Examining the role of OmpC porin in the β-lactam antibiotic resistance of *Serratia marcescens*

by

Olasunkanmi Famobio

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

Department of Microbiology
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Examining the Role of OmpC porin in the β-lactam Antibiotic Resistance of *Serratia marcescens*

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Of

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Abstract

Reduction in the amount, and/or modification of the permeability of porins is an important mechanism of antibiotic resistance in *Serratia marcescens*. The third eyelet loop is important for determining the size of pore channel of enterobacterial porins. At the tip of the loop, there is a consensus motif PEFGGD. *S. marcescens* OmpC deviates from this by having PEFDGM. Two single site-specific mutants, D112G and M114D, and one double mutant, with both mutations were created. Proteoliposomes reconstituted using mutant and wild-type porins were suspended in 18 mM glucose, 18 mM maltose, or 9 mM ampicillin, and permeability rates compared by liposome swelling assays. The double mutant had a higher permeability rate than the single mutants and wild-type *S. marcescens* OmpC, but less than *S. marcescens* OmpF. Permeability properties of the outer membrane of porin deficient *E. coli* BZB1107 bearing the mutant porins were also studied by measuring the net accumulation of ciprofloxacin, norfloxacin and ofloxacin, over ten minutes, using the fluoroquinolone accumulation assay. The strain bearing the double mutant porin (BZBGGD) accumulated more ciprofloxacin and norfloxacin, than strains bearing the single mutant porins (BZBD112G and BZBM114D) and wild-type *S. marcescens* OmpC (BZB8SX), but less than OmpF [BZBS1E(-)]. There was no difference in the accumulation of ofloxacin in *E. coli* strains bearing the mutant porins and *S. marcescens* wild-type OmpC, but it was less than that of wild-type OmpF.

The effects of environmental factors such as pH, osmolarity, salicylate and

temperature on the regulation of *S. marcescens* OmpF and OmpC porin expression were examined using the β-galactosidase assay. All environmental factors studied were found to regulate porin expression. Two or three of these conditions were also combined randomly, and regulation of porin expression examined. In all combinations used, there was always an increased expression of OmpF except when high temperature, 42°C, was used as one of the combined factors. Conversely, OmpC expression decreased in all combinations, but increased at high temperature.

The results presented in this thesis suggest that regulation of porin expression and mutations resulting in the constriction of the pore are strategies used by *S. marcescens* to cope with the presence of harmful substances like antibiotics, or when environmental conditions are unfavorable.

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List of Abbreviations

Å	angstrom
Asp	aspartate
bp	base pairs
EDTA	ethylenediamine-tetra-acetic acid
g	gram(s)
Glu	glutamate
Gly	glycine
Kb	kilobase pairs
KDa	kilodaltons
L	litre(s)
Lys	lysine
M	molar
mg	milligram
mL	millilitre
mM	millimolar
M_r	molecular mass
NaCl	sodium chloride
nm	nanometer
NaN_3	sodium azide
OD	optical density
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
SDS	sodium dodecyl sulphate
µg	microgram(s)
µL	microlitre(s)
v/v	volume/volume
w/v	weight/volume

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Serratia marcescens belongs to the family *Enterobacteriaceae*. It is a Gram-negative, facultatively anaerobic rod-shaped bacterium (Grimont & Grimont, 1984). It is motile by peritrichous flagella and found in a variety of environmental sources including soils, water, and plant surfaces. Certain strains of *S. marcescens* produce a red pigment, and this is known to be dependent on growth conditions e.g. incubation temperature (Williams *et al.*, 1971).

S. marcescens got its name from Bartolemeo Bizio, a young Italian Pharmacist who demonstrated in the early nineteenth century that the red pigment growing on cornmeal mush, which at that time was thought to be blood, was caused by a living organism. He named this organism *Serratia marcescens* in honor of an Italian Physicist, Serafino Serrati (Yu, 1979).

S. marcescens had earlier been thought to be a harmless saprophyte. The first clinical report involving *S. marcescens* came forward in 1913 when a patient with bronchiectiasis was described (Woodward & Clarke, 1913). By the 1950's, though it was still generally considered to be a harmless bacterium, more cases had emerged; and between 1968 and 1977 one hospital alone reported 76 cases of *Serratia* bacteremia (Yu *et al.*, 1979).

Presently, *S. marcescens* is recognized as a very important opportunistic pathogen having been implicated as the causative agent in various outbreaks resulting in all kinds of nosocomial infections, ranging from respiratory tract infections, urinary tract infections, meningitis, otitis media, peritonitis,

endocarditis, osteomyelitis, conjunctivitis to burns (Albers *et al.*, 2001; Byrne *et al.*, 2000; Jang *et al.*, 2001). The factors responsible for the spread of *S. marcescens* infection during outbreaks were identified in a study carried out in a pediatric intensive care unit. These factors include breaks in aseptic technique, reduced frequency of hand washing among health care workers before and between caring for patients, decreased attention to infection control practices, and environmental contamination (Manning *et al.*, 2001). The most susceptible to these infections have been the critically ill neonates and infants, immunocompromised patients that could be patients undergoing surgery, HIV/AIDS patients, and cancer patients undergoing treatments with immunosupressants. Various outbreaks of nosocomial infections due to *S. marcescens* have been reported. The first well-documented outbreak attributable to point-source dissemination of *S. marcescens* was that of eleven cases of meningitis, wound infection, and arthritis, which occurred in a pediatric ward (Rabinowitz & Schiffrin, 1952). In 1966, a nursery epidemic involving 27 babies was described and the source was traced to contamination of plastic caps of saline bottles from which fluid was used to moisten umbilical cords (McCormack & Kunin, 1966). More recent reports include outbreak of infections in neonatal intensive care units (Fleisch *et al.*, 2002; Jang, *et al.*, 2001), and outbreak of *S. marcescens* bacteremia in a surgical intensive care unit due to extrinsic contamination of a parenteral narcotic by a health care worker (Ostrowsky *et al.*, 2002).

Nosocomial infections due to *S. marcescens* are particularly important

because of the difficulty experienced when treating them. *S. marcescens* has an intrinsically high resistance to a broad spectrum of antibiotics. In the late 1970s *S. marcescens* was usually susceptible to kanamycin and always susceptible to gentamicin *in vitro* (Yu, 1979), but reports later emerged about strains that were resistant to a broad range of antibiotics, including gentamicin (Meyer *et al.*, 1976; Yu, *et al.*, 1979). Today, the literature is filled with cases of *S. marcescens*' resistance to almost every class of antibiotics in use, although some are more common than the others. Prevalence varies from country to country (Wenzel *et al.*, 2003). A group working in Japan reported a recent example of multidrug resistance in *S. marcescens*. They reported isolated clinical strains showing a fairly high resistance to norfloxacin, streptomycin, ampicillin, erythromycin, tetracycline, chloramphenicol, and antimicrobial dyes (Chen *et al.*, 2003).

The significance of the study of how this bacterium develops resistance to antibiotics, both old and new, cannot be overemphasized. If we are to gain an upper hand in the battle against antibiotic resistance, then we have to continue to explore the mechanisms of antibiotic resistance.

1.2. Antibiotic resistance of Gram-negative bacteria

1.2.1. Mechanism of action of β-lactam antibiotics

β-lactam antibiotics were introduced more than five decades ago, and are still prescribed in medicine today. β-lactam antibiotics comprise the penicillins, cephalosporins, monobactams, and the carbapenems. They are so called because they share a common core structure, the β-lactam ring (Fig 1.1). The

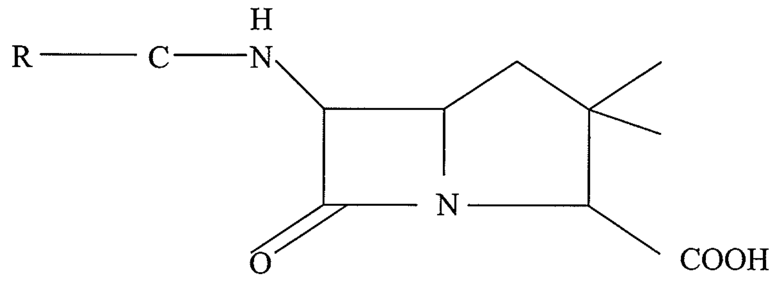
β-lactam antibiotics have structural similarities with the binding sites of bacterial substrates, which enable them to bind to and inactivate the transpeptidases involved in bacterial cell wall peptidoglycan synthesis (Williams, 1999). The peptidogylcan confers high tensile strength to the bacterial cell wall through a network of alternating strands of β-1,4-linked pyranoside N-acetylglucosamine and N-acetylmuramic acid residues, which form a chitin-like structure in which each alternate N-acetylglucosamine residue is ether-linked at C-3 to a lactyl group that has the D-configuration. The D-lactyl groups of N-acetylmuramic acid residues are amide-linked to L-alanyl-γ-D-glutamyl-L-R-D-alanine tetrapeptides and peptide-substituted glycan strands are connected by interpeptide bridges (Ghuysen, 1997). As these constitute very essential components of the cell machinery, bacterial cells have evolved ways of evading the effect of these agents by producing β-lactamases and altering the penicillin binding sites.

1.2.2. Mechanisms of resistance to β-lactam antibiotics

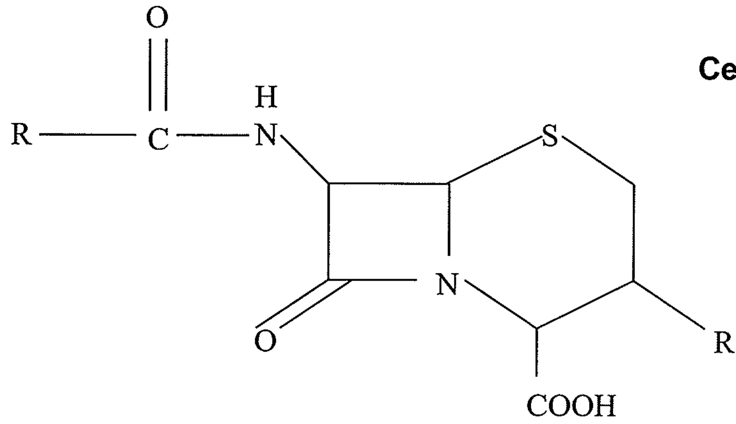
Generally, mechanisms of antibiotic resistance can be divided into six groups; namely, the presence of an enzyme which modifies the antibiotic, an alternative enzyme for the enzyme that is inhibited by the antibiotic, mutation of target of the antibiotic, modification of the target of the antibiotic, reduced uptake of the antibiotic, and active efflux of the antibiotic (Fluit & Visser, 1992). Although, in *S. marcescens* not all mechanisms have been examined, the most important and most widespread mechanism against β-lactam antibiotics is the production

Fig. 1.1. General structure of β-lactam antibiotics

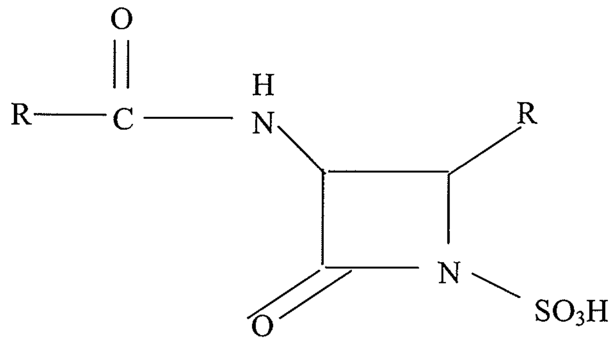
Penicillins



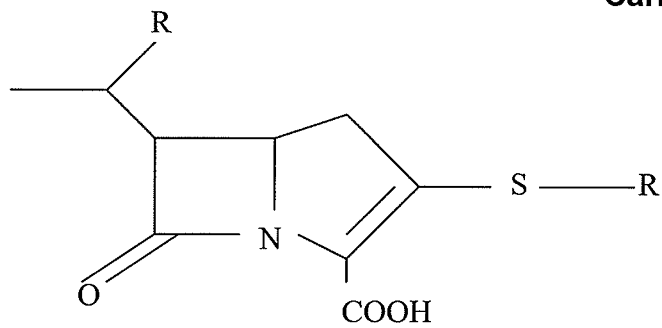
Cephalosporins



Monobactams



Carbapenems



of β-lactamases, which destroy the β-lactam ring (Raimondi *et al.*, 2001; Williams, 1999). Many genera of gram-negative bacteria possess naturally-occurring, chromosomally-encoded β-lactamases. β-lactamases are thought to have evolved from penicillin-binding proteins, since they share some sequence homology. Therefore it was suggested that this development was due to the selective pressure exerted by β-lactam-producing soil organisms found in the environment (Ghuysen, 1997). This is supported by the fact that the first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin into medical practice (Abraham & Chain, 1940). Examples of chromosomally encoded β-lactamases are the serine-based β-lactamases, SME-1 and SME-2, which confer resistance to carbapenems, aztreonam, cefamandole and cephalothin (Naas *et al*, 1994; Queenan *et al*, 2000). There has been a dramatic evolution of different β-lactamases since the description of the first plasmid mediated β-lactamase [TEM-1] (Datta & Kontomichalou, 1965). The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture of a patient named Temoniera in Greece, hence the designation TEM (Medeiros, 1984). Another plasmid mediated β-lactamase found in *S. marcescens* is the metallo-β-lactamase, closely related to bla_{IMP} (imipenem degrading β-lactamases) (Ito *et al*, 1995). Due to continual evolution of β-lactamases, variants now exist that have increased antibiotic spectrum, and they are called extended spectrum β-lactamases (ESBLs) (Bradford, 2001). The presence of ESBLs has been reported in *S. marcescens* (Luzzaro *et al*, 1998).

Antibiotic target modification has also been effectively utilized by *S. marcescens* against aminoglycosides through production of 16S rRNA methylase (Doi *et al.*, 2004). With respect to quinolones, mutational alterations in *gyrA*, which codes for the A subunit of DNA gyrase, are common (Kim *et al.*, 1998).

Outer membrane porins also play an important role in the antibiotic resistance of Gram-negative bacteria, as they permit entry of these agents, especially β-lactam antibiotics into the cell (Kumar & Worobec, 2003). Their role, although not yet very well studied in *S. marcescens*, is thought to be similar to those of *Enterobacteriaceae* family (e.g. *E. coli* and *K. pneumoniae*). Evidence indicates that porins, either alone or in combination with β-lactamases and/or active efflux play an important role in antibiotic resistance (Hechler *et al.*, 1989; Hernandez-Alles *et al.*, 1999; Hernandez-Alles *et al.*, 2000; Martinez-Martinez *et al.*, 2002; Martinez-Martinez *et al.*, 1999). Apart from *Pseudomonas aeruginosa*, *E. coli*, and perhaps *Salmonella typhimurium*, whose efflux systems have been extensively studied, until recently little was known about similar systems in other gram-negative bacilli like *K. pneumoniae* and *S. marcescens*. It has been well demonstrated that active efflux plays an important role in the intrinsic multiple antibiotic resistance of Gram-negative bacilli e.g. *E. coli*, most of the time, acting synergistically with reduced outer membrane protein expression (Poole, 2002). In *S. marcescens*, it has been shown that a proton gradient-dependent efflux mechanism utilizing RND efflux pumps is present (Kumar & Worobec, 2002; Kumar, 2004). Finally, porins serve as channels for the passage of substrates and wastes, and also constitute channels for antibiotics like the β-lactams to get

into the cell (Nikaido, 1992). They can undergo modification, which will result in decreased access of antibiotics or harmful substances into the cell (Sanders and Sanders, 1992). Porins have been shown to play a role in antibiotic resistance of *E. coli* (Nikaido, 2003), while, in *S. marcescens*, their role in antibiotic resistance is still being explored (Ruiz *et al*, 2003).

1.3. Outer membrane of Gram-negative bacteria

Apart from having a thin peptidoglycan layer, relative to the thick and robust peptidoglycan layer of Gram-positive bacteria, the cell envelope of Gram-negative bacteria is also surrounded by a highly lipophilic outer membrane, which is absent in Gram-positives.

The cell envelope of Gram-negative bacteria is composed of the cytoplasmic or inner membrane and the asymmetrical outer membrane, which are separated by the peptidoglycan containing periplasm (Benz & Bauer, 1988; Bos & Tommassen, 2004). While *S. marcescens* shares majority of the cell envelope properties described for Gram-negative organisms discussed in this section, it also produces and excretes extracellular enzymes, which include proteases, chitinases, lipases, nuclease, and a bacteriocin called marcescin (Guynn *et al.*, 1998).

The cytoplasmic membrane is a phospholipid bilayer fused with a variety of polypeptides, while the outer membrane consists of lipopolysaccharide (LPS) in its outer monolayer and phospholipids in its inner monolayer, in addition to various structural and pore forming proteins (Hancock, 1984). The cytoplasmic

membrane proteins perform numerous functions, the most prominent being energy generation, active and facilitated transport of nutrients and export of toxic metabolites, and enzymatic synthesis and translocation of cell envelope components.

The periplasmic space plays an important physiological role in Gram-negative bacteria as it harbors binding proteins for certain substrates, and it is known that β-lactamase activity is resides here (Benz, 1988). The periplasmic space occupies between 5 and 20% of the total cell volume, and it appears to have the same osmolarity as the cytoplasm.

The outer membrane is a permeability barrier, which allows the passage of hydrophilic molecules through embedded, water-filled proteins called porins; and the passage of hydrophobic compounds by passive uptake (Hancock, 1984). Its role in the physiology of *E. coli* and other enteric bacteria is very crucial as it enables them to survive in the hostile environment of their host by creating an important barrier against harmful substances like bile salts and digestive enzymes (Nikaido & Nakae, 1979).

The lipopolysaccharide component of the outer membrane is composed of a rough oligosaccharide core containing an unusual sugar, 3-deoxy-D-*manno*-octulosonic acid (also known as KDO), which is linked to a hydrophobic region, known as lipid A, and a hydrophilic O-polysaccharide (O-Antigen) (Hitchcock & Brown, 1983). There is a close association between lipopolysaccharides and outer membrane proteins thought to result from hydrophobic interactions or non-covalent cross-bridging (Yamada & Mizushima, 1980). The diffusion of

hydrophobic molecules through the outer membrane is hindered as a result of a strong barrier created by the association of the long oligosaccharide side chain attached to lipid A and the ionic bridges between charged groups in the polysaccharide (Lugtenberg & Van Alphen, 1983). Lipopolysaccharide is also important for virulence and pathogenicity in Gram-negative organisms (Straus *et al.*, 1985). *S. marcescens* has a variable LPS structure having more than 24 O-antigens (Gaston & Pitt, 1989).

The lipid composition of the outer membrane is considered to be very similar to that of the inner membrane, having as its major component in *E. coli* and *S. typhimurium*, phosphatidylethanolamine (Cronan & Vagelos, 1972). The most important proteins found in the outer membrane include the lipoprotein anchored to the outer membrane and the integral proteins known as the outer membrane proteins (Bos & Tommassen, 2004).

Lipoprotein (7.2 KDa) has a large number of copies per cell ($\approx 7 \times 10^5$). It exists in the outer membrane in two different forms. It occurs in a form covalently bound to the peptidoglycan through the ϵ-amino group of its C-terminal lysine, or as free protein (Braun, 1975). The main function of the protein is structural, stabilizing the architecture of the outer membrane-peptidogylcan complex (Nikaido & Vaara, 1985).

The outer membrane proteins (OMPs) primarily include the structural OMP OmpA and the pore-forming proteins (Nikaido & Vaara, 1985). OmpA protein is one of the major proteins found in the outer membrane of *E. coli*, occurring at about 100,000 copies/cell (Koebnik *et al.*, 2000). It provides a physical linkage

between the outer membrane and the underlying peptidoglycan layer, thus playing the important role of preserving the integrity of the cell surface (Sonntag *et al.*, 1978). It is also useful in conjugation (Schweizer & Henning, 1977), and serves as a receptor for bactriophages (Morona *et al.*, 1985) and colicins (Davies & Reeves, 1975). Resolution of the structure of OmpA has revealed that it has eight transmembrane β-strands with an antiparallel arrangement. It is, however, doubtful that OmpA plays a pore-forming role, as no continuous transmembrane channel could be detected, in spite of the presence of cavities (Koebnik*, et al.*, 2000). The pore-forming proteins are described in detail in the following section.

1.4. Porins

1.4.1. Introduction to porins

Non-specific protein channels were first described about three decades ago (Nakae, 1976). The first to be described was a 37 KDa protein in the outer membrane of *E. coli* B, which was later given the name OmpF. These proteins constitute the means by which hydrophilic solutes go through the lipid bilayer of the outer membrane of Gram-negative bacteria and some Gram-positive organisms (Nikaido, 2003).

Porins have been defined to mean only proteins that form nonspecific channels (Nikaido, 2003). This implies that channels like the LamB or the maltose channel are not to be referred to as porins, but specific channels. Unlike the specific channels, porins serve a general purpose in the outer membrane, where they allow the transmembrane passage of nutrient and other molecules,

which are usually small in size and hydrophilic. The best studied of these are OmpF, OmpC and PhoE, which together with their homologs are also called the classical porins, as they represent the foundation of our current understanding of porins. PhoE prefers anions as substrates, while OmpF and OmpC have preference for cations. OmpF has a wider pore diameter (≈ 1.2 Å) than OmpC, thus allowing the permeation of slightly larger solutes (≤ 600 KDa).

In *S. marcescens*, 40 KDa OmpC and 41 KDa OmpF porins have been characterized, and were shown to have high amino acid identity to OmpC (70.2%) and OmpF (67.7%) of *E. coli* (Hutsul & Worobec, 1994; Hutsul & Worobec, 1997). Homologs of *E. coli* OmpF and OmpC porins have been identified in *K. pneumoniae*, (i.e. OmpK35 and OmpK36 respectively) (Hernandez-Alles, *et al.*, 1999), in *Enterobacter aerogenes* (Omp35 and Omp36) (Bornet *et al.*, 2004), and in several other groups of bacteria outside the *Enterobacteriaceae*. Bearing in mind the unique architecture of the outer membrane of Gram-negative bacteria and other types of bacteria, which express porins, it is not surprising that porins serve the important function of allowing the influx of solutes into the cell and perhaps the extrusion of wastes out of the cell (Nikaido, 2003).

