Characterization of the outer membrane porins of *Serratia marcescens*

by

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CHARACTERIZATION OF THE OUTER MEMBRANE PORINS OF *Serratia marcescens*

BY

JO-ANNE MARIE HUTSUL

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

DOCTOR OF PHILOSOPHY

Abstract

Reductions in the amount of outer membrane porins in the opportunistic pathogen, *Serratia marcescens*, have been correlated with an increase in β-lactam resistance. In comparison to the well known *Escherichia coli* porins, the porins of *S. marcescens* have not been well characterized. In order to study the role of reduced porin copy number in β-lactam resistance of *S. marcescens* further characterization of its constituent porin(s) was first required. This thesis presents the molecular characterization of the *S. marcescens* porins. A comparative study of porins in *Serratia* spp. and other *Enterobacteriaceae* was performed to expand our knowledge of *Serratia* porins and determine the extent of homology between porins of *Serratia* and other enteric bacteria. Results of immunochemical, molecular biological and functional tests demonstrated the conservation of porin structure and function between *Serratia* species and throughout the *Enterobacteriaceae*. The outer membrane of *S. marcescens* strain UOC-51 was isolated and its proteins separated by electrophoresis. Two proteins, 40 and 41 kDa were identified as putative porins. The outer membrane concentration of these porins was shown to be regulated in response to external osmolarity and salicylate. Two genes were isolated from a constructed *S. marcescens* genomic DNA library using an oligonucleotide coding the N-terminal amino acid sequence of the major 41 kDa porin of *S. marcescens* UOC-69. Each cloned gene was sequenced and characterized. The two genes were found to be homologous to the *E. coli* porins, OmpF and OmpC. In addition, a

micF gene, important in the regulation of the OmpF porin, was identified upstream of the *ompC* gene. An OmpR binding site important in repression of *ompF* transcription was absent in the *S. marcescens ompF* when compared to *E. coli*. Amino acid sequence comparisons with other known general diffusion porins demonstrated conservation of protein sequence among this family of proteins. Although relatively conserved, important changes in sequence were noted for both *S. marcescens* porins. A functional examination of the two porins, using the liposome swelling assay, demonstrated that small uncharged solutes diffused through the *S. marcescens* OmpF porin faster than the OmpC. Specific sites were targeted for mutagenesis and an aspartic acid at position 112 of OmpC was shown to decrease the permeability through this pore channel.

Acknowledgments

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List of Abbreviations

Ap	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp	base pairs
Cm	chloramphenicol
Da	daltons
dATP	2'-deoxyadenosine 5'-triphosphate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine-tetra-acetic acid
g	gram(s)
IHF	Integration host factor
kb	kilobase pairs
kDa	kilodaltons
Km	kanamycin
L	litre(s)
LPS	lipopolysaccharide
M	molar
M_r	molecular mass
Mar	multiple antibiotic resistance
MDO	membrane derived oligosaccharide
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
NAG-NAMA	N-acetylglucosamine(β-1-4)N-acetylmuramic acid
ng	nanogram(s)
nS	nanoSiemens
OBS	OmpR binding sites
PBP	penicillin binding protein
PCR	polymerase chain reaction
PG	peptidoglycan
PVDF	polyvinyldifluoride
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
µg	microgram(s)
µl	microlitre(s)
UMCC	University of Manitoba Culture Collection
v/v	volume/volume
w/v	weight/volume

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Serratia marcescens is a Gram-negative facultative anaerobic rod-shaped bacterium in the *Enterobacteriaceae* family (Grimont & Grimont, 1984). It is motile by peritrichous flagella and found in soils, water and on plant surfaces. It was long thought to be a harmless saprophyte but is now recognized as an opportunistic human pathogen. The first time *S. marcescens* was isolated from an infected individual was in 1913 (Woodward & Clark, 1913), however, it wasn't until the 1960's that it was recognized as an important nosocomial pathogen (Clayton & von Graeventiz, 1966; Magnuson & Elston, 1966). The misconception that *S. marcescens* always produces red pigment (prodigiosin) resulted in erroneous identifications with many being reported as *Klebsiella*, *Hafnia*, *Enterobacter* or other paracolon bacteria (Franczek, *et al.*, 1986; Fulton *et al.*, 1959; Magnusson & Elston, 1966). In fact, most clinical isolates do not produce prodigiosin, and commonly 90% are non-chromogenic (Holmes & Gross, 1990). Once acknowledged that *S. marcescens* need not produce pigment it was easily and reliably identified using appropriate biochemical tests and flagella shape (Fulton, *et al.*, 1959).

As mentioned above *S. marcescens* is associated with nosocomial infections. That is, patients develop *Serratia* bacteremia while in the hospital. Common infections that this pathogen can be responsible for include urinary tract infections, pneumonia and other respiratory infections, meningitis, endocarditis,

and septicemia (Yu, 1979). During the 1960's the incidence of *S. marcescens* infection increased (reviewed in Saito *et al.*, 1989). In 1969 nosocomial infections due to *S. marcescens* were the second most common after *E. coli*. The use of indwelling devices provides one problem since *S. marcescens* has been found to adhere to medical devices such as catheters and has been isolated from salines and other solutions, even disinfectants (Yu, 1979). Endemics and epidemics within hospitals have been reported (Lewis *et al.*, 1983; Wilfert *et al.*, 1970). Frequently, spread has been found to be associated with hospital staff (Maki *et al.*, 1973).

Antibiotic resistance of *S. marcescens* is generally high. In the 1960's some reports indicated that gentamicin was the only effective antibiotic therapy for many *S. marcescens* bacteremias (Saito *et al.*, 1989; Wilfert *et al.*, 1970). By the 1980's the frequency of *S. marcescens* bacteremia declined and successful treatment of the infections improved with the introduction of newer antibiotics, such as, cephalosporins, aztreonam and imipenem (Saito *et al.*, 1989). More recently, *S. marcescens* isolates have been identified with the capability to resist one or more of these antibiotics (Osano *et al.*, 1994; Sanders & Sanders, 1992; Yang *et al.*, 1990). With increasing incidence of antibiotic resistance it is feared that common bacterial infections, including those of *S. marcescens*, will not be treatable.

In keeping with the prevalence of antibiotic resistant organisms, the importance for studying how their resistance arises increases. β-lactam therapy is a common treatment for *Serratia* infections and the development of resistance

has followed practically all new β-lactams used. Resistance to the β-lactam antibiotics can involve a number of mechanisms. One such mechanism arises from altered outer membrane permeability. β-lactams penetrate the Gram-negative outer membrane through pores in the membrane formed by porin proteins (Nikaido *et al.*, 1983). It is thought that altered porin structure and/or decreased porin expression may play a role in resistance by reducing the amount of antibiotic entering the cell (Nikaido, 1988). In *S. marcescens* the outer membrane porins have not been well characterized. A number of outer membrane proteins with a monomer molecular mass around 40 kDa, as identified by SDS-PAGE, have been suggested as being porins (Gutmann *et al.*, 1985; Hazishume *et al.*, 1993). There have also been reports demonstrating a decreased amount of these proteins in the outer membrane in antibiotic resistant strains (Gutmann *et al.*, 1985; Traub & Bauer, 1987). The characterization of the *S. marcescens* outer membrane and its porins is required as a first step to understanding the role altered outer membrane permeability may play in this organism's resistance to β-lactam antibiotics and is thus the aim of this study.

1.2. Antibiotic resistance of Gram negative bacteria

1.2.1. Mechanism of action of β-lactam antibiotics

There are a number of classes of β-lactam antibiotics. Some of the more common ones include penicillins, cephalosporins, monobactams and carbapenems (Dever & Dermody, 1991; Russel, 1992). The common element of

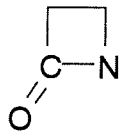
all the classes is the presence of the β-lactam nucleus which is a four membered ring including a peptide-type bond (CO-N). The differences in structure exist in the adjacent side ring, if it is present, and the various side chains present (Figure 1.1). β-lactams of all types will vary in potency because of variations in ability to permeate the cell wall, target affinity, inducibility of degradative β-lactamase enzymes and stability against β-lactamases (Dever & Dermody, 1991).

β-lactams function by preventing cell wall synthesis in Gram-negative and Gram-positive bacteria, more specifically, by inhibiting transpeptidation, the last step of peptidoglycan maturation (Dever & Dermody, 1991; Lambert, 1992). The targets of the β-lactam are transpeptidase and carboxypeptidase enzymes, commonly called penicillin binding proteins (PBPs). It is only the transpeptidase that is the lethal target. After addition of the peptidoglycan (PG) precursor to the existing peptidoglycan matrix a transpeptidase breaks the peptide bond between the terminal D-alanine and the adjacent D-alanine residue releasing the terminal amino acid. This leaves the PBP attached to the new terminal amino acid as an acyl-enzyme intermediate. Next the transpeptidase breaks its interaction and the energy released from it is used to form a new bond between the D-alanine (pos.4) and the *m*-diaminopimelic acid at position 3 of an adjacent PG chain. This cross-linking provides strength to the cell wall matrix.

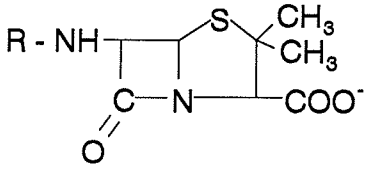
The β-lactam antibiotic is a structural analogue of D-alanyl-D-alanine (Lambert, 1992). The analogous β-lactam bond is attacked by the PBP forming an acyl-enzyme complex. This intermediate is more stable than the D-alanyl-enzyme complex thus preventing the PBP from performing its natural function. In

Fig. 1.1. Molecular structures of some β-lactam antibiotic groups. **A**, β-lactam nucleus common to all β-lactam antibiotics; **B**, penicillins; **C**, cephalosporins; **D**, carbapenems; **E**, monobactams

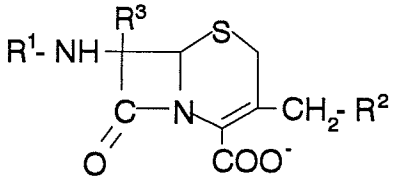
A



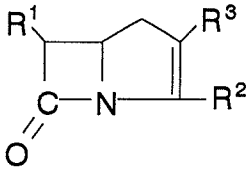
B



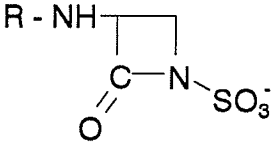
C



D



E



addition, autolysins or murein hydrolases continue to function degrading the peptidoglycan (Dever & Dermody, 1991). The overall effect of the antibiotic then is cell-wall lysis, disruption of cell shape, and inhibition of cell division. Clavulanic acid and sulbactam are β-lactam molecules that have poor antibacterial activity but are potent inhibitors of many β-lactamases. They function by binding irreversibly to β-lactamases. They are sometimes co-administered with β-lactam drugs during antimicrobial chemotherapy to improve efficacy of the β-lactam treatment by inhibiting β-lactamase activity.

1.2.2. Mechanisms of resistance to β-lactam antibiotics

Historically, the β-lactam antibiotic, penicillin, was the first antibiotic to be clinically used. Consequently, it was also the first antibiotic that a bacterium developed resistance to just a few years after its first use (Travis, 1994). That was the beginning of what was to become a major problem in treating bacterial infections.

Resistance to β-lactam antibiotics can occur by multiple mechanisms. The major mechanism recognized is the production of degradative β-lactamase enzymes (Franklin, 1992). A second mechanism involves alterations to PBPs such that the drug has a lower affinity to the PBP. This has been well characterized, for example, in the Gram-positive bacterium, *Staphylococcus aureus* (Archer & Climo, 1994). Alterations in outer membrane permeability have also been suggested to play a role in β-lactam resistance (Nikaido, 1988).

Many different β-lactamases exist and are produced by both Gram-

positive and Gram-negative bacteria. Gram-positive β-lactamases are distinct from those of the Gram-negative bacteria and the latter consist of a more heterogenous group (Franklin, 1992; Sykes, 1982). Enzymes can be distinguished from one another by their substrate and inhibitor profiles, gene location (chromosome or plasmid), isoelectric point and expression pattern. A number of typing methods exist based on these properties. The most recent and comprehensive typing scheme was proposed by Bush (1989). This method uses substrate and inhibitor profiles as the major determinant but also considers physical characteristics. β-lactamases are similar to PBPs, both are serine proteases and they carry out basically similar reactions (Sanders, 1992). The β-lactamase enzyme functions by targeting the β-lactam and forms an acyl-enzyme intermediate just as the PBP does. However, the deacylation step is efficient thus releasing an inactive antibiotic while the β-lactamase can now destroy another β-lactam molecule.

In order to combat resistant organisms new drugs are designed to produce more stable derivatives. However, with continued β-lactam use, β-lactamases have evolved to destroy the new drugs. In many cases a single amino acid change in a β-lactamase protein can alter the substrate specificity. There are numerous examples that have been sequenced and are summarized by Davies, (1994). Such events are frequent especially amongst the *Enterobacteriaceae*, including *S. marcescens*. Newly identified enzymes are also being characterized including a metallo β-lactamase effective on imipenem in *S. marcescens* (Yang *et al.*, 1990; Osano *et al.*, 1994). Another way that bacteria

respond to the use of β-lactams is to increase expression of β-lactamases through mutations in the regulation system (Chen & Clowes, 1987; Honoré *et al.*, 1986). With high levels of enzyme even relatively poor substrates can be inactivated leading to antimicrobial resistance.

PBP-mediated resistance is a less common mechanism than β-lactam inactivation in Gram-negative bacteria (Spratt, 1994). This may partially be due to the necessity to reduce β-lactam affinity without a major reduction in substrate affinity. Nevertheless, 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. In *Neisseria* and *Haemophilus* synergy with reduced outer membrane permeability means a less drastic reduction in PBP affinity is required to achieve resistance.

The mechanism of entry into the periplasmic space for β-lactam antibiotics is through pores in the outer membrane formed by porin proteins (Nikaido *et al.*, 1983). The rate of entry of these antibiotics can play a role in the resistance of the organism. For example, *Pseudomonas aeruginosa* does not produce the large general diffusion pores characteristic of the *Enterobacteriaceae* porins, but rather it produces a porin of another classification with much lower permeability

(Bellido *et al.*, 1992). Penetration across the *P. aeruginosa* outer-membrane is much slower, one hundredth the rate across the *E. coli* outer membrane (Nikaido *et al.*, 1991), due to the difference in porins. Consequently, *P. aeruginosa* has a higher natural resistance to many antibiotics. Other Gram-negative bacteria can increase their intrinsic or natural resistance by decreasing outer membrane permeabiltiy through a reduction in the expression of porins. Examples of decreased porin content, as evidenced by SDS-PAGE, in antibiotic resistant strains are numerous (Chow & Shlaes, 1991; Dang *et al.*, 1988; Mitsuyama *et al.*, 1987; Rice *et al.*, 1993). The exact role outer-membrane permeability plays in β-lactam resistance is uncertain. (In the case of *Pseudomonas aeruginosa* the specific channel, OprD, has a clear involvement in imipenem resistance (Huang & Hancock, 1993).) Permeability changes alone may only be of minimal importance in the overall resistance since the periplasmic levels of β-lactams may accumulate to high enough levels to saturate the PBPs. However, low permeability may have a synergistic effect on resistance when a β-lactamase is also produced (Sanders, 1992).

In addition to the mechanisms listed above, drug efflux is an important antimicrobial resistance mechanism. Drug efflux was first described by Levy and co-workers (McMurry *et al.*,1980) for tetracycline in *E. coli*. An active efflux pump in the cytoplasmic membrane essentially pumps out the antibiotics. It has become increasingly recognized as an important mechanism of resistance to many antibiotics in many bacteria (Nikaido, 1994a). The effectiveness in β-lactam resistance would be expected to be limited since the essential target is in

the periplasm. At least one β-lactam example is known, the *P. aeruginosa* MexA/B-OprM system, which includes a transporter, accessory protein and an outer membrane channel (Gotoh *et al.*, 1995; Li *et al.*, 1995). The three proteins of the system are thought to form a complex that involve both membranes and together remove a variety of antimicrobial agents from the cell. Even though the β-lactam target is in the periplasm the drug may become partially embedded in the cytoplasmic membrane and then pumped out (Li *et al.*, 1994).

In summary, resistance to β-lactams by Gram-negative bacteria can occur by one or more mechanisms. Decreased outer membrane permeability may be important since it may enhance resistance mediated by β-lactamases, PBPs and efflux.

1.3. The Outer membrane of Gram negative bacteria

1.3.1. Outer membrane structure/organization

The cell envelope of the Gram-negative bacterium consists of the cytoplasmic or inner membrane, the periplasmic space and the outer membrane. This is in contrast to the Gram-positive cell envelope which lacks the outer membrane.

The cytoplasmic membrane is similar in all eubacteria consisting of a lipid bilayer composed of phospholipids and proteins (Hancock, 1984). The functions of the cytoplasmic membrane include energy generation, signal transduction, transport and biosynthesis of membrane components. The cytoplasmic

membrane is a permeability barrier to the interior of the cell. Hydrophilic molecules cannot readily pass the membrane unless a specific uptake system exists for it, however hydrophobic molecules can pass relatively well.

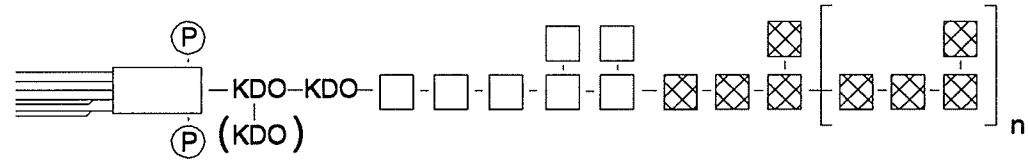
The gel-like periplasmic space in Gram-negative bacteria consists of peptidoglycan and protein (Hancock, 1984; Lambert, 1992). Peptidoglycan is made up of glycan strands of repeating units of the disaccharide N-acetylglucosamine(β-1-4)N-acetylmuramic acid (NAG-NAMA). Attached to the NAG-NAMA is a short peptide chain which may cross-link to neighbouring peptide chains via peptide bonds to form a molecular mesh. The peptidoglycan layer in Gram-negative bacteria is much thinner than in the Gram-positive cell wall. The peptidoglycan matrix provides strength to the cell wall maintaining cell shape and stability against osmotic pressure in dilute environments. In the periplasm are a variety of proteins involved in functions such as transport and processing. Among the many examples are, galactose binding protein involved in galactose transport and chemotaxis (Miller *et al.*, 1980), penicillin binding proteins involved in peptidoglycan synthesis (Spratt, 1994), and DsbA which catalyzes disulphide bond formation in exported proteins (Bardwell, 1994). Membrane derived oligosaccharides (MDO's) in the periplasm respond to and maintain isotonic osmolarity across the inner membrane (Kennedy, 1982). Because MDOs carry a net negative charge a membrane potential across the outer membrane exists and is referred to as the Donnan potential. It can reach as high as 80-100 mV in low osmotic media.

The outer membrane, the external most layer of the Gram-negative cell

wall in the absence of a capsule, consists of phospholipids, glycolipids and protein (Nikaido & Vaara, 1985). It is structurally and functionally different from the cytoplasmic membrane due, for the most part to a unique lipid in the outer leaflet layer of the lipid bilayer. This unique lipid is lipopolysaccharide (LPS) and is only found in the Gram negative bacterial outer membrane. The LPS molecule is divided into three regions: the lipid A component, the R-core oligosaccharide and the additional O-polysaccharide portion (Figure 1.2). The sugar content of the O-polysaccharide chains is highly variable (Hitchcock & Brown, 1983) while the R-core is generally invariable within a genus (Wright & Kanegasaki, 1971). The basic structure of the O-polysaccharide consists of repeating units of tri- to penta-saccharides. Variation of the O-polysaccharide occurs in the number of repeating units as well as the constituent sugars and linkage positions. The O-polysaccharide is highly immunogenic and the variability of this moeity is responsible for the different O-serotypes of Gram-negative bacteria. Strains without the O-polysaccharide are referred to as rough mutants due to their rough edged colonial morphology. Additionally, there are different R-mutant chemotypes since portions of the R-core may also be absent. Different R-mutants have been characterized and the minimum core structure required for LPS has been determined to contain two 2-keto-3-deoxy-octulosonic acid (KDO) residues attached to lipid A (Raetz, 1993).

Unlike phospholipids with two fatty acyl chains the LPS molecule has 6-7 fatty acyl chains (Nikaido & Vaara, 1985). In addition, these fatty acid chains are almost always saturated. The result is a less fluid bilayer and consequently the

Fig. 1.2. Lipopolysaccharide molecule. KDO, 2-keto-3-deoxy-octonic acid; □, monosaccharide unit of the R-core; ⊠, monosaccharide unit of the O-polysaccaride; Ⓟ, phosphate group.


Lipid A
R - core
O - polysaccharide
P
KDO
KDO
(KDO)
P
n

outer membrane is less permeable. Also contributing to reduced permeability is the association of the large head groups with divalent cations. This was evidenced when treatment with deacylpolymyxin, which displaces cations, increased the permeability of steroid probes by 100 fold (Plésiat & Nikaido, 1992). Long polysaccharides protruding out into the environment hinder lipophilic molecules and bacteriophage from reaching the lipid bilayer and ultimately from permeating the membrane effectively (van der Ley *et al.*, 1986; Plésiat & Nikaido, 1992). Thus, the outer membrane serves to reduce permeability of molecules into the cell.

Despite the low permeability of the outer membrane the cell must import nutrients and export waste products. In order to adapt to this situation they produce proteins, called porins, that form channels across the membrane (Nikaido, 1994b). Porins generally allow passage of molecules up to about 600 Da in size. This excludes many large toxic compounds such as basic dyes, bile salts and lysozyme while still allows essential nutrient passage. Not all unwanted molecules are excluded; many antibiotics including β-lactams will be able to pass through the channels. There exists different types of porins which will be discussed in detail in the next Section.

The Gram-negative bacterial cell can import some larger molecules such as, vitamin B12 and iron-siderophore complexes, which cannot penetrate porin channels. To do this energy-dependent uptake systems specific for their substrate are employed (Postle, 1993). The system requires a high affinity receptor and the TonB protein as well as accessory proteins. While the N-

terminal portion of TonB is integrated in the cytoplasmic membrane the C-terminal half of the protein reaches across the periplasm to interact with outer membrane receptors. The TonB protein couples energy from the cytoplasmic membrane for the energy-dependent uptake.

In addition to the proteins involved in transport there exists abundant structural proteins in the outer membrane important for maintaining cell shape and integrity. In *E. coli* this includes OmpA and Braun's lipoprotein (Sonntag *et al.*, 1978). Braun's lipoprotein is a 7.2 kDa integral membrane protein that stretches into the periplasm and associates with the peptidoglycan (Braun, 1975). Approximately one-third of the interactions are through covalent linkages. The remaining are through ion bridges. The OmpA protein attaches to the peptidoglycan via ion bridges (Endermann *et al.*, 1978). Both structural proteins have been shown to be non-essential for viability but mutants lacking either protein have an unstable outer membrane, releasing outer membrane vesicles and periplasmic components (Nikaido & Vaara, 1985 or Sonntag *et al.*, 1978). OmpA, with a size of 35 kDa (Chen *et al.*, 1980), has other functions in addition to maintaining cell shape and integrity. These include acting as a phage receptor (Datta *et al.*, 1977; Van Alphen *et al.*, 1977), a colicin-receptor (Chai & Foulds, 1974), and as a low permeability monomeric porin (Sugawara & Nikaido, 1992).

In addition to the *E. coli* OmpA many other outer membrane proteins also serve as receptor proteins. Receptors are required for some nutrients, bacteriophage, bacteriocins, and pilus-mediated conjugation. For example, in *E. coli* the OmpF porin acts as a receptor for colicins A and N (Fourel *et al.*, 1990)

and Tula (Datta *et al.*, 1977) and T2 phage. The LamB porin serves as a receptor for lambda phage (Charbit *et al.*, 1994). It has also been demonstrated that OmpA is involved in pilus reception (Van Alphen *et al.*, 1977). In addition, receptors for vitamin B12 (Kadner, 1990) and iron-siderophore complexes (Killmann *et al.*, 1993) are present in the outer membrane.

1.3.2. The outer membrane and the Gram negative pathogen

As was explained above the outer membrane is a permeability barrier to the external environment. This protects the cell from many toxic molecules encountered in the host (Nikaido & Vaara, 1985), such as bile salts, lysozyme and digestive enzymes. Since the outer membrane provides low permeability the Gram-negative cell has higher resistance than Gram-positive bacteria to many antibiotics. This is referred to as natural or intrinsic resistance. In fact, some antibiotics, such as vancomycin, are not at all effective on Gram-negative bacteria since they are too large to penetrate bacterial porins (Lambert, 1992). The negative charge on the outer membrane due to LPS serves to resist phagocytosis and complement action (Nikaido & Vaara, 1985). Also owing to the poor effectiveness of complement and phagocytosis is the steric hindrance of the long polysaccharide chains of the LPS (Finlay & Falkow, 1989). LPS rough strains of *Salmonella* are more susceptible to complement killing than smooth strains (Finlay & Falkow, 1989).

Gram-negative bacteria can also evade the host immune system by antigenic variation of surface structures. For example, *Neisseria gonorrhoeae*

can express several variations of its PII surface protein and pili (Finlay & Falkow, 1989). Also, several O-serotypes of *Salmonella* exist due to the variation in the O-polysaccharide (Wright & Kanegasaki, 1971).

Common symptoms of all Gram-negative type infections include fever and shock. LPS, or more specifically Lipid A is responsible for these effects (Raetz, 1993). LPS released from the outer membrane will bind to the host LPS binding protein which delivers it to the CD14 surface receptor of macrophage and certain other responsive cells. The CD14 receptor will in turn activate an unknown receptor which signals an increase in transcription of certain cytokines. The excessive production of these cytokines produces fever and shock. It is for this reason that LPS is also referred to as endotoxin.

1.4. Porins

1.4.1. Introduction to 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, 1976a). It was proposed that it be called porin due to its apparent pore-forming property. Since the initial characterization of the *E. coli* porin much work has been completed providing information on porin structure, function and regulation.

Porins form water-filled channels that span the outer membrane of Gram-negative bacteria. Porins may be classified into two types: non-specific porins,

which allow non-specific diffusion of substrates, and specific porins, which have substrate-binding sites (Nikaido, 1994b). In addition, they may also be distiniguished into trimeric and monomeric groups. Many porins form trimers as a functional unit. The trimers form a strong association that require heating at temperatures greater than 80°C for 10 minutes in SDS to dissociate them (Benz, 1988). Monomer molecular weights generally fall in the range of 30 to 48 kDa (Hancock, 1991).

Porins have been identified in many Gram-negative bacteria. Among the numerous examples are: *Salmonella typhi* (Puente *et al.*, 1989), *Pseudomonas aeruginosa* (Nikaido *et al.*, 1991), *Haemophilus influenzae* (Burns & Smith, 1987), *Neisseria* (Barlow *et al.*, 1989), *Bordetella pertussis* (Li *et al.*, 1991), and *Pasteurella multocida* (Chevalier *et al.*, 1993). Omp β is a porin identified in the phylogenetically primitive Gram-negative bacterium *Thermotoga maritima* suggesting a very early existence for porins (Engel *et al.*, 1993). A porin has even been demonstrated in the non-Gram-negative bacterium, *Mycobacterium chelonae* (Trias *et al.*, 1992). While, *Mycobacterium* is considered to be Gram-positive its cell wall is unique and can be considered similar to a Gram-negative cell wall. Similarity or conservation among the trimeric general diffusion porins has been demonstrated (Hofstra *et al.*, 1980; Jeanteur *et al.*, 1991). The widespread occurrence and conservation of structure of porins illustrates the important function they play in the Gram-negative bacterium.

There have been three common experimental techniques used to examine porin function: a) Efflux of radiolabelled solutes out of proteoliposomes prepared

with purified porins allows for the measurement of molecular weight exclusion limits (Nakae, 1975). b) The liposome swelling assay is used to measure rates of swelling of proteoliposomes. The proteoliposomes are prepared with an impermeable solute within and then diluted in isoosmotic test solutions. As the test solute moves in, water moves in with it, and the liposomes swell. The swelling can be detected by a reduction in light scattering in a spectrophotometer (Nikaido & Rosenberg, 1983). c) A third method, the black lipid bilayer technique, measures conductance through single channels incorporated in a lipid film (Benz *et al.*, 1978). The conductance through the channel is used to calculate pore size. Calculation of pore size by single channel conductance data made some assumptions now known to be false (Cowan *et al.*, 1992; Nikaido, 1994b). Firstly, it was assumed that the pore diameter was the same along the length of the pore. In fact, the pore is constricted at only a portion of its length. Secondly, the conductance through the pore channel was assumed to be the same as the bulk solution. It would be expected that there would be more resistance through the pore considering its small size. The methods for measuring pore activity are limited in use for calculating pore size, although they can be useful for relative comparisons of porins. As well, black lipid bilayer experiments can be designed to determine ion selectivity of porins.

1.4.2. *E. coli* porins

The porin originally described by Nakae (1976a) 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 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 complexly regulated such that as the number of one porin type increases the number of the other porin decreases (Csonka, 1989). The regulation of these two porins will be discussed in detail in a later section. The OmpF and OmpC porins are similar in amino acid sequence and structure (Hofstra *et al.*, 1980; Mizuno *et al.*, 1983). They both form cation selective pores with similar sizes (Benz *et al.*, 1985). The molecular weight exclusion limit of OmpF, OmpC and related porins of this type is about 600 Da (Decad & Nikaido, 1976). However, the OmpC porin forms a somewhat smaller pore than OmpF. The estimated pore sizes as determined by black lipid bilayer experiments are 1.2 and 1.1 nm for OmpF and OmpC, respectively (Nikaido & Rosenberg, 1983). The seemingly slight difference in channel size of OmpC compared to OmpF results in a twofold reduction in the permeability rate of glucose in liposome swelling experiments.

A third general diffusion porin 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; Overbeeke *et al.*, 1983). Opposite to OmpF and OmpC, however, PhoE is anion selective (Benz *et al.*, 1985) 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*. 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 (Schülein *et al.*, 1991). All three of these specific channels also exhibit some general diffusion activity.

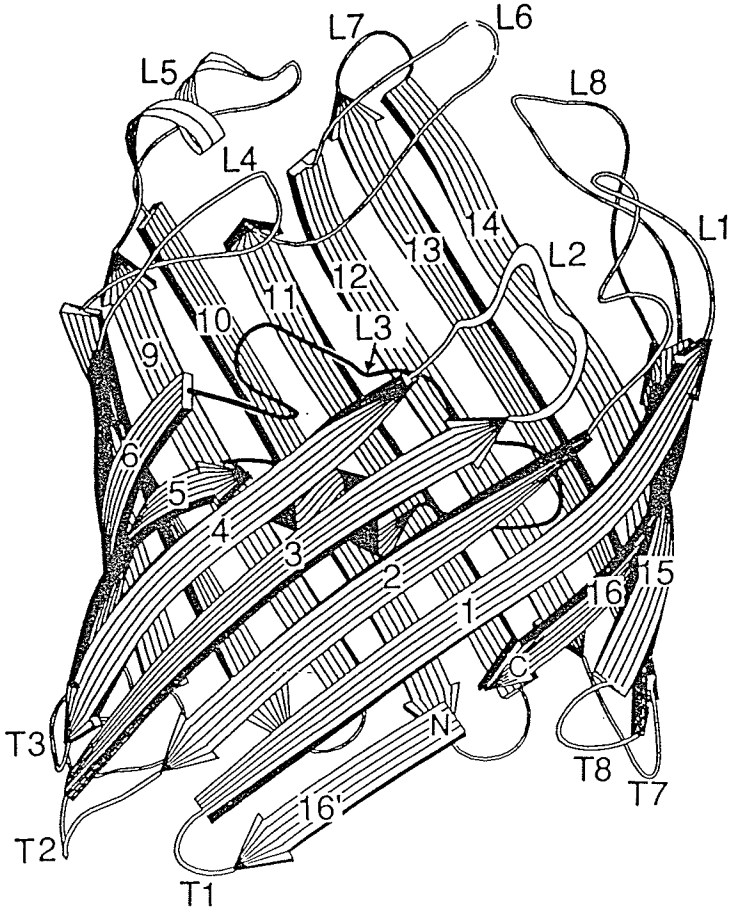
OmpA is an example of an *E. coli* monomeric porin. It was only recently discovered that OmpA can form non-specific diffusion channels (Sugawara & Nikaido, 1992) in the outer membrane. The OmpA channel forms a pore similar in diameter to the OmpC/OmpF porins, however, it has a much lower permeation rate. This is believed to be due to the possibility of open and closed states (Sugawara & Nikaido, 1994). It is estimated that only about 2 to 3% of OmpA channels are open at a given time. Interestingly, the OmpA protein appears to be analogous to the *Pseudomonas aeruginosa* OprF porin as determined by immunological cross-reactivity, amino acid sequence similarity and heat modifiability (Woodruff & Hancock, 1989). *P. aeruginosa* does not produce any classical porins and relies on the major porin, OprF and other specific porins for nutrient uptake (Bellido *et al.*, 1992). 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 & Nikaido, 1992).

1.4.3. Porin structure and function

The first porin crystallized was from *Rhodobacter capsulatus* (Nestel *et al.*, 1989; Weiss & Schulz, 1992). Since then the *E. coli* OmpF and PhoE porins have been crystallized (Figure 1.3) (Cowan *et al.*, 1992). While the primary structure of the *R. capsulatus* and *E. coli* porins were different, the tertiary structures were strikingly similar. Each barrel is formed by sixteen anti-parallel beta-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 surface. 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. Therefore the assumption of a uniform pore in pore size calculations using black lipid bilayer data was incorrect. Despite this, the experimental values obtained correlate well with that observed from crystallographic data. The diameter of the channel at the constriction zone measures 7 x 11Å. The design of the channel may be advantageous because the restriction allows size selection with less effect on diffusion rate by limiting it to only a small portion. Within the porin barrel 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 channel (Nikaido, 1994b).

The OmpF porins exist as trimers. Early models suggested the presence of three external pore openings which converged into one at the inner end (Engel

Fig. 1.3. Three-dimensional figure of the *E. coli* OmpF porin as determined by X-ray crystallographic data (Cowan *et al.*, 1992). 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 external loops are designated L1 through L8 whereas, short turns on the periplasmic side are designated T1 through T8. The third external loop, important in porin function, is designated L3 and can be seen folding down into the pore lumen. The second external loop, L2, extends out of the page toward the viewer where it would interact with a neighbouring porin monomer contributing to a strong trimer association.


L7
L6
L5
L8
L4
L1
14
13
L2
12
11
10
L3
9
6
5
4
3
2
1
16
15
C
N
T3
T8
T7
16'
T2
T1

et al., 1985). However, the crystallization data showed that each monomer had 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 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, 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. A side view of the porins illustrates bands of residues across the trimer unit. The pattern consists of polar, aromatic, hydrophobic, aromatic and once again polar amino acid regions. The two polar ends are the internal and external loops and the hydrophobic portion is the membrane spanning region. The narrow ring of aromatic amino acids at the hydrophobic-hydrophilic interface may function to hold the porin in place in the membrane by interacting with both neighbouring regions (Cowan, 1993).

