broth were diluted 1000-fold in fresh broth, grown at 37°C until $OD_{600}$=0.5-0.9 and 5 μL of the bacterial suspension was inoculated in MH containing serial dilutions of each antibiotic. Results were reported as MIC, the concentration of antibiotic that inhibited visible growth determined by absence of turbidity in MH after 18 hours of shaking at 37°C.

The accumulation of novobiocin and ciprofloxacin [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] by SDECDE1, SDECDE2 and SDECDE3 was measured using the method of Mortimer and Piddock, 1991 (Mortimer and Piddock 1991). Cultures in 3L batches were grown until $OD_{600}$ reaches 0.5-0.7. Cells were harvested by centrifugation at 4000 x *g* for 15 min at room temperature, resuspended to 30 ml in phosphate buffered saline (PBS) pH 7.5 and washed two times. Pellets were suspended in 1/10 volume of PBS. Ciprofloxacin and novobiocin were added to a final concentration of 10 μg/mL and 0.5 ml of culture was removed in 30 sec intervals for a period of 12 minutes and immediately diluted in 1 ml of cold PBS. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] was added to a final concentration of 100 μM after 5 minutes of antibiotic incubation. After incubation, a rapid centrifugation (13,000 x *g* at 4°C for 1 min) was carried out, followed by a wash in

cold PBS, suspension in 1 mL of HCl-glycine 0.1 M pH 3.0 to lyse the cells, and overnight incubation at room temperature. This suspension was centrifuged at 11,000 x *g* for 5 min to remove cellular debris and antibiotic concentration measured in a RF-1501 Shimadzu spectrofluorometer. The fluorescence of ciprofloxacin and novobiocin were measured at 279 and 420 nm excitation wavelengths, and 447 and 484 nm emission wavelengths, respectively. Ciprofloxacin and novobiocin concentrations were calculated using a standard curve (concentration ranging from 100 to 1000 ng) in 0.1 M HCl-glycine 0.1 M pH 3.0. The results were expressed as nanograms of ciprofloxacin incorporated per milligram (dry weight) of bacteria.

Using the same experimental details as above, DNP (dinitrophenyl) was used as the proton motive force collapser [Sigma-Aldrich Chemical Co. (St. Louis, Mo.)] and added to a final concentration of 100 μM after 5 minutes of antibiotic incubation.

## 5.4 Results

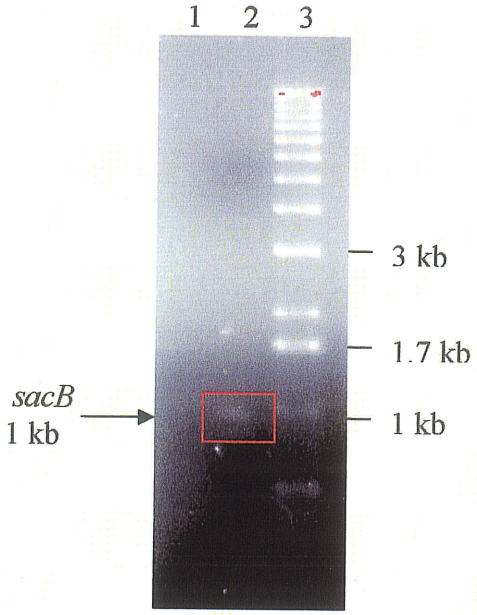
### 5.4.1 Construction of *S. marcescens sdeD* mutant strains

PCR amplification of *S. marcescens sdeD* insertion mutant genomic DNA using SdeD1f/r primers resulted in two products corresponding to a 2.6-kb insertion fragment and a 1.7-kb wild type *sdeD* gene after the single crossover event (Figure 5.4). Only the 2.6-kb insertion fragment was visible after the double crossover event (Figure 5.5). Additionally, a 1.1-kb band is depicted when SdeDGmf/r gentamicin cassette primers are used to examine the *sdeD* insertion mutant (Figure 5.6). PCR products obtained from genomic DNA from putative *S. marcescens sdeB and sdeD* double mutants ilustrate a 3.0-kb insertion band for *sdeB* and a 2.6-kb insertion band for *sdeD* using SdeD1f/r and SdeB1for/rev multiplex primers (Figure 5.7).

**Figure 5.4.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeD* mutants after the single crossover event.

a) Lane 1, ddH2O; Lane 2, *sacB* (SacBf/r) PCR product; Lane 3, 1 kb ladder. Molecular weight of product is 1 kb. b) Lane 1, 1 kb ladder; Lane 2, *sdeD* (SdeD1f/r) product; Lane 3, ddH2O. Important bands indicated by arrows; bands shown are around 1.7-kb and 2.6-kb. 3 μL of samples were loaded in each lane.

a)


1 2 3
3 kb
1.7 kb
sacB
1 kb
1 kb

b)

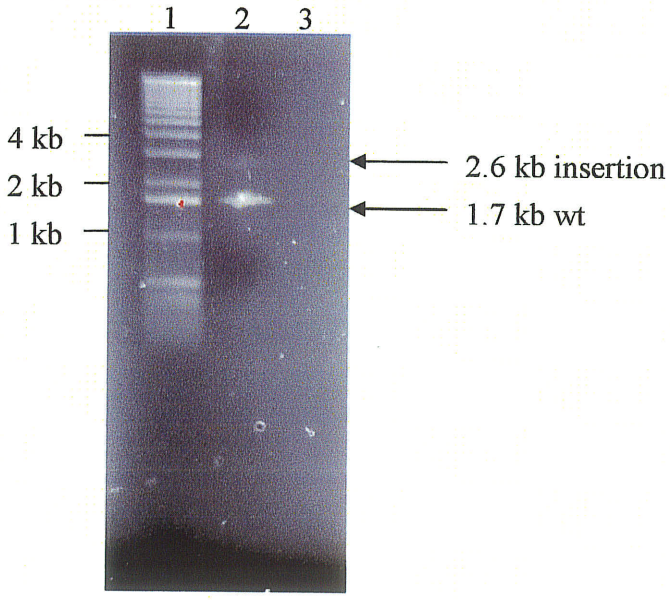

1 2 3
4 kb
2 kb
1 kb
2.6 kb insertion
1.7 kb wt

**Figure 5.5.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeD* mutants after sucrose selection.

Lane 1, 1 kb ladder; Lane 2, *sacB* product (SacBf/r; no band visible); Lane 3, ddH2O; Lane 4, *sdeD* (SdeD1f/r) product (2.6 kb band indicated); Lane 5, 1 kb ladder. 4 μL of samples were loaded in each lane.

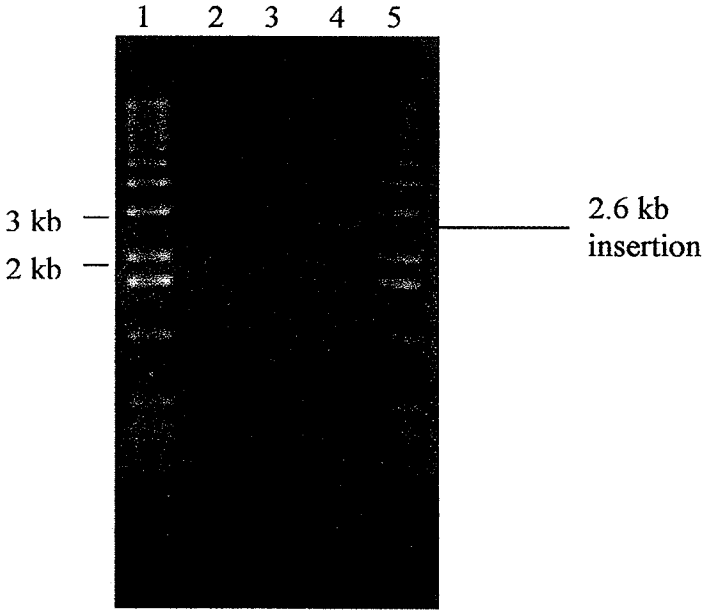

1
2
3
4
5
3 kb
2 kb
2.6 kb
insertion

**Figure 5.6.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeD* mutants using gentamicin$^r$ cassette primers.

Lane 1, 1 kb ladder; Lane 2, ddH2O; Lane 3, product using Gm$^r$ primers from within the cassette (650 bp amplified); Lane 4, larger fragment amplified (SdeDGmf/r, some of *sdeD* (around 500 bp) and some of the cassette (650 bp) yielding an approximate 1.1 kb product (boxed); Lane 5, 1 kb ladder. There is a shift in lane 4 compared to Lane 3 indicating the presence of the cassette. 5 μL of samples were loaded in each lane.

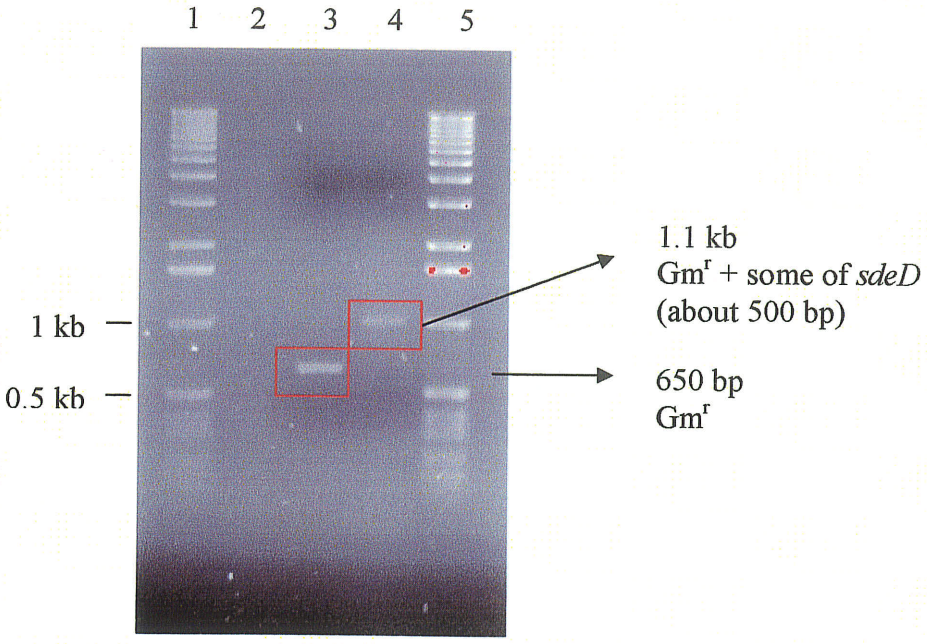
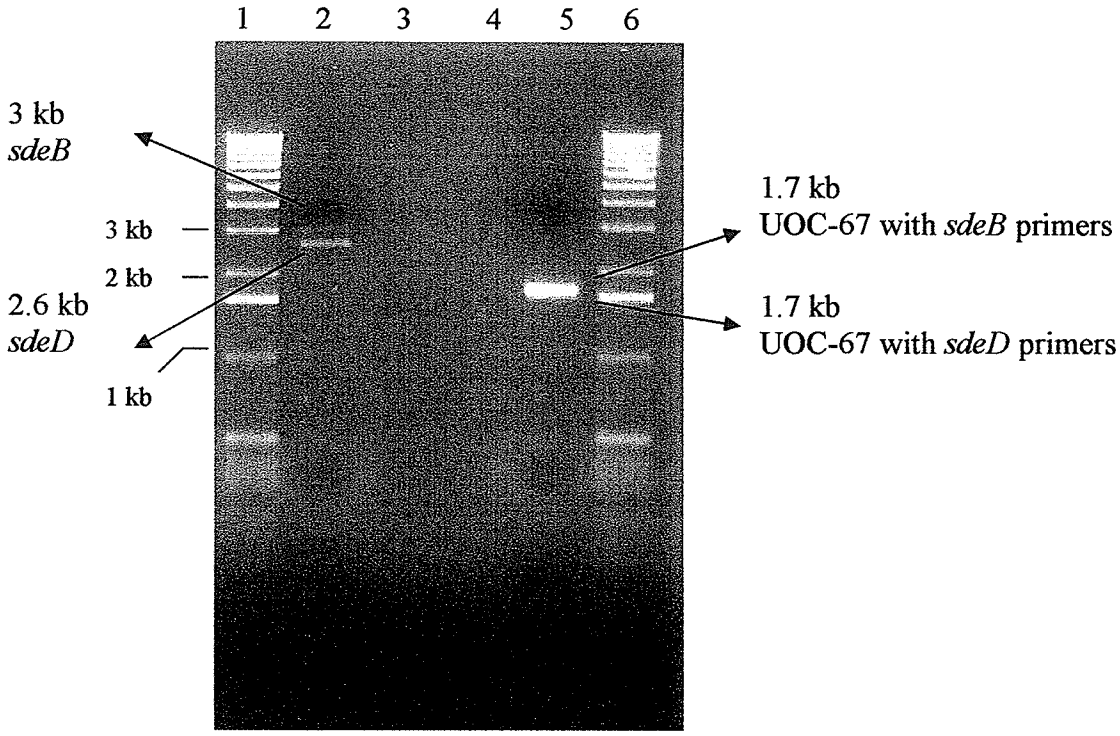

1 2 3 4 5
1 kb
0.5 kb
1.1 kb
Gm^r + some of sdeD
(about 500 bp)
650 bp
Gm^r

**Figure 5.7.** PCR products obtained from genomic DNA from putative *S. marcescens* *sdeB* and *sdeD* double mutants after sucrose selection.

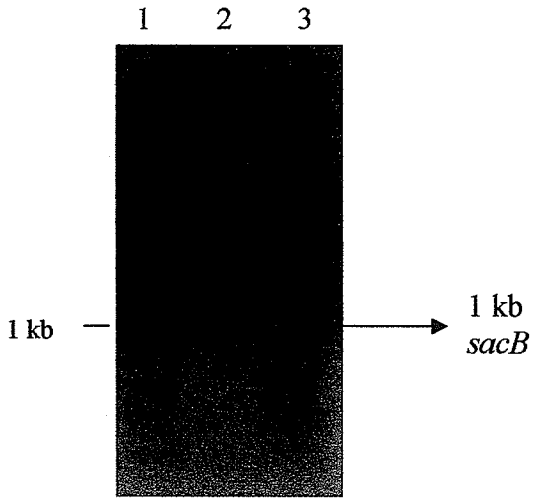
a) Lane 1, 1 kb ladder; Lane 2, *sdeD/sdeB* product (SdeD1f/r and SdeB1for/rev primers); Lane 3, *sacB* product; Lane 4, ddH2O; Lane 5, wt UOC-67 control (SdeD1f/r and SdeB1for/rev primers); Lane 6, 1 kb ladder . Molecular weights of PCR products are represented by 2.6 kb for *sdeD* and 3 kb for *sdeB*, respectively. Molecular weights of PCR products for UOC-67 are represented by a double 1.7 kb band (for each *sdeB* and *sdeD*). No band is obtained using *sacB* primers. 10 μL of samples were loaded in each lane.

b) After initial selection on kanamycin and gentamicin (no sucrose), *sacB* primers (SacBf/r) were used on the mutant for positive control as indicated by the presence of a 1.0-kb in Lane 2. Lane 1 and 3, 1 kb(+) ladder.

a)


1
2
3
4
5
6
3 kb
sdeB
3 kb
2 kb
2.6 kb
sdeD
1 kb
1.7 kb
UOC-67 with sdeB primers
1.7 kb
UOC-67 with sdeD primers

b)


1
2
3
1 kb
1 kb
sacB

### 5.4.2 Complementation of *sdeD*-deficient strains

Figure 5.8 shows a 7.4 vector band corresponding to pEX1.8 and the entire 4.9-kb wild type gene band for *sdeD*. The primers for the PCR reaction contained additional engineered *Eco*RI and *Pst*I sites (pEXDfor/rev). Figure 5.9 shows the stepwise digestion of pEXSD where the first digestion was performed with *Eco*RI (Step 1) to release the wild type *sdeB* gene, indicated by the 3.1-kb band, followed by a second digestion performed with *Pst*I (Step 2) resulting in the wild type *sdeD* gene release (4.9-kb). Additionally, please refer to the appendix for confirmation sequences throughout.

SDS-PAGE analysis analysis of whole cell lysates of SDECDE3 and SDECDE7 in Figure 5.10a confirms SdeB and SdeD absence in the SDECDE3 and SdeB and SdeD over-expression for SDECDE7 when induced by IPTG. SDS-PAGE analysis in Figure 5.10b confirms the presence of SdeD only in SDECDE5, presence of both, SdeB/SdeD in SDECDE4 due to SdeD over-expression, absence of SdeD in SDECDE1, presence of SdeB only in SDECDE6 and over-expression of both, SdeB/SdeD in SDECDE7 when induced by IPTG.

### 5.4.3 Antibiotic susceptibility tests

With the exception of novobiocin, no change in SDECDE1, SDECDE2 or SDECDE3 susceptibility in comparison to UOC-67, T-861 or SDEAB1, respectively, was seen for any other compounds tested (Table 5.3). For SDECDE1, SDECDE2 and SDECDE3, novobiocin susceptibility increased in comparison to UOC-67, T-861 and SDEAB1, respectively (Table 5.3). In addition, novobiocin susceptibility also increased for SDEAB1 in comparison to the wild type, accounting for the further increase in novobiocin susceptibility for SDECDE3, the *sdeB/sdeD* double mutant. MIC values for

**Figure 5.8.** Restriction digestion products of pEXD (*Eco*RI/*Pst*I digestion).

The primers for the PCR reaction contained additional engineered *Eco*RI and *Pst*I sites (pEXDfor/rev). The product from the PCR amplification (4.9-kb) was digested with *Eco*RI/*PstI* and cloned into the *Eco*RI/*Pst*I sites of pEX1.8. The vector band (7.4 kb) is the upper band (indicated by an arrow) and the insert wild type band is the lower band (indicated by a box) in Lane 2. 1 kb ladder (3 μL) is shown in Lane 1.

