Characterization of the OprB Porin of *Pseudomonas aeruginosa*

By

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CHARACTERIZATION OF THE OprB PORIN OF *Pseudomonas aeruginosa*

BY

JOHN L. WYLIE

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

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ABSTRACT

The OprB porin of *Pseudomonas aeruginosa* was first identified following growth of cells on glucose. The subsequent demonstration of facilitated diffusion of glucose into OprB-containing liposomes, suggested that OprB was a glucose-selective porin involved in facilitating the diffusion of glucose across the outer membrane of *P. aeruginosa*. However, the presence of a glucose binding site within the channel was not confirmed nor was the *in vivo* function of OprB demonstrated. This study was designed to characterize the OprB porin and to determine the role this porin plays in the physiology of *P. aeruginosa*. The OprB porin was purified by detergent extraction of the outer membrane of *P. aeruginosa*, followed by ion exchange chromatography. Analysis of purified OprB by circular dichroism spectropolarimetry, demonstrated a high ß sheet content (40%). Black lipid bilayer analysis revealed a single-channel conductance of 25 pS, the presence of a glucose binding site with a K_s for glucose of 380 ± 40 mM, and the formation of channels with a strong selection for anions. The gene encoding OprB was cloned and expressed in *E. coli*. The *oprB* gene was sequenced and revealed some homology to the maltodextrin-selective LamB porin of *E. coli*. This homology may be related to the presence of a sugar binding site in each protein. An unusual cluster of aromatic residues was identified in OprB, which may also play a role in determining the diffusion properties of the OprB channel.

Carbohydrate-inducible proteins potentially homologous to OprB were identified in several rRNA homology group I pseudomonads by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, Western immunoblotting, and N terminal amino acid sequencing. The cloned *oprB* gene was used to construct an OprB-deficient strain of *P. aeruginosa*. Analysis of this strain verified that *in vivo*, OprB does play a role in facilitating the diffusion of glucose across the outer membrane of *P. aeruginosa*. Additionally, OprB also facilitated the diffusion of fructose, mannitol, and glycerol into the periplasm. The results obtained during the course of this study, verify that OprB does contain a binding site for carbohydrates and that OprB actually functions as a central component of carbohydrate uptake in *P. aeruginosa*.

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Ω	antibiotic resistance cassette
ABC transporter	ATP binding cassette transporter
Ap	Ampicillin
ATCC	American Type Culture Collection
BM2	Basal Medium 2
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CD	Circular Dichroism
Cm	Chloramphenicol
CTAB	Hexadecyltrimethyl ammonium bromide
DEAE	diethylaminoethyl
DMSO	Dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropylthiogalactoside
KDO	3-deoxy-D-manno-octulosonic acid
Km	Kanamycin
LB	Luria broth
LPS	Lipopolysaccharide
MDO	Membrane derived oligosaccharide
NCTC	National Collection of Type Cultures
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
S	Siemens
sacB	Gene encoding levansucrase
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
Spc	Spectinomycin
SSC	sodium chloride/sodium citrate buffer
Tc	Tetracycline
TCA	Tricarboxylic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TNS	Tris, NaCl, SDS buffer
TTE	Tris, Triton X-100, EDTA buffer
UMCC	University of Manitoba Culture Collection

Porins

E. coli

OmpF	Nonspecific diffusion porin
OmpC	Nonspecific diffusion porin
PhoE	Anion selective porin
LamB	Maltodextrin-selective porin

Porins (con't)

P. aeruginosa

OprB	Carbohydrate-selective porin
OprC	Porin - function unknown
OprD	Basic amino acid-selective porin
OprE	Porin - function unknown
OprF	Nonspecific diffusion porin
OprP	Monophosphate-selective porin
OprO	Polyphosphate-selective porin

PUBLICATIONS ARISING FROM THIS THESIS

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Wylie, J. L., and E. A. Worobec. 1993. Cloning and nucleotide sequence of the Pseudomonas aeruginosa glucose-selective OprB porin gene and distribution of OprB within the family Pseudomonadaceae. Eur. J. Biochem. **220**:505-512.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Pseudomonas aeruginosa, the type species of the genus *Pseudomonas*, is a rod-shaped, polarly flagellated, nonsporulating, gram negative bacterium. Pseudomonads, in general, are characterized as chemoorganotrophs, typically showing an aerobic metabolism, however, nitrate can be used by some species as an alternate electron acceptor under anaerobic conditions. Pseudomonads are nutritionally versatile, able to utilize a wide range of compounds as carbon sources, generally without the need for any organic growth factors.

Applied as a definition, the above characteristics actually describe a large number of diverse, heterogeneous organisms. As the number of species within the genus *Pseudomonas* continually increased, it became apparent that the family *Pseudomonadaceae* did not describe a single natural group of organisms. Analysis of a wide range of phenotypic characteristics (Stanier *et al.*, 1966) coupled later with DNA-DNA and rRNA-DNA homology studies (Palleroni *et al.*, 1972; 1973) indicated that the family *Pseudomonadaceae* actually consisted of 5 groups of species, each only distantly related to any of the other groups. Analysis of catabolic pathways, cell wall composition, and 16S oligoribonucleotide catalogues produced results consistent with DNA hybridization analysis (Palleroni, 1983). The clear differentiation of the family *Pseudomonadaceae* into separate clusters is such that each rRNA

group is currently being assigned generic status, with the genus *Pseudomonas* being reserved for rRNA group I species. This group includes *P. aeruginosa* as the type species. rRNA group I is further subdivided into non-fluorescent (Ia) and fluorescent (Ib) subgroups. As the names imply, these subgroups are differentiated on the basis of pigment production. The fluorescent pigments produced by subgroup Ib, of which *P. aeruginosa* is a member, act as iron chelators and allow these bacteria to grow in media having a low iron content.

Most of the research dedicated to the genus *Pseudomonas* has been directed towards *P. aeruginosa*, mainly due to the prominence of this bacterium as an opportunistic human pathogen. *P. aeruginosa* is the leading cause of hospital acquired infections in patients otherwise compromised by illnesses or injuries such as cancer, burns, or cystic fibrosis. A number of factors contribute to the predominance of *P. aeruginosa* as a pathogen in comparison to other pseudomonads. These include: 1) an ability to survive in harsh environments (*P. aeruginosa* efficiently scavenges nutrients from distilled water and can survive in hospital sink traps [Vasil, 1986]), 2) production of a large number of toxins and enzymes many of which are released into surrounding host tissues rather than remaining associated with the outer membrane or periplasm (exotoxin A, exoenzyme S, Leukocidin, phospholipase C, elastase, alkaline protease, and rhamnolipid

[Nicas and Iglewski, 1986]), 3) the ability to express certain virulence factors in response to specific environmental conditions encountered in different kinds of compromised hosts (eg. the production of alginate in the lungs of cystic fibrosis patients [Vasil, 1986]), and 4) the natural resistance of this bacterium to many commonly used antibiotics (Yoshimura and Nikaido, 1982). Although, *P. aeruginosa* can produce ß-lactamase for the breakdown of ß-lactam antibiotics, the general antibiotic resistance of this bacterium is believed to be due mainly to the low outer membrane permeability of this bacterium, in comparison to most other gram-negative bacteria. The *P. aeruginosa* outer membrane is 100 - 500 times less permeable to antibiotics and other compounds than the *E. coli* outer membrane. This low permeability appears to be related to the unique properties of the porin molecules produced by this species of bacterium.

Porin proteins are responsible for the differential sieving properties of the outer membrane of most, if not all, gram-negative bacteria. Their properties allow the outer membrane of gram negative bacteria to act as a permeability barrier, preventing or slowing the passage of potentially toxic substrates, while allowing the rapid passage of nutrients or waste products into or out of the cell. Porins form water filled channels which span the outer membrane allowing the diffusion of substrates between the external environment and the periplasm of the cell. Two types of

porins have been recognized: nonspecific porins and substrate-selective channels. Nonspecific porins form channels which allow a nonspecific diffusion of substrates based on solute mass, charge, and polarity. Substrate-selective porins show some substrate specificity and possess a binding site for a given substrate within the channel.

The research presented in this communication centres on OprB, an outer membrane protein of *P. aeruginosa*. Preliminary evidence suggested this protein was a substrate-selective porin potentially involved in the diffusion of glucose across the outer membrane. The purpose of this research was to characterize the structure and function of this protein and to confirm its proposed role in glucose uptake by this bacterium. Since nutrient acquisition is necessary for survival of a pathogen within a host, and since OprB is expressed when *P. aeruginosa* is growing as a pathogen within human tissues (Brown *et al.*, 1984), an understanding of this porin may be of use in the development of strategies for combating *P. aeruginosa* infections. In general, the characterization of substrate-selective porins is of interest, since information regarding their structure/function relationships is lacking in comparison to nonspecific porins. Their origin and relationship to nonspecific porins is currently unknown and no high resolution structural information is available for the binding site of any of the substrate-selective porins currently identified.

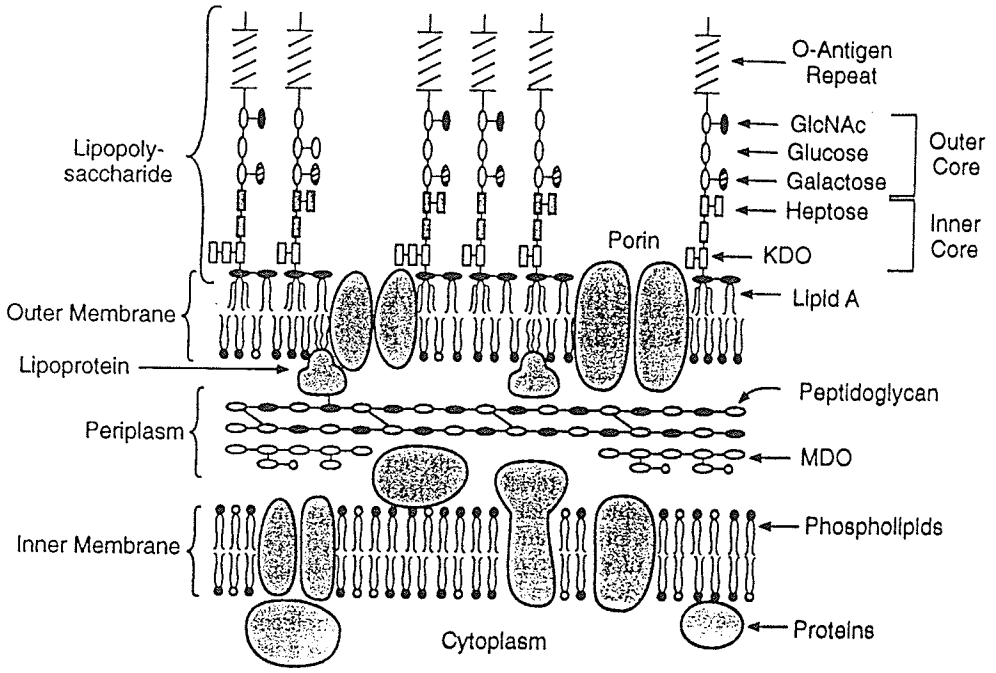
1.2. Bacterial Outer Membranes

1.2.1. Composition

The outer membrane of gram negative bacteria forms the outermost layer of the cell envelope (Fig. 1.1). Morphologically similar to the cytoplasmic membrane it forms a bilayer on the outer surface of the cell, however, unlike the cytoplasmic membrane, its layers are asymmetric (Smit *et al.*, 1975; Kamio and Nikaido, 1976). The inner monolayer consists of phospholipids similar to those found in the cytoplasmic membrane. These consist predominantly of phosphatidylethanolamine, with smaller amounts of acidic phospholipids, phosphatidylglycerol and cardiolipin (Meadow, 1975).

The outer monolayer consists of lipopolysaccharide (LPS). LPS is an amphiphilic molecule consisting of a hydrophobic lipid A region, a core oligosaccharide, and in some cases, an outer O-chain. The basic structure of the lipid A region is a diglucosamine backbone with six or seven fatty acyl chains esterified to the carbohydrate backbone. The core oligosaccharide contains 3-deoxy-D-manno-octulosonic acid (KDO) plus other heptose and hexose residues. The outer O-chain is made up of a variable number of repeating sugar units. Fatty acyl chains in the lipid A region and sugar composition in the core oligosaccharide and O-chain vary depending on species, strain, or serotype examined. The O-chain repeating units show the greatest diversity and can

Figure 1.1. Diagram of the cell envelope of a gram negative bacterium. MDO, membrane derived oligosaccharide; KDO, 3-deoxy-D-*manno*-octulosonic acid; GlcNAc, glucosamine. The core region depicted is that of *Escherichia coli* K-12. Reproduced from Raetz (1993).


O-Antigen
Repeat
GlcNAc
Glucose
Galactose
Heptose
Outer
Core
Inner
Core
Lipopoly-
saccharide
Porin
KDO
Lipid A
Outer Membrane
Lipoprotein
Peptidoglycan
Periplasm
MDO
Inner Membrane
Phospholipids
Proteins
Cytoplasm

consist of a diverse assemblage of sugars, including neutral, acidic, and amino, as well as various branched and substituted sugars. It is these sugars which are exposed on the external surface of the cell. This diversity may have developed as a means of evasion of host immune systems (Mayer *et al.* 1989). In a single strain each LPS molecule is capped by a different number of O chain repeats and many also occur as unsubstituted core oligosaccharides (rough LPS).

Interspersed within the LPS and lipids are proteins, which constitute approximately 50% of the outer membrane. The actual number of different types of protein is large if sufficiently sensitive methods of detection are used (Nikaido and Vaara, 1985), however, a relatively small number of proteins predominates resulting in relatively simple patterns on SDS-PAGE gels (Flammann and Weckesser, 1984; Mizuno and Kageyama, 1978; Lugtenberg *et al.* 1975; Nixdorf *et al.* 1977; Nakamura and Mizushima, 1976).

Outer membrane proteins consist of two general types: structural proteins and those involved in transport. Major structural proteins first identified in *Escherchia coli* include lipoproteins and a porin like protein (OmpA). Similar proteins have now been identified in other species of gram negative bacteria (Duchêne *et al.*, 1988; Braun and Cole, 1984, 1983, 1982; Verstreate *et al.*, 1982; Heckels, 1977). Lipoproteins are small proteins (7200 daltons in *E. coli*) associated either noncovalently or covalently with the

peptidoglycan and anchored to the outer membrane via a fatty acid residue and diglyceride attached to the C terminus of the protein (Braun, 1975; Braun *et al.*, 1976). This arrangement serves to anchor the outer membrane to the peptidoglycan via the lipoprotein.

The OmpA protein is similar to known porins based on its monomer molecular weight (33,000 daltons in *E. coli*) and a predominantly ß sheet structure (Nakamura and Mizushima, 1976). Additionally, like porins, this protein spans the outer membrane (shown by an association with the peptidoglycan layer [Endermann *et al.*, 1978] and by accessibility of OmpA to non-penetrating labelling reagents [Kamio and Nikaido, 1977]). Despite these similarities OmpA was generally believed to have no pore-forming ability, however, Sugawara and Nikaido (1992) recently demonstrated that OmpA does form channels of a similar size to other *E. coli* porins but which allow a much slower penetration of solutes. These inefficient channels would have a minimal effect on outer membrane permeability making the OmpA protein important largely in terms of a structural role (Sonntag *et al.*, 1978).

An exception is the OmpA homologue, OprF, in *P. aeruginosa*. This protein acts as the major porin of this bacterium despite its poor pore forming ability. The implications of this on outer membrane permeability, antibiotic resistance and evolution of substrate-selective porins in *P. aeruginosa* will be discussed in greater detail in

section 1.9.

Transport proteins of the outer membrane consist of three types: general diffusion porins, substrate-selective porins and high affinity energy dependent transport proteins (Nikaido, 1992). As indicated above, porins form open water filled channels spanning the outer membrane thus allowing the passage of solutes. These proteins will be discussed at greater length throughout this review. The third class of transport proteins functions in the specific transport of iron chelator complexes and vitamin B_{12}. Unlike porins, they do not form measurable open pores and bind specific substrates in an energy dependent fashion (Postle, 1990; Kadner, 1990). Since the outer membrane is unenergized, this class of transport protein must function in conjunction with a second protein, TonB. Although anchored in the cytoplasmic membrane, TonB spans the periplasm and serves to transfer energy from the cytoplasmic membrane to the energy dependent outer membrane transport proteins. Interaction of TonB with the outer membrane receptors is believed to result in a conformational change allowing release of the bound ligand from the receptor into the periplasm.

1.2.2. Bacterial Outer Membranes as a Permeability Barrier

Bacterial outer membranes serve to partially isolate and protect the cell from its external environment by acting as a permeability barrier. Penetration of harmful substances such

as lysozymes, digestive enzymes, antibiotics and hydrophobic detergents (eg. bile salts) are reduced due to the limited permeability of the membrane. Yet sufficient permeability must be maintained to ensure adequate movement of nutrients and waste products into and out of the cell. The presence of LPS coupled with transport proteins allows these potentially conflicting properties to be simultaneously maintained.

The low permeability of the outer membrane to most compounds arises from the amphiphilic nature of LPS. Similar to phospholipid bilayers, packing of the fatty acyl chains on the lipid A region of LPS coupled with the phospholipid content of the outer membrane creates a barrier to hydrophilic solutes. However, in contrast to the permeability properties of phospholipid bilayers, LPS also creates a permeability barrier to hydrophobic compounds such as hydrophobic antibiotics, detergents, and dyes (Cohen and Bangham, 1972; Nikaido, 1976; Leive, 1974). This property of LPS is related to the structural properties of the oligosaccharide portion. LPS carries a net negative charge due to the phosphate content of the core polysaccharide (Kropinski *et al.*, 1979). These negatively charged molecules are linked by noncovalent binding of divalent cations (Yamada and Mizushima 1980; Schindler and Osborn 1979; Schweitzer *et al.* 1978; Lieve, 1974, 1965) resulting in a tight network of LPS molecules in the outer membrane. It is this extensive network of divalent cation salt bridges between adjacent LPS molecules which have been

linked to the barrier properties of LPS to hydrophobic compounds. Analysis of rough LPS mutants in *S. typhimurium*, *E. coli*, and *P. aeruginosa* revealed that the degree of sensitivity to hydrophobic compounds correlates with the loss of phosphate groups in LPS and therefore the cross bridging potential of LPS (Nikaido and Vaara, 1985). Additionally, treatment of cells with chelating agents such as EDTA, which remove divalent cations from LPS, also increases sensitivity to hydrophobic reagents (Nicas and Hancock, 1983a). Polycationic antibiotics (eg. polymyxin), which bind to divalent cation binding sites of LPS (Moore *et al.*, 1986; 1984; Peterson *et al.* 1985), also increase outer membrane permeability to hydrophobic compounds (Hancock and Wong, 1984). It is believed that the stabilization of the outer membrane by divalent cation cross-bridging prevents lateral spatial displacement of outer membrane components and acts as a barrier to most solutes (Nikaido and Hancock, 1986).