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. 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 (Struyvé *et al.*, 1991). In PhoE the Glycine-144 residue is required for proper protein folding (de Cock *et al.*, 1994). One OmpF mutant resistant to colicins A and N was shown to have a single glycine at position 119 changed to aspartic acid (Fourel *et al.*, 1993). In addition to altered colicin binding this change affected the stability of the porin trimer since the temperature required to dissociate the trimers decreased. Also, the region between residues 115 and 144 in PhoE was demonstrated to be important in the formation of a stable trimer (Fourel *et al.*, 1994) with the use of various OmpF/OmpC chimeric proteins. This region includes loop 3 and part of the following transmembrane domain.

As mentioned earlier 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 PEFGG motif which is conserved in the enterobacterial porins (Jeanteur *et al.*, 1991). The motif is at the external tip of the third loop and is believed to be important for turn formation at this site in the loop. The glycine-114 to aspartic acid mutation characterized

by Fourel *et al.* (1993) occured in the PEFGG motif changing it to PEFDG. As mentioned this alteration affected porin stability, colicin binding, as well as, pore size (Jeanteur *et al.*, 1994). In another study, mutants lacking LamB and one of OmpF or OmpC but still able to grow on maltodextrins were isolated. Analysis of the functional OmpF or OmpC sequence revealed critical alterations that allowed the larger maltodextrins to pass. Deletions within the third loop were detected, as well as replacement of charged residues, aspartic acid and arginine, in the interior of the pore for uncharged amino acids (Benson *et al.*, 1988; Misra & Benson, 1988). Isolation of these mutants provided evidence for the charged amino acids within the channel and the third external loop in the size selectivity function of the porins.

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 & 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). Nikaido (1994b) has also argued that it would be unlikely to sustain a high potential across a membrane with so many large general diffusion channels. The voltage induced closure may serve as a safety mechanism in case a porin is mistakenly incorporated into the energized

cytoplasmic membrane, as suggested by Tommassen (Nikaido, 1994b).

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 therefore suspected. Chemical mutagenesis of the only histidine in OmpF and OmpC, H-21, resulted in a higher proportion of larger channels regardless of pH (Todt & McGroarty, 1992a). This suggested a role for histidine-21 in the pH-induced switch in channel size, possibly through an interaction with the third loop in the pore. Crystallographic data demonstrated that the H-21 residue was present on the external surface interacting with another monomer unit (Cowan *et al.*, 1992). Consequently, it is questioned how it would be able to function in regulating pore size. The pH induced size switch was also demonstrated *in vivo* in *E. coli* (Todt & McGroarty, 1992b). At higher pH the hydrolysis of cephalosporins by periplasmic β-lactamase was increased. This increase was shown not to be due entirely to increased β-lactamase activity and therefore, increased permeability was a factor. There is some confusion whether the porins are voltage-gated or size-regulated by pH, however, it does illustrate the possible dynamic nature of these potentiators of membrane permeability.

1.4.4. Porins in *S. marcescens*

In comparison to the *E. coli* porins, the porins of *S. marcescens* have not been well characterized. The *S. marcescens* outer-membrane was originally

thought to have at least one porin with an estimated molecular mass of 41 kDa (Malouin *et al.*, 1990). This porin has been shown to fall into the non-selective group, based on black lipid bilayer experiments, and has a calculated pore size of 1.1 nm. The average single channel conductance of 1.6 nS is intermediate to the *E. coli* OmpF and OmpC porins, with values 1.9 and 1.5 nS, respectively. Other outer membrane proteins in the 40 kDa range have been identified and classified as porins based on heat-modifiable mobility changes in SDS-PAGE and differential solubilization in NaCl/SDS/EDTA (Gutmann *et al.*, 1985; Hazishume *et al.*, 1993). Additional studies distinguish possible porins based on reduced outer membrane quantities in antibiotic resistant isolates (Goldstein *et al.*, 1983; Piddock & Traynor, 1991). These various reports identify a total of two or three possible porins in the *S. marcescens* outer membrane. No specific functional studies have been performed using these putative porins.

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

1.5.1. Introduction

It has long been known that the *E.coli* OmpF and OmpC porins are regulated in response to external osmolarity and temperature (Csonka, 1989). The total number of OmpF plus OmpC porins remains fairly constant while the ratios of 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 environment of the animal the less permeable porin, OmpC, predominates. However, where nutrients may be more scarce and the environment relatively 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 & Booth, 1992), and the presence of salicylate (Rosner *et al.*, 1991), tetracycline and chloramphenicol (Cohen *et al.*, 1988) have now been shown to regulate the levels of the OmpF and/or OmpC porins.

Two major mechanisms involved in the porin regulation have been characterized. These include the OmpR/EnvZ system and *micF*. The following sections will describe the mechanisms of the two systems and the roles each play in regulating the porins with respect to different conditions. As will be explained later, other factors may also affect the regulation of the two porins.

1.5.2. The EnvZ/OmpR system

Early genetic studies on *E. coli* OmpF and OmpC porins revealed three loci that were involved in the expression of the two porins, *ompF* (21 min), *ompC* (47 min), and *ompB* (74 min). Mutations in the *ompF* and *ompC* genes resulted in a total loss of outer membrane proteins IIa and IIb, while mutations in the *ompB* locus were more complex, providing different phenotypes of OmpF/OmpC expression (Hall & Silhavy, 1979). The *ompB* locus was postulated to be involved in the regulation of the structural genes, *ompF* and *ompC*. This was

supported by studies utilizing *lacZ-ompC/F* fusions and *ompB* mutants (Hall & Silhavy, 1981a). Further characterization of this locus demonstrated two gene products, *ompR* and *envZ* (Hall & Silhavy, 1981b). 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. Sequence analysis of the *ompR* gene demonstrated a 32.5 kDa soluble protein (Wurtzel, *et al.*, 1982). Within the sequence of the 44 kDa EnvZ protein a 17 amino acid hydrophobic stretch was found which supported the idea that it was a membrane protein (Mizuno *et al.*, 1982). Further studies concentrated on localization of the OmpR recognition sites and elucidation of the regulatory mechanism.

The EnvZ/OmpR system is described as a two component histidine kinase regulatory system (Stock *et al.*, 1989). These systems function by modulating the activity of a transcriptional regulator protein through phosphorylation 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. EnvZ probably exists as a dimer (Roberts *et al.*, 1994). The C-terminal end is in the cytoplasm and possesses both phosphatase and kinase activities. The way in which the EnvZ histidine kinase "senses" the osmolarity is unknown. The sensing domain has not been identified but may be in the periplasm or within the second membrane

spanning segment. Mutants defective in osmosensing have been isolated with alterations in either the transmembrane or cytoplasmic regions. The local anaesthetic, procaine, disrupts EnvZ function and is thought to interact with it through a transmembrane association. An EnvZ homologue from *Xenorhabdus nematophilus* appears not to possess a periplasmic domain. In place of this there is a larger transmembrane portion. In complementation experiments of an *E. coli envZ* mutant the *X. nematophilus* EnvZ osmoregulated the *E. coli* porins normally (Tabatabai & Forst, 1995). These results suggest that the periplasmic region is not responsible for osmosensing. However, various periplasmic deletion mutants suggested a positive role for the periplasmic domain of EnvZ (Tokishita *et al.*, 1991). An alternative view is that the deletions may have affected the protein conformation in other areas of the structure. For this reason and the *X. nematophilus* study it has been suggested that the periplasmic domain may function to allow proper spacing or conformation of the membrane portion. While the sensing domain has not been specifically localized the trigger is speculated to be a physical effect upon EnvZ and not a chemical interaction.

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 which activates it for transcriptional activation (Aiba *et al.*, 1989a; Forst *et al.*, 1989). 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 & Silhavy, 1991).

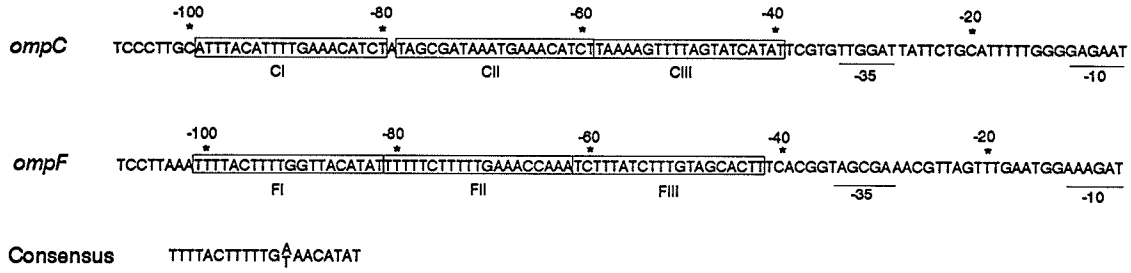
Two functional domains have been characterized in the OmpR protein which is consistent with previous genetic mapping of mutant phenotypes. A smaller fragment (16 kDa) identified as the C-terminal portion can arise spontaneously upon storage of purified OmpR protein (Tate *et al.*, 1988). The DNA binding domain was determined to be within this C-terminal portion as demonstrated by DNA protection assays using the spontaneously derived fragment. In addition to DNA binding, transcriptional activation (or repression) has been determined to be associated with the C-terminal domain (Pratt & Silhavy, 1994; Tsuzuki *et al.*, 1994). It has been demonstrated *in vitro* that EnvZ will transfer the phosphate at His-243 to the N-terminus of the OmpR protein (Aiba *et al.*, 1989a; Forst *et al.*, 1989). The specific site of transfer to the OmpR protein is primarily Asp-55 but some activity can be replaced by Asp-11 (Delgado *et al.*, 1993). The phosphorylation of OmpR facilitates its binding to the operator regions of the *ompF* and *ompC* genes (Aiba *et al.*, 1989b) and activates transcription of the each (Aiba & Mizuno, 1990; Forst *et al.*, 1990). Phosphorylation does not necessarily activate transcription directly but probably affects the conformation allowing OmpR to bind to DNA recognition sites and activate transcription (Kenney *et al.*, 1995; Tsuzuki *et al.*, 1994). The OmpR protein does not have a helix-turn-helix motif characteristic of bacterial DNA binding proteins (Nara *et al.*, 1986). Therefore, the nature of the binding is uncertain. The C-terminal portion involved in DNA binding has recently been

crystalized (Kondo *et al.*, 1994). Future crystalization data may provide insight into the nature of the DNA binding.

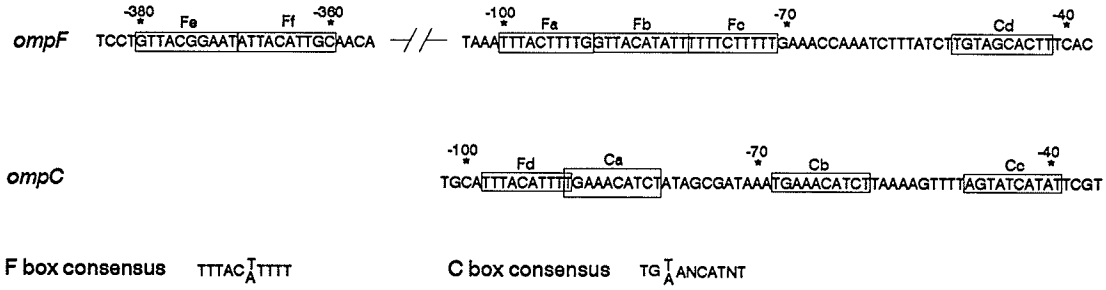
The site(s) of OmpR binding has been the subject of much debate with two main ideas suggested. Dnase I protection assays performed by Mizuno *et al.* (1988) located OmpR binding areas at -100 to -40 for both porin genes (*ompF* and *ompC*). The large region protected suggested that two or three OmpR molecules may interact with this area. This and further experiments led to the suggestion of an arrangement of three consecutive OmpR binding sites with the 20 bp consensus sequence TTTTACTTTTTG$^{A}_{T}$ AACATAT (Maeda *et al.*, 1991) (Fig. 1.4 A). In contrast another group found that approximately -100 to -60 were protected in Dnase I and chemical footprinting experiments (Norioka *et al.*, 1986; Tsung *et al.*, 1989). Two distinct 10 bp consensus sequences were proposed by the Inouye group (Norioka *et al.*, 1986; Tsung *et al.*, 1989). These are referred to as F and C boxes with consensus sequences of TTTAC$^{A}_{T}$ TTTT and TG$^{A}_{T}$ A-CAT-T, respectively (Fig. 1.4 B). In their model the *ompF* gene has three consecutive F boxes and one C box. The *ompC* gene has three C boxes spaced 10 bp apart and an F box just upstream. In addition, there are two F boxes or one 20 bp repeat upsream at the -380/-360 site of *ompF* (Huang *et al.*, 1994; Rampersaud *et al.*, 1994). Whatever the exact consensus sequence, there are at least two common properties between the two models. First, both involve multiple OmpR binding sites on the same side of the DNA as evidenced by the common denominator of 10 bp, one helical turn (Takayanagi & Mizuno, 1992). Second, OmpR binds to the OmpR binding sites (OBS) with differing affinities, the

Fig. 1.4. OmpR binding site (OBS) consensus sequences. **A**, OBS sequence as proposed by Maeda *et al.* (1991). A negative *ompF* OBS site had not been identified at this date and therefore not included in the figure. **B**, OBS sequence as proposed by Rampersaud *et al.* (1994). **C**, OBS sequence as proposed by Harlocker *et al.* (1995). The residues important in OmpR binding are underlined.

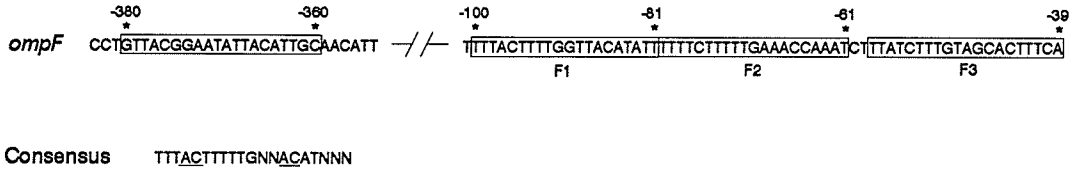
A


-100
-80
-60
-40
-20
ompC
TCCCTTGCATTTACATTTTGAAACATCTATAGCGATAAATGAAACATCTTAAAAGTTTTAGTATCATATTTCGTGTTGGATTATTCTGCATTTTTGGGGAGAAT
CI
CII
CIII
-35
-10
-100
-80
-60
-40
-20
ompF
TCCTTAAATTTTACTTTTGGTTACATATTTTTTCTTTTTGAAACCAAATCTTTATCTTTGTAGCACTTTCACGGTAGCGAAACGTTAGTTTGAATGGAAAGAT
FI
FII
FIII
-35
-10
Consensus TTTTACTTTTTG[A/T]AACATAT

B


-380
Fe
Ff
-360
-100
Fa
Fb
Fc
-70
Cd
-40
ompF
TCCTGTTACGGAATATTACATTGCAACA
TAAATTTACTTTTGGTTACATATTTTTTCTTTTTGAAACCAAATCTTTATCTTGTAGCACTTTCAC
-100
Fd
Ca
-70
Cb
Cc
-40
ompC
TGCATTTACATTTTGAAACATCTATAGCGATAAATGAAACATCTTAAAAGTTTTAGTATCATATTCGT
F box consensus TTTAC[T/A]TTTT
C box consensus TG[T/A]ANCATNT

C


-380
-360
-100
-81
-61
-39
ompF
CCTGTTACGGAATATTACATTGCAACATT
TTTTACTTTTGGTTACATATTTTTTCTTTTTGAAACCAAATCTTTATCTTTGTAGCACTTTCA
F1
F2
F3
Consensus TTTACTTTTTGNNACATNNN

significance of which will be discussed later.

A recent development by the Inouye group has cleared some of the controversy and connects the two models. They have found that 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 (Harlocker *et al.*, 1995) (Fig. 1.4 C). Two OmpR proteins will bind each unit consisting of two direct repeats of TTACATNTN and only in pairs. There is no evidence, however, that OmpR occurs in dimers prior to binding. The binding unit corresponds approximately with the 20 bp consensus proposed by Mizuno while the two subunits making up the binding unit correspond to the F and C boxes of the previous Inouye proposal. The upstream -380/-360 site also corresponds to the newly proposed consensus. While the investigation only involved the *ompF* gene the *ompC* regulatory region can be expected to be similarly structured.

Specific residues within the recognition sites have been identified that appear to be essential for OmpR binding. Mutation at these sites greatly impairs OmpR binding. These include the AC located in the binding motif with the C being particularly critical (Harlocker *et al.*, 1995; Mizuno & Mizushima, 1986; Pratt & Silhavy, 1995). See Fig. 1.4C. The first consensus of each unit is T-rich. Reversing the order of the two sites abolishes OmpR binding (Harlocker *et al.*, 1995). Therefore the asymmetry is thought to be important in coordinate binding. It is interesting to note that inverting the whole 20 bp unit still activates transcription (Maeda *et al.*, 1991). Another important feature is the periodicity of the sites. They occur every 10 bp or one helical turn. Consequently, the OmpR

molecules all bind on the same side of the DNA.

OmpR binds to the multiple sites with varying affinity. The F1 and C1 units have the strongest affinity and most resemble the consensus sequence (Harlocker *et al.*, 1995; Maeda *et al.*, 1991; Rampersaud *et al.*, 1994). 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 (six) 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. In addition, the F1 is a better binding site than C1. The ability of OmpR to bind to sites in a hierarchical manner allows differential expression in different conditions. At low levels of OmpR-P the higher affinity sites will be occupied allowing transcription of *ompF*. As the levels of OmpR-P rise the lower affinity sites of *ompC* will be bound and *ompC* will be activated. As previously stated the levels of OmpF are lower at high osmolarity (high OmpR-P), therefore this mechanism has not accounted for the repression of the *ompF* gene. The two additional OBS found upstream at -380 to -360 are essential for the negative expression of OmpF (Huang *et al.*, 1994; Rampersaud *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 interactions between the OmpR molecules (Rampersaud *et al.*,1994). 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 understood, however a loss of/or change in the interaction between OmpR and RNA-polymerase may be involved.

OmpR is required for transcriptional activation of both the *ompF* and *ompC* genes, transcription from the -35 and -10 regions alone is weak (Ozawa *et al.*, 1987). Several lines of evidence suggest that OmpR activates transcription through a direct interaction with the alpha-subunit of RNA polymerase (Bowrin *et al.*, 1994; Matsuyama & Mizushima, 1987; Sharif & Igo, 1993; Slauch *et al.*, 1991). Overproduction of the alpha-subunit interferes with *ompF* and *ompC* transcription without affecting house-keeping genes (Bowrin *et al.*, 1994). Certain mutants of both OmpR and RNA-Polymerase impair OmpR-dependent activation of *ompC* and *ompF* transcription (Aiba *et al.*, 1994; Russo *et al.*, 1993; Sharif & Igo, 1993). The OmpR interaction with RNA-Polymerase appears to be quite complex since activation, repression and DNA binding can be disrupted independently through mutations in OmpR (Russo *et al.*, 1993). In addition, mutations in the alpha subunit of RNA-Polymerase have been isolated that affect *ompF* and *ompC* transcription independently (Sharif & Igo, 1993).

1.5.3. *micF*

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 (Mizuno *et al.*, 1984; Schmidt *et al.*, 1995). *micF* was shown to decrease the levels of *ompF* mRNA when expressed in high copy number (Mizuno *et al.*, 1984). It is now believed that the mechanism involves destabilization of the *ompF* transcript by allowing accessibility of certain ribonucleases (Schmidt & Delihas, 1995). The *micF* and *ompF* RNA transcripts were found to form a stable complex at 37°C together with an unknown protein from *E. coli* (Andersen & Delihas, 1990). This complex was shown to be required for the destabilization of *ompF* mRNA.

micF appears to be under the control of the *ompB* locus via the same OmpR binding sites as *ompC* (Coyer *et al.*, 1990; Mizuno *et al.*, 1984). However, for years it was unclear what role *micF* played in the osmoregulation of the *E. coli* porins (Aiba *et al.*, 1987; Matsuyama & Mizushima, 1985; Mizuno *et al.*, 1984) considering the strong influence of the OmpR/EnvZ system. Recently it has been shown that *micF* is indeed a major factor in osmoregulation but only at low-to-intermediate levels of osmolarity (Ramani *et al.*, 1994). At higher levels of osmolarity transcriptional control by OmpR masks the effects of *micF*.

In addition, *micF* has been shown to be responsible for regulating the porins under a number of conditions. *micF* levels increase in response to salicylate (Rosner *et al.*, 1991), chloramphenicol, tetracycline, ethanol, oxidative stress and increased temperature (Andersen *et al.*, 1989) and thereby reduce OmpF levels in the membrane. Three other protein factors, Lrp, leucine responsive regulatory protein (Ferrario *et al.*, 1995), HRBF, heat resistant binding

factor and RSBF, redox sensitive binding factor (Gidrol & Farr, 1993) bind upstream to *micF*. Not much is known about HRBF and RSBF but Lrp decreases *micF* transcription. Lrp also interferes with *ompC* transcription so the effect is an increase in OmpF and a decrease in OmpC in the outer-membrane.

1.5.4. Multiple antibiotic resistant (Mar) *E. coli* strains

George & Levy (1983a) characterized multiple antibiotic resistance in *E. coli* after cultivating the organism on sub-inhibitory levels of tetracycline or chloramphenicol. Strains were shown to be resistant to many unrelated antibiotics including tetracycline, chloramphenicol, penicillins, cephalosporins and now fluoroquinolones (Cohen *et al.*, 1989). This phenotypic resistance could be reversed to low levels after removal of the drug. Resistance was partially explained by an active tetracycline efflux mechanism. This did not, however, explain resistance to other antibiotics. Further work identified a locus at 34 minutes on the *E. coli* chromosome that was responsible (George Levy, 1983b). A Tn*5* insertion at this site reversed the Mar phenotype. Interestingly, it was observed that OmpF levels were decreased in Mar mutants (Cohen *et al.*, 1988). The effect on OmpF was shown to occur because of increased *micF* transcription. Reduced OmpF levels only had a partial role in antibiotic resistance since *ompF* mutants were less resistant than *mar* mutants. Hachler *et al.* (1991) demonstrated that a 7.8 kb chromosomal fragment was needed to complement *mar* mutants unable to acquire the Mar phenotype. Finally, the *mar* locus was characterized with the cloning and sequencing of the region (Cohen *et*

al., 1993a; Gambino *et al.*, 1993). The resulting operon was referred to as *marRAB*. It was determined from complementation studies that it consisted of an operator sequence followed by three open reading frames termed, R, A and B. MarR acts as a repressor at the operator site. MarA has homology with a family of positive transcriptional activators including SoxS, TetD, and AraC and possesses a helix-turn-helix motif. MarB has yet no known function. Analysis of *mar* mutants revealed alterations in the operator or repressor sequence (Cohen *et al.*, 1993a). These mutations resulted in increased transcription of MarA. Overexpression of MarA was shown to be adequate to produce the Mar phenotype and decrease OmpF expression (Gambino *et al.*, 1993).

Salicylate is known to induce phenotypic antibiotic resistance in *E. coli* (Rosner, 1985). It has also been shown that salicylate increases *micF* transcription (Rosner *et al.*, 1991) therefore, it was logical to search for a link between salicylate and the *mar* operon. Cohen *et al.* (1993b) found that indeed salicylate increased transcription of the *marRAB* operon. The salicylate effect must also occur through a *mar*-independent pathway since increased drug resistance also occurred in *mar* deleted strains. Most recently it has been demonstrated that salicylate can bind to the MarR repressor and weaken its binding to the operator (Martin & Rosner, 1995). Neither tetracycline nor chloramphenicol were able to interact with MarR or otherwise inhibit it from binding the operator site.

The *mar* locus exists in other members of the *Enterobacteriaceae* as well (Cohen *et al.*, 1993c). Twenty seven genera from a variety of families were

assessed for the *mar* locus by hybridization with a *mar*-specific DNA probe. Members of the *Enterobacteriaceae* including *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter* and *Hafnia* hybridized. Hybridization was not detected with *Serratia* nor any bacteria outside of the *Enterobacteriaceae* that were tested. Since multiple antibiotic resistance has been detected in other bacteria a *mar* like mechanism may be present but less conserved.

1.5.5. Regulation of *S. marcescens* porins

Conflicting results are present in the literature on osmoregulation in *S. marcescens*. Sawai *et al.* (1987) have reported a lack of regulation of *S. marcescens* porins. Whereas, Puig *et al.* (1993) did observe regulation of 40 kDa and 39 kDa outer membrane proteins in response to medium osmolarity changes in a manner similar to *E. coli*. In addition, the amount of the 39 kDa protein decreased when bacteria were grown at a lower temperature of 18°C. Yet another report isolated only one porin from the *S. marcescens* outer membrane which appeared to be constitutively expressed under conditions tested (Malouin *et al.*, 1990).

The porins of *S. marcescens* do respond to the presence of salicylate in the growth medium (Sawai *et al.*, 1987). The 41 kDa protein was shown to decrease in expression whereas, the 40 kDa porin did not respond or only slightly increased in response to salicylate. Regulation of expression of *S. marcescens* porins have not been examined under other conditions.

1.6. Hypothesis and Objectives

Hypothesis: Reduced outer membrane permeability, by means of alterations to constituent porins, contributes to reduced susceptibility to β-lactam antibiotics observed in *S. marcescens*.

Specific Objectives

1. Identify the major porins in the *S. marcescens* outer membrane.
2. Evaluate the distribution and conservation of the porins in other *Serratia* species.
3. Characterize the identified *S. marcescens* porin(s) at the molecular level.
4. Evaluate the permeability of the *S. marcescens* porins .
5. Identify important amino acids in the porins that determine permeability to β-lactam antibiotics.

It is thought that a decrease in outer membrane permeability may contribute to β-lactam resistance in *S. marcescens*. In order to study the role that porins may play in β-lactam resistance it is essential first to understand the porins that are present. While numerous reports have identified putative porins in the outer membrane, it is not clear how many or which types are present. The most complete study of *S. marcescens* porins included the identification and functional characterization of one porin in the *S. marcescens* outer membrane (Malouin *et al.*, 1990). Cloning and sequencing of the *S. marcescens* porin genes will allow for a more complete understanding of its porins.

The *E. coli* OmpF and OmpC porins are osmoregulated. Osmoregulation of *S. marcescens* porins has been detected in some strains but not in others. Examination of the sequence of the *S. marcescens* porin genes may provide some clue as to why osmoregulation does not occur in some strains and if they are regulated by the same mechanisms.

The sequence obtained in this thesis will contribute to the list of known porin sequences. Amino acid sequence alignments of these porins will help determine conserved regions which may be expected to be important in porin structure or function. In addition, sequence alignments may identify other sites that are not as conserved, but where variation may contribute to different pore characteristics. From this information amino acids can be chosen for mutagenesis experiments. In addition, together with crystallographic data of the *E. coli* porins, prediction methods of protein folding in the outer membrane can be developed and existing methods tested more reliably with more sequences available.

The characterization of amino acids important for porin permeability properties will contribute to the understanding of the molecular function of porins as well as, outer membrane permeability. In turn, determining how molecular properties affect permeability will contribute to the understanding of antibiotic permeation and susceptibility.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions.

All bacterial strains and plasmids used are listed in Tables 2.1 and 2.2. For most experiments cells were grown in Luria-Bertani (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl). The top agarose used to obtain phage plaques was LB containing 0.7% agarose. Minimal media 9 (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.5 mM NaCl, 19 mM NH_4Cl, 2 mM $MgSO_4$, 1 mM thiamine supplemented with 0.2% (w/v) glucose) was used to maintain *E. coli* for single-stranded DNA preparations of pBluescript clones. For single-stranded DNA preparations used in site-directed mutagenesis experiments 2x YT broth (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl) was used. Antibiotics were added when required at 100 µg/ml for ampicillin and 20 to 30 µg/ml for chloramphenicol. Bacterial stocks were stored in 25% (v/v) glycerol or 7% (v/v) DMSO at -60°C.

2.2. DNA isolations.

Genomic DNA was isolated by the hexadecyltrimethylammonium bromide method (Ausubel *et al.*, 1987). Lambda DNA was isolated following the protocol of Kaslow (1986). Plasmid DNA was routinely isolated using the alkaline lysis method (Ausubel *et al.*, 1987). For sequencing, plasmid DNA was isolated using Magic Miniprep DNA purification kit (Promega, Madison, WI) following manufacturer's instructions. The Prep-A-Gene (Bio-Rad, Mississauga, ON) kit

Table 2.1. Bacterial Strains used in this study

Strain	Characteristic	Source/Reference
S. marcescens UOC-51	clinical isolate	T. Parr
S. marcescens UOC-67		ATCC 13880
S. odorifera		ATCC 33077
S. ficaria		ATCC 33105
S. liquefaciens		ATCC 27592
S. rubidaea		ATCC 27614
S. proteomaculans		ATCC 19323
S. plymuthica		ATCC 183
Bacillus subtilis		ATCC 6051
Salmonella typhimurium		UMCC
Shigella flexneri		UMCC
Edwardsiella tarda		UMCC
Citrobacter freundii		UMCC
Enterobacter cloacae		ATCC 222
Klebsiella pneumoniae		ATCC 13883
Proteus vulgaris		UMCC
Pseudomonas aeruginosa H103	wild type PAO1	Hancock & Carey, 1979; Holloway *et al.*, 1979
Escherichia coli HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	R.E.W. Hancock
E. coli LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	Bio/Can Scientific
E. coli NM522	*supE thi* Δ*(lac-proAB) hsd5* F' [*proAB*$^+$ *lacI*^{q} *lacZΔM15*]	Promega
E. coli CJ236	*dut*-1, *ung*-1,*thi*-1, *rel* A-1; pCJ105(Cm^r)	Kunkel *et al.*, 1987
E. coli BZB 1107	B^E, *ompF*::Tn5(Km^r)	Jeanteur *et al.*, 1994

Abbreviations: Cm, chloramphenicol; Km, kanamycin; B^E, *E.coli* B strain

Table 2.2 Bacteriophage and plasmids used in this study

Phage or plasmid	Characteristics	Source or Reference
Bacteriophage		
λgtWES·λB	λ*W*am403 *E*am1100 *inv* (*sr*Iλ1-*sr*Iλ2) Δ(*sr*Iλ2-*sr*Iλ3) *cIts*857 *sr*Iλ4° *nin*5 *sr*Iλ5°Sam100	Leder *et al.*, (1977)
λgtS1	λgtWES·λB; 8 kb cloned *S. marcescens* genomic DNA; *ompF*	This study
λgtS8	λgtWES·λB; 11 kb cloned *S. marcescens* genomic DNA; *ompC*	This study
R408	helper phage	Promega
Plasmids		
pTZ	pMB1 replicon; Ap^r; *lacZ'*	Mead *et al.*, (1986)
pBluescriptKS(+)	ColE1 replicon; Ap^r; *lacZ'*;	Stratagene
pBluescriptKS(-)	ColE1 replicon; Ap^r; *lacZ'*;	Stratagene
pMKS(+)	pKS(+) with *Kpn*I in multiple cloning site removed	This study
pS1E(-)	pKS(-) with 8 kb *Eco*RI fragment from λgtS1; *ompF*	This study
pS1KE(-)	pKS(-) with 3.5 kb *Kpn*I/*Eco*RI fragment; *ompF*	This study
pS1H(-)	pKS(-) with 0.9 kb *Hind*III fragment; *ompF*	This study
pK2.8F(-)	pKS(-) with 2.8 kb *Kpn*I fragment with gene next to Forward primer; *ompF*	This study
pK2.8R(-)	pKS(-) with 2.8 kb *Kpn*I fragment in reverse direction; *ompF*	This study
p8HK	pTZ with 4.5 kb *Hind*III/*Kpn*I fragment; *ompC*	This study
p8SX	pTZ with 6.1 kb *Sst*I/*Xba*I fragment; *ompC*	This study
p8BP(-)	pKS(-) with 1.7 kb *Bam*HI/*Pst*I fragment; *ompC*	This study
p8PK(-)	pKS(-) with 0.48 kb *Pst*I/*Kpn*I fragment; *ompC*	This study
p8BK(+)	pKS(+) with 2.2 kb *Bam*HI/*Kpn*I fragment; *ompC*	This study
pM8BH(+)	pMKS(+) with 6.6 kb *Bam*HI/*Hind*III fragment; *ompC*	This study

pM8BH12(+)	pM8BH(+) with deletion from *Hind*III leaving a 2.5 kb insert; *ompC*	This study
pM1.2(-)	p8PK(-) with D^{112} to G	This study
pM2.4(-)	p8PK(-) with M^{114} to D	This study
pMBH(D112G)	pMBH12(+) with D^{112} to G	This study
pMBH(M114D)	pMBH12(+) with M^{114} to D	This study

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin

was used after Promega changed the formulation of their kit. Manufacturer's instructions were followed except that the spin filters were replaced with Promega's minicolumns.

Single stranded DNA was also used for sequencing templates. Cells with an appropriate plasmid were inoculated in LB-ampicillin and incubated at 37°C until slight growth was seen, approximately 2 hours. At this time cells were infected with approximately 10^{10} R408 helper phage particles (Russel *et al.*, 1986) and incubated overnight. Cells were removed by centrifuging twice for 2 minutes each. The phage were precipitated by incubating at room temperature for 15 minutes in 4% (w/v) polyethylene glycol-8000, 0.3 M NaCl followed by a 15 minute centrifugation. The phage pellet was resuspended in 200 µl TE buffer and extracted with equal volumes of phenol, phenol/chloroform and chloroform and precipitated with 1/50 volume 5 M NaCl and 2 volumes of 95% (v/v) ethanol. Single stranded DNA was resuspended in water.

2.3. Genomic library production.

Genomic DNA from *S. marcescens* was isolated as described above and 100 µg digested with *Eco*RI. After 1 hour digestion at 37°C the sample was precipitated with one-half volume of 7.5 M ammonium acetate and 2.25 volumes of 95% (v/v) ethanol. After centrifugation and drying, the DNA was resuspended in 100 µl STE buffer (1 M NaCl, 20 mM Tris-HCl, pH7.5, 5 mM EDTA) and loaded on a 20 to 40% continuous sucrose gradient in the same buffer (Ausubel *et al.*, 1989). The gradient was centrifuged overnight at 75,000 x*g*. Two hundred

microlitre fractions were collected from the gradient and 20 µl samples from every third fraction were loaded on a 0.7% agarose gel. Fractions were collected that contained approximately 5 to 13 kb sized fractions which would include the porin genes. In a similar way the λgtWES.λB vector DNA arms were separated from the stuffer fragment. Water was added to dilute the sucrose present and the DNA precipitated with ammonium acetate and ethanol. Ligation of 240 ng of fractionated DNA into the lambda arms was accomplished with a 6:1 vector to insert ratio. One half of the ligated λ-*S. marcescens* DNA was packaged using the Can/Pack single extract lambda DNA packaging system (Bio/Can Scientific, Mississauga, ON) and transfected into *E. coli* LE392. The titre of the packaged library was 4×10^4 plaque forming units/ml. To amplify the library, the phage were infected into *E. coli* LE392 and plated on a total of 5 LB plates as described in the plaque blotting procedure below. After 9 hours of incubation at 37°C, 3 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin) was added to each plate and stored overnight at 4°C. The next day the plates were rotated for 2 hours at room temperature to elute the phage particles. The phage containing buffer was collected into Teflon tubes, chloroform added to 5% (v/v) and shaken for 15 minutes to lyse cells followed by a centrifugation at 2K rpm for 5 minutes. The resulting lysate was aliquoted and a few drops of chloroform added to store the library at 4°C. For long term storage of the library, DMSO was added to 7% (v/v) and the library was frozen at -20°C. The titre of the amplified library was 4×10^8 pfu/ml.