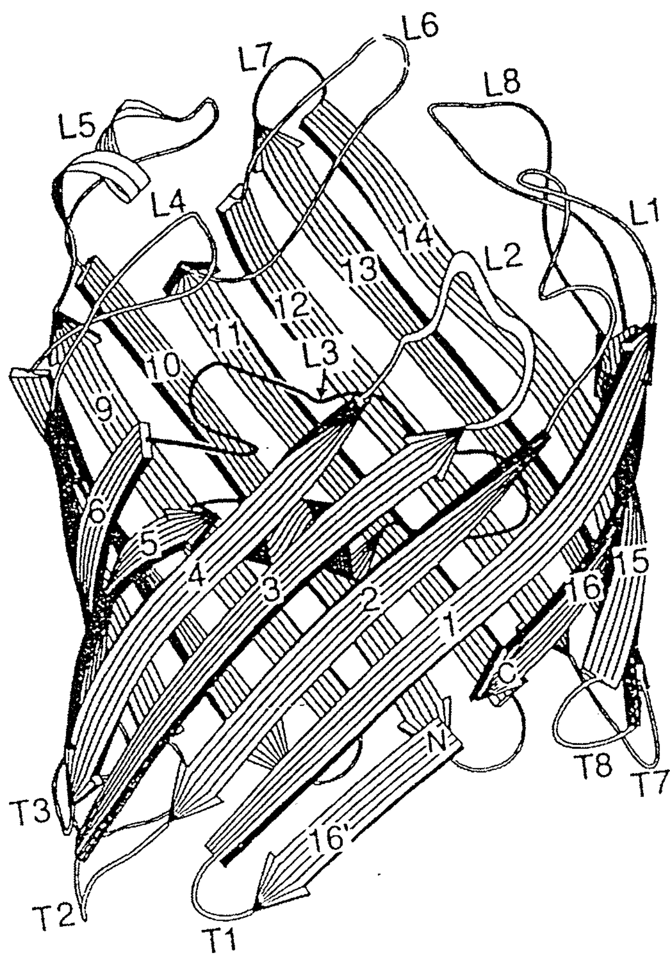
Various methods have been used to study the function of porins. In one method, porins are isolated and reconstituted into planar bilayer lipids. This allows the measurement of conductance through single channels incorporated in a lipid film (Nikaido, 1992). Another method involves the efflux of radiolabelled solutes out of proteoliposomes prepared with purified porins, which makes

possible the measurement of molecular weight with exclusion limits (Nakae, 1975). Recently, a group used quinolone accumulation assay to study the difference in permeability of the cell envelope of *S. marcescens* strains expressing wild-type and mutant porins (Ruiz *et al.*, 2003). The function of porins has also been studied by reconstituting them into multilayered proteoliposomes. The rate of diffusion of solutes through porins is measured by the rate of optical density decrease of the liposomes (Nikaido & Rosenberg, 1983). Finally, diffusion rates through porin channels have been measured in intact cells by coupling the influx of the solute e.g. a cephalosporin to its hydrolysis by periplasmic β-lactamases (Nikaido *et al.*, 1983).

1.4.2. Structure and function of porins

The characterized structures of members of the non-specific pore proteins reveal homotrimeric proteins with subunits between 250-450 aminoacyl residues in length. These proteins form 16 stranded anti-parallel β-barrel structures, with all β-strands hydrogen-bonded to their nearest neighbors along the chain. The trimer comprises three water-filled channels, each with the β-barrel perpendicular to the plane of the membrane. Each β-strand is linked to the other by polypeptide loops that are formed in between them. These loops control the width of the channel formed by the β-barrel. The third loop, L3 (Fig. 1.2.) contributes significantly to the permeability properties of the pore channel (Koebnik, *et al.*, 2000). The third loop folds back into the pore lumen forming a constriction zone at half the height of the channel, usually referred to as the eyelet region.

Fig. 1.2. Three-dimensional structure of *E. coli* OmpF porin as revealed by X-ray crystallographic data (Cowan*, et al.*, 1992). Thick arrows designate beta-strands while loops are shown as thin strands. The extracellular side of the porin is at the top of the figure. The external loops are designated L1 through to L8 whereas short turns on the periplasmic side are designated T1 through to 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 neighboring porin monomer contributing to a strong trimer association.


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

Characteristic of the third loop, in the enterobacterial porins, is a sequence motif, PEFGG. The nature of residues lining the wall of the pore confers on it different diffusion characteristics (Cowan *et al.*, 1992). For instance, the negative charge preference of PhoE has been shown to be mainly due to the replacement of Gly131 in the eyelet region of OmpF with a positively charged Lys125. The channels often have diameters in the range of 1nm, and thus the penetration rate of solutes through porin channels is likely to be affected strongly by what seems to be minor differences in the size, shape, hydrophobicity or charge of the solute molecule (Nikaido, 1992).

The presence of porins in the outer membrane allows the permeation of useful and toxic substrates across the outer membranes. Comparison of the permeability properties of bacterial strains with different porin profiles using β-lactam antibiotics has confirmed this role of porins (Hancock, 1987).

1.4.3. *Serratia marcescens* porins

As earlier mentioned, two major porins have been identified in *Serratia marcescens*: 40 KDa OmpC and 41 KDa OmpF (Hutsul & Worobec, 1994; Hutsul & Worobec, 1997). These are given their names based on the level of similarity they share with respective porins in *E. coli*. Alhough we are still in the process of fully characterizing *S. marcescens* porins, one important difference recognized between *S. marcescens'* OmpC and other enterobacterial porins lies in the conserved sequence motif, PEFGG(D) of the eyelet region. The motif sequence in *S. marcescens'* OmpC is PEFDGM. Initial study of this change through site-

directed mutagenesis and subsequent analysis of permeability properties of mutant porins by the liposome swelling assay revealed that the aspartate residue is important for size exclusion and not ionic selection (Hutsul, 1996).

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

1.5.1. Introduction

E. coli faces a continuous challenge of low pH, presence of bile salts, osmolarity changes, anaerobiosis, temperature fluctuation and presence of toxic substances in the intestinal tract of its host organism (Nikaido, 2003). One of the ways of survival *E. coli* uses is through regulation of expression of its porins. The regulation of porins is carried out through a complex network of mechanisms (Pratt *et al.*, 1996).

Functionally, porins allow the transmembrane diffusion of small solutes, usually hydrophilic molecules, and show no particular substrate specificity, despite some selectivity for either cations or anions. In response to a variety of environmental conditions, the total amount of OmpC and OmpF proteins combined remains fairly constant, whereas the relative levels of the two proteins fluctuate in a reciprocal manner. Under conditions of low osmolarity, OmpF expression is favored; conversely, OmpC expression is favored in high osmolarity (Van Alphen & Lugtenberg, 1977). Salicylate reduces OmpF expression, but the expression of OmpC is greatly increased (Rosner *et al.*, 1991). It has been shown in the past that the expression of the porin genes is affected by the pH of the growth medium (Heyde *et al*, 1987). The proportion of

the major porins also changes with temperature, presence of antibiotics and presence or absence of oxygen (Pratt, *et al.*, 1996).

There are two main mechanisms through which environmental factors influence the regulation of porins. These mechanisms do not act independently, but rather interact with one another. These include the extensively studied and most understood, two-component regulatory system, EnvZ-OmpR, mediating osmoregulation; and the *micF*-RNA (mRNA interfering complementary RNA) system involved in the post-transcriptional negative regulation of *ompF*, resulting from increased temperature. In addition, many environmental parameters influence *micF* expression through DNA binding proteins like Rob, Lrp, MarA and SoxS, to either prevent or enhance *ompF* translation (Pratt, *et al.*, 1996).

1.5.2. EnvZ-OmpR

Two genes, *ompR* and *envZ*, together constitute the *ompB* locus mapped at 75 minutes on the *E. coli* chromosome (Sarma & Reeves, 1977). The products of these genes, OmpR and EnvZ, are known to control osmoregulation of OmpF and OmpC. EnvZ is a 45,000 Da integral cytoplasmic membrane protein, which plays the role of a sensor, monitoring the external osmolarity and passing on this signal to OmpR by phosphorylation and dephosphorylation. OmpR is a 27,355 Da DNA binding protein and the response regulator located in the cytoplasm (Forst & Inouye, 1988; Kenney, 1997). Resulting OmpR-phosphate either activates the transcription of *ompF* under conditions of low osmolarity, or represses the transcription of *ompF* and activates the transcription of *ompC*

under conditions of high osmolarity.

1.5.3. *micF*-RNA

The *micF* gene RNA is one of several antisense RNAs that control gene expression in response to environmental signals (Lease & Belfort, 2000). It is located immediately upstream of *ompC*, and transcribed in the opposite direction (Pratt, *et al.*, 1996). It is involved in the post-transcriptional negative regulation of *ompF*. Although *micF* is not located anywhere near *ompF*, when activated its transcript binds to *ompF* mRNA and inhibits its translation (Delihas & Forst, 2001). The sequence of *micF* RNA is partially complementary to *ompF* RNA (Fig. 1.3). Therefore, inhibition of translation of *ompF* mRNA results from shielding of the Shine-Dalgarno sequence and AUG translation start site on the ribosome (Andersen, *et al.*, 1989). The significance of this is that *E. coli* and other bacteria become more resistant to antibiotics as a result of the increased expression of the less permeable OmpC porin or its homologs (Martinez-Martinez, *et al.*, 1999; Nikaido, 2003). High temperature is a key challenge *E. coli* faces in the intestinal environment, and this is known to increase the transcription of *micF*.

A number of environmental factors comprising oxidative stress, hydrogen peroxide, osmolarity increase, temperature increase, nutrients, weak acids, antibiotics, cationic peptide antibiotics, λ phage infection, bile salts, ethanol, copper ions and hypochlorous acid are known to stimulate *micF* through transcriptional activators (Delihas & Forst, 2001). Apart from being a component of the EnvZ-OmpR transduction system, OmpR has been reported to control

micF expression in a manner similar to osmotic regulation of *ompC* (Forst & Inouye, 1988). MarA is another transcriptional regulator of *micF*, which is activated by weak acids, and certain antibiotics (Pratt, *et al.*, 1996). *marA* is a part of *marRAB* regulon which is controlled by the repressor MarR (Miller & Sulavik, 1996). In the presence of weak acid like salicylate, MarR is inactivated causing the derepression of *marA* (Martin & Rosner, 1995), and MarA binds to *micF* promoter and activates *micF* transcription. *soxS* is part of the *soxRS* locus. SoxR is a sensor protein activated by oxidative stress, which in turn activates transcription of *soxS*. SoxS also binds to the *micF* promoter to activate its transcription (Li & Demple, 1994). The role of Rob, another DNA binding protein, in *micF* regulation is still unclear. Little is known about the suggested activation of *rob* by cationic peptide antibiotics. Although, in an *in vitro* experiment, Rob has been shown to bind the *micF* promoter. Rob has 100 residues at its N-terminus that are closely related to those found in MarA and SoxS (Ariza *et al.*, 1995). Interdependence of the different regulatory mechanisms is supported by the loss of activation of *micF* by peptide antibiotics in a *rob* deletion strain but not in *soxS* or *marA* deletion background, implying that Rob needs intact and fully functional SoxS and MarA to perform its roles (Oh *et al.*, 2000). Finally, the level of Lrp (leucine-responsive regulator protein) in conditions of famine is high, and it positively regulates *ompF* expression post-transcriptionally by decreasing the *micF* transcription (Pratt, *et al.*, 1996).

1.6. Porin regulation in *S. marcescens*

There are conflicting reports on the observed osmoregulation in *S. marcescens.* In two earlier studies (Hutsul, *et al.*, 1993; Sawai *et al.*, 1987), it was reported that osmoregulation did not exist in *S. marcescens*. In later studies, it has been shown that there is osmoregulation in *S. marcescens* (Hutsul & Worobec, 1997; Puig *et al.*, 1993). However, there is still disparity in the observed pattern vis-à-vis *E. coli* osmoregulation. In studies where osmoregulation was observed, urea-SDS-PAGE method was used for the separation of *S. marcescens* porins. Therefore, the inability to observe osmoreguation in the studies mentioned might be due to the use of ordinary SDS-PAGE separation method (Hutsul & Worobec, 1997).

Serratia marcescens porins have also been shown to be regulated by salicylate, with the larger pore OmpF decreasing in its presence, and the narrower pore OmpC increasing in its presence (Hutsul & Worobec, 1997; Sawai, *et al.*, 1987). Previous studies observed little or no change in the OmpC porin of *S. marcescens* or OmpC of *E. coli* (Rosner, 1985).

From the analyses performed on the *S. marcescens ompF* sequence it has been concluded that the OmpR-EnvZ system of regulation as described in *E. coli*, cannot take place in *S. marcescens* (Hutsul & Worobec, 1997). Hence, it has been suggested that osmoregulation in *S. marcescens* involves *micF*-RNA. The *micF* gene from *S. marcescens* has been described (Hutsul & Worobec, 1994), and the secondary structure of the *S. marcescens* 4.5S *micF*- RNA has been predicted (Schmidt *et al.*, 1995). On the basis of this predicted structure

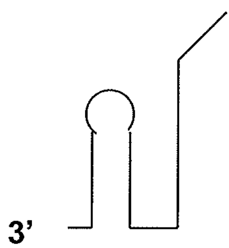
and the known hybridization pattern of *E. coli ompF* mRNA/*micF*-RNA, the hybridization of *S. marcescens ompF* mRNA/*micF*-RNA was predicted (Fig.1.3.). It has therefore been predicted that *micF* could function in regulating the OmpF porin in *S. marcescens* (Hutsul & Worobec, 1997) and account for what was observed experimentally.

Fig. 1.3. Predicted hybridization of the *S. marcescens micF*-RNA with the 5' end of the *S. marcescens ompF* mRNA (Hutsul & Worobec, 1997)

ompF-RNA

5' ······ AAUGAGGGUAAUAAUGAUGAAGC········ 3'
UUACUUCCAUUAUUACUACUUCG

U U
A A
C U
U U
U



3'

micF-RNA

1.7. Hypothesis and Objectives

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

Specific Objectives:

1. Construction and characterization of OmpC porin-deficient strains of *S. marcescens*.
2. Construction and analysis of genetically altered OmpC porin.
3. Examination of the regulation of *S. marcescens* OmpF and OmpC porin expression.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Tables 2.1 and 2.2, respectively. All strains of *S. marcescens* were grown in either Luria-Bertani (LB) or Tryptic Soy (T-Soy) Broth at 28°C or 37°C, depending on the procedure. All strains of *E. coli* were grown in LB medium at 37°C for most experiments. All media were supplemented when necessary with ampicillin (100 μg), chloramphenicol (25 μg), kanamycin (40 μg) and streptomycin (40 μg). M9 minimal medium, made from Difco™ M9 Minimal Salts, was used alone or supplemented with salicylate and sucrose, and adjusted to different pHs for the β-galactosidase assays. Freezer stocks of bacterial cultures were prepared by addition of dimethyl sulfoxide (DMSO) to a final concentration of 7% (v/v) to mid-log phase cultures.

2.2. DNA isolation, digestion and purification

Procedures described in the Current Protocols in Molecular Biology (Ausubel *et al.*, 1989) were followed to isolate plasmid and genomic DNA, to perform restriction enzyme digestions and agarose gel electrophoresis, to purify DNA, and to carry out DNA ligation.

2.3. Preparation of competent cells and transformation

Cells were made competent using a $CaCl_2$ treatment method (Ausubel *et al.*, 1989). They were incubated overnight in LB broth, and subcultured into fresh LB

Table 2.1. Bacterial strains used in this study

Strain	Characteristic	Source/Reference
S. marcescens UOC-67	Wild-type strain	ATCC 13880
E. coli NM522	*supE thi Δ (lac-proAB) hsd5 F' {proAB*$^+$ *lacI*^{q} *lacZΔM15}*	Promega
E. coli BZB 1107	B^E, *ompF::Tn5* (Km^r)	(Jeanteur *et al.*, 1994)
E. coli MT616	MT607 *(pro-82 thi-l hsdR17 supE44)*:pRK600	(Finan *et al.*, 1986)
E. coli CC118	*ara D 139 Δ (ara, leu) 7697 Δ lacX74 phoA20 galE galK thi rpsE rpoB argEam recA1*	Dr. C. Manoil, University of Washington, Seattle, USA
S. marcescens UOCTC	*S. marcescens* UOC-67 *ompC*::pKNGBAK	This study

Km^r = kanamycin resistance

Table 2.2. Plasmids used in this study

Plasmids	Characteristics	Source/Reference
pUC18	ColE1 replicon, Ap^r; *lacZ*'	Invitrogen
p8SX	pTZ with 6.1 Kb *SstI*/*Xba*I fragment; *ompC*	(Hutsul, 1996)
p8BK(+)	pKS(+) with 2.2 Kb *Bam*HI/*Kpn*I fragment; *ompC*	(Hutsul, 1996)
p8BAK	p8BK(+) missing *Aat*II 400 bp in the *Bam*HI/*Kpn*I fragment; *ompC*	This study
pM8BH12(+)	pM8BH(+) with deletion from *Hind*III leaving a 2.5 Kb insert; *ompC*	(Hutsul, 1996)
pAKIN	pM8BH12(+) with D^{112} to G and M^{114} to D	This study
pM2.4(-)	p8PK(-) with M^{114} to D	(Hutsul, 1996)
pM3.6(-)	pM2.4(-) with D^{112} to G	This study
pKNG101	Suicide vector, *pir*$^-$ *oriR6K* *mobRK2* *sacB* Sm^r	(Kaniga *et al.*, 1991)
pKNGBAK	pKNG101 with 1.8 Kb *ompC* deletion fragment	This study
pRK600	ColE1 replicon, with RK2 transfer gene, Cm^r	(Finan, *et al.*, 1986)
pOY009	Contains promoter- deleted *lacZ*-*lacY* operon from pKM005	(Ozawa *et al.*, 1987)
pOY012	Contains *E. coli ompF* promoter-*lacZ* fusion	(Ozawa, *et al.*, 1987)

Table 2.2. continued

Plasmids	Characteristics	Source/Reference
pOYL338W	Contains *E. coli ompC* promoter-*lacZ* fusion	(Ozawa, *et al.*, 1987)
pK98C	Contains *S. marcescens ompC* promoter-*lacZ* fusion	Ayush Kumar, University of Manitoba
pOY005	Contains *S. marcescens ompF* promoter-*lacZ* fusion	Sanela Begic, University of Manitoba

Ap^r = ampicillin resistance, Cm^r = chloramphenicol resistance,

Sm^r = streptomycin resistance

broth in the morning and incubated for about 3 hours at 37°C. Cells were harvested by centrifugation at 1000 x g for 5 minutes at 4°C. The cells were resuspended in ice cold 0.1 M $CaCl_2$, and incubated on ice for 30 minutes. Cells were collected by centrifugation at 1000 x g for 5 minutes at 4°C, and finally resuspended in ice cold 0.1 M $CaCl_2$. This was sometimes used immediately, or stored at -70°C after the addition of 600 µL 50% (w/v) glycerol.

To carry out transformation, 100 µL of competent cells were mixed with 0.01-1 µg of DNA and incubated on ice for 30 minutes. Cells were heat shocked for 3 minutes at 42°C and cooled on ice for 5 minutes. One millimeter of LB broth was added and cells were incubated at 37°C for about 1 hour. Finally, 100 µL of culture was spread plated on selective LB agar medium.

2.4. Conjugation

Conjugation was done using triparental mating as described by Goldberg and Ohman (Goldberg & Ohman, 1984). Strains were incubated overnight; donor [*E. coli* CC118λ (pKNGBAK)] and helper [*E. coli* MT616 (pRK600)] strains at 30°C, and recipient strain (*S. marcescens* UOC-67) at 42°C. The following morning, 0.1 mL of each was added to 2 mL L-broth and mixed. The mixture was filtered using a 0.45 µm Nalgene filter unit. Filter was removed and placed on LB agar, and incubated overnight at 28°C. The cells from the filter were then resuspended in 2-5 mL of 0.9% (w/v) NaCl and one part of this was suspended in 100 parts of same solution. One hundred microliter each of diluted and concentrated cell suspensions was spread plated on selective medium and

incubated at 37°C for 24-48 hours.

2.5. DNA Amplification by PCR (Polymerase Chain Reaction)

PCR was done following standard procedures (Ausubel *et al.*, 1989). Primers OMPCF (forward) and OMPCR (reverse) shown in Table 2.3 were used to confirm successful deletion of ≈ 400 bp within the *ompC* sequence, and transconjugants after the integration of pKNGBAK into the genome of *S. marcescens* (UOC-67). For further confirmation of transconjugants, primers SACBF and SACBR were used. Primer D112G was used in site-directed mutagenesis by PCR to change Asp to Glu in the PEFDGM motif of the third eyelet loop of OmpC porin. Primer MMR was used to analyze transformants after site-directed mutagenesis. Analysis of transformants was done by MAMA (Mismatch Amplification Mutation Assay) PCR (Qiang et al., 2002). The unique feature of this is the use of a reverse primer that has a single nucleotide mismatch at the 3' extremity, which renders *Taq* polymerase unable to extend the primer.

For routine analysis, *Taq* polymerase from New England Biolabs was used in PCR reactions. For site- directed mutagenesis, Platinum© *Pfx* polymerase from Invitrogen was used.

2.6. Cell envelope preparation

Cell envelopes were prepared following the method described by Lugtenberg *et al.* (Lugtenberg *et al.*, 1975). Fifty millimeters of culture was grown

Table 2.3. Primers used in this study

Name	Sequence	Description
OMPCF (forward)	CTC CAG CAA CAA CGG CGT GGA	Amplification of ≈0.8 Kb sequence within *ompC*
OMPCR (reverse)	TAG CCG ACG AAC GGA CGC AGA	Same as above
D112G	CCT GAG TTC GGT GGT GAC ACC TAC G	Site directed mutagenesis within PEFDGM motif of *ompC*
MMR	CGG CGC CGT AGG TGT CAC TAC	Analysis of mutants from above by MAMA-PCR (Mismatch Amplification Mutation Assay-PCR)
SACBF	GTT CAG CAG GAA GCT AGG CG	Analysis of transconjugants after *S. marcescens-E. coli* conjugation
SACBR	CCT TTA CTA CCG CAC TGC TG	Same as above

overnight, and cells were harvested by centrifugation. Pellet was resuspended in 20 mL of 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 centrifugation at 1200 x g for 10 minutes. The supernatant was collected and centrifuged at 100,000 x g for 1 hour to remove cell membranes. Cell membranes were resuspended in 50 mM Tris-HCl pH 7.9. Resuspension of cell membranes was not carried out in situations where the purpose of the cell envelope preparation was porin isolation. The method of porin isolation used is outlined in detail below.