The low permeability of the outer membrane must be countered sufficiently to allow influx of nutrients and efflux of waste products. Gram negative bacteria possess porins and high affinity energy dependent transport proteins in the outer membrane to create a selectively permeable outer membrane. Porins form water filled channels bridging the outer membrane and allow the passage of small hydrophilic molecules. Evidence establishing the role of porins in diffusion of these solutes is based on model membrane studies (Hancock, 1986)

and studies involving porin deficient mutants (Poole and Hancock, 1986a; Nicas and Hancock, 1983b; Lutkenhaus, 1977). Most nonspecific porins possess a channel diameter of 0.6 to 2.3 nm such that diffusion of small nutrients such as amino acids, carbohydrates, and inorganic ions can be accommodated through these channels (Hancock, 1987). By forming water filled channels, significant passage of hydrophobic molecules is prevented and formation of relatively small channels also excludes large hydrophilic molecules. Substrate-selective porins possessing a binding site for a given substrate, facilitate uptake of large substrates (eg. maltodextrins through the *E. coli* LamB porin) or uptake of substrates at low substrate concentrations when nonspecific diffusion would occur at very low rates (Benz, 1988). High affinity energy dependent transport proteins are required for very large substrates such as iron bearing siderophores or vitamin B12, which exceed the molecular exclusion limit of porins and occur at such low environmental concentrations that higher affinity systems are required to acquire these nutrients (Postle, 1990). In this way, gram negative bacteria are able to maintain an effective barrier against hydrophobic and large hydrophilic molecules through the properties of LPS, yet still acquire nutrients via nonspecific diffusion of small solutes through porins or by specifically recognizing larger nutrients and selectively allowing their passage through the outer membrane.

1.3. Porin channel properties

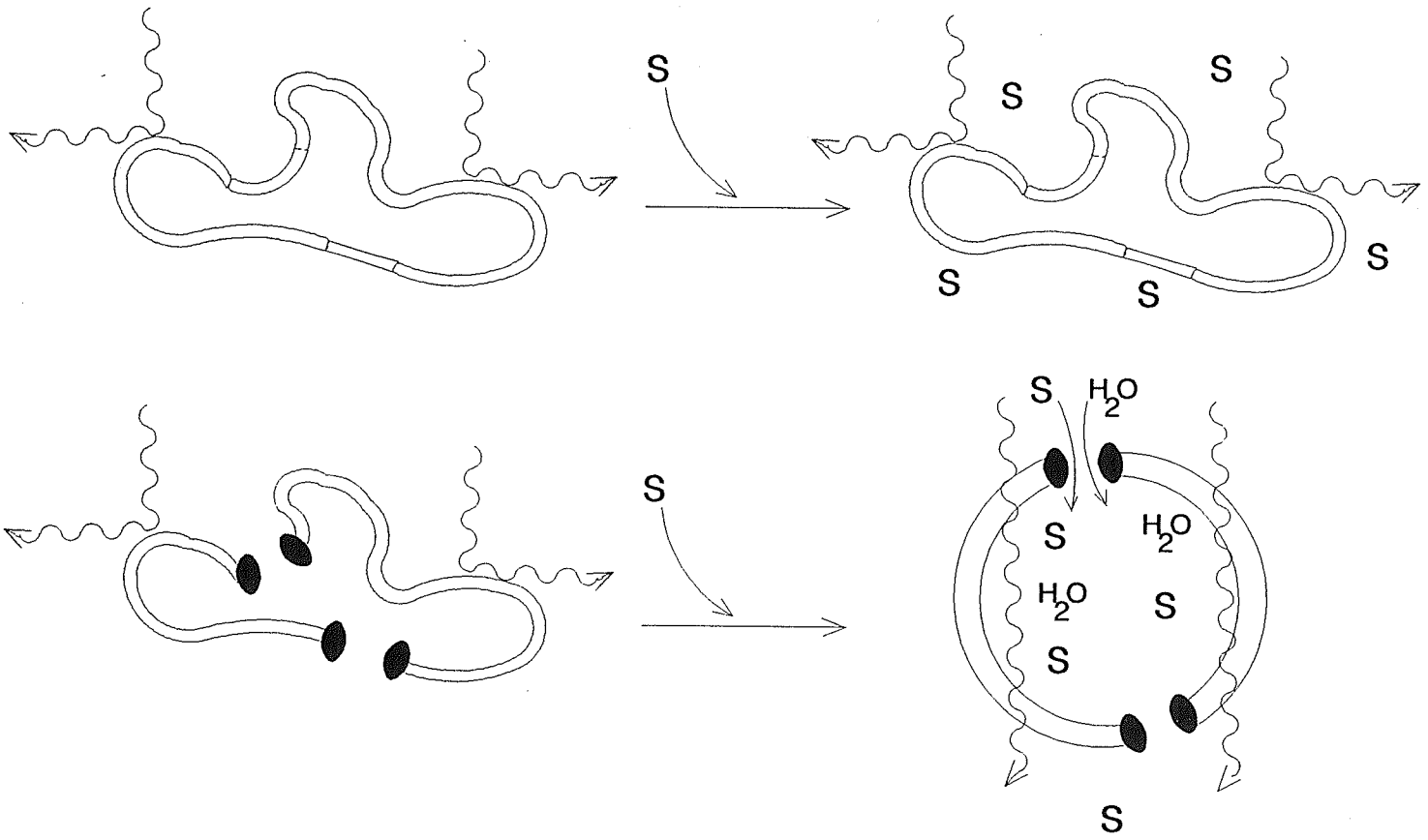
1.3.1. Model Membrane techniques

Descriptions of the following model membrane techniques are based on reviews by Hancock (1986) and Nikaido (1983).

One of the first techniques developed for analysis of porins was radioisotope efflux from liposomes (Nakae, 1975). This technique involves the reconstitution of a dried solution of phospholipids, LPS, and porin in a solution of a small, permeable (with respect to the porin channel) ^{14}C-labelled sugar and a large, impermeable ^{3}H-labelled polysaccharide (eg. dextran). Reconstituted liposomes containing the labelled sugars are diluted to create a concentration gradient across the lipid bilayer. Under these conditions the more permeable sugar preferentially diffuses out of the liposome through the porin channel. Liposomes are collected by column chromatography and the relative retention of the two sugars assessed by scintillation analysis. Analysis of the ratio of ^{14}C-labelled sugar retained to ^{3}H-labelled sugar retained verifies the identity of a protein as a porin, and provides an estimate of the M_r cutoff of a porin channel. Because of the time required for chromatography of the liposome solution the technique provides no information on the rate of diffusion of a solute through a porin pore. This technique is no longer commonly used, being largely replaced by the liposome swelling assay and black lipid bilayer analysis.

Liposome swelling assay (Fig. 1.2) was developed as an

Figure 1.2. Diagram of the liposome swelling assay. Liposomes are represented as either not containing (A) or containing (B) porin proteins. Depicted are the events following addition of liposomes to an isotonic solute solution. When porins are present, solute molecules diffuse through the porin pore, followed by an influx of water and swelling of the liposome (Hancock, 1986).


S
S
S
S
S
S
S
S
S
H_2O
H_2O
S
S
S

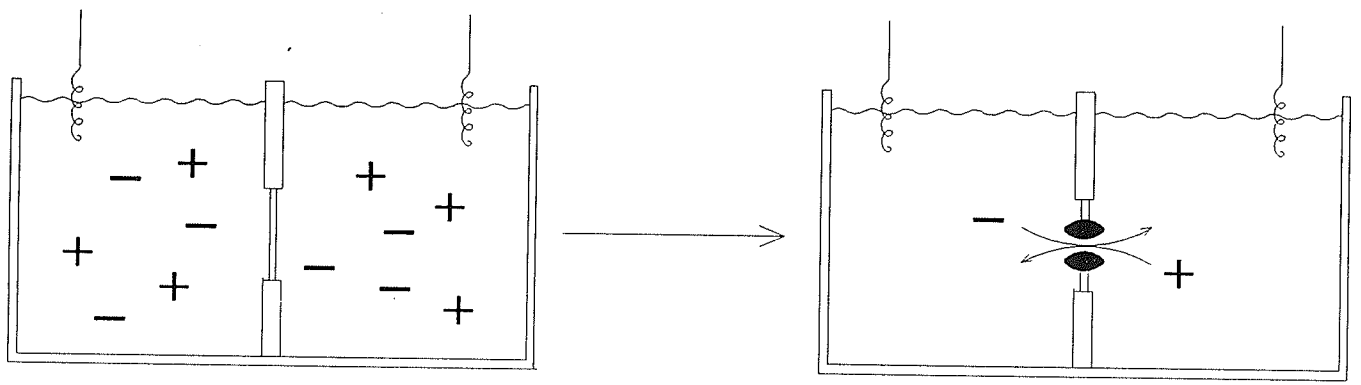
assay for measuring substrate movement through porin channels (Luckey and Nikaido, 1980; Nikaido and Rosenberg, 1983). This assay is based on a technique originally described by Bangham *et al.* (1974). Liposomes are reconstituted from a dried lipid film in the presence of an aqueous solution of purified porin or isolated outer membrane. Liposomes containing the porin are again dried and reconstituted in the presence of an impermeable sugar such as stachyose (666 Da) or dextran (dextrans of 20 or 40 kDa are commonly used). Dextran is required for porins forming a large diameter channel, such as OprF of *P. aeruginosa*, as stachyose is able to slowly permeate the channels of these proteins (Nikaido *et al.*, 1991). The irregular shape of the reconstituted liposomes effectively scatters light and, as a result, the liposome solution has a high optical density. To assess solute permeability, liposomes are diluted into a solution of the test solute, previously adjusted to be isotonic with the liposome interior. If the solute does not diffuse through the pore, the system will remain at osmotic equilibrium. If the solute is able to permeate the pore, it will diffuse down a concentration gradient into the interior of the liposome, followed by an influx of water as the osmotic pressure of the liposome interior changes with respect to the exterior. As the liposome swells to a spherical shape, it is less able to scatter light and the optical density of the solution decreases. The initial rate of change of the optical density

is taken as an indication of the rate of solute penetration. The liposome swelling assay is used to verify the porin function of a purified protein, estimate porin channel size, and compare the relative rates of diffusion of a series of solutes (the rate of diffusion of the smallest solute is taken as 100%). The assay is widely used and a detailed description of the technique and potential problems has recently been published by Nikaido *et al.* (1991).

The third model membrane technique commonly used to investigate porin channel properties is the black lipid bilayer analysis. This technique is designed to characterize the channel properties of a porin by measuring the conductivity of ions through the porin pore. The apparatus consists of a small teflon container separated into two buffer chambers by a teflon divider (Fig. 1.3). A small hole in the teflon divider connects the two compartments. The teflon chamber is filled with a buffer solution and a lipid solution is painted across the hole to form a lipid bilayer. An electrode is placed in each compartment through which a voltage can be applied. In the absence of any porin, resistance of the lipid bilayer to ion flow is high and the measured current is low. If a dilute porin solution is added, porins insert in the membrane and ions will flow through the porin in response to an applied voltage.

Depending on the dilution of the porin solution, either single channel conductance or macroscopic conductance can be

Figure 1.3. Representation of the black lipid bilayer apparatus. Depicted is the teflon chamber divided into two compartments by a teflon divider. A lipid bilayer is contained in the centre of the divider. Ovals within the bilayer represent a porin protein. Coiled lines represent electrodes immersed in the salt solution in each chamber. Positive and negative signs represent ions in the buffer solution. In response to an applied voltage ions pass through the pore of the porin molecule.



measured. In single channel conductance analysis, a sufficiently dilute porin solution is used such that increases in ion flow are observed as small stepwise increases in the current, each step consisting of a single porin molecule inserting in the membrane (Fig. 1.4). In macroscopic conductance measurements a more concentrated solution of porins is added resulting in a continuous rapid rise in conductance, slowly decreasing with time (Fig. 1.5). Typically, the concentration of the porin solution is adjusted such that the conductance increases approximately 100 - 1000 fold over 20 - 30 min.

Macroscopic conductance measurements are used to measure the ion selectivity of porin channels (zero current membrane potential measurements) or the half saturation constant of substrate binding to a substrate-selective porin. For ion selectivity measurements, once the porins have inserted in the membrane, the applied voltage is removed and a concentrated salt solution is added to one side of the membrane and an equal amount of buffer added to the second chamber. This creates a chemical potential gradient across the membrane, driving ion diffusion through the porin channels to the dilute side of the membrane (Fig. 1.6). If the porin shows some degree of ion selectivity a preferential diffusion of either positive or negative ions will occur creating a charge differential across the membrane. When the electrical potential balances the chemical potential, movement of ions

Figure 1.4. Stepwise increases in conductance of a lipid bilayer in response to the addition of a dilute solution of the OprP porin of *Pseudomonas aeruginosa*. Reproduced from Hancock and Benz (1986).

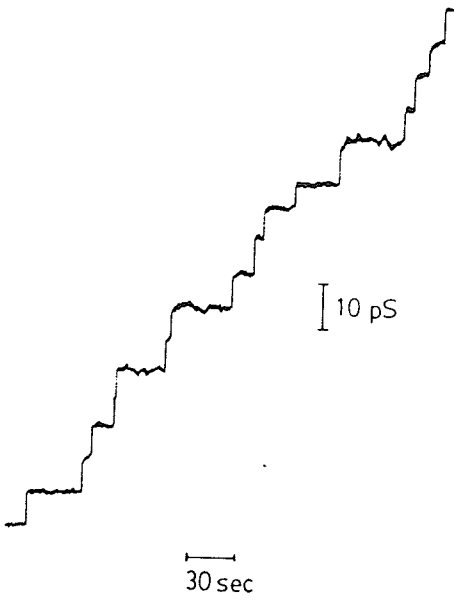

10 pS
30 sec

Figure 1.5. Macroscopic conductance increase of a lipid bilayer following addition of a solution of *P. aeruginosa* OprP porin to the buffer chamber. OprP concentration was 3 orders of magnitude higher than that used to generate the results shown in Figure 1.4. Reproduced from Hancock and Benz (1986).

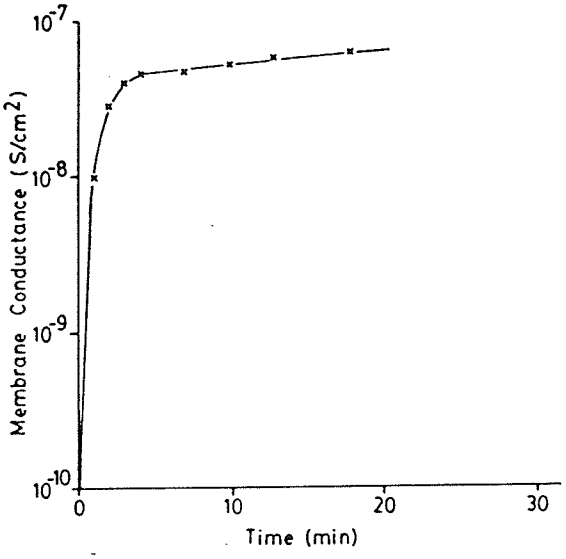
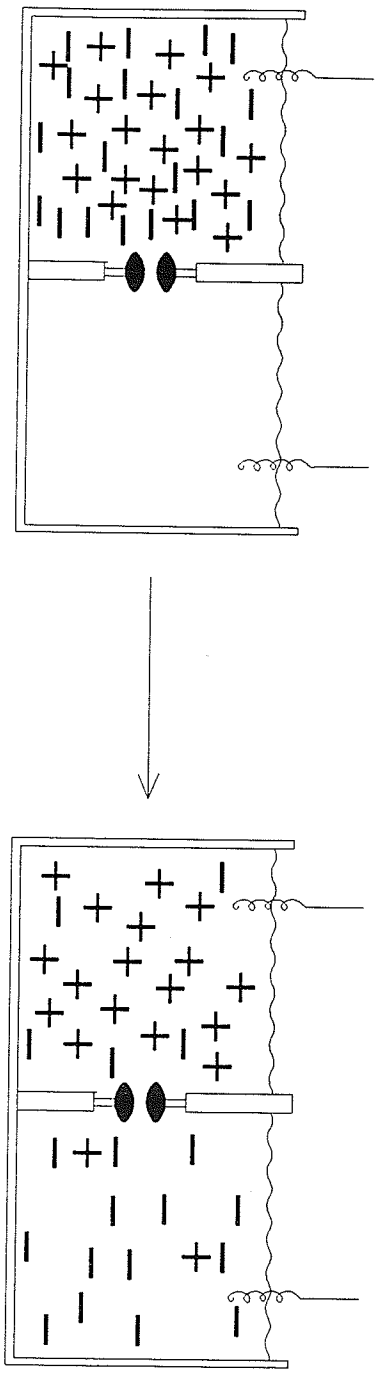

10^{-7}
10^{-8}
10^{-9}
10^{-10}
Membrane Conductance (S/cm^2)
0
10
20
30
Time (min)

Figure 1.6. Representation of the events occurring during ion selectivity analysis using the black lipid bilayer technique. The diagram is identical to that shown in Figure 1.3 except a voltage has not been applied and the concentration of the buffer solution differs on each side of the lipid bilayer. Ions begin to move down a chemical gradient with preferential movement of anions or cations depending on the ion selectivity of the porin. When the chemical potential is balanced by the electrical potential the charge on the dilute side of the membrane is an indication of the ion selectivity of the channel.



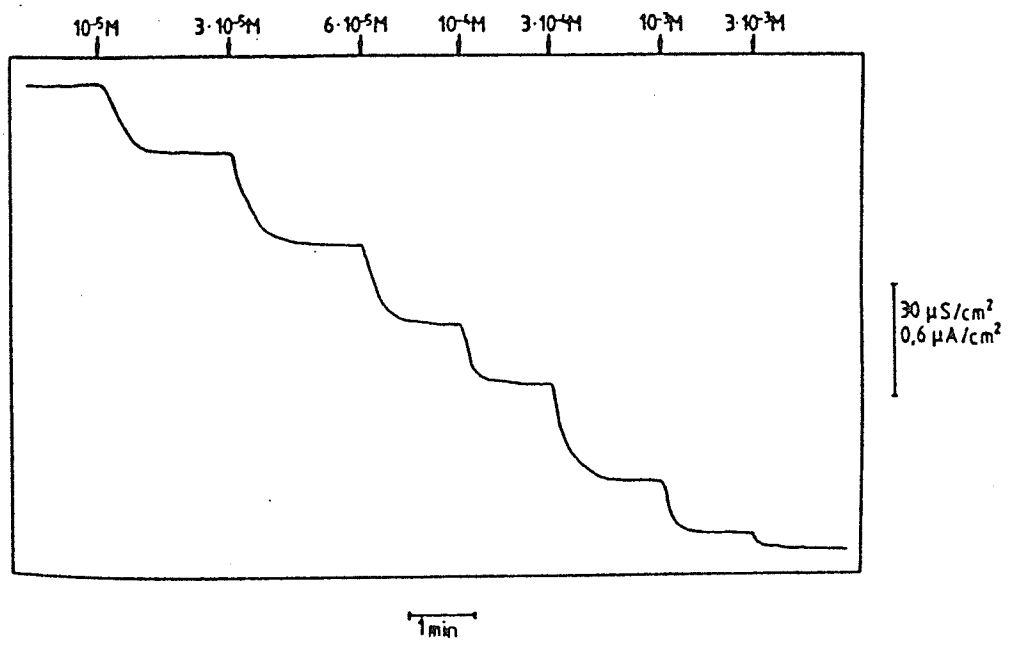
will stop. At this point, measurement of the electrical potential (zero current potential) will indicate the ion selectivity of the porin. An example of the analysis of the ion selectivity of a series of porins can be found in Benz *et al.* (1985).