2.4. Southern Blotting.

For agarose electrophoresis the DNA samples were prepared in loading buffer (6% (v/v) glycerol, 2 mM EDTA, 0.05% (w/v) bromophenol blue). Samples were routinely run in 0.7 to 1.0% (w/v) agarose gels using 1xTAE buffer (40 mM Tris-acetate, pH 7.6, 1 mM Na_2.EDTA). Before transferring DNA to nylon membranes the gel was soaked in 0.2 N HCl for 10 minutes with gentle shaking to depurinate DNA for ease of transfer of larger DNA fragments. After rinsing the gel with water an alkaline Southern transfer was set-up as described by Reed and Mann (1985). After transfer was complete the membrane was neutralized in 2x SSC (0.3 M NaCl, 30 mM sodium citrate) for 2 minutes.

2.5. Plaque blotting.

Appropriate dilutions of phage (10^3/plate for initial screening and 10^2 or less for secondary screening) were incubated with 0.3 ml of an overnight culture of LE392 at 37°C for 15 minutes to allow phage adsorption. Three millilitres of top agarose was added and the suspension was poured onto LB plates and incubated at 37°C overnight. Plates were cooled at 4°C for at least 2 hours before plaque lifting. Nylon membranes were placed on plates for 1 min and 2 min for a second filter to lift plaques. Phage were lysed and DNA denatured with 0.5 M NaOH/1.5 M NaCl for 30 to 60 seconds then neutralized with 0.5 M Tris-HCl, pH 7.5/1.5 M NaCl for 5 minutes and finally rinsed with 2x SSC for 2 minutes. After the filters dried the DNA was cross-linked to the nylon by exposure to UV light for 5 minutes.

2.6. DNA Hybridizations.

Ten picomoles of oligonucleotide were 5' end-labelled with T4 polynucleotide kinase and the equivalent amount of picomoles of [γ-^{32}P]ATP (3000 Ci/mmol) (Dupont Canada, Mississauga, ON) and incubated at 37°C for 30 minutes. The manufacturer's instructions were followed when using the Random-primer labelling kit (Gibco BRL, Burlington ON) to prepare larger probes. Hybridizations were carried out at 42°C when using oligonucleotide probes and 65°C when using random-primer labelled probes. In both cases the hybridization buffer consisted of 5x SSC (0.75 M NaCl, 75 mM sodium citrate) and 0.5% (w/v) SDS. Wash stringency was controlled by changing the salt (SSC) concentration at hybridization temperature. Washes started at 5x SSC, 0.5% (w/v) SDS, followed by 3x SSC, 0.1% (w/v) SDS and if necessary, 0.5x SSC, 0.1% (w/v) SDS. The level of radioactivity on the membranes was monitored every fifteen minutes. Hybridization membranes were exposed to X-OMAT RP (Kodak, New Haven, CT) film at -70°C with an intensifying screen.

2.7. DNA subcloning.

Restriction enzyme digestions were generally incubated for one hour at 37°C using manufacturer's buffers. DNA ligations were performed either at 15°C overnight or at room temperature for 4 hours. To isolate DNA fragments for subcloning, restriction enzyme digested samples were electrophoresed in 0.7 to 1.0 % agarose gels with 1xTAE buffer and the appropriate bands cut out of the gel. These gel slices were then "freeze-squeezed" (Tautz & Benz, 1983). The

gel slice was frozen and DNA extracted from it by sandwiching it in Parafilm (American National Can, Greenwich, CN), applying gentle pressure and collecting the liquid that was expressed. The DNA sample was then extracted with phenol, phenol/chloroform and chloroform until the interface was clear and finally precipitated with 1/50 volume 5 M NaCl and 2 volumes of 95% (v/v) ethanol.

2.8. Preparation of deletion clones.

Deletion clones were generated with the Erase-a-Base System (Promega). Manufacturer's instructions were followed except that the DNA was treated with T4 DNA ligase for 2 hours at room temperature prior to the procedure to repair nicks in the plasmid. Generally 5 to 10 µg of DNA was used. For the p8BK(+) set of deletion clones *Sst*I was used to protect an end from Exonuclease III digestion and *Xba*I was used as the deletion start site. For the p8BP(-) deletion set *Kpn*I was used for protection and *Eco*RI was used for the deletion. In both cases the Exo III deletion step was performed at 40°C and samples were taken every 15 seconds for 10 to 13 samples. At this temperature I found a deletion rate of approximately 600bp min^{-1}. For the generation of pM8BH(+) deletions the plasmid was digested with *Xho*I and ends filled with α-phosphorothioate dioxynucleotides (Promega) using Klenow, for ExoIII protection. *Hind*III was used as the site of deletion origination. In this case ExoIII deletion was performed at 40°C and after 2.5 minutes 4 samples were taken at 30 second intervals followed by 9 more samples at 15 second intervals. The

timing schedule was selected to obtain deletions that concentrated farther from the start site.

2.9. Preparation of competent cells and transformation.

Competent cells were prepared using $CaCl_2$ treatment (Ausubel *et al.*, 1987). Log phase cultures (2.5 to 3 hours incubation at 37°C) were harvested by centrifugation at 1000 x*g* for 10 minutes at 4°C. The cells were resuspended in 1/2 the original culture volume of ice-cold $CaCl_2$ solution (60 mM $CaCl_2$, 10 mM PIPES, pH7.0, 15% (v/v) glycerol) and kept on ice for 20 minutes. These steps were repeated with a 1/5 volume $CaCl_2$ solution resuspension and a final 1/10 volume resuspension. Competent cells were aliquoted and frozen at -70°C.

To transform competent cells 200 µl of cells prepared above were mixed with 10 ng to 1 µg DNA in a 10 µl volume and kept on ice for a minimum of 20 minutes. The cells were then heat shocked at 37°C for 5 minutes or 42°C for 60 seconds and placed on ice for an additional 5 minutes. To allow expression of β-lactamase before plating on ampicillin plates 1 ml of LB broth was added to cells and cells were incubated at 37°C for 45 minutes.

2.10. Oligonucleotide Preparation.

Oligonucleotides for sequencing and mutagenesis experiments were synthesized on an Applied Biosystems 391 DNA synthesizer (Mississauga, ON) by Jack Switala (Dept. of Microbiology, University of Manitoba) and removed from the synthesizing column by passing 1 ml of ammonium hydroxide through

the column ten times and incubating for 15 minutes. After repeating this three times the solute was collected and incubated overnight at 55°C. The oligonucleotide was then dried in a SpeedVac (Savant SC110 Speedvac, Farmingdale, NY) to remove the ammonium hydroxide and resuspended in 0.5 ml water. For site-directed mutagenesis the primers were phosphorylated using T4 polynucleotide kinase (Gibco BRL).

2.11. Amplification of DNA: Polymerase chain reaction (PCR).

PCR (Saiki *et al.*, 1988) was used for DNA amplification or as a tool for screening colonies from Erase-a-Base experiments. A final reaction mixture of 50 µl was used and contained final concentrations of the following: 1x Taq DNA polymerase reaction buffer (Promega), 1.5mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer and 1 unit of Taq DNA polymerase in storage buffer A (Promega). The amount of template DNA used depended on the source of DNA; 50 ng of genomic DNA or 10 ng of plasmid DNA was used per reaction. For colony screening a small amount of cells were picked off of an agar plate and resuspended in 50 µl of water. The cell suspension was vortexed for 10 seconds and centrifuged at 12,000 rpm for 2 minutes. From this lysate 2 µl were used as a source of template per reaction. Reaction mixtures generally went through 25 to 30 cycles of denaturation at 93°C for 1 min., annealing at 50°C for 1 min. and polymerization at 72°C for 2 min. unless otherwise specified. The polymerization time varied depending on the expected size of the amplified product following a general rule of thumb of 1 kb per minute. A final polymerization at 72°C for 10

minutes followed the last cycle to ensure complete products. Reactions were performed in a Perkin Elmer-Cetus DNA thermal cycler (Norwalk, CN)

2.12. Site-directed mutagenesis.

The method used for generating site-directed mutants was originally described by Kunkel (1985). Single-stranded templates were isolated from the *dut⁻ ung⁻* strain, CJ236, which contained a high proportion of uracils in place of thymidine bases. A mutagenic primer was annealed to the template by heating at 70°C for 2 minutes in annealing buffer (20 mM Tris-HCl, pH 7.5, 2 mM Mg Cl_2, 50 mM NaCl) and then slowly cooled to room temperature. Second strand synthesis was completed in a buffer with the following components: 0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.9, 3.75 mM $MgCl_2$, 0.5 mM DTT, 5 units T4 DNA ligase, and 1 unit unmodified T7 DNA polymerase (New England Biolabs, Beverly, MA). The 10 µL mixture was first incubated on ice for 5 minutes, followed by room temperature for 5 minutes and finally at 37°C for 90 minutes. The reaction was stopped with 90 µL stop buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA). 10 to 20 µL of the final reaction was transformed into *E. coli* NM522.

2.13. DNA sequencing.

Sequencing was performed with single-stranded or double-stranded templates by the Sanger dideoxy method (Sanger *et al.*, 1977) using the Sequenase Version 2.0 DNA Sequencing Kit (USB Biochemical, Cleveland,

Ohio) and [^{35}S]dATPαS (1000-1500Ci/mmol) (DuPont Canada). Subclones, deletion clones and synthesized primers were used to complete the sequences. A quick denaturing step for double-stranded DNA sequencing was employed by boiling the template DNA and primer with Sequenase buffer and 5% (v/v) formamide for 2 minutes and immediately submerging in a -70°C ethanol bath to freeze the sample. After this step the procedure was the same as single-stranded sequencing and manufacturers instructions were followed. To enable sequencing through compressed areas due to secondary structure the 7-deaza-dGTP DNA Sequencing Kit (USB) was used. In addition, for strong compressions that were not resolved with this kit 30% (v/v) formamide was included in the sequencing gel as described in the Sequenase manual. Sequencing samples were electrophoresed in 6 to 8% polyacrylamide gels for 2 to 6 hours. Following electrophoresis the gels were fixed in a solution of 12% (v/v) methanol/10% (v/v) acetic acid for 20 minutes and then dried. Exposure to Kodak X-Omat AR film ranged from overnight to several days at room temperature.

2.14. Cell envelope preparation.

Cell envelopes were prepared following the procedure of Lugtenberg *et al.* (1975). Fifty ml cultures were grown overnight and harvested by centrifugation. Cells were resuspended in 50 mM Tris-HCl pH 8.5, 2 mM EDTA and passed through a French Pressure Cell twice at 18,000 psi. Unlysed cells and debris were removed by centrifuging at 1200 x*g* for 10 minutes. Cell envelopes were

collected by centrifuging the supernatant at 100,000 x*g* for 1 hour. The cell membranes were resuspended in 50 mM Tris-HCl, pH 8.0 or 2 mM Tris-HCl, pH 7.8. For cell envelopes the procedure was stopped here. Depending on the purpose of the sample, a number of routes may have been employed for further purification, as described below.

2.15. SDS-insoluble fraction.

Crude porin preparations were obtained following a method described by Nakae (1976b). After cell envelopes were obtained the cytoplasmic membrane and some outer membrane proteins were solubilized from the envelopes with 2% (w/v) SDS, at 32°C for 30 min. and centrifuged at 100,000 x *g* for 30 min. The supernatant was discarded and the pellet was resuspended in 50 mM Tris-HCl, pH 8.0.

2.16. Outer membrane preparation.

Outer membranes were isolated on a discontinuous sucrose gradient (Malouin *et al.*, 1990). First, cell envelopes were obtained as described above and resuspended in 50mM Tris-HCl, pH 7.9 buffer. Alternatively, the cell lysate may also have been loaded without precipitating cell envelopes first. The sample was loaded on a two step sucrose gradient (54%; 70% (w/v) sucrose in 50 mM Tris-HCl, pH 7.9) and centrifuged at 100,000 x*g* overnight, 5°C. The lowest band, the outer membranes, was collected, diluted with water and centrifuged at 100,000 x*g* for 1 hour. Outer membranes were resuspended in 50 mM Tris-HCl,

100,000 x*g* for 1 hour. Outer membranes were resuspended in 50 mM Tris-HCl, pH 8.0.

2.17. Porin isolation.

Porins were isolated as described by Malouin *et al.* (1990). First, the SDS-insoluble fraction was obtained. The resulting pellet was washed with water and recentrifuged as above. To further remove OmpA the pellet was resuspended in 50 mM Tris-HCl, pH 7.2, 1% (w/v) SDS, and 5 mM EDTA. After solubilization at 37°C, the sample was centrifuged at 100,000 x*g* for 1 hour. The porin was 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. Alternatively, the same procedure can be started with outer membranes that have been separated on a discontinuous sucrose gradient as described above.

2.18. Polyclonal antibody production.

For production of antibodies, the UOC-51 porin(s) was electroeluted from polyacrylamide gel slices as described by Parr *et al.* (1986). After emulsifying in Freund's Complete Adjuvant, the porin was injected subcutaneously into a female New Zealand white rabbit. Two booster injections with Freund's incomplete adjuvant were given at two week intervals. Two weeks later blood was collected, allowed to clot overnight at 4°C and serum collected by centrifuging in a table top centrifuge. To effectually remove anti-LPS antibodies the serum was incubated with a half volume of isolated *S. marcescens* UOC-51

LPS at room temperature for one hour. In a similar manner contaminating anti-OmpA antibodies were removed using electroeluted UOC-51 OmpA.

2.19. LPS Isolation.

LPS was isolated by the method of Darveau & Hancock (1983) except that a (2:1) chloroform/methanol extraction was added as a final step. Cells were harvested after reaching mid-logarithmic phase and washed by resuspending in 10 mM Tris-HCl, pH 7.9 and recentrifuging. After drying under vacuum, cells were resuspended in 10 mM Tris-HCl, pH 7.9, 2 mM $MgCl_2$, 100 µg/ml DNase and 25 µg/ml RNase and passed through the French Pressure Cell twice at 18,000 psi. To further break cells the suspension was sonicated at 60% probe intensity for two bursts of 30 seconds each. DNase and RNase were added to 200 µg/ml and 50 µg/ml respectively, and the suspension allowed to incubate for 2 hours at 37°C to digest nucleic acids. To solubilize the membranes, EDTA and SDS in 10 mM Tris-HCl, pH 8.0 were added to a final concentration of 0.1 M EDTA, 2% (w/v) SDS, and 10 mM Tris-HCl with a final pH of approximately 9.5 and the sample vortexed. Peptidoglycan was removed by centrifugation at 50,000 x*g* for 30 min. at 20°C. Pronase was added to the supernatant to a final concentration of 200 µg/ml and incubated at 37°C overnight with shaking. LPS was then precipitated with two volumes of 0.375 M $MgCl_2$ in 95% (v/v) ethanol by placing at -20°C for 20 minutes and centrifuging at 12,000 x*g* for 15 minutes, 4°C. The pellet was resuspended in 0.1 M EDTA, 2% (w/v) SDS, 10 mM Tris-HCl, pH 7.9 and sonicated as above. The solution was then incubated at 85°C

for 30 minutes to ensure denaturation of SDS-resistant proteins. Pronase was then added to the sample at a final concentration of 25 µg/ml and incubated overnight at 37°C with shaking. The LPS was precipitated as above with two volumes of 0.375 M $MgCl_2$ in 95% (v/v) ethanol. The resulting pellet was resuspended in 10 mM Tris-HCl, pH 7.9, sonicated as above and centrifuged at 1000 rpm for 5 minutes to remove insoluble Mg/EDTA complexes. The LPS was then recovered by adding $MgCl_2$ to a final concentration of 25 mM and centrifuging at 200,000 x*g* for 2 hours. The final pellet was resuspended in dH_2O. To remove phospholipids the sample was extracted twice with an equal volume of chloroform:methanol (2:1). The final LPS sample was then dialyzed against three changes of 5 mM Hepes, pH 7.4, 5 mM EDTA followed by one change of 5 mM Hepes, pH 7.4, 50 mM NaCl and one of water.

2.20. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Samples were subjected to SDS-PAGE by the Lugtenberg system (Lugtenberg *et al.*, 1975) at a polyacrylamide concentration of 11% (w/v). Prior to loading, the samples were heated at 90 to 100°C for 10 min in 12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 2% (v/v) glycerol and 1% (v/v) β-mercaptoethanol. In order to separate porin samples an urea-SDS-PAGE system was employed as described by Uemura and Mizushima (1975) which was modified to contain 4M urea instead of 8M. Proteins were stained for 15 minutes using 0.4% (w/v) Coomassie Brilliant Blue R-250 (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 15 minutes and then overnight with fresh destain solution. The gels were destained with fresh solution until the background was clear. Stained SDS-PAGE gels were wrapped in cellophane (Bio-Rad) and air-dried.

2.21. Whole cell lysis.

Whole cell lysates were used as a quick test for expression of recombinant porins in *E. coli* (Hitchcock & Brown, 1983). 1.5 ml of cultures were microfuged for 1.5 minutes to pellet cells. The pellets were solubilized in 50 µl lysing buffer (2% (w/v) SDS, 4% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 1 M Tris-HCl, pH 6.8). Prior to loading on SDS-PAGE samples were heated at 100°C for 10 minutes. Samples were microfuged for 1 min. and 20 µl loaded per lane.

2.22. Western blotting and immunodetection.

After SDS-PAGE the proteins were transferred to nitrocellulose using a Bio-Rad Trans-Blot Cell at 10 mA constant current (Bio-Rad) as described by Burnette (1981). Immunodetections were performed as outlined by Towbin *et al.* (1979). Anti-porin antibodies were generally used at a dilution of 1:200 in 1% skim milk in phosphate buffered saline while peroxidase conjugated goat anti-rabbit antibodies (Sigma) were used at a 1:1500 dilution in the same buffer.

2.23. Amino acid sequencing.

Porin samples were separated using SDS-PAGE and Western blotted onto PVDF Immobilon-P membrane (Millipore Corporation, Bedford, MA) using CAPS (3-[cyclo-hexylamino]-1-propanesulfonic acid) transfer buffer (10% (v/v) methanol, 10 mM CAPS, pH 11 (Sigma)). Proteins were stained using 0.2% (w/v) Ponceau S (Sigma) in 3% (w/v) trichloroacetic acid (TCA) and destained briefly with 5% (v/v) acetic acid followed by a few rinses with water. Protein bands were cut out and sequenced by automated Edman degradation using either an Applied Biosystems model 477A pulsed liquid sequencer or a model 470A gas phase sequencer (Matsudaira, 1987). Amino terminal sequencing was performed by Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

2.24. Planar lipid bilayer experiments.

Functional characterization of porin activity was done using the black lipid bilayer technology and equipment as previously detailed (Benz *et al.*, 1985). By using dilutions of about 1/10,000, the porin preparations were added at concentrations of 0.1 ng ml^{-1} to the Teflon chamber. The artificial lipid membrane used was composed of 2% oxidized cholesterol in *n*-decane and was generously provided by L. C. Blaszczak (Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.). Experiments were done at 22°C by T. Parr, Jr. at Eli Lilly and Co.

2.25. Liposome swelling assay.

The liposome swelling assay was performed as previously described (Nikaido *et al.*, 1991; Nikaido & Rosenberg, 1983). Wild-type and mutant porins were expressed in the *E. coli* porin deficient strain, BZB1107 and isolated as described above in Section 2.17. Liposomes were prepared as follows: dicetyl phosphate and phosphatidylcholine were dried in the bottom of a 1.5 mL eppendorf tube with mild heat. A 200 µL aqueous solution of purified porin was added to the tube and vortexed gently to resuspend. The samples were then sonicated for three 15 second bursts. The liposomes were dried in a Speed-vac under medium heat and stored overnight in the dark in a vacuum dessicator. The dried lipid-protein film was reconstituted with 400 µL of 12 mM stachyose, 4 mM Na-NAD, 1 mM imidazole-NAD, pH 6.0. This mixture was allowed to sit undisturbed for 1.5 hours, then mixed gently by hand and left for an additional hour. The suspension was filtered through an 8 µm filter (Millipore) to remove large aggregates. To determine the isosmotic concentration of proteoliposome suspensions, which is necessary for the assay, 10 µL were diluted in various concentrations of the impermeable solute, stachyose. When the liposomes are in their isosmotic concentration of stachyose there is no change in optical density at 400 nm. Proteoliposomes were tested in various solutes by diluting 20 µL of the suspension in 600 µL of the test solution and the optical density at 400 nm was recorded at 10 second intervals over a period of 90 seconds. As a control, 20 µL of the proteoliposome suspensions were mixed with hypo- (water) and hyper-osmotic (50 mM stachyose) solutions and appropriate shrinking or swelling

observed. Each experiment was repeated a minimum of four and up to eight times.

2.26. Computer analyses.

Sequence analysis was performed with various programs included in BIRCH (Biological Research Computer Heirarchy), a collection of programs within the SUN Unix system at the University of Manitoba (Fristensky, 1991). Sequences were retrieved from the EMBL/GenBank/DDBJ sequence data libraries using the FETCH program. Amino acid sequences were aligned with the CLUSTALV program and then manipulated manually for the best alignments using MASE (Multiple Aligned Sequence Editor). A distance matrix was calculated for the multiple sequence alignment by PROTDIST (Dayhoff PAM Matrix). The NEIGHBOR program (neighbor-joining option) was used to generate a distance dendogram. The confidence level of the dendogram was determined by repeating the same procedure except that 100 bootstrap replicates generated by SEQBOOT were used in PROTDIST followed by NEIGHBOR and concluded with CONSENSE. The percent identity between porin amino acid sequences were determined by PALIGN in the PC/GENE genetics analysis package (Intelligenetics Inc., Mountain View, CA).

3. RESULTS AND DISCUSSION

3.1. *Serratia marcescens* UOC-51 outer membrane

3.1.1. Introduction

Previous reports examining the *S. marcescens* outer membrane porins provide conflicting data. There have been anywhere between one and three porins identified with only one study providing any porin functional data (Gutmann *et al.*, 1985; Hazishume *et al.*, 1993; Malouin *et al.*, 1990). In *E. coli* the OmpF and OmpC porins exhibit a characteristic response to osmoregulation and salicylate (Csonka, 1989; Rosner *et al.*, 1991). One study demonstrated porin osmoregulation in *S. marcescens* (Puig *et al.*, 1993) while another reported an absence of such regulation (Sawai *et al.*, 1987). The reduction of a 41 kDa outer membrane protein was demonstrated in response to salicylate in *S. marcescens* (Sawai *et al.*, 1987). This section addresses objective #1 outlined in Section 1.6: to identify the major porins in the *S. marcescens* outer membrane. As well, the regulatory response of the identified porins to salicylate and osmolarity was evaluated.

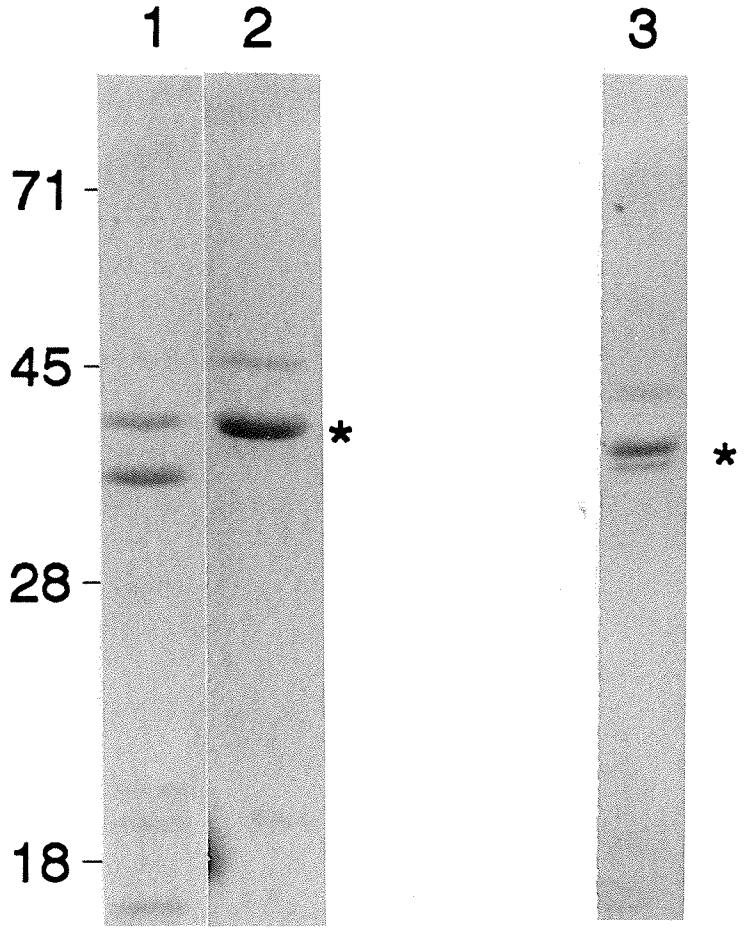
3.1.2. Examination of the *S. marcescens* outer membrane

Serratia marcescens UOC-51, a non-pigmented clinical blood isolate, was the strain studied in this thesis. As a start to studying *S. marcescens* porins, the UOC-51 outer membrane was examined. The outer membrane was isolated on

a discontinuous sucrose gradient and subjected to SDS-PAGE. The sample was heated for 10 min at 95°C in SDS prior to electrophoresis and can be seen in Fig. 3.1, lane 1. Two major bands are seen in the outer membrane. The approximately 38 kDa protein is the OmpA protein. It demonstrates heat modifiability (data not shown) with a slower mobility after heating, as described for *E. coli* OmpA protein (Heller, 1978). The *S. marcescens ompA* gene has been previously cloned and sequenced (Braun & Cole, 1984). The other major band at approximately 41 kDa represents what we suspected to be a porin. The protein in this SDS-PAGE band exhibits heat modifiability as described with other porins, that is, it migrates as multiple high molecular weight bands when not heated (data not shown). Also characteristic of porins, this protein was extracted by differential salt extraction as shown in lane 2. Subsequent amino acid sequencing of the protein from this 41 kDa band (Section 3.2) revealed an amino terminal sequence that closely resembled other porins sequenced, including the *S. marcescens* UOC-69 porin (Malouin *et al.*, 1990).

From the separation on SDS-PAGE (Fig. 3.1, lane 2) it would appear that only one porin is produced however, it is known that the *E. coli* OmpF and OmpC porins are only well separated when urea is added to the gel system (Uemura & Mizushima, 1975). Indeed the *S. marcescens* porin separated into two bands when run in a 4M urea-SDS-PAGE system (Fig. 3.1, lane 3). The higher molecular weight band seen in lanes 2 and 3 was not investigated, but may be the LamB porin which is 47 kDa in *E. coli* (Werts *et al.*, 1993). Two other *S. marcescens* porin studies have revealed the presence of three approximately 40

Fig 3.1. Electrophoretic separation of the *S. marcescens* UOC-51 outer membrane proteins. Samples (approximately 3 µg per lane) were heated at 100°C for 10 min before loading. The migration of the protein molecular weight standards are indicated in kDa at the left of the figure. Lane 1, outer membrane proteins separated by SDS-PAGE; Lane 2, *S. marcescens* porins separated by SDS-PAGE; Lane 3, *S. marcescens* porins separated by 4 M urea-SDS-PAGE. The asterisk indicates the separation of the stronger band on conventional SDS-PAGE into two bands on urea-SDS-PAGE. The molecular weight markers do not apply to the urea-SDS-PAGE sample as they do not migrate as expected.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*

kDa porins in the outer membrane when using urea-SDS-PAGE (Hazishume *et al.*, 1993; Puig *et al.*, 1993). These investigators classified the protein bands observed on their gels based on heat-modifiability, or association with peptidoglycan in differential salt extractions. In a third study only two porins were identified by visualization on conventional SDS-PAGE (Sawai *et al.*, 1987). Malouin *et al.* (1990) reported the presence of only one porin in the outer membrane of *S. marcescens* UOC-69. This group did not use the urea-SDS-PAGE system to check for the presence of more than one porin species but functional studies and N-terminal amino acid sequencing supported their report of a single porin.

The difference in number of porins observed among the various studies may be due to different SDS-PAGE systems, growth conditions and/or strains used. In this thesis, I have demonstrated that two porins exist in UOC-51 based on differential salt extraction, heat modified SDS-PAGE mobility, and amino acid sequencing (see following Section, Fig. 3.8), however, the possibility of a third porin is not ruled out. The amino acid sequence was obtained from the protein in the indicated band on non-urea SDS-PAGE (Fig. 3.1, lane 2) before it was known that two proteins migrated to this location. Since a mixed signal was not obtained, the two proteins probably have the same N-terminal sequence. This was later determined to be so upon DNA sequencing of the two different porin genes (Sections 3.3 and 3.4).

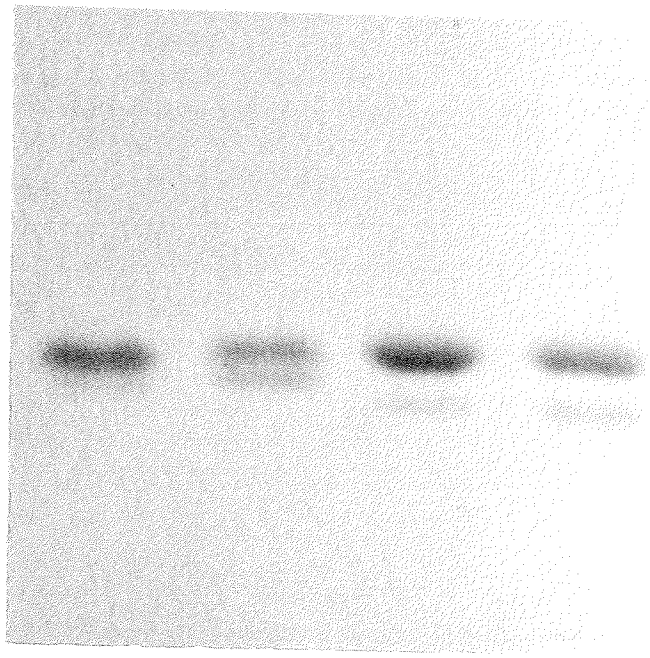
3.1.3. Regulation of porins by external osmolarity and salicylate

One of the distinguishing features of the *E. coli* OmpF and OmpC porins is that they are osmoregulated (Csonka, 1989). In the presence of low osmolarity OmpF is the major porin seen in the outer membrane while OmpC is the predominant species in high osmolarity. In order to compare the *S. marcescens* porins with *E. coli* porins osmoregulation was examined.

S. marcescens UOC-51 and the type strain, ATCC 13880, were grown in high (0.3 M NaCl) and low (no NaCl added) osmolarity and the porins examined on urea-SDS-PAGE (Fig. 3.2). For both strains tested the concentration of the 41 kDa protein decreased with increased osmolarity (lanes 2 and 4) while the concentration of the 40 kDa protein increased, thus both strains appear to exhibit porin osmoregulation. The pattern of osmoregulation however, does not match that reported for *E. coli* where characteristically the quantity of OmpC is greater than OmpF at high osmolarity. In *S. marcescens* the 41 kDa porin remained more abundant than the 40 kDa porin despite the changing ratio of the protein. Puig *et al.* (1993) observed osmoregulation in the *S. marcescens* strains examined and the pattern was similar to that seen in *E. coli*. Sawai *et al.* (1987) did not observe osmoregulation of *S. marcescens* porins in their study. Interestingly, early examinations of the UOC-51 porins of cells grown in the two osmolarity levels suggested an absence of osmoregulation in this strain. The discrepancy was due to electrophoretic problems, first the lack of urea in the gels and later poor resolution in the urea-SDS-PAGE system.

Salicylate is a natural substance that bacteria may encounter in some

Fig. 3.2. Urea-SDS-PAGE of *S. marcescens* UOC-51 and ATCC 13880 porins isolated in the SDS-insoluble fractions after growth in low (no NaCl added) and high (0.3 M NaCl) osmolarity media. Samples (approximately 5 µg per lane) were heated at 100°C before loading. Lane 1, UOC-51, no NaCl; Lane 2, UOC-51, 0.3 M NaCl; Lane 3, ATCC 13880, no NaCl; Lane 4, ATCC 13880, 0.3 M NaCl.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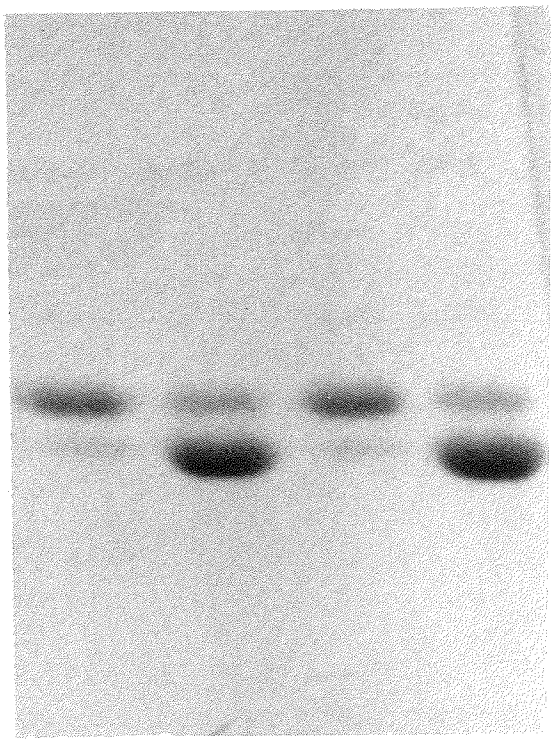
plants or in the human host after oral intake of foods or aspirin (Budavari, 1989; Rosner, 1985). It is also used topically for its antiseptic and keratolytic properties. The concentration of *E. coli* OmpF porin is known to decrease in response to salicylate and a reduction in a 41 kDa porin has also been observed in *S. marcescens* (Sawai *et al.*, 1987). Therefore, regulation of the UOC-51 porins in response to salicylate was examined.

Following growth in the presence and absence of 5 mM salicylate the outer membranes were isolated from both UOC-51 and ATCC 13880. Samples were heated at 100°C for 10 min and loaded on 4 M urea-SDS-PAGE gel (Fig. 3.3). For both UOC-51(lane 2) and ATCC 13880 (lane 4) the higher molecular weight protein, approximately 41 kDa, was reduced when the cells were grown in 5 mM salicylate. This is the same result obtained by Sawai *et al.* (1987). In addition, for both strains tested the 40 kDa protein increased when cells were grown in the presence of salicylate. Previous studies observed little or no change in the 40 kDa porin of *S.marcescens* or OmpC of *E. coli* (Rosner *et al.*, 1991; Sawai *et al.*, 1987). The reason for the difference in OmpC regulation is unclear, however, the OmpF result is as expected.