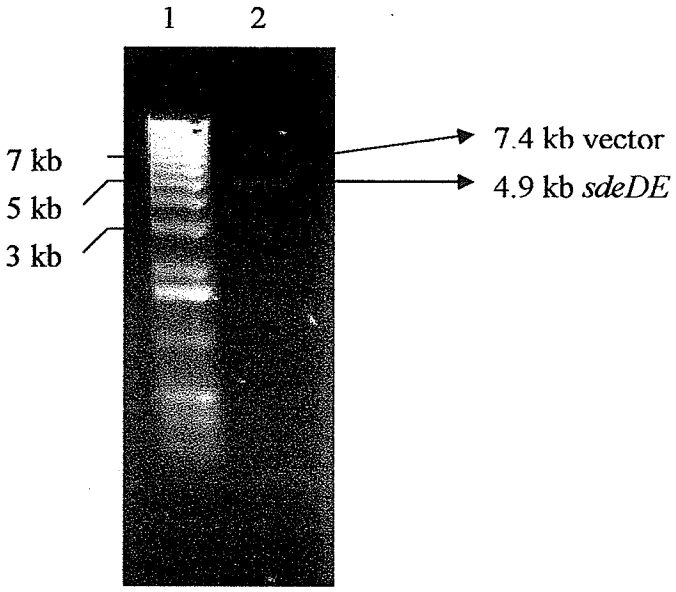
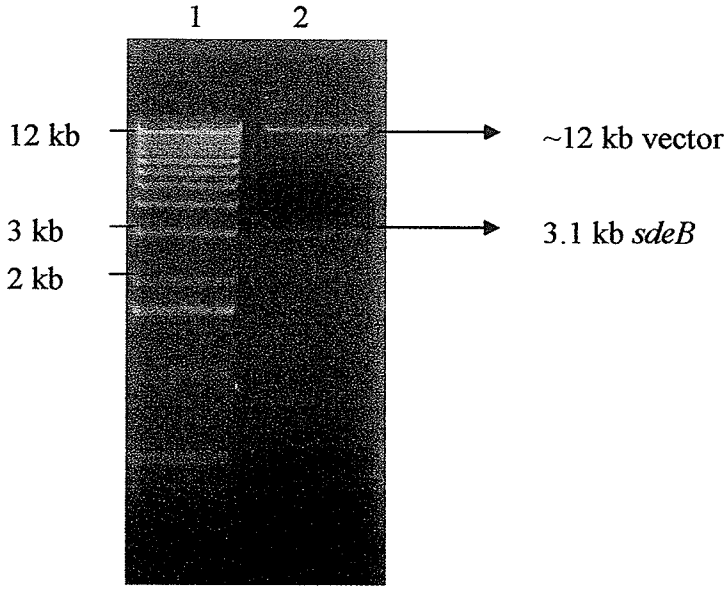

1
2
7 kb
5 kb
3 kb
7.4 kb vector
4.9 kb sdeDE

**Figure 5.9.** Restriction digestion products of pEXSD. To test for the presence of both wild type genes in pEX1.8JH, a stepwise digestion was done where first digestion was performed with *Eco*RI (Step 1) to release the *sdeB* gene (3.1 kb, indicated by arrow in Lane 2). The remaining 12 kb vector contains the gel purified wild type *sdeDE*. A second digestion performed with *Pst*I (Step 2) resulting in the wild type *sdeDE* gene (4.9 kb, indicated by arrow in Lane 2) and the approximate 7.4 kb vector band is indicated. 1 kb ladder (3 μL) is shown in Lane 1 of both Step 1 and Step 2.

Step 1


1
2
12 kb
3 kb
2 kb
~12 kb vector
3.1 kb sdeB

Step 2

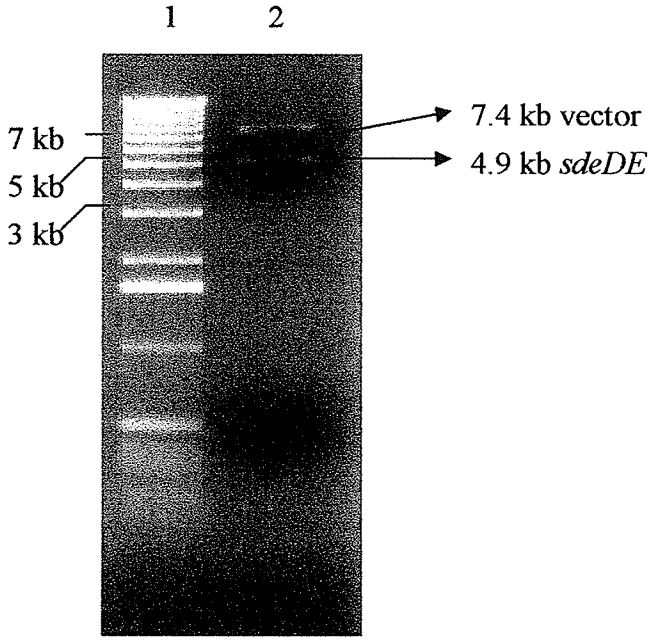
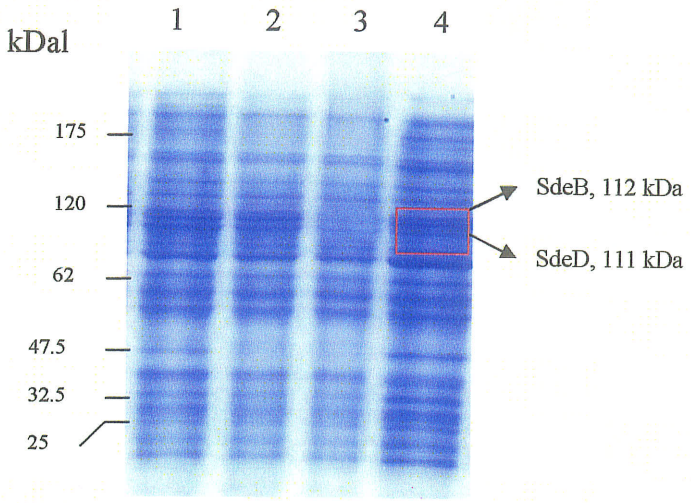

1
2
7 kb
5 kb
3 kb
7.4 kb vector
4.9 kb sdeDE

**Figure 5.10.** SDS-PAGE of whole cell lysates from *S. marcescens* SdeB and SdeD knockout and complemented strains.

a) Lane 1, whole cell lysates of SDECDE7 (*sdeB/sdeDE* double complemented strain) induced by 0.5 mM IPTG; Lane 2, UOC-67; Lane 3, SDECDE3 (*sdeB/sdeD* deficient); and Lane 4, SDECDE7 (*sdeB/sdeDE* double complemented strain) induced by 1 mM IPTG. SdeB and SdeD are of similar molecular weight; 112 kDa for SdeB and 111 kDa for SdeD. Molecular weights of markers are indicated along the side of the gel.

b) Lane 1, whole cell lysates of UOC67; Lane 2, SDECDE5 (*sdeB* deficient, complemented *sdeDE*) induced by 0.5 mM IPTG; Lane 3, SDECDE4 (*sdeDE* complemented strain) induced by 0.5 mM IPTG; Lane 4, SDECDE1 (*sdeD* deficient); Lane 5, SDECDE6 (*sdeD* deficient, complemented *sdeB*) induced by 0.5 mM IPTG; and Lane 6, SDECDE7 (*sdeB/sdeDE* double complemented strain) induced by 0.5 mM IPTG.

a)


kDal
1
2
3
4
175
120
62
47.5
32.5
25
SdeB, 112 kDa
SdeD, 111 kDa

b)

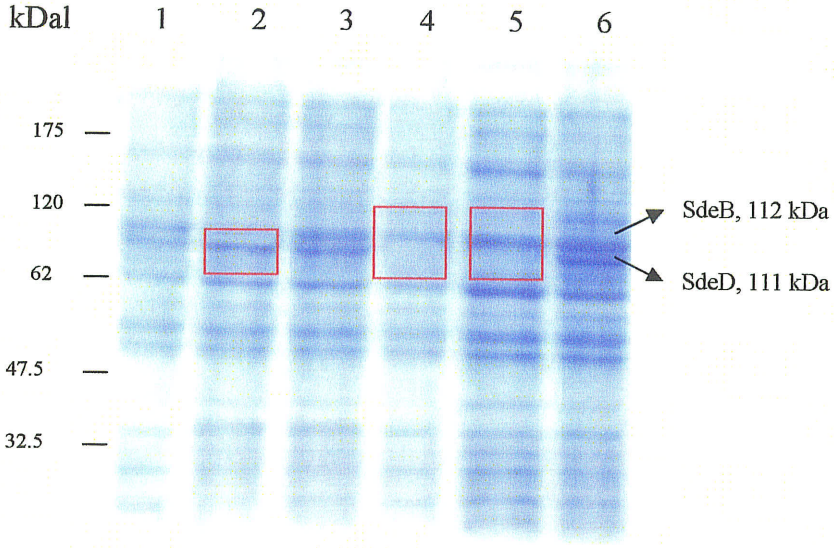

kDal
1
2
3
4
5
6
175
120
62
47.5
32.5
SdeB, 112 kDa
SdeD, 111 kDa

**Table 5.3.** The susceptibilities of wild type *S. marcescens*, clinical isolate and various mutant *S. marcescens* strains to a range of antibiotics.

| | MIC (μg/ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Cip | Nor | Ofl | Chl | DA | CA | Nov | SDS | EtBr |
| UOC-67 (wild type) | 1 | 1 | 1 | 32 | 1 | 1 | 64 | 1 | 1 |
| SDECDE1 (*sdeD*-deficient in UOC-67) | 1 | 1 | 1 | 32 | 1 | 1 | 8 | 1 | 1 |
| T-861 (clinical isolate) | 128 | 128 | 128 | 256 | 1 | 1 | 256 | 4 | 8 |
| SDECDE2 (*sdeD*-deficient T-861) | 128 | 128 | 128 | 256 | 1 | 1 | 32 | 4 | 8 |
| SDEAB1 (*sdeB* deficient UOC-67) | 0.125 | 0.125 | 0.125 | 4 | 1 | 1 | 16 | 0.25 | 0.25 |
| SDECDE3 (*sdeD*-deficient SDEAB1) | 0.125 | 0.125 | 0.125 | 4 | 1 | 1 | 2 | 0.25 | 0.25 |
| SDECDE4 (*sdeDE* complemented SDECDE1) | 1 | 1 | 1 | 32 | 1 | 1 | 64 | 1 | 1 |
| SDECDE5 (*sdeDE*-complemented SDECDE3) | 0.125 | 0.125 | 0.125 | 4 | 1 | 1 | 64 | 0.25 | 0.25 |
| SDECDE6 (*sdeB*-complemented SDECDE3) | 1 | 1 | 1 | 32 | 1 | 1 | 64 | 1 | 1 |
| SDECDE7 (*sdeB* and *sdeDE*-complemented SDECDE3) | 1 | 1 | 1 | 32 | 1 | 1 | 64 | 1 | 1 |

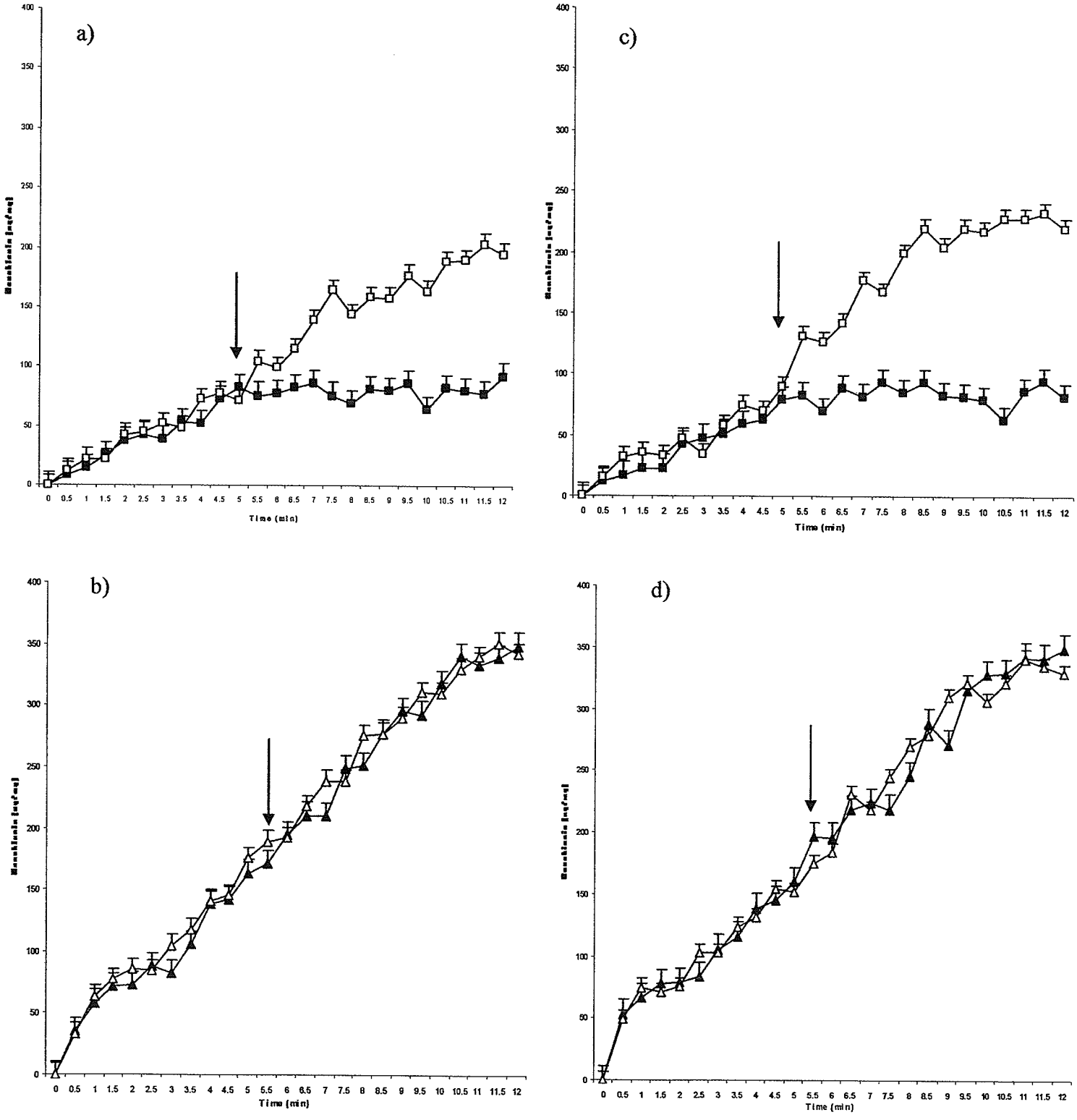
Note:
For complemented strains (SDECDE4, SDECDE5, SDECDE6 and SDECDE7) MIC values reported are in the presence of 1 mM IPTG.
Abbreviations: Nor, norfloxacin; Ofx, ofloxacin; Cip, ciprofloxacin; Chl, chloramphenicol; DA, deoxycholic acid sodium salt; CA, cholic acid sodium salt; Nov, novobiocin; SDS, sodium dodecyl sulphate; EtBr, ethidium bromide. Results are a representation of what was found for 3 trials.

SDECDE4, the SDECDE1 *sdeDE* complemented strain, and SDECDE7, the SDECDE3 *sdeDE* and *sdeB* complemented strain, were identical to those of wild-type UOC-67 for all antibiotics tested, whereas the values for SDECDE5, the SDECDE3 *sdeDE* complemented strain, were identical to UOC-67 only for novobiocin (the values for other compounds remain unchanged from those for SDECDE3). MIC values for SDECDE6, the SDECDE3 *sdeB* complemented strain, were identical to UOC-67 for all compounds. Therefore, the values suggest that both SdeB and SdeD are important for *S. marcescens* multidrug resistance; SdeD for novobiocin and SdeB for all antibiotics tested.

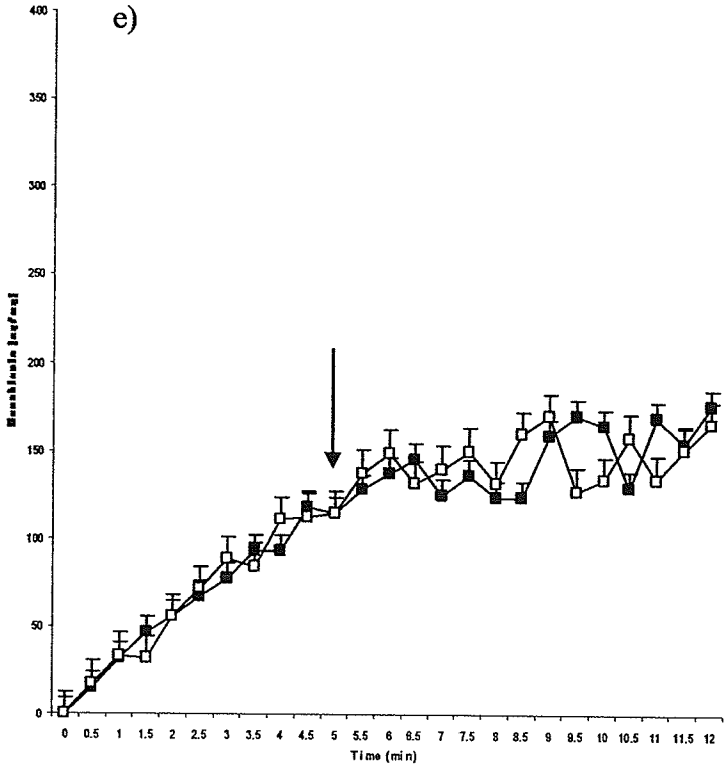
**5.4.4 Novobiocin accumulation in the presence of CCCP**

The loss of the SdeCDE efflux pump (SDECDE1 [Figure 5.11b], SDECDE2 [Figure 5.11d] and SDECDE3 [Figure 5.11f]) resulted in a dramatic increase in novobiocin accumulation as compared to UOC-67 (Figure 5.11a), T-861 (Figure 5.11c) and SDEAB1 (Figure 5.11e) parental strains, respectively. Addition of CCCP increased the accumulation of novobiocin for UOC-67 (Figure 5.11a) and T-861 (Figure 5.11c), but had no significant effect upon novobiocin accumulation in the knockout mutant strains (Figure 5.11b, d, e and f). No change in ciprofloxacin accumulation for the *sdeCDE* knockout strain SDECDE1 (Figure 5.12b), as compared to UOC-67 was seen (Figure 5.12a). Table 5.4 shows the initial rate of novobiocin uptake for the 30 sec and 1 min time intervals and agrees with our overall accumulation data in that, SDECDE1 and SDECD2 display a higher initial diffusion rate into the cell than UOC-67 and T-861, respectively. Also, SDECDE3, which is deficient in both SdeAB and SDECDE efflux pumps, shows a higher initial diffusion rate in comparison to SDEAB1 which is deficient

**Figure 5.11.** Accumulation of novobiocin by wild type *S. marcescens*, clinical isolate and various mutant strains: a) accumulation by UOC-67 in absence of CCCP (■); UOC-67 in presence of CCCP (□); b) SDECDE1 in absence of CCCP (▲); SDECDE1 in presence of CCCP (Δ); c) accumulation by T-861 in absence of CCCP (■); T-861 in presence of CCCP (□); d) SDECDE2 in absence of CCCP (▲); SDECDE2 in presence of CCCP (Δ); e) accumulation by SDEAB1 in absence of CCCP (■); SDEAB1 in presence of CCCP (□) and f) SDECDE3 in absence of CCCP (▲) and SDECDE3 in presence of CCCP (Δ). Proton motive force uncoupler CCCP (100 μM) was added 5 minutes after incubation (indicated by the arrow). All trials were performed in triplicate. Results were graphed by the EXCEL computer program.