For measurement of the half saturation constant of binding to a substrate-selective porin, the black lipid bilayer apparatus is setup as described for macroscopic conductance measurement. The concentrated salt solution, used for ion selectivity measurements, is replaced by a concentrated solution of the potential ligand. Binding of the substrate to the binding site in the interior of the channel results in a blockage of ion flow through the pore (Fig. 1.7). The decrease in conductivity is measured and the process repeated with increasing concentrations of substrate. The data can be analyzed by means of a Lineweaver-Burke plot to determine the half saturation constant of binding. A detailed description of the analysis of substrate-selective porins by black lipid bilayer analysis can be found in Benz *et al* (1987).

1.3.2. Porin channel properties

Porins are widespread in gram-negative eubacterial genera having been identified in 3 of the 4 (α, ß, γ) subgroups of the purple bacteria (Gerbl-Reiger *et al*, 1991; Jeanteur *et al.*, 1991), spirochetes (*Spirochaeta aurantia* [Kropinski *et*

Figure 1.7. Titration of a substrate-selective-porin-induced conductance of a lipid bilayer. Depicted is the titration of LamB-induced conductance in response to the addition of increasing concentrations of maltopentaose. Maltopentaose binding to the LamB channels results in blockage of the pores and a dose-dependent decrease in conductance. Reproduced from Benz (1988).


10⁻⁵M
3·10⁻⁵M
6·10⁻⁵M
10⁻⁴M
3·10⁻⁴M
10⁻³M
3·10⁻³M
30 µS/cm²
0,6 µA/cm²
1 min

al, 1987], and *Leptospira alstoni* [Haake *et al.*, 1993]), *Chlamydia* (Stephens *et al.*, 1986), cyanobacteria (*Anabaena variabilis* [Benz and Böhme, 1985], and *Synechococcus* [Hansel *et al.*, 1994), and *Thermotoga maritimus* (Rachel *et al.*, 1990). The widespread appearance of porins and their presence in one of the deepest phylogenetic branches of the bacterial kingdom suggests that porin-type molecules may be present in all bacteria possessing a permeability barrier on the outside of the cell. Porins are generally not found in gram-positive bacteria given their lack of a permeability barrier on the exterior of the peptidoglycan layer. One exception is *Mycobacterium chelonae*, in which a porin has recently been identified (Trias *et al.*, 1992). However, this gram-positive bacterium is unusual in that it possesses an outer membrane and therefore requires some means of allowing permeation of solutes across the outer membrane.

Although widespread in eubacterial genera, all bacterial porins show a high degree of structural similarity. The molecular mass of porin monomers ranges from approximately 28,000 - 48,000 (Hancock, 1986). Crosslinking experiments (Palva and Randall, 1978; Angus and Hancock, 1983), SDS-PAGE analysis (Ishii and Nakae, 1980; Rachel *et al.*, 1990), electron microscopy (Chalcroft *et al.*, 1987; Lepault *et al.*, 1988; Rachel *et al.*, 1990), and X-ray crystallography (Cowan *et al.*, 1992; Weiss and Schulz, 1992), suggest all porins exist as a trimer in their native state, composed of 3 monomer

subunits. The trimers of most porins are resistant to high temperatures, pH extremes, ionic detergents, and urea (Rosenbusch, 1974; Yu *et al.*, 1979; Schindler and Rosenbusch, 1984; Rocque and McGroarty, 1990). Most porins will migrate as a trimer with a very low mobility on SDS-PAGE gels. Generally, trimers must be heated to high temperatures in the presence of SDS to dissociate the native protein to individual monomers. This treatment also results in denaturation of the monomers to a non-functional state. The extreme stability of these proteins is believed to be due to the ß sheet content of porins (Mizushima, 1974; Nakamura and Mizushima, 1976). Most porins have a strong, noncovalent association with the peptidoglycan layer of the periplasm and the LPS of the outer membrane (Hancock, 1986). Despite this association porins are functional in the absence of peptidoglycan and the specific lipid environment surrounding the porin does not appear to affect the porin channel properties (Benz *et al.*, 1978).

Porin channels have been estimated to range in size from 0.6 - 2.3 nm (Hancock, 1987). The channels are water-filled based on the permeability of the channels to hydrophilic solutes when incorporated in liposomes (Nakae, 1975; Nikaido and Rosenberg, 1983), and by black lipid bilayer analysis in which the conductivity of ions through porin channels is almost identical to the conductivity of the salt solution in the absence of a membrane (Benz *et al.*, 1978). Porin channels

contain charged residues and as such are weakly anion or cation selective. The presence of charged residues within porin channel interiors and their role in ion selectivity is demonstrated by changes in the ion selectivity of a channel as a function of pH (Benz *et al.*, 1979; Hancock *et al.*, 1983; Darveau *et al.*, 1984) and by chemical modification of carboxyl and amino groups within the pore interior (Tokunaga *et al.*, 1981; Darveau *et al.*, 1984; Hancock *et al.*, 1983; Hancock *et al.*, 1986).

Although the physical characteristics of many porins have been well documented and confirmed by different groups of investigators, the question of whether porins are voltage-regulated has remained unclear. Several groups investigating voltage-regulation of bacterial porins regularly report a voltage-induced closure of porins when applied voltages in black lipid bilayer analysis exceed approximately 100 mV (Schindler and Rosenbusch, 1978, 1981; Morgan *et al.*, 1990). However, other groups report that channel closure does not occur or is a very infrequent event (Benz *et al.*, 1978; Lakey *et al.*, 1985; Xu *et al.*, 1986). Lakey and Pattus (1989) have presented evidence that procedural differences in the setup of the black lipid bilayer apparatus influences the results obtained. These authors examined channel closure using bilayer membranes formed in three different ways. They found that channel closure was observed with all of the membranes, however, it was more prevalent with the membrane formation

technique used by Schindler and Rosenbusch (1978). The deciding factor is believed to be the thickness of the bilayer membrane formed. Results demonstrating no voltage-regulation typically use a membrane formation technique which produces thicker membranes (Lakey and Pattus, 1989). The thicker membrane may serve to stabilize porins in an open state.

Even if porin closure does occur, it has been questioned whether this characteristic of porins has any relevance *in vivo*, or whether it is strictly an *in vitro* phenomenon. This question will be discussed in greater detail in section 1.5 dealing with porin function *in vivo*.

1.4. Porin structure

1.4.1. Primary structure

The genes encoding several porin proteins have been sequenced, most from genera within the family *Enterobacteriaceae*. Some sequence data is also available for species within the genera *Neisseria, Comomonas, Leptospira, Haemophilus, Pseudomonas, Bordetella, Chlamydia and Rhodobacter* (Stephens *et al.*, 1986; Gerbl-Reiger *et al.*, 1991; Jeanteur *et al.*, 1991; Li *et al.*, 1991; Schiltz *et al.*, 1991; Haake *et al.*, 1993).

Analysis of available nucleotide sequence data has resulted in the identification of several general characteristics of porin primary structure. Molecular mass determination based on DNA sequence information indicates

porin monomers range in size from 30-50 kDa. Molecular mass determination by sequence analysis shows close agreement to that determined by protein analytical techniques (Inokuchi *et al.*, 1982; Schiltz *et al.*, 1991). All porins examined contain typical procaryotic signal sequences for export of proteins across the cytoplasmic membrane. Unlike inner membrane proteins, porins show no long stretches of hydrophobic residues which could potentially form α-helical membrane spanning regions; rather, charged and uncharged residues are distributed evenly throughout the molecule. The lack of long hydrophobic regions may be necessary to ensure porins are exported across the inner membrane without becoming permanently anchored in the inner membrane (Davis and Model, 1985; MacIntyre *et al.*, 1988). Porins contain high levels of glycine residues and, in general, contain no cysteine residues (Gerbl-Reiger *et al.*, 1991). The high level of glycine may relate to the prevalence of glycine residues in ß turns (Schiltz *et al.*, 1991), a secondary structure common to porins. Typically, porins and other outer membrane proteins possess phenylalanine as the C terminal residue (Struyvé *et al.*, 1991). The presence of this residue appears to be necessary for correct outer membrane incorporation of the protein and formation of stable trimers.

Nucleotide sequence comparison of porins does not generally indicate extensive global homology between porin molecules (Nikaido and Wu, 1984; Schiltz *et al.*, 1991). Only

porins that have arisen as a result of a recent gene duplication within a species (eg. OmpF, OmpC, PhoE of *E. coli* [Mizuno *et al.*, 1983]) or homologous porins identified in closely related species (eg. LamB within the enteric bacteria [Francoz *et al.*, 1990; Werts *et al.*, 1993]) have any significant degree of homology. However, several authors have determined that porins previously considered as nonhomologous actually contain regions of local homology. Comparisons of porins from *Neisseria meningitidis, N. gonorrhoeae, E. coli, Shigella dysenteriae, Chlamydia trachomatis,* and the mitochondrial porin of *Neurospora crassa,* identified up to 8 regions of local homology ranging in size from 10 to 20 amino acid residues (Gerbl-Reiger *et al.*, 1991; Jeanteur *et al.*, 1991). The local homologies were most prevalent in the N terminal third and, to a lesser extent, the C terminal portion of the proteins. Mutant analysis and hybrid porin molecules have suggested that it is primarily the N terminus that determines the functional properties of a given porin while the C terminus plays a role in localization of a porin to the outer membrane (Gerbl-Reiger *et al.*, 1991). The conserved regions are believed to correspond to areas of the protein critical for proper formation of channel walls.

1.4.2. Secondary structure

Prior to the recent extensive investigation of porin proteins, structural analyses of inner membrane proteins had

suggested that hydrophobic α-helices were a common structural motif in membrane proteins, allowing them to span the hydrophobic environment of the membrane. When nucleic acid sequence analysis of porin genes failed to identify any regions which could code for long continuous stretches of hydrophobic residues, the applicability of inner membrane protein structural models to outer membrane proteins was questioned.

A variety of spectroscopic techniques (circular dichroism, infrared spectroscopy, and Raman spectroscopy) indicated that porins contained extensive ß sheet structure (Rosenbusch, 1974; Schindler and Rosenbusch, 1984; Kleffel *et al.*, 1985; Vogel and Jähnig, 1986). Vogel and Jähnig (1986) found the ß sheet content of outer membrane proteins (OmpF, LamB, OmpA) to range from 50% to 68%. High ß sheet content has also been determined for *P. aeruginosa* and *P. putida* porins (Siehnel *et al.*, 1990a; Saravolac *et al.*, 1991). The similarity in secondary structure content of outer membrane proteins, despite an absence of primary sequence homology suggests that a ß sheet conformation represents the common folding pattern for outer membrane proteins.

Further topological information for porins was obtained by identifying cell-surface-exposed regions of the proteins. Some porins act as bacteriophage receptors (eg. LamB, lambda receptor [Randall-Hazelbauer and Schwartz, 1973]; PhoE, TC45 receptor [Korteland *et al.*, 1985]). By selecting *E. coli*

mutants which produced a functional PhoE protein but were resistant to phage TC45, Korteland *et al.* (1985) were able to identify one region of the protein exposed to the cell surface. Similarly, monoclonal antibodies recognizing cell-surface-exposed epitopes of PhoE were used to select mutant PhoE proteins (van der Ley, 1986). Mutant proteins failing to bind the antibodies were selected using the bactericidal action of complement. Based on these analyses, 4 regions of PhoE were identified as cell-surface-exposed, each approximately 40 residues apart.

It was found that these regions corresponded to hydrophilic maxima on a hydropathy plot and also corresponded to several variable regions previously identified by amino acid sequence comparisons between PhoE, OmpC, and OmpF of *E. coli*. Given the nature of the cell-surface-exposed regions, further analysis of the amino acid sequence of PhoE identified a further 4 regions which showed variability in their amino acid sequence and corresponded to hydrophilic maxima, each approximately 40 residues apart. Kleffel *et al.* (1985), using X-ray diffraction analysis, had previously determined a length of 10 - 12 residues for the ß strands of OmpF. Based on this information the regions between the cell-surface-exposed portions of PhoE was sufficient to form two transmembrane ß strands.

The regions of PhoE predicted to form ß strands were notably amphipathic, providing an explanation for the lack of

extensive hydrophobic regions in porins despite their transmembrane location. Folding of the PhoE protein would expose the hydrophobic residues of a ß sheet to the lipid environment on the exterior of the channel while hydrophilic residues would face the aqueous channel interior. However, the lack of complete amphipathicity in predicted ß strands of PhoE and other porins (Tommassen, 1988), the inaccessibility of some amino and carboxyl groups of OmpF to polar, pore-permeant reagents (Tokunaga *et al*, 1981; Schindler and Rosenbusch, 1982; Schlaeppi *et al.*, 1985) and abnormal pK values for conformational transition of OmpF (Schindler and Rosenbusch, 1984), suggested some ionizable residues of porins were actually buried in the hydrophobic environment (Paul and Rosenbusch, 1985). It is believed that ion pair formation between these residues allows for localization of some hydrophilic residues to the hydrophobic environment of the membrane.

Analysis of other *E. coli* outer membrane proteins (Morona *et al.*, 1984; Tommassen, 1988) and porins from *Neisseria* (Jeanteur *et al.*, 1991; van der Ley *et al.*, 1991) and *Haemophilus* (Srikumar *et al.*, 1992) by similar techniques, suggested the folding pattern described for PhoE is common to all porins despite showing little primary sequence homology. The consensus folding pattern appears to consist of antiparallel ß strands separated by short ß turns on the periplasmic side of the protein and longer hydrophilic loops

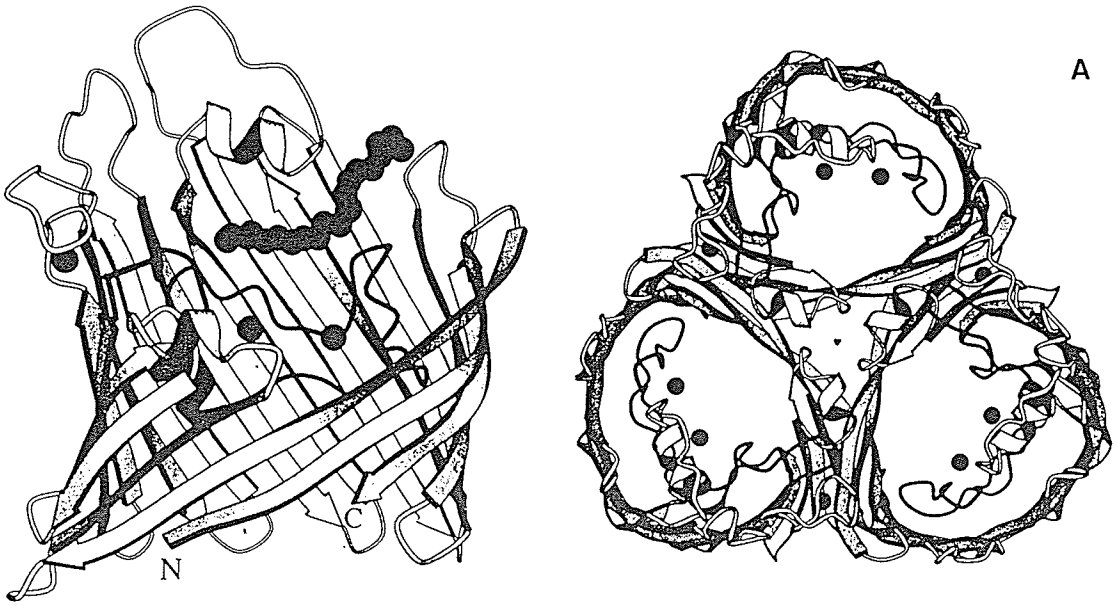
on the external surface of the protein.

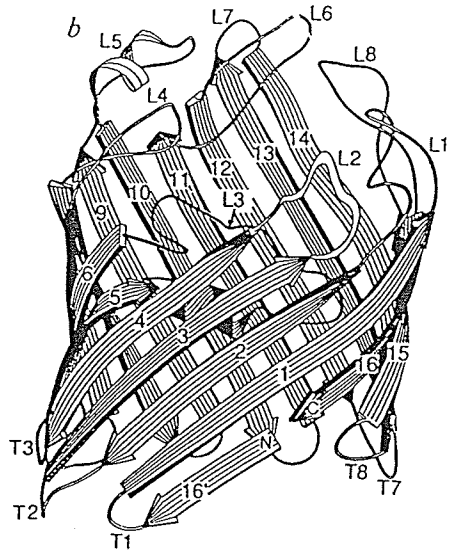
1.4.3. Tertiary and Quaternary Structure

Currently, the structure of *E. coli* porins, OmpF and PhoE, and *Rhodobacter capsulatus* porin have been solved by X-ray crystallography. Several other porins such as LamB and ScrY of *E. coli* (Forst *et al.*, 1993; Keller *et al.*, 1994) and OprP of *P. aeruginosa* (Worobec *et al.*, 1988) have been crystallized, but high resolution structures are not yet available. Despite a large evolutionary distance between *E. coli* and *R. capsulatus*, and a lack of extensive sequence homology between their respective porins, the structures of the porins from these bacteria are found to be remarkably similar. Resolution of the three dimensional structure of these proteins have confirmed early topological analyses discussed in section 1.4.2.

The crystallization and structural analysis of the *R. capsulatus* porin has been described in a series of papers (Weiss *et al.*, 1989; Weiss *et al.*, 1990; Weiss *et al.*, 1991; Weiss and Schulz, 1992). Cowan *et al.* (1992) have described the structural analysis of the *E. coli* OmpF and PhoE porins. The structure of the *R. capsulatus* porin and the *E. coli* OmpF porins are shown in Fig. 1.8. Each porin trimer consists of three identical monomer subunits, each monomer consisting of a 16 stranded antiparallel ß barrel. The *R. capsulatus* porin monomer contains 57% of the residues in ß sheet, 6% α helix,

Figure 1.8. Diagrammatic representation of the structure of the *Rhodobacter capsulatus* (A) and *Escherichia coli* OmpF (B) porin monomer. The tops of the monomers as shown are extracellular. Arrows represent ß strands and are numbered 1-16 for the OmpF monomer. The long extracellular loops of OmpF are labelled L1-L8, the periplasmic ß turns T1-T8. The isolated black circles in (A) represent calcium ions. The string of black circles in (A) represent an unidentified ligand binding to the *R. capsulatus* monomer. Reproduced from Cowan *et al.* (1992) and Cowan (1993).


A
N
C


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

and the remainder in ß turns or loops. Short ß turns connect the ß strands on the periplasmic side of the protein, while longer loops (from 5-44 residues in *R. capsulatus* porin) connect the ß strands on the extracellular side of the protein. Corresponding numbers for *E. coli* OmpF are 57% ß sheet, 4% α helix, and the remainder in ß turns and loops. Any α helical content present in these porins is found within the external loops.