There has been some controversy in the literature regarding the number of porins in the *S. marcescens* outer membrane and whether any are osmoregulated. In both UOC-51 and ATCC 13880 two porins are observed and they were inversely regulated in response to media osmolarity. This was only evident when a urea-SDS-PAGE system was employed. A previous report suggesting the absence of osmoregulation of *S. marcescens* porins did not use

Fig. 3.3. Urea-SDS-PAGE of *S. marcescens* UOC-51 and ATCC 13880 porins after growth in the presence and absence of salicylate. Samples (approximately 5 µg per lane) were heated at 100°C before loading. Lane 1, UOC-51, no salicylate; Lane 2, UOC-51, 5 mM salicylate; Lane 3, ATCC 13880, no salicylate; Lane 4, ATCC 13880, 5 mM salicylate.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5mM
salicylate
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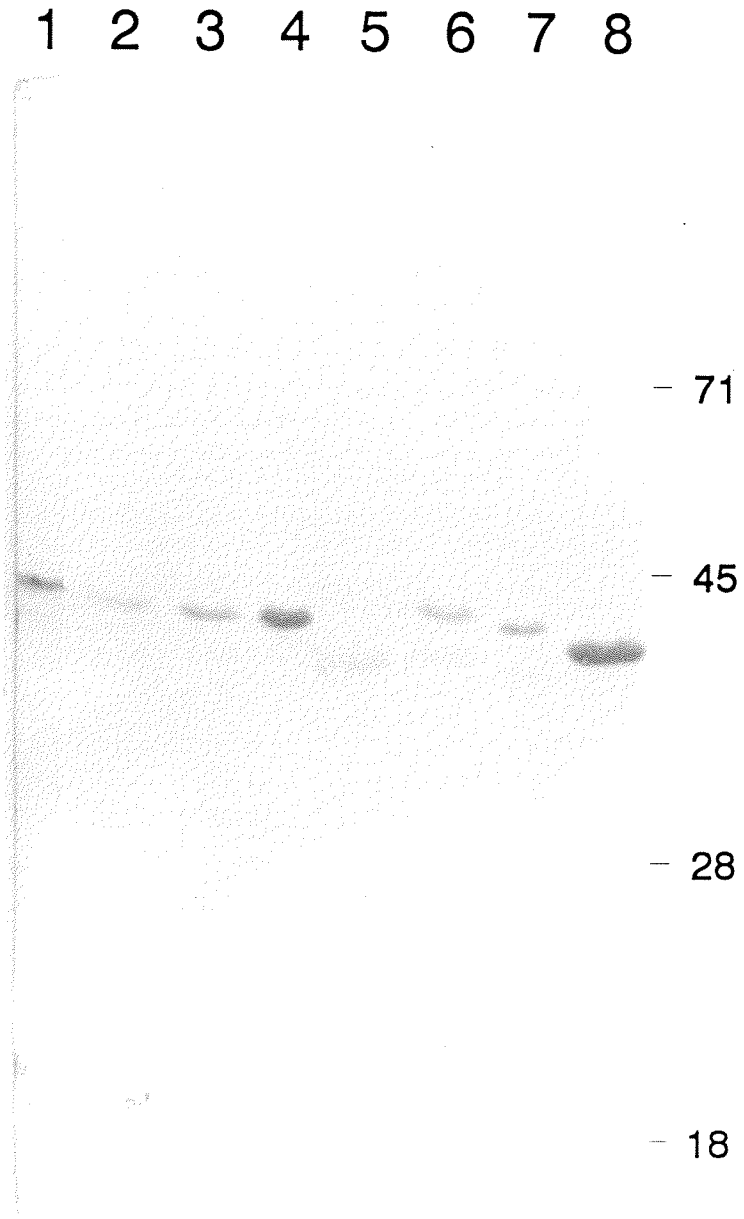
the urea-SDS-PAGE method and it is possible that the strains studied do osmoregulate. In addition, the porins in this study responded to salicylate. The mechanism of decreased OmpF expression due to salicylate in *E. coli* involves *micF*, which is specific for the *ompF* transcript. Considering that the larger 41 kDa outer membrane protein was reduced in salicylate grown cells it would indicate that it is equivalent to OmpF. The pattern of expression of the two porins in high and low osmolarity also indicates that the larger 41 kDa protein may be equivalent to OmpF while the 40 kDa protein may be OmpC. The results obtained in this section form the basis of the investigation in Sections 3.3 and 3.4.

3.2. Comparative analyses of *Serratia* porins

Much information is available for the *E. coli* OmpF, OmpC and PhoE porins, yet in comparison, little is known about *Serratia* porins. This Section describes various comparative analyses among *Serratia* spp. as well as, other members of the *Enterobacteriaceae*, which were performed to expand our knowledge of *Serratia* porins and determine the extent of homology between the *Serratia* spp. porins and other enteric porins (Objective #2, Section 1.6). By establishing the extent of homology between the *S. marcescens* porins and those produced by evolutionarily related species, we can augment our study of the *S. marcescens* porins by comparison with other, well characterized porins.

Polyclonal antibodies were raised against the two *S. marcescens* UOC-51 porins. At the time it was not known that the one band observed on SDS-PAGE actually represented two porin species, however, this does not interfere with our interpretation. Any anti-OmpA and anti-LPS antibodies present in the antiserum were removed by adsorption with purified *S. marcescens* OmpA and LPS. The anti-porin antiserum reacted specifically with the *S. marcescens* UOC-51 porin(s) (Fig. 3.4 lane 1). Cell envelopes were isolated from eight species of *Serratia*, separated on SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-UOC-51-porin antibodies (Fig. 3.4). The cells were grown in intermediate osmolarity (0.17M NaCl) and temperature (28°C) to minimize preferential expression of certain porins. Each of the eight species tested had at least one cross-reactive band. *S. proteomaculans* (lane 6) and *S. plymuthica* (lane 7) exhibited two cross-reactive bands perhaps analogous to the *E. coli*

Fig. 3.4. Western immunoblot of various cell envelopes from *Serratia* species. Samples (50 µg per lane) were run on SDS-PAGE, transferred to nitrocellulose, and probed with *Serratia marcescens* UOC-51 porin monomer specific antibodies (1:100 dilution). Samples were heated at 100°C for 10 min prior to electrophoresis. Lane 1, *Serratia marcescens* UOC-51; lane 2, *Serratia odorifera*; lane 3, *Serratia ficaria*; lane 4, *Serratia liquefaciens*; lane 5, *Serratia rubidaea*; lane 6, *Serratia proteomaculans*; lane 7, *Serratia plymuthica*; lane 8 *Serratia marcescens* ATCC 13880.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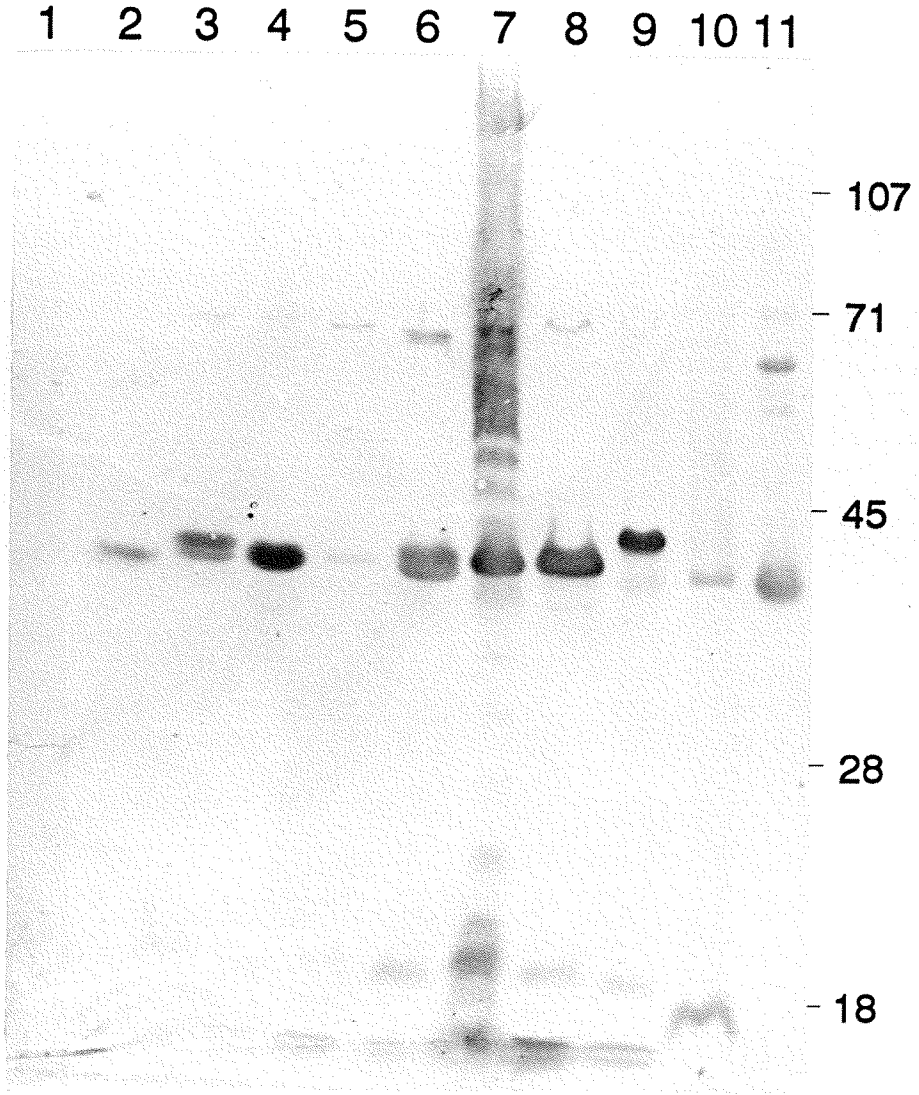
OmpF and OmpC. While attempts were made not to favor conditions for one porin type over another, the difference in numbers of porins detected between species may have be influenced by culture conditions or lack of electrophoretic separation. The molecular masses of the proteins ranged from 37.5 to 41 kDa. The two *S. marcescens* strains (lanes 1 and 8) tested had proteins that were different by approximately 2 kDa. UOC-51(lane 1) has smooth LPS whereas, ATCC 13880 (lane 8) has rough LPS which may affect the mobility of the respective porins. Differences in lipopolysaccharide has been shown to affect mobility of OmpF trimers in SDS-PAGE (Diedrich *et al.*, 1990). The weakest reaction was observed with *S. rubidaea* (lane 5). This band was also lower in molecular weight than the others.

Cell envelopes of nine members of the *Enterobacteriaceae*, another Gram negative organism, *Pseudomonas aeruginosa*, and one Gram positive organism, *Bacillus subtilis*, were also probed with the anti-porin antibodies (Fig. 3.5). *B. subtilis* (lane 1) was included as a negative control and as expected there was no reaction with the antiserum. All other cell envelopes tested had at least one cross-reactive species, including *Ps. aeruginosa* (lane 11). The molecular mass of each of the cross-reactive bands are similar with sizes ranging between 38 and 42.6 kDa. *Salmonella typhimurium* (lane 3) and *Citrobacter freundii* (lane 6) both have a second cross-reactive band with slightly faster mobility, presumably due to a second porin. A weaker reacting band of lower molecular mass was also detected for *Enterobacter cloacae* (lane lane 7), *Klebsiella pneumoniae* (lane 8) and *S. marcescens* (lane 9). An additional higher molecular weight band

Fig. 3.5. Western immunoblot of cell envelopes from members of the *Enterobacteriaceae*. Samples (50 µg per lane) were run on SDS-PAGE, transferred to nitrocellulose, and probed with *Serratia marcescens* UOC-51 porin monomer specific antibodies (1:100 dilution). Samples were heated at 100°C for 10 min prior to electrophoresis. Lane 1, *B. subtilis*; lane 2, *Escherichia coli* HB101; lane 3, *Salmonella typhimurium*; lane 4, *Shigella flexneri*; lane 5, *Edwardsiella tarda*; lane 6, *C. freundii*; lane 7, *Enterobacter cloacae*; lane 8, *K. pneumoniae*; lane 9, *Serratia marcescens* UOC-51; lane 10, *Proteus vulgaris*; lane 11, *Pseudomonas aeruginosa*.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seen in all strains, except *E. coli* and *Proteus vulgaris*, may be due to porin multimers. *E. coli* possesses two porins, OmpF and OmpC, however, only one cross-reactive band was seen. The absence of a second reaction may be due to a lack of separation on the urea-free SDS-PAGE gel. Culture conditions may also have affected the number of porins expressed by, not only *E. coli*, but each of the strains tested. The multiple bands seen in the *Enterobacter cloacae* sample are probably due to a cross-reaction with LPS. Anti-LPS antibodies were removed by adsorption with isolated LPS, however, not all may have been removed. The cross-reactive band in the *Ps. aeruginosa* cell envelope appears to be the OprF porin since it exhibits heat- and 2-mercaptoethanol-modifiable mobility in SDS-PAGE (data not shown) (Hancock & Carey,1979). This was surprising since there is no sequence homology between OprF and the enteric porins (Duchêne *et al.*, 1988). Sequence homology between OprF and *E. coli* OmpA has been observed (Woodruff & Hancock, 1989) thus, for the same reasoning as above for the LPS cross-reaction seen in the *Enterobacter cloacae* sample, the OprF may be reacting with anti-OmpA antibodies that were not completely removed. On the other hand, the cross-reaction may represent a common structural epitope between the enteric porins and OprF. Struyvé *et al.* (1991) reported that the last membrane spanning segment of outer membrane proteins, including the enteric porins, *E. coli* OmpA, and *Ps. aeruginosa* OprF, have some similarity.

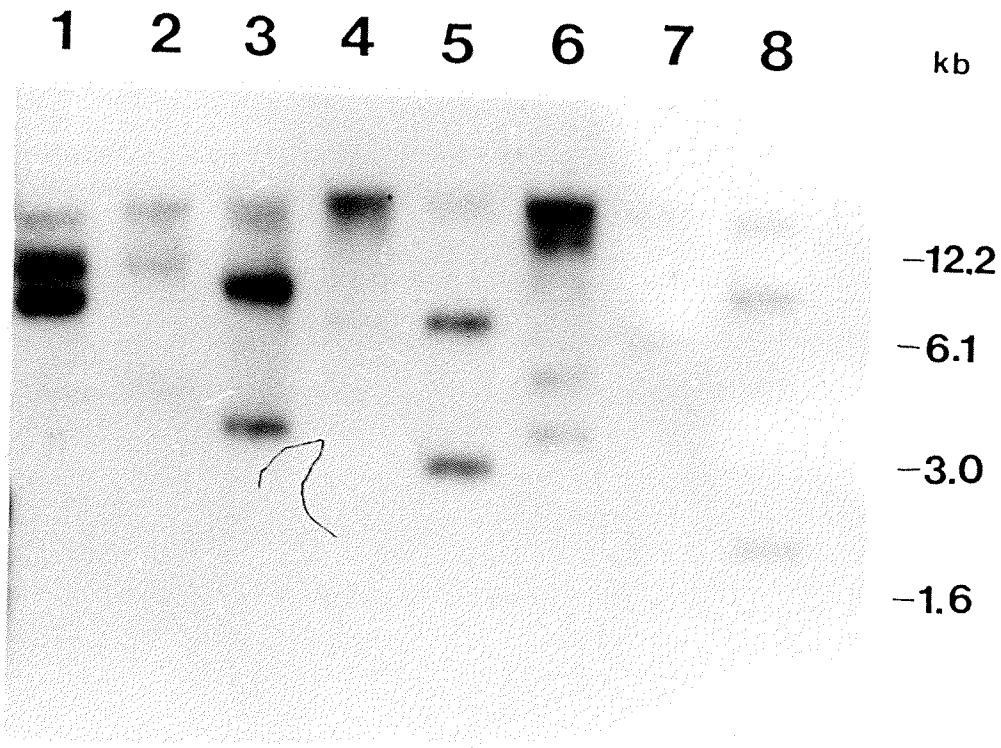
The presence of proteins that cross-react with the *S. marcescens* anti-porin antibodies in all the *Enterobacteriaceae* tested confirms other reports that

enterobacterial porins are to some extent structurally conserved (Hofstra *et al.*, 1980; Jeanteur *et al.*, 1991). Additionally, the porins of the genus *Serratia* are structurally conserved as detected by the cross-reactions in the immunoblot experiments however, variations occur in the size and number of porins among the members tested.

Alignments of the N-terminal amino acid sequence of the *S. marcescens* UOC-69 41 kDa porin with the *E. coli* porins, OmpF, OmpC and PhoE, demonstrated that the region appeared to be conserved (Malouin *et al.*, 1990). An oligonucleotide probe (GAAAT$^{A}_{C}$ TATAATAAAGA$^{T}_{C}$ GG) was designed based on the UOC-69 41 kDa porin N-terminal amino acid sequence and used to probe the same strains used in the above described immunoblot experiments. Results from the hybridization of the eight *Serratia* species are shown in Fig. 3.6. All species tested hybridized with the oligonucleotide probe. Each also hybridized more than once suggesting the presence of more than one related porin gene.

Chromosomal DNA from members of the *Enterobacteriaceae*, *Ps. aeruginosa*, and *B. subtilis* were also Southern blotted and probed with the N-terminal oligonucleotide (Fig. 3.7). *S. marcescens* UOC-51(lane 3), *Citrobacter freundii* (lane 6), *Shigella flexneri* (lane 8) and *E. coli* (lane 10) hybridized strongest with the probe. *Salmonella typhimurium* (lane 9) and *K. pneumoniae* (lane 4) hybridized more weakly. *S. marcescens* had two fragments that hybridized to the probe whereas, only one fragment hybridized for all other strains tested. Although not seen on this figure, *Enterobacter cloacae* had a 0.5 kb fragment that could hybridize. While DNA from other genera of this family

Fig. 3.6. Southern hybridization of chromosomal DNA from *Serratia* species. *Eco*RI-digested chromosomal DNA samples were transferred to nylon membranes after agarose electrophoresis. Hybridization was at 42°C with a 5' end-labelled oligonucleotide probe specific to the *Serratia marcescens* UOC-69 41 kDa porin. Washes were performed at 42°C with 3x SSC, 0.1% (w/v) SDS. Lane 1, *Serratia marcescens* UOC-51; lane 2, *Serratia odorifera*; lane 3, *Serratia ficaria*; lane 4, *Serratia liquefaciens*; lane 5, *Serratia rubidaea*; lane 6, *Serratia proteomaculans*; lane 7, *Serratia plymuthica*; lane 8, *Serratia marcescens* ATCC 13880.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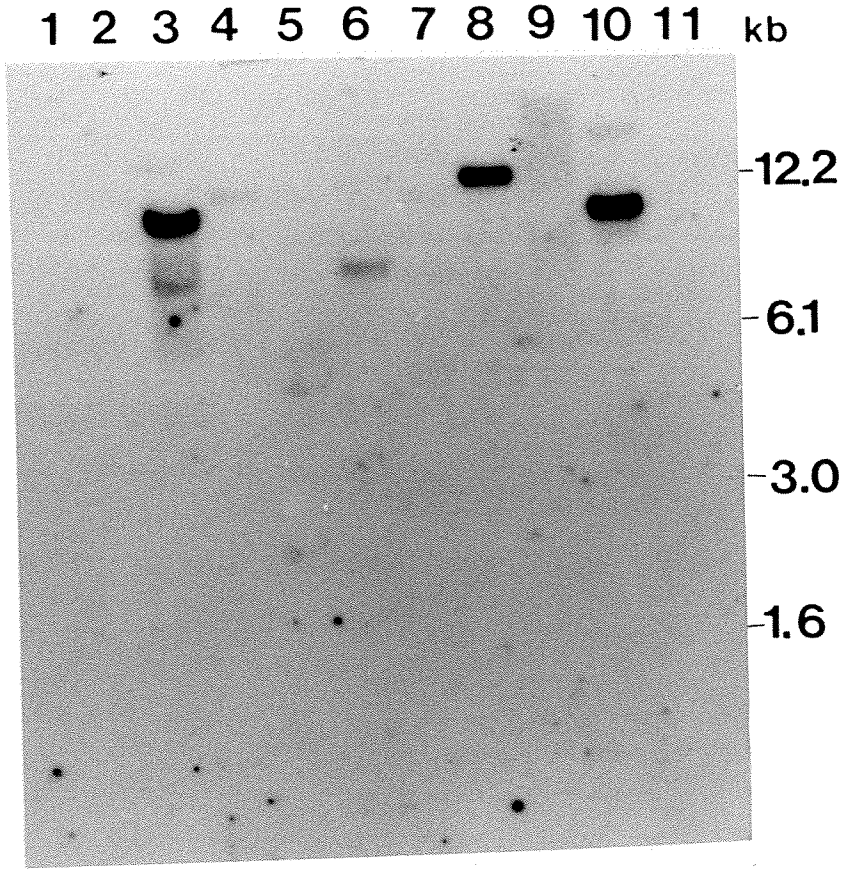
12.2
6.1
3.0
1.6

Fig. 3.7. Southern hybridization of chromosomal DNA from members of the *Enterobacteriaceae*. *Eco*RI-digested chromosomal DNA samples were transferred to nylon membranes after agarose electrophoresis. Hybridization was at 42°C with a 5' end-labelled oligonucleotide probe specific to the *Serratia marcescens* UOC-69 41 kDa porin. Washes were performed at 42°C with 3x SSC, 0.1% (w/v) SDS. Lane 1, *Pseudomonas aeruginosa*; lane 2, *Proteus vulgaris*; lane 3, *Serratia marcescens* UOC-51; lane 4, *K. pneumoniae*; lane 5, *Enterobacter cloacae*; lane 6, *C. freundii*; lane 7, *Edwardsiella tarda*; lane 8, *Shigella flexneri*; lane 9, *Salmonella typhimurium*; lane 10, *Escherichia coli* HB101; lane 11, *B. subtilis*.

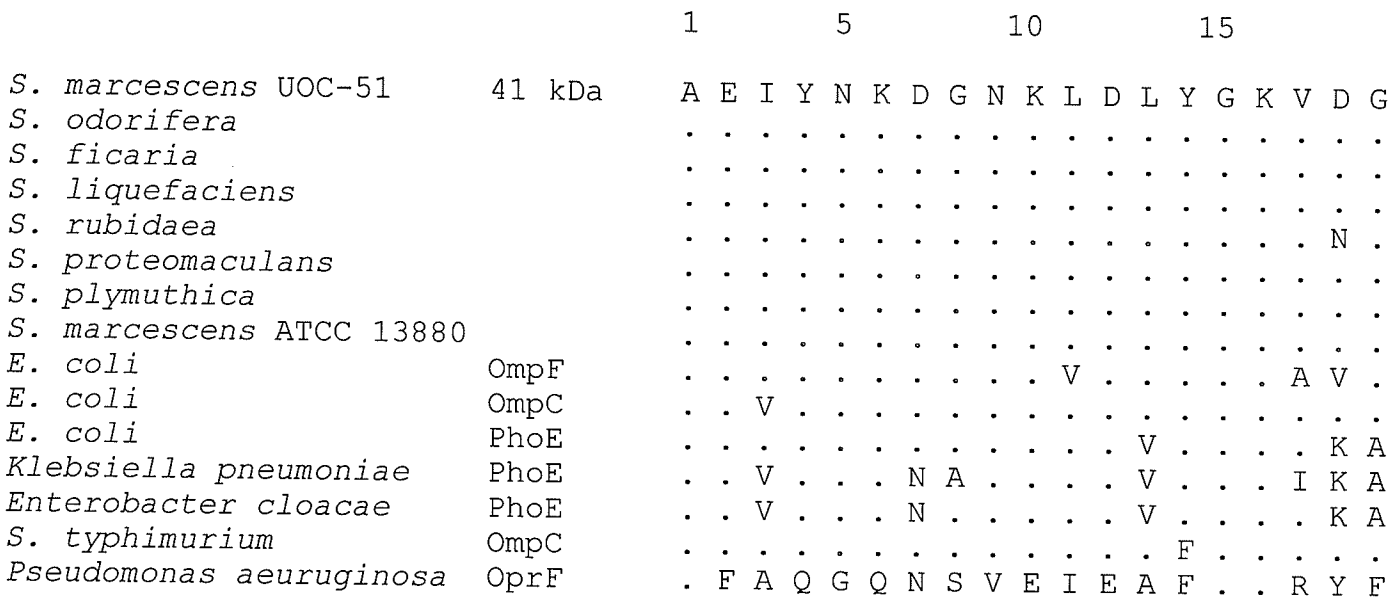

1 2 3 4 5 6 7 8 9 10 11 kb
12.2
6.1
3.0
1.6

would be expected to hybridize it is likely that conditions were too stringent for any sequences that diverge enough from that of the probe to hybridize. For example, *Salmonella* is known to produce three porins, OmpC, OmpD, OmpF and *E. coli* produces two, OmpC and OmpF (Mizuno *et al.*, 1983; Pai *et al.*, 1992). In addition, the phosphate-starvation induced porin, PhoE, is homologous to the OmpF and OmpC porins and has been identified in a number of *Enterobacteriaceae*, including *E. coli*, *Salmonella typhimurium*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Overbeeke *et al.*, 1983; Spierings *et al.*, 1992a; Spierings *et al.*, 1992b; van der Ley *et al.*, 1987). The probe used in this experiment is only 20 bp and if this region is not conserved in all porins it may not detect all related genes.

In comparing the results of *S. marcescens* UOC-51 obtained from the two Southern hybridization experiments it can be seen that in one case two (Fig. 3.7) and in the other three DNA fragments (Fig. 3.6) hybridize with the UOC-69 N-terminal oligonucleotide probe. The high molecular weight fragment seen in Fig. 3.6 but not in Fig. 3.7, has occurred variably in Southern experiments but usually only two bands hybridize. This may be due to partially undigested genomic DNA, non-specific hybridization, or hybridization with a weakly conserved third porin, possibly a *phoE* gene.

The amino acid sequence was determined for the first 19 residues of the major porin from each of the eight *Serratia* strains used in Western and Southern blot analyses (Fig. 3.8). All of the *Serratia* species had identical sequences except for an aspartic acid to aspargine change at residue 18 of *S. rubidaea*.

Fig. 3.8. N-terminal amino acid sequences of *Serratia* spp. porins. Amino acids that are identical to the *S. marcescens* UOC-51 porin sequence are indicated by dots. Amino acid sequences from *Escherichia coli* OmpC and OmpF porins, the PhoE porins from *Escherichia coli*, *K. pneumoniae*, and *Enterobacter cloacae*, the *Salmonella typhi* OmpC porin (Jeanteur *et al.*, 1991), and the *Pseudomonas aeruginosa* OprF porin (Duchene *et al.*, 1988) are from previously published sequences.


1 5 10 15
S. marcescens UOC-51 41 kDa A E I Y N K D G N K L D L Y G K V D G
S. odorifera . . . . . .
S. ficaria . . . . . .
S. liquefaciens . . . . . .
S. rubidaea . . . . . N .
S. proteomaculans . . . . . .
S. plymuthica . . . . . .
S. marcescens ATCC 13880 . . . . . .
E. coli OmpF . . . . . V . . . . A V .
E. coli OmpC . . V . . . . . .
E. coli PhoE . . . . . V . . . . K A
Klebsiella pneumoniae PhoE . . V . . . N A . . . . V . . . I K A
Enterobacter cloacae PhoE . . V . . . N . . . . . V . . . . . K A
S. typhimurium OmpC . . . . . F . . . . . .
Pseudomonas aeuruginosa OprF . F A Q G Q N S V E I E A F . . R Y F

Very strong homologies were found with published sequences for the *E. coli* OmpF and OmpC porins, and the PhoE porins from *K. pneumoniae*, *Enterobacter cloacae*, and *E. coli* (Jeanteur *et al.*, 1991).

The major porin from each of the *Serratia* strains used in this study was characterized for porin function using the planar lipid bilayer system (Benz *et al.*, 1985). This analysis measures the conductance of ions through channels incorporated into an artificial membrane. The resulting conductance, measured in nS, correlates to the relative size of the porin channel that allowed the passage of ions (the smaller the conductance measured, the smaller the channel). Prior to the experiments, the system was calibrated with the purified *E. coli* OmpC porin. Similar single-channel conductances in the presence of 1.0 M KCl were observed for all porins ranging from 1.72 to 2.00 nS (Table 3.1). Results obtained for the *S. marcescens* porins were consistent with those obtained by Malouin *et al.* (1990) for the *S. marcescens* UOC-69 41-kDa porin. These results are found in Table 3.1, along with published single channel conductances for some other porins from enteric bacteria (Benz *et al.*, 1985). The *Serratia* porins appear to be very similar to the *E. coli* porins OmpF and OmpC. A second smaller pore was also detected for *S. liquefaciens* and *S. plymuthica* (1.25 and 1.00 nS, respectively), which was not further investigated.

In summary, using immunochemical and molecular biological screening systems the presence of outer membrane proteins analogous to the 41-kDa major nonspecific porin protein from *S. marcescens* in various members of the enteric family of Gram negative bacteria were identified. In addition, direct amino

TABLE 3.1 Average single channel conductance[a] of isolated porins

Species and strain	Porin	$\overline{\Lambda}$ (nS)
S. marcescens UOC-51	41 kDa	1.72
S. odorifera UOC-61		1.86
S. ficaria UOC-62		1.7
S. liquefaciens UOC-63		1.25
		2.0
S. rubidaea UOC-64		1.92
S. proteomaculans UOC-65		2.0
S. plymuthica UOC-66		1.0
		2.0
E. coli	OmpF	1.9
	OmpC	1.5
	PhoE	1.8
Salmonella typhimurium	OmpF	2.2
	OmpC	2.4

[a]Average single channel conductance was measured on membranes made from 2.0% oxidized cholesterol in *n*-decane, bathed in 1 M KCl.

[b]Data for *E. coli* and *S. typhimurium* porins are from Benz *et al* (1985).

acid sequence analysis revealed the presence of a highly homologous protein in all species of *Serratia* examined. These results strongly point to the conservation of porin structure and function between *Serratia* species and throughout the *Enterobacteriaceae*.

3.3. Cloning, sequencing, and characterization of the *S. marcescens ompC* porin gene

E.coli K-12 strains produce two constitutive non-selective porins, OmpF and OmpC (Mizuno *et al.*, 1983). The closely related *S. typhimurium* is known to produce three constitutive non-selective porins known as OmpF, OmpC and OmpD (Pai *et al.*, 1992). As described in the previous two sections, examination of the *S. marcescens* UOC-51 outer membrane revealed the presence of two possible porins. Like the *E. coli* OmpF and OmpC porins the two putative porins in the *S. marcescens* outer membrane displayed osmoregulation. In order to fully characterize the *S. marcescens* porins the structural genes for the two porins were cloned, sequenced, and analyzed as will be described in the following sections fulfilling Objective #3, Section 1.6.

In Section 3.2 an oligonucleotide probe deduced from the N-terminal amino acid sequence of the *S. marcescens* UOC-69 41 kDa porin (Malouin *et al.*, 1990) was used to probe genomic DNA from various *Serratia* species. Two *Eco*RI fragments of *S. marcescens* UOC-51 genomic DNA routinely hybridized with the oligonucleotide probe in the Southern hybridization experiments. See Fig. 3.7, lane 3 or Fig. 3.6, lane 1 in Section 3.2. The sizes of the two *Eco*RI fragments were 8 and 11 kb. A subgenomic lambda library of *Eco*RI-digested UOC-51 chromosomal DNA was prepared including 6 to 12 kb sized fragments. The library was screened using the oligonucleotide above and positive plaques with 8 and 11 kb inserts were obtained. This section describes the

characterization of the 11 kb fragment. Characterization of the 8 kb fragment is described in Section 3.4. The cloned 11 kb fragment was mapped with restriction endonucleases and hybridized against the oligonucleotide to identify smaller fragments for subcloning. Initially, a 2.1 kb *Kpn*I/*Bam*HI fragment which hybridized, was subcloned into pTZ19 creating the recombinant plasmid p8BK, for further analysis. The map of the 11 kb lambda library clone, λgtS8, is presented in Fig 3.9 along with the subclones created during this study.

Outer-membranes were isolated from *E. coli* NM522/p8BK, and analysed by SDS-PAGE; but there was no expressed protein detected. This indicated that either a regulatory region and/or some other portion of the gene may be missing from the 2.1 kb *Kpn*I/*Bam*HI fragment and therefore, a larger fragment would be required for complete expression. A 6 kb *Sst*I/*Xba*I fragment from λgtS8 (that also hybridized to the probe) was then selected and subcloned into the plasmid, pTZ19, producing the plasmid p8SX. When p8SX was transferred into *E. coli* NM522, an outer-membrane protein with a size of 40 kDa was visualized by SDS-PAGE. This protein also reacted with anti-*S. marcescens*-porin specific antibodies as seen in Fig. 3.10. The *E. coli* porins, OmpF and OmpC also react with these antibodies. However, this cross reaction does not in any way influence the interpretation of the results from this experiment since the *E. coli* porins are easily separated from the *S. marcescens* porin by SDS-PAGE, due to different molecular weights. Such a cross reaction has been previously demonstrated (Section 3.2) and is primarily due to the strong conservation of porin structure (Hofstra *et al.*, 1980; Hutsul *et al.*, 1993).

Fig. 3.9. Restriction endonuclease map of the λgtS8 isolate and subsequent plasmid subclones. The location of the coding region of the porin gene is boxed with the direction of transcription indicated. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sst*I; X, *Xba*I.