a)
c)
b)
d)
Time (min)

e)


400
350
300
250
200
150
100
50
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5 11 11.5 12
Time (min)

f)

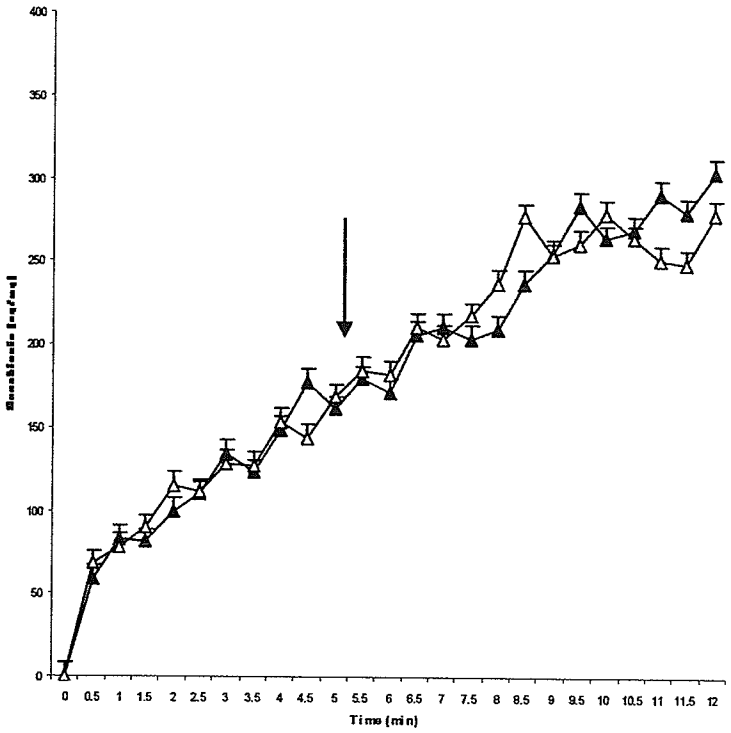
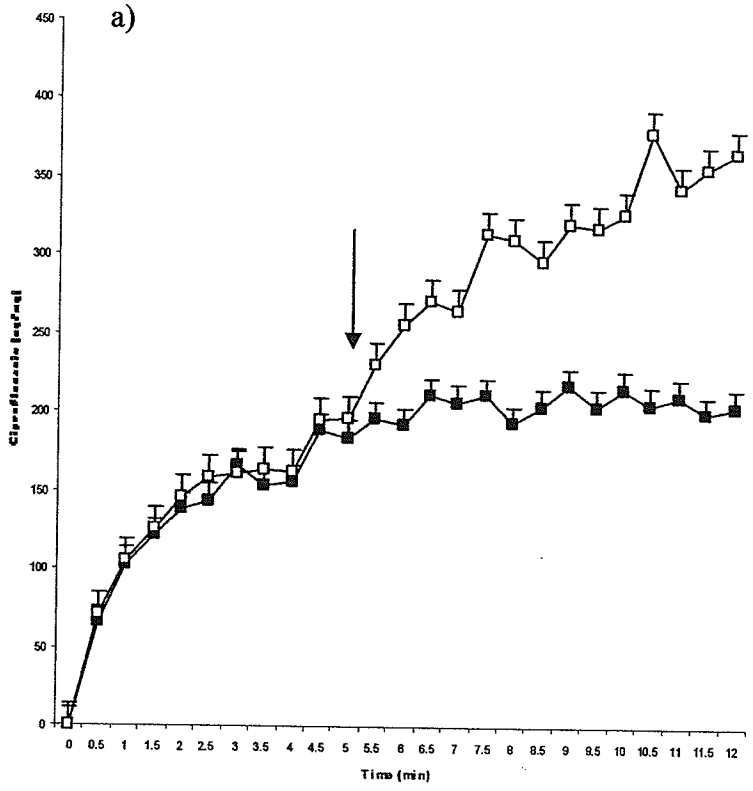

400
350
300
250
200
150
100
50
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5 11 11.5 12
Time (min)

**Figure 5.12.** Accumulation of ciprofloxacin by wild type *S. marcescens* and SDECDE1 mutant strains: a) accumulation by UOC-67 in absence of CCCP (■); UOC-67 in presence of CCCP (□) and b) SDECDE1 in absence of CCCP (▲) and SDECDE1 in presence of CCCP (Δ). Proton motive force uncoupler CCCP (100 μM) was added 5 minutes after incubation (indicated by the arrow). All trials were performed in triplicate. Results were graphed by the EXCEL computer program.

a)




b)

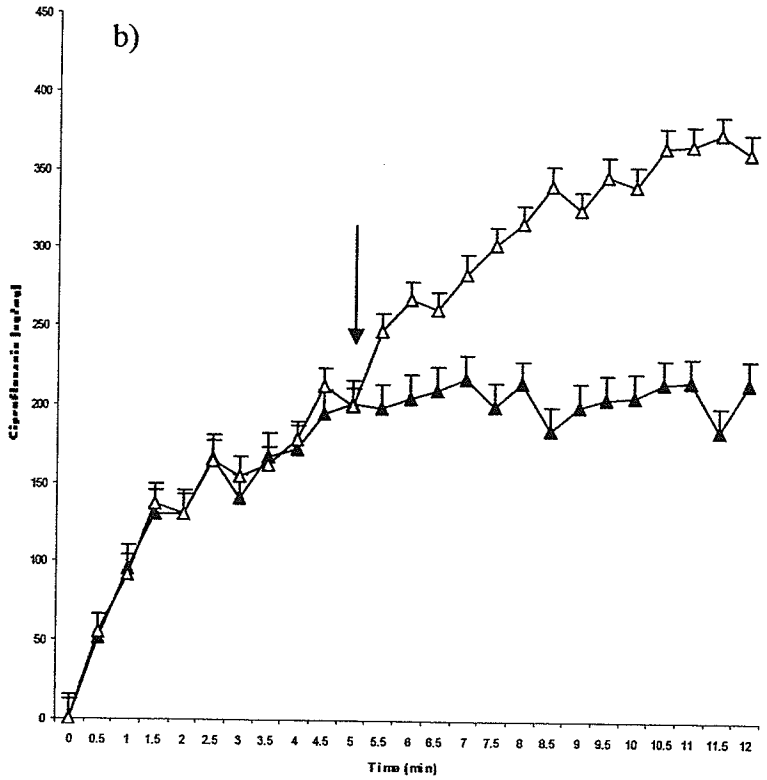


**Table 5.4.** Initial novobiocin uptake (initial diffusion into cells for the rate per minute taken after the first 30 second time interval and the 1 minute interval) for the wild type and mutant *S. marcescens* strains.

| Novobiocin uptake rate of diffusion (ng/mg/min) | | |
|---|---|---|
| | Initial rate at 30 seconds | Rate at 1 minute |
| wt UOC-67 | 16.46 (1.95) | 22.32 (4.72) |
| SDECDE1 | 70.12 (5.77) | 58.04 (8.09) |
| SDEAB1 | 29.20 (3.40) | 31.91 (4.84) |
| SDECDE3 | 118.42 (6.33) | 82.88 (8.28) |
| T-861 | 29.84 (2.38) | 31.76 (6.13) |
| SDECDE2 | 105.58 (8.58) | 66.04 (9.16) |

Note: Standard deviation is indicated in brackets.

The results were expressed as nanograms of novobiocin per milligram (dry weight) of cells.

Y = Fluorescence

W = Dry weight (mg)

Z = ng antibiotic accumulated

= Y/slope of the standard curve for given antibiotic

ng/mg of cells = Z/W

Mortimer and Piddock, 1991.

in SdeAB alone, suggesting the continuation of novobiocin net uptake due to the absence of a functioning SdeCDE pump. Therefore, SdeCDE plays an important role in accumulation of novobiocin.

### 5.4.5 Novobiocin accumulation in the presence of DNP

DNP (dinitrophenyl) was used as a protonophore (Figure 5.13), instead of CCCP to see its effect on UOC-67 and T-861 novobiocin accumulation. Similar results were obtained with DNP as compared to CCCP where the novobiocin accumulation for both UOC-67 (Figure 5.13 a and b) and T-861 (Figure 5.13 c and d) increased upon DNP addition, however accumulation was faster for T-861 than UOC-67 suggesting a more effective active efflux system. In T-861, an efflux mechanism may be overexpressed, but this possibility was not examined in this thesis.

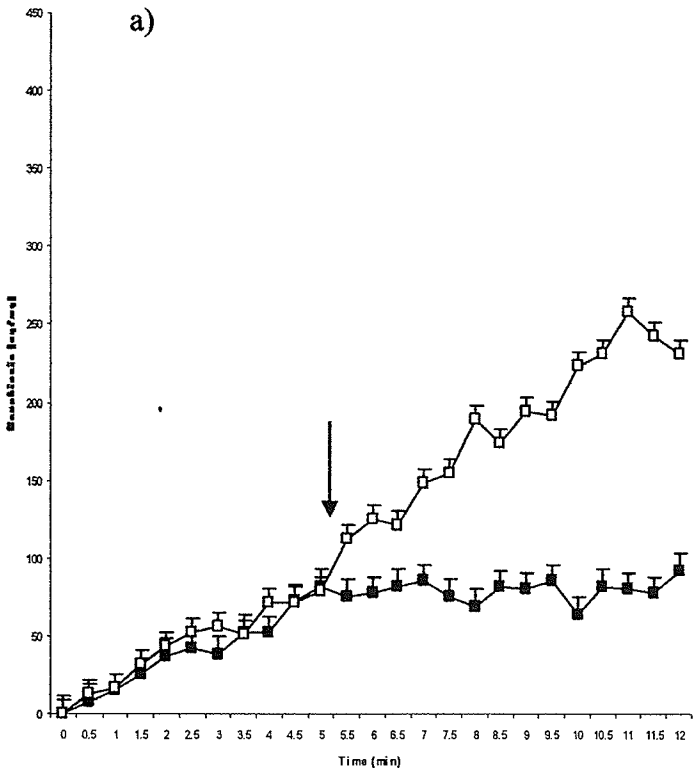
## 5.5 Discussion

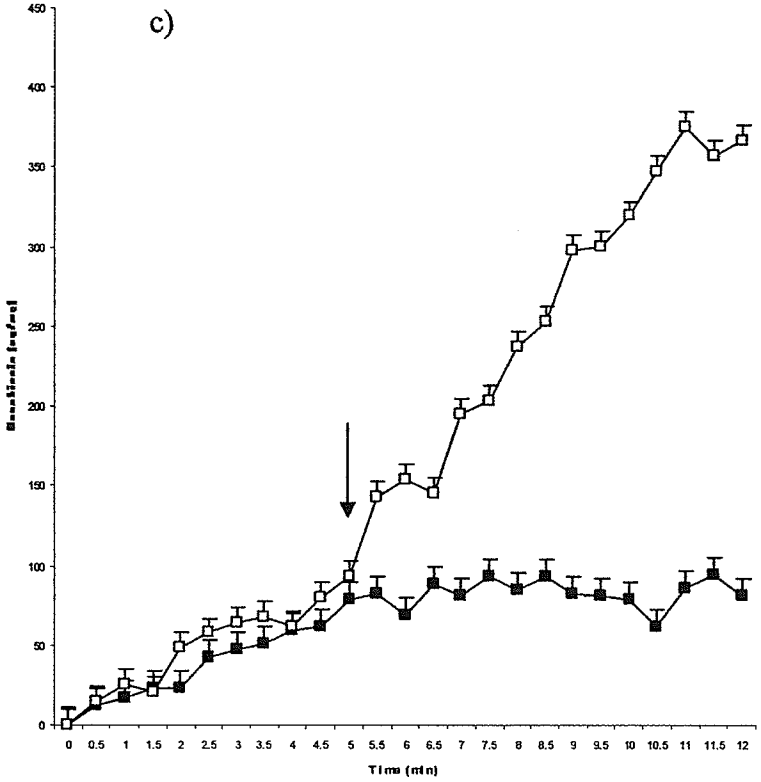
Based on the limited number of substrates examined in this study, we conclude that SdeCDE has a restrictive selectivity. SdeCDE conferred resistance only against novobiocin, a competitive inhibitor of DNA gyrase (Maxwell and Lawson 2003) which disables the ATPase reaction catalyzed by GyrB. Its potency is significantly higher than that of fluoroquinolones that target the GyrA subunit of DNA gyrase (Maxwell and Lawson 2003). Similar results have been reported for the SdeCDE homolog, MdtABC of *E. coli* (Nagakubo et al. 2002). MdtABC only confers resistance against novobiocin and bile salt derivatives (Nagakubo et al. 2002). SdeCDE differs in that bile salt derivatives are not substrates (Table 5.3).

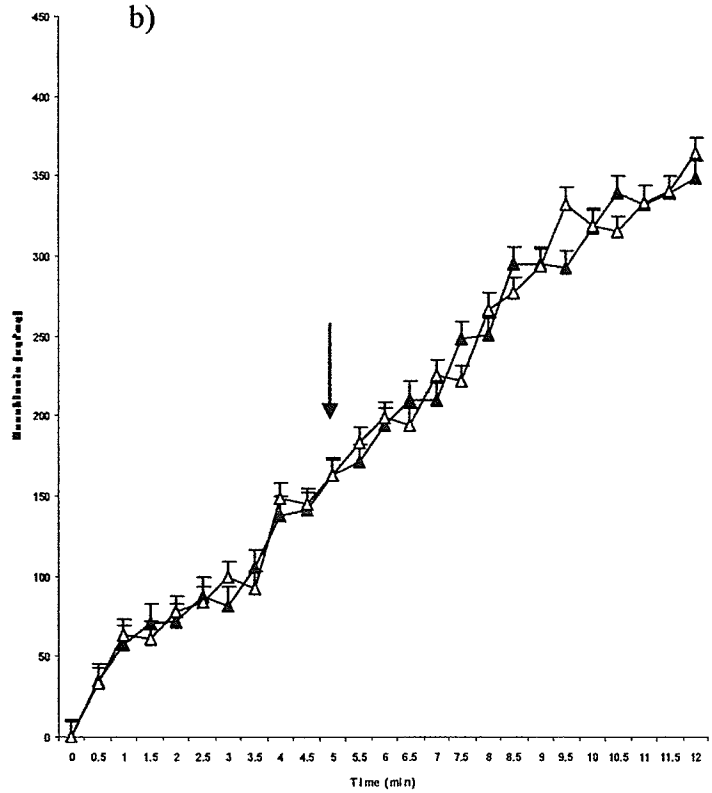
Conclusions from this series of experiments are based in part on accumulation assays. It is often difficult to make quantitative estimations from accumulations assays.

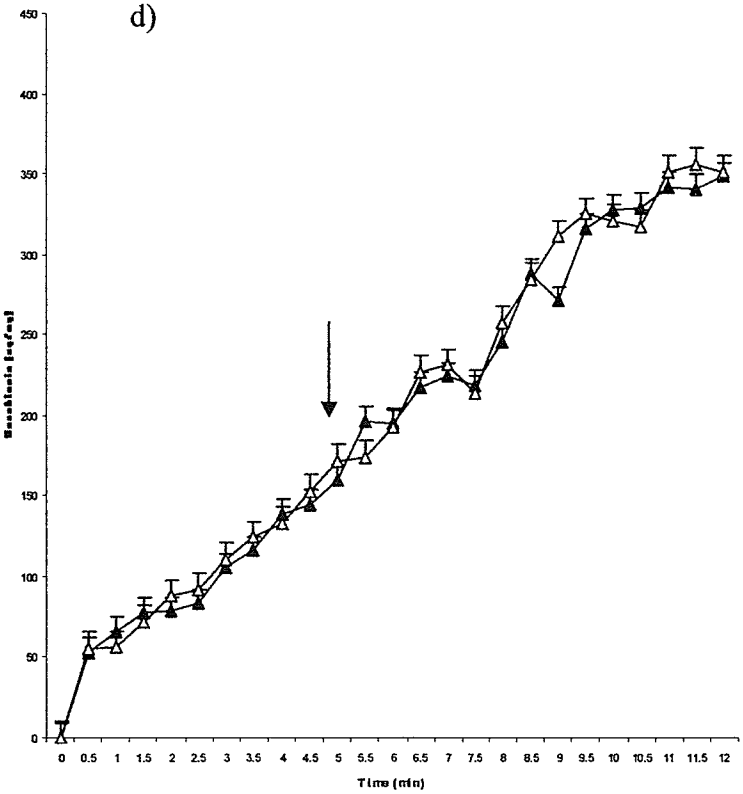
**Figure 5.13.** Accumulation of novobiocin by wild type and clinical *S. marcescens* strains using DNP (dinitrophenyl) as the protonophore (100 μM).

a) accumulation by UOC-67 in absence of DNP (■); UOC-67 in presence of DNP (□); b) SDECDE1 in absence of DNP (▲); SDECDE1 in presence of DNP (Δ); c) accumulation by T-861 in absence of DNP (■); T-861 in presence of DNP (□) and d) SDECDE2 in absence of DNP (▲) and SDECDE2 in presence of DNP (Δ). PMF uncoupler DNP (100 μM) was added 5 minutes after incubation (indicated by the arrow). All trials were performed in triplicate. Results comparable to the assay where the CCCP was used as the PMF uncoupler (Figure 5.11). Results were graphed by the EXCEL computer program.


a)
Time (min)


c)
Time (min)


b)
Time (min)


d)
Time (min)

However, qualitative interpretations regarding the presence/absence of proton gradient-dependent efflux mechanism are a consistent and valid way to observe accumulation differences for wild type versus knockout mutant strains. For UOC-67, there is an equilibration of antibiotic level inside the cell, and after the CCCP addition (T=5 min), accumulation of antibiotic resumes. UOC-67 accumulation levels out if the CCCP is not added as early as 5 minutes after addition of antibiotic (Figure 3.8) suggesting that the presence of a functioning efflux pump is responsible for the gradual depletion of antibiotic accumulation within the cell. However, when CCCP is added 10 minutes after addition of antibiotic, accumulation once again continues (Figure 3.8). CCCP appears to have an effect on UOC-67 accumulation, inconsistent with previous observations that lab-derived strains that were generated by serial passaging of UOC-67 exclusively in the presence of a certain fluoroquinolone show a strong CCCP effect (Kumar and Worobec 2005a). For T-861, there is also a lower rate of antibiotic accumulation inside the cell, but upon CCCP addition, novobiocin accumulation rate increases somewhat more rapidly than UOC-67 (Figure 5.11 a and c), demonstrating the CCCP effect. This indicates the presence of a relatively active efflux system in the quinolone resistant strain (T-861) while UOC-67 has an active but slightly weaker efflux pump. This difference is more pronounced in Figure 5.13 where in the presence of DNP, T-861 accumulation rate is more rapid in comparison to UOC-67 demonstrating an active efflux system in T-861.