2.7. Isolation of porins

Using the method described by Malouin *et al.* (Malouin *et al.*, 1990), cell envelope preparations obtained from above were resuspended in 2% w/v SDS and incubated for 30 minutes at 32°C for solubilization of cytoplasmic membrane and non-porin outer membrane proteins. This was followed by centrifugation at 100,000 x g for 30 minutes. To further remove OmpA, the pellet was resuspended in 50 mM Tris-HCl, pH 7.2, 1% w/v SDS, and 5 mM EDTA. Following solubilization at 37°C for 1-2 hours, sample was centrifuged as above for 1 hour. Using NaCl buffer: 50 mM Tris-HCl, pH 7.7, 1% w/v SDS, 5 mM EDTA, 0.4 M NaCl and 3 mM NaN_3, porins were solubilized from the pellet at 37°C for 2 hours. Protein concentration was determined through optical density measurement (1 mg/mL protein has absorbance of 1 at OD_{280}), and samples

were analyzed by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

2.8. Liposome swelling assay

Liposome swelling assay was carried out as previously described (Nikaido & Rosenberg, 1983). Phosphatidycholine (6.2 μmole) and dicetylphosphate (0.2 μmole) were placed in a test tube and allowed to dry using mild heat. Dried lipid film was suspended in 200 μL (equivalent to 10μg of porin) of aqueous suspension of isolated porin from above. Lipid was dispersed first by shaking, and then by sonication using a Fisher Sonic Dismembrator Model 300 for three 15 seconds bursts at 35%. Liposomes were dried in a Speed-Vac under medium heat and stored overnight in the dark in a vacuum desicator. Next day, 400 μL of 12mM stachyose, 4mM Na-NAD (pH 6.0), 1mM imidazole-NAD (pH 6.0) was added to the dried liposomes, and left undisturbed at room temperature for at least 2 hours. Resuspension was completed by gently shaking with the hand. The suspension was then filtered through a 8 μm Millipore membrane filter to remove large aggregates. To measure liposome swelling, an isotonic concentration of solutes was prepared in 1 mM Na-NAD (pH 6.0), 1 mM imidazole-NAD (pH 6.0). Twenty microliters of liposome suspension was added to a cuvette containing 600 μL of solute, mixed quickly and liposome swelling was measured following optical density change at 400 nm every 10 seconds over a period of 90 seconds.

2.9. SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Lugtenberg *et al.* (Lugtenberg*, et al.*, 1975) at a polyacrylamide concentration of 12% (w/v). Before loading, samples were heated at 90-100°C for 10 minutes in 12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 2% (v/v) glycerol, 0.0125% bromophenol blue, and 1% (v/v) mercaptoethanol. Samples were separated at 100-200 Volts with a 3% acrylamide stacking gel over the separating gel. Prestained broad range markers (New England Biolabs) were used for the determination of molecular weights. Gels were stained for 1 hour using Coomassie Blue staining solution {30% (v/v) ethanol, 10% (v/v) acetic acid, 0.05 (w/v) Coomassie Blue R-250}. Gels were destained using a mixture of 15% (v/v) methanol and 7% (v/v) glacial acetic acid, initially for 1 hour, and then overnight with fresh destaining solution.

2.10. β-galactosidase assay

The method of Miller (Miller, 1972) was followed with little modification. A single colony of the test organism was used to inoculate 3 mL of LB and incubated at 37°C overnight with gentle shaking. The following day, 100 µL of overnight culture was subcultured into 4 mL of fresh M9 minimal medium, supplemented with $MgSO_4$ (2 mM), Vitamin B1 (20 µg/mL), $CaCl_2$ (0.1 mM), glucose [0.4% (w/v)], and ampicillin (100 µg/mL). Further supplementation with sucrose [5, 8, & 10% (w/v)] or salicylate (1, 5, & 8 mM), and adjustment of pH (pH 6, 7 & 8) was carried out depending on environmental conditions to be

tested. Cultures were incubated at 37°C with gentle shaking until 2-5 × 10^8 cells/mL (OD_{600}:0.28-0.70) was reached. Once this was achieved, cultures were cooled down by immersing in an ice bucket containing a mixture of ice and water for 20 minutes, and final OD_{600} taken using Milton Roy Spectronic 601 spectrophotometer.

To assay the level of β-galactosidase enzyme produced under different growth conditions, 900 μL of Z-Buffer [$Na_2HPO_4.7H_2O$ (60 mM), $NaH_2PO_4.H_2O$ (40 mM), KCl (10 mM), $MgSO_4.7H_2O$ (1 mM) & β-mercaptoethanol (50 mM)] was placed in an eppendorf tube, along with 100 μL of each culture. One drop of toluene was added to this mixture and immediately vortexed for 10 seconds. Toluene was allowed to evaporate by placing tubes on a shaker at room temperature for not less than 2 hours. Tubes were place in a water bath at 28°C for 5 minutes, removed, and 0.2 mL of 4 mg/mL o-Nitrophenyl β-D-galactopyranoside [ONPG] (Sigma-Aldrich) was added to each and mixed rapidly for a few seconds. The reaction was stopped by adding 500 μL of a 1M Na_2CO_3 solution after sufficient yellow color has developed. Finally, the content of the tubes were spun to remove cells and debris, and the OD_{420} of supernatant was read.

2.11. Fluoroquinolone accumulation assays

Fluoroquinolone accumulation assays were carried out using a method described by Mortimer and Piddock (Mortimer & Piddock, 1991) with minor modifications. T-soy broth (300 mL) was inoculated with the appropriate bacterial

strain and incubated overnight at 37°C. Cells were harvested by centrifugation at 12,000 x g for 5 minutes. The pellet was washed with 50 mM sodium phosphate buffer (pH 7.0) and centrifuged as above. The supernatant was discarded, and the pellet resuspended in same buffer to obtain a cell suspension with OD_{600} of 20.0. Cells were equilibrated for 10 minutes by placing in an incubator at 37°C. Antibacterial agents to be tested were added to a final concentration of 10 µg/mL, and 500µL of culture was removed at the end of a ten minute period, and added to cold 50 mM sodium phosphate buffer, pH 7.0. The samples were centrifuged at 19,600 x g for 10 minutes. The pellet was washed with thesame buffer as above, centrifuged, and resuspended in 1 mL of 0.1 M glycine hydrochloride, pH 3.0. After incubation for at least 15 hours at room temperature, samples were centrifuged at 19,600 x g for 10 minutes, and fluorescence of the supernatant was measured using a Shimadzu RF-1501 spectrofluorometer. Excitation (Ex) and emission (Em) wavelengths for the fluoroquinolones tested are as follows: ciprofloxacin (Ex=279, Em=447), norfloxacin (Ex=281, Em=440), and ofloxacin (Ex=292, Em=496). The amount of the accumulated fluoroquinolone was calculated by referring to a standard curve for respective antibacterial agent. Standard curves were constructed by making serial dilution of fluoroquinolone (100-700ng/µL) in 0.1 M glycine hydrochloride (pH 3.0), followed by measurement of fluorescence. Results were expressed as amount in nanogram (ng) of fluoroquinolone accumulated per dry weight in milligram (mg) of bacteria. Dry weight was obtained after drying pellet in vacuum desicator overnight.

3. RESULTS AND DISCUSSION

3.1. Construction of OmpC porin deficient strain of *S. marcescens*

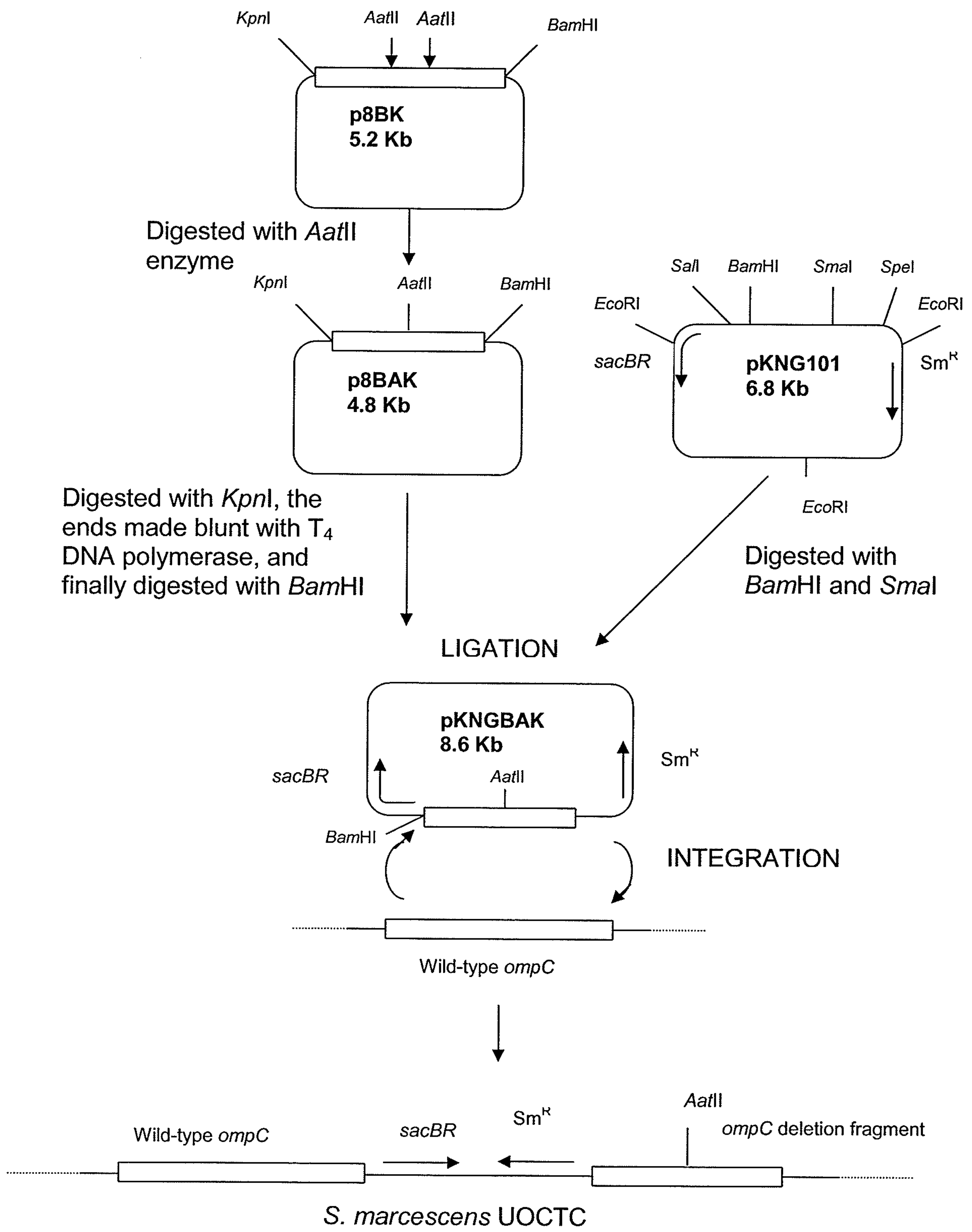
3.1.1. Introduction

The pore size of *E. coli* OmpF and OmpC were estimated by black lipid bilayer experiments to be 1.2 nm and 1.1 nm respectively. This very small difference was shown to account for a twofold reduction in the permeability rate of glucose through OmpC when compared to OmpF in liposome swelling experiments (Nikaido & Rosenberg, 1983). Similarly, the pore sizes of *S. marcescens* OmpF and OmpC have been estimated, using the same method, to be comparable to those of the *E. coli* porins (Hutsul, 1996; Malouin, *et al.*, 1990). It has always been expected that loss of one or both of the porins will result in increased resistance to certain antibiotics, as was observed in a clinical isolate of *S. marcescens* that was resistant to both aminoglycosides and β-lactams (Goldstein *et al.*, 1983). Recently, another study showed that clinical isolates of *S. marcescens* had a 30 to 200-fold reduced permeability coefficients due to porin deficiency when compared with wild type strains (Ruiz, *et al.*, 2003). In *K. pneumoniae*, loss of OmpK36 (homolog of OmpC) has been associated with both cefoxitin resistance and increases in the Minimum Inhibitory Concentrations (MICs) of other cephalosporins and quinolones (Martinez-Martinez *et al.*, 1996). In another experiment, restoration of OmpK36, in OmpK36 deficient clinical isolates of *K. pneumoniae*, lowered MICs of tested cephalosporins (Martinez-Martinez, *et al.*, 1999). With these previous results in mind, I planned to further examine the role of porins in antibiotic resistance of *S. marcescens*.

3.1.2. Cloning a of deletion mutant fragment of the *S. marcescens ompC* gene

From the library of clones of the *ompC* coding region created earlier (Hutsul, 1996), p8BK(+) was selected. This plasmid consists of a 2.2 Kb fragment of *ompC* cloned into pBluescript. Two unique *Aat*II restriction sites separated by about 400 bases were identified to be a feasible target for creating a deletion mutation in the *ompC* coding region. p8BK(+) was digested with *Aat*II, the fragments gel isolated and remaining fragment religated. This was transformed into *E. coli* NM522 and named *E. coli* p8BAK. After transformation, mutant clone *E. coli* p8BAK was confirmed by restriction digest, PCR and sequencing. The deletion fragment was isolated from *E. coli* p8BAK and transferred into replacement vector pKNG101 (Kaniga, *et al.*, 1991). pKNG101 contains a conditional origin of replication (*oriR6K*), the *strAB* genes encoding streptomycin phosphotransferase (Sm^R), an origin of transfer (*mobRK2*), the *sacB* gene mediating sucrose sensitivity, and a few multiple cloning sites. The deletion fragment was cloned into pKNG101 by digesting pKNG101 with *SmaI* and *BamHI*. p8BAK was digested with *KpnI*, the ends made blunt using T_4 DNA polymerase, and digested with *Bam*HI. Ligation of both fragments gave pKNGBAK, and was cloned in *E. coli* CC118 λ *pir*. pKNGBAK was confirmed by PCR, using primers OMPCF and OMPCR (Table 2.3.), and restriction digest. (Fig. 3.1.)

Fig. 3.1. Construction of a deletion fragment of *ompC* and the integration of pKNGBAK into *S. marcescens* UOC-67 genome. Descriptions of plasmids are given in Table 2.2.

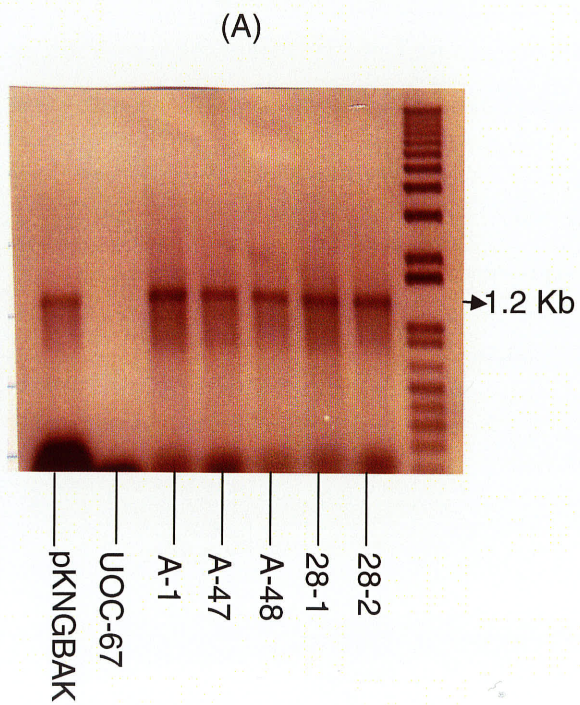

KpnI
AatII
AatII
BamHI
p8BK
5.2 Kb
Digested with AatII enzyme
KpnI
AatII
BamHI
p8BAK
4.8 Kb
SalI
BamHI
SmaI
SpeI
EcoRI
EcoRI
sacBR
pKNG101
6.8 Kb
Sm^R
EcoRI
Digested with KpnI, the ends made blunt with T4 DNA polymerase, and finally digested with BamHI
Digested with BamHI and SmaI
LIGATION
pKNGBAK
8.6 Kb
sacBR
AatII
Sm^R
BamHI
INTEGRATION
Wild-type ompC
AatII
Sm^R
Wild-type ompC
sacBR
ompC deletion fragment
S. marcescens UOCTC

3.1.3. Integration of *ompC* deletion fragment into the *S. marcescens* genome

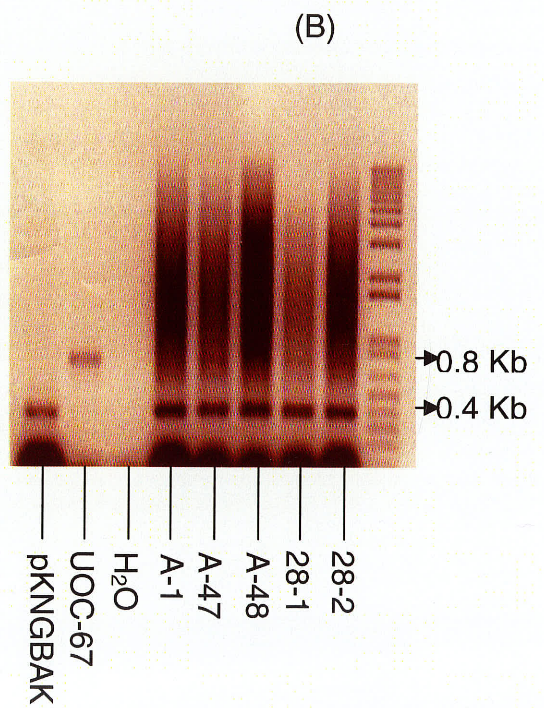
Conjugation between *E. coli* strain harboring pKNGBAK and *S. marcescens* was carried out using *E. coli* strain MT616 as a helper (Goldberg & Ohman, 1984). Transconjugants were selected on LB agar plates supplemented with ampicillin and streptomycin. Analysis of transconjugants was done by PCR using both *sacB* and *ompC* forward and reverse primers (Table 2.3). Transconjugants gave expected bands with both primers confirming the integration of the entire plasmid into the chromosome (Fig. 3.2.). To knock out *ompC* from the genome of *Serratia marcescens*, transconjugants were streaked on LB agar supplemented with 5, 7.5, and 10% sucrose. This did not result in excision of suicide vector, pKNG101, mediating sucrose sensitivity, and loss of the wild-type *ompC* gene. The transconjugants should be sensitive to sucrose due to the synthesis of levans, a lethal compound, catalyzed by levansucrase, a product of *sacB* gene (Kaniga *et al.*, 1991). It is not understood why the transconjugants were resistant to sucrose, as this system has been used in another investigation to knock out the *luxS* gene of *S. marcescens* (Coulthurst *et al.*, 2004). One would not predict that the knock out of *ompC* gene would be lethal to the cell since there is another major outer membrane porin OmpF, which should compensate (Hancock, 1984). The absence of sensitivity to sucrose in transconjugants might have been due to inadequate or total lack of expression of *sacB* gene due to spontaneous mutations or some rearrangement that took place in the genome (Hanson *et al.*, 1998). This experiment was repeated a number of

Fig. 3.2. Agarose gel electrophoresis of PCR products obtained from *S. marcescens* genomic preparations. (A) PCR products obtained using *sacB* primers; (B) PCR products obtained using *ompC* primers. Plasmid pKNGBAK [*ompC*Δ-pKNG101] is a +ve control; UOC-67 [wild-type *S. marcescens*] is a –ve control; A-1, A-47, A-48, 28-1, and 28-2 are selected *S. marcescens* transconjugants [*ompC*Δ-pKNGBAK], H_20 = Water [negative control]. Molecular weights of PCR products are represented by 1.2 Kb (*sacB*), 0.8 Kb (*ompC*), and 0.4 Kb (*ompC*Δ).

(A)



(B)



times without any success. Hence, I decided to forgo this strategy and focus on mutated porin production and analysis as discussed below in 3.2.

3.2. Construction and analysis of genetically altered OmpC porin

3.2.1. Introduction

Transmembrane strands of outer membrane porins are connected on the external sides by "loops" which are often long (Nikaido, 2003). Loop 3 connects strands 5 and 6, and is different than other loops as a result of its unique length (33 residues in *E. coli* OmpF). Loop 3 folds into the barrel to produce a narrowing of the channel, known as the eyelet region. This region is very important in determining the size and ion selectivity of the porin channels (Koebnik, *et al.*, 2000). At the tip of this loop, a conserved motif PEFG^{112}GD, exists in most enterobacterial porins. This motif has been proposed to aid in formation of a turn at the tip of the third external loop (Cowan, *et al.*, 1992). The *Serratia marcescens* OmpC deviates from this motif, having instead the sequence PEFD^{112}GM. In addition, a group in France recently reported a clinical strain of *Enterobacter aerogenes* with very high β-lactam resistance producing an Omp36 (homolog of OmpC) porin with a G^{112}D substitution in its eyelet region (Thiolas *et al.*, 2004). The importance of the third external loop for size and ion selectivity determination has led us to postulate that this unique arrangement in the *S. marcescens* OmpC porin might be correlated with its unusually high antibiotic resistance.

3.2.2. Site-directed mutagenesis of *ompC* gene

Earlier, two separate mutations were introduced into the PEFDGM motif of *S. marcescens* OmpC to create PEF**G**GM and PEFDG**D** (Hutsul, 1996). These were introduced individually into a porin-deficient *E. coli* strain, BZB1107. Comparison of the relative permeability of *S. marcescens* wild-type porin OmpF and OmpC and the mutant OmpC porins to cephaloridine, cephalothin, cefotaxime and glucose by liposome swelling assays showed that OmpC with the PEF**G**GM mutation and wild-type OmpF both allowed more rapid diffusion of uncharged solutes than wild-type OmpC porin. No change in permeability was seen with OmpC with PEFDG**D** mutation (Hutsul, 1996). Based on the results obtained from the analysis of the permeability rates of solutes through the single mutant porins, it was proposed that a double mutant with the conserved motif PEFGGD be created, with the rational being that there would be a synergistic effect of the two mutations.

To create the double mutant, pM2.4(-) was selected from our library. pM2.4(-) is made up of pBluescript(-) containing a 0.48 Kb *PstI*/*KpnI* fragment of *ompC* with M114D single mutation. This was sequenced, to confirm the presence of the single mutation, and used as a template in site-directed mutagenesis by PCR. Platinum© *Pfx* DNA polymerase from Invitrogen was used in the PCR setup, along with its PCRx Enhancer solution. Primer D112G (Table 2.3) was used to introduce the change into the DNA sequence of *ompC*. Double mutants were confirmed by MAMA (Mismatch amplification mutation assay) PCR (Qiang *et al.*, 2002) and sequencing. The rationale behind MAMA PCR is that a single

nucleotide mismatch at the 3' extremity of the annealed reverse primer renders *Taq* polymerase unable to extend the primer. The 0.48 Kb *PstI/KpnI* fragment with the double mutation was recloned into pM8BH12(+) and transformed into the porin-deficient *E. coli* strain BZB1107 for porin expression. Cell envelope preparations of BZB1107 and recombinants were prepared and used for porin isolation (Malouin *et al.*, 1990). Cell envelope and isolated porins were analyzed by SDS-PAGE (Fig. 3.3).