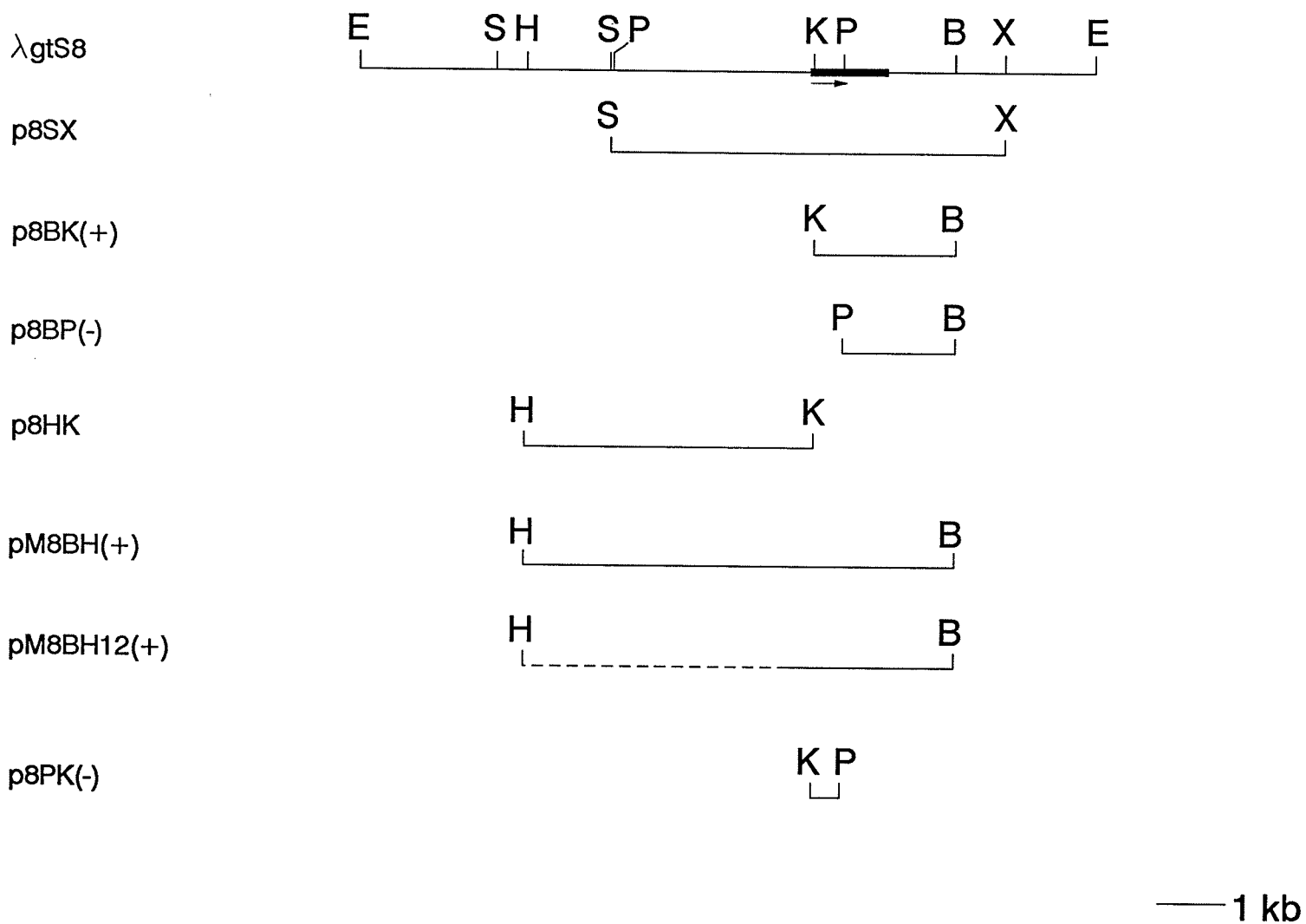
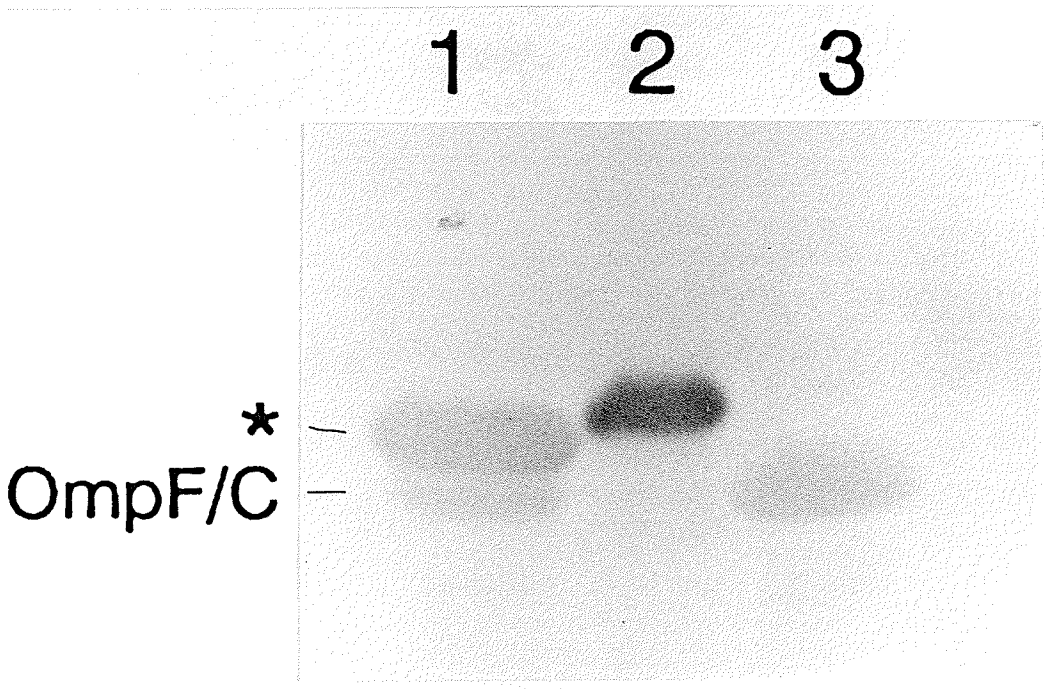

λgtS8
E
S H
S P
K P
B
X
E
p8SX
S
X
p8BK(+)
K
B
p8BP(-)
P
B
p8HK
H
K
pM8BH(+)
H
B
pM8BH12(+)
H
B
p8PK(-)
K P
1 kb

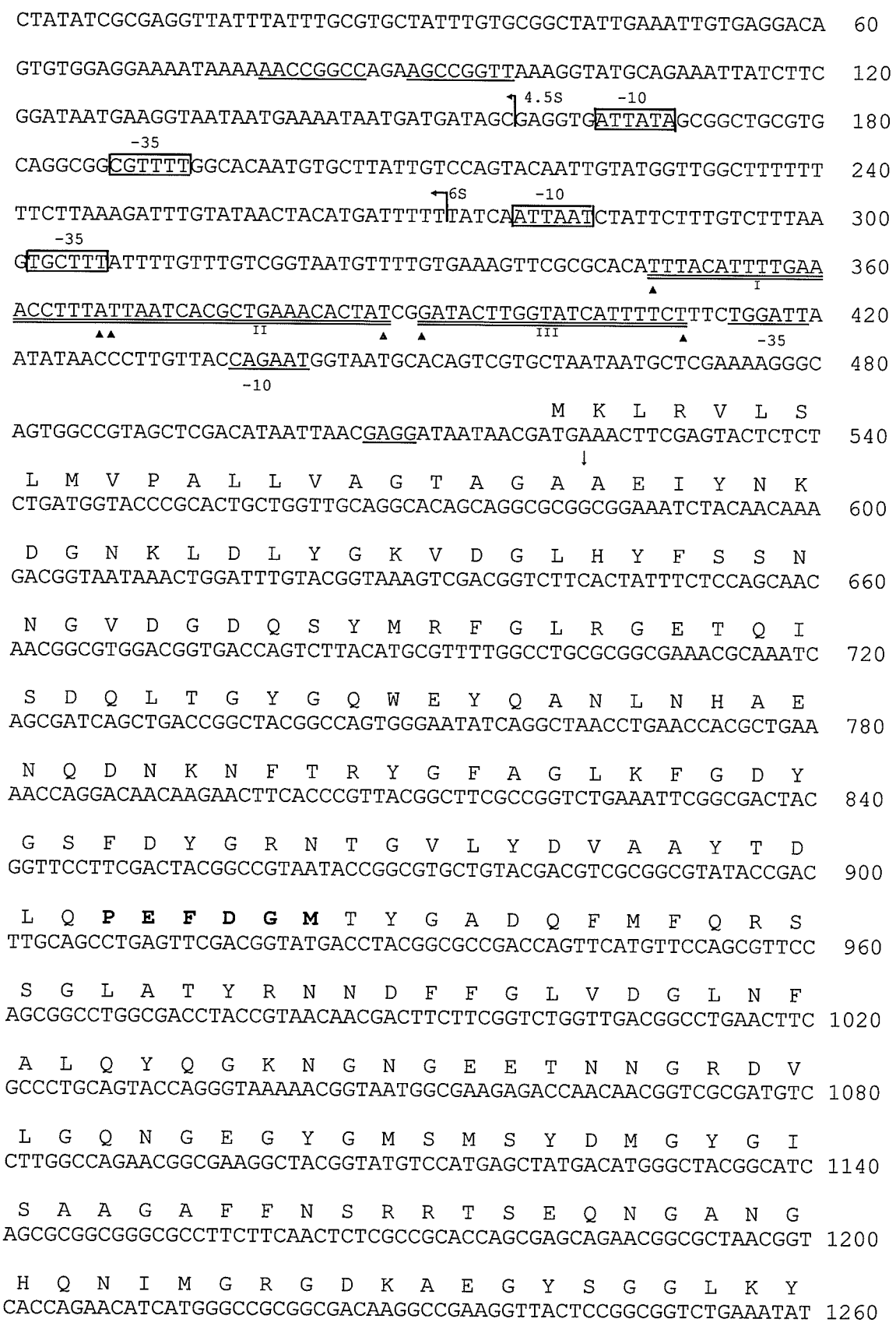
Fig. 3.10. Expression of the cloned *S. marcescens* 40 kDa porin in *E. coli*. Immunoblot of the outer membrane proteins (approximately 50µg per lane) probed with the *S. marcescens* porin specific antibodies. Antibodies were used at a 1:200 dilution. Lane 1, *E. coli* NM522/p8SX; lane 2, *S. marcescens* UOC-51; lane 3, *E. coli* NM522. The asterisk denotes the cloned product seen in the *E. coli* outer membrane.


1
2
3
*
OmpF/C

The cloned *S. marcescens* porin gene product, when expressed in the *E. coli* outer membrane, appeared to run approximately 1 kDa faster by SDS-PAGE than the major 41 kDa porin(s) observed in the *S. marcescens* UOC-51 outer membrane. This difference in SDS-PAGE mobility may be due to the differences in LPS from *E. coli* and *S. marcescens* since LPS remains tightly associated with porin proteins. Alternatively, this may be the second porin. Recall that, when the porin isolated from the *S. marcescens* outer membrane was run on a 4 M urea-SDS-PAGE system it separated into two bands, the lower of which had the same mobility as the cloned product (Fig. 3.10, lane 1). The amount of the 40 kDa porin in the *S. marcescens* outer membrane is low, which may make it indistinguishable from the highly expressed 41 kDa porin on conventional SDS-PAGE due to similarity in size. The 40 and 41 kDa porins may be analogous to the OmpC and OmpF porins of *E. coli* which have similar N-terminal amino acid sequences (Malouin *et al.*, 1990). It is, therefore, not surprising that the oligonucleotide derived from the 41 kDa porin may have identified the 40 kDa porin gene in the subgenomic library.

The 6 kb *Sst*I/*Xba*I clone, p8SX, expressing the porin protein was sequenced in both directions using various subclones, deletion clones and synthesized primers. The nucleotide sequence of the cloned *S. marcescens* porin and its translation are presented in Fig. 3.11. The oligonucleotide probe deduced from the UOC-69 41 kDa porin N-terminal sequence used to clone the gene was found to have only 2 to 4 mismatches present within the degenerate 20 base probe sequence. Amino acid residues 22 to 41 are identical to the N-

Fig. 3.11. Nucleotide sequence of the *S. marcescens* 40 kDa porin gene. The complete coding sequence and its translation plus upstream and downstream elements are shown. The Shine-Dalgarno box and -10 and -35 regions of the *ompC* gene are underlined. Possible OmpR-binding sites are double underlined with arrow heads bordering each one. A putative rho-independent terminator (underlined) is seen after the coding sequence. An arrow in the amino acid sequence is used to show the cleavage site for removal of the leader peptide. The *micF* 4.5S and 6S transcription start sites are indicated with small arrows showing the direction of transcription. The -10 and -35 regions of each are boxed. The termination hairpin of *micF* is also underlined.




```
CTATATCGCGAGGTTATTTATTTGCGTGCTATTTGTGCGGCTATTGAAATTGTGAGGACA  60

GTGTGGAGGAAAATAAAAAACCGGCCAGAAGCCGGTTAAAGGTATGCAGAAATTATCTTC 120

GGATAATGAAGGTAATAATGAAAATAATGATGATAGCGAGGTGATTATAGCGGCTGCGTG 180

CAGGCGGCGTTTTGGCACAATGTGCTTATTGTCCAGTACAATTGTATGGTTGGCTTTTTT 240

TTCTTAAAGATTTGTATAACTACATGATTTTTTATCAATTAATCTATTCTTTGTCTTTAA 300

GTGCTTTATTTTGTTTGTCGGTAATGTTTTGTGAAAGTTCGCGCACATTTACATTTTGAA 360

ACCTTTATTAATCACGCTGAAACACTATCGGATACTTGGTATCATTTTCTTTCTGGATTA 420

ATATAACCCTTGTTACCAGAATGGTAATGCACAGTCGTGCTAATAATGCTCGAAAAGGGC 480

                                M  K  L  R  V  L  S
AGTGGCCGTAGCTCGACATAATTAACGAGGATAATAACGATGAAACTTCGAGTACTCTCT 540

 L  M  V  P  A  L  L  V  A  G  T  A  G  A  A  E  I  Y  N  K
CTGATGGTACCCGCACTGCTGGTTGCAGGCACAGCAGGCGCGGCGGAAATCTACAACAAA 600

 D  G  N  K  L  D  L  Y  G  K  V  D  G  L  H  Y  F  S  S  N
GACGGTAATAAACTGGATTTGTACGGTAAAGTCGACGGTCTTCACTATTTCTCCAGCAAC 660

 N  G  V  D  G  D  Q  S  Y  M  R  F  G  L  R  G  E  T  Q  I
AACGGCGTGGACGGTGACCAGTCTTACATGCGTTTTGGCCTGCGCGGCGAAACGCAAATC 720

 S  D  Q  L  T  G  Y  G  Q  W  E  Y  Q  A  N  L  N  H  A  E
AGCGATCAGCTGACCGGCTACGGCCAGTGGGAATATCAGGCTAACCTGAACCACGCTGAA 780

 N  Q  D  N  K  N  F  T  R  Y  G  F  A  G  L  K  F  G  D  Y
AACCAGGACAACAAGAACTTCACCCGTTACGGCTTCGCCGGTCTGAAATTCGGCGACTAC 840

 G  S  F  D  Y  G  R  N  T  G  V  L  Y  D  V  A  A  Y  T  D
GGTTCCTTCGACTACGGCCGTAATACCGGCGTGCTGTACGACGTCGCGGCGTATACCGAC 900

 L  Q  P  E  F  D  G  M  T  Y  G  A  D  Q  F  M  F  Q  R  S
TTGCAGCCTGAGTTCGACGGTATGACCTACGGCGCCGACCAGTTCATGTTCCAGCGTTCC 960

 S  G  L  A  T  Y  R  N  N  D  F  F  G  L  V  D  G  L  N  F
AGCGGCCTGGCGACCTACCGTAACAACGACTTCTTCGGTCTGGTTGACGGCCTGAACTTC 1020

 A  L  Q  Y  Q  G  K  N  G  N  G  E  E  T  N  N  G  R  D  V
GCCCTGCAGTACCAGGGTAAAAACGGTAATGGCGAAGAGACCAACAACGGTCGCGATGTC 1080

 L  G  Q  N  G  E  G  Y  G  M  S  M  S  Y  D  M  G  Y  G  I
CTTGGCCAGAACGGCGAAGGCTACGGTATGTCCATGAGCTATGACATGGGCTACGGCATC 1140

 S  A  A  G  A  F  F  N  S  R  R  T  S  E  Q  N  G  A  N  G
AGCGCGGCGGGCGCCTTCTTCAACTCTCGCCGCACCAGCGAGCAGAACGGCGCTAACGGT 1200

 H  Q  N  I  M  G  R  G  D  K  A  E  G  Y  S  G  G  L  K  Y
CACCAGAACATCATGGGCCGCGGCGACAAGGCCGAAGGTTACTCCGGCGGTCTGAAATAT 1260
```

```
 D  A  N  D  V  Y  L  A  V  M  F  T  Q  S  Y  N  A  A  R  F
GACGCCAACGACGTCTACCTGGCGGTGATGTTCACCCAGTCCTACAACGCGGCGCGTTTC 1320

 G  S  S  D  S  S  V  Y  G  Y  A  N  K  A  Q  S  F  E  A  Y
GGCAGCTCCGACAGCAGCGTTTACGGCTACGCCAACAAGGCGCAGAGCTTCGAAGCCTAC 1380

 A  H  Y  Q  F  D  F  G  L  R  P  F  V  G  Y  N  Q  T  K  G
GCGCACTACCAGTTCGATTTCGGTCTGCGTCCGTTCGTCGGCTATAACCAGACCAAAGGT 1440

 K  D  L  G  R  A  G  N  G  K  D  Y  G  D  Q  D  L  V  K  F
AAAGACCTGGGCCGCGCCGGTAACGGCAAGGACTACGGCGATCAAGACCTGGTCAAATTC 1500

 V  D  L  G  A  T  Y  F  F  N  K  N  M  S  T  Y  V  D  Y  K
GTCGACCTGGGTGCGACCTACTTCTTCAACAAAAACATGTCTACCTATGTTGATTACAAA 1560

 I  N  L  V  D  N  N  D  F  T  D  A  A  G  I  N  T  D  N  V
ATCAACCTGGTGGACAACAACGACTTCACCGACGCTGCCGGGATCAACACCGACAACGTG 1620

 V  A  V  G  L  V  Y  Q  F  -
GTTGCCGTGGGCCTGGTTTACCAGTTCTAAGCACCTCAGTGTGAACGTCATCGCGCGGCC 1680

TTCGGGCCGCGTTTTTTTTGCCTGTAAACCGGTAAAAAGCACGGATTGATCCGCTATCGG 1740

CACGGGAAGCACCGTGGTTTTCCTCCTCTAAGCCTACGCTGTCAGTACAAAAAAGAGGGG 1800

GATGATATGGAACTCAAGATCGATAAGGTGATCGAAACGGTG                    1842
```

terminal amino acid sequence determined by Edman degradation of the native protein (Malouin *et al.*, 1990; Hutsul *et al.*, 1993).

The nucleotide sequence contains an ORF of 1128 nucleotides which translates into 376 amino acids, including a 21 amino acid signal peptide. A characteristic Shine-Dalgarno sequence, GAGG, can be seen centred approximately 11 bases upstream from the initiation codon. A putative transcription terminator consisting of a hairpin with a free energy of -17 kcal, followed by a string of T residues, is present downstream from the ORF. The calculated M_r of the mature monomer is 39.5 kDa which is close to the molecular mass of 40 kDa for the native protein estimated by SDS-PAGE.

The regulatory region of the cloned *S. marcescens* 40 kDa porin gene is very similar to that of the *E. coli ompF* and *ompC* genes. The location of the promoter region of the *S. marcescens* gene was determined by comparison to the *E. coli ompF* and *ompC* upstream sequences and the -10 and -35 sequences were found to be almost identical to that of the *E. coli ompC* gene. The -10 and -35 regions from these three genes do not follow the bacterial promoter consensus sequences well (Ozawa *et al.*, 1987). However, the efficient transcription of the *E. coli ompF* and *ompC* genes are controlled by three consecutive *cis*-acting activation sequences upstream from the -35 region, to which the transcriptional activator, OmpR, will bind (Maeda *et al.*, 1991). Putative OmpR binding regions are also found upstream from the -35 region of the *S. marcescens* 40 kDa porin gene.

Another regulatory element that is found upstream to the *E. coli ompC*

gene is the *micF* transcript (Mizuno *et al.*, 1984). *micF* is a short RNA transcript, transcribed in the opposite direction from *ompC*, which can hybridize to the 5' end of the *E. coli ompF* transcript allowing increased degradation of the *ompF* transcript by specific ribonucleases. *micF* definitely plays a role in the regulation of OmpF under a number of conditions (Chou *et al.*, 1993). A *micF*-like sequence is seen upstream to the cloned *S. marcescens* 40 kDa porin gene. Two transcription products, 4.5S and 6S, are produced from two different promoters in *E. coli*, however, the 4.5S transcript is the major one produced (Andersen *et al.*, 1987). Fig. 3.12 illustrates the alignment obtained with the *S. marcescens* and *E. coli micF* genes. The secondary structure of the 6S *E. coli micF* includes three hairpin structures, with the last one functioning as a terminator. The *S. marcescens micF* is similar, but it does not appear to have an analogous 5' end hairpin. The major transcription product, the 4.5S *micF*, remains more conserved between the two genera. The differences noted between the two *micF* genes are generally within the secondary structure of the transcript while the region which functions in binding to the *ompF* transcript is well conserved. Perhaps more importantly, the *S. marcescens ompF* gene sequence is necessary to determine the conservation of function of the *micF* gene.

The protein sequence of the cloned *S. marcescens* porin was compared with other enterobacterial OmpF and OmpC porins. The PALIGN program was used to obtain the percent identity between each porin sequence, as illustrated in Table 3.2. The two OmpC sequences, from *E. coli* and *S. typhi*, are 84.2%

Fig. 3.12. Alignment of the *micF* genes of *E. coli* and *S. marcescens*. The transcription start sites of the 4.5S and 6S products of *E. coli* and their promoter regions (boxed) are indicated. Hairpin structures are designated with underlying and overlying arrows. The region of *E. coli micF* that binds to *ompF* mRNA is also shown. Corresponding putative structures are also designated in *S. marcescens*. | denotes conserved bases.

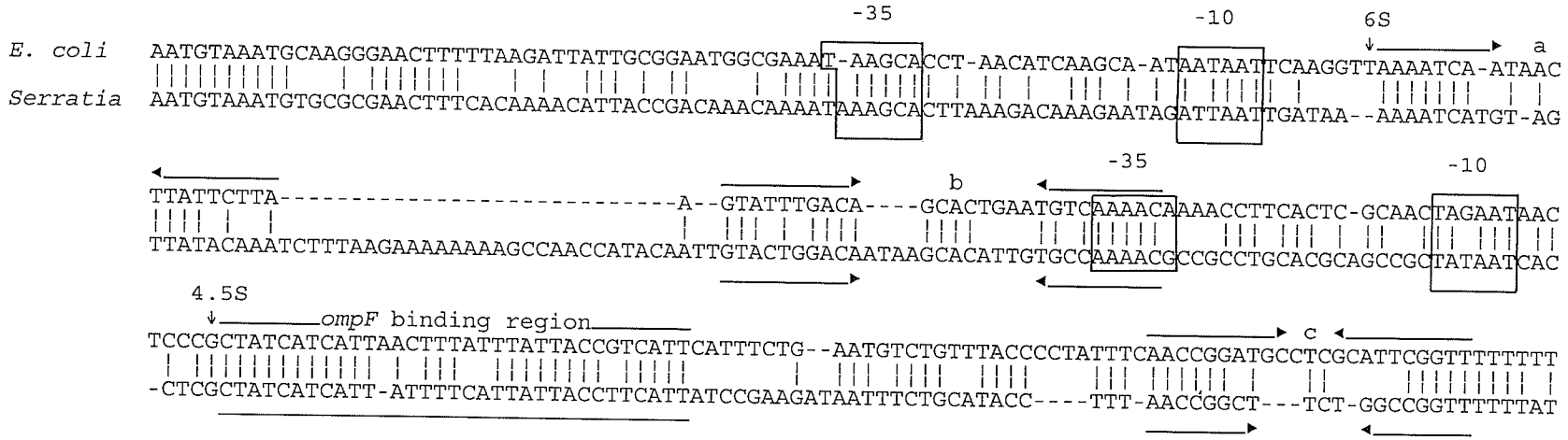

-35
-10
6S
a
E. coli
Serratia
AATGTAAATGCAAGGGAACTTTTTAAGATTATTGCGGAATGGCGAAAT-AAGCACCT-AACATCAAGCA-ATAATAATTCAAGGTTAAAATCA-ATAAC
AATGTAAATGTGCGCGAACTTTCACAAAACATTACCGACAAACAAAATAAAGCACTTAAAGACAAAGAATAGATTAATTGATAA--AAAATCATGT-AG
-35
-10
b
TTATTCTTA----------A--GTATTTGACA----GCACTGAATGTCAAAACAAAACCTTCACTC-GCAACTAGAATAAC
TTATACAAATCTTTAAGAAAAAAAAGCCAACCATACAATTGTACTGGACAATAAGCACATTGTGCCAAAACGCCGCCTGCACGCAGCCGCTATAATCAC
4.5S
ompF binding region
c
TCCCGCTATCATCATTAACTTTATTTATTACCGTCATTCATTTCTG--AATGTCTGTTTACCCCTATTTCAACCGGATGCCTCGCATTCGGTTTTTTTT
-CTCGCTATCATCATT-ATTTTCATTATTACCTTCATTATCCGAAGATAATTTCTGCATACC----TTT-AACCGGCT---TCT-GGCCGGTTTTTTAT

Table 3.2 Amino acid sequence comparisons between the *S. marcescens* 40 kDa porin and other OmpF and OmpC porins

	SM40KDA	ECOOMPF	ECOOMPC	KPNOMPC	STYOMPC
SM40KDA	*	60.1	70.2	71.3	68.6
ECOOMPF	*	*	64.9	64.5	65.9
ECOOMPC	*	*	*	81.8	84.2
KPNOMPC	*	*	*	*	82.7
STYOMPC	*	*	*	*	*

Values are percentage identity between porins as determined by PALIGN.
SM40KDA, *S. marcescens* UOC-51 40 kDa porin; ECOOMPF, *E. coli* OmpF (Inokuchi *et al.*, 1982); ECOOMPC, *E. coli* OmpC (Mizuno *et al.*, 1983); KPNOMPC, *Klebsiella pneumoniae* OmpC (Albertí *et al.*, 1995); STYOMPC, *Salmonella typhi* OmpC (Puente *et al.*, 1989).

identical whereas, the identity between OmpF and the two OmpC sequences are 64.9% and 66.0%, respectively. The cloned *S. marcescens* porin has the highest identity with the OmpC sequences; 68.6% with *S. typhi* OmpC and 70.3% with *E. coli* OmpC, although these values are not as high as that obtained between *S. typhi* and *E. coli* OmpC sequences.

A conserved feature of most outer membrane proteins, including porins, is a C-terminal phenylalanine (Struyvé *et al.*, 1991). The terminal phenylalanine was shown to be important for the correct assembly of the outer membrane proteins into the outer membrane. In keeping with this function, the *S. marcescens* 40 kDa porin also has a C-terminal phenylalanine.

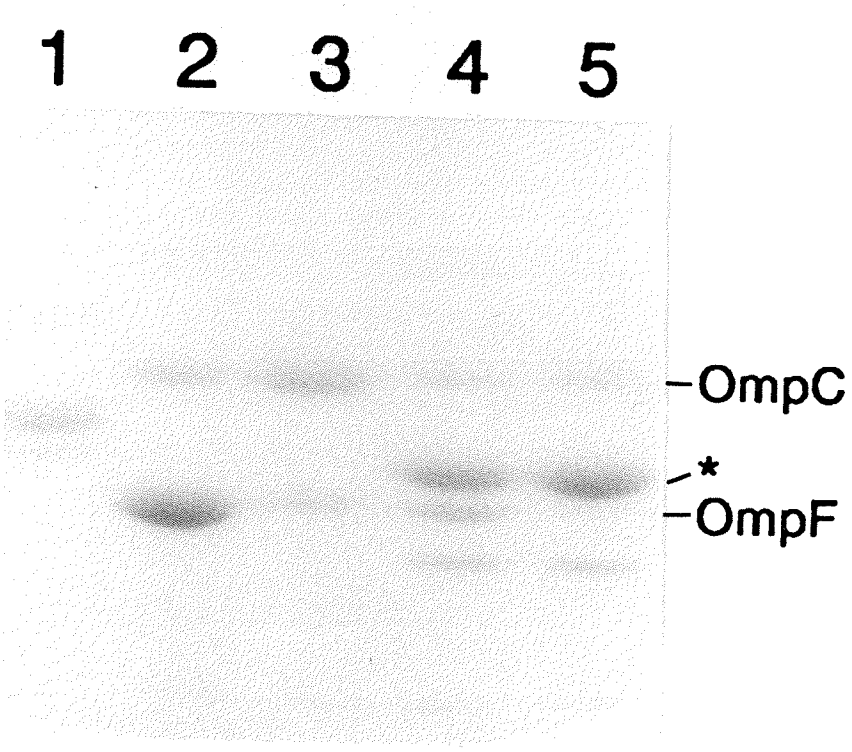
The third external loop of enterobacterial porins plays a role in determining size and ion selectivity of the porin channels (Bauer *et al.*, 1989; Cowan *et al.*, 1992). A conserved motif, PEFGG followed by D, has been observed at the tip of this important loop . The *S. marcescens* 40 kDa porin has diverged from this motif with the sequence, PEFDGM. It has been proposed that the GG sequence has been conserved in enterobacterial porins because of the unique torsion angles it would form, thus aiding in turn formation of the loop (Cowan *et al.*, 1992). Jeanteur *et al.* (1994) reported the alteration of the PEFGG motif to PEFDG in a colicin resistant OmpF mutant in *E. coli*. The change also affected the size selection of the OmpF pore. It will be interesting therefore, to examine the permeability properties of the *S. marcescens* 40 kDa porin.

When outer membranes were first isolated from *E. coli* NM522/p8SX, which expresses the *S. marcescens* cloned porin, there appeared to be a

decrease in the *E. coli* OmpF and/or OmpC content in the outer membrane. Therefore, the effect osmotic strength of the media may have on expression of the cloned *S. marcescens* porin, as well as, the effect the expression of this cloned porin may have on the expression of the two *E. coli* porins was examined. With no added NaCl in the medium the expression of *E. coli* OmpF is increased over that of the *E. coli* OmpC (Fig. 3.13, lane 2), whereas, in 0.3M NaCl OmpC is preferentially expressed (Fig. 3.13, lane 3), as would be expected (Csonka, 1989). When the *S. marcescens* cloned porin is expressed in *E. coli*, OmpF still exhibits regulation in response to high osmotic strength (Fig. 3.13, lane 5), although there is an apparent decrease in the overall content of OmpF in the membrane under both conditions. The amount of *E. coli* OmpC does not appear to increase in the high salt condition (Fig. 3.13, lane 5) when the *S. marcescens* cloned porin is expressed, however, a slight increase in the amount of the *S. marcescens* cloned porin is observed in the *E. coli* NM522/p8SX outer membrane. These results suggest that the OmpR binding sites, located upstream to the *S. marcescens* cloned porin gene, are functional in *E. coli* NM522. The lower bands seen in lanes 4 and 5 are OmpA which were not removed completely during the porin isolation of these samples.

OmpR binding sites found upstream to the *S. marcescens* porin gene suggest a potential for regulation by a system similar to *E. coli* EnvZ/OmpR (Waukau & Forst, 1992). Indeed, we observed that when grown in medium containing high salt (0.3M NaCl) the concentration of the cloned *S. marcescens* 40 kDa porin in *E. coli* appeared to increase.

Fig. 3.13. Osmoregulation of the *S. marcescens* OmpC porin. SDS-insoluble cell envelope fractions of cells grown in the absence or presence of NaCl were run on a 4 M urea/SDS-PAGE system. Samples (approximately 2 to 4 µg per lane) were loaded after heating at 100°C for 10 min. Lane 1, *S. marcescens* in 0.086 M NaCl; lane 2, *E. coli* NM522, no NaCl; lane 3, *E. coli* NM522, 0.3 M NaCl; lane 4, *E. coli* NM522/p8SX, no NaCl; lane 5, *E. coli* NM522/p8SX, 0.3 M NaCl. The asterisk denotes the cloned *S. marcescens* porin in the *E. coli* outer membrane.


1
2
3
4
5
OmpC
*
OmpF

Under high osmotic stress *E. coli* OmpC levels normally rise. In our osmoregulation experiments little or no increase in *E. coli* OmpC was detected in the outer membrane when the *S. marcescens* 40 kDa porin gene was present. The similarity of the regulatory regions, or OmpR binding sites, between the *E. coli* and *S. marcescens* porin genes and the fact that there are more copies of the cloned *S. marcescens* gene present in the clone could explain the absence of an increase in *E. coli* OmpC expression. A fraction of the *E. coli* OmpR protein could bind to *S. marcescens* DNA thus leaving a smaller than normal proportion of the OmpR pool free to bind to the binding sites preceding the *E. coli ompC* gene. Similarly, the lower content of the *E. coli* OmpF in the outer-membrane under both osmotic growth conditions could be explained by the abundance of *S. marcescens* OmpR binding sites. Probably more important than the OmpR effect is the high copy number of *micF* that would be present if it is expressed. It has been shown that when present in high quantities it can inhibit OmpF synthesis (Matsuyama & Mizushima, 1985). *E. coli* OmpF is repressed in the presence of salicylate and *micF* has been shown to play a role in this regulation (Rosner *et al.*, 1991). Repression of the 41 kDa porin in *S. marcescens* by salicylate has also been reported (Sawai *et al.*, 1987). The *micF* sequence seen upstream to the *S. marcescens* 40 kDa porin gene may therefore play a role in the repression of the 41 kDa porin by salicylate as determined in *E. coli*.

As determined by the cloning and sequencing of a porin structural gene from *S. marcescens* UOC-51 genomic DNA we have found that *S. marcescens* produces a porin with a molecular mass of 39.5 kDa for the mature peptide. The

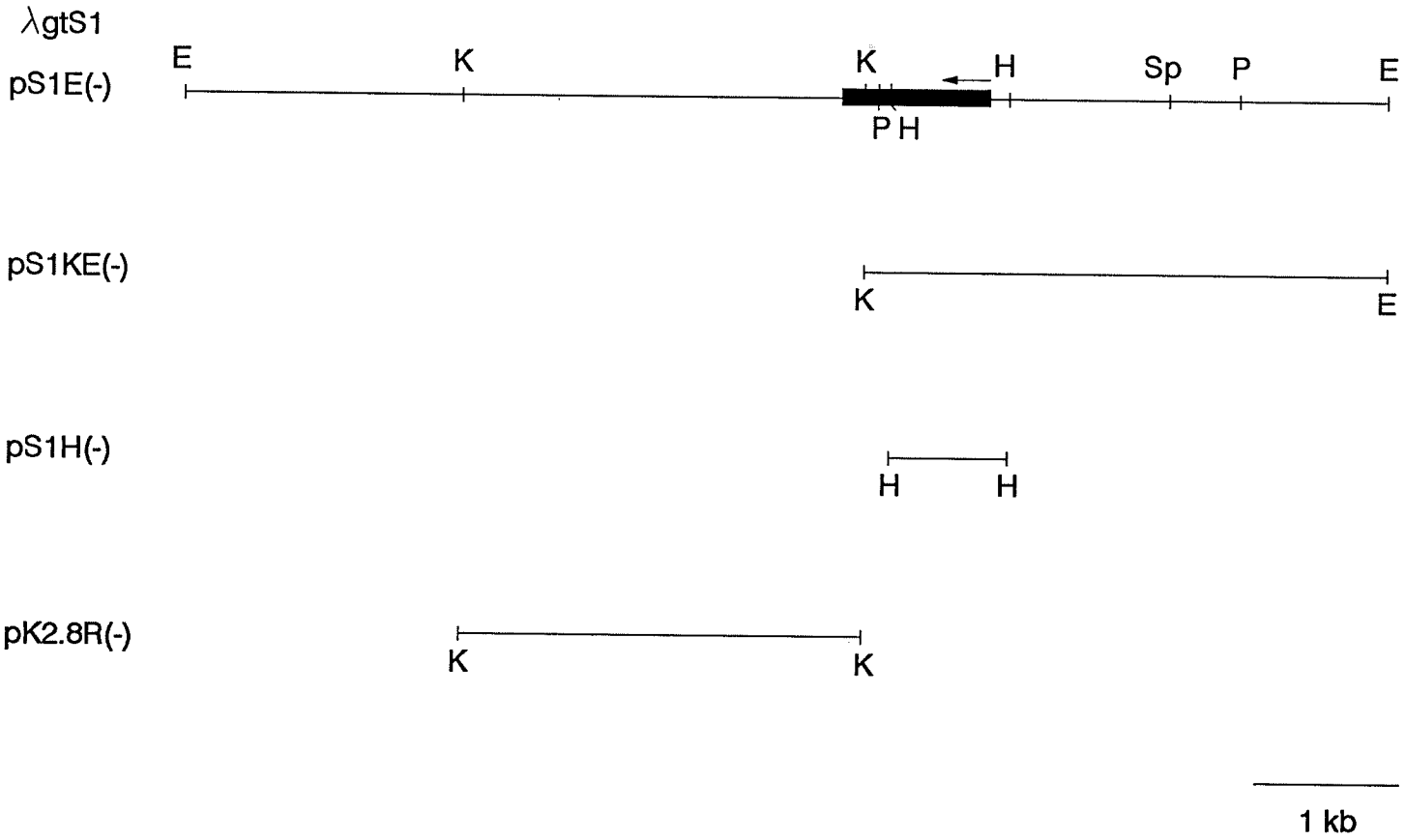
sequence of this porin has the highest similarity, ~70%, with *E. coli* and *Salmonella typhi* OmpC porins. The upstream sequence of the *S. marcescens* porin gene also resembles the *ompC* genes, with three consecutive OmpR binding regions and an upstream sequence resembling *micF* which is also found upstream from *E. coli* and *Salmonella typhi ompC* genes. The *S. marcescens* porin is very similar in size to the *E. coli* OmpC and *Salmonella typhi* OmpC porins, which are 38.5 and 40 kDa, respectively. Like the *E. coli* OmpC, the expression of the cloned *S. marcescens* porin appears to be regulated in *E. coli* in response to NaCl concentration. Taking all of these factors into consideration, this cloned *S. marcescens* porin gene was called *ompC*.