In regards to susceptibilities of *sdeDE* complemented strains, such as SDECDE4 and SDECDE7, MIC values are identical to those of UOC-67 for all antibiotics tested. One may expect higher MICs for these strains due to IPTG induction resulting in more copies of SdeD and SdeE in the cell. Wild type MICs were likely observed because of the

limited number of copies of the outer-membrane component, *hasF*. *hasF* was not overexpressed, nor was *sdeC*, leaving the overexpressed SdeD and SdeE with a limited amount of outer membrane proteins to use to expel the drug. No other homolog of *hasF* was found in the genome search of *S. marcescens*. It is very likely that *hasF* is the only efflux pump associated outer membrane component in this organism. This is similar to *E. coli* where TolC is the only functional outer membrane component known to be acting in concert with different efflux pumps.

In summary, we have used knockout mutagenesis to establish the contribution of the SdeCDE efflux pump to the intrinsic antibiotic resistance of *S. marcescens* UOC-67 and T-861 strains. I conclude that the SdeCDE pump has a limited substrate specificity based on the substrates examined.

## CHAPTER 6 General Discussion

### 6.1 Recent Developments

Several new developments have occurred in the past few years which have enhanced the scope of the research I performed. Bacterial adaptation to environmental stress is a key step in the emergence of multidrug-resistant strains that are a threat to human health. Although I attempted to describe some of the potential regulators involved in antibiotic resistance mechanisms (Begic and Worobec 2005), more research needs to be done in this area, especially in the area of bacterial response to external stresses. Recently, a study was done in this area (Dupont et al. 2007), where the expression of *ompX* porin in *Enterobacter aerogenes* is increased in response to environmental stress, much in the same way as *S. marcescens ompC* expression and at the same time, the level of *E. aerogenes ompF* porin expression was negatively affected, similar to our findings (Begic and Worobec 2005). The study carried out by Dupont et al. 2007 concluded that because of the declining *ompF* expression, response in the presence of environmental stressors, such as salicylate and osmotic pressure and because of the role of these porins in membrane permeability, OmpF and OmpX are involved in the control of the penetration of antibiotics such as fluoroquinolones (as per Chapter 3). Many parallels can be formed between the *E. aerogenes* study and what I have presented in this thesis.

To date, only three RND *S. marcescens* pumps have been characterized, two of which have been described in this work, and SdeXY, which confers norfloxacin and tetracycline resistance and is a close homologue of the *E. coli* AcrAB-TolC pump (Chen et al. 2003). Evidence is emerging for non-RND, but rather the MF (Major Facilitator) family of pumps in *S. marcescens*, such as the SmfY (Shahcheraghi et al. 2007) and

SsmE (Minato et al. 2008) pumps which are both considered to be multidrug efflux pumps showing resistance to many structurally unrelated antibiotics.

**6.2 Methodology for measuring antibiotic resistance**

Throughout the thesis, antibiotic accumulation has been used as a method for detecting the activity of efflux pumps. RND pumps, such as SdeAB and SdeCDE are energized by the PMF and CCCP is a protonophore that acts as a pump PMF inhibitor. I would like to address several anomalies associated with this method. One unanswered question is why a high concentration of CCCP is used in these antibiotic accumulation assays (Chapters 3, 4 and 5).

With respect to antibiotic resistance and efflux pumps, one of the lowest concentrations reported in comparison to 100 μM, has been 50 μM (Tavio et al. 2004). In this case, effect of salicylate, diazepam and benzoate on norfloxacin uptake was observed in *K. pneumoniae* strains. No change in norfloxacin uptake was reported when the higher CCCP concentration was used, suggesting that CCCP concentration does not have an effect on antibiotic uptake itself and 100 μM assay CCCP concentration would not have an adverse effect that would skew assay results further.

Additionally, I have performed antibiotic uptake assays for select knockout mutants with another protonophore, DNP (dinitrophenyl) at 100 μM. Others have used as high as 1000 μM of DNP in their accumulation assays (Piddock et al. 2000; Williams et al. 1998). I observed similar trends for the DNP inhibition as seen with CCCP inhibition (Figure 5.13).

Evidence exists that CCCP may be excreted out of the cell using efflux pumps (Yin et al. 2006), specifically with the aid of the accessory protein (equivalent to *S.*

*marcescens* SdeA or SdeC). This may explain why a high concentration of CCCP needs to be applied. It has also been reported that CCCP has cytotoxic effects on quinolone sensitive *E. coli* strains (Cho et al. 2001). Here the authors only required 10 μM CCCP to inhibit ofloxacin accumulation in quinolone-susceptible strains. In this same study, 100 μM CCCP was used to collapse the efflux pump in quinolone-resistant strains and prevent ofloxacin from being excreted out of the cell (Cho et al. 2001). It was hypothesized that the higher CCCP concentration would have a harmful effect on cells because it dissipates the proton gradient and the quinolone-susceptible bacterial cells are more easily damaged by CCCP than the quinolone-resistant bacteria resulting in leakage of the intracellular materials; therefore less intracellular quinolone would be accumulated (Cho et al. 2001). CCCP efflux functions by being intercepted by the efflux transporter on the inner membrane as it crosses towards the cytoplasm. In the absence of drug efflux, CCCP diffuses across the inner membrane from the periplasmic space in the protonated form, disrupting the pH differential as it moves into the cytoplasm (Yin et al. 2006). The molecule next releases its proton to become a lipophilic soluble anion that rapidly diffuses back into the periplasm. The possibility remains that CCCP may bind to the inner membrane transporter (eg. with *S. marcescens* SdeB or SdeD/E) through hydrophobic interactions on the inner leaflet side acting as a competitive inhibitor. However, it is speculated that proton translocation and drug transport may occur at different locations, in which case there would be no competition for the same transporter binding site and the drug would be effluxed (Yin et al. 2006). However, this could explain why a high CCCP concentration is used.

Two methods for measuring antibiotic uptake in gram negative organisms that have been reported, the fluorescence measurement approach we have employed, and measuring uptake of a radiolabelled antibiotic using a vacuum filtration method (Zhanel et al. 1995; Lowry et al. 1951). In the later case, the cells are harvested on filters, washed with buffer, the filters dried and the radioactivity determined by the scintillation counting. This method does have disadvantages such as non-specific binding of the labeled antibiotic to the filter and radiolabelled antibiotics are very expensive. I have used the fluorescence method previously described by Mortimer and Piddock, 1991. It was difficult to make reproducible quantititative estimations from these assays mainly due to variations between culture batches, especially for norfloxacin, one of the fluoroquinolones used in my studies. I experienced much less difficulty with ciprofloxacin, which is why all accumulation data reported in the thesis is that of ciprofloxacin. In spite of their limitations, these assays serve as a solid method to make qualitative interpretations regarding the presence or absence of proton gradient-dependent efflux. My rationale for using this assay was to observe antibiotic accumulation differences between wild type and pump knockout strains. I was able to consistently obtain these qualitative results for each of the trials. CCCP in each case was added at minute 5 of the accumulation assay as I noticed in the absence of CCCP it is around this time point that the start of a plateau is reached (Figure 3.1) suggesting equilibrium between rate of efflux and uptake had been reached. UOC-67 accumulation does level out at approximately 5 minutes after the addition of antibiotic (Figure 3.8) suggesting that the presence of a functioning efflux pump is responsible for the gradual depletion of antibiotic accumulation within the cell. However, when CCCP is added to the assay even

10 minutes after the addition of antibiotic, accumulation once again increases (Figure 3.8) as the energy driving the efflux pump is collapsed. One could thus conclude that antibiotic accumulation is time-based (Berlanga et al. 2000), but also depends on the concentration of the antibiotic used (10 μg/ml, which I use, and has been widely reported).

I measured antibiotic uptake for 12 minutes in 30 sec intervals. Figure 4.25 shows an extended ciprofloxacin accumulation assay for wild type UOC-67 strain and the T-861 clinical strain where uptake measurements were extended to 30 minutes (in 1 min intervals). For the T-861 strain, accumulation past minute 20 decreases gradually due to the efflux pump activity, while in the presence of CCCP, accumulation continues past 30 minutes. For UOC-67, CCCP clearly also has the same effect, however the accumulation did not decrease as slowly and gradually as for T-861 in the absence of CCCP, and showed more of a plateau. Additionally, for UOC-67, accumulation did not increase as rapidly in the presence of CCCP as for T-861. This reflects T-861 being more resistant to quinolones presumably due to increased pump production and as such would efflux more antibiotic than a more susceptible strain such as UOC-67.

Another concern with CCCP is that it is prepared in an ethanol solution which may have a physiological effect. In our Chapter 3 studies, CCCP was solubilized in ethanol (70%) and then further diluted in 10 mM NaOH to dilute the amount of ethanol in the medium. Studies have shown that prepared in this fashion, ethanol has no significant effect on bacterial viability (Lambert and Le Pecq 1984). Ciprofloxacin accumulation by UOC-67 conducted in the presence of ethanol alone (no CCCP) was also studied (Figure 3.7). 70% ethanol alone did not have an effect on ciprofloxacin accumulation within our

experimental parameters and accumulation studies in Chapters 4 and 5 were performed without CCCP dilution in NaOH.

Due to concerns with the CCCP-based technique and the differences in the appearance of graphical plots of accumulation even within publications from the same group (Kumar and Worobec 2005a; Berlanga and Vinas, 2000), many investigators are opting not to use graphs in publications, but rather express data in a table format instead (Piddock et al. 2000). Others rely on MIC data alone to interpret their results (Schurek et al. 2005; van Amsterdam et al. 2005).

One has to keep in mind that the methodology for antibiotic accumulation assays is dependant on the growth conditions of the organism. It was thought that proton motive force is necessary for the growth of bacteria which have a redox pump for protons (Kinoshita et al. 1984). However, it is possible that proton motive force is not obligatory for the growth of *E. coli* at pH 7.5 when glucose is used an as energy source (Kinoshita et al. 1984). In any case, more studies need to be done regarding antibiotic accumulation assays employing CCCP, specifically regarding the optimization of CCCP concentration used in order to detect efflux, especially in studies that deal with both, quinolone-susceptible and quinolone-resistant strains.

## CHAPTER 7 Conclusions and Future Directions

### 7.1 Conclusions

**Objective 1.** Site-directed mutagenesis studies to probe the role of specific residues in the external loop (L3) of OmpF and OmpC porins in susceptibility of *Serratia marcescens* to antibiotics (Chapter 2).

1. The amino acid at position 114 of OmpC porin is not responsible for either antibiotic size or ionic selection, the amino acid at position 112 is responsible for size selection only, and position 124 is involved in both, size and ionic selection within the porin third loop.

From these experiments, I have demonstrated that porin alterations can result in increased antibiotic resistance due to a modification in porin permeability. Larger side-channel amino acids within a porin (if at a turn-forming, loop 3 location) may extend into the pore channel contributing to size constriction by conferring a smaller channel in the eyelet loop region of a porin resulting in a slower antibiotic passage. These results agree with those reported in other organisms, namely the porins of *E. coli* (Jeanteur et al. 1994) and *E. aerogenes* (Thiolas et al. 2004).

**Objective 2.** To study the fluoroquinolone resistance of *Serratia marcescens* in terms of the osmolarity, salicylate, temperature and pH induction of phenotypic resistance (Chapter 3).

1. Accumulation of fluoroquinolones, specifically ciprofloxacin, decreased when *S. marcescens* was grown in high concentrations of salicylate (8 mM) and sucrose (10% w/v), high temperature (42°C) and pH 6, and was restored only in

the presence of CCCP, due to the collapse of proton gradient-dependent efflux in *S. marcescens*. More importantly, the diffusion rate varied under a variety of different growth conditions, such as pH, temperature, salicylate and osmotic stress.

2. Although nalidixic acid accumulation was observed, it was not affected by salicylate, sucrose, pH or temperature changes.

3. CCCP effect on disruption of equilibrium concentrations of antibiotic implies that proton gradient efflux is responsible for controlling the final concentration of antibiotic in the cell.

I have demonstrated that an increase in antibiotic resistance is partially a result of decreased production of OmpF. Osmotic pressure, salicylate, high temperature and an acidic pH can reduce ciprofloxacin uptake due to reduced porin expression.

**Objective 3.** To study the role of *Serratia marcescens* SdeAB multidrug efflux pump and TolC-homolog in fluoroquinolone resistance studied using gene knockout mutagenesis (Chapter 4).

1. *sdeB*, *hasF* and *sdeR* knockout strains were consistently more susceptible to antibiotics than the parent strains, with the *sdeB/hasF* double knockout strain showing the highest susceptibility.

2. A marked increase in fluoroquinolone (ciprofloxacin) accumulation was observed for strains deficient in either the *sdeB* or *hasF* genes when compared to the parental strains, with the highest ciprofloxacin accumulation observed for the *sdeB/hasF* double knockout.

3. Antibiotic accumulation assays for the *sdeB* knockout mutant strains performed in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore, confirmed previous results that SdeAB mediated efflux is proton motive force dependent.

4. Due to the comparable susceptibility of the *sdeB* and the *hasF* individual knockouts, I conclude that *S. marcescens* HasF is the sole outer membrane component of the SdeAB pump.

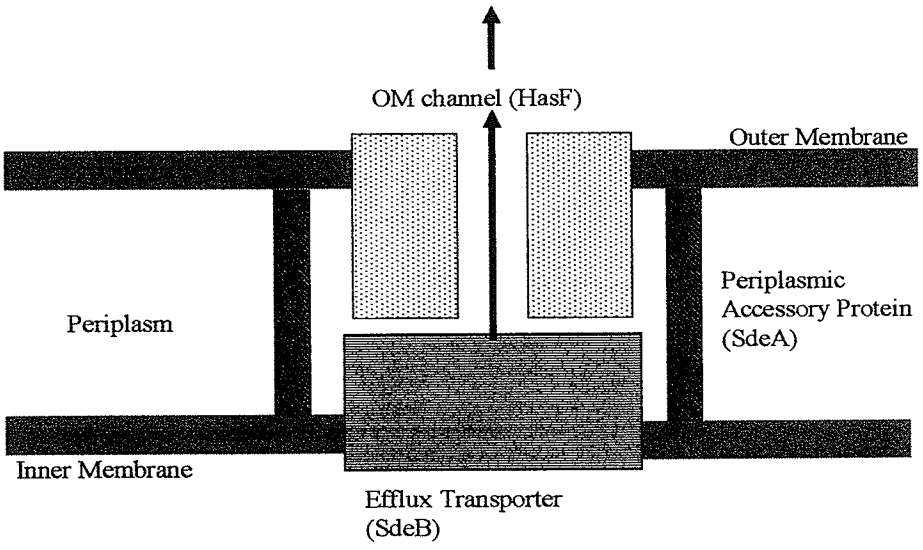
5. MIC data for *sdeR*-deficient and overexpressing strains confirm that SdeR is an activator of *sdeAB*.

The summary of the results from Objective 3 can lead to the predicted model below. In regards to Model A, the SdeAB efflux pump would form a three-component system, consisting of an inner membrane component (SdeB), a periplasmic component (SdeA), and an outer membrane component (HasF). Presumable, as for the *E. coli* model, when the end of the HasF tunnel contacts the top of the periplasmic domain of SdeB, a 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.

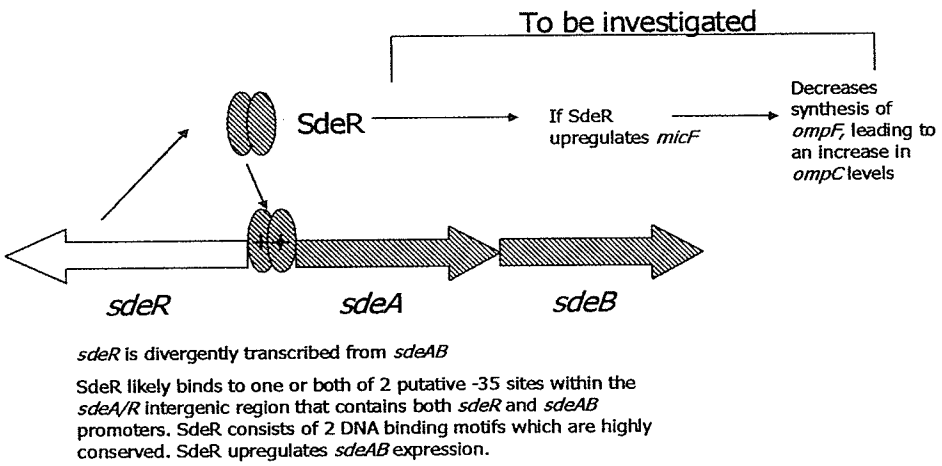
In regards to Model B, work is on-going to determine the precise SdeR binding sites within the *sdeA/R* intergenic region and its role on upregulation of genes other than *sdeAB,* for instance *micF*, which would enhance this schematic representation. As mentioned in Chapter 4, at the amino acid level, SdeR is 40% homologous to the MarA protein of *E. coli*, a transcriptional activator of the AcrAB-TolC drug efflux pump (Alekshun and Levy 1997; Barbosa and Levy

2000; Hachler et al. 1991). Amino acid prediction and 3-dimensional structural prediction also showed similarity to the MarA protein of *E. coli* with high degree of conservation of the DNA binding helices. The global nature of MarA has been demonstrated using gene array analysis, in which the protein was shown to constitutively regulate the expression of over 60 *E. coli* genes (Barbosa and Levy 2000), including *micF*. Production of *micF* would in turn downregulate the expression of *ompF* (Delihas and Forst 2001). Further studies need to be conducted to determine if SdeR can regulate gene expression globally, in much the same way as MarA, including as an activator of the *micF* transcription.

A)



B)




**Objective 4.** To characterize *Serratia marcescens* SdeCDE multidrug efflux pump using gene knockout mutagenesis (Chapter 5).

1. *sdeCDE* knockout strains showed no change in antibiotic susceptibility in comparison to the parental strains for any of the substrates tested with the exception of novobiocin. Novobiocin was the only antibiotic to be accumulated by *sdeCDE* deficient strains. My results demonstrate that SdeCDE is a highly specific pump belonging to the Resistance-Nodulation-Cell Division family.