3.2.3. Analysis of mutated *S. marcescens* OmpC porin

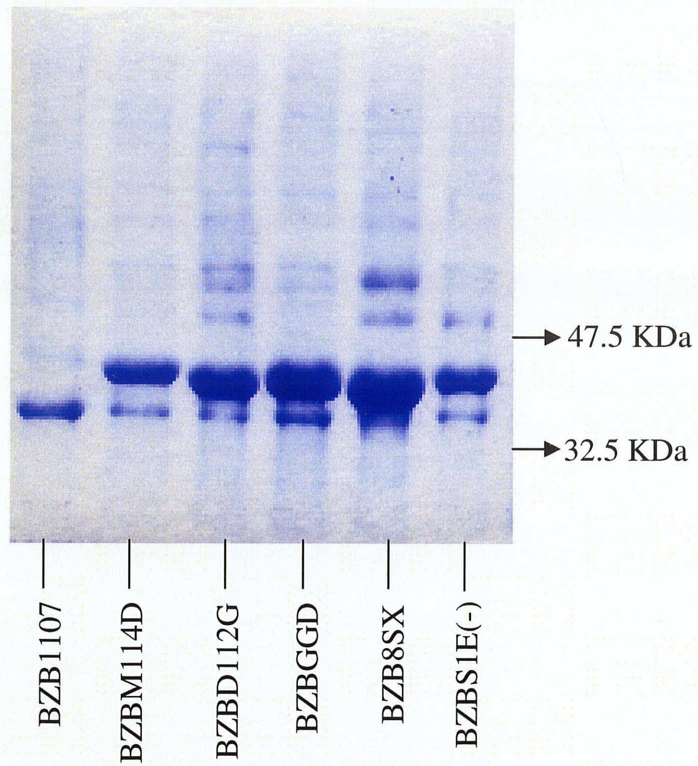
Two methods, liposome swelling and fluoroquinolone accumulation assays, were used to examine the function of mutant porins, vis-à-vis the wild-type OmpF and OmpC porins.

3.2.3.1. Liposome swelling assays

The results obtained from liposome swelling assays conducted are presented in Figs. 3.4. to 3.9. Data used to construct these plots are found in Tables 7.1 to 7.3 in the Appendix. All results of liposome swelling assays presented are representative of five different assays performed for all solutes. Figs. 3.4, 3.5 and 3.6 illustrate the rate of swelling of proteoliposomes with time i.e. the rate of optical density decrease, with time, while Figs. 3.7, 3.8 and 3.9 compare the permeability rates of all solutes through mutant and wild-type porins. The slopes of curves illustrated in Figs. 3.4, 3.5 and 3.6 and presented in Table 3.1 were used to determine permeability rates. The slopes were

Fig. 3.3. (A) SDS-PAGE of cell envelope preparations of BZB1107 (porin deficient *E. coli* B strain), plus various recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. (B) SDS-PAGE of specified porins isolated from the above envelopes. Molecular weights of porins fall within 32.5 - 47.5 KDa.

(A)



(B)

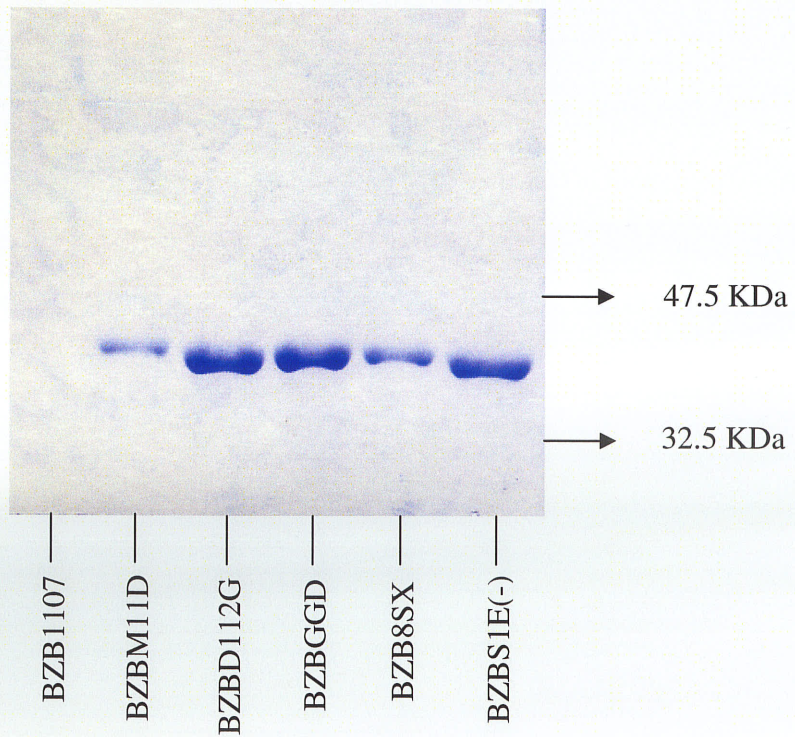


Fig. 3.4. Liposome swelling assay results of reconstituted porins isolated from the cell envelopes of porin deficient *E. coli* BZB1107 recombinant strains in 18 mM glucose. To assess swelling rate, 20 µL of prepared proteoliposome was added to 600 µL of 18 mM glucose, mixed rapidly, and optical density decrease measured every 10 seconds over 90 seconds. Results are representative of five other assays. Liposomes without porins, represented by w/o on the plot, were used as a negative control.

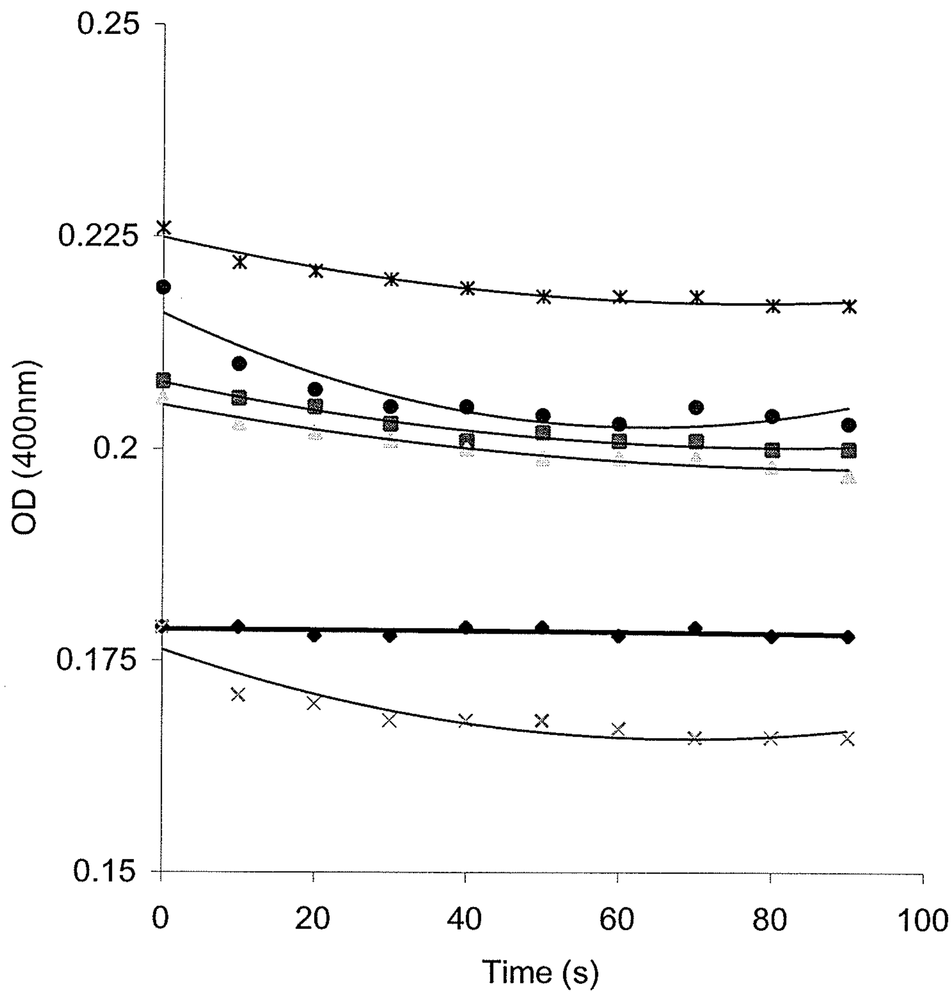

0.25
0.225
0.2
0.175
0.15
OD (400nm)
0
20
40
60
80
100
Time (s)
w/o
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E(-)

Fig. 3.5. Liposome swelling assay results of reconstituted porins isolated from the cell envelopes of porin deficient *E. coli* BZB1107 recombinant strains in 18 mM maltose. To assess swelling rate, 20 µL of prepared proteoliposome was added to 600 µL of 18 mM maltose, mixed rapidly, and optical density decrease measured every 10 seconds over 90 seconds. Results are representative of five other assays. Liposomes without porins, represented as w/o on the plot, was used as a negative control.

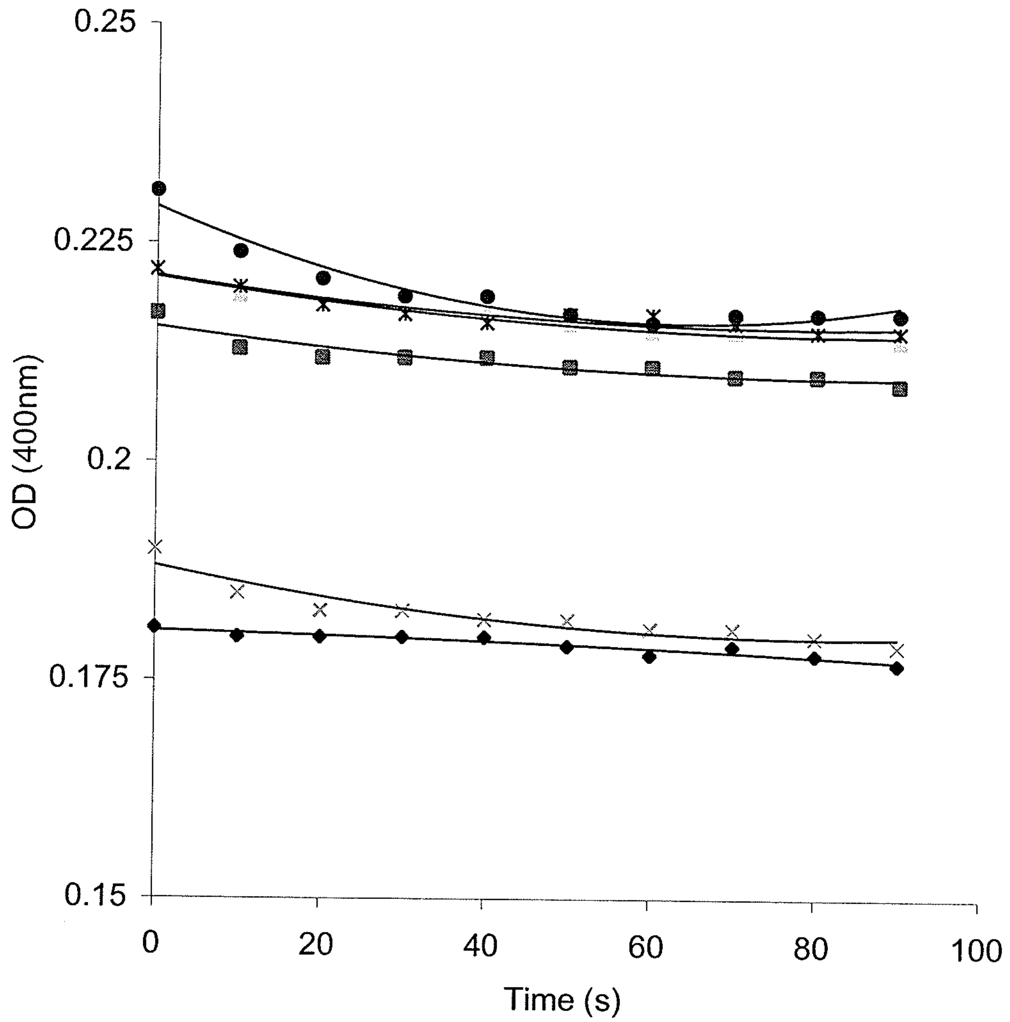

0.25
0.225
0.2
0.175
0.15
OD (400nm)
0
20
40
60
80
100
Time (s)
w/o
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E

Fig. 3.6. Liposome swelling assay results of reconstituted porins isolated from the cell envelopes of porin deficient *E. coli* BZB1107 recombinant strains in 9 mM ampicillin. To assess swelling rate, 20 µL of prepared proteoliposome was added to 600 µL of 9 mM ampicillin, mixed rapidly, and optical density decrease measured every 10 seconds over 90 seconds. Results are representative of five other assays. Liposomes without porins, represented as w/o on the plot, was used as a negative control.

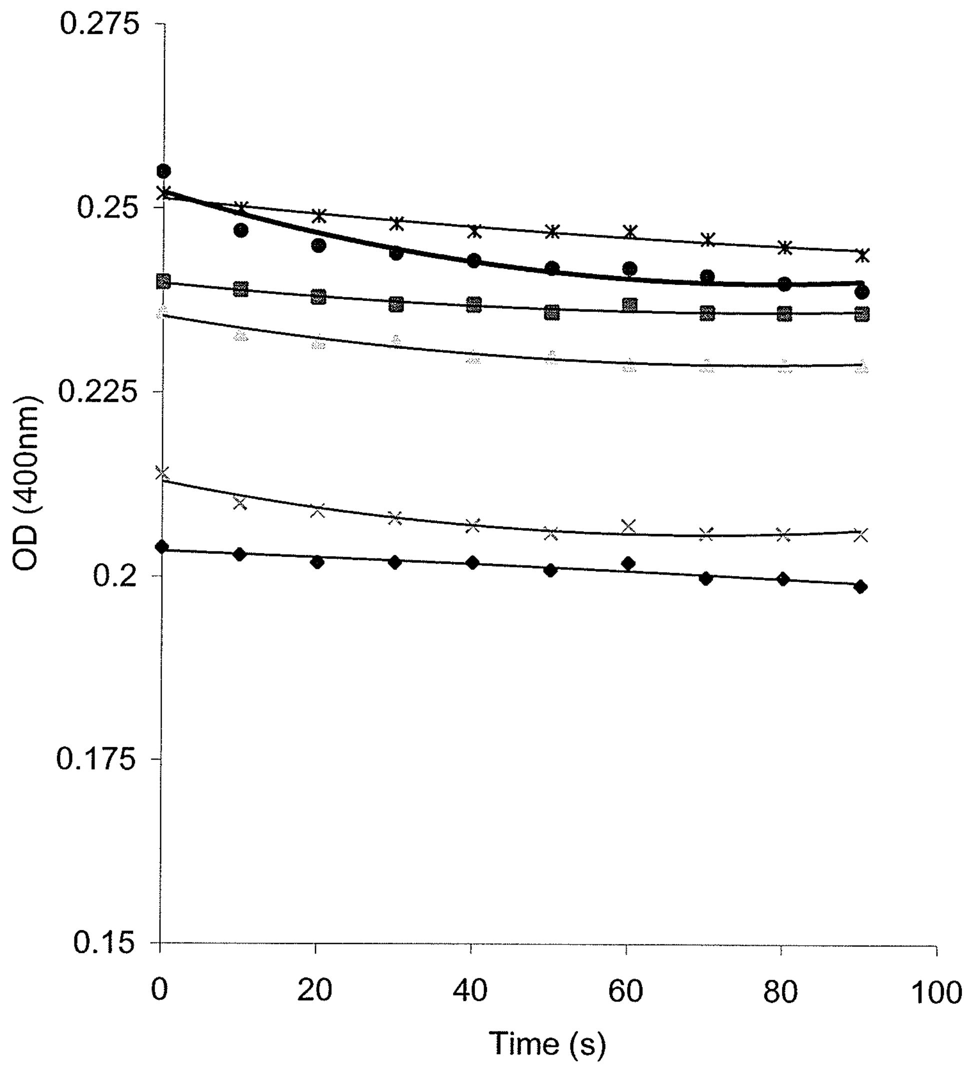

0.275
0.25
0.225
0.2
0.175
0.15
OD (400nm)
0
20
40
60
80
100
Time (s)
w/o
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E(-)

Table 3.1. Permeability rates of proteoliposomes suspended in 18 mM glucose, 18 mM maltose or 9 mM ampicillin

Strains	Glucose	Maltose	Ampicillin
BZBM114D	2×10^{-4}	1×10^{-4}	1×10^{-4}
BZBD112G	2×10^{-4}	2×10^{-4}	2×10^{-4}
BZBGGD	3×10^{-4}	2×10^{-4}	2×10^{-4}
BZB8SX	2×10^{-4}	1×10^{-4}	1×10^{-4}
BZBS1E(-)	4×10^{-4}	4×10^{-4}	3×10^{-4}

Rates are derived from the slopes (coefficient of x) of curves in Figs. 3.4, 3.5 and 3.6 using the formular $y = ax^2 + bx + c$, where a, b, and c are contants, and $y=OD_{400}$ (Optical density of proteoliposome suspension at 400 nm).

w/o = Without porins, BZBM114D = M114D OmpC mutant, BZBD112G = D112G OmpC mutant, BZBGGD = M114D and D112G double mutant, BZB8SX = wild-type OmpC, BZBS1E(-) = wild-type OmpF. Results are representative of five other assays for all solutes used.

N. B. All recombinants and wild-type *S. marcescens* porins are expressed in BZB1107: porin deficient *E. coli* B strain

determined from the coefficient of x of the line equations represented by $y = ax^2 + bx + c$.

The permeability rate of glucose (M_r = 180.16 Da) through the porins of BZBS1E(-) was twice of that observed for BZBM114D, BZBD112G and BZB8SX. The permeability rate through the porins of BZBGGD, which was proposed would be the same as BZBS1E(-) expressing OmpF, was 25% less, but was 50% more than the rates seen for BZBM114D, BZBD112G and BZB8SX. In the case of maltose (M_r = 360.32 Da), BZBS1E(-) still had the highest penetration rate, twice that of BZBD112G and BZBGGD, but four times that of BZBM114D and BZB8SX. The pattern observed for ampicillin (M_r = 371.39 Da) is similar to maltose, but the rate of penetration through BZBS1E(-) was 50% more than that of BZBD112G and BZBGGD and three times that of BZBM114D and BZB8SX.

The penetration rates of glucose through the porins of BZBM114D, BZBD112G, BZB8SX and BZBS1E(-) is similar to those observed in an earlier study (Hutsul, 1996). This confirms further that the change $M^{114}D$ did not confer any increase on pore size, since the penetration rate through BZBM114D porins is not different than BZB8SX expressing the wild-type porins. The presence of both $D^{112}G$ and $M^{114}D$ seemed to be synergistic as the penetration rate of glucose through BZBGGD porins is 50% more than BZBD112G porins. The earlier study (Hutsul, 1996) referred to above showed a difference between BZBM114D and BZBD112G porins with the latter allowing more penetration of glucose. The study also investigated the penetration of cephaloridine (M_r = 415.5 Da), cephalothin (M_r = 418.4 Da) and cefotaxime (M_r = 477.4), revealing that the

diffusion of uncharged cephaloridine occurred more slowly than glucose in all porins tested, and the diffusion of the charged cephalothin and cefotaxime occurred more slowly without differences in their rates of diffusion through tested porins.

Comparing the results of the liposome swelling assays for the different solutes tested, it was observed that the penetration rate of glucose and maltose through wild-type *S. marcescens* OmpF porin is the same (Figs 3.7-3.9). This is unusual, as the molecular size effect should play a role in the permeability rate of the solutes. The smaller the size of the molecule the higher the penetration rate should be, as demonstrated by an experiment involving the transmembrane pores of *E. coli* (Nikaido & Rosenberg, 1981), meaning that the rate of penetration of glucose should be about twice that of maltose. However, the penetration rate of both solutes through *S. marcescens* OmpF is higher than its wild-type OmpC and recombinant OmpC porins of BZBM114D, BZBD112G and BZBGGD. The rate of penetration of glucose through BZBGGD porins is 25% less than OmpF. This was surprising since both porins now have the same consensus sequence PEFGGD in the third eyelet loop. This difference may be due to a pore channel conformational difference that cannot be explored within the scope of the present study. No difference was seen in the penetration rates (Table 3.1) of glucose through the recombinant *S. marcescens* OmpC porins of BZBM114D, BZBD112G, and its wild-type OmpC. The earlier study showed that BZBD112G had a higher penetration rate than *S. marcescens* wild-type OmpC porin and BZBM114D porin. The rate of penetration of maltose through the

Fig. 3.7. Comparison of permeability rates of glucose through liposome reconstituted porins isolated from the cell envelopes of *E. coli* BZB1107 recombinants. BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. Results are representative of five assays and data used to construct the plot are presented in Table 3.1.

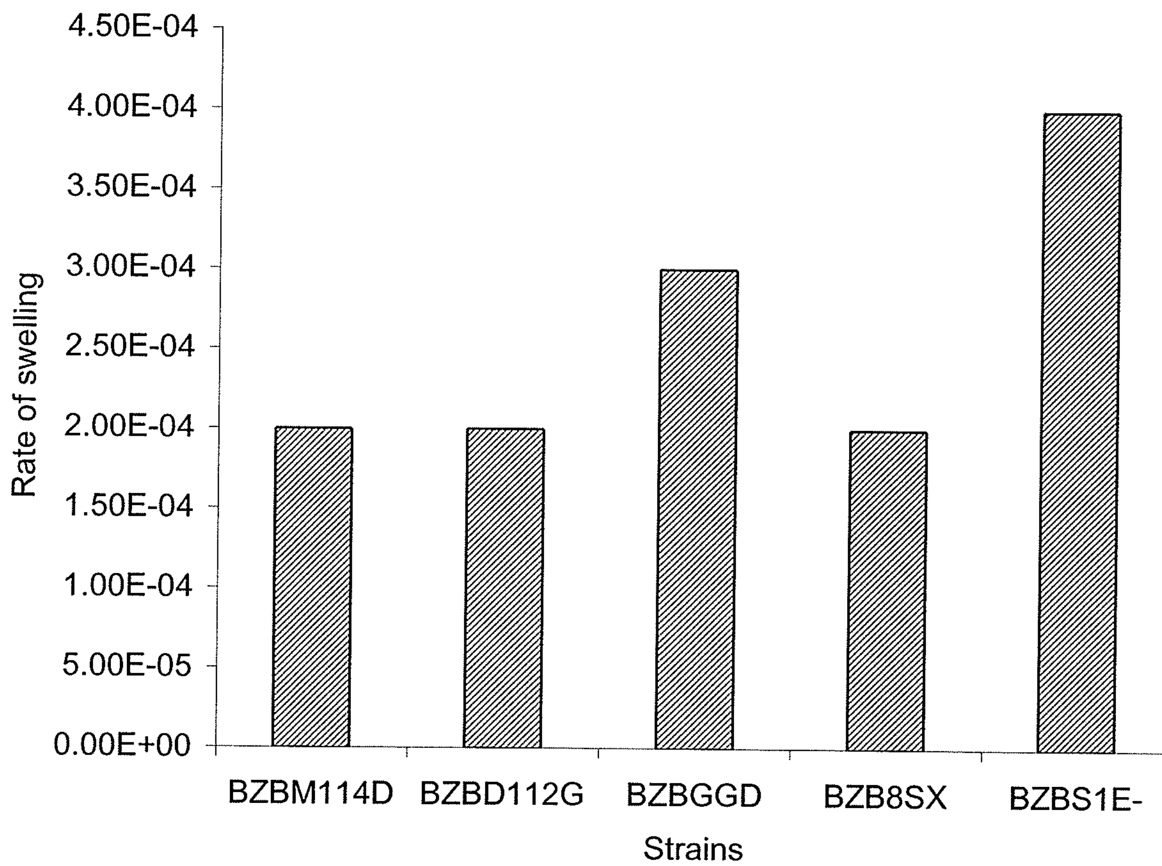

4.50E-04
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Rate of swelling
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E-
Strains

Fig. 3.8. Comparison of permeability rates of maltose through liposome reconstituted porins isolated from the cell envelopes of *E. coli* BZB1107 recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. Results are representative of five assays and data used to construct the plot are presented in Table 3.1.