3.4. Cloning, sequencing, and characterization of the *S. marcescens ompF* porin gene

As mentioned in the previous Section (3.3), two different positive clones were isolated from the *S. marcescens* UOC-51 sub-genomic library when probed with the oligonucleotide deduced from the N-terminal amino acid sequence of the *S. marcescens* UOC-69 41 kDa porin. The size of the two phage isolates, λgtS8 and λgtS1, corresponded to the two *Eco*RI fragments that were detected in Southern hybridizations with the UOC-69 N-terminal oligonucleotide (Fig. 3.7, lane 3). The porin gene cloned in λgtS8 was characterized in Section 3.3 and determined to be analogous to the *E. coli ompC* gene. This section describes the characterization of the second porin gene cloned in λgtS1.

The *Eco*RI fragment contained in the phage isolate, λgtS1, was determined to be 8 kb in size. This fragment was mapped with restriction endonucleases and shown to be unique from the 11 kb fragment described earlier. The map of this isolate can be seen in Fig.3.14. Southern hybridization with the aforementioned oligonucleotide occurred with a 0.9 kb *Hind*III segment. Subsequently a 3.5 kb *Kpn*I/*Eco*RI fragment was subcloned into pKS(-), creating the plasmid pS1KE(-), for further analysis. A cross-reactive protein was detected in whole cell lysates but not in the outer membrane suggesting that the whole gene may not have been contained on that fragment. Using the *S. marcescens ompC* gene as a probe the neighbouring 2.8 kb *Kpn*I fragment was also found to hybridize in Southern analysis of the phage isolate. The entire 8kb *Eco*RI

Fig. 3.14. Restriction endonuclease map of the 8 kb *S. marcescens* genomic DNA isolate, λgtS1, and its subclones. The pS1E(-) plasmid contains the entire 8 kb *Eco*RI genomic DNA fragment of λgtS1. The location of the porin gene is boxed with the direction of transcription indicated. E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I.


λgtS1
pS1E(-)
E
K
K
H
Sp
P
E
P H
pS1KE(-)
K
E
pS1H(-)
H
H
pK2.8R(-)
K
K
1 kb

fragment was transferred into pKS(-), creating the plasmid pS1E(-), to allow expression and examination of the cloned protein. An approximately 41 kDa product was detected by SDS-PAGE in the *E. coli* NM522/pS1E(-) outer membrane (Fig. 3.15, lane 2) which was not seen in the *E. coli* outer membrane without the plasmid (lane 3). The UOC-51 porin specific polyclonal antibodies reacted with this protein in immunoblot experiments (data not shown) confirming that a porin gene was cloned. The protein produced appeared to be the same size as the major band in the *S. marcescens* outer membrane (lane1). In addition, this protein migrated more slowly than the cloned *S. marcescens* OmpC, appearing to be about 1 kDa larger. These results suggest that this may be the major porin seen in the *S. marcescens* outer membrane. Interestingly, the quantity of *S.marcescens* OmpC protein found in the *E. coli* outer membrane is much higher than the *S. marcescens* OmpF protein (data not shown). The reason for this is unclear and is opposite to that seen in the *S. marcescens* outer membrane if this is indeed the major 41 kDa porin.

The cloned gene was sequenced from pS1KE(-) and pK2.8R(-) plasmids (Fig. 3.14) using various synthesized primers. The nucleic acid sequence along with the deduced amino acid sequence is illustrated in Fig. 3.16. The gene has an ORF of 1122 kb, with a typical Shine-Dalgarno sequence centred 9 nucleotides upstream, and potential terminator hairpins downstream. The sequence matched the UOC-69 derived oligonucleotide for 16 to 18 sites and was identical to the *S. marcescens ompC* N-terminal sequence. The amino acid sequence from 22 to 41 was identical to that determined in Section 3.2 and to the

Fig. 3.15. Expression of the cloned *S. marcescens* OmpF porin in *E. coli*. Approximately 3 to 5 µg samples were heated at 100°C for 10 min prior to loading on SDS-PAGE. Lane 1, *S. marcescens* UOC-51 porins; Lane 2, *E. coli* NM522/pS1E- SDS-insoluble fraction; Lane 3, *E. coli* NM522 outer membrane. The migration of the protein molecular weight standards are indicated in kDa at the left of the figure. a, *S. marcescens* OmpF porin; b, *E. coli* OmpF/C porins; c, *E. coli* OmpA protein.

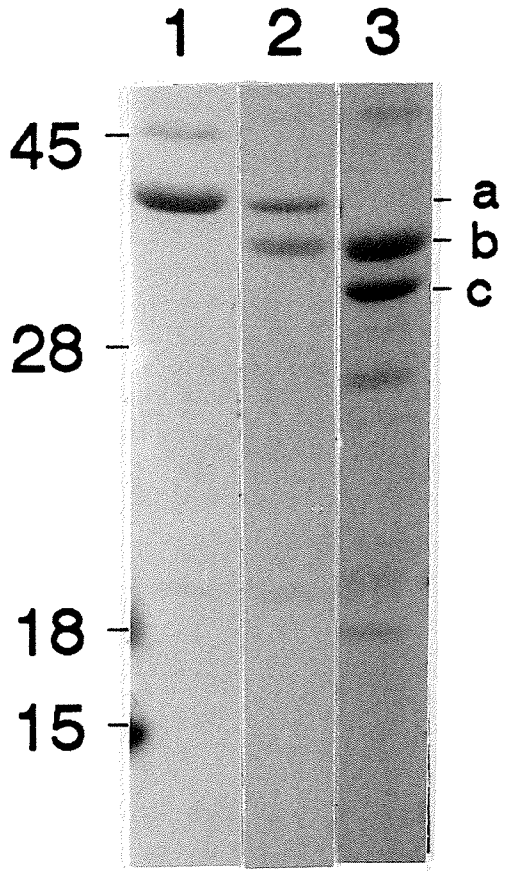

1
2
3
45
28
18
15
a
b
c

Fig. 3.16. Nucleotide sequence of the *S. marcescens* OmpF porin gene. The complete coding sequence and its translation plus upstream and downstream elements are shown. The Shine-Dalgarno box and -10 and -35 regions are underlined. Possible OmpR-binding sites are double underlined with arrow heads bordering each one. A putative transcription terminator (underlined) is seen after the coding sequence. An arrow in the amino acid sequence is used to show the cleavage site for removal of the leader peptide.

```
TTTCGGCCTGGGCTTTGAACGTTTAATCGCCTATGTCACCGGCGTGCAAAACGTGCGCGA 60

TGTGATTCCATTCCCGCGCACGCCGCGTAACGCAAGCTTCTAATTTATACGATCACACGT 120

TTTTTAAACGATTTCGTTACAAAACAAAGGCCAGCATCGCTGGCCTTTCGCATTTTTTAA 180

TATTGACGCACGTCACAAAGTTCCCGAAAAATTACATTTGGTTACACATACTTTCCTTTT 240
                                  ▲        I     ▲▲      II
GCAACCTGATTGGGACATTGGTATCATTTTCGTCCTAGATTAACCCGCCTGTGAATGGAA 300
          ▲  ▲       III      ▲    -35
CACTGCGTTCAGACACAGGACGACACCAATCTATCAACAATAGTTCCCAAAGGATTATTG 360
-10
                                                M  M  K  R  N
GCGGCAGTGGCAAAGGTGTCCGAATAACACCAATGAGGGTAATAATGATGAAGCGCAACA 420
                                                      ↓
I  L  A  V  V  I  P  A  L  L  A  A  G  A  A  N  A  A  E  I
TTCTTGCAGTGGTAATCCCGGCTCTGTTGGCTGCTGGTGCAGCAAACGCAGCTGAAATCT 480

Y  N  K  D  G  N  K  L  D  L  Y  G  K  V  D  G  L  H  Y  F
ACAACAAAGACGGCAACAAGCTGGATCTGTACGGCAAAGTTGACGGTCTGCACTACTTCT 540

S  K  D  K  G  N  D  G  D  Q  T  Y  V  R  F  G  F  K  G  E
CCAAAGACAAAGGTAATGACGGCGATCAGACCTATGTTCGTTTCGGCTTCAAAGGTGAAA 600

T  Q  I  T  D  Q  L  T  G  Y  G  Q  W  E  Y  N  V  Q  S  N
CCCAGATTACTGACCAACTGACCGGTTACGGCCAGTGGGAATACAACGTTCAGTCCAACC 660

H  A  E  S  Q  G  T  E  G  T  K  T  R  L  G  F  A  G  L  K
ACGCCGAATCTCAGGGCACCGAAGGCACCAAAACCCGTCTGGGCTTCGCCGGTCTGAAAT 720

F  A  D  Y  G  S  F  D  Y  G  R  N  Y  G  V  L  Y  D  V  E
TCGCTGACTACGGCTCCTTCGACTACGGCCGTAACTACGGCGTACTGTACGACGTGGAAG 780

G  W  T  D  M  L  P  E  F  G  G  D  T  Y  T  Y  S  D  N  F
GCTGGACCGATATGCTGCCAGAGTTCGGTGGCGATACTTACACCTACTCCGACAACTTCA 840

M  T  G  R  T  N  G  V  A  T  Y  R  N  N  N  F  F  G  L  V
TGACCGGCCGTACCAACGGCGTTGCGACCTATCGTAACAACAACTTCTTCGGTCTGGTTG 900

D  G  L  N  F  A  L  Q  Y  Q  G  K  N  Q  N  D  G  R  D  V
ACGGTCTGAACTTCGCTCTGCAATACCAGGGCAAAAACCAGAACGACGGCCGTGACGTCA 960

K  K  Q  N  G  D  G  W  G  I  S  S  T  Y  D  I  G  E  G  V
AAAAACAAAACGGCGACGGCTGGGGCATTTCCTCTACTTATGACATCGGCGAAGGCGTAA 1020

S  F  G  A  A  Y  A  S  S  N  R  T  D  D  Q  K  L  R  S  N
GCTTCGGCGCTGCATACGCTTCTTCCAACCGTACCGACGACCAGAAACTGCGTTCAAACG 1080

E  R  G  D  K  A  D  A  W  T  V  G  A  K  Y  D  A  N  N  V
AGCGTGGCGACAAGGCTGACGCATGGACCGTGGGCGCGAAATATGACGCCAACAACGTCT 1140

Y  L  A  A  M  Y  A  E  T  R  N  M  T  P  F  G  G  G  N  F
ACCTGGCGGCAATGTATGCAGAAACCCGTAACATGACTCCGTTCGGTGGCGGTAACTTCA 1200

T  N  T  C  A  A  T  E  N  C  G  G  F  A  S  K  T  Q  N  F
CCAACACTTGCGCAGCGACTGAAAACTGCGGCGGCTTCGCTAGCAAAACTCAGAACTTCG 1260

E  V  T  A  Q  Y  Q  F  D  F  G  L  R  P  E  V  S  Y  L  Q
AAGTGACTGCTCAGTATCAGTTCGACTTCGGTCTGCGCCCAGAAGTGTCTTACCTGCAGT 1320
```

S K G K N L N V P G V G S D Q D L V K Y
CTAAGGGTAAAAACCTGAACGTCCCAGGCGTGGGTTCTGACCAAGATCTGGTTAAATATG 1380

V S V G T T Y Y F N K N M S T Y V D Y K
TTTCTGTTGGTACCACTTACTACTTCAACAAAAACATGTCCACCTATGTTGATTACAAAA 1440

I N L L D D N D F T K A T G I A T D D I
TCAACCTGCTGGATGACAACGACTTCACCAAAGCAACCGGCATCGCAACTGACGATATCG 1500

V G V G L V Y Q F -
TAGGCGTTGGTTTGGTATACCAGTTCTAAGTTGTCTCGCTTATCGGCGGTTCACCGTCGA 1560

CTAAGTTAAAAAAACAGGGCTTCGGCCTGTTTTTGTTTGTATCAGCCGGTAACTTTTTAT 1620

CCTGCCGATTCCCCCTTCCCCATCATTTTTTCTGTCGAATCCTTCACGTTCATAAGATCG 1680

CGGTGTTTTTATCGCAAACGG 1701

OmpC porin. The calculated Mr of the the mature protein is 39.0 kDa, which is 0.5 kDa smaller than the mature *S. marcescens* OmpC porin despite a slower SDS-PAGE mobility. The primary sequence exhibited characteristics of other porins including a C-terminal phenylalanine and the conserved PEFGGD motif. An interesting feature seen in this porin sequence is the presence of two cysteines at positions 269 and 275. A comparison with the *E. coli* OmpF amino acid sequence showed that the cysteines would be located in the sixth external loop of the *E. coli* porin structure. Never has a cysteine been reported in the sequence of any classical trimeric diffusion porins. However, cysteines have been identified in the *Ps. aeruginosa* porins, OprF (Duchêne *et al.*, 1988) and OprB (Wylie & Worobec, 1994), and the LamB porin of *E. coli* (Clément & 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 & Stretton).

Amino acid sequence comparisons between the 41 kDa porin and other OmpF and OmpC porins are presented in Table 3.3. The *S. marcescens* porin exhibits 60 to 70% identity with other enterobacterial OmpF and OmpC porins, the same level of identity between the two major porin types. The *S. marcescens* 41 kDa porin therefore, is no more similar to one porin type than another. Amino

Table 3.3 Amino acid sequence comparisons between the *S. marcescens* 41 kDa porin and other OmpF and OmpC porins

	SM41KDA	ECOOMPF	ECOOMPC	KPNOMPC	STYOMPC
SM41KDA	*	67.7	68.4	71.5	70.3
ECOOMPF	*	*	64.9	64.5	65.9
ECOOMPC	*	*	*	81.8	84.2
KPNOMPC	*	*	*	*	82.7
STYOMPC	*	*	*	*	*

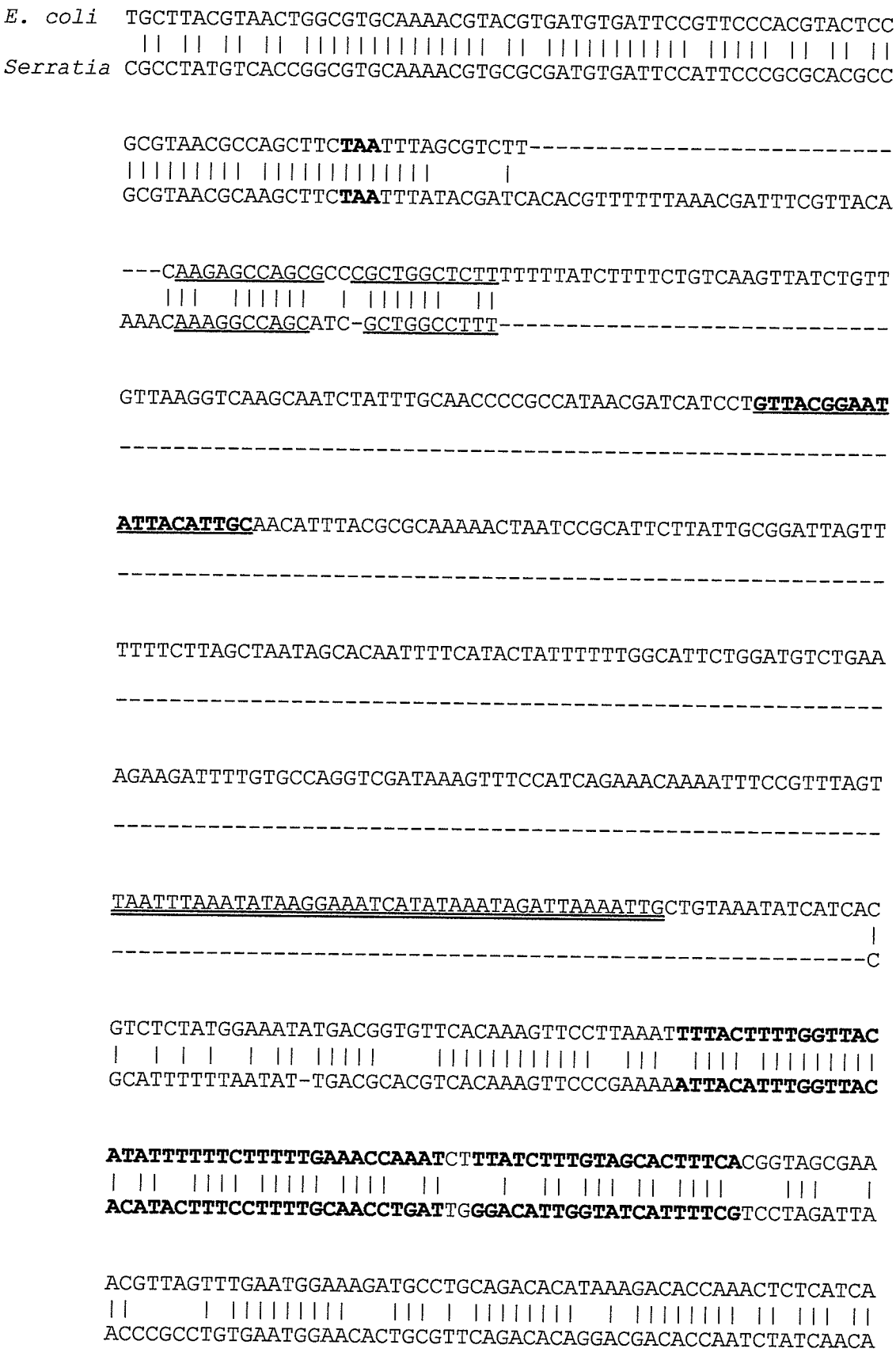
Values are percentage identity between porins as determined by PALIGN.
SM41KDA, *S. marcescens* UOC-51 41 kDa porin; ECOOMPF, *E. coli* OmpF (Inokuchi *et al.*, 1982); ECOOMPC, *E. coli* OmpC (Mizuno *et al.*, 1983); KPNOMPC, *Klebsiella pneumoniae* OmpC (Albertí *et al.*, 1995); STYOMPC, *Salmonella typhi* OmpC (Puente *et al.*, 1989).

acid sequence comparisons hence, do not indicate to which category of porin (OmpF or OmpC) it belongs.

The upstream sequence of the cloned gene most closely resembles the *E. coli ompF* upstream sequence. With the *E. coli* sequence as a guide the OmpR binding sites (OBS) and promoter sequences were localized. However, it is interesting to note that similarity ends just before the three consecutive OBS. In *E. coli*, the *ompF* gene is located after *asnS*, a gene encoding asparaginyl tRNA synthetase. The intergenic region is 600 bp in size and contains regulatory components including the negative OBS at -380 to -360 and an integration host factor binding site. An *asnS* gene was also identified upstream to the cloned *S. marcescens ompF* gene, however the intergenic region is approximately 300 bp shorter. Further examination shows that a region of DNA after the *asnS* terminator to just upstream from OBS is absent (Fig. 3.17). Included in the segment would be OBS and integration host factor (IHF) recognition sequences crucial for repression of *ompF* transcription. Therefore, osmoregulation via the OmpR/EnvZ system cannot take place in *S. marcescens* and must involve *micF*. This result was also recently observed in *Xenorhabdus nematophilus* (Forst *et al.*, 1995).

The hypothetical secondary structure of the *E. coli* 4.5S *micF* RNA and its hybridization with *ompF* has been determined (Schmidt *et al.*, 1995). In addition, the predicted secondary structure of the *S. marcescens* 4.5S *micF* was presented. Based on this structure and the *E. coli ompF/micF* hybridization, the hybridization of the *S. marcescens micF* with the *S. marcescens ompF* sequence

Fig. 3.17. Alignment of *E. coli* and *S. marcescens ompF* upstream DNA sequence. The sequences shown are from within the *asnS* genes to approximately 70 bp after the translation start of the *ompF* genes. The termination codon, TAA, of each of the *asnS* genes is in bold followed shortly by the terminator hairpin which is underlined. OmpR binding sites are in bold with the OBS involved in repression of *ompF* transcription also underlined. The integration host factor binding region is double underlined. A | is used to show sites that are conserved between the two genes. Deletions are represented by a dashed line.


E. coli TGCTTACGTAACTGGCGTGCAAAACGTACGTGATGTGATTCCGTTCCCACGTACTCC
Serratia CGCCTATGTCACCGGCGTGCAAAACGTGCGCGATGTGATTCCATTCCCGCGCACGCC
GCGTAACGCCAGCTTCTAATTTAGCGTCTT----------
GCGTAACGCAAGCTTCTAATTTATACGATCACACGTTTTTTAAACGATTTCGTTACA
---CAAGAGCCAGCGCCCGCTGGCTCTTTTTTTATCTTTTCTGTCAAGTTATCTGTT
AAACAAAGGCCAGCATC-GCTGGCCTTT----------
GTTAAGGTCAAGCAATCTATTTGCAACCCCGCCATAACGATCATCCTGTTACGGAAT

ATTACATTGCAACATTTACGCGCAAAAACTAATCCGCATTCTTATTGCGGATTAGTT

TTTTCTTAGCTAATAGCACAATTTTCATACTATTTTTTGGCATTCTGGATGTCTGAA

AGAAGATTTTGTGCCAGGTCGATAAAGTTTCCATCAGAAACAAAATTTCCGTTTAGT

TAATTTAAATATAAGGAAATCATATAAATAGATTAAAATTGCTGTAAATATCATCAC
----------C
GTCTCTATGGAAATATGACGGTGTTCACAAAGTTCCTTAAATTTTACTTTTGGTTAC
GCATTTTTTAATAT-TGACGCACGTCACAAAGTTCCCGAAAAATTACATTTGGTTAC
ATATTTTTTCTTTTTGAAACCAAATCTTTATCTTTGTAGCACTTTCACGGTAGCGAA
ACATACTTTCCTTTTGCAACCTGATTGGGACATTGGTATCATTTTCGTCCTAGATTA
ACGTTAGTTTGAATGGAAAGATGCCTGCAGACACATAAAGACACCAAACTCTCATCA
ACCCGCCTGTGAATGGAACACTGCGTTCAGACACAGGACGACACCAATCTATCAACA

was predicted and is shown in Fig 3.18. Sites in the *S. marcescens micF* sequence that were not conserved with the *E. coli micF* would still allow hybridization with the *S. marcescens ompF*. Therefore, it is predicted that *micF* would function in regulating expression of the OmpF porin in *S. marcescens*.

Expression of the *S. marcescens* OmpF in *E. coli* was tested under high and low osmolarity and the amount of the cloned OmpF increased when the cells were grown in the high osmolarity condition (Fig. 3.19, lane 2). The OBSs present appear to function in *E. coli* since the cloned gene responds to osmolarity. The amount of *S. marcescens* OmpF porin would be expected to decrease in response to higher osmolarity in *E. coli*. The result obtained is not surprising as there are no -380 to -360 negative regulatory sites upstream of the *S. marcescens ompF* gene. The *micF* produced by the two organisms are homologous and we may expect the *E. coli micF* to reduce the amount of *S. marcescens* OmpF produced. This may not be observed because of the high copy number of the cloned *ompF*. *micF* may actually reduce the amount of *ompF* transcript, however, with the increased OmpR-P in higher osmolarity conditions, more *ompF* RNA may be produced and the reduction by *micF* may be masked.

The porin samples isolated from cells grown in different osmolarity were run on non-urea SDS-PAGE. Therefore the OmpF and OmpC of *E. coli* were not distinguished, but, in general there is less native *E. coli* porin present in the high salt condition. This is similar to the situation in Section 3.3 where the amounts of OmpF and OmpC are generally lower when the *S. marcescens* OmpC is produced. Once again the abundance of OBS due to the *S. marcescens ompF*

Fig. 3.18. Predicted hybridization of the *S. marcescens micF* transcript with the 5' end of the *S. marcescens ompF* mRNA.

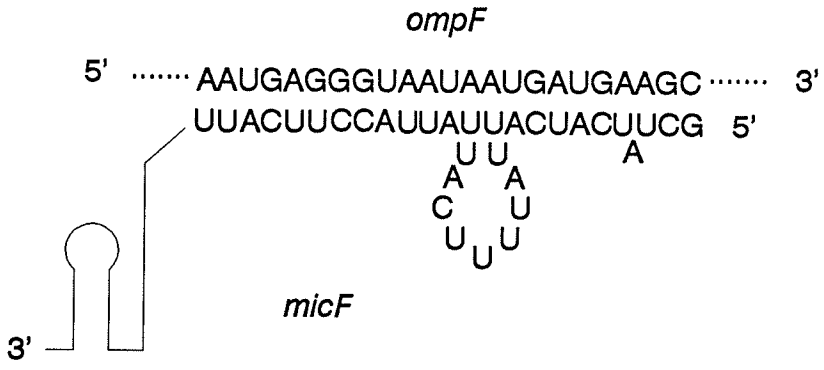
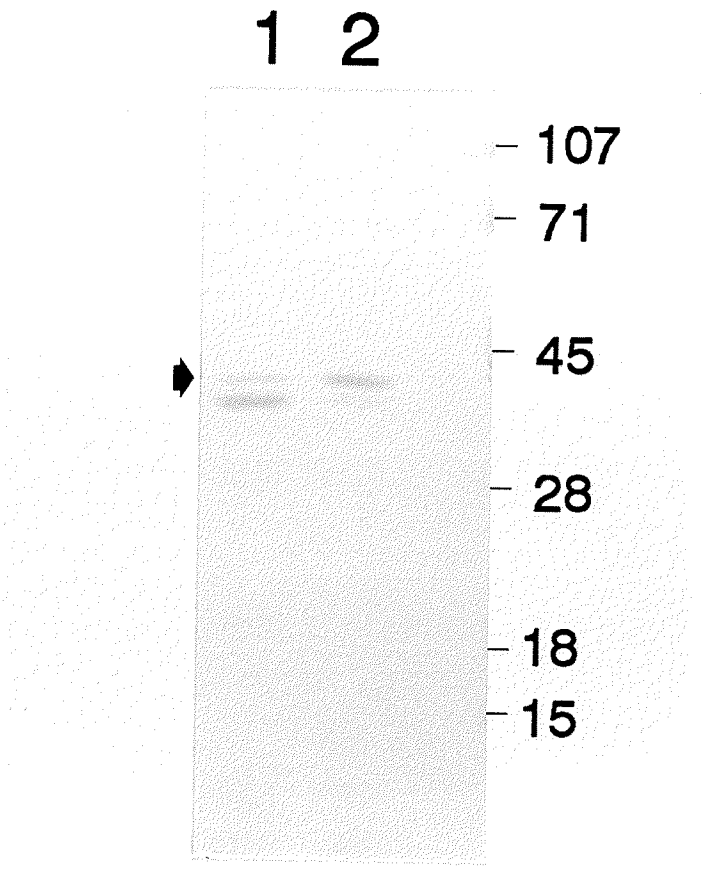

ompF
5'
AAUGAGGGUAAUAAUGAUGAAGC
3'
UUACUUCCAUUAUUACUACUUCG
5'
U U
A
A A
C U
U U U
micF
3'

Fig. 3.19. Osmoregulation of the *S. marcescens* OmpF porin. SDS-insoluble cell envelope fractions of cells grown in the absence or presence of NaCl were run on SDS-PAGE. Samples (approximately 3 µg per lane) were loaded after heating at 100°C for 10 min. Lane 1, *E. coli* NM522/pS1E(-), no NaCl; lane 2, *E. coli* NM522/pS1E(-), 0.3 M NaCl. The arrow indicates the cloned *S. marcescens* porin in the *E. coli* outer membrane while the band below it consists of the *E. coli* OmpF and OmpC porins.


1 2
107
71
45
28
18
15

could be depleting the free OmpR. In addition the *ompF* OBSs have a higher affinity for OmpR than *ompC* sites and therefore OmpR would preferentially bind to *ompF* OBS reducing the expected OmpC levels. A limit to the number of porins that could be accomodated in the outer membrane may also reduce the *E. coli* porin content when the cloned porin is overexpressed.

The observation of osmoregulation of OmpF in *S. marcescens*, which lacks a repressor OBS, supports the suggestion that *micF* plays a major role in osmoregulation (Ramani *et al.*, 1994). The possibility remains however, that the observed changes in expression of the OmpF porin may have occurred through an alternate negative-OBS. OmpR is required for efficient transcription of either porin gene regardless of medium osmolarity, but if *micF* can replace OmpR in osmoregulation function, what is the need for OmpR/EnvZ for this purpose in *E. coli*? *micF* decreases the amount of OmpF produced but has no effect on OmpC production. Increased *ompC* transcription in high osmolarity occurs through OmpR. *micF* is under the control of the OmpR/EnvZ system and its transcription increases in high osmolarity in concert with *ompC*. The OmpR/IHF-mediated loop mechanism of OmpF repression combined with *micF* may therefore enhance the response of decreased OmpF production. Furthermore, the sole use of *micF* to alter *ompF* expression uses more cell energy by its function at the translational level as compared to OmpR preventing synthesis of a transcript.

The amino acid sequence comparisons presented in this Section do not strongly indicate which *E. coli* porin the *S. marcescens* 41 kDa porin is analogous to. However, the upstream nucleic acid sequence and the location of the gene

suggest that it is an OmpF porin. The cloned porin does not decrease in high osmolarity when expressed in *E. coli*, but this can be explained by the absence of DNA that includes the site of negative regulation found in the *E. coli ompF* gene. The *ompF mRNA* is predicted to be able to hybridize with the *micF* transcript described in the previous chapter and thus be negatively regulated in conditions that activate *micF* transcription.

micF is involved in reducing the amount of *E. coli* OmpF in the outer membrane under a number of environmental conditions, including increased growth temperature, oxidative stress and exposure to certain antibiotics (Andersen *et al.*, 1989). The phenomenon of multiple antibiotic resistant strains of *E. coli* occurs through a complicated mechanism of global transcriptional regulation which includes elevated transcription of *micF* (Gambino *et al.*, 1993). A survey of the *mar* operon in a number of Gram negative bacteria demonstrated the presence of the operon in a variety of members of the *Enterobacteriaceae*, although not *S. marcescens* (Cohen *et al.*, 1993c). This does not mean that it is necessarily absent from *S. marcescens* since it is possible that the hybridization conditions were too stringent to detect the operon in a bacterium with some divergence from *E. coli*. Reduced levels of the 41 kDa porin have been observed in strains of *S. marcescens* with high levels of antibiotic resistance (Gutmann *et al.*, 1985). It is possible that the mechanism of reduction of this protein could occur by a mechanism that is at least similar to that observed for MAR strains.

3.5. Amino acid sequence alignments and comparisons of enterobacterial porins

The enterobacterial general diffusion porins are a homologous group of proteins with a reported average homology of 79% (Jeanteur *et al.*, 1991). The neisserial porins are less closely related to the enterobacterial porins, with approximately 30% average homology existing between the two groups. Together they form the bacterial porin superfamily. Sequence information is now available for two *S. marcescens* outer membrane porins. Sequence comparisons were performed to assess the relationship between the *S. marcescens* porins and other enterobacterial porins (Objective #3, Section 1.6).

The PALIGN program in the PC/GENE package (Intelligenetics) was used to obtain the percent identity between each porin sequence and the results are illustrated in Table 3.4. The anionic selective PhoE proteins form a cluster with identities ranging from 84.3 to 91.1% and the OmpC sequences of *E. coli*, *K. pneumoniae*, and *S. typhi* have identities ranging from 81.8 to 84.2%. The percent identity obtained between porins outside the two groups (the PhoE and OmpC porins form separate groups) range from 57.2 to 66.3%. The cloned *S. marcescens* OmpC porin has the highest similarity with the OmpC sequences; 68.6% with *S. typhi* OmpC, 70.3% with *E. coli* OmpC, and 71.3% with *K. pneumoniae*, although these values are intermediate to those found within a group of similar porins and those found outside that group. There is less conservation of sequence among the OmpF group of porins. The percent identity between the three OmpF porins range from 60.5 to 67.5%, which is

Table 3.4. Amino acid sequence comparisons between enterobacterial porins†

	SM OMPC	KPN OMPC	STY OMPC	ECO OMPC	SM OMPF	ECO OMPF	XEN OPNP	ECO PHOE	STY PHOE	CFR PHOE	KPN PHOE	ENT PHOE
SMOMPC	100	71.3	68.6	70.2	64.0	60.1	57.2	61.6	59.6	59.8	58.0	59.7
KPNOMPC	*	100	82.7	81.8	71.5	64.5	61.6	65.3	63.7	64.1	65.4	66.0
STYOMPC	*	*	100	84.2	70.3	65.9	61.1	64.8	63.0	64.1	64.6	65.9
ECOOMPC	*	*	*	100	68.4	64.9	60.7	64.1	61.0	62.6	62.1	63.3
SMOMPF	*	*	*	*	100	67.7	66.5	67.0	66.5	65.8	64.6	66.3
ECOOMPF	*	*	*	*	*	100	60.5	64.8	60.3	62.9	62.0	63.7
XENOPNP	*	*	*	*	*	*	100	61.4	58.6	60.6	60.0	60.0
ECOPHOE	*	*	*	*	*	*	*	100	88.6	90.9	84.3	87.4
STYPHOE	*	*	*	*	*	*	*	*	100	91.1	84.6	86.0
CFRPHOE	*	*	*	*	*	*	*	*	*	100	84.3	86.3
KPNPHOE	*	*	*	*	*	*	*	*	*	*	100	85.7
ENTPHOE	*	*	*	*	*	*	*	*	*	*	*	100

† Values are percentage identity between porins as determined by PALIGN.
Acronyms are as in Fig. 3.20.

similar to that found between different groups as stated above. That is, the percent identities obtained, when each OmpF porin is compared with each of the other porins, is roughly the same for all porin types tested.

The sequences were aligned using the CLUSTALV program and then manipulated manually for the best alignments using MASE (multiple aligned sequence editor) (Fig. 3.20). The alignments illustrate that there is a strong conservation between the *S. marcescens* OmpC and OmpF porins and all porins of the *Enterobacteriaceae* that have been sequenced thus far. This is not surprising since previous studies by Jeanteur *et al.* (1991), using sequence alignments demonstrated the similarities between enterobacterial porins. Also, we previously demonstrated the similarity of porins between *Serratia* spp. and other *Enterobacteriaceae* through Southern and Western analyses, N-terminal amino acid sequencing and porin functional analyses (Hutsul *et al.*, 1993). The majority of sequence variability occurs in clusters of which would be the external loop regions.

A distance tree was created from the multiple sequence alignments using PROTDIST (Dayhoff PAM matrix) and NEIGHBOR (neighbor-joining option) as described in Materials and Methods, Section 2.26. The confidence level was determined using 100 bootstrap replicates. The distance tree obtained (Fig. 3.21) generally supports the percentage identity results obtained from individual sequence alignments. The PhoE proteins cluster as a conserved group at node 9. Three OmpC proteins (ECOOMPC, KPNOMPC, and STYOMPC) cluster at node 2 while the *S. marcescens* OmpC is positioned more distant at node 3.