I speculate that the SdeCDE is mostly inactive. It is possible that, from an evolutionary point of view, SdeD is derived from SdeE and that the transporter is slowly gaining an extension of substrate range by the replacement of an ancestral SdeE homomultimer with the SdeDE heteromultimer. In our studies, SdeCDE

conferred resistance only against novobiocin, a competitive inhibitor of DNA gyrase (Maxwell and Lawson 2003) which disables the ATPase reaction catalyzed by GyrB. Its potency is significantly higher than that of fluoroquinolones that target the GyrA subunit of DNA gyrase (Maxwell and Lawson 2003). Novobiocin (612 Da) is larger than most fluoroquinolones, which range in molecular mass from 300-380 Da. This suggests that an SdeD/SdeE heteromultimer may be required to accommodate this large substrate. Similar results have been reported for the SdeCDE homolog, MdtABC of *E. coli* (Nagakubo et al. 2002).

In conclusion, results from objectives 1-5 were essential to further the understanding of the mechanisms of antibiotic resistance in *S. marcescens*. Over the last ten years, an increasing number of antibiotic resistant *S. marcescens* strains have been isolated, making treatment challenging. The basis of this resistance is typically multifactorial, and the research streaming from these experiments clearly demonstrated the importance of two of these resistance mechanisms: a) decreased outer membrane permeability and b) resistance-nodulation-cell division (RND) pump mediated active efflux.

**7.2 Future Directions**

Logically, the next step in this research needs to readdress the topic of porin regulation in more detail. Specifically, one would need to find out the role of the EnvZ-OmpR system as well as the MicF system in the regulation of *ompF* and *ompC* porin genes. Deletion mutants of the *S. marcescens envZ, ompR* homologs and *micF* should be

constructed. Suicide vectors to knock out these genes need to be constructed and introduced into various *S. marcescens* strains, such as UOC-67 and T-861, either by tri-parental mating or electroporation. The construction of these knockout mutants would provide conclusive evidence as to the role of each regulatory system in *S. marcescens.* Additionally, recently, *micC,* a novel small-RNA gene encoded by the *E. coli* genome was identified that encodes a ~100-nucleotide small-RNA transcript (Chen et al. 2004). Twenty-two nucleotides at the 5' end of this transcript have the potential to form base pairs with the leader sequence of the mRNA encoding the outer membrane protein OmpC (Chen et al. 2004). It is presumed that in *E. coli*, MicC hinders the translation of OmpC in much the same way it has been described for MicF/OmpF hybrid, therefore playing a significant role in porin regulation. It is also necessary to knock out the *S. marcescens micC* homolog to confirm this finding and look into *micC* gene expression via β–galactosidase assays. The work on this objective is under way by MSc student Jalil Nasiri.

Secondly, more needs to be discovered about certain key amino acid residues that may play a structural or functional porin role. For instance, two cysteines are present in external loop 6 of the *S. marcescens* OmpF porin that are absent in other OmpF sequences. However, cysteines have been identified in the *Ps. aeruginosa* porins, OprF (Duchene et al. 1988) and OprB (Wylie and Worobec, 1994) and the LamB porin *of E. coli* (Clement and Hofnung, 1981). In the non-selective monomeric porin, OprF, the four cysteines present are proposed to function in switching between the two functional pore sizes observed for this porin through alternate disulphide bonds (Hancock 1987). However, in the glucose-selective OprB and maltodextrin-selective LamB porins, the

cysteines are not thought to play any functional role. In the case of LamB, replacement of the two cysteines with serine had no effect on maltodextrin binding (Ferenci and Stretton, 1989). Through site-directed mutagenesis, altering one or both of these sites through PCR, as was done in Chapter 2, one could see if these play a significant role in OmpF porin function.

Thirdly, I have only begun to explore the SdeCDE pump. Analysis of the *sdeE* downstream sequence revealed that there is a MFS (Major Facilitator superfamily) pump-encoding gene present. Such a gene is present downstream of the *E. coli mdtABC* locus as well, however it is not required for the functioning of the pump. It is important to investigate the role of this MFS protein in the functioning of the SdeCDE pump to see if the effect of the SdeCDE may be overshadowed by that of the Major Facilitator Superfamily pump.

The MdtABC system (Nagakubo et al. 2002) has been shown to be controlled by an adjacently encoded two-component system, and thus the 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. Site-specific mutants should also be created in order to understand the mechanism of functioning of multicomponent RND pumps. Candidates for site-directed mutagenesis can be selected by doing an alignment search between, MdtB and SdeD, and MdtC and SdeE residues that are conserved between these four proteins but not in other RND pumps. Results from such an experiment would help in further characterization of the SdeCDE pump in *S. marcescens*.

Lastly, we have shown that SdeR is an activator of SdeAB expression. Data from sequence analysis shows that it is most likely a transcriptional activator of *sdeAB*. A

SdeR binding site must be experimentally confirmed by nuclease protection assays. A His-tag clone of *sdeR* must be constructed and it should be used to purify the SdeR protein, for the nuclease protection assay. These assays will help to identify the binding site for SdeR within the intergenic region of *sdeA* and *sdeR*. It is highly likely that there are two binding sites present, one for each, the *sdeA* and *sdeR* genes. Once SdeR is confirmed to be an activator of MDR systems in *S. marcescens*, expression of porin genes should be monitored upon over-expression of *sdeR* in order to determine whether this protein is a global regulator.

## CHAPTER 8 Appendix

NCBI GenBank nucleotide output for *Serratia marcescens* outer membrane porin (*ompF*) gene

Accession number U81967, size: 1701 bp

*ompF* gene found between 405 and 1529 bp (1.1 kb)

```
   1  tttcggcctg ggctttgaac gtttaatcgc ctatgtcacc ggcgtgcaaa acgtgcgcga
  61  tgtgattcca ttcccgcgca cgccgcgtaa cgcaagcttc taatttatac gatcacacgt
 121  tttttaaacg atttcgttac aaaacaaagg ccagcatcgc tggcctttcg catttttaa
 181  tattgacgca cgtcacaaag ttcccgaaaa attacatttg gttacacata ctttcctttt
 241  gcaacctgat tgggacattg gtatcatttt cgtcctagat taacccgcct gtgaatggaa
 301  cactgcgttc agacacagga cgacaccaat ctatcaacaa tagttcccaa aggattattg
 361  gcggcagtgg caaaggtgtc cgaataacac caatgagggt aataatgatg aagcgcaaca
 421  ttcttgcagt ggtaatcccg gctctgttgg ctgctggtgc agcaaacgca gctgaaatct
 481  acaacaaaga cggcaacaag ctggatctgt acggcaaagt tgacggtctg cactacttct
 541  ccaaagacaa aggtaatgac ggcgatcaga cctatgttcg tttcggcttc aaaggtgaaa
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 661  acgccgaatc tcagggcacc gaaggcacca aaacccgtct gggcttcgcc ggtctgaaat
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 781  gctggaccga tatgctgcca gagttcggtg gcgatactta cacctactcc gacaacttca
 841  tgaccggccg taccaacggc gttgcgacct atcgtaacaa caacttcttc ggtctggttg
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 961  aaaaacaaaa cggcgacggc tggggcattt cctctactta tgacatcggc gaaggcgtaa
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1081  agcgtggcga caaggctgac gcatggaccg tgggcgcgaa atatgacgcc aacaacgtct
1141  acctggcggc aatgtatgca gaaacccgta acatgactcc gttcggtggc ggtaacttca
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1261  aagtgactgc tcagtatcag ttcgacttcg gtctgcgccc agaagtgtct tacctgcagt
1321  ctaagggtaa aaacctgaac gtcccaggcg tgggttctga ccaagatctg gttaaatatg
1381  tttctgttgg taccacttac tacttcaaca aaaacatgtc cacctatgtt gattacaaaa
1441  tcaacctgct ggatgacaac gacttcacca aagcaaccgg catcgcaact gacgatatcg
1501  taggcgttgg tttggtatac cagttctaag ttgtctcgct tatcggcggt tcaccgtcga
1561  ctaagttaaa aaaacagggc ttcggcctgt ttttgtttgt atcagccggt aacttttttat
1621  cctgccgatt ccccccttccc catcattttt tctgtcgaat ccttcacgtt cataagatcg
1681  cggtgttttt atcgcaaacg g
```

NCBI GenBank nucleotide output for *Serratia marcescens* outer membrane porin (*ompC*) gene

Accession number L24960, size: 1842 bp

*ompC* gene found between 507 and 1650 bp (1.1 kb)

```
   1  ctatatcgcg aggttattta tttgcgtgct atttgtgcgg ctattgaaat tgtgaggaca
  61  gtgtggagga aaataaaaaa ccggccagaa gccggttaaa ggtatgcaga aattatcttc
 121  ggataatgaa ggtaataatg aaaataatga tgatagcgag gtgattatag cggctgcgtg
 181  caggcggcgt tttggcacaa tgtgcttatt gtccagtaca attgtatggt tggctttttt
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 601  gacggtaata aactggattt gtacggtaaa gtcgacggtc ttcactattt ctccagcaac
 661  aacggcgtgg acggtgacca gtcttacatg cgttttggcc tgcgcggcga aacgcaaatc
 721  agcgatcagc tgaccggcta cggccagtgg gaatatcagg ctaacctgaa ccacgctgaa
 781  aaccaggaca acaagaactt cacccgttac ggcttcgccg gtctgaaatt cggcgactac
 841  ggttccttcg actacggccg taataccggc gtgctgtacg acgtcgcggc gtataccgac
 901  ttgcagcctg agttcgacgg tatgacctac ggcgccgacc agttcatgtt ccagcgttcc
 961  agcggcctgg cgacctaccg taacaacgac ttcttcggtc tggttgacgg cctgaacttc
1021 gccctgcagt accagggtaa aaacggtaat ggcgaagaga ccaacaacgg tcgcgatgtc
1081 cttggccaga acggcgaagg ctacggtatg tccatgagct atgacatggg ctacggcatc
1141 agcgcggcgg gcgccttctt caactctcgc cgcaccagcg agcagaacgg cgctaacggt
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1561 atcaacctgg tggacaacaa cgacttcacc gacgctgccg ggatcaacac cgacaacgtg
1621 gttgccgtgg gcctggttta ccagttctaa gcacctcagt gtgaacgtca tcgcgcggcc
1681 ttcgggccgc gttttttttg cctgtaaacc ggtaaaaagc acggattgat ccgctatcgg
1741 cacgggaagc accgtggttt tcctcctcta agcctacgct gtcagtacaa aaaagagggg
1801 gatgatatgg aactcaagat cgataaggtg atcgaaacgg tg
```

NCBI GenBank nucleotide output for *Serratia marcescens sdeAB* operon

Accession number AY168756, size: 4356 bp

*sdeA* gene can be found between 1 and 1188 bp (1.1 kb)

*sdeB* gene can be found between 1213 and 4356 bp (3.1 kb)

```
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 121 gtcgcgcaca atgccccgcc gccgccgccg gtggtcagcg ccgccagcgt ggtggtcaag
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1741 gtgcagatct tcggcgccgg tgaatacgcg atgcgcatct ggctcgatcc gaacaaggtc
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2401 agtctgttcg ggctggtgct ggcgatcggc atcgtggtgg atgacgccat cgtggtggtg
2461 gagaacgtcg agcgcaacat cgaagagggg ctgtcgccgc tggcggcggc gcaccaggcg
2521 atgcgcgagg tctccggccc gatcatcgcc atcgccgtgg tgctgtgcgc ggtgttcgta
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2641 gccatctcga cggtgatctc cgccatcaac tcgctgaacc tgtcgccggc gctggcggcg
2701 cgcttgctga aaccgcacgg cgcgccgaaa gatatgccgt cgcgcctgat cgacaggctg
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2941 aagctgtatc tgattggcgg cgtgaagatg ccggaaggcg cctcgctgga gcgcaccgat
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3181 aacgcgcgca tttcgcagat ccaggaaggt ttcgccttct cgatcatgcc gccgccgatc
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NCBI Gen Bank nucleotide output for *Serratia marcescens sdeCDE* operon

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NCBI GenBank nucleotide output for *Serratia marcescens hasF* gene

Accession number X98513, size: 2571 bp

*hasF* gene found between 656 and 2158 bp (1.5 kb)

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NCBI GenBank nucleotide output for *Serratia marcescens sdeR* gene
Accession number AY623133, size: 405 bp
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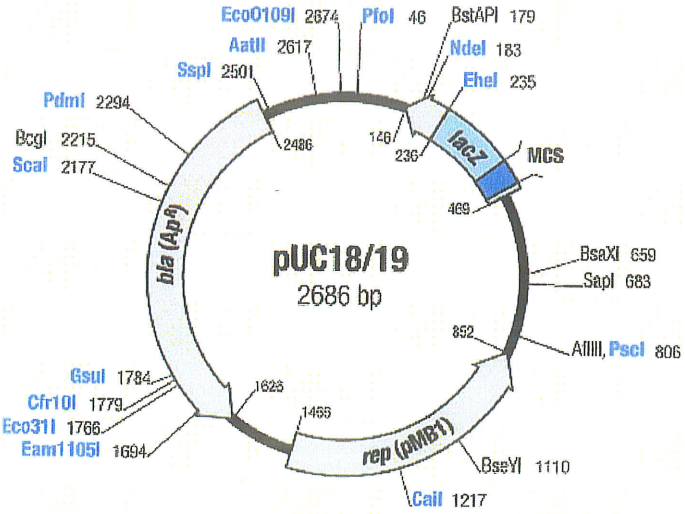
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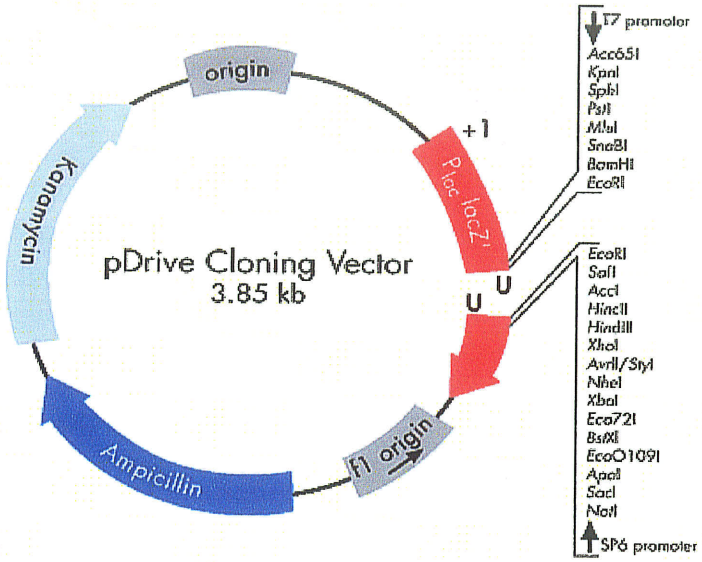
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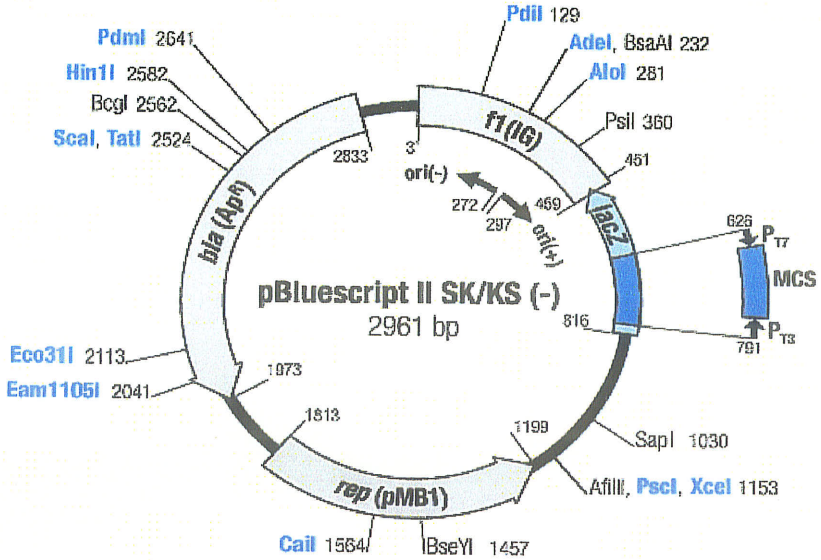
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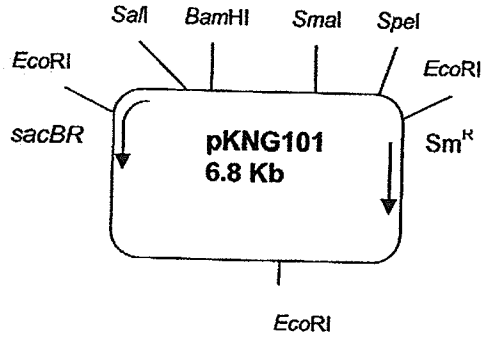
AloI 281
PsiI 360
451
PdmI 2641
Hin1I 2582
BcgI 2562
ScaI, TatI 2524
2833
3
ori(-)
272
297
469
ori(+)
626
816
791
Eco31I 2113
Eam1105I 2041
1973
1813
1199
SapI 1030
AflIII, PscI, XceI 1153
CaiI 1564
BseYI 1457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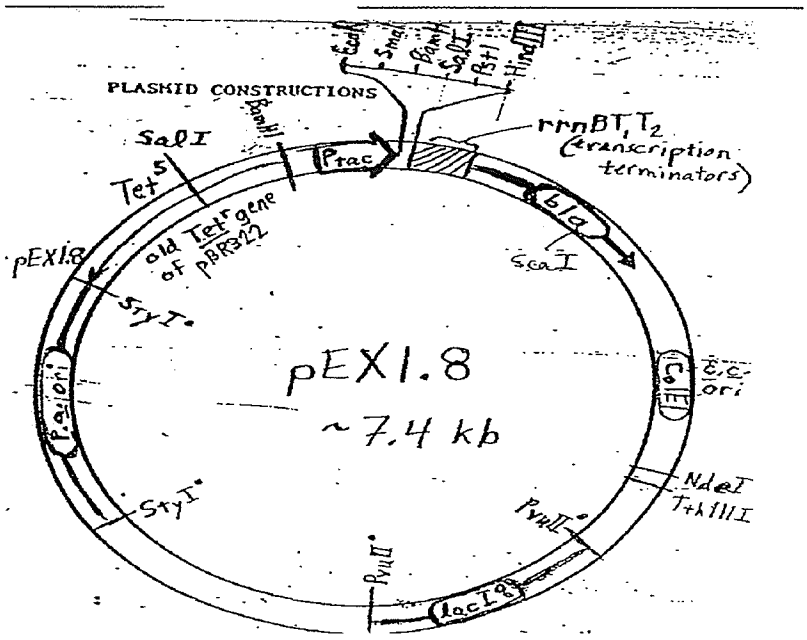
BamHI
SmaI
SpeI
EcoRI
EcoRI
sacBR
pKNG101
6.8 Kb
Sm^R
EcoRI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Ptac
rrnBT1T2
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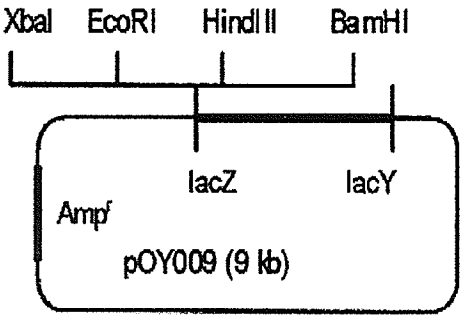
ScaI
StyI
StyI
PvuII
PvuII
NdeI
lacI^q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HindIII
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Amp^r
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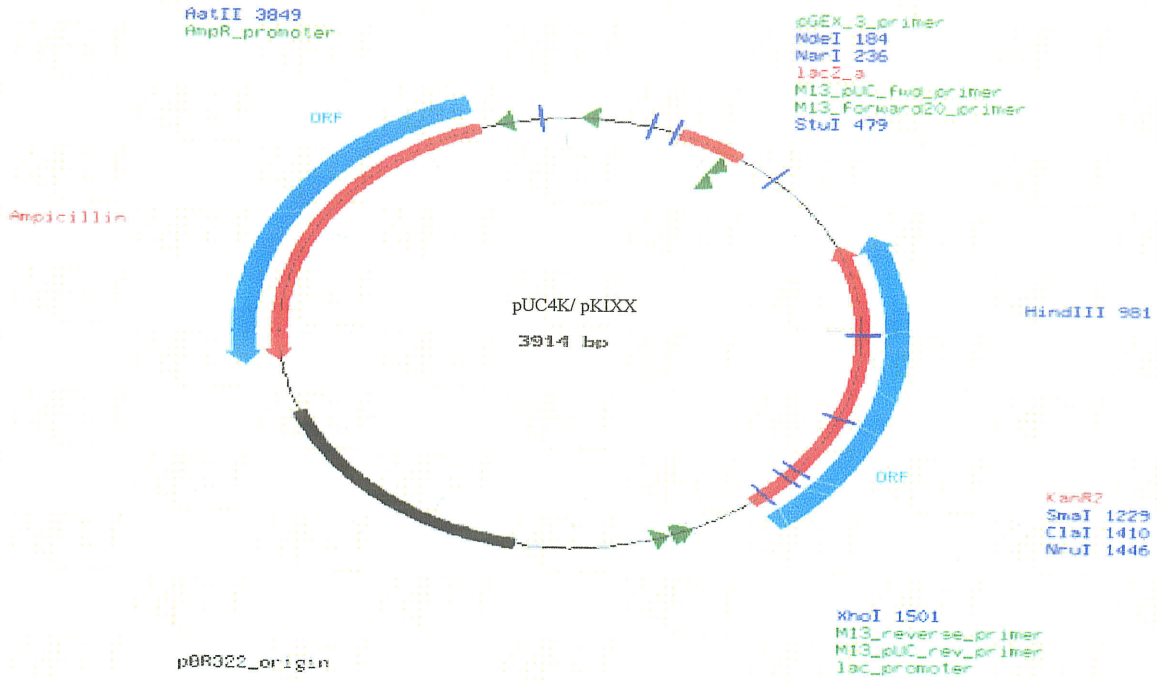
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AmpR_promoter
pGEX_3_primer
NdeI 184
NarI 236
lacZ_a
M13_pUC_fwd_primer
M13_Forward20_primer
StuI 479
ORF
Ampicillin
pUC4K/ pKIXX
3914 bp
HindIII 981
ORF
KanR?
SmaI 1229
ClaI 1410
NruI 1446
XhoI 1501
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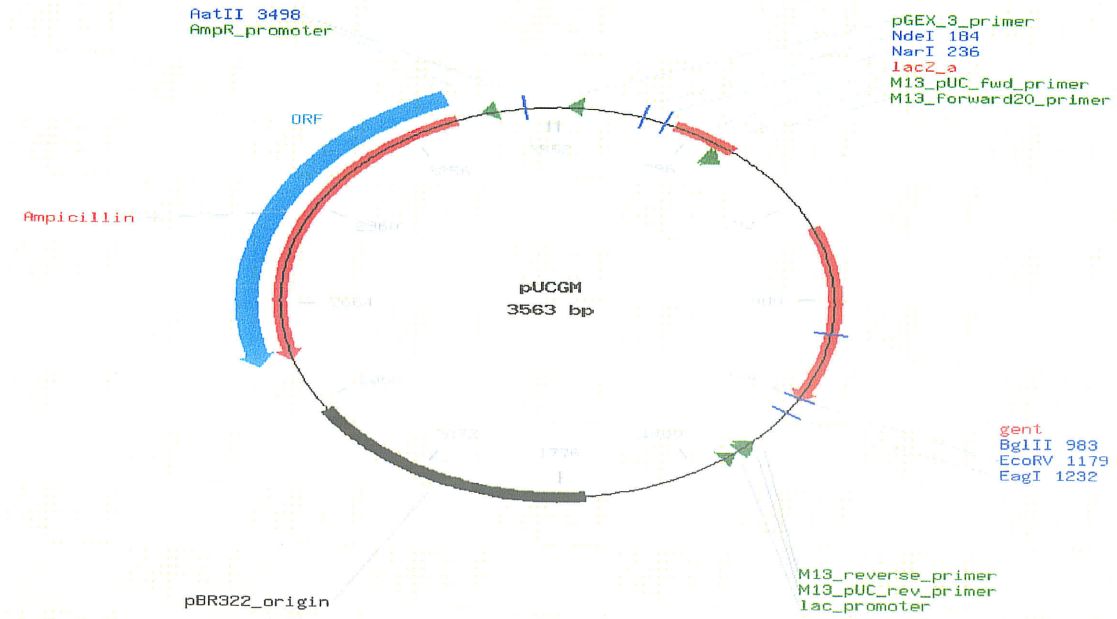
lac_promoter
pBR322_origin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AmpR_promoter
pGEX_3_primer
NdeI 184
NarI 236
lacZ_a
M13_pUC_fwd_primer
M13_Forward20_primer
ORF
Ampicillin
pUCGM
3563 bp
gent
BglII 983
EcoRV 1179
EagI 1232
M13_reverse_primer
M13_pUC_rev_primer
lac_promoter
pBR322_origin

PARTIAL BLAST sequence alignment for *ompF* insertional knockout blasted with the kanR marker from pUC4K/pKIXX (Accession number: X06404)
Score = 962 bits (500)
Identities = 500/500 (100%), Gaps = 0/500 (0%)
Strand=Plus/Plus