The exterior of the *E. coli* and *R. capsulatus* porins can be divided into 4 zones. Near the periplasmic surface, a ring of aromatic residues encircles each protein. In all cases Phe residues point up into the hydrophobic environment of the membrane, whereas Tyr residues point down towards the hydrophilic environment of the periplasm. Following this aromatic region is a hydrophobic band composed primarily of Leu, Val, and Ala residues. A second less extensive aromatic region follows the hydrophobic band. In *R. capsulatus* porin this aromatic region is composed of Tyr and Trp, but no Phe. The exclusion of Phe from this band does not occur in the *E. coli* porins. As in the first band of aromatic residues all Tyr residues point away from the non-polar membrane. The area occupied by the two aromatic bands and the intervening hydrophobic band is approximately equal to the thickness of the non-polar interior of the membrane. The remainder of the exterior surface of the porin consists of a polar region made up of the extracellular loops. The polar nature of this

region of the protein allows the loops to extend into the extracellular media. Negative charges on the external loops of the porins may result in interaction between porins and LPS via Ca^{2+} cross-bridges making the proteins an integral part of the outer membrane structure.

The aromatic regions present in these porins are also found to a lesser extent in inner membrane bacterial proteins (Henderson *et al.*, 1990) and eukaryotic membrane proteins (Landolt-Marticorena *et al.*, 1993; Sipos and von Heijne, 1993), although generally only Tyr and Trp are found at the hydrophobic/hydrophilic boundary of these proteins. It is believed that the aromatic residues may help to position transmembrane proteins within the membrane. The polarizable aromatic rings of Tyr and Trp are especially suited for hydrogen bonding with the hydrophilic areas at the membrane boundary while simultaneously interacting with hydrophobic moieties in the lipid core. In bacterial outer membranes aromatic residues may additionally interact with sugar residues in LPS, similar to the role these residues have in contributing to the binding of carbohydrates by periplasmic carbohydrate binding proteins (Quiocho, 1989).

An asymmetric charge distribution is evident within the channel of the *R. capsulatus* and *E. coli* porins. Most of the charged amino acid residues present within the pore of each monomer are located in the upper half of the channel toward the extracellular surface of the porin. Of these residues

there is an asymmetric distribution of positive residues on the side of the channel closest to the monomer-monomer interface, with the opposite wall being strongly negative. This is especially evident in the *R. capsulatus* porin. Two Lys, 3 Arg, and 1 His are located opposite 7 Asp and 4 Glu residues. Some of the excess negative charge of the *R. capsulatus* porin is compensated by the presence of two Ca^{2+} ions within the channel interior (Fig. 1.8). The result is a relatively even distribution of 7 negative and 6 positive charges on opposite walls of the channel. The opposition of positive and negative charges also occurs in the *E. coli* porins although no strong excess of negative charge is found and no Ca^{2+} ions are present.

In each of the *E. coli* and *R. capsulatus* porin channels, a vertical row of 4 stacked basic residues are found (3 Arg and 1 Lys in both *R. capsulatus* and *E. coli* porins). Electrostatic interactions between the negative and positive residues on opposite walls of the channel allow the guanidinium groups of the Arg to extend into the channel and form a parallel line within van der Waal's distance of each other. The extension of the side chains into the channel interior partially determines pore size. Selection of OmpF and OmpC mutants with increased pore size resulted in replacement of each of the 3 stacked Arg residues with residues possessing a smaller side chain (Benson *et al.*, 1988; Misra and Benson, 1988). The size of the side chain was

inversely proportional to the size of the resultant channel in OmpC (Lakey *et al.*, 1991).

The second determinant of pore size is the third extracellular loop (L3) connecting ß strands 4 and 5. In both the *R. capsulatus* porin and the *E. coli* porins, L3 extends down into the interior of the channel. L3 helps brace the channel to maintain an open configuration and also contributes to both pore size and ion selectivity. Pore size is determined by the degree of constriction of the channel caused by the presence of the loop within the pore. In selecting pore size mutants of OmpF, Benson *et al.* (1988), also identified small deletions in L3, in addition to the changes in Arg residues as mentioned above. Deletion of several residues from L3 reduced the degree of constriction within the channel, leading to increased pore diameter. Ion selectivity is influenced by the charge of the residues located on L3. The presence of Lys on L3 of PhoE, rather than the Gly found in OmpF, greatly influences the ion selectivity of the pore (Bauer *et al.*, 1989). Alteration of charged residues at the mouth of the pore can also influence ion selectivity but not to the extent observed by changes in residues located directly on L3.

Due to the presence of L3 within the porin interiors, the channels of the *E. coli* OmpF and PhoE porins, and the *R. capsulatus* porin each narrow approximately midway down the channel. The actual diameter of the narrowest part of the

channel of the *E. coli* OmpF porin is 0.7 x 1.1 nm, increasing to 1.5 x 2.2 nm past the constriction zone. The diameter of the constriction zone in the *R. capsulatus* porin is 0.3 x 0.6 nm. The constriction of the *E. coli* OmpF porin is large enough for a glucose molecule to pass through the channel without losing its hydrating shell of water (Schindler and Rosenbusch, 1978). Prior to the solving of the *E. coli* OmpF structure, the channel diameter of this porin had been estimated at 0.26 - 1.16 nm (Nikaido and Rosenberg, 1983; Jap and Walian, 1990; Nikaido, 1992). These values are derived from exclusion limits determined using radioisotope efflux, rates of substrate diffusion measured by liposome swelling assay, and single channel conductance measurements from black lipid bilayer analysis. Problems associated with estimating pore diameter by any of these methods are many, including, errors in estimating the true radii of solutes, the lack of true spherical solutes for assaying size exclusion (large substrates used in these assays, such as raffinose, are linear rather than spherical), and deviation of the true channel geometry from the assumed constant-diameter cylinder. However, in comparison to the diameter of the narrowest part of the *E. coli* OmpF channel, measured from the resolved crystal structure, calculation of pore diameter based on size exclusion and permeability rates appears more reliable than measurements based on single channel conductance measurements.

The interaction of 3 monomers to form the trimeric porin

protein involves approximately 30% of the surface of each monomer. Residues interact via salt bridges, hydrogen bonds, and hydrophobic interactions. Additionally, loop L2 of one porin monomer extends into the gap between $L2^*$ and $L4^*$ (* indicates structural units of a neighbouring subunit) left by the folding of $L3^*$ into the monomer interior. In the case of the *R. capsulatus* porin a third Ca^{2+} ion, located in the centre of the porin trimer, is also involved in trimer stabilization. As a result purification of functional *R. capsulatus* porin monomers is possible in the presence of EDTA (Nestel *et al.*, 1989). No Ca^{2+} ions are known to be involved in trimer formation of the *E. coli* porins and functional monomers cannot be isolated. Monomers of the *E. coli* porins can only be isolated by heat dissociating the trimer in the presence of detergent.

In *R. capsulatus* porin the individual channels converge into one channel on the periplasmic side of the trimeric protein. The ß strands at the monomer/monomer subunit interface of this porin are significantly shorter than the overall length of the pore cylinder resulting in a merging of the channel. This does not occur to any great extent with the OmpC and PhoE porins and the individual channels remain essentially separate throughout the length of the trimeric protein. The difference is slight and it is likely that porins in general will show a range of conformations from complete separation of the channels to convergence, depending

on the length of the ß strands found in any given porin protein.

1.5. Porin function

1.5.1. Nonspecific diffusion porins

The majority of porins examined to date form nonspecific diffusion channels. Diffusion through these channels is characterized by: 1) a decrease in diffusion rate as solute size increases (Nikaido and Rosenberg, 1983), 2) decreased diffusion with increasing hydrophobicity of a solute (Nikaido and Rosenberg, 1983; Nikaido *et al.*, 1983), and 3) a linear increase in diffusion as solute concentration outside the cell increases (Nikaido and Rosenberg, 1981). These characteristics are consistent with the physical properties of porins as measured by *in vitro* model membrane techniques (i.e. porins are open, transmembrane, water-filled channels).

Diffusion through all nonspecific porins follows these general patterns. However, in comparing different porins, the actual rate of diffusion of a specific solute through a given porin depends on the physical environment the solute encounters in the pore interior. The production of multiple porin types by enteric bacteria is illustrative of how differences in the physical properties of porins can influence the diffusion rate of solutes across the outer membrane. Most enteric bacteria produce 3 major porins, OmpF, OmpC, and PhoE. Temperature and osmotic pressure affect the ratio of OmpF to

OmpC in the outer membrane, with OmpC production favoured by increases in temperature and osmotic strength of the media (Nikaido and Vaara, 1985). Although the pore of OmpF is only slightly larger than the OmpC channel, diffusion of large or hydrophobic compounds through the OmpC channel is greatly reduced, while diffusion of small nutrient molecules is relatively unaffected (Nikaido and Rosenberg, 1983). In this way, a slight change in the predominant pore size of the outer membrane produces a low level of resistance to larger, potentially toxic compounds. The growth conditions favouring OmpC production mimic those experienced by bacteria growing in a warm-bodied host. Exclusion of larger solutes would protect the bacteria from potentially inhibitory compounds present in the bodies of animals, such as bile salts or digestive enzymes. In dilute environments, production of OmpF would be advantageous when maximizing the rate of solute diffusion may be critical for survival.

Unlike the constitutive production of OmpF and OmpC, PhoE is only produced in response to phosphate starvation (Overbeeke and Lugtenberg, 1980). In contrast to OmpF and OmpC, PhoE is anion selective (Nikaido and Rosenberg, 1983; Benz *et al.*, 1985). The anion selectivity of the channel interior facilitates the diffusion of phosphate compounds into the cell. PhoE does not have a saturable binding site for phosphate in the channel, and, therefore, is a nonspecific diffusion porin like OmpF and OmpC (Benz *et al.*, 1985; Bauer

et al., 1988). Although, its channel size is similar to OmpF and OmpC, its role in the physiology of the cell differs from OmpF and OmpC due to the opposite ion selectivity it possesses.

Although the ion selectivity of many porins has been well characterized, the role, if any, of ion selectivity in the *in vivo* function of most porins is not clear at this time. Of the porins currently examined, cation selectivity is more common than anion selectivity. Cation selectivity has been found for the major porins of *P. aeruginosa* (Benz and Hancock, 1981), *Yersinia pestis* (Benz *et al.*, 1985), *Thermotoga maritima* (Engel *et al.*, 1993), *Haemophilus influenzae* (Vachon *et al.*, 1986), *Mycobacterium chelonae* (Trias and Benz, 1993), *Rhodopseudomonas blastica* (Butz *et al.*, 1993), *Aeromonas hydrophilia* (Jeanteur *et al.*, 1992), *Campylobacter jejuni* and *C. coli* (Page *et al.*, 1989), *Thiobacillus versutus* (Woitzik *et al.*, 1989), and species of the family *Enterobacteriaceae* (Benz *et al.*, 1985). The only anion selective porins identified are the PhoE porin of the *Enterobacteriaceae* (Benz *et al.*, 1985), OprP of *P. aeruginosa* (Siehnel *et al.*, 1990), and the major porin species of *Wolinella recta* (Kennell *et al.*, 1992), *Neisseria gonorrhoeae* (Young *et al.*, 1983), *Acidovorax delafieldii* (Brunen *et al.*, 1991), and *Comamonas acidovorans* (Brunen *et al.*, 1991). The role of anion selectivity in porin function is well understood for only two of these porins, PhoE and OprP. Both are induced by phosphate starvation and *in*

vivo and *in vitro* investigations have verified that the anion selectivity of these porins results in facilitated phosphate transport across the outer membrane (Korteland *et al.*, 1982; Hancock and Benz, 1986; Poole and Hancock, 1986a). A role for the anion selective porins of *A. delafieldii* and *C. acidovorans* in the physiology of these bacteria has been proposed by Brunen *et al.* (1991). These authors have speculated that the ion selectivity of these porins facilitates the diffusion of organic acids, the primary carbon source of these bacteria.

The physiological function of cation selectivity in porins has also not been resolved at this time. The negative charges producing the cation selectivity may not necessarily be present to facilitate the diffusion of a specific solute. Instead, negatively charged resides may participate in the formation of divalent cation bridges with LPS, making the porin an integral part of the outer membrane (Cowan *et al.*, 1992; Weiss and Schulz, 1992). Alternatively, the cation selectivity of porins may play a role in osmoregulation by bacteria. The periplasm and cytoplasm of gram-negative bacteria are in osmotic equilibrium (Stock *et al.*, 1977). Osmoregulation of the periplasm in most bacteria is regulated by the production and transport of negatively charged oligosaccharides (membrane derived oligosaccharides - MDO [Kennedy, 1982]) to the periplasm. The negative charges on the MDO trap cations in the periplasm and it is these cations

which largely determine the osmotic pressure of the periplasm. Like the negative charges of the MDO, the cation selectivity of porins may facilitate the diffusion of cations into the periplasm.

One other aspect of *in vivo* porin function that remains unresolved is the role that voltage-regulated channel closure may play in regulating diffusion across the outer membrane. Although closure of channel pores in response to an applied voltage is frequently observed *in vitro* (Lakey and Pattus, 1989), it is generally considered that channel closure does not occur in intact cells. The only known electrical potential across the outer membrane of gram-negative bacteria is the Donnan potential (Stock *et al.*, 1977; Kennedy, 1982) created by the production of the negatively charged MDO, mentioned above. The production of negatively charged molecules in the periplasm, unless balanced by an influx of cations, can create a voltage potential across the outer membrane of approximately 100 mV. Voltage regulated closure of porins is generally seen at higher voltages so channel closure *in vivo* is unlikely. In support of this, Sen *et al.* (1988) were unable to detect changes in solute permeability across the outer membrane in response to changes in the Donnan potential of intact cells. As pointed out by these authors, the highest Donnan potentials in cells are created during growth in dilute media, and channel closure under these conditions would presumably be disadvantageous to the cell.

Recently, however, Xu *et al.* (1986) have demonstrated that voltages required to close channels are reduced at low pH. This suggests that under some conditions porins may close *in vivo* to protect the cell from extreme conditions. Alternatively, Tommassen has suggested that voltage-regulated channel closure may be a safety mechanism for bacterial cells (Nikaido, 1992). Accidental insertion of a porin in the inner membrane would be a lethal event for a cell, unless some means of channel closure existed. Predatory Bdellovibrio actually appear to kill their prey in this manner, through transfer of a porin from their own outer membrane to the inner membrane of its prey (Tudor and Karp, 1994).

In general, solutes diffuse across the outer membrane of gram-negative bacteria via the transmembrane porin channels. Diffusion follows certain general patterns characteristic of nonspecific diffusion porins. Diffusion rate can depend on both the concentration and nature of the solute, and the physical environment of the porin channel.

1.5.2. Substrate-selective porins

Substrate-selective porins differ from nonspecific porins in that they contain a saturable binding site within the channel interior. These porins do act as nonspecific diffusion channels to some degree (Luckey and Nikaido, 1980; Trias *et al.*, 1988). However, since they are only induced under certain conditions, and are generally produced at lower

levels than nonspecific porins, they do not play a major role in nonspecific diffusion of solutes into the cell.

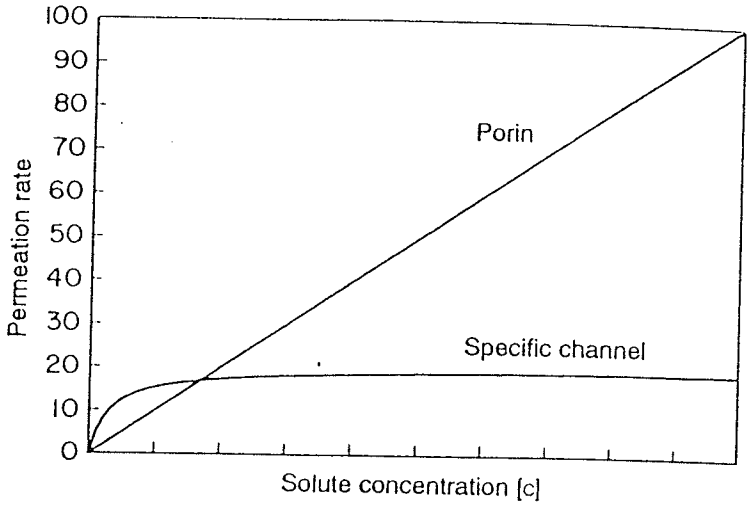
The advantage to a cell of producing a substrate-selective porin centres on the changes in solute diffusion that occur as a result of the presence of a binding site. Diffusion through a nonspecific porin follows Fick's first law of diffusion (Nikaido and Vaara, 1985), such that rate of diffusion is dependent mainly on the permeability of a given solute (i.e. an increase in size or hydrophobicity of a solute decreases the diffusion rate) and the external concentration of that solute (diffusion increases linearly as solute concentration increases). For instance, the diffusion of disaccharides through OmpF is approximately 100 fold less than that of arabinose (Nikaido and Rosenberg, 1983). Although these solutes are within the exclusion limit of the channel, the small diameter of the channel coupled with the increased size and length of a disaccharide, makes it much less likely that disaccharides will successfully negotiate the channel. Similarly, for a solute such as glucose, a drop in the external concentration from 28 mM to 1 μM can reduce diffusion 10,000 fold (Nikaido and Vaara, 1985). Depending on the V_{max} of the cytoplasmic transport system, outer membrane diffusion of large substrates or solutes present at low concentrations may become rate limiting for transport. Substrate-selective porins become advantageous under these conditions, due to the presence of a substrate binding site. The binding site

affects the rate of diffusion such that diffusion as a function of concentration is no longer a linear relationship. Although diffusion saturates at high concentrations of substrate, it is accelerated at low concentrations (Fig. 1.9), increasing the likelihood that the cytoplasmic transport system will operate at full capacity.

Currently, 8 types of substrate-selective porins have been identified. LamB, ScrY and Tsx found in the *Enterobacteriaceae* (Benz *et al.*, 1986; Maier *et al.*, 1988; Schmid *et al.*, 1991), TolC recently identified in *E. coli* (Benz *et al.*, 1993a), and OprB, OprP, OprO, and OprD found in *P. aeruginosa* and its relatives (Hancock and Carey, 1980; Poole and Hancock, 1986a; Trias and Nikaido, 1990a,b; Hancock *et al.*, 1992). In general, these porins facilitate the diffusion of large substrates (maltodextrins, LamB; sucrose, ScrY; peptides, TolC). These substrates would normally enter nonspecific channels very slowly and diffusion across the outer membrane would quickly become the rate limiting step for transport as the external concentration of substrate decreased. The porins of *P. aeruginosa* and its relatives are exceptions in that they facilitate the diffusion of small substrates which would normally diffuse through a nonspecific channel in other species of bacteria. This is believed to be due to the unusual permeability properties of the *P. aeruginosa* outer membrane and is discussed in greater detail in section 1.9.

In general, all porins function to create a selective

Figure 1.9. Solute diffusion through a nonspecific and a substrate-selective porin. Diffusion of a solute through a nonspecific channel increases linearly as solute concentration increases. In contrast, diffusion through a substrate-selective porin saturates at high solute concentrations but is accelerated at low concentrations due to the presence of a solute binding site. Reproduced from Nikaido (1992).


100
90
80
70
60
50
40
30
20
10
0
Permeation rate
Porin
Specific channel
Solute concentration [c]

permeability barrier across the outer membrane of gram-negative bacteria, allowing the influx and efflux of small nutrient molecules and waste products, respectively, while excluding larger, potentially toxic substrates. The presence of a substrate-selective porin is advantageous in that it accelerates the diffusion of large substrates or substrates present at low concentrations in the media; otherwise diffusion across the outer membrane would become the limiting process for transport of these substrates into the cytoplasm.