Fig. 3.20. Alignment of the *S. marcescens* OmpC and OmpF porins with the porins of the *Enterobacteriaceae*. The initial alignment was made using CLUSTALV and then completed manually for the best alignment. Asterisks and dots are used to indicate perfectly and well conserved amino acids, respectively. The PEFGGD motif is boxed. Cysteine residues in *S. marcescens* OmpF are circled. SMOMPC, *S. marcescens* OmpC porin; SMOMPF, *S. marcescens* OmpF; ECOOMPF, *E. coli* OmpF (Inokuchi *et al.*, 1982); ECOOMPC, *E. coli* OmpC (Mizuno *et al.*, 1983); KPNOMPC, *Klebsiella pneumoniae* OmpC (Albertí *et al.*, 1995); STYOMPC, *Salmonella typhi* OmpC (Puente *et al.*, 1989); STYPHOE, *Salmonella typhimurium* PhoE (Spierings *et al.*, 1992a); CFRPHOE, *Citrobacter freundii* PhoE (Spierings *et al.*, 1992b); KPNPHOE, *Klebsiella pneumoniae* PhoE, and ENTPHOE, *Enterobacter cloacae* PhoE (van der Ley *et al.*, 1987); ECOPHOE, *E. coli* PhoE (Overbeeke *et al.*, 1983); XENOPNP, *Xenorhabdus nematophilus* OpnP (Forst *et al.*, 1995).

```
SMOMPC     M-KLRVLSLMVPALLVAGTAGAAEIYNKDGNKLDLYGKVDGLHYFSSNN----- 48
ECOOMPC    M-KVKVLSLLVPALLVAGAANAAEVYNKDGNKLDLYGKVDGLHYFSDNK----- 48
STYOMPC    M-KVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLFGKVDGLHYFSDDK----- 48
KPNOMPC    M-KVKVLSLLVPALLVAGAANAAEIYNKDGNKLDLYGKIDGLHYFSDDK----- 48
SMOMPF     MMKRNILAVVIPALLAAGAANAAEIYNKDGNKLDLYGKVDGLHYFSKDK----- 49
ECOOMPF    MMKRNILAVIVPALLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKGNGENSY 54
XENOPNP    M-KRNILAVVIPALLVAGTANAAEIFNKDGNKLDLYGKVDVRHQFADKRSSE-- 51
ECOPHOE    M-KKSTLALVVMGIVASASVQAAEIYNKDGNKLDVYGKVKAMHYMSDNASK--- 50
CFRPHOE    M-KKSTLALVVMGITASASVQAAEVYNKNGNKLDLYGKVKAMHYMTDYDSK--- 50
KPNPHOE    M-KKSTLALMMMGFVASTATQAAEVYNKNANKLDVYGKIKAMHYFSDYDSK--- 50
ENTPHOE    M-KKSTLALVVMGVVASASVHAAEVYNKNGNKLDVYGKVKAMHYISDDDTK--- 50
STYPHOE    M-NKSTLAIVV-SIIASASVHAAEVYNKNGNKLDVYGKVKAMHYMSDYDSK--- 49
           * . ..*.... .  ..... ***..**..**.*..**    * ...

SMOMPC     GVDGDQSYMRFGLRGETQISDQLTGYGQWEYQANLNHAENQDNK-N-FTRYGFA 100
ECOOMPC    DVDGDQTYMRLGFKGETQVTDQLTGYGQWEYQIQGNSAENENN--S-WTRVAFA 99
STYOMPC    GSDGDQTYMRIGFKGETQVNDQLTGYGQWEYQIQGNQTEGSND--S-WTRVAFA 99
KPNOMPC    DVDGDQTYMRLGVKGETQINDQLTGYGQWEYNVQANNTESSSDQ-A-WTRLAFA 100
SMOMPF     GNDGDQTYVRFGFKGETQITDQLTGYGQWEYNVQSNHAESQGTE-GTKTRLGFA 102
ECOOMPF    GGNGDMTYARLGFKGETQINSDLTGYGQWEYNFQGNNSEGADAQTGNKTRLAFA 108
XENOPNP    --DGDDSYARIGIKGETQISDQLTGFGRWEYNVKAKGTEAAVAESS--TRLAFA 101
ECOPHOE    --DGDQSYIRFGFKGETQINDQLTGYGRWEAEFAGNKAESDTAQ--QKTRLAFA 100
CFRPHOE    --DGDQSYIRLGFKGETQINDELTGYGRWEAEFAGNKAESDSNQ--QKTRLAFA 100
KPNPHOE    --DGDQTYVRFGIKGETQINEDLTGYGRWESEFSGNKTESDSSQ--QKTRLAFA 100
ENTPHOE    --DGDQTYVRFGFKGETQINDQLTGYGRWEAEFAGNKAESDSSQ---KTRLAFA 99
STYPHOE    --DGDQSYVRFGFKGETQINDQLTGYGRWEAEFASNKAESDSSQ--QKTRLAFA 99
             .** .* *.* .****....***.*.** .   . .*.  .     ** .**

SMOMPC     GLKFGDYGSFDYGRNTGVLYDVAAYTDLQPEFDGMTYG-ADQFMFQRSSGLATY 153
ECOOMPC    GLKFQDVGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYG-SDNFMQQRGNGFATY 152
STYOMPC    GLKFADAGSFDYGRNYGVTYDVTSWTDVLPEFGGDTYG-ADNFMQQRGNGYATY 152
KPNOMPC    GLKFGDAGSFDYGRNYGVVYDVTSWTDVLPEFGGDTYG-SDNFLQSRANGVATY 153
SMOMPF     GLKFADYGSFDYGRNYGVLYDVEGWTDMLPEFGGDTYTYSDNFMTGRTNGVATY 156
ECOOMPF    GLKYADVGSFDYGRNYGVVYDALGYTDMLPEFGGDTAY-SDDFFVGRVGGVATY 161
XENOPNP    GLKFANYGSLDYGRNYRVNYDVNAWTDVLPIFGGDPMAQTDNFMTGRSTGLLTY 155
ECOPHOE    GLKYKDLGSFDYGRNLGALYDVEAWTDMFPEFGGDSSAQTDNFMTKRASGLATY 154
CFRPHOE    GSKLKNLGSFDYGRNLGALYDVEAWTDMFPEFGGDSSAQTDNFMTKRASGLATY 154
KPNPHOE    GVKLKNYGSFDYGRNLGALYDVEAWTDMFPEFGGDSSAQTDNFMTKRASGLATY 154
ENTPHOE    GLKLKDFGSLDYGRNLGALYDVEAWTDMFPEFGGDSSAQTDNFMTKRASGLATY 153
STYPHOE    GLKLKDIGSFDYGRNLGALYDVEAWTDMFPEFGGDSSAQTDNFMTKRASGLATY 153
           * *  . **.*****  . **. ..**. * *.* .   .*.*.  * .*  **

SMOMPC     RNNDFFGLVDGLNFALQYQGKNGN--GEE-----TNNGRDVLGQNGEGYGMSMS 200
ECOOMPC    RNTDFFGLVDGLNFAVQYQGKNGNPSGEGFTSGVTNNGRDALRQNGDGVGGSIT 206
STYOMPC    RNTDFFGLVDGLDFALQYQGKNGSVSGEN------TNGRSLLNQNGDGYGGSLT 200
KPNOMPC    RNSDFFGLVDGLNFALQYQGKNGSVSGEG----ATNNGRGALKQNGDGFGTSVT 203
SMOMPF     RNNNFFGLVDGLNFALQYQGKNQN-----------DGRDVKKQNGDGWGISST 198
ECOOMPF    RNSNFFGLVDGLNFAVQYLGKNER----------DTARRSNGDGVGGSIS 201
XENOPNP    RNTDFFGLVDGLNFRLQYQGQNSDRT--------KNKGRDTERSNGDGYGLSST 201
ECOPHOE    RNTDFFGVIDGLNLTLQYQGKNEN----------RDVKKQNGDGFGTSLT 194
CFRPHOE    RNTDFFGVVDGLDLTLQYQGKNQD----------RDVKKQNGDGFGTSVT 194
KPNPHOE    RNTDFFGLVDGLDLTLQYQGKNEG----------REAKKQNGDGVGTSLS 194
ENTPHOE    RNTDFFGAIDGLDMTLQYQGKNEN----------RDAKKQNGDGFGTSLT 193
STYPHOE    RNTDFFGIVDGLDLTLQYQGKNED----------RDVKKQNGDGFGTSVS 193
           **..*** .***.. .** *.*                .    **.* * * .
```

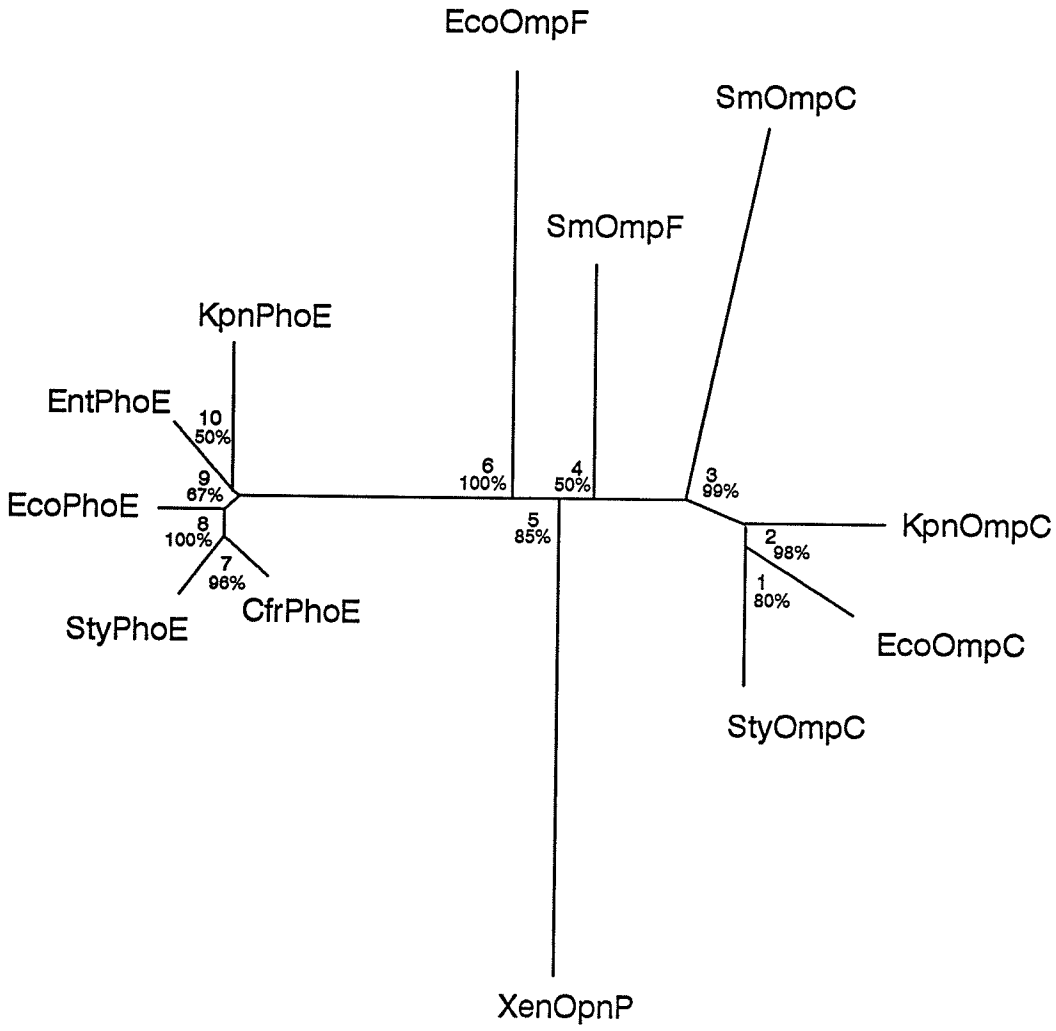
```
SMOMPC     YDMGYG-ISAAGAFFNSRRTSEQNGANGHQNIMGRGDKAEGYSGGLKYDANDVY 253
ECOOMPC    YDY-EG-FGIGGAISSSKRTDAQNTAA----YIGNGDRAETYTGGLKYDANNIY 254
STYOMPC    YAIGEG-FSVGGAITTSKRTADQNNTA-NARLYGNGDRATVYTGGLKYDANNIY 252
KPNOMPC    YDIFDG-ISAGFAYANSKRTDDQNQL-----LLGEGDHAETYTGGLKYDANNIY 251
SMOMPF     YDIGEG-VSFGAAYASSNRTDDQKLRSNE-----RGDKADAWTVGAKYDANNVY 246
ECOOMPF    YEY-EG-FGIVGAYGAADRTNLQEAQPL-----GNGKKAEQWATGLKYDANNIY 248
XENOPNP    YDVGYG-ITVGGSYANSARTADQKEKVSD----AYGKRAEAWNIGAKYDANNVY 250
ECOPHOE    YDFGGSDFAISGAYTNSDRTNEQNLQSR-----GTGKRAEAWATGLKYDANNIY 243
CFRPHOE    YDFGGSDFAVSGAYTNSDRTNQQNLQTR-----GTGDKAEAWATGLKYDANDIY 243
KPNPHOE    YDFGGTDFAVSAAYTSSDRTNDQNLLAR-----AQGSKAEAWATGLKYDANNIY 243
ENTPHOE    YDFGGTDFAVSGAYTNSDRTNAQNLLAR-----AQGQKAEAWATGLKYDANDIY 242
STYPHOE    YDFGGSDFAVSGAYTLSDRTREQNLQRR-----GTGDKAEAWATGVKYDANDIY 242
           *.      .   .   . **  *.           * .*. .. * *****..*

SMOMPC     LAVMFTQSYNAARFGSSDSS----VY----GYANKAQSFEAYAHYQF-DFGLRP 298
ECOOMPC    LAAQYTQTYNATRVG---------SL----GWANKAQNFEAVAQYQF-DFGLRP 294
STYOMPC    LAAQYSQTYNATRFGTSNGSNPSTSY----GFANKAQNFEVVAQYQF-DFGLRP 301
KPNOMPC    LATQYTQTYNATRAG---------SL----GFANKAQNFEVAAQYQF-DFGLRP 291
SMOMPF     LAAMYAETRNMTPFGGGNFTNTCAATENCGGFASKTQNFEVTAQYQF-DFGLRP 299
ECOOMPF    LAANYGETRNATPITNK-FTNTS-------GFANKTQDVLLVAQYQF-DFGLRP 293
XENOPNP    LAAMYGETRNMTRYTR-----TIADT-DATLIANKTQNIELTAQYLFSDLGLKP 298
ECOPHOE    LATFYSETRKMTPIT--------------GGFANKTQNFEAVAQYQF-DFGLRP 282
CFRPHOE    IATFYSETRNMTPIS--------------GGFANKTQNFEAVVQYQF-DFGLRP 282
KPNPHOE    LATMYSETRKMTPIS--------------GGFANKAQNFEAVAQYQF-DFGLRP 282
ENTPHOE    LAAMYSETRNMTPIS--------------GGFANKAQNFEVVAQYQF-DFGLRP 281
STYPHOE    IATFYSETRNMTPVS--------------GGFANKTQNFEAVIQYQF-DFGLRP 281
           .*. .... . .. .                  *.*.*.     .* * *.**.*

SMOMPC     FVGYNQTKGKDLGRAGNGKDYGDQDLVKFVDLGATYFFNKNMSTYVDYKINLVD 352
ECOOMPC    SLAYLQSKGKNLGR-GYD----DEDILKYVDVGATYYFNKNMSTYVDYKINLLD 343
STYOMPC    SVAYLQSKGKDISN-GYGASYGDQDIVKYVDVGATYYFNKNMSTYVDYKINLLD 354
KPNOMPC    SVAYLQSKGKDLN--GYG----DQDILKYVDVGATYYFNKNMSTYVDYKINLLD 339
SMOMPF     EVSYLQSKGKNLNVPGVGS---DQDLVKYVSVGTTYYFNKNMSTYVDYKINLLD 350
ECOOMPF    SIAYTKSKAKDVE--GIG----DVDLVNYFEVGATYYFNKNMSTYVDYIINQID 341
XENOPNP    SLAYVQSKGKDLTE-GKGF---NGDLVKYVSVGTYYYFNKNLSTYVDYKINLLK 348
ECOPHOE    SLGYVLSKGKDIE--GIG----DEDLVNYIDVGATYYFNKNMSAFVDYKINQLD 330
CFRPHOE    SLGYVLSKGKDIE--GVG----NEDLVNYIDVGATYYFNKNMSAFVDYKINQLD 330
KPNPHOE    SLGYVLSKGKDIE--GVG----SEDLVNYIDVGLTYYFNKNMNAFVDYKINQLK 330
ENTPHOE    SLGYVQSKGKDNE--GIG----DEDLVKYIDVGATYYFNKNMSAFVDYKINQID 329
STYPHOE    SLGYVLSKGKDIE--GVG----SEDLVNYIDVGATYYFNKNMSAFVDYKINQLD 329
            ..*  .*.*. .  *      . *.... ..*  *.****....*** ** ..

SMOMPC     NNDFTDAAGINTDNVVAVGLVYQF 376
ECOOMPC    DNQFTRDAGINTDNIVALGLVYQF 367
STYOMPC    KNDFTRDAGINTDDIVALGLVYQF 378
KPNOMPC    DNSFTRSAGISTDDVVALGLVYQF 363
SMOMPF     DNDFTKATGIATDDIVGVGLVYQF 374
ECOOMPF    SDN---KLGVGSDDTVAVGIVYQF 362
XENOPNP    KDN---ELGVNARNVFGVGLTYQF 369
ECOPHOE    SDN---KLNINNDDIVAVGMTYQF 351
CFRPHOE    SDN---KLGINNDDIVAVGMVYQF 351
KPNPHOE    SDN---KLGINDDDIVALGMTYQF 351
ENTPHOE    DDN---KLGVSSDDIVAVGMTYQF 350
STYPHOE    SDN---TLGINDDDIVAIGLTYQF 350
           ..      .... .. ..*..***
```

Fig. 3.21. Unrooted distance tree based on alignments of 12 enterobacterial porins constructed by the neighbor joining method. The numbers at the branches represent the nodes. The percentages below the node numbers represent the number of times out of 100 replicates that a given branch appeared among the 100 bootstrap replicates. See Fig. 3.20 for acronyms.


EcoOmpF
SmOmpC
SmOmpF
KpnPhoE
EntPhoE
10
50%
9
67%
EcoPhoE
8
100%
7
96%
StyPhoE
CfrPhoE
6
100%
4
50%
5
85%
3
99%
2
98%
1
80%
KpnOmpC
EcoOmpC
StyOmpC
XenOpnP

However, the *S. marcescens* OmpC still appears to be closer to the other OmpCs than to OmpFs or PhoEs. The OmpF porins appear to form a more diverse group. The OmpF porins (nodes 4, 5, and 6) do not form a distinct cluster and are distant from all of the other porins. They are positioned slightly closer to other OmpFs than to OmpCs or PhoEs and are at a relatively equal distance between OmpCs and PhoEs. This divergence may reflect the fact that the OmpF porin sequences are derived from distantly related species within the *Enterobacteriaceae* family. The *S. marcescens* OmpF was placed closer to the other OmpFs than the other groups, supporting its classification as an OmpF porin despite a slightly higher percent identity with OmpCs (Table 3.4).

Although considerable similarity has been observed between the *S. marcescens* porins and those from the *Enterobacteriaceae*, a change in a conserved region of the third external loop of the *S. marcescens* OmpC protein in the sequence alignments was detected. This loop folds down into the pore and serves to constrict the pore size with its bulkiness. At the tip of the loop is a turn formed by the PEFXG motif. The PEFXG motif is seen in all enterobacterial and neisserial porins (Jeanteur *et al.*, 1991) with X = glycine (G) in those of enterobacterial origin and X = alanine (A) or serine (S) in neisserial porins. The G-G sequence of enterobacterial porins is thought to be conserved because of the unique torsion angles it forms which would aid in the turn formation of the PEFXG region (Cowan *et al.*, 1992). In the *S. marcescens* OmpC the X is an aspartate (D) not a G, as normally found with the other enterobacterial porins. Aspartic acid is also turn promoting (Paul & Rosenbusch, 1985) which therefore

may not be expected to disrupt the function of the PEFXG motif. On the other hand, an *E. coli* OmpF porin with the sequence PEFDG displayed reduced permeability through the porin (Jeanteur *et al.*, 1994). X-ray crystallography of the mutant OmpF porin showed that the aspartic acid side-chain protruded into the pore lumen. The motif is followed by an aspartic acid residue in enterobacterial porins and a hydrophobic phenylalanine residue in neisserial porins (Jeanteur *et al.*, 1991). Again the *S. marcescens* OmpC porin differs from the enterobacterial porins as the motif is followed by the hydrophobic residue, methionine. We do not intend to suggest that the *S. marcescens* 40 kDa porin is more similar to neisserial porins (the average identity is 37%) than to enterobacterial porins, but the changes seen in this conserved motif may be a reflection of the divergence this porin has taken from other OmpC porins. In addition, these changes may indicate a possible difference in the pore characteristic of the *S. marcescens* porin.

The third external loop is important because it is responsible for the size constriction of porin pores by folding down into the pore (Cowan *et al.*, 1992). It is also the region where a lysine residue responsible for the anion specificity of PhoE porins is located (Bauer *et al.*, 1989). This can be seen in Fig. 3.20 at position 146 (numbering relative to *E. coli* PhoE) in bold. At this location in the OmpF porins a glycine replaces the lysine residue. In OmpC proteins the site is occupied by glutamine except in *K. pneumoniae* where it is replaced by serine. From the PhoE structure, determined by X-ray crystallography, the lysine side-chain was shown to protrude into the pore lumen. Therefore, the glutamine (or

serine) side chain may also extend into the pore channel conferring a smaller channel in OmpC porins in comparison to OmpF which has a glycine.

An interesting feature seen in the *S. marcescens* OmpF porin is the presence of two cysteine residues. Cysteine is not present in any of the other porins presented. The cysteines would be present in the sixth external loop of the porin assuming it has the same structure as the *E. coli* OmpF porin (Cowan *et al.*, 1992). Cysteines present in the *E. coli* LamB and the *Ps. aeruginosa* OprB porins are not predicted to play any functional role (Ferenci & Stretton, 1989; Wylie & Worobec, 1994). Four cysteines present in the *Ps. aeruginosa* OprF porin have been proposed to have a functional role although this has not been investigated (Hancock, 1987). The above three porins are structurally and functionally distinct from the classical enterobacterial porins, including the *S. marcescens* OmpF. It will be interesting to see if altering the cysteines in the *S. marcescens* OmpF porin have an effect on pore characteristics.

In summary, it has been demonstrated that the *S. marcescens* OmpC porin is more similar to other enteric OmpCs than it is to OmpF or PhoEs. However, it is less well conserved than the other OmpCs within the OmpC group. The *S. marcescens* OmpF porin is relatively distant from all the porins. A comparison of identities suggests a slightly higher likeness to OmpCs while the distance matrix places the the OmpF closer to other OmpFs. However, the other two OmpFs are also placed distantly from all porins and it appears the OmpFs may not be as well a conserved group.

3.6. Analysis of site-specific *S. marcescens* OmpC mutants

The results presented in this thesis thus far have demonstrated that *S. marcescens* UOC-51 produces two constitutive porins regulated by external osmolarity. The two UOC-51 porin genes were cloned and characterized. The identified porins were shown to be analogous to the *E. coli* OmpF and OmpC porins based on amino acid sequence comparisons and DNA sequence organization and similarity in the regulatory regions. To set the groundwork for future examination into the role of porins in the β-lactam resistance observed in *S. marcescens*, the relative permeabilities of the two porins were examined (Objective #4, Section 1.6). In addition, specific amino acids were altered by site-directed mutagenesis to analyze their role in determining pore characteristics (Objective #5).

From the alignments of enterobacterial porins in Section 3.5 two sites within a key region were targeted for site-directed mutagenesis. The PEFGG(D) motif is conserved in enterobacterial porins and forms a turn at the tip of the third external loop (Cowan *et al.*, 1992; Jeanteur *et al.*, 1991). The PEFGGD motif was not conserved in the *S.marcescens* OmpC porin being replaced with the sequence, PEFD^{112}GM^{114}. Therefore, D^{112} and M^{114} were selected for individual alteration to glycine (G) and aspartic acid (D), respectively, to reflect the enterobacterial consensus sequence and determine the effect of these changes on porin permeability.

For mutagenesis experiments a 0.5 kb *Pst*I/*Kpn*I fragment was subcloned into KS(-), creating the plasmid p8PK(-), to minimize the amount of sequencing

needed after site-directed mutagenesis. After confirmation of the single amino acid change with no other sites altered, the *Pst*I/*Kpn*I fragment from pM8BH12(+) was replaced with the mutant *Pst*I/*Kpn*I fragment (Fig. 3.22). pM8BH12(+) was created by deleting approximately 4.1 kb from the *Hind*III end of pM8BH(+) with Promega's Erase-a-Base kit. This allowed the removal of a *Pst*I site which would have interfered with subcloning of the *Pst*I/*Kpn*I fragment. Once again the modification was confirmed by sequence analysis.

Fig. 3.23 illustrates the nucleic acid changes and consequent amino acid change in each of the two mutants. Wild-type *ompC* DNA sequence of the region is shown in panel A. The change from D^{112} to G was obtained by changing the sequence GAC to GGC and is shown in Fig. 3.23 panel B. The $M^{114}D$ alteration was obtained by changing the codon ATG to GAC as seen in Fig. 3.23 panel C. Once mutants were confirmed and subcloned they were transformed into the *E. coli* porin deficient strain, BZB1107 (Jeanteur *et al.*, 1994). BZB1107 was constructed by transposon insertion into the *ompF* gene of an *E. coli* B strain, which naturally only produces the OmpF porin. The SDS-insoluble fraction isolated from cell envelopes when analyzed by SDS-PAGE showed that the mutant porins were produced and were the only porins present in the outer membrane (Fig. 3.24). The *E. coli* BZB1107 strain itself did not produce any porins visible by SDS-PAGE in the size range of the *E. coli* OmpF or *S. marcescens* OmpC porins (38 to 40 kDa) (lane 1). A peptide of ~25 kDa is observed, however, it is not seen in any of the strains producing the recombinant porins. It is possible that in response to a low outer membrane permeability from

Fig. 3.22. Construction of site-directed mutants of the *S. marcescens ompC* gene. The dashed lines indicate the region that was deleted by Erase-a-Base procedure creating the pM8BH12(+) clone (solid line). The coding region of the *ompC* gene is shown as a thick line. The mutation was created using p8PK(-). The wild-type (wt) *Pst*I/*Kpn*I fragment was removed and replaced with the mutated (m) fragment from p8PK(-). The asterisk represents a DNA sequence alteration.

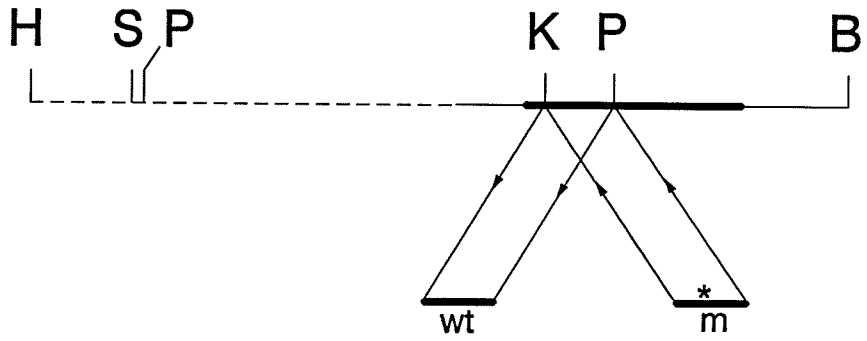
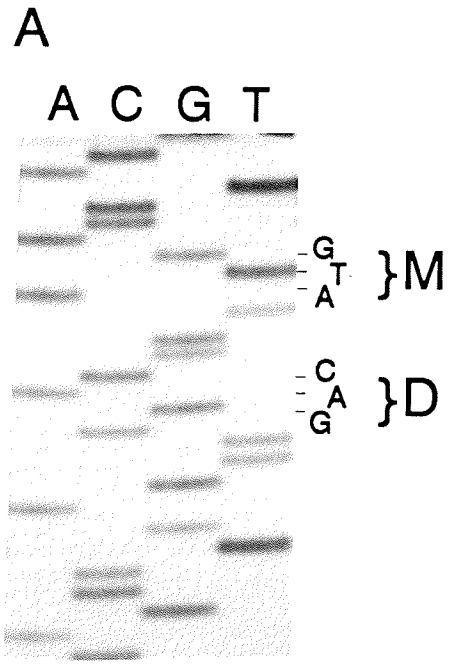
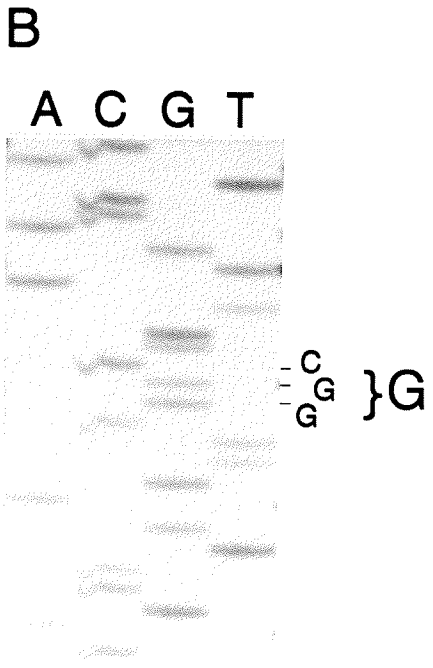

H
S
P
K
P
B
wt
*
m

Fig. 3.23. Site-directed sequence alterations of the *S. marcescens ompC* gene. A, wild-type *ompC* sequence in the PEFDGM region illustrating the Asp (GAC) and Met (ATG) codons. B, sequence alteration GAC to GGC constructing Asp to Gly mutation. C, sequence alteration ATG to GAC constructing Met to Asp mutation.


A
A C G T
G
T
A
}M
C
A
G
}D


B
A C G T
C
G
G
}G

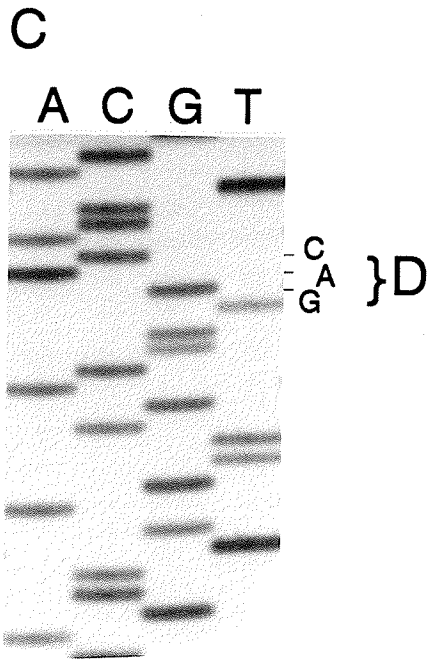
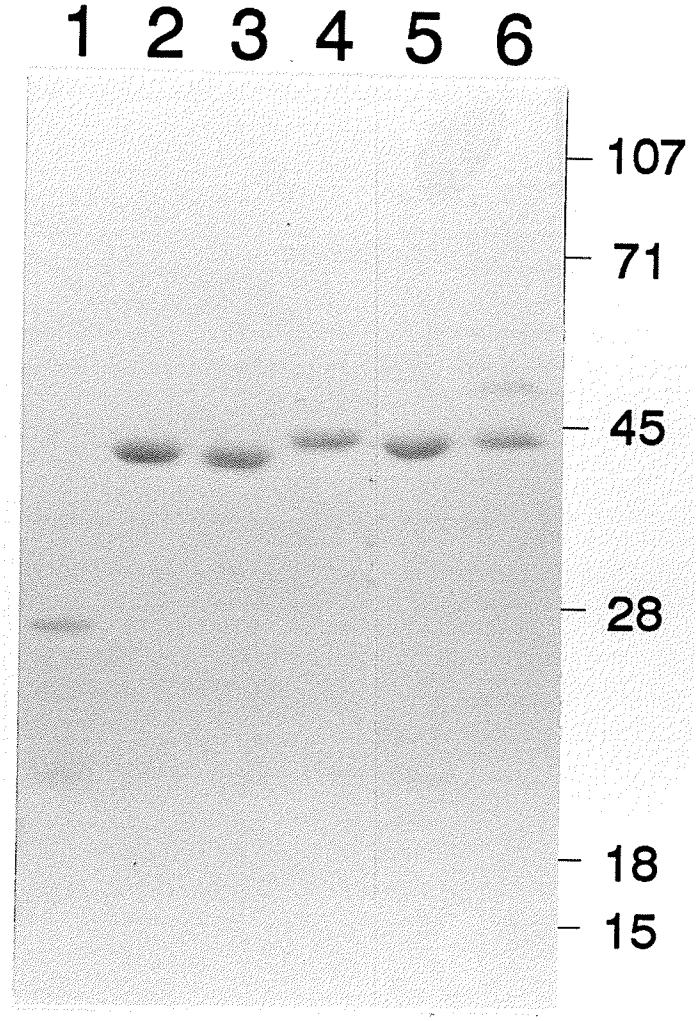

C
A C G T
C
A
G
}D

Fig. 3.24. Expression of *S. marcescens* wild-type and altered porins in the porin deficient strain of *E. coli*, BZB1107. A 5 µg sample of each SDS-insoluble fraction containing porin was heated at 100°C for 10 min before loading on SDS-PAGE. Lane 1, *E. coli* BZB1107; lane 2, *S. marcescens* OmpC; lane 3, D112G; lane 4, M114D; lane 5, *S. marcescens* OmpF; lane 6, purified porins from *S. marcescens* outer membrane.