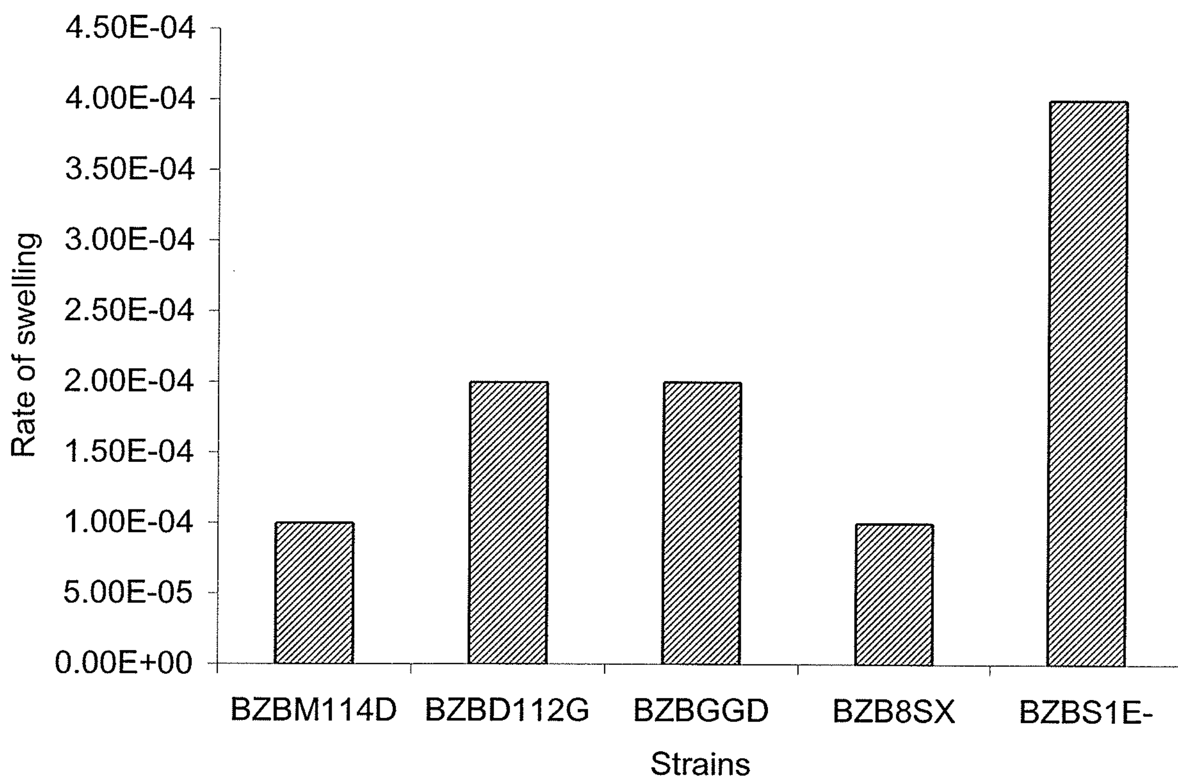
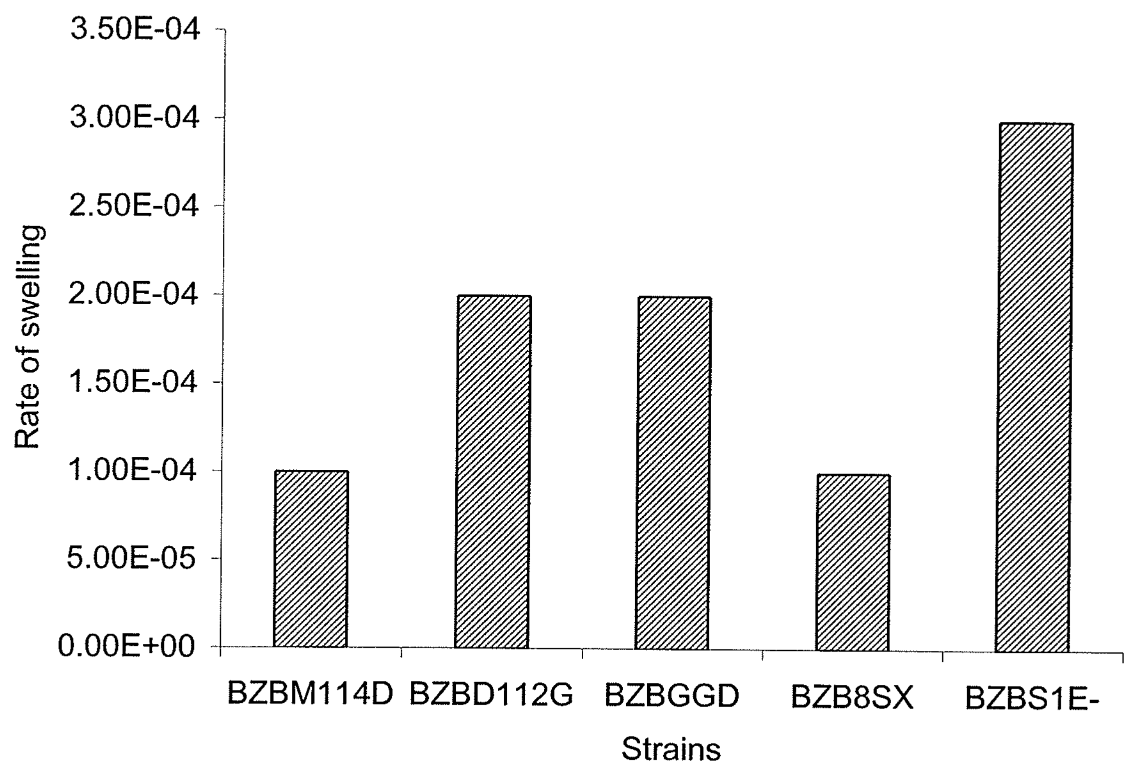

4.50E-04
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Rate of swelling
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E-
Strains

Fig. 3.9. Comparison of permeability rates of ampicillin through liposome reconstituted porins isolated from the cell envelopes of porin deficient *E. coli* BZB1107 recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S* *marcescens* OmpF wild-type]. Results are representative of five assays and data used to construct the plot are presented in Table 3.1.


3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
Rate of swelling
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E-
Strains

porins of BZBD112G and BZBGGD is the same, and 50% less than the *S. marcescens* wild-type OmpF. It is possible that no difference can be seen between both porins because the difference in pore sizes between them is not large enough to permit the permeability rate difference with respect to maltose. This is also true for ampicillin, considering that it has roughly the same size as maltose but is negatively charged. Penetration rates through porins of BZBM114D and wild-type OmpC are the same, and are 75% less than wild type OmpF. This agrees with earlier observation (Hutsul, 1996). The pattern of penetration rates of ampicillin through the porins is very similar to maltose with the exception that the penetration rate through OmpF is 25% less than that of maltose. The existence of ampicillin in an anionic form might be responsible for this slow rate, since OmpF and OmpC are known to prefer cations slightly over anions (Nikaido, 2003). As mentioned earlier, there was no observed difference in the penetration rates of ampicillin through porins of BZBD112G and BZBGGD. The liposome swelling assay did not show any difference between OmpC with the single mutation, $D^{112}G$, and that with a second mutation, $M^{114}D$. As will be seen later, this is not the case with respect to fluoroquinolone accumulation.

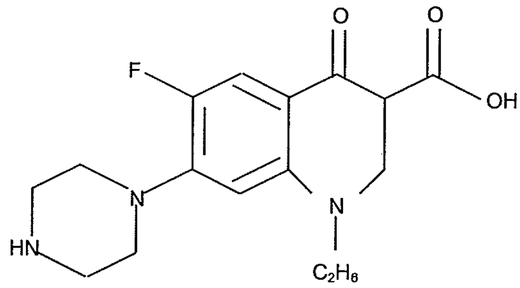
3.2.3.2. Fluoroquinolone accumulation assays

Although, fluoroquinolone accumulation is the most commonly used assay in the study of efflux pumps, there are not many examples of studies where it has been used to evaluate porin function (Ruiz*, et al.*, 2003). Considering that porins are known channels for the passage of fluoroquinolones, accumulation assays should provide some useful information about the role of porins in the entrance of

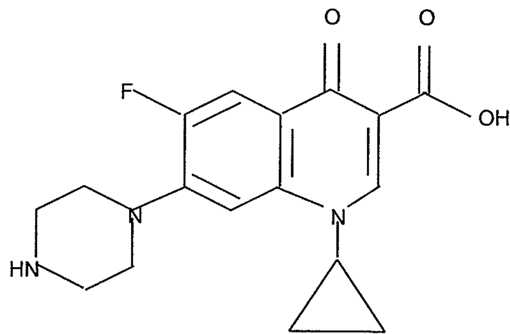
antibiotics. One should note that liposome swelling assays are different than fluoroquinolone accumulation assays in that liposome swelling examines the rate at which solutes get trapped in reconstituted proteoliposomes, while the latter examines rate of accumulation and/or the total amount of solutes accumulated in cells at the end of a period. Results of fluoroquinolone accumulation assays conducted are presented in Figs. 3.11 to 3.13, and Table 7.4 in the Appendix.

In this study, I examined the accumulation of ciprofloxacin (M_r = 331.4 Da), norfloxacin (M_r = 319.34 Da), and ofloxacin (M_r = 361.4 Da) [Fig. 3.10] using the porin deficient *E. coli* strain BZB1107 as a negative control. For all three antibiotics, the accumulation was highest in *E. coli* strain expressing the wild-type OmpF. The patterns of accumulation of ciprofloxacin and norfloxacin were very similar. The introduction of a double mutation to the PEFD^{112}GM^{114} motif of *Serratia marcescens* OmpC, as expressed by BZBGGD, led to a twofold increase in accumulation of ciprofloxacin and norfloxacin. The results obtained for ofloxacin accumulation are not consistent with ciprofloxacin and norfloxacin accumulation studies. Apart from not obtaining a pattern similar to the two fluoroquinolones, ofloxacin's net accumulation in terms of concentration per dry weight in *E. coli* strains used is about one tenth of ciprofloxacin and norfloxacin accumulation. BZBS1E(-) had the highest ofloxacin accumulation, but the levels are still not as significant in comparison to what was observed for ciprofloxacin and norfloxacin accumulation. Corresponding results were obtained from a different study in our laboratory (Kumar, 2004) where *S. marcescens* efflux pumps were examined, and another that examined the accumulation of

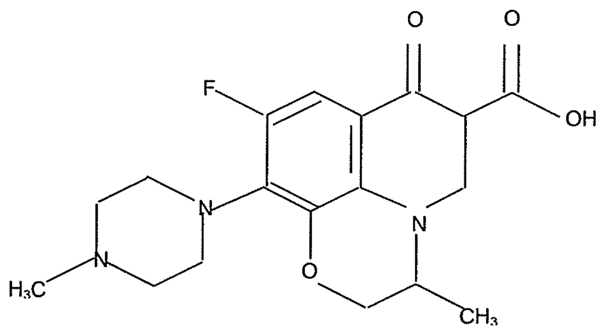
Fig. 3.10. Structures of fluoroquinolones used in this study.



Norfloxacin



Ciprofloxacin



Ofloxacin

Fig. 3.11. Net accumulation of ciprofloxacin in porin deficient *E. coli* BZB1107 and recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. Ciprofloxacin was added to a final concentration of 10 µg/mL to harvested late log phase cultures. Fluorescence was measured as described in Section 2.11 in Materials and Methods. (n = 3).

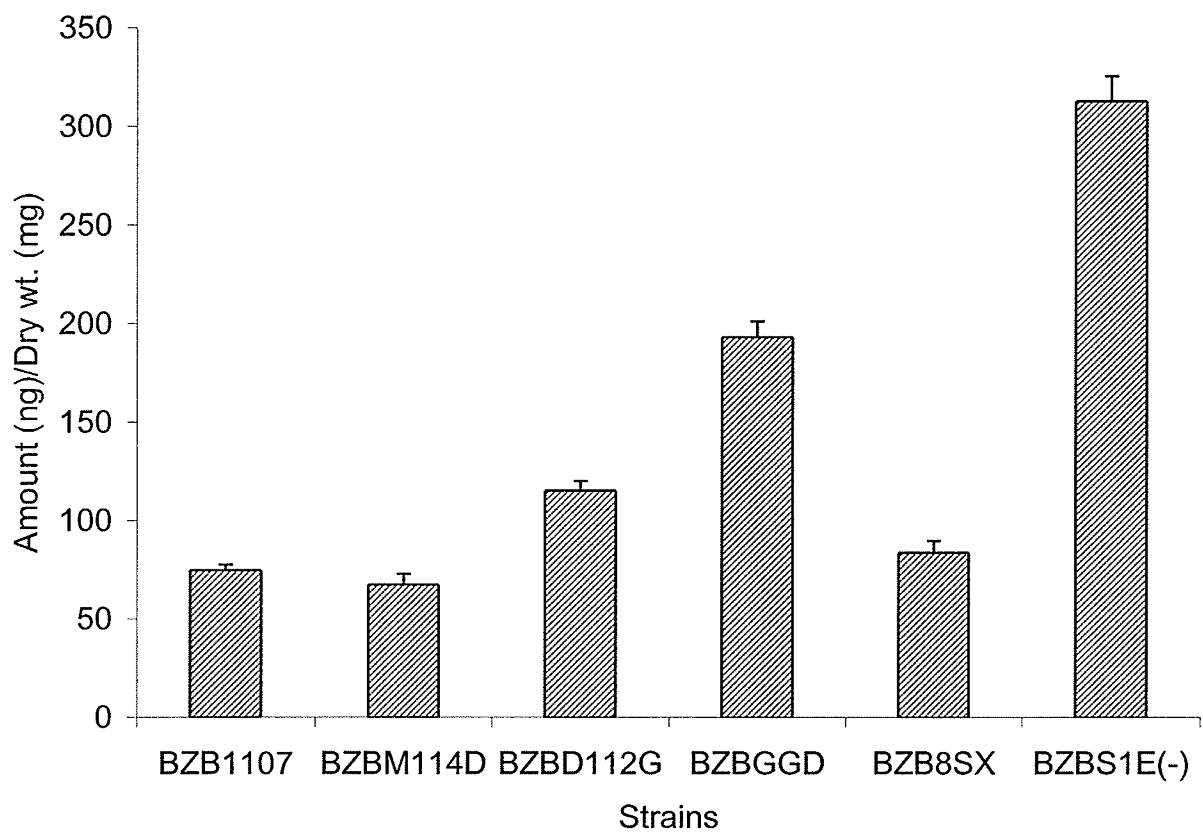

350
300
250
200
150
100
50
0
Amount (ng)/Dry wt. (mg)
BZB1107
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E(-)
Strains

Fig. 3.12. Net accumulation of norfloxacin in porin deficient *E. coli* BZB1107 and recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. Norfloxacin was added to a final concentration of 10 µg/mL to harvested late log phase cultures. Fluorescence was measured as described in Section 2.11 in Materials and Methods. (n = 3).

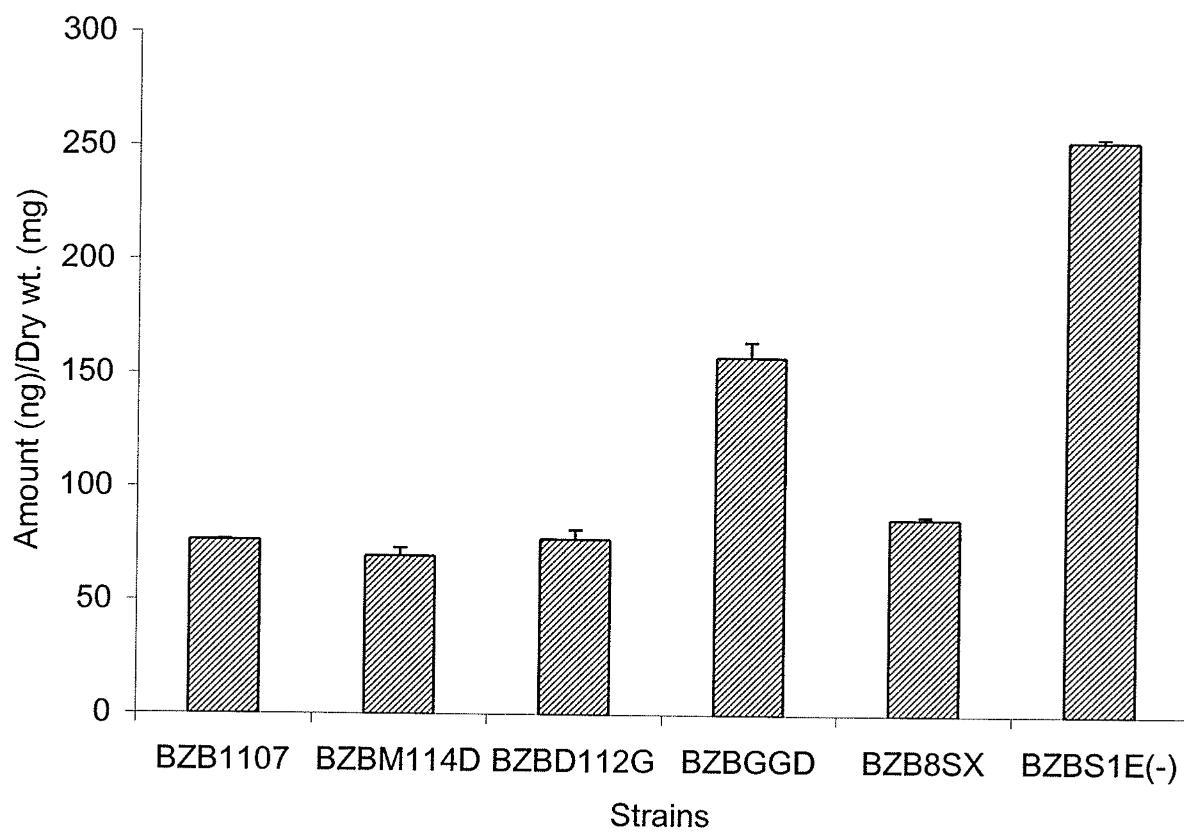
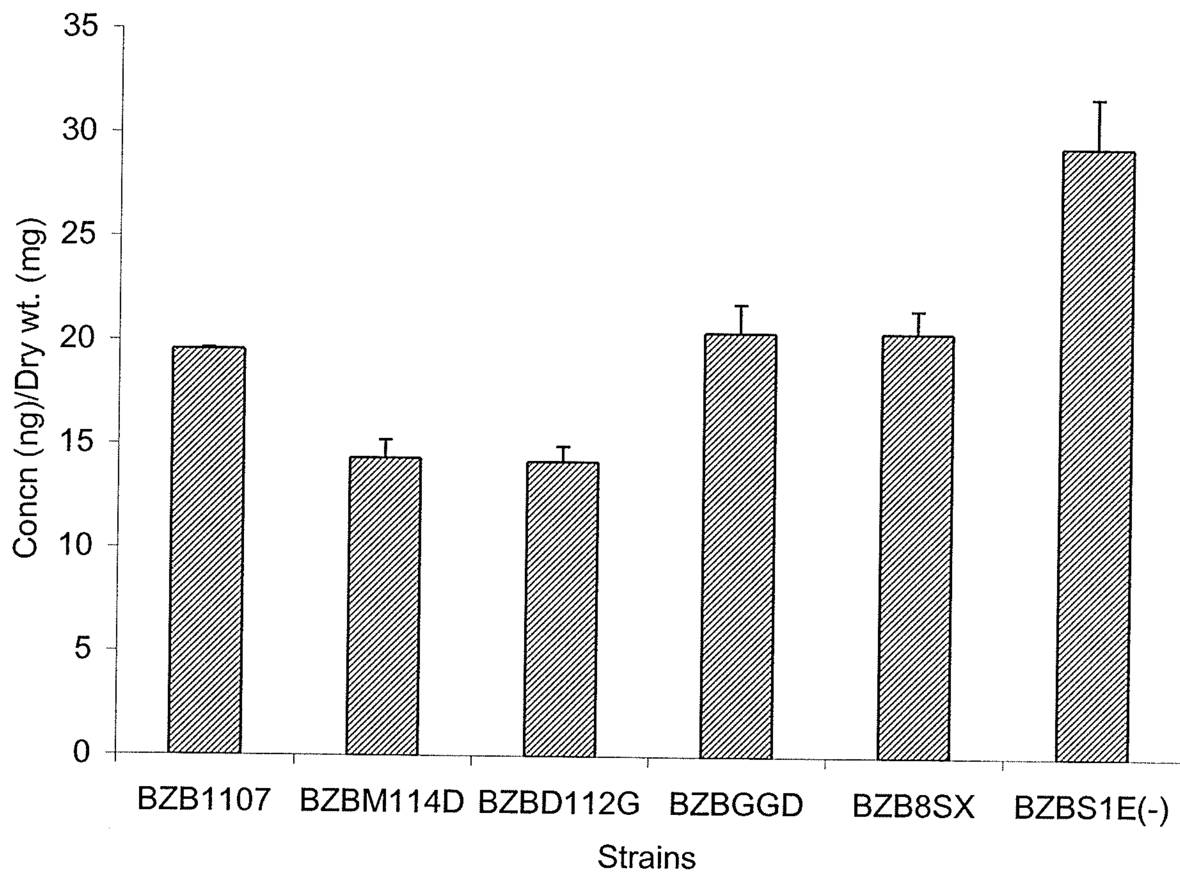

300
250
200
150
100
50
0
Amount (ng)/Dry wt. (mg)
BZB1107
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E(-)
Strains

Fig. 3.13. Net accumulation of ofloxacin in porin deficient *E. coli* BZB1107 and recombinants BZBM114D [*S. marcescens* OmpC, $M^{114}D$], BZBD112G [*S. marcescens* OmpC, $D^{112}G$], BZBGGD [*S. marcescens* OmpC, $M^{114}D$ & $D^{112}G$], BZB8SX [*S. marcescens* OmpC wild-type] and BZBS1E(-) [*S marcescens* OmpF wild-type]. Ofloxacin was added to a final concentration of 10 µg/mL to harvested late log phase cultures. Fluorescence was measured as described in Section 2.11 in Materials and Methods. (n = 3).