1.6. ABC transport systems

The OprB porin of *P. aeruginosa* was first identified following growth of cells on glucose (Hancock and Carey, 1980) and was co-induced with a periplasmic glucose binding protein (GBP), originally identified by Stinson *et al.* (1977). Hancock and Carey (1980) proposed that OprB and GBP form part of the high affinity glucose transport system of *P. aeruginosa*, one of two glucose transport systems known to occur in *P. aeruginosa* (Midgley and Dawes, 1973). Given the presence of a periplasmic binding protein, the high affinity glucose transport system of *P. aeruginosa* appears to belong to a family of transport systems found in procaryotic and eukaryotic cells. Originally, called binding-protein dependent transport systems, binding proteins are now known to be associated with only a subset of these transporters, therefore the names Traffic ATPases (Ames *et al.*, 1990) and

ABC (ATP-binding-cassette) transporters (Higgins *et al.*, 1990) have been proposed to emphasize features common to all transporters of this type.

ABC transporters have been identified in eubacterial and eukaryotic cells and are involved in transport of substrates both into and out of cells (reviewed by Ames *et al.*, 1990; Higgins *et al.*, 1990). One of the distinguishing features of these transporters is the presence of 4 cytoplasmic membrane associated protein domains. Two of the domains cross the cytoplasmic membrane and consist of either a homodimer composed of two identical proteins, a heterodimer composed of two related but distinct proteins, or a single fusion protein derived from the apparent fusion of two identical or similar membrane proteins. Associated with the transmembrane proteins, are 2 peripheral membrane domains located on the cytoplasmic side of the inner membrane. Like the integral membrane proteins, the peripheral proteins are present as either heterodimers, homodimers, or a single fusion product. Additionally, in some eukaryotic cells, higher order fusion products are observed such that the entire membrane transport complex may be composed of a single protein in the form of a fused heterotetramer. Two eukaryotic transporters of medical importance fall in this latter class, the multidrug resistance and the cystic fibrosis transport proteins found in mammalian cells.

Each of the peripheral-membrane domains contains an ATP-

binding motif, one cassette per protein in the case of a dimer, two per protein if the domains are fused to a single polypeptide. The presence of the 4 membrane associated domains and an ATP-binding motif are the distinguishing features of an ABC transporter. The presence of an ATP-binding motif in the membrane associated domains suggests that ATP is the energy source used for transport. This has been confirmed by the examination of energy use by transport complexes which have been reconstituted in liposomes using purified proteins (Davidson and Nikaido, 1990; Hoshino *et al.*, 1992).

In addition to the components described above, some bacteria produce a periplasmic binding protein as part of an ABC transporter. This is unique to procaryotes, as no homologous protein has been identified as a part of any eukaryotic ABC transporter (reviewed by Tam and Saier, 1993). In procaryotes, binding proteins are only associated with ABC transporters involved in the transport of substrates into the cytoplasm; no binding proteins are known to form a part of an export transporter. Nucleotide sequence analyses of binding proteins have identified some sequence conservation and resulted in the grouping of binding proteins into families (Tam and Saier, 1993). Binding proteins within a given family generally show similarities in their molecular size and substrate-binding specificities. The family of binding proteins showing specificity for hexoses and pentoses also

show some similarity to the lactose, galactose, and fructose repressors of *E. coli*, suggesting a possible common origin for both transport and regulatory proteins (Müller-Hill, 1983; Tam and Saier, 1993).

Periplasmic binding proteins exhibit a remarkable degree of structural similarity. High resolution crystal structures are now available for 7 periplasmic binding proteins, some in both liganded and unliganded forms (Quiocho, 1990; Spurlino *et al.*, 1991; Sharff et. al., 1992; Oh *et al.*, 1993). Their predominant features are two similar globular domains, each domain composed of both N and C terminal segments. The globular domains are connected by a flexible hinge consisting of portions of the polypeptide chain which cross 3 times between the two domains. The cleft between the two domains forms the substrate binding site. The presence of a substrate in the binding site brings about a conformational change in the protein, causing a narrowing of the cleft and trapping of the substrate within the protein.

Although rare, some bacteria also produce a substrate-selective porin as part of an ABC transporter. The best characterized of these porins is the LamB maltodextrin-selective porin forming part of the maltodextrin ABC transporter of the *Enterobacteriaceae*. The *lamB* gene is co-regulated with the other components of the transport system and is found within the same operon lying upstream of *malK*, the gene for the peripheral membrane protein (Saurin *et al.*,

1989). Production of a substrate-selective porin as part of an ABC transporter or any other type of transport system, is uncommon as the substrates of most transport systems diffuse through the nonspecific diffusion porins produced constitutively by bacteria. Substrate-selective porins are advantageous in that they ensure rapid diffusion of large substrates across the outer membrane. These porins are not generally essential for transport unless external substrate levels are very low. OprB is believed to form part of the glucose ABC transporter in *P. aeruginosa*, facilitating the diffusion of glucose across the outer membrane (Hancock and Carey, 1980). Classification of the high affinity glucose transport system of *P. aeruginosa* as an ABC transporter is based only on the presence of GBP, as yet no cytoplasmic membrane components have been identified.

A general model, proposed by Ames *et al.* (1990), for transport of substrates by a procaryotic ABC transporter into the cytoplasm of a cell begins with diffusion of the substrate across the outer membrane either through a nonspecific channel or a substrate-selective pore. The substrate is bound in the periplasm by the periplasmic binding protein. The conformational change in the binding protein, following binding of substrate, increases the affinity of the binding protein for the membrane protein complex. The precise role of the binding protein is not clear, as it is now known that some bacteria carrying mutations in some of the inner membrane

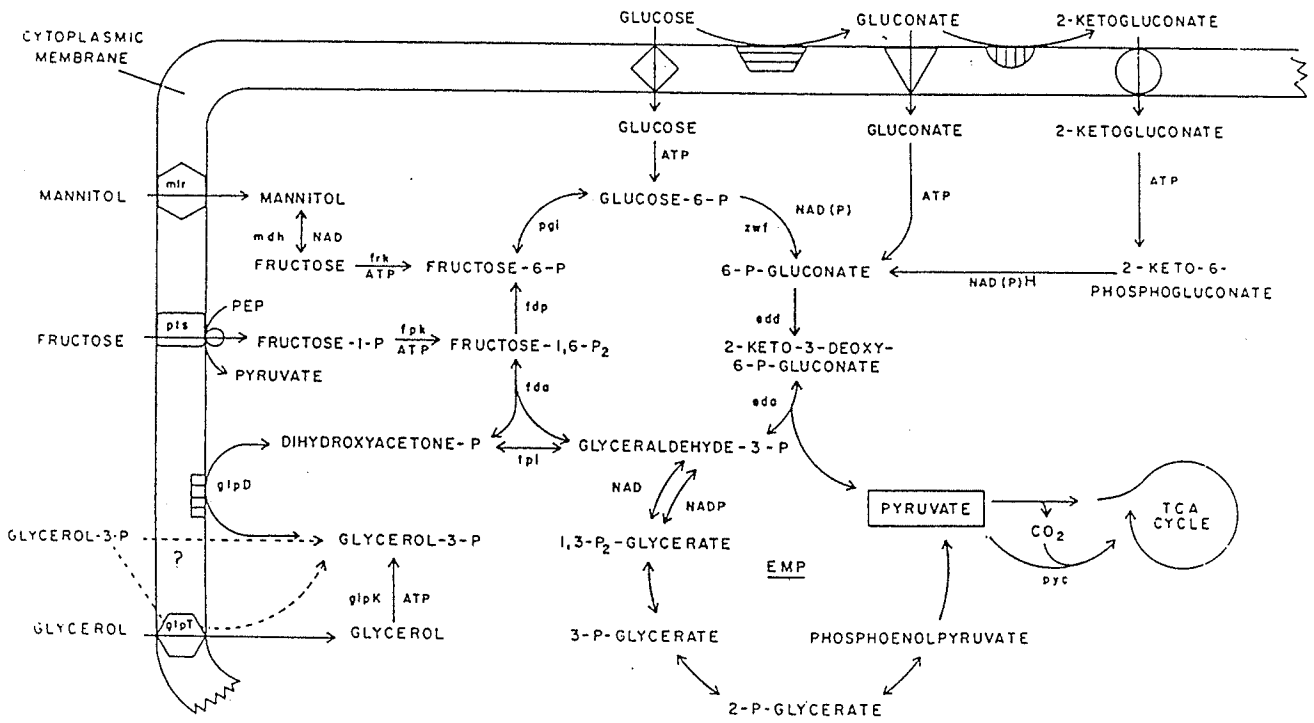
proteins, are able to transport substrate in the absence of the binding protein (Shuman, 1982; Speiser and Ames, 1991). However, the high periplasmic concentration of binding proteins (approximately 1 mM [Dietzel *et al.*, 1978]) coupled with the high binding affinity (typically 0.01 - 1 μM) presumably ensures the maintenance of a concentration gradient across the outer membrane to facilitate diffusion of the substrate, and ensures the efficient delivery of substrate to the cytoplasmic components. Following interaction of the binding protein with the cytoplasmic transport components a conformational change is believed to occur in these proteins, such that substrate is released from the binding protein and transported into the cytoplasm, possibly through formation of a transmembrane pore or exposure of a binding site. The presence of a binding site on the inner membrane proteins is supported by examination of binding protein independent mutants of the histidine and maltose ABC transporters which still show specificity for their respective substrates (Shuman, 1982; Speiser and Ames, 1991). The exact role of ATP hydrolysis is unknown at this time, and may be involved in either conformational changes of the cytoplasmic membrane proteins or release of the binding protein from the membrane complex. In addition to interacting with the cytoplasmic transport proteins, binding proteins can also interact with cytoplasmic membrane signal transducer proteins as part of a chemotaxis response.

1.7. Carbohydrate metabolism of *Pseudomonas aeruginosa*

Although *P. aeruginosa* is able to use a diverse assemblage of compounds as carbon sources, this bacterium is only able to grow on a limited number of carbohydrates (Stanier *et al.*, 1966). Preferred substrates for growth appear to be organic acids, as succinate transport and production of TCA cycle enzymes is constitutive. Also, succinate and other organic acids repress the uptake systems and catabolic enzymes needed for sugar metabolism (Tiwari and Campbell, 1969). Recently, a gene involved in the catabolite repression of sugar uptake by organic acids has been identified, although its mode of action is unknown at this time (MacGregor *et al.*, 1991; Wolff *et al.*, 1991).

All of the known carbohydrate catabolic pathways of *P. aeruginosa* are illustrated in Figure 1.10. This bacterium possesses two inducible pathways for the uptake of glucose: a high affinity pathway with a K_m for glucose of 8 μM (phosphorylative pathway) and a low affinity pathway with a K_m of approximately 2 mM (oxidative pathway) (Eagon and Phibbs, 1971; Midgley and Dawes, 1973; Stinnett *et al.*, 1973). The phosphorylative pathway is equivalent to the glucose ABC transporter discussed earlier, of which OprB and GBP are believed to be components. The oxidative pathway converts glucose extracellularly to gluconate and 2-ketogluconate via membrane-associated glucose dehydrogenase and gluconate dehydrogenase (Midgley and Dawes, 1973; Stinnett *et al.*,

Figure 1.10. Carbohydrate transport and catabolism in *Pseudomonas aeruginosa*. glt, glucose transport; gnuT, gluconate transport; gnuK, gluconokinase; glk, glucokinase, zwf, glucose-6-phosphate dehydrogenase; edd, 6-phosphogluconate dehydratase; eda, 2-keto-3-deoxy-6-phosphogluconate aldolase; fda, fructose-1,6-diphosphate aldolase; fdp, fructose-1,6-diphosphatase; pgi, phosphoglucoisomerase; frk, fructokinase; mdh, mannitol dehydrogenase; fpk, fructose-1-phosphate kinase; glpD, glycerol-3-phosphate dehydrogenase; glpK, glycerol kinase;, glpT, glycerol transport; pts, phosphotransferase system; mtr, mannitol transport system; EMP, Embden-Meyerhof pathway. Reproduced from Temple *et al*. (1990).


CYTOPLASMIC MEMBRANE
GLUCOSE
GLUCONATE
2-KETOGLUCONATE
GLUCOSE
GLUCONATE
2-KETOGLUCONATE
ATP
ATP
ATP
MANNITOL
mtr
MANNITOL
mdh
NAD
GLUCOSE-6-P
pgi
zwf
NAD(P)
FRUCTOSE
frk
ATP
FRUCTOSE-6-P
6-P-GLUCONATE
NAD(P)H
2-KETO-6-PHOSPHOGLUCONATE
PEP
fdp
edd
FRUCTOSE
pts
FRUCTOSE-1-P
fpk
ATP
FRUCTOSE-1,6-P2
2-KETO-3-DEOXY-6-P-GLUCONATE
PYRUVATE
fda
edo
DIHYDROXYACETONE-P
tpi
GLYCERALDEHYDE-3-P
glpD
NAD
NADP
PYRUVATE
CO2
TCA CYCLE
GLYCEROL-3-P
?
GLYCEROL-3-P
1,3-P2-GLYCERATE
EMP
pyc
glpK
ATP
GLYCEROL
glpT
GLYCEROL
3-P-GLYCERATE
PHOSPHOENOLPYRUVATE
2-P-GLYCERATE

1973). Glucose dehydrogenase and gluconate dehydrogenase are regulated independently. Glucose is believed to be the inducer of glucose dehydrogenase, while gluconate induces gluconate dehydrogenase and the respective uptake systems and intracellular catabolic enzymes necessary for conversion of gluconate and 2-ketogluconate to 6-phosphogluconate (Hunt and Phibbs, 1981; 1983). Uptake of glucose via the oxidative pathway is active under aerobic conditions, but only when external glucose concentrations are high. As the glucose concentration of the medium decreases, uptake occurs primarily by the phosphorylative pathway (Whiting *et al.*, 1976a; Hunt and Phibbs, 1981; 1983). Glucose is transported into the cytoplasm by the phosphorylative pathway in an unaltered state. In the cytoplasm glucose transported in this manner is phosphorylated to glucose-6-phosphate and converted to 6-phosphogluconate via glucose-6-phosphate dehydrogenase. 6-phosphogluconate is the common intermediate of glucose, gluconate, and 2-ketogluconate catabolism.

The enzymes 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, form the Entner-Doudoroff pathway and convert 6-phosphogluconate to pyruvate for entry into the TCA cycle. Glucose cannot be metabolized by the Embden-Meyerhoff pathway in *P. aeruginosa* due to a lack of phosphofructokinase for conversion of fructose-6-phosphate to fructose-1,6-diphosphate (Tiwari and Campbell, 1969). Fructose and glycerol catabolism could potentially occur

through the Embden-Meyerhoff pathway, however, activity of these enzymes is very low in *P. aeruginosa* and all carbohydrate metabolism by *P. aeruginosa* is primarily via the Entner-Doudoroff pathway (Blevins *et al.*, 1975; Phibbs *et al.*, 1978). The various enzymes and proteins involved in the glucose ABC transporter, gluconate uptake/catabolism, the Entner-Doudoroff pathway, and extracellular glucose oxidation (glucose dehydrogenase) appear to be regulated independently of each other (Hylemon and Phibbs, 1972; Whiting *et al.*, 1976a,b; Phibbs *et al.*, 1978; Hunt and Phibbs, 1983), although no information is available on transcriptional control of the operons encoding these proteins. The genes encoding the enzymes of the Entner-Doudoroff pathway, the glucose binding protein (or possibly a regulatory protein) and glucose-6-phosphate dehydrogenase have been cloned and are known to be tightly clustered on the *P. aeruginosa* genome (Roehl *et al.*, 1983; Cuskey and Phibbs, 1985; Cuskey *et al.*, 1985), however, sequence analysis of the cloned genes has not yet been reported.

Like glucose, mannitol uptake also occurs via a phosphorylative pathway (Eagon and Phibbs, 1971). The presence of a mannitol binding protein in the periplasm of *P. aeruginosa* suggests transport is via a mannitol ABC transporter (Eisenberg and Phibbs, 1982). Following transport, mannitol is converted intracellularly to 6-phosphogluconate (Phibbs *et al.*, 1978) where catabolism

proceeds via the Entner-Doudoroff pathway.

Unlike other carbohydrates transported by *P. aeruginosa*, fructose is internalized via a phosphoenolpyruvate phosphotransferase (PTS) system. This is the only known PTS transport system in *P. aeruginosa* (Phibbs *et al.*, 1978; Durham and Phibbs, 1982; Roehl and Phibbs, 1982). Initially, fructose obtained extracellularly is catabolized via a different pathway than fructose originating intracellularly through mannitol catabolism. Fructose from the external environment is converted to fructose-1-phosphate by the action of the PTS transport system, followed by conversion to fructose-1,6-diphosphate and fructose-6-phosphate (Phibbs *et al.*, 1978). Once converted to fructose-6-phosphate, mannitol and fructose catabolism is identical.

Glycerol is transported in an unaltered state, possibly via a glycerol ABC transporter (Tsay *et al.*, 1971). Glycerol is converted to glycerol-3-phosphate and dihydroxyacetone-phosphate. Dihydroxyacetone-phosphate is converted to fructose-1,6-diphosphate from which its catabolism is identical to that of fructose.

1.8. Porin proteins of *Pseudomonas aeruginosa*

1.8.1. OprF

OprF is the major outer membrane protein of *P. aeruginosa*. The protein is bifunctional acting as the main general diffusion channel of this bacterium (Hancock *et al.*,

1979) and also playing an important role in maintaining the structural integrity of the cell (Gotoh *et al.*, 1989; Woodruff and Hancock, 1989). The protein is well conserved within rRNA group I of the family *Pseudomonadaceae* (Ullstrom *et al.*, 1991). Crosslinking experiments indicate that OprF exists as a trimer *in vivo* (Angus and Hancock, 1983), however, unlike most porins identified to date, OprF readily dissociates to native functional monomers in the presence of SDS (Yoshimura *et al.*, 1983). Native monomers are evident on SDS-PAGE gels by their more rapid mobility in comparison to heat denatured monomers (as an example see Fig. 3.1 of OprB, which also shows this type of mobility on SDS-PAGE gels, page 109).

Black lipid bilayer analysis indicates that the OprF protein is capable of forming two types of channels. The larger channels possess a single channel conductance greater than 4 nS, while the smaller channels demonstrate a conductance of 0.36 nS (Woodruff *et al.*, 1986). Quantitative analysis of individual insertion events in bilayers suggests formation of the larger pore is rare (<1%). OprF purified from either *P. aeruginosa*, or from *E. coli* expressing the cloned *oprF* gene, both demonstrate a dual pore size, suggesting it is the OprF protein itself which forms the large channels rather than a minor contaminating protein (Woodruff *et al.*, 1986). Formation of two different channel types is unusual as no other porin characterized to date shows this property.