```
Query  221
CATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTT  280

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Sbjct  1
CATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTT  60

Query  281
GAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAA  340

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Sbjct  61
GAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAA  120

Query  341
GATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCC  400

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Sbjct  121
GATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCC  180

Query  401
CCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG  460

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Sbjct  181
CCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG  240

Query  461
AGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCT  520

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Sbjct  241
AGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCT  300

Query  521
CGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGA  580

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Sbjct  301
CGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGA  360

Query  581
GACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGC  640

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Sbjct  361
GACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGC  420
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Query  641
GCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATA  700

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Sbjct  421
GCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATA  480

Query  701  CCTGGAATGCTGTTTTCCCG  720
            ||||||||||||||||||||
Sbjct  481  CCTGGAATGCTGTTTTCCCG  500
```

PARTIAL BLAST sequence alignment for *ompC* insertional knockout
blasted with the gmr marker from pUCGM (Accession number: U04610)
Score = 1088 bits (566)
Identities = 580/580 (100%), Gaps = 0/580 (0%)
Strand=Plus/Plus

```
Query  254
ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAAC  313

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Sbjct  1
ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAAC  60

Query  314
TGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG  373

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Sbjct  61
TGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG  120

Query  374
GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTA  433

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Sbjct  121
GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTA  180

Query  434
TGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGA  493

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Sbjct  181
TGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGA  240

Query  494
GCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA  553

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Sbjct  241
GCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA  300

Query  554
AAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTC  613

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Sbjct  301
AAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTC  360
```

PARTIAL BLAST sequence alignment for *hasF* insertional knockout
blasted with the gmr marker from pUCGM (Accession number: U04610)
Score = 1127 bits (586), Expect = 0.0
Identities = 600/600 (100%), Gaps = 0/600 (0%)
Strand=Plus/Plus

```
Query  93
ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAAC  152

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Sbjct  1
ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAAC  60

Query  153
TGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG  212

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Sbjct  61
TGTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCG  120

Query  213
GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTA  272

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Sbjct  121
GTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTA  180

Query  273
TGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGA  332

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Sbjct  181
TGCCTCGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGA  240

Query  333
GCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA  392

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Sbjct  241
GCAGCAACGATGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACA  300

Query  393
AAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTC  452

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Sbjct  301
AAGTTAGGTGGCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTC  360

Query  453
AAATCCATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTAC  512

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Sbjct  361
AAATCCATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTAC  420

Query  513
TCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACATTCATC  572
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Sbjct  421
TCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACATTCATC  480

Query  573
GCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCC  632

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Sbjct  481
GCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTGGCGCTCTCGCGGCTTACGTTCTGCCC  540

Query  633
AGGTTTGAGCAGCCGCGTAGTGAGATCTATATCTATGATCTCGCAGTCTCCGGCGAGCAC  692

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Sbjct  541
AGGTTTGAGCAGCCGCGTAGTGAGATCTATATCTATGATCTCGCAGTCTCCGGCGAGCAC  600
```

PARTIAL BLAST sequence alignment for *sdeB* insertional knockout
blasted with the kanR marker from pUC4K/pKIXX(Accession number:X06064)
Score = 1346 bits (700), Expect = 0.0
Identities = 700/700 (100%), Gaps = 0/700 (0%)
Strand=Plus/Plus

```
Query  220
GTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCTGAATCGCCCCATCATCCA  279

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Sbjct  1
GTCTGCCTCGTGAAGAAGGTGTTGCTGACTCATACCAGGCCTGAATCGCCCCATCATCCA  60

Query  280
GCCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGA  339

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Sbjct  61
GCCAGAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGA  120

Query  340
TTTTGAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGAT  399

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Sbjct  121
TTTTGAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGAT  180

Query  400
CCTTCAACTCAGCAAAAGTTCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGT  459

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Sbjct  181
CCTTCAACTCAGCAAAAGTTCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGT  240

Query  460
AATGCTCTGCCAGTGTTACAACCAATTAACCAATTCTGATTAGAAAAACTCATCGAGCAT  519

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Sbjct  241
AATGCTCTGCCAGTGTTACAACCAATTAACCAATTCTGATTAGAAAAACTCATCGAGCAT  300

Query  520
CAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCG  579

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Sbjct  301
CAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCG  360

Query  580
TTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTA  639

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Sbjct  361
TTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTA  420

Query  640
TCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA  699
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Sbjct  421
TCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAA  480

Query  700
AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAA  759

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Sbjct  481
AATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAA  540

Query  760
AAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAA  819

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Sbjct  541
AAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAA  600

Query  820
ATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATAC  879

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Sbjct  601
ATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATAC  660

Query  880  GCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAA  919
             |||||||||||||||||||||||||||||||||||||||
Sbjct  661  GCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAA  700
```

PARTIAL BLAST sequence alignment for *sdeR* insertional knockout
blasted with the kanR marker from pUC4K/pKIXX(Accession number:X06064)
Score = 904 bits (470), Expect = 0.0
Identities = 470/470 (100%), Gaps = 0/470 (0%)
Strand=Plus/Plus

```
Query  81
CATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTT  140

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Sbjct  1
CATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTT  60

Query  141
GAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAA  200

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Sbjct  61
GAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAA  120

Query  201
GATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCC  260

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Sbjct  121
GATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCC  180

Query  261
CCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG  320

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Sbjct  181
CCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTG  240

Query  321
AGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCT  380

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Sbjct  241
AGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCT  300

Query  381
CGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGA  440

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Sbjct  301
CGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGA  360

Query  441
GACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGC  500

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Sbjct  361
GACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGC  420

Query  501  GCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATAT  550
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATAT  470
```

PARTIAL BLAST sequence alignment for *sdeD* insertional knockout
blasted with the gmr marker from pUCGM (Accession number: U04610)
Score = 877 bits (456), Expect = 0.0
Identities = 470/470 (100%), Gaps = 0/470 (0%)
Strand=Plus/Plus

```
Query  317
ATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAA  376

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Sbjct  1
ATCCCCGGGTACCGAGCTCGAATTGACATAAGCCTGTTCGGTTCGTAAACTGTAATGCAA  60

Query  377
GTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGG  436

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Sbjct  61
GTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAACGCAGCGGTGGTAACGG  120

Query  437
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGC  496

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Sbjct  121
CGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTTTTGTACAGTCTATGCCTCGGGC  180

Query  497
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGA  556

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Sbjct  181
ATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAACGA  240

Query  557
TGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTG  616

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Sbjct  241
TGTTACGCAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGTG  300

Query  617
GCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCCATGC  676

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Sbjct  301
GCTCAAGTATGGGCATCATTCGCACATGTAGGCTCGGCCCTGACCAAGTCAAATCCATGC  360

Query  677
GGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTACTCCCAACATC  736

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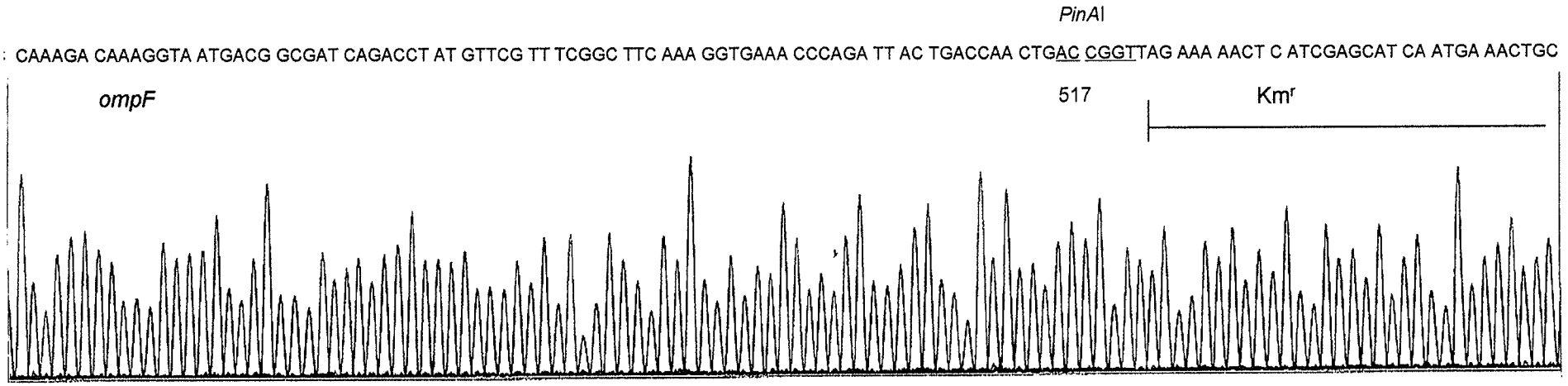
Sbjct  361
GGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCCACCTACTCCCAACATC  420

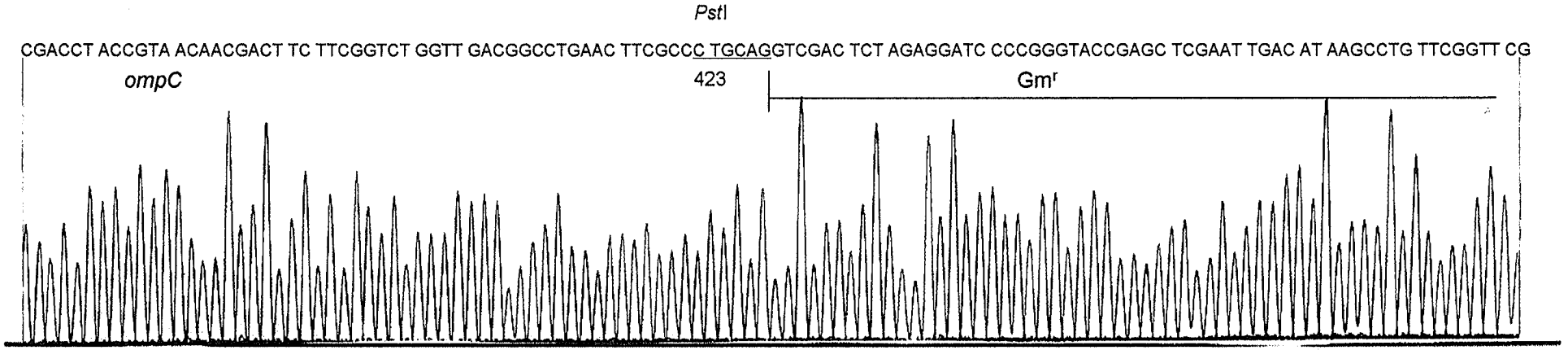
Query  737  AGCCGGACTCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACATTCATC  786
             ||||||||||||||||||||||||||||||||||||||||||||||||||
```

Sbjct 421 AGCCGGACTCCGATTACCTCGGGAACTTGCTCCGTAGTAAGACATTCATC 470



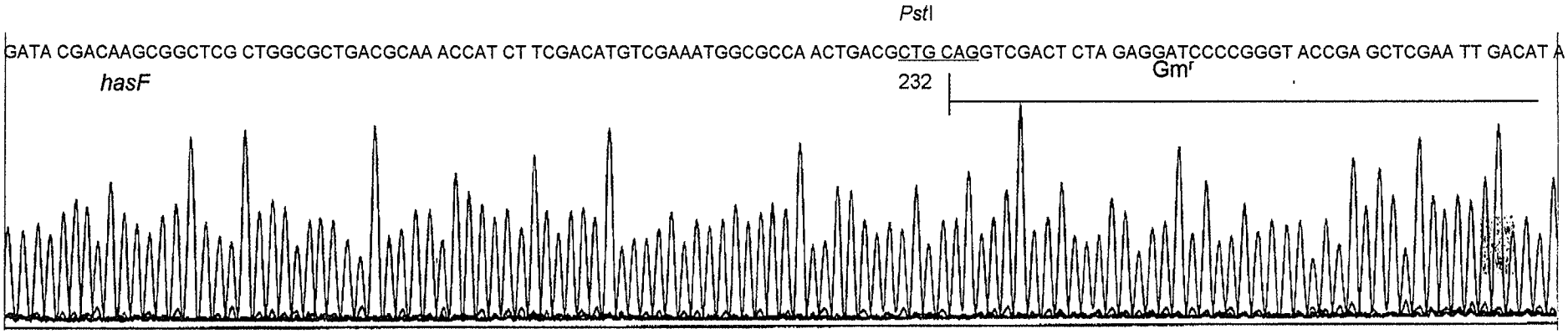

*ompF* insertional mutant

Km$^r$ cassette insertion site underlined, Km$^r$ cassette is inserted into a *PinAI* site approximately 520 bp within the *ompF* gene



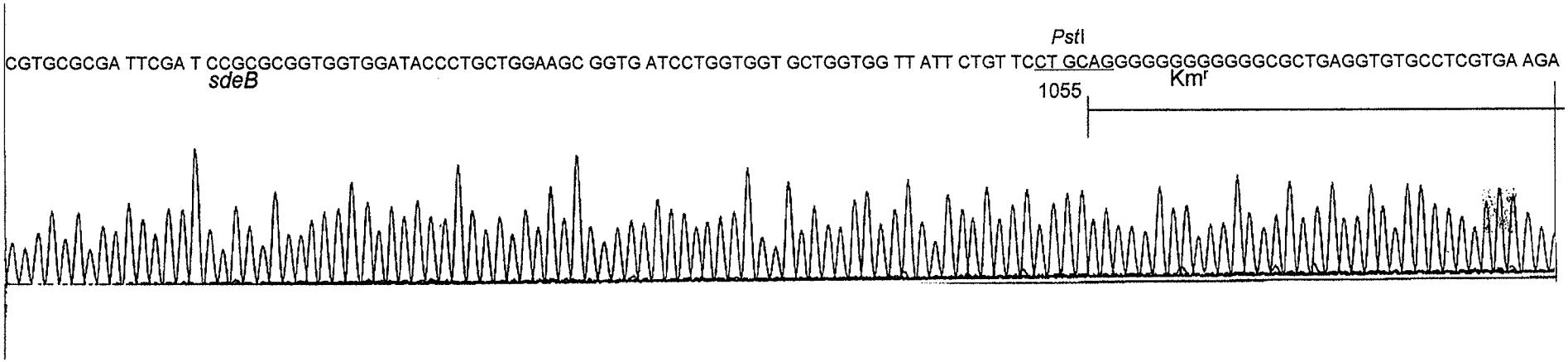

*ompC* insertional mutant

Gm$^r$ cassette insertion site underlined, Gm$^r$ cassette is inserted into a *Pst*I site approximately 430 bp within the *ompC* gene



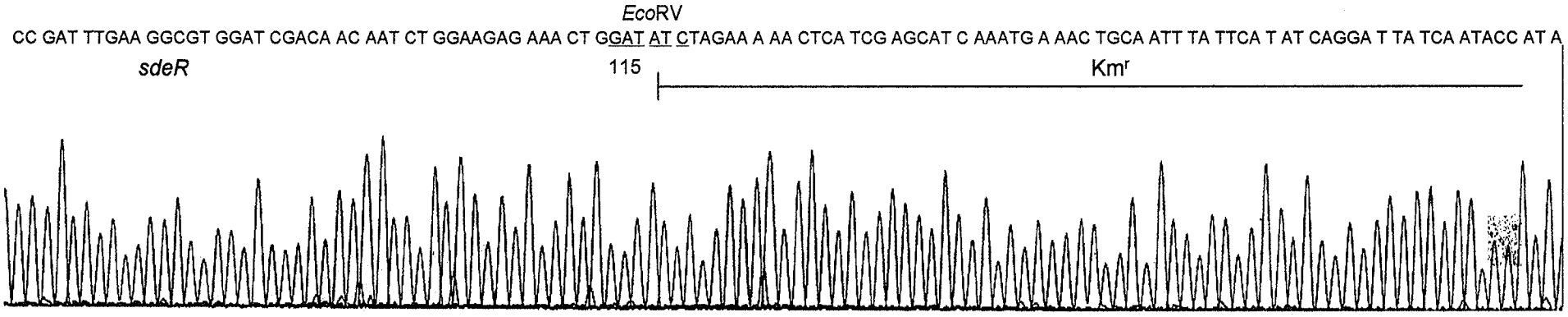

*hasF* insertional mutant

Gm$^r$ cassette insertion site underlined, Gm$^r$ cassette is inserted into *Pst*I site approximately 230 bp within the *hasF* gene.



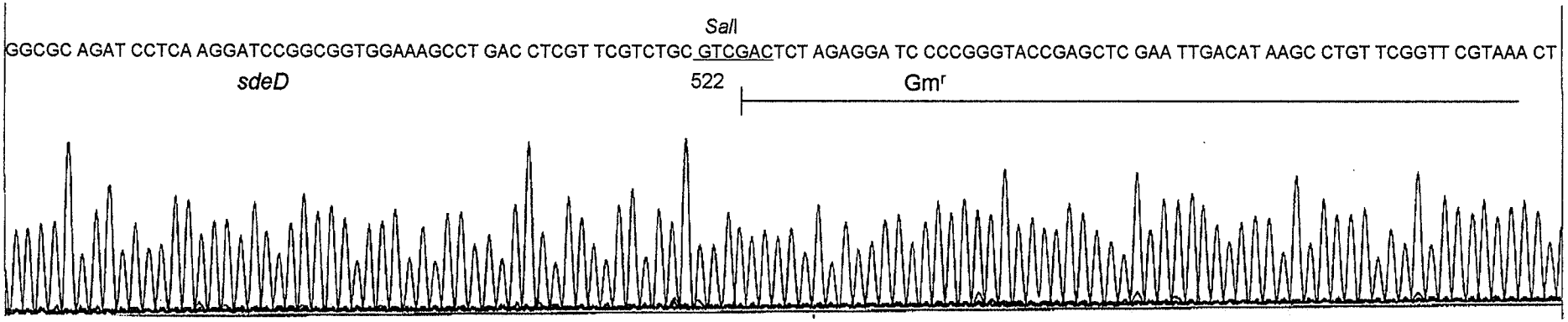

*sdeB* insertional mutant

Km$^r$ cassette insertion site underlined, Km$^r$ cassette inserted into *Pst*I site approximately 1050 bp within the *sdeB* gene.



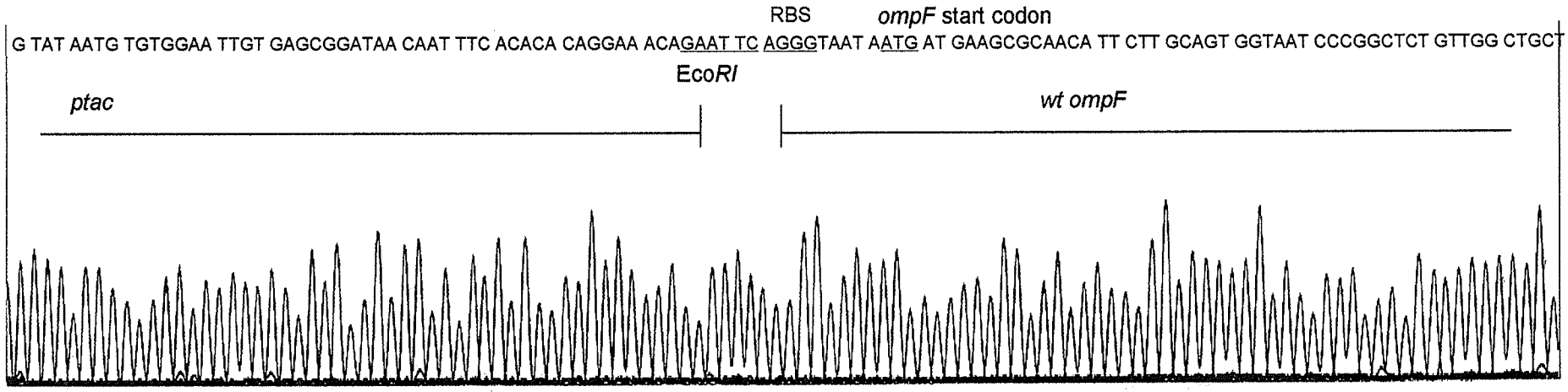

*sdeR* insertional mutant

Km$^r$ cassette insertion site underlined, Km$^r$ cassette inserted into the *Eco*RV site approximately 120 bp within the *sdeR* gene.



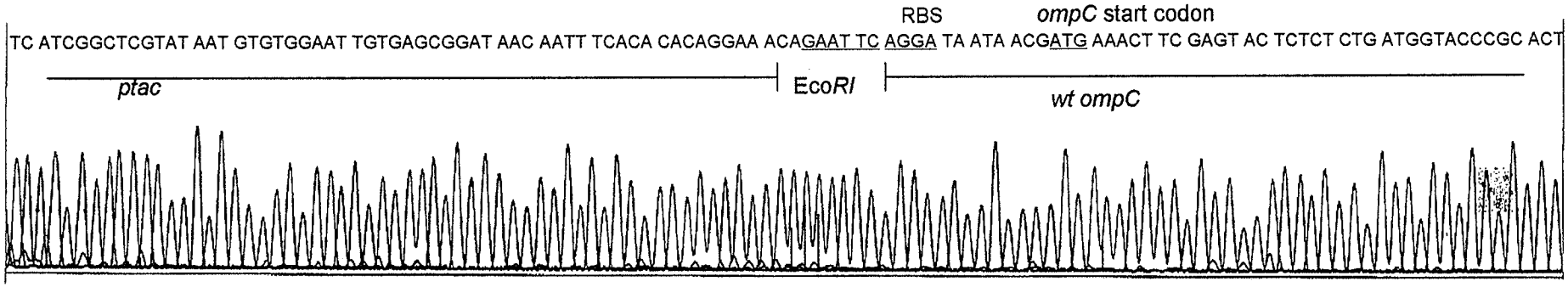

*sdeD* insertional mutant

Gm$^r$ cassette insertion site underlined, Gm$^r$ cassette inserted into the *Sal*I site approximately 520 bp within the *sdeD* gene



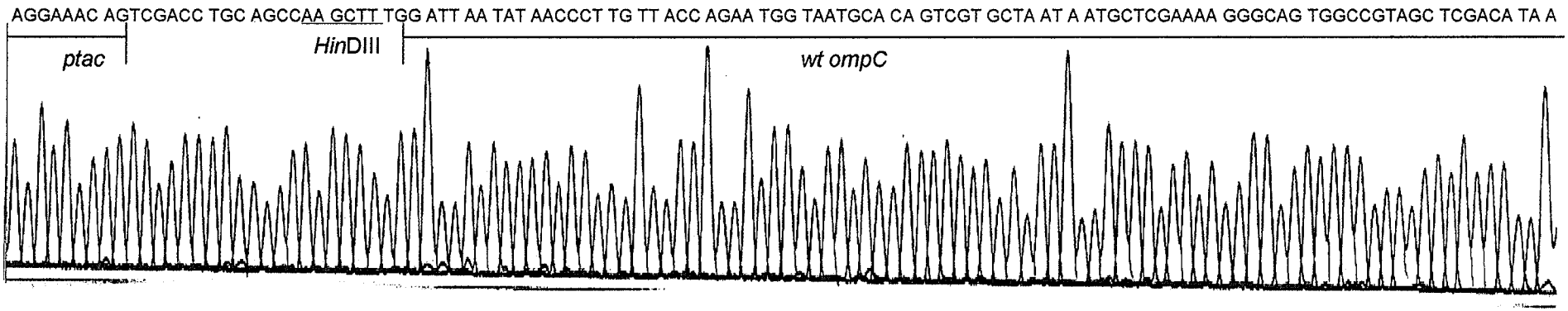

*ompF* pEX1.8 vector for complementation

wt *ompF* (1.1 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *ompF* start codon are also indicated.



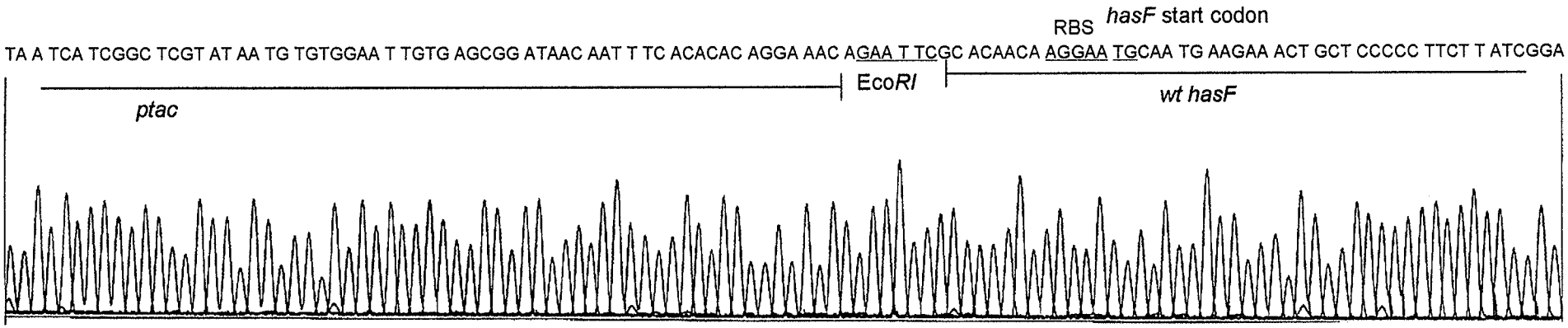

*ompC* pEX1.8 vector for complementation (pEXC)

wt *ompC* (1.1 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *ompC* start codon are also indicated.



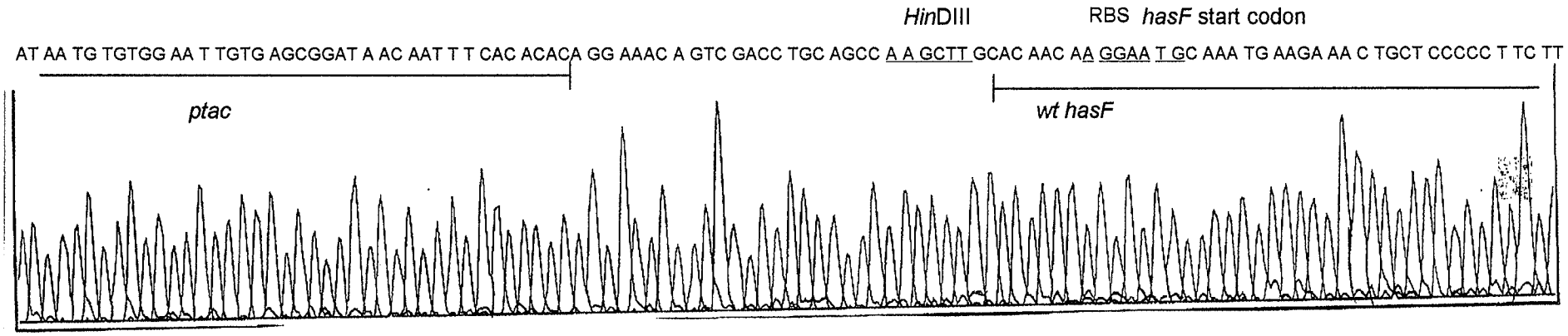

*ompC* pEX1.8JH1 vector for double complementation (pEXCH/H)

wt *ompC* (1.1 kb) was inserted into a unique *Hin*DIII site (underlined) within the pEX1.8JH1 multiple cloning site. RBS and *ompC* start codon are further downstream from where the wt *ompC* sequence is indicated.



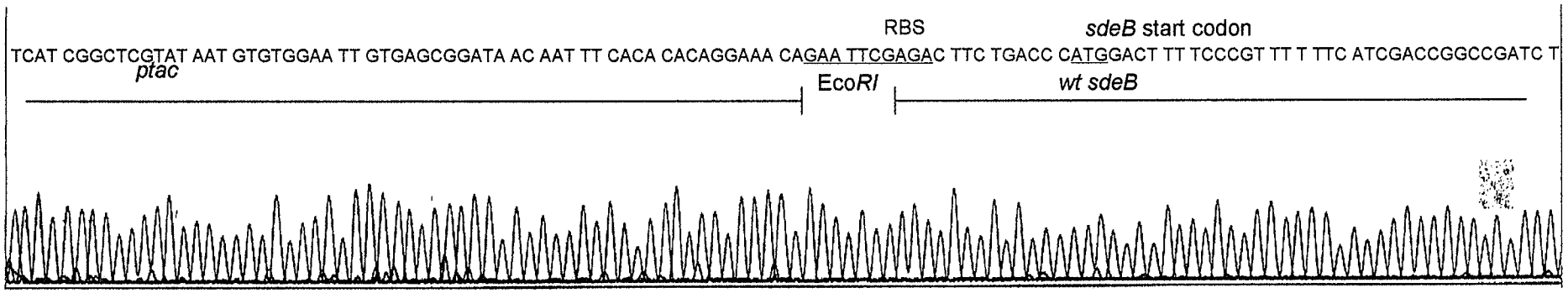

*hasF* pEX1.8 vector for complementation (pEXH)

wt *hasF* (1.5 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *hasF* start codon are also indicated.



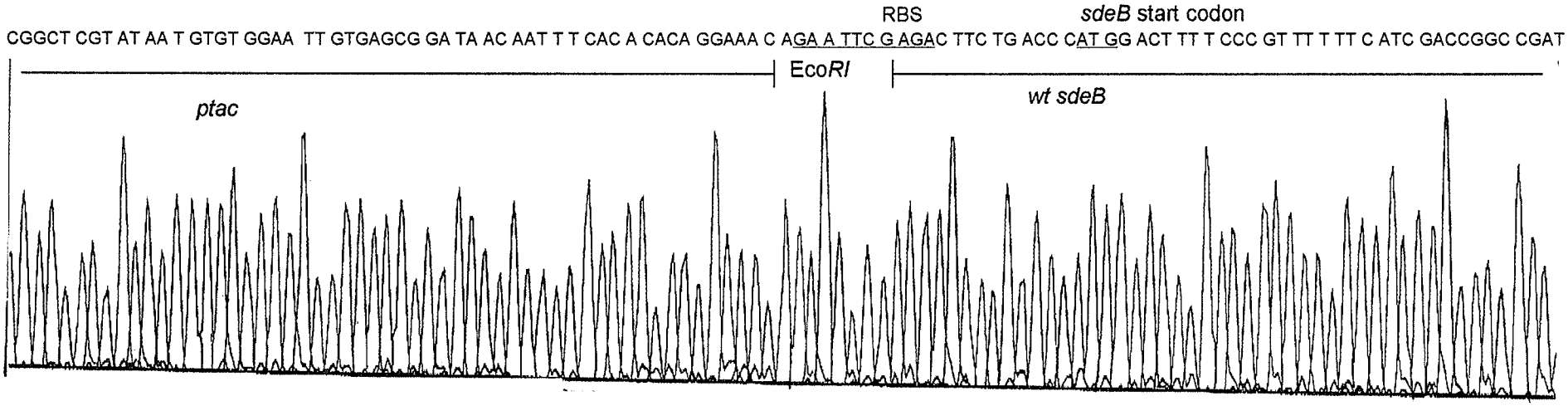