35
30
25
20
15
10
5
0
Concn (ng)/Dry wt. (mg)
BZB1107
BZBM114D
BZBD112G
BZBGGD
BZB8SX
BZBS1E(-)
Strains

fluoroquinolones by wild-type and efflux mutant *Streptococcus pneumoniae* (Piddock & Johnson, 2002). Of all three fluoroquinolones, ofloxacin has the highest molecular weight, and it is the most hydrophobic. Molecular weight and hydrophobicity are important factors that affect the accumulation of fluoroquinolones (Piddock *et al* 2001), and these are partly responsible for the observed accumulation of ofloxacin. In addition, ofloxacin is an optically active compound usually supplied as a racemate, i.e. containing equal mixtures of its levorotatory and dextrorotatory forms. Given that one conformation is more stable than the other (Morrison & Boyd, 1983), it is not unlikely that this was responsible for our observation of reduced accumulation of ofloxacin. This is supported by another study that compared the accumulation of ofloxacin and levofloxacin (D-isomer of ofloxacin), in which the former is accumulated to higher concentrations in strains tested than the latter. This led the authors to suggest that the L-isomer is accumulated to a higher concentration than the D-isomer (Piddock & Johnson, 2002).

3.3. Examination of the regulation of *S. marcescens* porins expression

Osmoregulation of the porins of *E. coli* has been well established (Csonka, 1989). However, in *S. marcescens*, there are conflicting reports about the regulation of its porins by external osmolarity (Sawai *et al*, 1987; Puig *et al*, 1993).

In this study, the β-galactosidase assay (Miller, 1972) was used to examine the regulation of *S. marcescens* porins. The promoter regions of *S.*

marcescens ompC and *ompF* were cloned to a promoterless *E. coli lacZ* vector and transformed into *E. coli* NM522. The resulting strains are referred to as pK98C and pOY005, respectively. pOY009, a plasmid having the *lacZ* gene without a promoter, was used as a negative control. pOY012 and pOYL338W carry the promoter regions of *E. coli ompF* and *ompC*, respectively fused to *lacZ* gene, and were used as positive controls. Single and combination of two or three conditions were tested as illustrated in the figures that follow (Figs 3.14-3.26). Tables 7.5-7.17 in the Appendix contain the data used to construct these charts.

The highest concentration of sucrose used (10% w/v) in the medium led to a three-fold decrease in expression of *S. marcescens* OmpF and a two-fold increase in expression of its OmpC porin (Fig. 3.14). This is consistent with the results of an earlier study on osmoregulation of *S. marcescens* porins (Hutsul, 1996).

Weak acids, like salicylate, trigger an increased transcription of *micF* leading to an increase in the threshold levels of 4.5S *micF* RNA available for binding to *ompF* RNA, thereby causing a reduction in the expression of OmpF (Pratt, *et al.*, 1996). In this study, salicylate resulted in a two-fold decrease in expression of *S. marcescens* OmpF and about a two-fold increase in *S. marcescens* OmpC expression (Fig. 3.15).

Environmental pH is also an important factor to consider when studying the regulation of porins. It can influence porin gene expression due to pH-elicited changes in the cytoplasmic level of cAMP (Pratt, *et al.*, 1996). The levels of cAMP have been reported to affect porin synthesis, with high cAMP levels

elicited by alkaline pH, suppressing *ompC* expression, and vice versa (Scott & Harwood, 1980). This is supported by an observation where glucose, a carbon source associated with low cAMP was used at acidic pH, it resulted in the expression of *ompC*; but when cAMP was added there was a partial reversal of *ompC* expression. With this in mind, when glycerol or succinate, associated with high cAMP, was the sole carbon source at pH 6, there was expression of *ompF* rather than *ompC*. Therefore, these have led to the assumption that changes in cytoplasmic levels of cAMP may be a result of a change in external pH (Thomas & Booth, 1992) leading to an influence on gene regulation. In addition, acidic pH can switch porins from large to smaller sized pores (Heyde *et al*., 1987), and increased pH can switch from narrow to a wider pore channel, without resulting in a global change in porin secondary structure (Todt & McGroarty, 1992). Porin expression at slightly acidic pH (pH 6) and slightly basic pH (pH 8) were examined (Fig. 3.16). At pH 8, the expression of OmpF is twice its expression at pH 6. This is the opposite for OmpC, having a three-fold decrease in expression at pH 8, when compared to pH 6. Similarly, there was a rapid induction of *E. coli* OmpC expression in response to acidification of the medium (Thomas & Booth, 1992).

Effect of temperature on the expression of *S. marcescens* porins was also studied (Fig. 3.17). When compared to expression of OmpF at 28°C, there was about 10-fold decrease in expression at 42°C. OmpC expression was higher at 42°C (4-fold) than at 28°C.

Most of the published porin regulation studies only consider the effects of

Fig. 3.14. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 5, 8, 10% sucrose. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

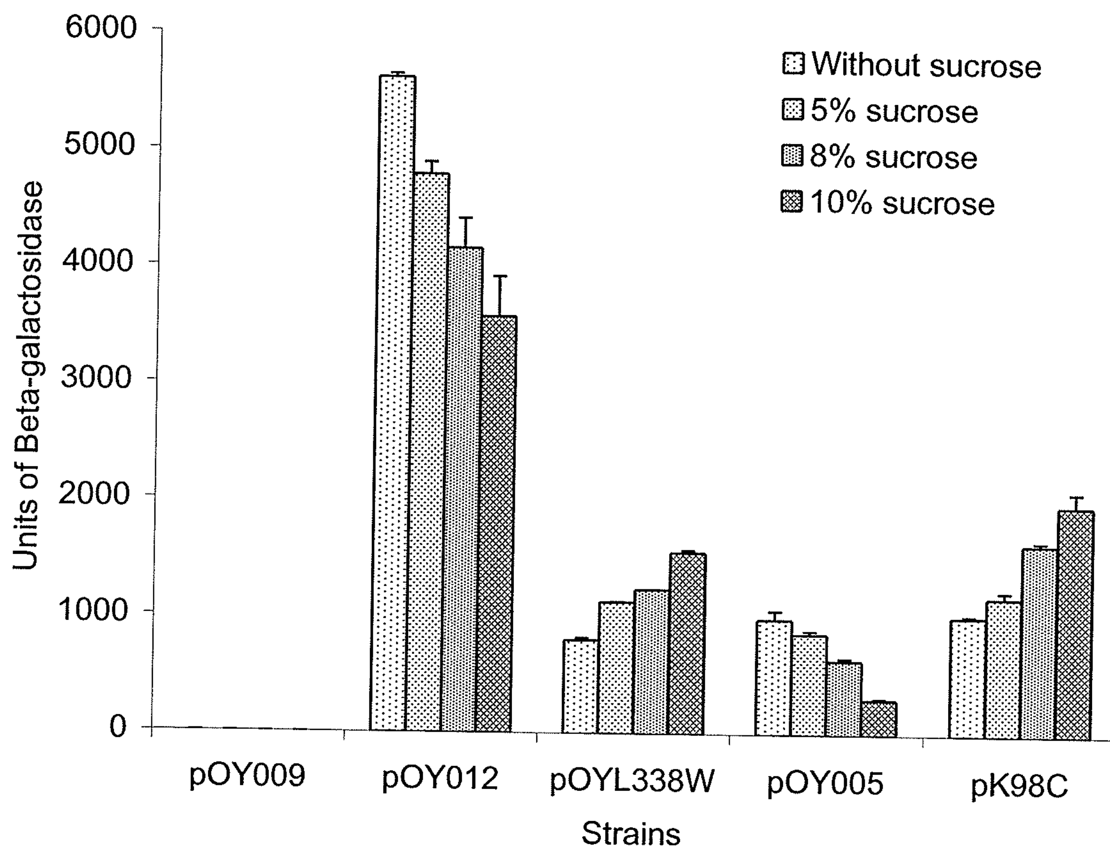

6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
Without sucrose
5% sucrose
8% sucrose
10% sucrose
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.15. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 1, 5, 8 mM Salicylate. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

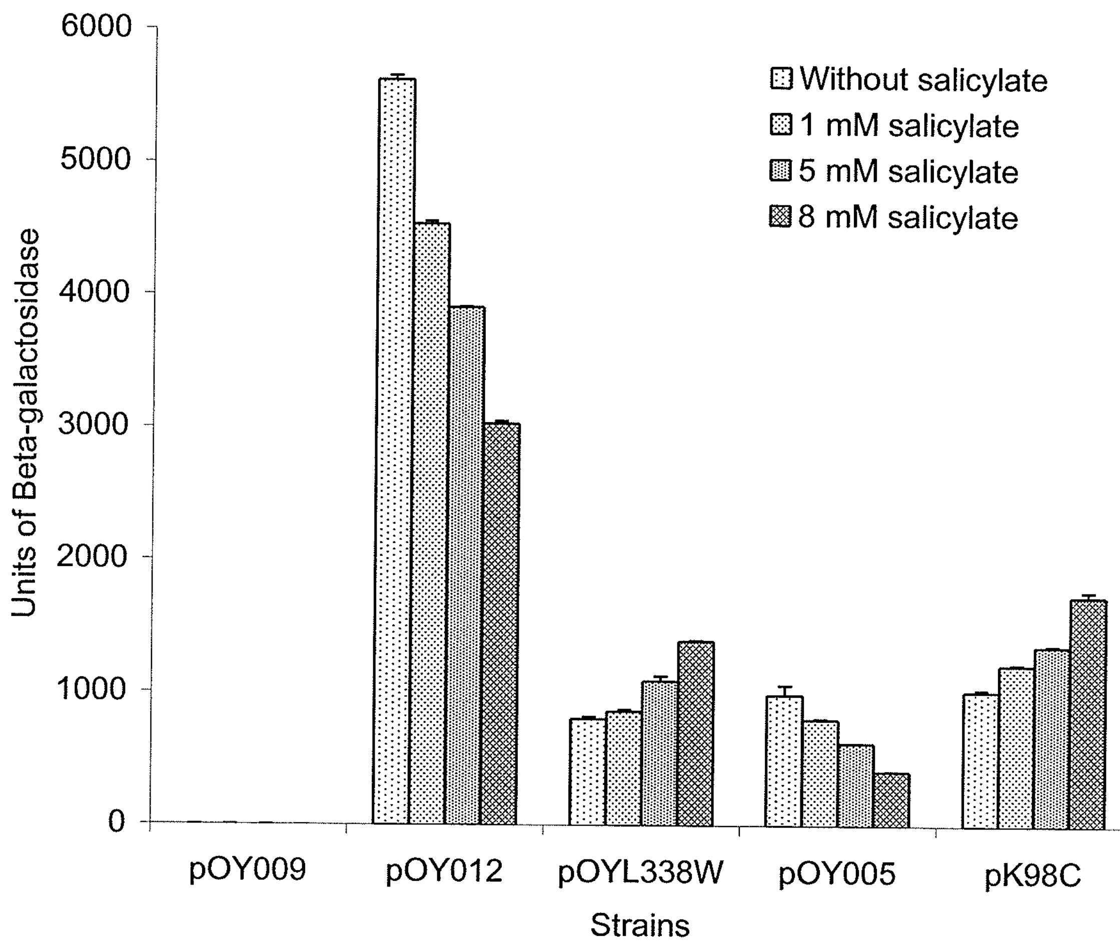
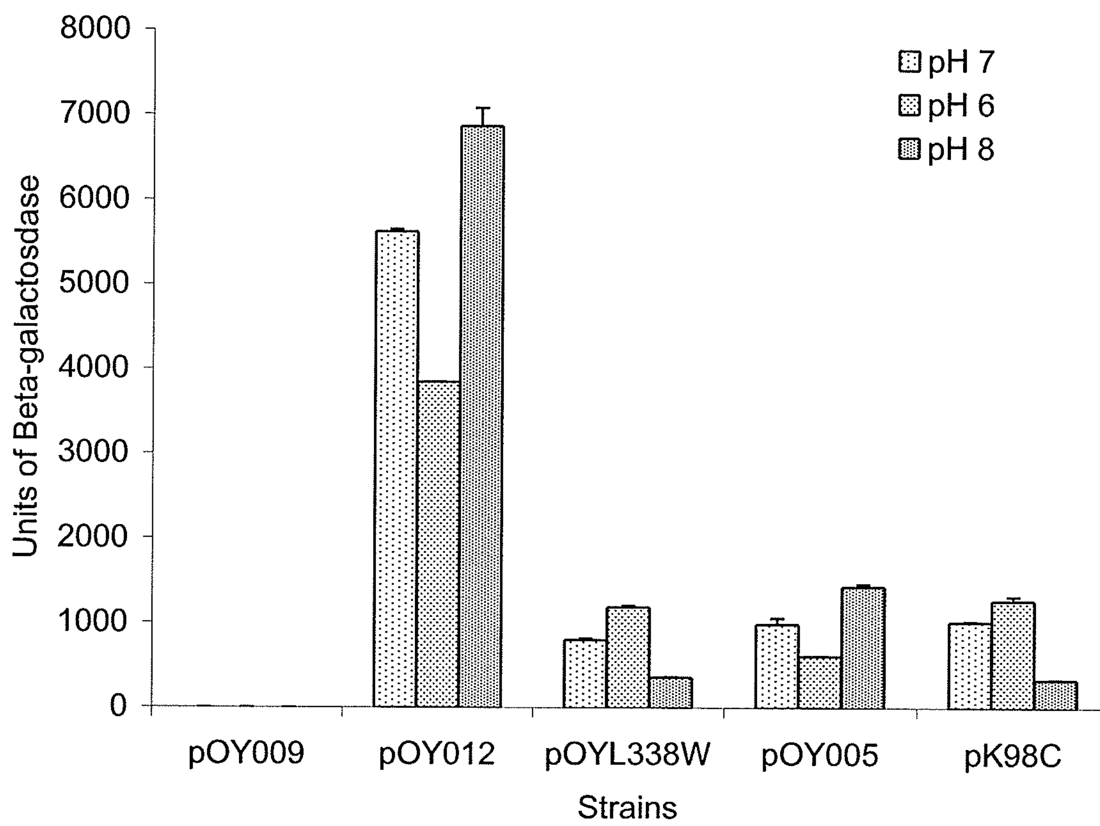
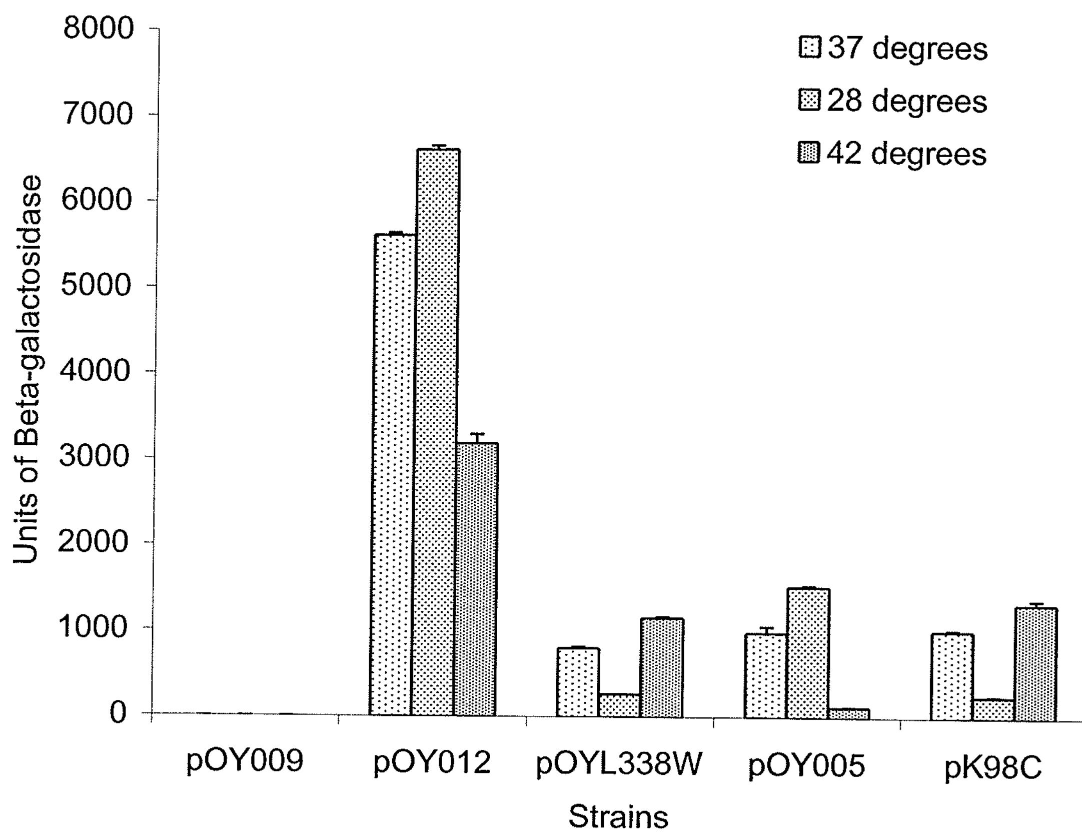

6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
Without salicylate
1 mM salicylate
5 mM salicylate
8 mM salicylate
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.16. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media at pH 6, 7, and 8. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).


8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosdase
pH 7
pH 6
pH 8
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

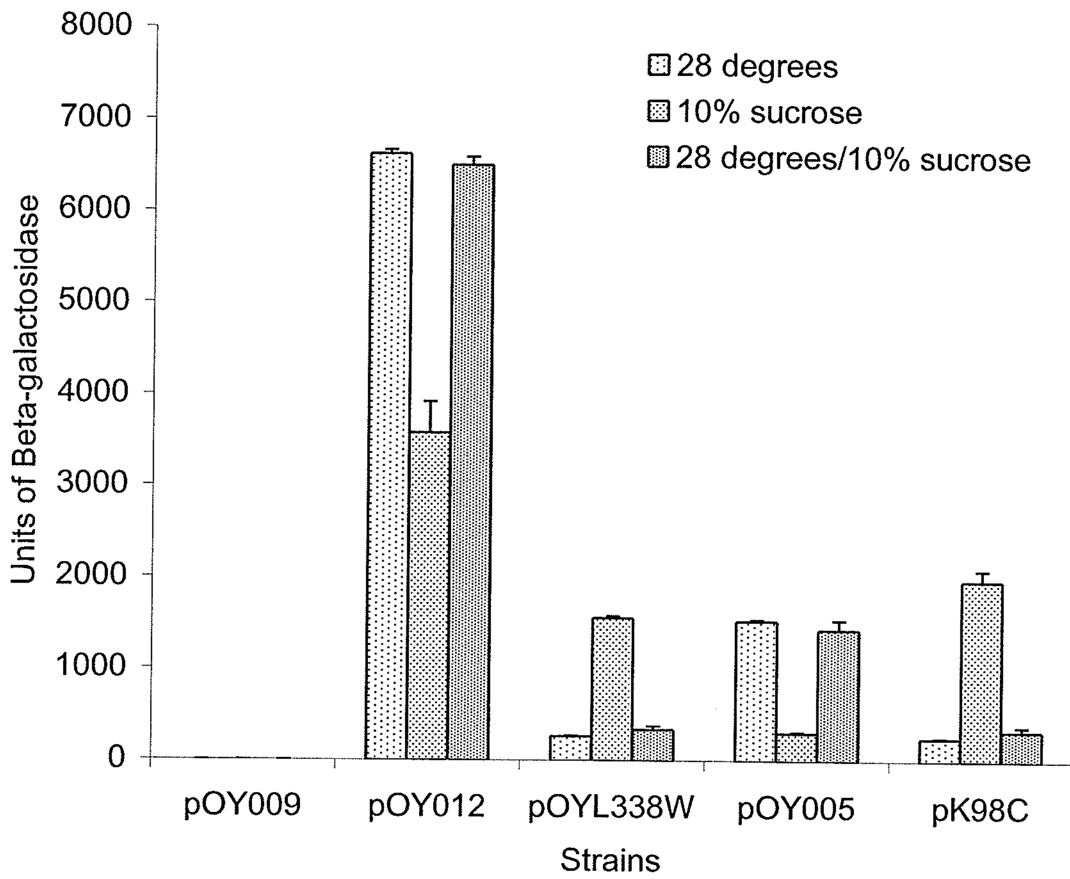
the individual conditions mentioned above. In this study, random combination of the above tested single conditions was examined. The rationale for this complex study was that *S. marcescens* could be faced with the challenge of high osmolarity, low pH and high temperature in its host environment during infection. Fig. 3.18 illustrates the effect of combining 28°C and 10% sucrose. This shows that temperature had a more pronounced effect on OmpF and OmpC expression than did osmolarity. The observation is the same when 28°C and 8 mM salicylate were combined (Fig. 3.19). When 42°C and pH 8 were combined (Fig. 3.20), temperature had a more obvious effect on porin expression. In the case of pH 8 and 10% sucrose (Fig. 3.21), pH had a more pronounced effect on porin expression than osmolarity. Fig. 3.22 shows the results of growing the cells in a medium supplemented with 5% sucrose and 8 mM salicylate. The effect of osmolarity on porin expression is higher than salicylate. pH 8 and 8 mM salicylate were also combined (Fig. 3.23), and effect of pH on porin expression regulation was more obvious than the salicylate in the growth medium. The effect of combining three conditions on porin expression was also examined. As was evident when two conditions were combined, one of the factors had a dominant effect on expression than the others. When 28°C, 10% sucrose and 8 mM salicylate were combined temperature appeared to have the dominant effect on porin expression (Fig. 3.24). pH had the most dominant effect when pH 8, 10% sucrose and 1 mM salicylate were combined (Fig. 3.25). Finally, the combination of 28°C, pH 6 and 10% sucrose was used (Fig. 3.26). Temperature had more dominant effect on regulation of porin expression in spite of slightly acidic pH

Fig. 3.17. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media at 28°C, 37°C and 42°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).