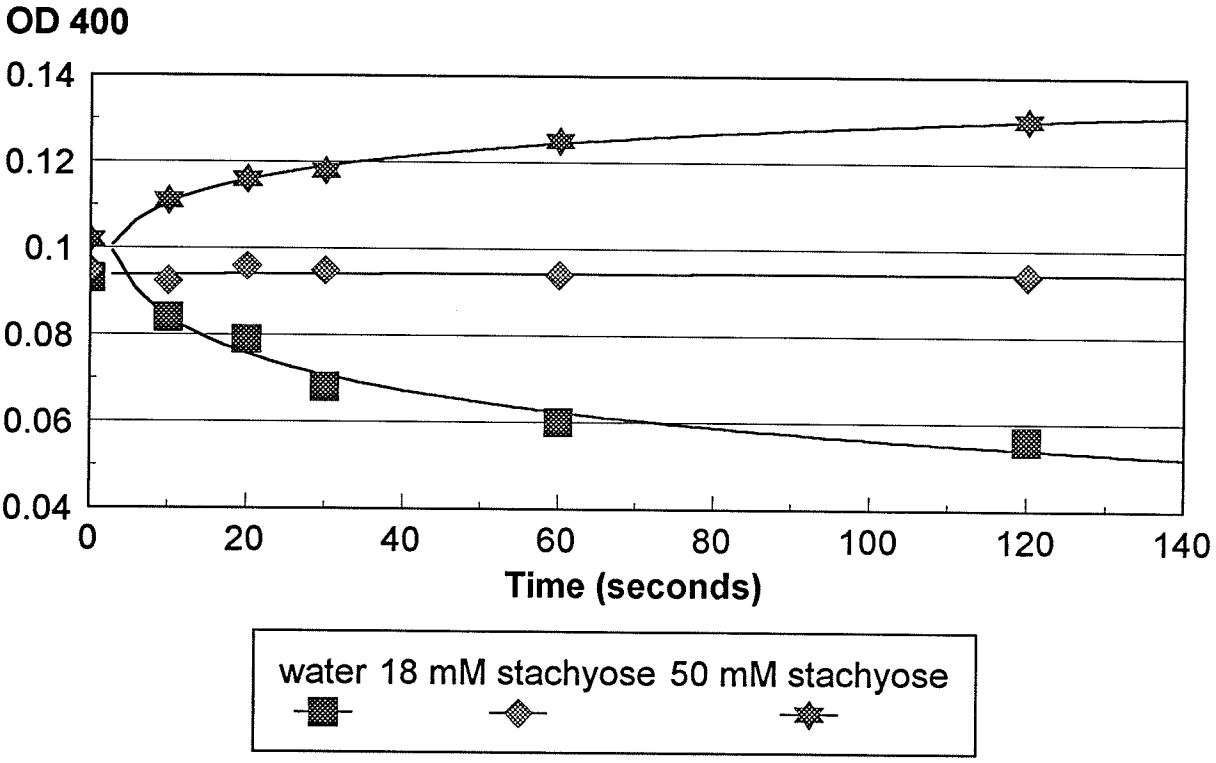

1 2 3 4 5 6
107
71
45
28
18
15

a lack of porins that another repressed porin was derepressed (Hancock, 1984).

The relative permeability of wild-type and constructed mutant porins was tested using the liposome swelling assay (Nikaido & Rosenberg, 1983). Fig. 3.25 is presented as an example of how the assay works. Liposomes with porin are prepared with an impermeable or very slowly permeable solute inside, in this case stachyose. When proteoliposomes are diluted in a hypotonic solution (water) a net flow of water moves into the liposomes to dilute the solute within. As this occurs the liposomes swell diffracting less light and a decrease in optical density will be recorded. If the same proteoliposomes are diluted into a hypertonic solution (50 mM stachyose) a net flow of water moves out of the liposome and the liposomes shrink further. The shrunken proteoliposomes diffract more light and an increase in optical density will be recorded. There will be no net flow of water into or out of the proteoliposome if the test solution is isotonic (18 mM stachyose) to the solution inside the liposomes and consequently no change in optical density will be recorded. To measure liposome swelling due to the permeation of solutes through the porins, and not initial water movement, the test solution must be iso-osmotic to the solute inside the liposome.

In keeping with our interest in the role of porins in *S. marcescens* β-lactam resistance, the permeability of three cephalosporin antibiotics as well as, glucose were tested. Porins isolated from the SDS-insoluble fraction of various cell envelopes were reconstituted into liposomes and the rates of swelling in iso-osmotic solutions of the test molecules were measured. Glucose, a common

Fig. 3.25. Dilution of proteoliposomes in hyper-, hypo-, and iso-tonic solutions. Optical density at 400 nm was recorded and plotted against time.


OD 400
0.14
0.12
0.1
0.08
0.06
0.04
0
20
40
60
80
100
120
140
Time (seconds)
water
18 mM stachyose
50 mM stachyose

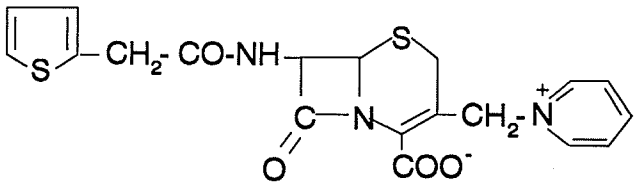
nutrient, is a relatively small non-ionic molecule, with a molecular mass of 180 Da. In comparison, most β-lactam molecules are larger and the zwitterionic cephalosporin, cephaloridine (Mr = 415.5 Da) was chosen as a non-ionic representative. Many cephalosporins used in therapy are anionic and the *E. coli* OmpF and OmpC porins are known to be cation selective, therefore the anionic cephalosporins, cephalothin (M_r, 418.4 Da) and cefotaxime (M_r, 477.4 Da) were also tested. Structures of the cephalosporins used are provided in Fig. 3.26. Results obtained for each of the porins tested in 18 mM glucose are presented in Fig. 3.27. The fastest rate of optical density decrease, or liposome swelling, occurred with liposomes containing the *S. marcescens* OmpF porin followed closely by the $D^{112}G$ constructed porin. Liposome swelling of proteoliposomes reconstituted with the *S. marcescens* OmpC porin occurred at a slower rate than the OmpF porin. The rate of swelling from the $M^{114}D$ porin was the same as the wild type OmpC porin.

Results of the liposome swelling experiments in 18 mM cephaloridine for the different porins were similar to glucose (Fig. 3.28). OmpF had the fastest rate of swelling, whereas OmpC and $M^{114}D$ had the slowest rate of change. The $D^{112}G$ porin had a greater rate of swelling compared to the wild-type OmpC porin, but the increase in swelling rate was not as great as that observed when glucose was used (Fig. 3.27). In comparison to glucose, diffusion of cephaloridine into liposomes occurred at an overall lower rate (compare Fig. 3.27 and 3.28).

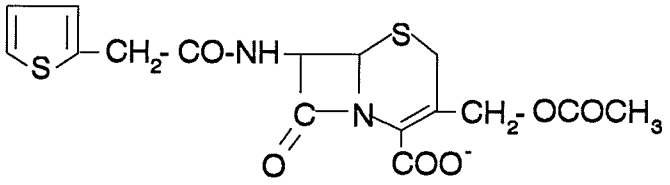
For both anionic cephalosporins tested, cephalothin (Fig. 3.29) and cefotaxime (Fig. 3.30), the rate of swelling of all proteoliposomes occurred very

Fig. 3.26. Molecular structures of cephalosporin antibiotics used in liposome swelling experiments. A, cephaloridine; B, cephalothin; C, cefotaxime.

A


S
CH2- CO-NH
S
C
N
CH2- N+
O
COO-

B


S
CH2- CO-NH
S
C
N
CH2- OCOCH3
O
COO-

C

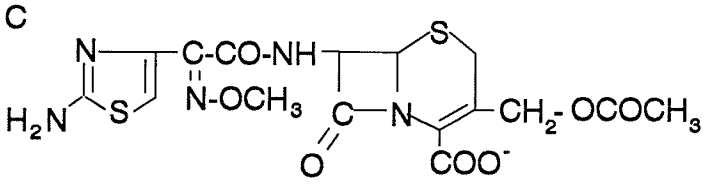

N
C-CO-NH
S
N-OCH3
H2N
S
C
N
CH2- OCOCH3
O
COO-

Fig. 3.27. Liposome swelling of wild-type and mutant proteoliposomes in 18 mM glucose. The change in optical density is recorded as a measure of time.

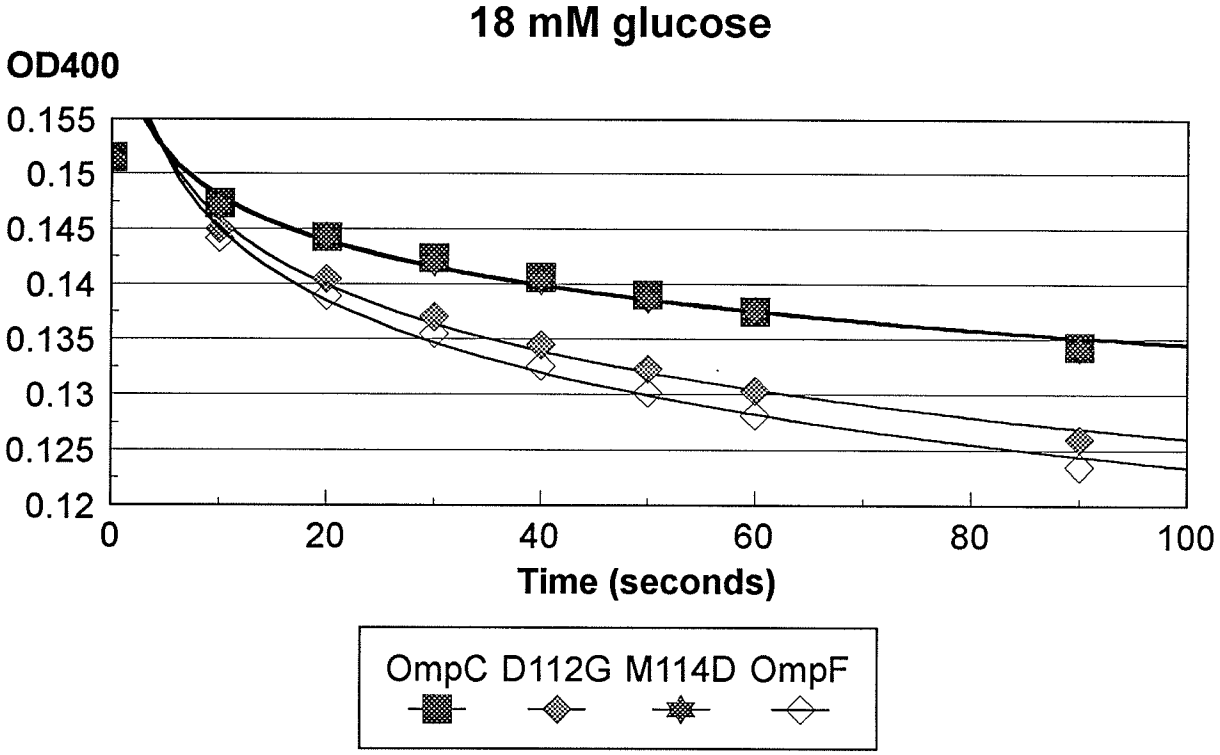

18 mM glucose
OD400
0.155
0.15
0.145
0.14
0.135
0.13
0.125
0.12
0
20
40
60
80
100
Time (seconds)
OmpC D112G M114D OmpF

Fig. 3.28. Liposome swelling of wildtype and mutant porin proteoliposomes in 18 mM cephaloridine.

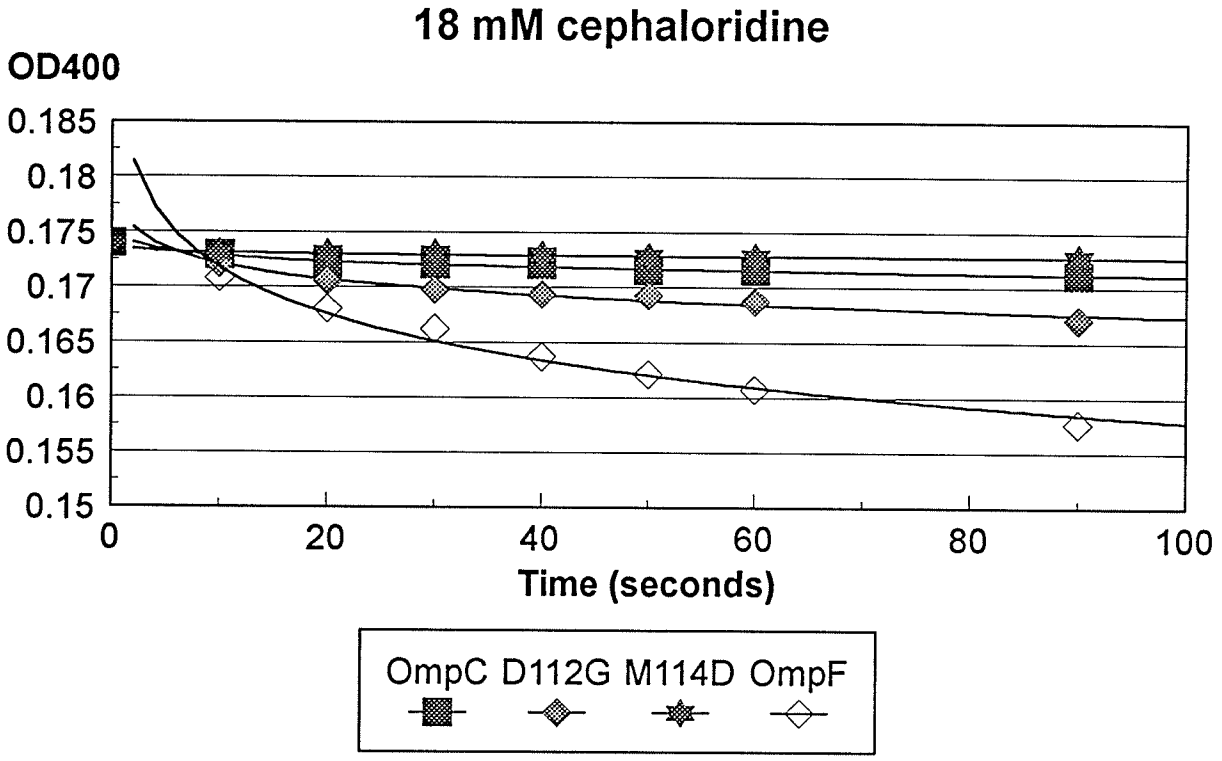

18 mM cephaloridine
OD400
0.185
0.18
0.175
0.17
0.165
0.16
0.155
0.15
0
20
40
60
80
100
Time (seconds)
OmpC D112G M114D OmpF

Fig. 3.29. Liposome swelling of wild-type and mutant porin proteoliposomes in 9 mM cephalothin.

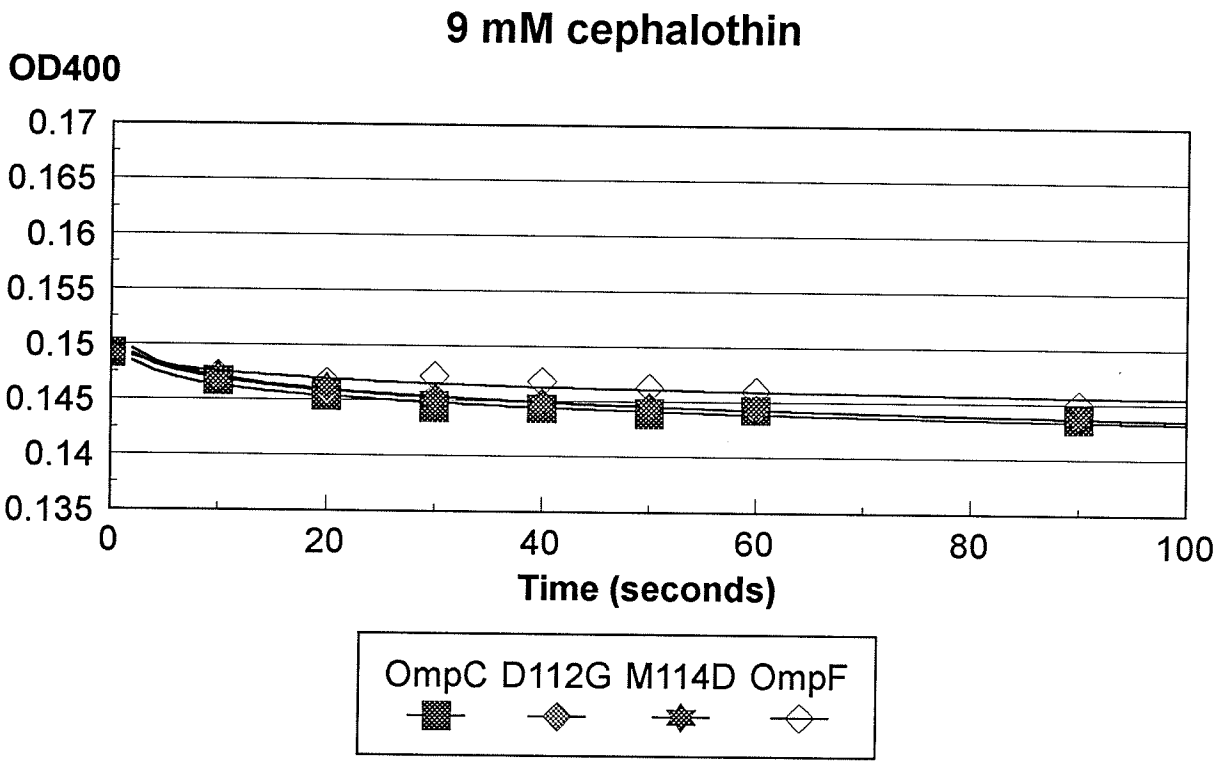
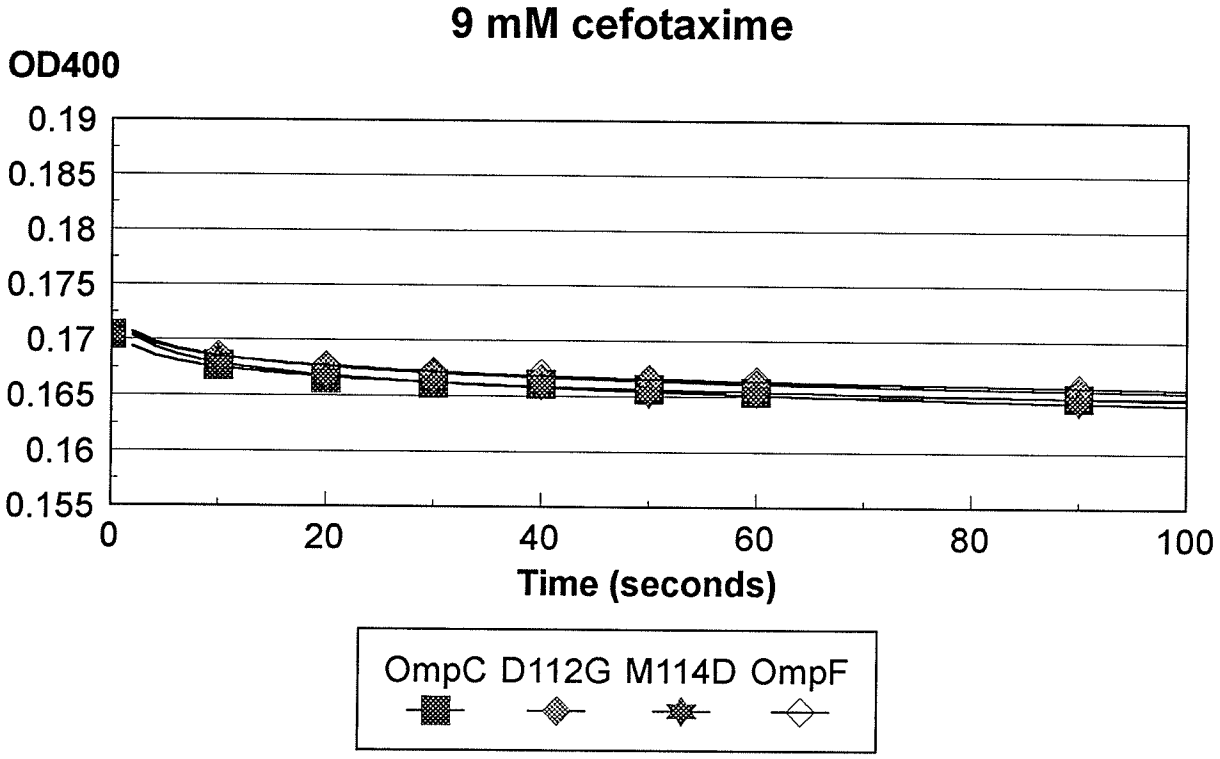

9 mM cephalothin
OD400
0.17
0.165
0.16
0.155
0.15
0.145
0.14
0.135
0
20
40
60
80
100
Time (seconds)
OmpC D112G M114D OmpF

Fig. 3.30. Liposome swelling of wild-type and mutant porin proteolipsomes in 9 mM cefotaxime.


9 mM cefotaxime
OD400
0.19
0.185
0.18
0.175
0.17
0.165
0.16
0.155
0
20
40
60
80
100
Time (seconds)
OmpC D112G M114D OmpF

slowly. There were no significant differences seen among the four porins tested.

From the results of liposome swelling experiments it can be seen that the *S. marcescens* OmpF porin allows more rapid diffusion of uncharged solutes than does the OmpC porin. Diffusion of the larger uncharged solute, cephaloridine, occurred more slowly than glucose in all porins tested and the difference in rate of cephaloridine diffusion by the OmpF and OmpC porins was greater than observed with glucose. The diffusion of charged solutes, cephalothin and cefotaxime, occurred more slowly and no differences between porins were detected. These results are similar to liposome swelling experiments using *E. coli* OmpC and OmpF porins reported by Nikaido *et al.* (1983).

The change at residue 112 of the *S. marcescens* OmpC porin from aspartic acid to glycine allowed a more rapid diffusion of glucose, with the rate coming close to OmpF. This rate difference was not as significant when the larger solute, cephaloridine, was tested and no difference was detected in liposome swelling experiments with charged solutes. This suggested that amino acid at position 112 may play an important role in pore constriction, but not ionic selection. Fourel *et al.*, (1993) isolated a colicin resistant OmpF mutant of *E. coli* with an amino acid change at G^{114} to D (this is equivalent to 112 in the *S. marcescens* OmpC porin). This mutant lost the ability to bind colicin N and was less stable in the membrane since trimers dissociated at a lower temperature. More recently it was demonstrated that this pore also had reduced permeability using liposome swelling and single channel conductance experiments (Jeanteur *et al.*, 1994). In addition, the mutant porin was crystallized and it was determined

that the aspartic acid side chain protrudes into the pore lumen constricting the pore.

In our case, D^{112} appears to be important in permeability of small hydrophilic molecules since a larger difference between D^{112} G and OmpC was seen when glucose versus the larger cephaloridine was tested. It is possible that the amino acid at position 112, or others in the constriction zone, confers the first line of size selectivity. In the case of large uncharged solutes the site will slow passage but is less critical since other amino acids of the pore channel wall will impede passage.

The *S. marcescens* OmpC porin diverges from the PEFGGD motif of enterobacterial porins as the aspartic acid at position 114 is replaced with methionine. It may be predicted that such a dramatic change in a residue in the third loop, and close to D^{112}, may affect porin function, possibly by affecting the confirmation of the third loop. The $M^{114}D$ porin demonstrated no change from OmpC in liposome swelling experiments, suggesting that the substitution of aspartic acid for methionine in the *S. marcescens* OmpC porin plays no role in affecting pore size selectivity. However, considering the effect $D^{112}G$ had on pore characteristics it is likely that any effect $M^{114}D$ may have in pore constriction would be masked by the aspartic acid at position 112. In order to confidently say that M^{114} has no effect on pore constriction a double mutant with both $D^{112}G$ and $M^{114}D$ should be constructed and evaluated.

This study, as well as that of Jeanteur *et al.* (1994), demonstrated the importance of an aspartic acid in replacing glycine-112 in determining pore size

constriction. The *S. marcescens* OmpC porin can be predicted to have lower permeability than other OmpCs provided that other sequence differences haven't altered the porin permeability. A functional comparison of the *E. coli* and *S. marcescens* OmpCs was not performed to confirm this. The sequence of the third loop was determined in one other *S. marcescens* isolate and was also found to have the sequence PEFDGM (data not shown) suggesting that the UOC-51 OmpC porin is not a mutant and the sequence alteration is probably common to *S. marcescens*.

Reduced permeability through OmpC may contribute to an overall lower outer membrane permeability in *S. marcescens*, but porin function in the outer membrane is probably more complex than that. OmpF, with a larger pore size, may mask a reduction in permeability by OmpC leading to no detectable difference in membrane permeability. In the human host, with higher temperature and osmolarity, OmpC would predominate and altered outer membrane permeability may become an issue. Even if the permeation of β-lactam antibiotics are slower in *S. marcescens*, will it result in a lower susceptibility to β-lactams? Gutmann *et al.* (1985) reported the reduction of a 41 kDa protein and an increase in a 40 kDa protein in the outer membrane of a strain of *S. marcescens* with reduced susceptibility to three antibiotics. These resistant strains showed reduced glucose and chloramphenicol uptake suggesting a reduction in outer membrane permeability. On the other hand, a loss of the OprF porin in *Ps. aeruginosa* resulted in a decrease in permeability to β-lactams by six-fold but only had a small effect on antibiotic sensitivity

(Hancock, 1984). The role of reduced outer membrane permeability in β-lactam resistance of *S. marcescens* is unclear. The work presented in this thesis has provided some background information to allow future examination of this problem.

4. Conclusions

Prior to the onset of this work little information was available for *S. marcescens* porins except for conflicting reports regarding the number of porins and osmoregulation, and for observations of reduced content of proteins suspected of being porins in the outer membrane of strains with high β-lactam resistance. The objective of this thesis was to further the understanding of *S. marcescens* porins allowing future examination into the role of porins in the β-lactam resistance observed in this organism. The specific objectives outlined in Section 1.6. were satisfied as described below.

Objective 1: Identify the major porins in the *S. marcescens* outer membrane.

It was determined that *Serratia marcescens* UOC-51 produces at least two constitutive general diffusion porins in its outer membrane with M_r of 41 and 40 kDa. This was determined by differential salt extraction, heat modified SDS-PAGE mobility, and amino acid sequencing (Sections 3.1 and 3.2). The separation of the two proteins required the addition of urea to the gel electrophoresis system. In addition, the level of each porin was regulated in response to medium osmolarity and salicylate similar to *E. coli*. DNA hybridization and cloning confirmed the presence of two genes encoding outer membrane porins (Sections 3.2 through 3.4). These are homologous to the well known *E. coli* OmpF and OmpC porins.

Objective 2: Evaluate the distribution and conservation of the porins in other

Serratia species.

A survey of other *Serratia* spp., as well as other genera of the *Enterobacteriaceae* family, demonstrated the presence of outer membrane proteins analogous to the 41-kDa major nonspecific porin protein from *S. marcescens* in various members of the enteric family of Gram negative bacteria (Section 3.2). In addition, direct amino acid sequence analysis revealed the presence of a highly homologous protein in all species of *Serratia* examined. All porins tested had very similar porin function with single channel conductance ranging between 1.72 and 2.00 nS. These results demonstrate the conservation of porin structure and function among *Serratia* species and throughout the *Enterobacteriaceae*.

Objective 3: Characterize the identified *S. marcescens* porin(s) at the molecular level.

An *ompC* gene was identified in *S. marcescens* UOC-51 which codes for a 39.5 kDa mature peptide (Section 3.3). Three consecutive OmpR binding sites (OBS) are present upstream of the coding region. In *E. coli* these function in transcriptional activation of the *E. coli ompC* gene. When the cloned *S. marcescens* OmpC protein was expressed in *E. coli*, the membrane levels increased with higher medium osmolarity suggesting that the OBS are functional. In addition, a *micF* gene was identified upstream to the *ompC* gene which would be transcribed in the opposite direction of *ompC* transcription.

A second porin gene, *ompF*, was also identified in *S. marcescens* (Section

3.4). The calculated molecular weight of the mature product was 39.0 kDa. The two gene products of the *S. marcescens ompF* and *ompC* genes are very close in molecular size and therefore it is not surprising that they were difficult to separate in SDS-PAGE. OmpR binding sites are present upstream to *ompF* and the gene is located after the *asnS* gene similar to its homolog in the related bacterium *E. coli*. However, 300 bp of the region between *asnS* and *ompF* in *E. coli* is not present in *S. marcescens*. In *E. coli*, this region carries recognition sites for OmpR and integration host factor, which together function to repress *ompF* transcription. Osmoregulation was observed in *S. marcescens* however, the reduction of OmpF expression is not expected to occur through OmpR but rather, through *micF*. The *micF* transcript encoded by the gene upstream to *ompC* is complementary to the 5' end of the *ompF* transcript and is therefore predicted to be functional.

Amino acid sequence comparisons illustrated a conservation of both *S. marcescens* porins with other porins of the *Enterobacteriaceae* (Section 3.5). The PEFGGD motif of the enteric porins was not conserved in the *S. marcescens* OmpC porin, however, being replaced by PEFDGM. In *S. marcescens* OmpF the motif was conserved however, unique to this general diffusion porin was the presence of two cysteines predicted to be within the sixth external loop.

From the percent identity obtained between porin amino acid sequences, the *S. marcescens* OmpC was shown to be more similar to other OmpC's (~70%) than to PhoE's or OmpF's (~60%). The OmpF sequence however, did not clearly fall into one of the porin types. Distance analysis confirmed the placement of the

S. marcescens 40 kDa porin with OmpCs. The *S. marcescens* OmpF was distant from all porin types (OmpF, OmpC or PhoE) but was placed slightly closer to other OmpFs on the dendogram. Both analyses showed the PhoE porins cluster as a well conserved group, with approximately 90% identity. The OmpC porins also remain conserved in a group (80%), except the *S. marcescens* OmpC which displays more divergence from the others (70%). The OmpF porins are a less conserved group (60 to 70%).

Objective 4: Evaluate the permeability of the *S. marcescens* porins.

Functional analysis of the two native porins of *S. marcescens* demonstrated that the OmpF porin has a faster diffusion rate for uncharged molecules than OmpC (Section 3.6). Relatively large anionic molecules penetrate slowly and no rate difference is detected between the two porins. In order to reduce outer membrane permeability to anionic β-lactams a reduction in number of porins may be required whereas, single amino acid alterations may affect the permeation of uncharged β-lactam molecules.

Objective 5: Identify important amino acids in the porins that determine permeability to β-lactam antibiotics.

Recall that the third external loop of the general diffusion porins folds down into the pore channel and is important in determining size and ion selectivity. The D^{112} residue in the PEFDGM motif located in the third external loop of the *S. marcescens* OmpC porin conferred a slower penetration rate of

uncharged molelcules but had no effect on the passage of anionic molecules (Section 3.6). This suggested that the amino acid at position 112 may play an important role in size constriction, but not particularly ionic selection. The *S. marcescens* OmpC is expected to be less permeable than the OmpCs characterized in other enteric bacteria, which all have a glycine at that location, with possible implications in the outer membrane permeability of this organism.

The $M^{114}D$ constructed porin did not have altered permeability of any molecules tested. Thus, M^{114} appears not to play a role in pore constriction or selectivity however, D^{112} may have masked any effects from the $M^{114}D$ construction and a double mutant needs to be examined in order to make a more definite conclusion about M^{114}.

5. Future studies

This thesis presented the characterization of two outer membrane porins in *S. marcescens*. Further research discussed in this section will cover four areas: 1) Construction of *S. marcescens* porin deficient mutants; 2) regulation of *S. marcescens* porins; 3) structure/function of the porins; and 4) role of *S. marcescens* porins and outer membrane permeability in β-lactam resistance.

1) Construction of *S. marcescens* porin deficient mutants.

Porin deficient mutants of *S. marcescens* could be constructed by homologous recombination. The procedure requires the use of a suicide vector to drive the recombination event. My previous attempts with two different plasmids were unsuccessful because of various problems with the plasmids employed. Therefore, the establishment of a suitable vector system for *S. marcescens* would first be required. The construction of individual omp knock-out mutants would definitively link each identified gene with a protein band observed in the outer membrane. Since the porins migrate close together in SDS-PAGE a third porin, if one exists, may be detected when the 40 and/or 41 kDa porins are removed. Such porin deficient *S. marcescens* strains would be useful in examining the effects of reduced outer membrane permeability on β-lactam susceptibility in this organism. In addition, they would be a suitable host background for examining site-specific porin mutants.

2) Regulation of *S. marcescens* porins.

Osmotic regulation of the *S. marcescens* constitutive porins was observed however, the negative regulation of the OmpF porin probably occurs through *micF*. The regulation of the *S. marcescens* porins is an area that requires more examination. The regulatory regions of both *S. marcescens* porin genes, *ompF* and *ompC*, could be cloned into a promoter-less *lacZ* vector to quantitate transcriptional activity from these promoters under different growth conditions. It would be expected that the *S. marcescens ompC* promoters would result in increased β-galactosidase activity when cells are grown in high osmolarity. If the OmpF levels decrease via *micF* as proposed in this thesis, the transcriptional activity should not change or increase in high osmolarity. This would not prove that *micF* decreases OmpF levels in high osmolarity, but it eliminates the possibiltiy of an OmpR-mediated mechanism in the *ompF* upstream region.

A *micF* deletion mutant of *S. marcescens* would be useful to examine whether *micF* is responsible for the observed osmoregulation. This can be done by examining whether the deletion of *micF* results in a loss of OmpF osmoregulation by visualization of outer membranes in SDS-PAGE or more precisely by pulse-labelling experiments. Further, *micF* and *ompF* mRNA synthesis in wild-type and the *micF* mutant of *S. marcescens* could be monitored after switching the cells from low to high osmolarity. If *micF* is exclusively responsible for OmpF reduction then a decrease in *ompF* mRNA in the *micF* deletion mutant should not be seen. Results from such experiments will not only test whether OmpF is regulated by *micF* but will also confirm that *micF* is actively

transcribed in *S. marcescens*.

In addition, regulation of *S. marcescens* porins to other environmental conditions, such as temperature, should be investigated.

3) Structure/function of porins.

The third external loop of enterobacterial porins folds down into the pore and functions to restrict the size of molecules which can pass through the channel. The *S. marcescens* OmpC porin was shown to have two modifications of a conserved region within this external structure. In this thesis the two sites within the third loop, D^{112} and M^{114}, were altered back to the consensus (G^{112} and D^{114}) for investigation into the role these changes may play in pore permeability. D^{112} was shown to decrease the diffusion rate of uncharged molecules through the OmpC pore channel in comparison to G^{112} of the conserved motif. On the other hand, M^{114} did not appear to affect pore constriction. In considering the large difference D^{112} has on solute permeability it is possible that any difference in diffusion rate of the $M^{114}D$ porin may have been masked. Therefore, a double mutant preserving the entire motif, PEFGGD should be constructed and the difference in function between $D^{112}G$ and $D^{112}G/M^{114}D$ porins should be evaluated. This would allow a more definite conclusion about the role of M^{114}. Evaluation of the same sites in OmpF would confirm results obtained for OmpC.

A second site that should be investigated in both *S. marcescens* porins is at position 124 (according to numbering of *S. marcescens* OmpC). In PhoE porins this site is occupied by lysine which has been shown to protrude into the

channel and confer the anion selectivity characteristic of this porin. In all three OmpF sequences known this site is a glycine and in three of the four OmpCs it is a glutamine. The glutamine side-chain may contribute to size constriction if it protrudes into the pore lumen as lysine does in PhoE. Therefore, $Q^{124}G$ in OmpC and the reverse, $G^{124}Q$ in OmpF should be constructed in the respective porins and the permeability evaluated by liposome swelling experiments. Keeping in mind that D^{112} reduces pore size, these experiments should be done with site-112 as glycine as explained above.

The *S. marcescens* OmpF porin sequence was presented in this thesis and it was shown that two cysteine residues are present. By comparison to the *E. coli* OmpF structure the two cysteines would be expected to be in the sixth external loop. Site-directed mutagenesis altering one or both of these sites would test if they have any functional, structural or other role.

4) Examination of the role of porins in β-lactam resistance.

D^{112} in the third loop was shown to reduce permeability of uncharged cephalosporins through the *S. marcescens* OmpC porin. However, would this translate into increased antibiotic resistance? Using *S. marcescens* or *E. coli* porin deficient strains (BZB1107), antibiotic susceptibility of the strain with no porin, with wild-type porins and constructed mutant porins can be compared. Since the β-lactamases are a major mechanism of resistance, antibiotic susceptibility tests should be done with and without a β-lactamase inhibitor. This may indicate whether a reduction in number of porin molecules in the outer

membrane or reduced permeability through an unaltered number of porins can affect the levels of β-lactam resistance attained.

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