Currently, the mechanism behind alternative pore size formation in OprF is unknown, however, examination of the primary sequence of OprF suggests two possibilities. First, 4 closely spaced cysteine residues (C176, C185, C191, and C205 in *P. aeruginosa* OprF) are present in one of the proposed external loops of the protein (Siehnel *et al.*, 1990a). Disulfide bond formation does occur in this protein (Hancock and Carey, 1979) and Siehnel *et al.* (1990a) have proposed that alternative cystine disulfide bonds may alter the pore size of the OprF channel. As previously indicated, relatively minor changes in the third external loop of *E. coli* OmpF and OmpC are known to be able to alter the channel characteristics of these porins.

Second, dual pore size formation by OprF may result from the presence of an alanine- and proline-rich region spanning residues 165 to 173 of the mature OprF protein (AAPAPEPVA). This cluster of residues resembles the hinge region of immunoglobulins (Duchêne *et al.*, 1988). These residues are also present in the OmpA structural protein of enteric bacteria and affect the folding of this protein. In OmpA, the N terminal residues up to the hinge domain form an 8 stranded ß barrel embedded in the outer membrane. The C terminal portion is located in the periplasm. The ß barrel portion of the OmpA protein does show some porin activity with diffusion properties suggesting a small constricted channel diameter (Sugawara and Nikaido, 1992). The majority of the OprF

channels may fold in a manner analogous to OmpA, such that most of the pores are small 8 stranded ß barrels. However, a small number of OprF molecules may form a large 16 stranded ß barrel, typical of most porins. Monoclonal antibodies, specific for the carboxy terminal region of OprF, do bind to whole cells of *P. aeruginosa* (Hughes *et al.*, 1992), indicating that at least some of the molecules have a structure different than that exhibited by OmpA. This analysis is not quantitative and it is not known if all or only a part of the OprF molecules fold in this way.

The channel forming properties of OprF explain the unusual permeability properties of the *P. aeruginosa* outer membrane. *P. aeruginosa* is naturally resistant to antibiotics due to diffusion rates across the outer membrane being 100 to 500 times lower than those measured for the outer membrane of enteric bacteria (Yoshimura and Nikaido, 1982). Previously, Nakae and several other groups had argued that the antibiotic resistance of this bacterium was due to the small diameter of its porins (Caulcott *et al.*, 1984; Yoneyama *et al.*, 1986; Yoshihara *et al.*, 1988; Yoshihara and Nakae, 1989); they argued that OprF was strictly a structural protein unable to form large diameter channels. Recently, however, Nikaido *et al.* (1991) have presented evidence that the purification procedure utilized by Nakae actually inactivates OprF. Additionally, Bellido *et al.* (1992) have demonstrated that in plasmolysis experiments conducted by Nakae, he did not allow

sufficient time for diffusion of large tri- and tetrasaccharides to occur across the outer membrane of *P. aeruginosa*. The outer membrane of *P. aeruginosa* is actually permeable to very large substrates (eg. pentamethionine [Miller and Becker, 1978], raffinose [Bellido *et al.*, 1992], and large antibiotics [Siehnel *et al.*, 1990a]) if sufficient time is allowed for diffusion to occur.

The dual pore size of OprF can explain both the low diffusion rates across the outer membrane and the permeation of very large substrates. Since 99% of the OprF channels are very small, diffusion across the outer membrane is slow, however, since a small fraction of the channels form large diameter pores, large substrates can diffuse if a sufficiently long time frame is allowed for permeation to occur.

1.8.2. OprP and OprO

The OprP porin of *P. aeruginosa* is produced under conditions of phosphate limitation (Hancock *et al.*, 1982). *In vivo*, OprP forms part of a high affinity phosphate transport system in *P. aeruginosa* and facilitates the diffusion of phosphate across the outer membrane (Poole and Hancock, 1986a). OprP is present as a trimer in the outer membrane (Angus and Hancock, 1983), however, unlike many *P. aeruginosa* porins, OprP must be heated to temperatures greater than 60°C in the presence of SDS before the trimer will dissociate to monomers (Worobec *et al.*, 1988). Based on genetic,

biochemical, and immunological data, homologous proteins are common in rRNA group I pseudomonads (Poole and Hancock, 1986b; Poole *et al.*, 1986; Siehnel *et al.*, 1990b).

OprP forms small (single channel conductance 160 pS) anion selective channels in black lipid bilayer analysis (Hancock *et al.*, 1982). OprP is considered a substrate-selective porin as it possesses a saturable binding site for anions within the channel interior (Hancock and Benz, 1986). Of the anions tested, OprP showed the greatest affinity for monophosphate (Benz *et al.*, 1993b). Examination of the effect of pH changes and chemical modification on the conductivity of OprP in black lipid bilayer suggests lysine residues within the channel form the phosphate binding site (Hancock *et al.*, 1983; Hancock and Benz, 1986; Hancock *et al.*, 1986).

Sequence analysis of the region surrounding the *oprP* gene revealed the site of a second related gene (Siehnel *et al.*, 1988). Named *oprO*, this gene codes for a polyphosphate-selective porin (Hancock *et al.*, 1992; Siehnel *et al.*, 1992). OprO was induced following phosphate starvation but only in cells which had reached the stationary growth phase. In contrast to OprP, which shows a preference for monophosphate, OprO shows a 10 fold higher affinity for polyphosphate over monophosphate. The OprO and OprP proteins are highly homologous, with amino acid residues showing 74% identity and 16% similarity.

1.8.3. OprD

Attention was first focused on the OprD porin of *P. aeruginosa* when it was noted that strains resistant to the ß-lactam imipenem lacked an outer membrane protein (Büscher *et al.*, 1987; Quinn *et al.*, 1988). Trias and Nikaido (1990a) purified this protein (OprD) from wild-type *P. aeruginosa* strains and demonstrated that it showed a specificity for imipenem in the liposome swelling assay. Additionally, if OprD was present in the outer membrane, imipenem diffusion across the outer membrane increased and eventually saturated as concentration increased, verifying that a saturable binding site for imipenem was present in the OprD channel (Trias *et al.*, 1989).

Since it was unlikely that OprD would evolve as a means of facilitating the diffusion of an antibiotic across the outer membrane, Trias and Nikaido (1990b) investigated the role of OprD in the physiology of *P. aeruginosa*. Based on the similarity in structure between imipenem and basic amino acids, they examined the potential role of OprD in the diffusion of these amino acids across the outer membrane. Using purified protein reconstituted in liposomes, and intact cells lacking OprD, they verified that OprD contained a binding site for basic amino acids and di- and tripeptides containing these amino acids. Additionally, Huang and Hancock (1993), using OprD-defective *P. aeruginosa* strains, found that OprD also facilitated the diffusion of gluconate.

Induction of OprD is unusual in that it appears to be induced by growth in minimal media on a variety of substrates (eg. glucose, gluconate, pyruvate [Hancock and Carey, 1980]), yet, it is known that OprD does not facilitate the diffusion of glucose and pyruvate across the outer membrane (Huang and Hancock, 1993). Several genes encoding potential regulatory proteins for OprD have been identified (Huang *et al.*, 1992), however, control of OprD expression is not understood at this time.

1.8.4. OprC and OprE

The porin activity of the OprC and OprE proteins of *P. aeruginosa* was first demonstrated by Yoshihara and Nakae (1989) using the liposome swelling assay. However, the small amounts of OprC produced by *P. aeruginosa* have made it difficult for other groups to identify OprC and verify its activity (Hancock *et al.*, 1990; Nikaido *et al.*, 1991). Porin activity of OprE has been verified by Nikaido *et al.* (1991), although purified OprE appeared to consist of two similarly sized proteins and it is not clear which protein Yoshihara and Nakae (1989) purified, and if both or only one of the proteins is a porin (Hancock *et al.*, 1990; Nikaido *et al.*, 1991). Recently, Yamano *et al.* (1993) reported that production of OprE increased under anaerobic conditions although its physiological role in nutrient uptake is still unknown.

1.8.5. OprB

The OprB porin of *P. aeruginosa* was first identified following growth of cells on glucose (Hancock and Carey, 1980). It is repressed by growth in the presence of several TCA cycle intermediates such as succinate and citrate, and induced by a variety of sugars, some of which *P. aeruginosa* is unable to utilize for growth (Hancock and Carey, 1980). OprB is present as a trimer in the outer membrane of *P. aeruginosa* (Angus and Hancock, 1983) and, like the OprF porin, dissociates to a monomer in the presence of SDS without heating. In the liposome swelling assay, OprB specifically facilitated the diffusion of xylose and glucose (Trias *et al.*, 1988), suggesting that a sugar binding site is present. Using glucose molecules with substitutions at various positions, these authors suggested that the hydroxyl groups at C2 and C4 were important for binding to OprB. A protein homologous to *P. aeruginosa* OprB has also been identified in *P. putida* (Saravolac *et al.*, 1991).

1.9. Permeability properties of the *Pseudomonas aeruginosa* outer membrane

The homology between the OprF porin of *P. aeruginosa* and the OmpA structural protein of enteric bacteria (Woodruff and Hancock, 1989), coupled with the role of OprF as the main porin of *P. aeruginosa* (Hancock *et al.*, 1979) suggests that *P. aeruginosa* and its close relatives are analogous to porin-

deficient mutants of other species of bacteria. Some porin-deficient, mutant bacterial strains have been identified, that have lost one or all of their major porins as a result of selection pressure from exposure to antibiotics, either under experimental *in vitro* conditions or in patients undergoing antibiotic treatment. In general, this increases antibiotic resistance by reducing the rate of diffusion of antibiotics across the outer membrane of these bacterial strains.

In the soil environment where Pseudomonads are normally found, antibiotics produced by other species of soil bacteria and fungi may have acted as a selective force for the loss of one or more "typical" porins from the outer membrane of these organisms. The low residual porin activity of the OmpA homologue, OprF, in these bacteria, may have been adequate for sufficient permeation of solutes across the outer membrane to allow growth of *P. aeruginosa* under most conditions, yet provide an effective mechanism for increasing the antibiotic resistance of this bacterium. Although sufficient for survival, the low rates of diffusion may still have acted as the driving force for evolution of the substrate-selective porins found in *P. aeruginosa* and other rRNA group I pseudomonads. Group I pseudomonads possess substrate-selective porins recognizing very small substrates (eg. glucose, amino acids, phosphate). In other species, substrates of this size are believed to diffuse across the outer membrane via nonspecific porins since in most species

these porins permit higher rates of diffusion than the OprF porin of *P. aeruginosa*. Substrate-selective porins are found in other species of bacteria, but these porins are generally regarded as being specific for substrates which are relatively large and unable to diffuse efficiently through general diffusion channels (eg. maltodextrins, sucrose, peptides). The evolution, in *P. aeruginosa* and its relatives, of a range of substrate-selective porins specific for small solutes, coupled with inefficient nonspecific diffusion across the outer membrane, provides these bacteria with an effective means of antibiotic resistance without having a major impact on the diffusion of solutes necessary for growth.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

All of the bacterial strains and plasmids used during the course of this research are listed in Tables 2.1 and 2.2, respectively. Portions of these tables are repeated in the appropriate chapters. Restriction maps of plasmids are summarized in Appendix 1.

Rich medium for culturing cells was Luria broth (LB) (1% [w/v] tryptone [Difco, Detroit, MI], 0.5% [w/v] yeast extract [Difco], 0.5% [w/v] NaCl). Defined medium was Basal medium 2 (BM2) minimal medium (40 mM K_2HPO_4, 22 mM KH_2PO_4, 7 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 10 μM $FeSO_4$, pH 7), routinely supplemented with either 0.4% (w/v) glucose or 20 mM potassium succinate as a carbon source (Hancock and Carey, 1979). M63 medium (100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1.8 μM $FeSO_4$, 1 mM $MgSO_4$, pH 7) supplemented with 0.2% (w/v) maltose was used to induce production of LamB in *E. coli* (Ausubel *et al.*, 1989). TN medium, used for preparing competent *P. aeruginosa* cells, was 5% (w/v) tryptone, 1% (w/v) dextrose, 2.5% (w/v) yeast extract (Olsen *et al.*, 1982). VB medium, used for triparental mating, was 0.8 mM $MgSO_4$, 10 mM citric acid, 57 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$ (Vogel and Bonner, 1956). Long term storage of strains was in 7% dimethyl sulfoxide (0.1 ml DMSO and 1.3 ml culture) at -70°C.

Antibiotic concentrations for *E. coli* were 12.5 μg/ml tetracycline, 50 μg/ml ampicillin, 20 μg/ml streptomycin, 30

Table 2.1. Bacterial strains used in this study

Strain	Characteristics	Source/reference
Pseudomonas aeruginosa		
H103	wild-type PAO1 prototroph reference strain	R.E.W. Hancock/Hancock and Carey, 1979; Holloway *et al.*, 1979
H234	H103 rough LPS mutant	R.E.W. Hancock
H673	H103 *opdE*::Tn501[1]	R.E.W. Hancock/Huang *et al.*, 1992
PFB311	PAO1 *gcd gnd*[2]	P.V. Phibbs/Cuskey and Phibbs, 1985
WW100	H103 *oprB*::Ω (Sm^r)	This study
WW151	PFB311 *oprB*::Ω (Sm^r)	This study
P. putida		ATCC 12633
P. chlororaphis		ATCC 9446
P. fluorescens		UMCC
P. stutzeri		ATCC 17588
P. cepacia		NCTC 10661
Comamonas acidovorans		NCTC 10683
Xanthomonas maltophilia		ATCC 13637
Escherichia coli		
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	Promega
MM294	*supE44 hsdR endA1 thi*; carries helper plasmid pRK2013 for triparental mating	R.E.W. Hancock
NM522	*supE thi* Δ*(lac-proAB) hsd5 F'[proAB' lacI^q lacZ*ΔM15]	UMCC

Abbreviations: ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; UMCC, University of Manitoba Culture Collection; Sm, Streptomycin.

[1] *opdE* is a putative regulatory gene controlling OprD porin production. H673 contains an intact *oprD* gene but does not produce OprD.

[2] PFB311 contains mutations in glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gnd*). Therefore, of the two glucose transport pathways known in *P. aeruginosa*, only the high affinity pathway is functional.

Table 2.2. Plasmids used in this study.

Plasmid	Characteristics	Reference/Source
pLAFR1	Tc^R; cosmid vector	Friedman et al., (1982)
pE7(9)	pE7(9) to pF7(36) each contain approximately 25 kb of P. aeruginosa genomic DNA cloned in pLAFR1	This study
pD6(10)		This study
pF9(14)		This study
pH6(20)		This study
pE6(30)		This study
pF7(36)		This study
pTZ19R	Ap^R	Mead et al., (1986)
pJBB34	Ap^R; pTZ19R with 3.4 kb *Bgl*II/*Bgl*II fragment from pE7(9)	This study
pJEB54	Ap^R; pTZ19R with 5.4 kb *Eco*RI/*Bgl*II fragment from pE7(9)	This study
pJEK67	Ap^R; pTZ19R with 6.7 kb *Eco*RI/*Kpn*I fragment from pE7(9)	This study
pJHS6	Ap^R; pTZ19R with 0.6 kb *Hinc*II/*Sca*I fragment from pJBB34	This study
pJPS8	Ap^R; pTZ19R with 0.8 kb *Pst*I/*Sca*I fragment from pJBB34	This study
pJES34	Ap^R; pTZ19R with 3.4 kb *Eco*RI/*Sph*I fragment from pJEK67	This study
pJSS26	Ap^R; pTZ19R with 2.6 kb *Sph*I/*Sph*I fragment from pJBB34	This study
pJSB18Δ1	Ap^R; pTZ19R with 1.2 kb *Sph*I/*Hinc*II and 0.6 kb *Sca*I/*Hind*III fragments from pJBB34	This study
pJSB1	Ap^R; pTZ19R with 0.6 kb *Sca*I/*Hind*II fragment from pJBB34	This study
pJSH12	Ap^R; pTZ19R with 1.2 kb *Sph*I/*Hinc*II fragment from pJBB34	This study
pJSS14	Ap^R; pTZ19R with 1.4 kb *Sal*I/*Sal*I fragment from pJBB34	This study
pBR322	Ap^R Tc^R	Bolivar (1979)
pHP45ΩSm/Spc	Ap^R Sm^R Spc^R	Fellay et al. (1987)
pMOB3	Km^R Cm^R *sacBR oriT*	Schweizer (1992)
pNOT19	Ap^R; pUC19 with *Not*I site	Schweizer (1992)
pNOT322	Ap^R Tc^R; pBR322 with *Not*I site	Schweizer (1992)
pNOTSB18	Ap^R; pNOT19 with 1.8 kb *Sph*I/*Hind*III fragment from pJSB18Δ1	This study
pMOBSB18	Ap^R Cm^R *sacBR oriT*; pNOTSB18 with 5.0 kb *Not*I/*Not*I fragment from pMOB3	This study

pBR322$_\Delta$	Ap^R Tc^R; Deletion of 0.64 kb *Ava*I/*Pvu*II fragment from pBR322	This study
pBRSS14	Tc^R; pBR322$_\Delta$ with 1.4 kb *Eco*RI/*Pst*I fragment from pJSS14	This study
pBRSS14::Sm	Tc^R Sm^R; pBRSS14 with 1.4 *Sma*I/*Sma*I fragment from pHP45ΩSm/Spc	This study
pNOTSS14::Sm	Tc^R Sm^R; pNOT322 with 3.2 kb *Eco*RI/*Pst*I site from pBRSS14::Sm	This study
pMOBSS14::Sm	Tc^R Sm^R Cm^R *sacBR oriT*; pNOTSS14::Sm with 5.0 kb *Not*I/*Not*I fragment from pMOB3	This study
pPZ375	Ap^R; Broad host range deriative of pGEM-3Z(+)	P.V. Phibbs
pPZSAC	Ap^R *sacBR*; pPZ375 with 2.6 *Pst*I/*Pst*I fragment from pMOB3	This study
pPZEH34	Ap^R; pPZ375 with 3.4 kb *Eco*RI/*Hind*III fragment from pJBB34	This study
pPZXS27	Ap^R; pPZ375 with 2.7 kb *Xba*I/*Sph*I fragment from pJBB34	This study
pPZSH27	Ap^R; pPZ375 with 2.7 kb *Sph*I/*Hind*III fragment from pJBB34	This study
pRK2013	Km^R	Goldberg and Ohman (1984)

Abbreviations: Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; *sacBR*, levansucrase gene and its regulatory region; *oriT*, origin of transfer from RP4.

μg/ml chloramphenicol, and 25 μg/ml kanamycin. For *P. aeruginosa*, concentrations were 500 μg/ml carbenicillin, 100 μg/ml tetracycline, 500 μg/ml kanamycin, 500 μg/ml streptomycin and 15 μg/ml $HgCl_2$.

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Analysis of proteins by SDS-PAGE used the 11% (w/v) gel system of Lugtenberg *et al.* (1975). Running gels consisted of 4.71 ml of 44:0.8 acrylamide (44% [w/v] acrylamide and 0.8% [w/v] methylene-bisacrylamide [Gibco-BRL, Gaithersburg, MD], 0.45 ml of 1% (w/v) ammonium persulfate; 0.38 ml of 10% (w/v) SDS (Fisher Biotech, Fairlawn, NJ); 4.68 ml of 1.5 M Tris, pH 8.8; 0.32 ml of 5 M NaCl; 0.075 ml of Temed; 8.27 ml of H_2O. The stacking gel was 0.5 ml of 30:0.8 acrylamide; 0.12 ml of 1% (w/v) ammonium persulfate; 0.05 ml of 10% SDS; 1.25 ml of 0.5 M Tris, pH 6.8; 0.03 ml of Temed; 3.08 ml of H_2O. Running buffer was 25 mM Tris, 210 mM glycine, 0.1% (w/v) SDS.