8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
37 degrees
28 degrees
42 degrees
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.18. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 10% sucrose at 28°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).



8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
28 degrees
10% sucrose
28 degrees/10% sucrose
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.19. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 8 mM Salicylate at 28°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

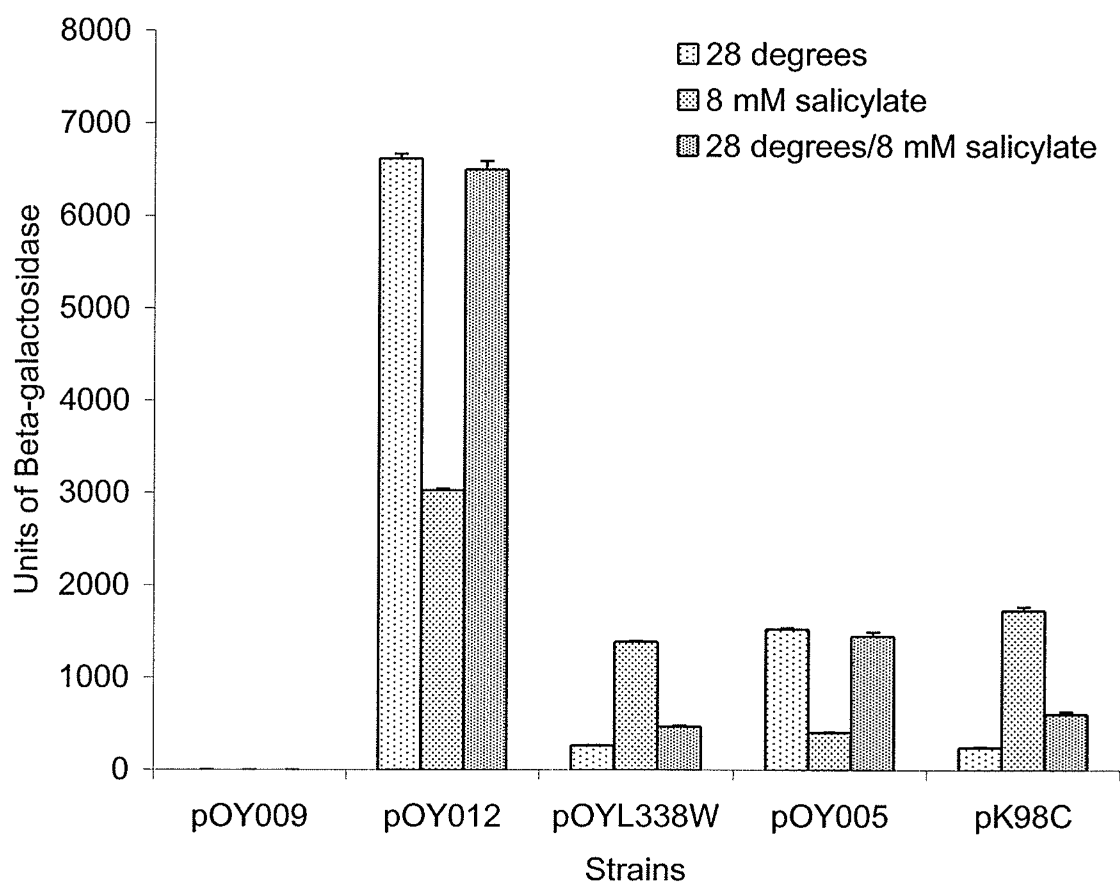
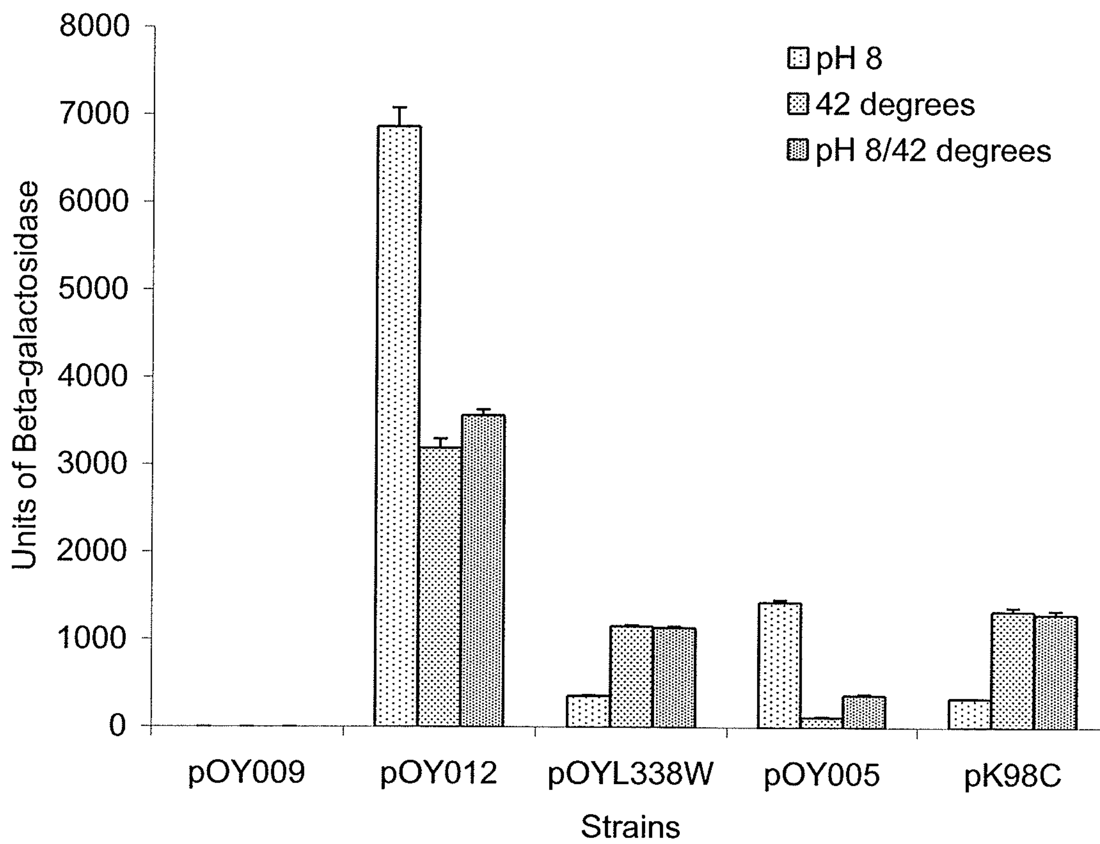

8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
28 degrees
8 mM salicylate
28 degrees/8 mM salicylate
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.20. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media at pH 8 and 42°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).


8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
pH 8
42 degrees
pH 8/42 degrees
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

and high osmolarity, which singly resulted in a decrease in expression of OmpF and increase in expression of OmpC.

The initial assumption, before studying the effect of growing cells in the presence of multiple environmental conditions, was that these would result in some kind of synergy between conditions. The presence of any stressful condition among the combinations studied would lead to reduction of expression of the porin with the wider pore channel i.e. OmpF, while the OmpC expression would be increased. The results that are presented in this thesis do not support this hypothesis. When cells were grown in the presence of a combination of conditions such as 28°C/10% sucrose, 28°C/8 mM salicylate, 28°C/10% sucrose/8 mM salicylate, and 28°C/pH 6/10% sucrose, the effects of high osmolarity or presence of salicylate and slightly acid pH were overshadowed by the growth temperature. In spite of the presence of high osmolarity, high concentration of salicylate, acid pH or high temperature in all of the combinations studied, there was an increased expression of OmpF. Considering the fact that the cells were grown in a defined media, in which some nutrients might be scarce, increasing the expression of the larger pore porin (Pratt *et al*, 1996), but reducing its pore size through mutation, might be a way to cope with the stress. This may be compared to an experiment in which lactose was the sole carbon source in a minimal medium. Strains evolved that overproduced the larger pore channel porin OmpF of *E. coli* over OmpC. In addition, these strains produced *ompF* containing mutations that altered the residues in the constriction region or

Fig. 3.21. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 10% sucrose at pH 8. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

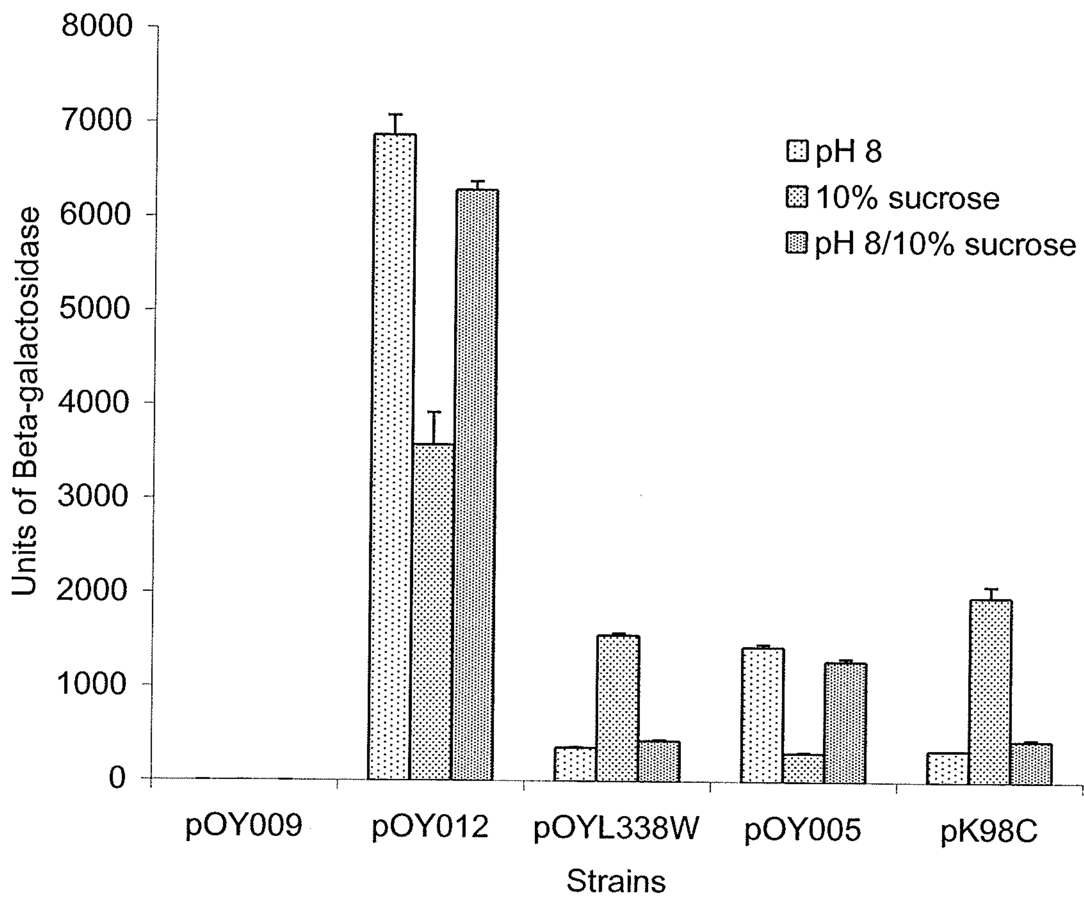

8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
pH 8
10% sucrose
pH 8/10% sucrose
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.22. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 5% sucrose and 8 mM Salicylate. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

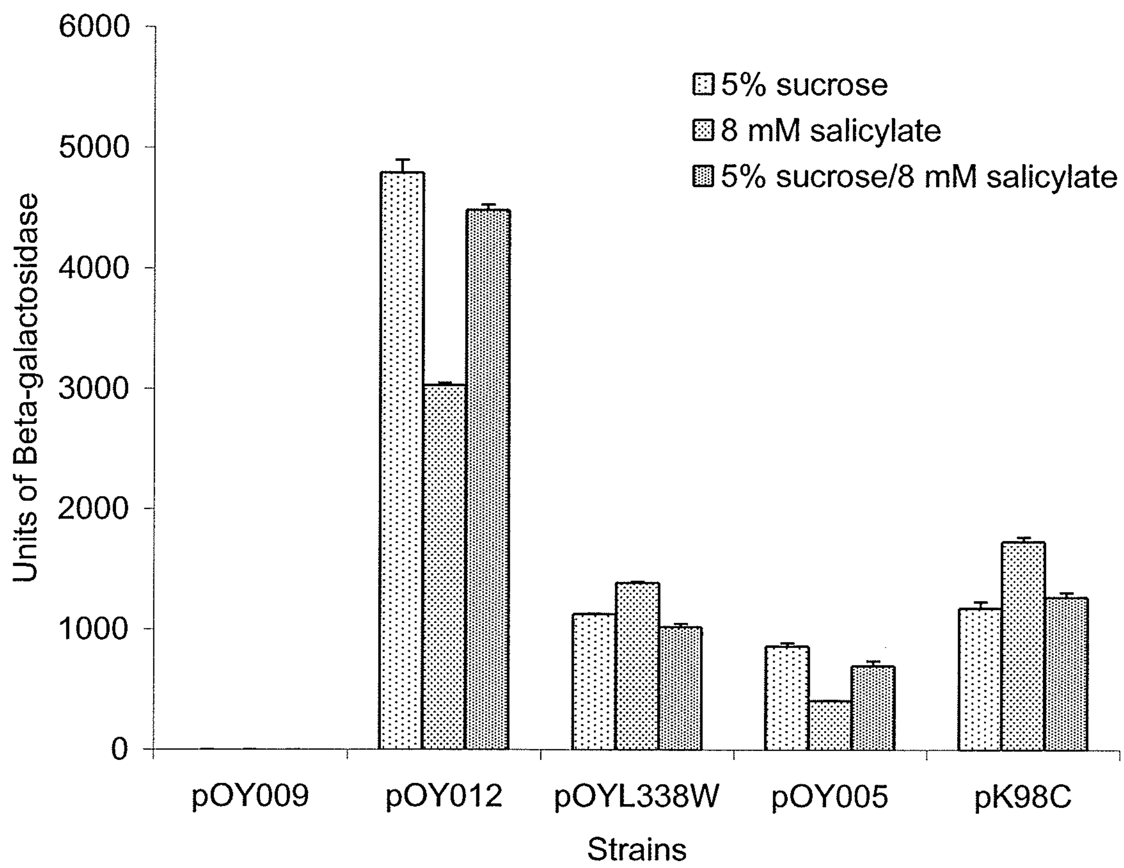

6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
5% sucrose
8 mM salicylate
5% sucrose/8 mM salicylate
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.23. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 8 mM Salicylate at pH 8. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

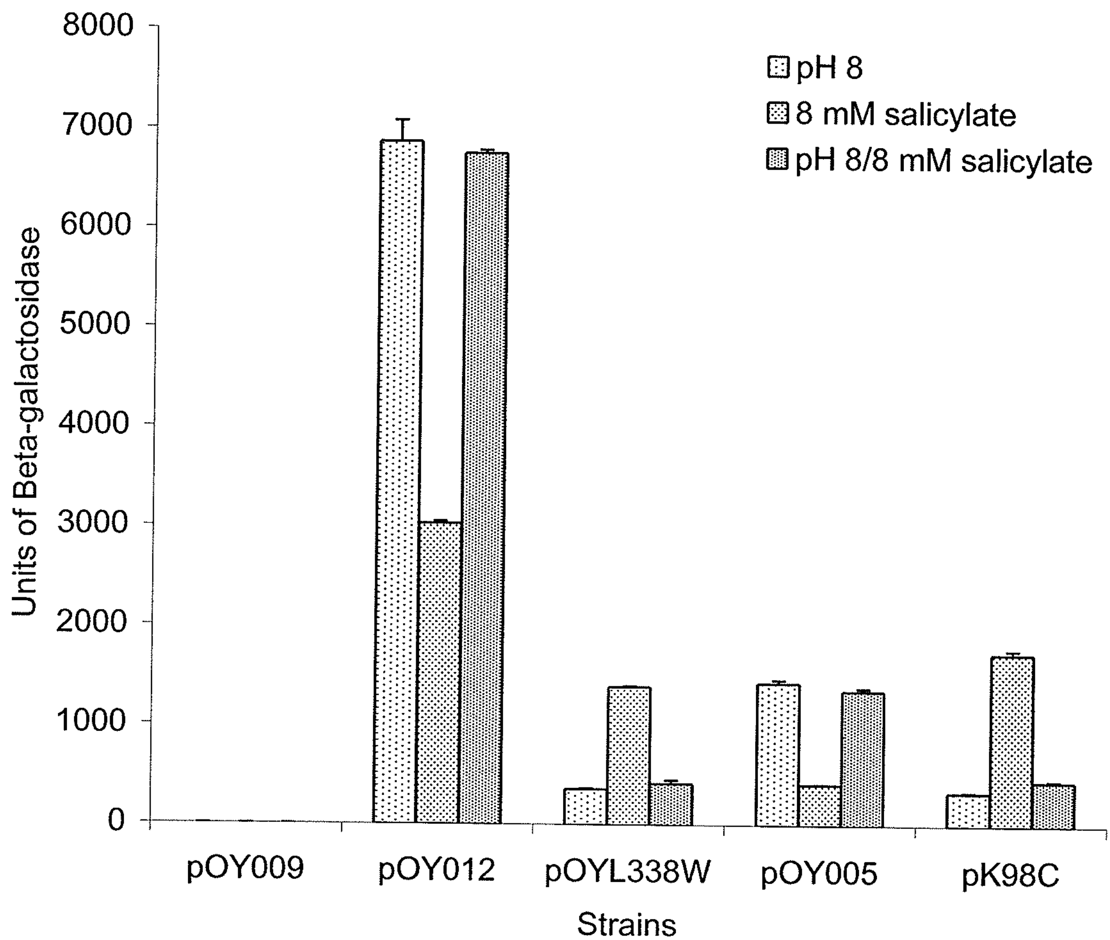
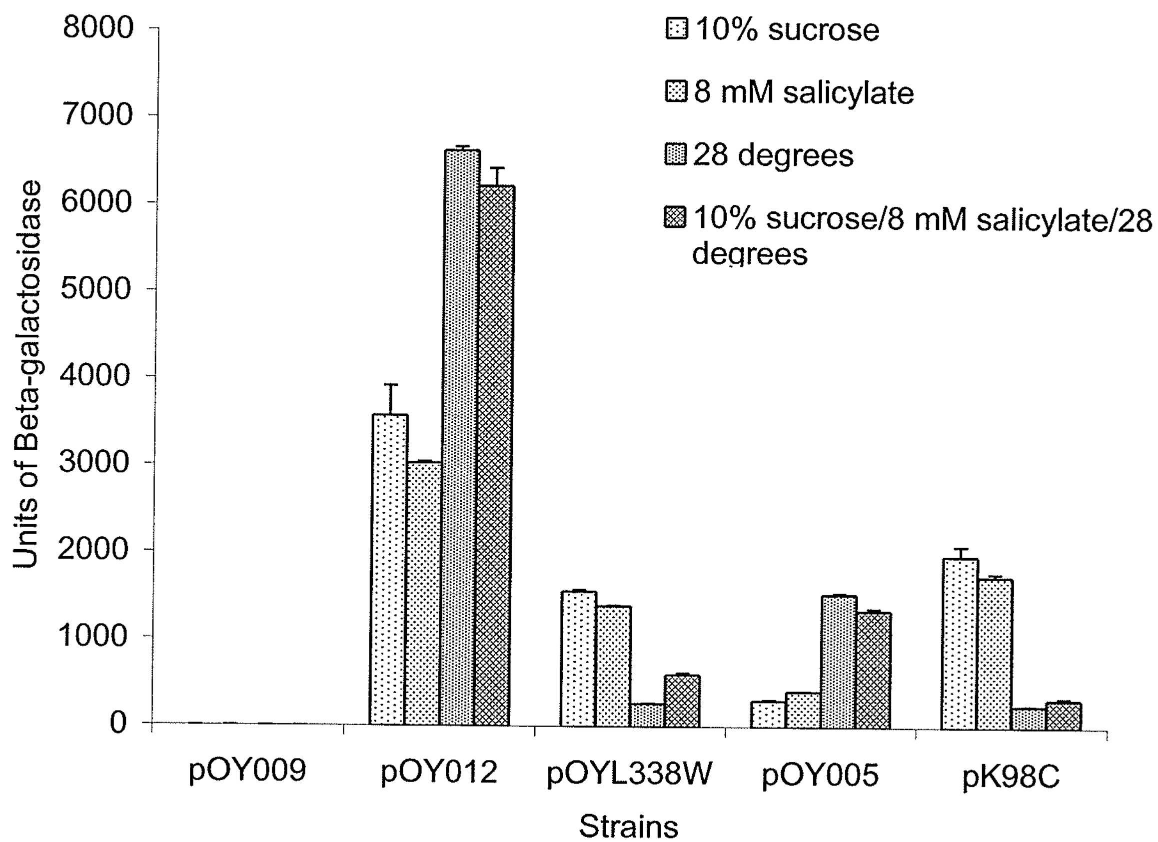

8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
pH 8
8 mM salicylate
pH 8/8 mM salicylate
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.24. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 10% sucrose and 8 mM Salicylate at 28°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).



8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
10% sucrose
8 mM salicylate
28 degrees
10% sucrose/8 mM salicylate/28 degrees
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.25. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 10% sucrose and 1 mM Salicylate at pH 8. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).