*hasF* pEX1.8JH1 vector for double complementation (pEXHH/H)

wt *hasF* (1.5 kb) was inserted into a unique *Hin*DIII site (underlined) within the pEX1.8JH1 multiple cloning site. RBS and *hasF* start codon are also indicated.



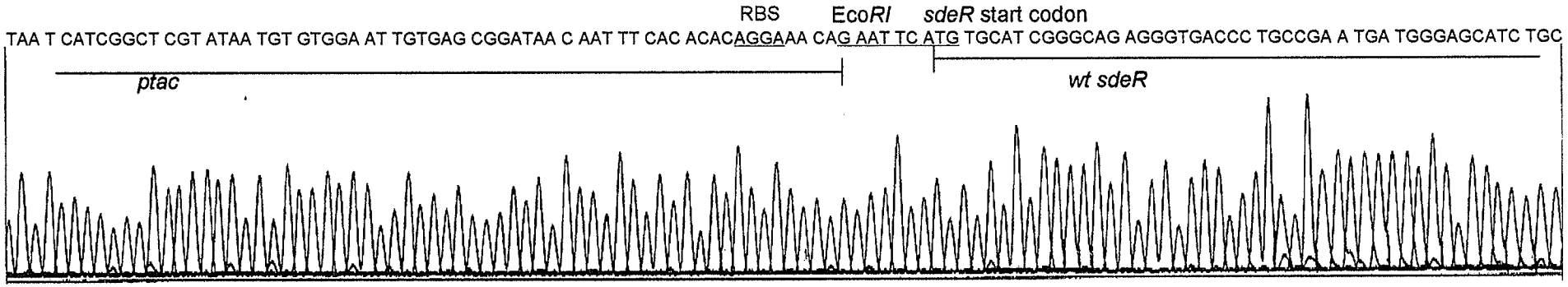

*sdeB* pEX1.8 vector for complementation (pEXS)

wt *sdeB* (3.1 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *sdeB* start codon are also indicated.



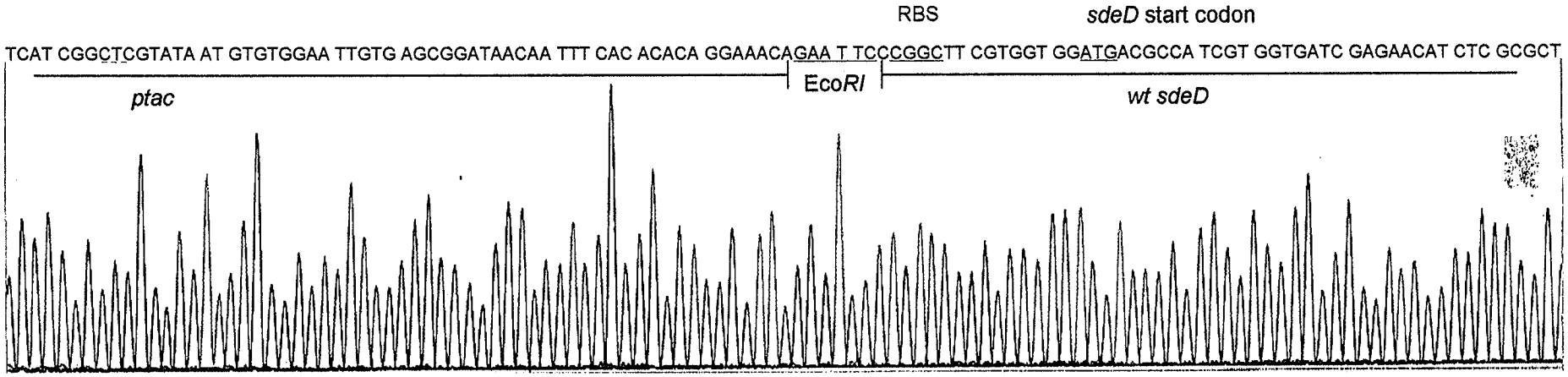

*sdeB* pEX1.8JH vector for double complementation (pEXSE/E)

wt *sdeB* (3.1 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8JH multiple cloning site. RBS and *sdeB* start codon are also indicated.



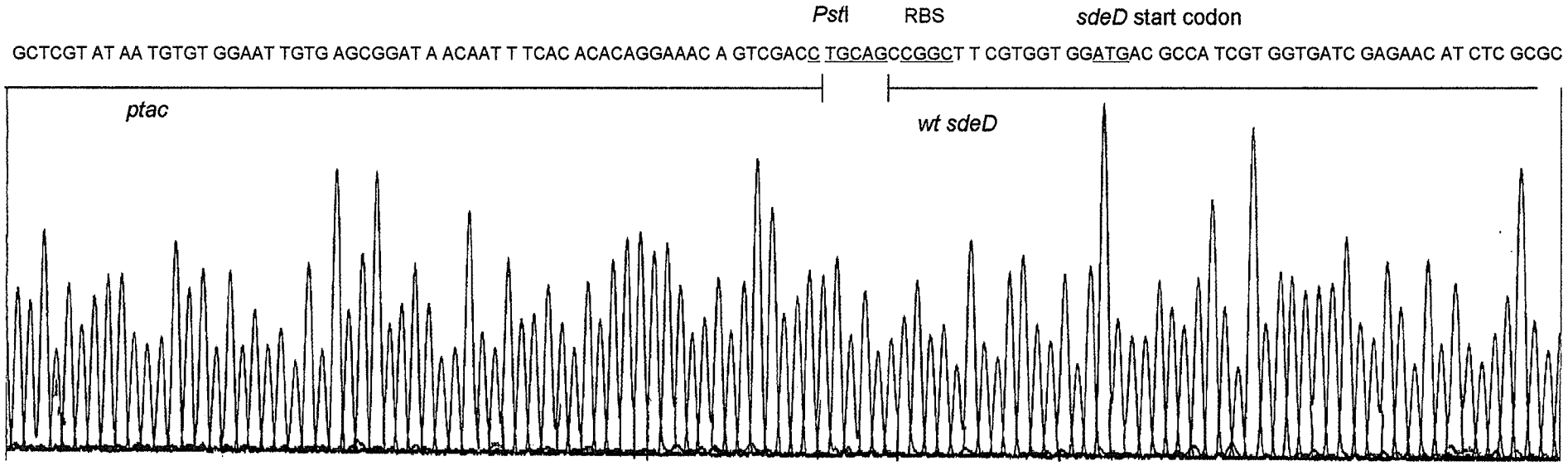

*sdeR* pEX1.8 vector for complementation

wt *sdeR* (0.4 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *sdeR* start codon are also indicated.



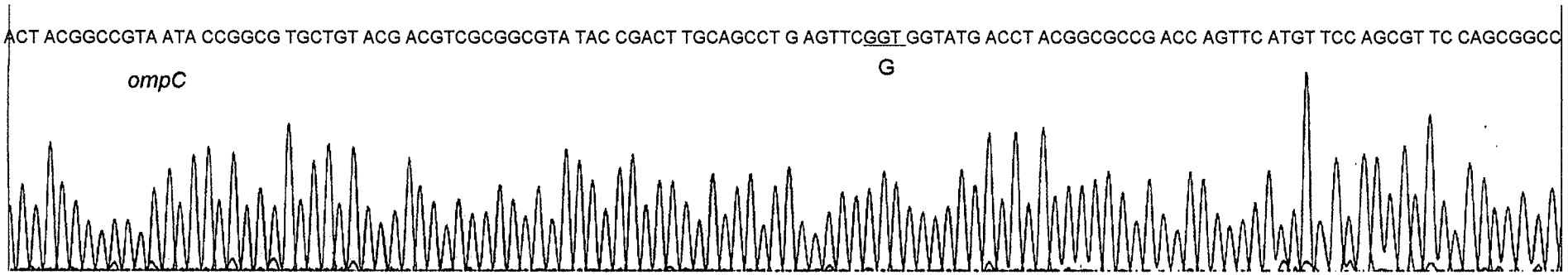

*sdeDE* pEX1.8 vector for complementation

wt *sdeDE* (4.9 kb) was inserted into a unique *Eco*RI site (underlined) within the pEX1.8 multiple cloning site. RBS and *sdeDE* start codon are also indicated.



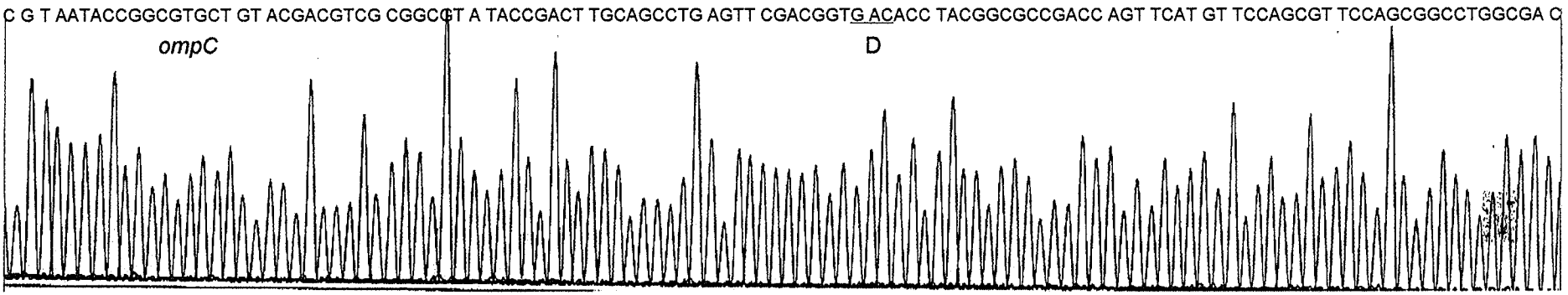

*sdeDE* pEX1.8JH1 vector for double complementation (pEXDP/P)

wt *sdeDE* (4.9 kb) was inserted into a unique *PstI* site (underlined) within the pEX1.8JH1 multiple cloning site. RBS and *sdeDE* start codon are also indicated.



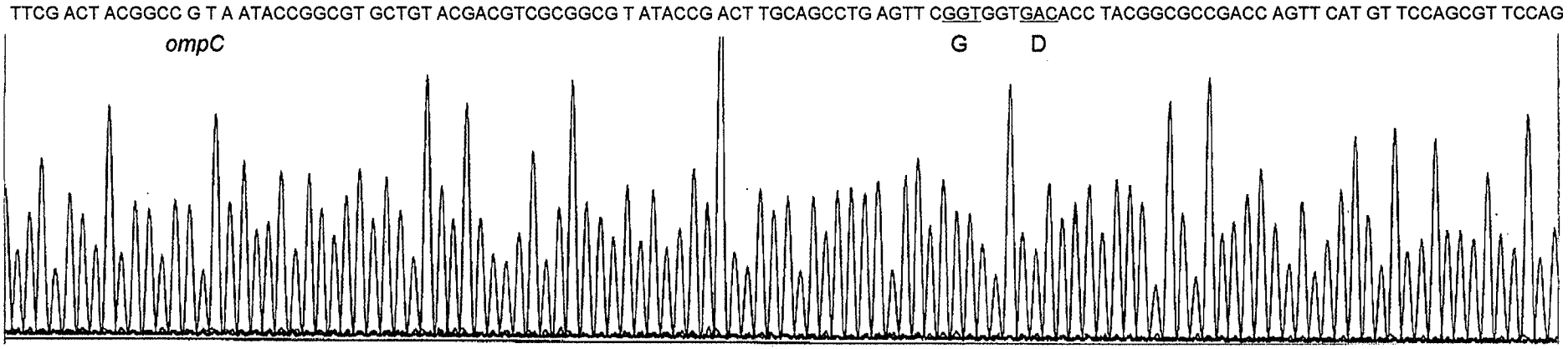

D112G single site-directed mutant within *ompC*

Glycine (G) site underlined




M114D single site-directed mutant within *ompC*

Aspartic acid (D) site underlined



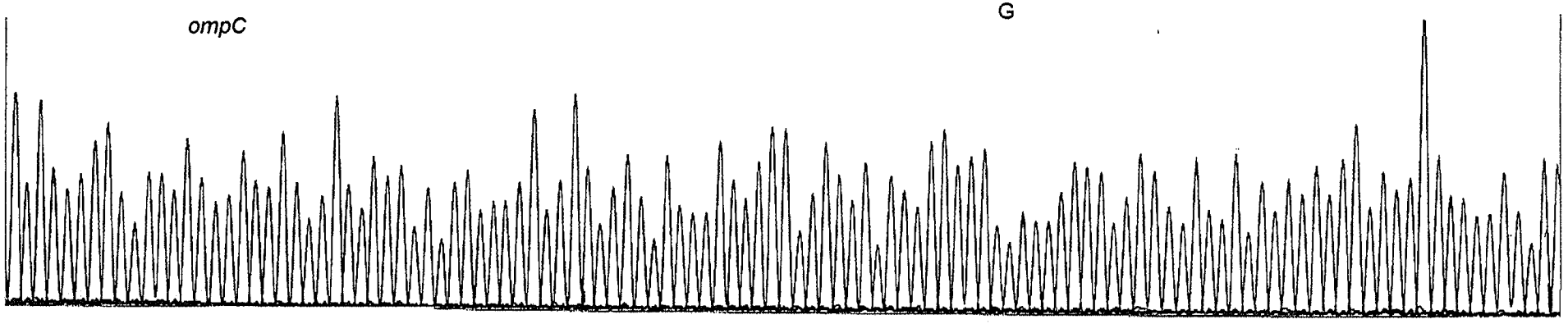

D112G/M114D double site-directed mutant within *ompC*

Glycine (GGT) and Aspartic acid (GAC) sites underlined

ACG ACGT CGCGGCG TA TACCG ACT TGCAGCC TG AGTTCG ACGGTA TG ACC TACGGCGCCG ACC AGTT CAT GT TCGGGCGTTCCAG CGGCCTG GCGACCT ACCG TA AC AACGA CT TC

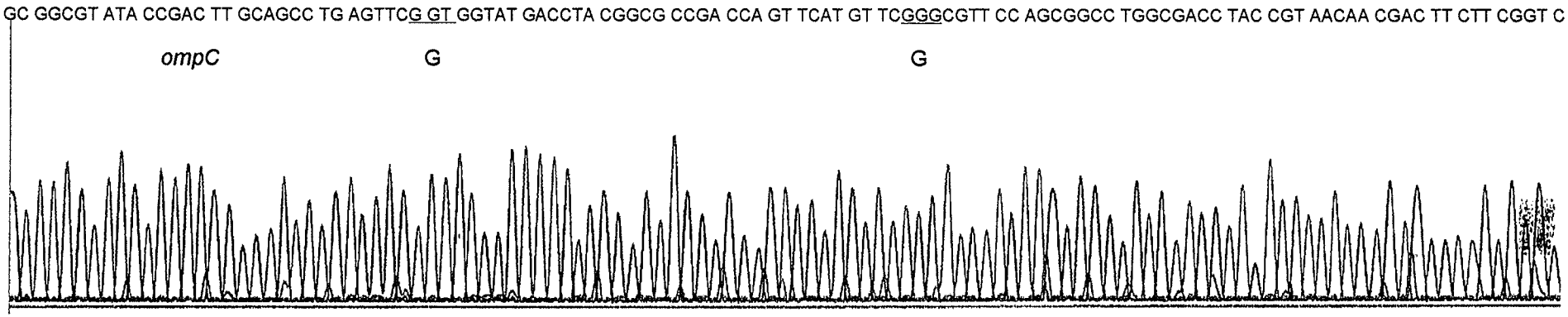
G

*ompC*



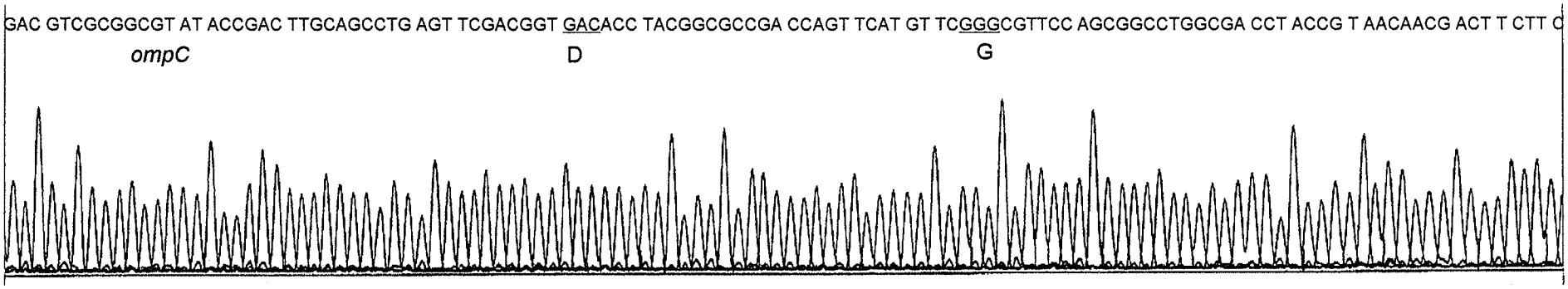
Q124G single site-directed mutant within *ompC*

Glycine (G) site underlined



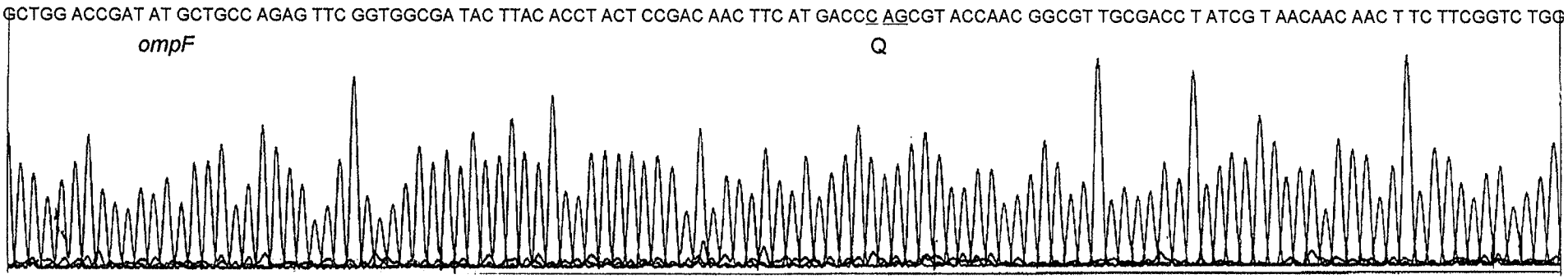

D112G/Q124G double site-directed mutant within *ompC*

Glycine (GGT, GGG) sites underlined




M114D/Q124G double site-directed mutant within *ompC*

Aspartic acid (GAC) and Glycine (GGG) sites underlined




G124Q single site-directed mutant within *ompF*

Glutamic acid (Q) site underlined

**CHAPTER 9 References**

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