Samples of heat unmodified protein were prepared by solubilization at 20°C in 0.2% SDS (w/v), 20% (v/v) glycerol, 125 mM Tris, pH 6.8 prior to loading on the gel. Heat modified proteins were prepared by solubilization at 95°C for 10 min in the same buffer containing 3% (v/v) dithiothreitol.

Gels were stained for 15 min in 25% (v/v) isopropyl alcohol, 10% (v/v) acetic acid, 0.4% (w/v) Coomassie Brilliant

Blue R-250 (Sigma, St. Louis, MO). Dye was removed by placing the gels in destaining solution (27% (v/v) methanol, 10% (v/v) acetic acid) for 15 min. Fresh destain solution was then added and the gels were left to destain overnight.

2.3. Western Blot transfer

Western blotting of proteins to Nitro ME nitrocellulose filters (MSI, Westboro, MA) for immunodetection followed the procedure of Burnette (1981). Transfer was conducted in a Bio-rad (Richmond, CA) Western blotting chamber. Transfer buffer consisted of 192 mM glycine, 20% (v/v) methanol, 28 mM Tris, pH 8.3. Buffer was cooled to 4°C prior to use. Protein transfer was conducted overnight at 10 mA constant current.

2.4. OprB purification

Purification of *P. aeruginosa* OprB was based on a protocol developed by Hancock and Carey (1980). Fourteen grams of cells (wet wt.), collected from 2 L of culture, were routinely used for OprB purification. Cells were harvested by centrifugation at 10,000 x g at 4°C and resuspended in 20 ml of 10 mM Na_3PO_4, pH 7.4. The cell suspension was French pressed at 15,000 PSI and intact cells removed by a subsequent centrifugation step at 3,500 x g for 10 min. Cell envelopes were collected by centrifugation at 145,000 x g for 1 hr. and resuspended in 8 ml H_2O. Resuspended cell envelopes were layered on a two step sucrose gradient composed of 70% (w/v)

and 54% (w/v) sucrose (2 ml of resuspended cell envelopes layered on 6 ml of sucrose in a 13 ml centrifuge tube). Following centrifugation for 18 hr at 75,000 x g, the lower outer membrane band was collected, washed free of sucrose and resuspended by sonication in 40 ml of 2% (v/v) Triton X-100, 20 mM Tris, pH 8.0 and left at 4°C for 30 min. Outer membranes were collected by centrifugation at 184,000 x g for 1.5 hr. This step was repeated. Finally OprB was dissociated from outer membrane fragments by resuspension in 8 ml 2% (v/v) Triton X-100, 10 mM EDTA, 20 mM Tris, pH 8.0. Outer membrane fragments were pelleted by centrifugation at 184,000 x g for 1.5 hr and the supernatant containing OprB was loaded onto a 1 x 20 cm DEAE-Sephacel (Pharmacia, Baie d'Urfe, PQ) column equilibrated with 0.1% (v/v) Triton X-100, 10 mM EDTA, 20 mM Tris, pH 8.0 (TTE). The column was washed with 20 ml TTE, then 25 ml of 0.1 M NaCl in TTE and OprB eluted using 80 ml of a 0.1-0.25 M NaCl gradient in TTE. Fractions were screened by staining with Coomassie Blue following SDS-PAGE. Fractions containing OprB were pooled and concentrated in an ultrafiltration cell (Amicon, Beverley, MA) with a 30,000 Da cutoff membrane (Diaflo PM30, Amicon).

2.5. Polyclonal antibody production and immunodetection experiments

OprB collected by ion exchange chromatography was further purified by electroelution from SDS-PAGE gels with the

Bio-Rad 422 electroeluter using a 12,000 Da cutoff membrane cap. OprB was injected intramuscularly into female New Zealand white rabbits (obtained from University of Manitoba breeding stock). OprB was in a partially unfolded form following electroelution as it showed the same mobility on SDS-PAGE gels as heat modified OprB. The first injection consisted of 60 μg of OprB suspended in 400 μl of 192 mM glycine, 25 mM Tris, 0.1% SDS (w/v) mixed with 1000 μl of Freund's complete adjuvant. The solution was mixed by repeated passes through an 18 gauge needle. A second injection, 3 weeks later, consisted of an identical quantity of OprB mixed with 600 μl of Freund's incomplete adjuvant.

Serum was collected 1 week later from the ear vein of the rabbit. Prior to use in immunodetection experiments the serum was preadsorbed with *P. aeruginosa* heat-treated cell envelopes (100°C, 10 min.) isolated from succinate-grown *P. aeruginosa* cells. This was done by mixing 100 μl of OprB antiserum with approximately 0.8 mg of *P. aeruginosa* cell envelopes (100 ul volume) at 37°C for 1 hr. For use in immunodetection experiments, this mixture was diluted 1:50 with PBS (137 mM NaCl, 1.5 mM KH_2PO4, 16.6 mM K_2HPO4, 5.4 mM KCl, pH 7.4) containing 1% (w/v) skim milk (Difco). This treatment removed antibodies reacting with LPS or other *P. aeruginosa* outer membrane proteins.

Immunodetection experiments followed the method of Towbin *et al.* (1979). Nitrocellulose filters carrying bound protein

samples were immersed for 0.5 hr in PBS containing 1% (w/v) skim milk. Filters were rinsed with PBS, and OprB antisera, prepared as described above, was added to the nitrocellulose filter. Filters were placed on a rotary shaker for approximately 2 hours at room temperature. Following this, antisera solution was collected and frozen. This solution still contained unused antibodies and could be reused 2-3 more times. Goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) were diluted 1:1000 in PBS/skim milk and added to the rinsed filters. Antibodies were allowed to bind approximately 1-2 hr at room temperature. Filters were rinsed with PBS followed by the addition of 50 ml PBS containing 20 μl 30% (v/v) H_2O_2 and 30 mg 4-chloro-1-napthol (4-chloro-1-napthol was first dissolved in 10 ml methanol). Following colour development filters were rinsed in water and dried overnight between paper towels.

2.6. Cell envelope preparation

Bacterial cell envelopes were prepared following the method of Lugtenberg *et al.* (1975). A 100 ml overnight culture of cells was harvested by centrifugation and resuspended in 15 ml 50 mM Tris, 2 mM EDTA, pH 8.5. Cells were French pressed twice at 15,000 PSI and intact cells removed by a subsequent centrifugation step at 3,500 x g for 10 min. Supernatant was centrifuged for 60 min at 145,000 x g for 1 hr at 4°C. Pelleted cell envelopes were resuspended

in 7.5 ml of the same buffer and centrifuged as above. Cell envelopes were resuspended overnight at 4°C in 500-1000 μl of 2 mM Tris, pH 7.8.

2.7. Periplasmic protein extraction

Preparation of bacterial periplasmic proteins followed the procedure of Hoshino and Kageyama (1980). An overnight 1 L culture of cells was harvested and resuspended in 200 mM $MgCl_2$, 50 mM Tris, pH 7.3 (80 ml per L of original culture). Cells were incubated in a 30°C water bath for 10 min and transferred to a shaking ice water bath for 15 min. This temperature shift was repeated once more. The suspension was centrifuged at 10,000 x g and the supernatant concentrated 10x in an ultrafiltration cell with a 30,000 Da cutoff membrane (Diaflo PM30, Amicon). The supernatant was dialysed overnight at 4°C in 0.05 M Tris, pH 7.3 and concentrated another 2-4 times.

2.8. Amino terminal sequencing

The Amino terminal sequences of proteins were determined by S. Kielland at the Protein Microchemistry Centre, U. of Victoria, Victoria, British Columbia. Outer membrane proteins were separated by SDS-PAGE and transferred by Western blotting onto PVDF membranes (Millipore, Bedford, MA) using CAPS (3-[cyclo-hexylamino]-1-propanesulfonic acid) buffer (10% [v/v] methanol, 10 mM CAPS, pH 11 [Sigma]) as the transfer buffer.

Membrane strips containing the desired protein were cut out following visualization of proteins with Ponceau S (Sigma). Staining with Ponceau S was conducted as follows: membranes were rinsed briefly in H_2O and stained for 5 min in Ponceau S solution (0.2% [w/v] Ponceau S in 3% [w/v] trichloroacetic acid). Membranes were again rinsed in H_2O prior to destaining twice for 5 min in 5% (v/v) acetic acid. Membranes were rinsed twice for 1-2 min in H_2O and dried overnight between sheets of Whatman filter paper.

2.9. Whole cell Lysis

Bacterial cells from a 1.5 ml culture were pelleted in a microcentrifuge and resuspended in 100 ul of cell lysis buffer (2% [w/v] SDS, 4% [v/v] dithiothreitol, 10% [v/v] glycerol, 1 M Tris, pH 6.8). The suspension was heated for 10 min and stored at -20°C. Typically, 3-5 μl were loaded on an SDS-PAGE gel.

2.10. Circular dichroism spectroscopy

OprB, purified by ion exchange chromatography, was transferred to TNS buffer (0.1% SDS [w/v], 100 mM NaCl, 10 mM Tris, pH 8.0) by acetone precipitation (Schlaeppi *et al.*, 1985). OprB was precipitated with ice-cold acetone (60% final concentration), washed with 80% (v/v) ethanol and resuspended overnight at 4°C in TNS.

Spectra were recorded on a Jasco J-500A

spectropolarimeter. Cuvettes of 1 mm pathlength contained protein solutions at a concentration of 112 μg/ml. Analyses were based on an average trace from 4 scans. Baseline readings due to the solvent (0.1% SDS (w/v), 100 mM NaCl, 10 mM Tris, pH 8.0) were subtracted. The instrument was calibrated with d(+)-1-camphorsulfonic acid as described by Hennessey and Johnson (1982). The concentration of camphorsulfonic acid was determined based on its molar extinction coefficient (Σ) of 34.5 l mol^{-1} cm^{-1} at 285 nm. The concentration of OprB was determined using its extinction coefficient at 205 nm determined with the method of Scopes (1974). Protein concentration (P) based on the Scopes method is determined using the formula:

$$P\ (mg/ml) = A_{205}/(27 + 120[A_{280}/A_{205}]).$$

The secondary structure content of OprB was determined using the convex constraint algorithm (CCA) of Perczel *et al.* (1992). This algorithm deconvolutes a CD spectrum into its pure components consisting of α helix, ß sheet, ß turn, etc. Pure component spectra are assigned to specific types of secondary structure by comparison with the CD spectrum characteristic for a given type of secondary structure. The CD spectrum of the protein to be analyzed is appended to the reference data set of 25 proteins supplied with the program. For OprB analysis, program input was in 1 nm intervals from 197 to 240 nm.

2.11. Black lipid bilayer analysis

Black lipid bilayer analysis was conducted following protocols described by Benz *et al.* (1985, 1987). Membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidycholine in *n*-decane. For single channel experiments OprB was used at a concentration of approximately 1 ng/ml (diluted in 0.1% (v/v) Triton X-100). Single channel measurements were recorded with 1 M KCl in the buffer chamber. Applied membrane potential was 20 mV. For zero current membrane potential measurements membranes were formed in a 100 mM salt solution and OprB added so that conductance increased approximately 100- to 1000-fold within 20 to 30 minutes. The voltage was then switched off and the salt concentration raised on one side of the membrane in 100 mM increments by adding 100 μl of a 3 M salt solution. The zero current membrane potential was measured after 5-10 minutes. Macroscopic inhibition experiments were initiated as for zero current membrane potentials. Once the conductance had increased 100- to 1000-fold, inhibition following titration with glucose was measured.

2.12. Transport assays

P. aeruginosa was grown overnight in BM2 medium supplemented with appropriate substrates and antibiotics. Cells were washed twice and resuspended in substrate-free BM2. Cells used for carbohydrate uptake were resuspended to an A_{600}

of approximately 0.04 (1-2 x 10^8 cells/ml). For arginine uptake a cell concentration of approximately 0.2 x 10^8 cells/ml was necessary to obtain linear uptake due to the higher affinity *P. aeruginosa* has for arginine in comparison to carbohydrates. Cells used for examining inhibition of glucose uptake were resuspended to an A_{600} of 0.20 (for experiments with 70 μM [^{14}C] glucose] or 0.04 (for experiments with 0.8 μM [^{14}C] glucose]. Different cell suspensions were used to ensure uptake was linear over the time course of the experiment. The different concentrations of labelled glucose were chosen so that transport by the high affinity glucose pathway of P. aeruginosa would be either near the V_{max} of the transport system or approaching the lower limits for uptake.

Resuspended cells were maintained on ice and warmed for 10 min to 30°C prior to measuring uptake. Following addition of radiolabelled substrates, samples were taken at 20, 40, and 60 sec. Uptake was linear over this time interval. Samples of 0.250 ml were removed, filtered on 0.45 μm filters (Supor-450, Gelman Sciences, Ann Arbor, MI) using a Millipore filter manifold, and washed with 5 ml BM2. Regression lines were fit to the data and used to calculate uptake at 60 sec. Background radioactivity due to non-specific binding to bacterial cells and filters was assessed using formalin killed cells (18% formaldehyde final concentration). Counts due to background radioactivity were subtracted from data obtained from uptake assays.

For measurement of glucose transport inhibition, the above procedure was repeated with the following exceptions. Inhibiting substrates were added 20 sec prior to addition of labelled glucose. Inhibiting substrates were added at a 50 fold excess to labelled glucose (3.5 mM for glucose concentrations of 70 μM and 40 μM for 0.8 μM glucose). Samples were removed at 20, 40, and 60 sec following addition of labelled glucose. Regression lines were fit to the uptake data and changes in slope were used as a measure of inhibition, with uptake in the absence of any inhibitor used as a standard.

Filters were placed in scintillation vials followed by the addition of 8 ml of Ecolume scintillation fluor (ICN, St. Laurent, PQ). Samples were allowed to equilibrate for 1 hour prior to scintillation counting. Samples were analyzed in a Rackbeta liquid scintillation counter (LKB Wallac, Washington, DC).

Radiolabelled substrates (Dupont Canada, Mississauga, ON) and their activities are as follows: glucose (320.0 mCi/mmol); glycerol (147.8 mCi/mmol), fructose (302.0 mCi/mmol), mannitol (56.7 mCi/mmol), and arginine (339.4 mCi/mmol).

2.13. DNA isolation

Plasmid DNA was initially prepared using an alkaline lysis miniprep method (Ausubel *et al.* 1989). Later preparations employed a Magic Miniprep DNA purification system

(Promega, Madison, WI). Manufacturer's instructions were followed when using the Magic minipreps system. Plasmid DNA was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Genomic DNA was prepared using the CTAB (hexadecyltrimethyl ammonium bromide) purification protocol as outlined by Ausubel *et al.* (1989). Routinely, 1.5 ml of an overnight culture was used for preparation of genomic DNA. Purified DNA was resuspended in a final volume of 100 μl TE buffer. This protocol was suitable for isolating genomic DNA from *E. coli* and all of the pseudomonads examined during the course of this study.

Single stranded DNA for sequencing was prepared using the helper filamentous phage R408 (Russel *et al*, 1986). *E. coli* NM522 containing recombinant pTZ19 plasmids was grown in LB media for 2 hr, infected with R408 (approximately 10^{10} phage) and grown overnight. The supernatant of this culture was collected and DNA precipitated with polyethylene glycol (20% w/v). DNA was resuspended in TE buffer, extracted with phenol and followed by a second extraction with chloroform (final concentration for both phenol and chloroform was 40% [v/v]). The DNA was then ethanol precipitated with 95% ethanol. The dried pellet was stored at -20°C and resuspended in water to an approximate concentration of 0.1 μg/μl, just prior to use.

2.14. Restriction enzyme digestion and agarose gel electrophoresis

Restriction enzyme digests were performed with either One-phor-all buffer PLUS (Pharmacia) or REact buffer (Gibco BRL). Plasmid DNA (approximately 1 μg, as judged from agarose gel electrophoresis) was digested for 1 hour at 37°C using 5 U of enzyme. Chromosomal DNA was digested 3-4 hr at 37°C with 15 U enzyme. 1 μl of a 1 mg/ml solution of RNase (Sigma), made DNase free by boiling for 20 min, was added to the reaction mixture if necessary. Final reaction volume was 15 μl.

Agarose gels were made with Agarose (Gibco BRL) at a final concentration of 0.7% (w/v) with TBE (89 mM Tris, 89 mM Boric acid, 0.7 mM EDTA, pH 8) or TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) as the electrophoresis buffer. Ethidium bromide was added to the gel at a ratio of 5 μl (10 mg/ml solution) per 50 ml of agarose gel solution. A Foto/prepI UV transilluminator (Bio/Can Sci., Mississauga, ON) was used to detect the DNA fragments in the agarose gel.

2.15. DNA ligation

DNA fragments (digested as described above) were separated by agarose gel electrophoresis and removed from the gel using the Geneclean II kit (Bio 101, Mississauga, ON) following manufacturer's instructions. Fragments were ligated using 0.5 U T4 DNA ligase and T4 DNA ligase buffer (Gibco BRL)

in a final reaction volume of 20 μl. Ligation was conducted overnight at 12°C. Following overnight incubation the ligation mixture was diluted 5x with distilled water to dilute the polyethylene glycol present in the ligase buffer. 25 μl of the diluted mix was used to transform 100 μl of competent *E. coli* cells.

2.16. Preparation and transformation of competent bacterial cells

Competent *E. coli* cells (NM522) were prepared using the $CaCl_2$ procedure of Ausubel *et al.* (1989). $CaCl_2$ solution consisted of 60 mM $CaCl_2$, 15% (v/v) glycerol, 10 mM Pipes (Sigma), pH 7. Cells to be made competent were harvested from 400 ml of a 3 hour culture grown in a baffle flask on a rotary shaker (previously inoculated with 4 ml of an overnight culture). Competent cells were frozen at -70°C and stored for up to six months.

The procedure of Ausubel *et al.* (1989) was used for transforming competent *E. coli* cells. Approximately 10 ng DNA in a volume of 25 μl was used to transform 100 μl of competent cells. Cells stored at -70°C were thawed rapidly by warming between hands. Cells were left on ice for 30 min instead of the 10 min outlined by Ausubel *et al.* (1989). Following transformation, 1 ml of room temperature LB was added and the cells were allowed to grow 1 hr with no rotation. The cell suspension was then centrifuged in a microfuge for 30 sec.

The cells were resuspended in 300 µl LB and plated on appropriate solid media.