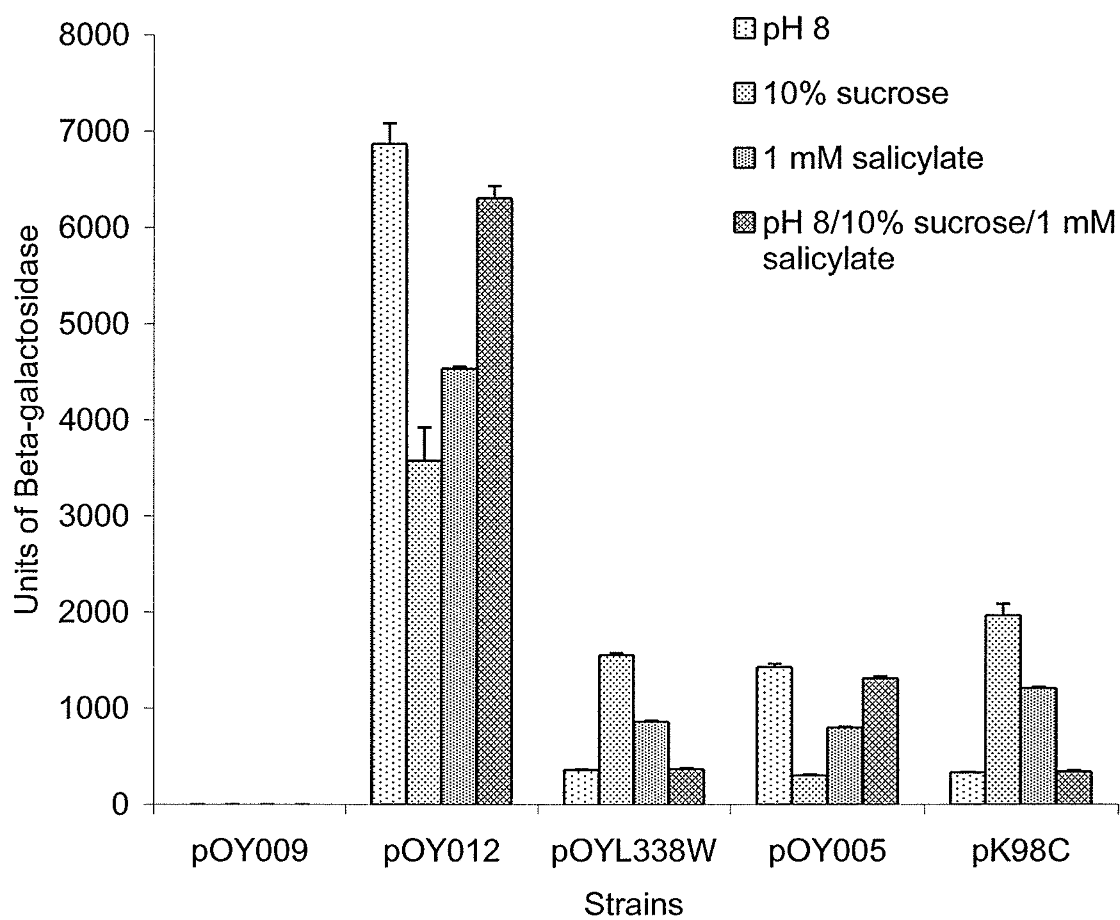
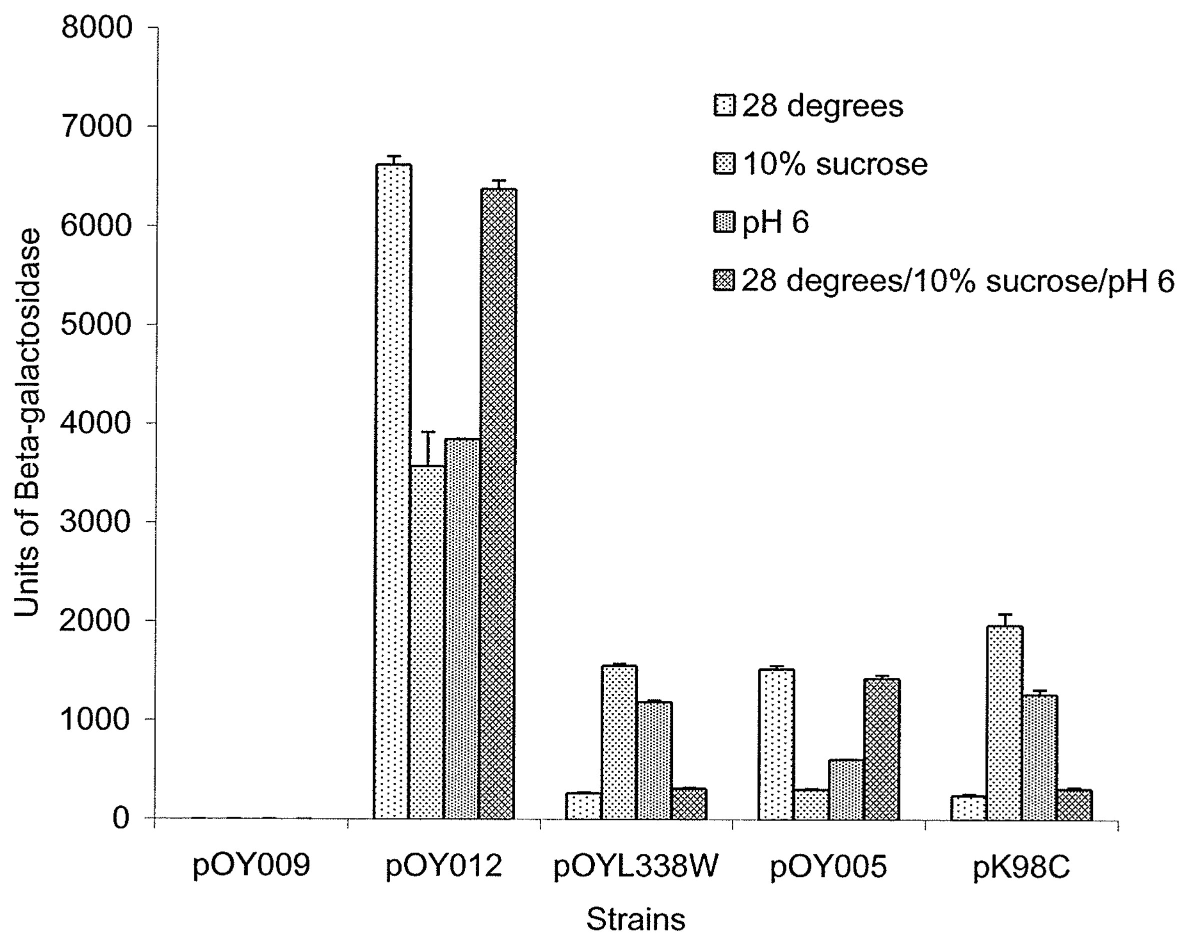

8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
pH 8
10% sucrose
1 mM salicylate
pH 8/10% sucrose/1 mM salicylate
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

Fig. 3.26. Units of β-galactosidase produced by *E. coli* strains pOY009 (promoterless *lacZ-lacY*), pOY012 (*E. coli ompF* promoter-*lacZ* fusion), pOYL338W (*E. coli ompC* promoter-*lacZ* fusion), pOY005 (*S. marcescens ompF* promoter-*lacZ* fusion), and pK98C (*S. marcescens ompC* promoter-*lacZ*), grown in M9 minimal media supplemented with 10% sucrose at pH 6 and 28°C. The assay was initiated once cultures reached OD_{600}=0.28-0.70. See Section 2.10 in Materials and Methods for experimental details. (n = 3).


8000
7000
6000
5000
4000
3000
2000
1000
0
Units of Beta-galactosidase
28 degrees
10% sucrose
pH 6
28 degrees/10% sucrose/pH 6
pOY009
pOY012
pOYL338W
pOY005
pK98C
Strains

resulted in a short deletion in the channel-constricting loop L3 (Zhang & Ferenci, 1999). The incubation of cell cultures until the optimal optical density for the performance of β-galactosidase assay is achieved took up to between 48 and 72 hours when two or three conditions were combined. At this time cells could have undergone some physiological changes by developing resistance to the toxic molecules and stressful conditions, such as salicylate, low pH and high osmolarity. Once resistance developed, cells had to increase the expression of OmpF in the nutrient-scarce environment to increase their chances of survival, which is likely the case in this study. If larger pore size porins can be altered to reduce their pore size (Heyde *et al.* 1987), the observation of an increased expression of OmpF in the combination of 28°C/pH 6/10% sucrose would promote the survival of the cells under this stressful condition.

Except for when cells were grown at 42°C, in all situations when different conditions were combined, there was always an increase in expression of OmpF. The decrease in the expression of OmpF seen in this case is due to the fact that temperatures above 37°C will stimulate *micF* transcription up to ten-fold (Pratt *et al.*, 1996) which ultimately will result in the post-transcriptional negative regulation of *ompF*. In this instance, high temperature (42°C) was combined with pH 8, which alone increased the expression of OmpF (Fig.3.16). Hence, high temperature, combined with favorable pH level for OmpF expression, would naturally decrease the expression of OmpF.

4. CONCLUSION

The production of the double mutation $D^{112}G$ and $M^{114}D$ in the consensus motif of the third eyelet loop of *S. marcescens* OmpC causing it to conform to the enterobacterial consensus, has shown that the simultaneous presence of both mutations has a combined effect of widening the pore lumen to increase permeability, while having no effect on ion selectivity. However, the permeability of this mutant OmpC porin is still lower than that of wild-type OmpF porin of *S. marcescens*. This may be due to differences in steric conformation of residues lining the pores of each porin.

Examination of the regulation of expression of the *S. marcescens* porins using the β-galactosidase assay has shown that these porins are regulated by pH, osmolarity and temperature. In conditions of high osmolarity, acidic pH, and high temperature there is a reduced expression of the larger pore size porin OmpF, while there is an increased expression of the narrower pore OmpC. In the face of increased environmental stress caused by combining two or more environmental conditions, in spite of growth in minimal medium, there was always an increase in expression of OmpF instead of OmpC, as was hypothesized. Also, rather than the two or three combined environmental conditions having a synergistic effect, one of the factors has a more prominent or pronounced effect on porin expression than the other(s). This has been explained to be due to the production of OmpF, with reduced pore size, resulting from mutations in the constriction region (Zhang & Ferenci, 1999); or physiological adaptation of cells to toxic and stressful conditions due to

incubation for up to 72 hours, and limitation of nutrients in M9 minimal medium (Pratt *et al.*, 1996).

5. FUTURE STUDIES

The effort to delete the *ompC* gene from the genome of *S. marcescens* must continue. When this is accomplished, possibly by trying out more efficient and effective methods of making deletion mutants, such as the Flp-*FRT* recombination system (Sadowski, 1995), the strain will be a suitable host background for examining site-specific porin mutants; although the use of *E. coli* porin deficient strains is acceptable at the moment. The ideal aspect of studying a porin protein is the elucidation of its crystal structure. This may sound a little bit ambitious, but a great deal of information may become available perhaps revealling some important difference between the well studied *E. coli* porins and the *S. marcescens* porins.

There is need to study the third eyelet loop motif, PEFDGM, of *S. marcescens* OmpC in more detail. Having mutated the $D^{112}G$ and $M^{114}D$, causes the porin to share the enterobacterial consensus, however this porin is not as permeable as wild-type OmpF. This again indicates that the steric conformation of residues lining the wall of both porins differs, and is responsible for the difference in permeability. In addition, the lysine residue, which confers anion selectivity property on PhoE porin has been shown to protrude into the pore channel. This site on the *S. marcescens* OmpC, at position 124, is occupied by glutamine, but in OmpF it is occupied by glycine. Therefore, a change from $Q^{124}G$ by site-directed mutatgenesis, and evaluation of permeability by liposome swelling assay will yield some useful information about this difference in both porins.

From the studies that have been done so far on the examination of the regulation of *S. marcescens* porins as presented in this thesis and the work of another student on the role of *micF* RNA on porin expression, it is becoming clear that *micF*-RNA may not be involved with osmoregulation of *S. marcescens* porins. Although, pH, salicylate and temperature were found to influence *micF* expression in this study on the role of *micF* RNA, constructing a *micF* deletion mutant might be able to confirm this observation. The upstream region of *E. coli ompF* , which contains the OmpR binding site (OBS), closely resembles that of *S. marcescens ompF.* Analysis of both have been carried out, and has revealed some important differences leading to the conclusion that the classic Env-OmpR system does not exist in *S. marcescens*. The search is on to find the system involved in osmoregulation of this organism.

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7. APPENDIX

Table 7.1. Optical densities obtained from proteoliposomes suspended in 18 mM glucose for a swelling period of 90 seconds

Time	w/o	BZBM114D	BZBD112G	BZBGGD	BZB8SX	BZBS1E(-)
0	0.179	0.208	0.206	0.179	0.226	0.219
10	0.179	0.206	0.203	0.171	0.222	0.21
20	0.178	0.205	0.202	0.17	0.221	0.207
30	0.178	0.203	0.201	0.168	0.22	0.205
40	0.179	0.201	0.2	0.168	0.219	0.205
50	0.179	0.202	0.199	0.168	0.218	0.204
60	0.178	0.201	0.199	0.167	0.218	0.203
70	0.179	0.201	0.199	0.166	0.218	0.205
80	0.178	0.2	0.198	0.166	0.217	0.204
90	0.178	0.2	0.197	0.166	0.217	0.203

w/o = Without porins, BZBM114D = M114D OmpC mutant, BZBD112G = D112G OmpC mutant, BZBGGD = M114D and D112G double mutant, BZB8SX = wild-type OmpC, BZBS1E(-) = wild-type OmpF

N. B. All recombinants and wild-type *Serratia marcescens* porins are expressed in BZB1107: porin deficient *E. coli* B strain

Table 7.2. Optical densities obtained from proteoliposomes suspended in 18 mM maltose for a swelling period of 90 seconds

Time	w/o	BZBM114D	BZBD112G	BZBGGD	BZB8SX	BZBS1E
0	0.181	0.217	0.222	0.19	0.222	0.231
10	0.18	0.213	0.219	0.185	0.22	0.224
20	0.18	0.212	0.218	0.183	0.218	0.221
30	0.18	0.212	0.217	0.183	0.217	0.219
40	0.18	0.212	0.217	0.182	0.216	0.219
50	0.179	0.211	0.216	0.182	0.217	0.217
60	0.178	0.211	0.215	0.181	0.217	0.216
70	0.179	0.21	0.215	0.181	0.216	0.217
80	0.178	0.21	0.215	0.18	0.215	0.217
90	0.177	0.209	0.214	0.179	0.215	0.217

w/o = Without porins, BZBM114D = M114D OmpC mutant, BZBD112G = D112G OmpC mutant, BZBGGD = M114D and D112G double mutant, BZB8SX = wild-type OmpC, BZBS1E(-) = wild-type OmpF

N. B. All recombinant and wild-type *Serratia marcescens* porins are expressed in BZB1107: porin deficients *E. coli* B strain

Table 7.3. Optical densities obtained from proteoliposomes suspended in 9 mM ampicillin for a swelling period of 90 seconds

Time	w/o	BZBM114D	BZBD112G	BZBGGD	BZB8SX	BZBS1E(-)
0	0.204	0.24	0.236	0.214	0.252	0.255
10	0.203	0.239	0.233	0.21	0.25	0.247
20	0.202	0.238	0.232	0.209	0.249	0.245
30	0.202	0.237	0.232	0.208	0.248	0.244
40	0.202	0.237	0.23	0.207	0.247	0.243
50	0.201	0.236	0.23	0.206	0.247	0.242
60	0.202	0.237	0.229	0.207	0.247	0.242
70	0.2	0.236	0.229	0.206	0.246	0.241
80	0.2	0.236	0.229	0.206	0.245	0.24
90	0.199	0.236	0.229	0.206	0.244	0.239

w/o = Without porins, BZBM114D = M114D OmpC mutant, BZBD112G = D112G OmpC mutant, BZBGGD = M114D and D112G double mutant, BZB8SX = wild-type OmpC, BZBS1E(-) = wild-type OmpF

N. B. All recombinants and wild-type *Serratia marcescens* porins are expressed in BZB1107: porin deficient *E. coli* B strain

Table 7.4. Accumulation of ciprofloxacin, ofloxacin and norfloxacin, over a period of ten minutes

Strains	Ciprofloxacin (ng)/Dry weight	Norfloxacin (ng)/Dry weight	Ofloxacin (ng)/Dry weight
BZB1107	74.76±2.94	76.55±0.22	19.58±0.08
BZBM114D	67.38±5.47	69.57±3.66	14.36±0.88
BZBD112G	115.24±4.50	77.23±4.27	14.21±0.75
BZBGGD	193.18±8.14	157.70±6.79	20.46±1.38
BZB8SX	83.68±5.94	86.53±1.24	20.47±1.11
BZBS1E(-)	312.82±12.70	253.08±1.61	29.44±2.40

BZB1107 = porin deficient *E. coli* B strain, BZBM114D = M114D OmpC mutant, BZBD112G = D112G OmpC mutant, BZBGGD = M114D and D112G double mutant, BZB8SX = wild-type OmpC, BZBS1E(-) = wild-type OmpF

N. B. All recombinants and wild-type *Serratia marcescens* porins are expressed in BZB1107.

Table 7.5. Units of β-galactosidase produced by cells grown in M9 minimal media at different concentrations of sucrose

Strains	Control	5% sucrose	8% sucrose	10% sucrose
pOY009	0	0	0	0
pOY012	5623.77±30.35	4794.29±103.37	4159.38±256.88	3576.43±345.43
pOYL338W	802.63±17.25	1129.17±3.10	1235.54±3.58	1556.56±21.49
pOY005	987.13±72.42	860.77±27.92	635.98±22.77	303.35±11.01
pK98C	1013.89±13.39	1179.49±51.28	1634.92±27.50	1968.25±119.83

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.6. Units of β-galactosidase produced by cells grown in M9 minimal media at different concentrations of salicylate

Strains	Control	1 mM salicylate	5 mM salicylate	8 mM salicylate
pOY009	0	0	0	0
pOY012	5623.77±30.35	4537.98±21.48	3908.33±4.17	3029.81±20.67
pOYL338W	802.63±17.25	862.00±11.63	1088.12±39.22	1389.49±8.30
pOY005	987.13±72.42	800.34±8.51	621.48±3.00	409.30±2.43
pK98C	1013.89±13.39	1211.11±11.11	1353.46±9.35	1731.58±38.23

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.7. Units of β-galactosidase produced by cells grown in M9 minimal media at different pH

Strains	pH 7	pH 6	pH 8
pOY009	0	0	0
pOY012	5623.77±30.35	3849.62±4.68	6869.78±214.12
pOYL338W	802.63±17.25	1189.50±18.52	359.69±6.87
pOY005	987.13±72.42	609.456±4.20	1431.49±30.85
pK98C	1013.89±13.39	1268.43±49.43	332.98±5.52

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.8. Units of β-galactosidase produced by cells grown in M9 minimal media at different temperatures

Strains	37°C	28°C	42°C
pOY009	0	0	0
pOY012	5623.77±30.35	6623.95±45.95	3193.15±107.92
pOYL338W	802.63±17.25	265.57±6.06	1158.06±15.12
pOY005	987.13±72.42	1525.07±14.64	118.39±5.46
pK98C	1013.89±13.39	247.14±7.73	1327.84±46.84

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.9. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 10% glucose at 28°C

Strains	28°C	10% sucrose	28°C/10% sucrose
pOY009	0	0	0
pOY012	6623.95±45.95	3576.43±345.43	6502.34±90.72
pOYL338W	265.57±6.06	1556.56±21.49	340.96±37.01
pOY005	1525.07±14.64	303.35±11.01	1430.22±105.07
pK98C	247.14±7.73	1968.25±119.84	329.54±47.39

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.10. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 8 mM salicylate at 28°C

Strains	**28°C**	**8 mM salicylate**	**28°C/8 mM salicylate**
pOY009	0	0	0
pOY012	6623.95±45.95	3029.81±20.70	6502.67±90.23
pOYL338W	265.57±6.06	1389.49±8.30	473.32±7.86
pOY005	1525.07±14.64	409.30±2.43	1448.93±46.47
pK98C	247.14±7.73	1731.58±38.23	605.83±28.44

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.11. Units of β-galactosidase produced by cells grown in M9 minimal media at pH 8 and 42°C

Strains	pH 8	42°C	pH 8/42°C
pOY009	0	0	0
pOY012	6869.78±214.12	3193.15±107.92	3566.04±68.27
pOYL338W	359.69±9.87	1158.06±15.12	1138.81±15.05
pOY005	1431.49±30.85	118.39±5.46	370.55±15.27
pK98C	332.98±5.52	1327.84±46.84	1291.41±45.07

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.12. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 10% sucrose at pH 8

Strains	pH 8	10% sucrose	pH 8/10% sucrose
pOY009	0	0	0
pOY012	6869.78±214.12	3576.43±345.43	6288.62±89.31
pOYL338W	359.69±6.87	1556.56±21.49	432.14±11.55
pOY005	1431.49±30.85	303.35±11.01	1281.31±30.88
pK98C	332.98±5.52	1968.25±119.84	440.14±16.85

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.13. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 5% sucrose and 8 mM salicylate

Strains	5% sucrose	8 mM salicylate	5% sucrose/8 mM salicylate
pOY009	0	0	0
pOY012	4794.29±103.37	3029.81±20.70	4484.35±43.18
pOYL338W	1129.17±3.10	1389.49±8.30	1022.32±25.60
pOY005	860.77±27.92	409.30±2.43	700.30±38.35
pK98C	1179.49±51.28	1731.58±38.23	1269.56±40.34

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.14. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 8 mM salicylate at pH 8

Strains	pH 8	8 mM salicylate	pH 8/8 mM salicylate
pOY009	0	0	0
pOY012	6869.78±214.12	3029.81±20.70	6754.91±30.95
pOYL338W	359.69±6.87	1389.49±8.30	417.67±33.12
pOY005	1431.49±30.85	409.30±2.43	1351.34±29.02
pK98C	332.98±5.52	1731.58±38.23	445.38±13.37

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.15. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 10% sucrose and 8 mM salicylate at 28°C

Strains	10% sucrose	8 mM salicylate	28°C	10% sucrose/8 mM salicylate/28°C
pOY009	0	0	0	0
pOY012	3576.43±345.43	3029.81±20.70	6623.95±45.95	6218.30±208.59
pOYL338W	1556.56±21.49	1389.49±8.30	265.57±6.06	599.25±18.82
pOY005	303.35±11.01	409.30±2.43	1525.07±14.64	1339.69±22.72
pK98C	1968.25±119.84	1731.58±38.23	247.14±7.73	319.68±17.69

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.16. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 10% sucrose and 1 mM salicylate at pH 8

Strains	pH 8	10% sucrose	1 mM salicylate	pH 8/10% sucrose/1 mM
pOY009	0	0	0	0
pOY012	6869.78±214.12	3576.43±345.43	4537.98±21.48	6306.43±127.84
pOYL338W	359.69±6.87	1556.56±21.49	862.00±11.63	370.79±9.13
pOY005	1431.49±30.85	303.35±11.01	800.34±8.51	1313.74±18.45
pK98C	332.98±5.52	1968.25±119.84	1211.11±11.11	343.41±12.32

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.

Table 7.17. Units of β-galactosidase produced by cells grown in M9 minimal media supplemented with 10% sucrose at 28°C and pH 6

Strains	28°C	10% sucrose	pH 6	28°C/10% sucrose/pH 6
pOY009	0	0	0	0
pOY012	6623.95±45.95	3576.43±345.43	3849.617±4.68	6218.30±208.59
pOYL338W	265.57±6.06	1556.56±21.49	1189.503±18.53	599.25±18.82
pOY005	1525.07±14.64	303.35±11.01	609.45±4.20	1339.69±22.72
pK98C	247.14±7.73	1968.25±119.84	1268.431±49.43	319.68±17.69

Table 2.2 gives the description of pOY009, pOY012, pOYL338W, pOY005 and pK98C.