The protocol for preparing competent *P. aeruginosa* cells was based on the $MgCl_2$ procedure of Olsen *et al.* (1982). 100 ml of TN or LB media in a 500 ml baffle flask was inoculated with 1 ml of an overnight culture of *P. aeruginosa* and grown for 2.5 hr (approximately 10^8 cells/ml). The 0.15 M $MgCl_2$ solution used originally by Olsen *et al.* (1982) was replaced with 0.15 M $MgCl_2$, 15% (w/v) glycerol, 10 mM Pipes (Sigma), pH 7. Use of this solution allowed competent cells to be stored at -70°C. Cells were harvested by centrifugation at 1000 x g at 4°C and resuspended in 50 ml of 4°C $MgCl_2$ solution. Cells were incubated in an ice water bath for 5 min. Following incubation, this step was repeated with the ice water incubation extended to 20 min. Cells were then pelleted at 1000 x g and resuspended in 10 ml of 4°C $MgCl_2$ solution. Cells could be transformed at this point or frozen at -70°C.

DNA (approximately 0.2 µg) in a volume of 25 µl was used to transform 200 µl of competent *P. aeruginosa* cells. Cells were incubated in an ice bath for 60 min and heat shocked for 3 min at 37°C while gently rocking the tube. Cells were placed in an ice water bath for 5 min. 0.5 ml of room temperature TN or LB broth was added and cells incubated at 37°C for 2.5 hr with no rotation. Cells were concentrated by centrifugation and plated on appropriate media.

2.17. Protein expression from cloned genomic DNA

E. coli, containing cloned genomic DNA in pTZ19, was grown overnight in 5 ml LB broth containing the appropriate antibiotics and inoculated the next day into 50 ml of the same media. Cells were grown for 2.5 hr on a rotary shaker. 5 ml were removed and isopropyl ß-D-thiogalactoside (Sigma) added to a final concentration of 0.4 mM. A separate 5 ml aliquot with no addition of IPTG was treated in the same way to compare expression in the absence of IPTG induction. After 3 hr a 1 ml sample was removed and whole cell lysis performed, as described above. This procedure was also used for *P. aeruginosa* (containing genomic fragments cloned in pPZ375) with the exception that IPTG was not used since the *lac* repressor gene is not present in *P. aeruginosa*.

2.18. Oligonucleotide preparation and labelling

Oligonucleotides were synthesized by Jack Switala (Dept. of Microbiology, University of Manitoba) on an Applied Biosystems (Mississauga, ON) DNA synthesizer. DNA was removed from the synthesis column with concentrated ammonium hydroxide and incubated overnight at 55°C. Ammonium hydroxide was subsequently removed by lyophilization in a Speedvac evaporator (Savant SC110 Speedvac, Farmingdale, NY). Samples were resuspended in 200 µl H_2O and DNA was precipitated with 600 µl of 95% (v/v) ethanol and 20 µl of 3 M sodium acetate, pH 4.8. Oligonucleotides were collected by centrifugation and

washed twice with 70% (v/v) ethanol. The concentration was determined by measuring absorbance at 260 nm.

Oligonucleotides were 5' end labelled with [γ-^{32}P] ATP (3000 Ci/mmol) (Dupont Canada) using T4 polynucleotide kinase following the protocol of Ausubel *et al.* (1989). Typically, the reaction mixture (75 μl) contained 50 pmol [γ-^{32}P] ATP, 25 pmol oligonucleotide, 7.5 μl One-phor-all buffer PLUS (Pharmacia) and 20 U T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 1 hr and the reaction was stopped by the addition of 5 μl of 0.25 M EDTA.

2.19. Random primer labelling

Double stranded DNA fragments were labelled by random priming using the Random Primers DNA labelling system (Gibco BRL). Manufacturer's instructions were followed. Approximately 25 ng of DNA in 20 μl distilled H_2O and 5 μl of [α-^{32}P] dATP (3000 Ci/mmol) were used in the reaction mixture.

2.20. Colony blot preparation

Lysis and washing of bacterial colonies on nitrocellulose filters (Nitro ME, MSI) followed the procedure of Woods (1984). Bacterial colonies were grown overnight on appropriate solid media and stored at 4°C for 1-2 hr prior to transfer. Colonies were transferred to filters by laying the dry filter on the colonies and removing once the filter was

completely wet. Filters, bacteria side up, were placed on a sheet of Whatman paper soaked with 0.5 M NaOH. After 5 min filters were blotted on dry Whatman paper and transferred for 5 min to a sheet of Whatman soaked with 1 M Tris, pH 7.5. Finally, the filter was placed for 5 min on Whatman paper soaked with 1.25 M NaCl, 0.5 M Tris, pH 7.5. Prior to storage the filters were dried and baked (Blue M Electric Co. oven, Blue Island, IL) at 80°C for 90 min. Before use in hybridization experiments, bacterial debris was removed from the filter by washing the filter in 3x SSC and 0.1 % (w/v) SDS in a shaking water bath at 65°C for 16 hours.

2.21. Southern Blotting

DNA from agarose gels was transferred to nylon membranes (Zeta-Probe Blotting membrane, BioRad) by alkaline transfer following the protocol outlined by Ausubel *et al.* (1989). Agarose gels were depurinated for 10 min in 0.2 N HCl and rinsed in H_2O prior to transfer. DNA was allowed to transfer for approximately 16 hours after which filters were rinsed in 2x SSC and air dried.

2.22. Hybridization with radioactive probes

Hybridization of radioactive probes to DNA bound to nylon or nitrocellulose membranes followed standard procedures (Ausubel *et al.*, 1989). Pre-hybridization solution for nitrocellulose and nylon membranes was 6x SSC, 1x Denhardt's

solution (0.2% [w/v] each Ficoll 400, polyvinylpyrrolidone, Bovine Serum Albumin [Fraction V]), 0.05% (w/v) sodium pyrophosphate, and 0.5% SDS. Routinely, 50 ml of solution was used for prehybridizing filters. Hybridization solution for nitrocellulose membranes was 6x SSC, 1x Denhardt's solution, and 0.05% sodium pyrophosphate. The same solution was used for nylon membranes with the addition of 0.1% SDS. 30 ml of hybridization solution was used.

Hybridization with oligonucleotide probes was conducted overnight in a shaking water bath at 37-48°C depending on the size of the oligonucleotide. Initially, filters were washed at 58°C with 6x SSC, and 0.05% (w/v) sodium pyrophosphate, however, this protocol was changed and washes were subsequently conducted at the hybridization temperature with decreasing concentrations of SSC. Filters were first rinsed in 6x SSC, then washed for 10 min each in 6x SSC, 3x SSC, 1x SSC, 0.5x SSC and, if necessary, 0.1x SSC. Each wash solution contained 0.05% sodium pyrophosphate. 0.1% (w/v) SDS was also included if nylon filters were being used (0.5% (w/v) for 6x SSC solutions).

Hybridization with probes prepared by random primer labelling was conducted overnight in a shaking water bath at 65°C. Formamide (7% v/v) was included in the hybridization solution. Washes were conducted at the hybridization temperature with decreasing concentrations of SSC, as described above. Radioactivity was visualized on Kodak X-Omat

RP film (Kodak, Rochester, NY). Films were exposed to the filters for 4-16 hr.

2.23. DNA sequencing

DNA was sequenced by the chain termination DNA sequencing method of Sanger *et al.* (1977) using the Sequenase™ version 2.0 DNA sequencing kit (USB,) or the ^{T7}Sequencing™ kit (Pharmacia). Manufacturer's instructions were followed in each case. Compressions were resolved by substituting 7-deaza dGTP (Mizusawa *et al.*, 1986) for dGTP. The sequence of any restriction sites used for subcloning were verified by sequencing across such sites on larger restriction fragments. Double stranded sequencing was used except for a region of approximately 100 nucleotides near the centre of the *oprB* gene. I was consistently unable to sequence this region from double stranded preparations, therefore, single stranded preparations of DNA were used instead. Double stranded DNA was prepared for sequencing by alkali denaturation as outlined in the Sequenase™ instruction manual. Sequencing gels were 8% (w/v) acrylamide. Sequence reactions were performed with [α-^{35}S] dATP (Dupont Canada). Radioactivity was visualized using Kodak X-Omat AR film (Kodak). Nucleotide sequence analysis was performed with the PC/Gene program (Intelligenetics).

2.24. Triparental Mating

The procedure used for triparental mating was based on that of Goldberg and Ohman (1984). *E. coli* MM294 (pRK2013), NM522 (carrying the plasmid to be transformed), and *P. aeruginosa* were grown overnight separately in LB with antibiotics added as necessary. One hundred microliters of each culture was mixed in 2 ml of LB and filtered onto a 0.2 μm filter. The filter was placed cell side up on LB-agar and incubated at 37°C overnight. Following incubation cells were washed off the filter with 2 ml of sterile saline. 100 μl was plated on either VB- or BM2-agar with appropriate antibiotics. Plates were incubated from 1-3 days until colonies were visible.

3. OPRB PURIFICATION

3.1. Introduction

Most research on porins has centred on the OmpF and OmpC porins of *E. coli*. These porins are easily purified due to their tight association with the peptidoglycan. Contaminating proteins can be removed from the outer membrane/peptidoglycan complex by exposure to detergent. OmpF and OmpC are unaffected by this treatment and remain associated with the peptidoglycan, unless heated to temperatures greater than 60°C or exposed to high concentrations of NaCl (Rosenbusch, 1974; Benz and Bauer, 1988). Application of techniques of this kind to *P. aeruginosa* does not produce satisfactory results as some of the porins of this species show only limited, or no, association with the peptidoglycan (Hancock *et al.*, 1981). Additionally, purification techniques developed for *P. aeruginosa*, but which use EDTA at each step of the protocol (eg. Mizuno and Kageyama, 1978) are also unsatisfactory as some outer membrane proteins are solubilized in the presence of EDTA (Hancock *et al.*, 1981). However, EDTA can be used to advantage if potential contaminating proteins are first removed from the outer membrane by detergent solubilization, followed by solubilization of the protein of interest by exposure to detergent and EDTA. In this way detergent and a combination of detergent and EDTA, can be used to partially purify some outer membrane porins of *P. aeruginosa*. The protocol used to purify OprB utilizes this approach and is

based on a purification protocol developed by Hancock and Carey (1980). The actual steps involved in purification of OprB have been described in Materials and Methods. This chapter describes the rationale behind the purification procedure and some of the physical characteristics of the purified protein.

3.2. Results and Discussion

3.2.1. OprB purification

Strain H673 (Hg^R *opdE*::Tn*501*) was used for preparation of purified OprB. Due to insertion of Tn*501* into a putative regulatory protein gene (*opdE*) controlling OprD expression (Huang *et al.*, 1992), H673 does not produce OprD. OprD migrates with approximately the same mobility as OprB on SDS-PAGE gels, and use of this strain simplifies purification of OprB.

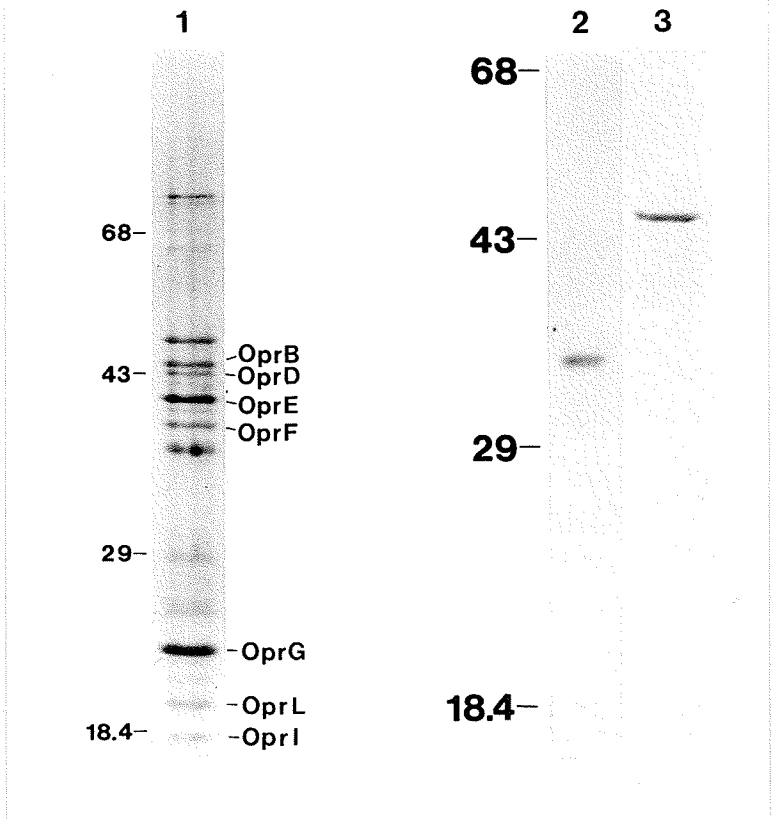
Harvested cells were broken by French pressing, and cell envelopes (inner and outer membranes and peptidoglycan) were collected by ultracentrifugation. The volumes used for resuspension of cell envelopes and loading on the sucrose gradient were critical for obtaining good separation of the inner and outer membranes. The outer membrane and associated peptidoglycan (outer membrane complex) formed the lowest band on the sucrose gradient. Following removal of the outer membrane complex from the sucrose gradient, treatment with Triton X-100 solubilized any contaminating cytoplasmic or

inner membrane proteins, some outer membrane proteins, and approximately 40% of the LPS (Hancock *et al.*, 1981). This step was repeated, otherwise the final product contained too many contaminating proteins.

The partially solubilized outer membrane complex was treated with Triton X-100 in the presence of EDTA. This released several outer membrane proteins from the outer membrane complex, including the porins OprB and OprD, and OprG (the function of OprG is not clear at this time [Hancock *et al.*, 1990]). Major proteins remaining associated with the peptidoglycan following this treatment are the general diffusion porin, OprF, an uncharacterized porin, OprE, and the lipoproteins, OprL and OprI (the major outer membrane proteins of *P. aeruginosa* are shown in Figure 3.1 and reviewed by Hancock *et al.*, 1990). The solubilization of proteins OprB, D, and G by EDTA may be due to disruption of a divalent cation mediated association of these proteins with LPS. However, disruption of a weak association with the peptidoglycan (in comparison to the tight association that Triton X-100/EDTA insoluble proteins show with the peptidoglycan), cannot be ruled out. Hasegawa *et al.* (1976) has demonstrated that divalent cations are involved in the interaction of OmpF and OmpC with the peptidoglycan.

Following treatment with Triton X-100/EDTA, the peptidoglycan-associated proteins are removed by centrifugation. Solubilized OprB and other Triton X-100/EDTA

Figure 3.1. Cell envelope proteins and purified OprB from *P. aeruginosa*. Lane 1, cell envelope proteins isolated from glucose grown *P. aeruginosa* H103 cells. Lanes 2 and 3 show, respectively, nonheat modified and heat modified OprB purified by ion exchange chromatography.


1
2
3
68
43
29
18.4
OprB
OprD
OprE
OprF
OprG
OprL
OprI

soluble proteins remain in the supernatant. Further purification of OprB was accomplished by ion exchange chromatography.

OprB purified by ion exchange chromatography is shown in Figure 3.1 (Lanes 2 and 3). OprB migrated on SDS-PAGE gels with an apparent M_r of 30,000 or 47,000. The mobility was dependent on heat treatment of the samples prior to loading on the SDS-PAGE gel. The more rapidly migrating form of OprB resulted from solubilization at 20°C. Heating to 95°C for 10 min decreased the mobility of OprB. The addition of dithiothreitol to samples had no effect on mobility of OprB on SDS-PAGE gels, suggesting no disulfide bonds were present. *P. aeruginosa* OprF is the only known porin to contain disulfide bonds, detectable by changes in mobility on SDS-PAGE in response to dithiothreitol treatment (Hancock *et al.*, 1979).

Heat-associated changes in mobility (heat modifiability) are commonly found with most porins, however, unlike OprB, most porins show an increase in mobility on SDS-PAGE gels following heat treatment. In these cases, increased mobility is due to the dissociation of a porin trimer to monomers. The characteristic heat modifiability properties of OprB are demonstrated by at least two other *P. aeruginosa* porins, OprF and OprD (Hancock and Carey, 1979). Although OprF and OprB are known to exist as trimers *in vivo* (Angus and Hancock, 1983), the rapid mobility of the unheated forms of these proteins on SDS-PAGE gels suggest that the trimers readily

dissociate to monomers upon isolation from the outer membrane. The nonheat modified monomer presumably has a more compact globular form in comparison to the heat modified form and therefore migrates more rapidly.

The isolated heat-unmodified monomers of these porins are believed to be capable of forming a functional unit. Yoshimura *et al.* (1983) found that liposome permeability was proportional to the amount of OprF porin added. If trimer formation was necessary for porin function, diffusion into liposomes might be expected to initially increase exponentially as protein concentration increased. Isolated monomers of all porins may likewise be able to form pores, however, since most porin trimers must be heated before dissociation occurs, the pore forming ability of their porin monomers is unknown. Xu *et al.* (1986) have found that exposure of *E. coli* OmpF to acidic conditions weakens the association between monomers, resulting in the porin channels apparently functioning individually in black lipid bilayer analysis. This result suggests that a porin monomer can potentially form an open functioning pore. Formation of trimers may not be strictly necessary for formation of a functional channel, but instead may increase protein stability by essentially creating a hydrophobic core at the monomer/monomer interface, analogous to the hydrophobic core of globular proteins.

Only two other porins are known to be dissociable to monomers in the absence of heat, the *Rhodobacter capsulatus* (Nestel *et al.*, 1989) and the *Rhodopseudomonas sphaeroides* porins (Weckesser *et al.*, 1984). The crystal structure of the *R. capsulatus* porin revealed a calcium ion at each monomer/monomer interface (Weiss and Schulz, 1992). Purification of this porin in the presence or absence of EDTA resulted in isolation of monomers or trimers, respectively (Nestel *et al.*, 1989). Since EDTA is used in the purification of *P. aeruginosa* porins, it is possible that removal of calcium ions may also be responsible for trimer dissociation in this species.

3.2.2. Polyclonal antibody production

Polyclonal antibodies were raised against OprB in New Zealand white rabbits. To try to limit production of antibodies against LPS and other minor contaminating proteins, OprB was further purified by preparative SDS-PAGE. Guide strips were stained with Coomassie Blue to identify OprB-containing portions of the gel. Removal of OprB from the polyacrlyamide gel by electroelution and subsequent injection produced a strong immune response. Electroelution appeared to denature the native monomer of OprB as the protein now migrated with a mobility identical to that of heat modified OprB. Antibodies against LPS and some other outer membrane proteins were also produced, however, they were easily removed

prior to immunodetection experiments by preincubation of the antisera with *P. aeruginosa* cell envelopes isolated from succinate grown cells. OprB is not produced following growth on succinate, therefore, this treatment did not affect antibodies specific for the OprB protein. The antisera/cell envelope mixture was used directly in immunodetection experiments. The antibodies reacted strongly with the heat modified form of OprB, however, a weak reaction with the heat unmodified form of OprB could also be detected. This may be due to conservation of a conformational epitope on both the heat modified and unmodified forms of the protein or the presence of a linear epitope accessible on both the heat modified and unmodified forms.

The OprB antibodies were used for detecting homology between potential OprB homologues identified in other species of bacteria (chapters 5 and 8), verifying the expression of OprB from the cloned gene in *E. coli* (chapter 6) and verifying plasmid-directed expression of OprB in OprB-deficient strains of *P. aeruginosa* (chapter 9). Examples of typical immunodetection results can be found in Figures 8.3 (page 217) and 9.6 (page 244).

4. SUBSTRATE SPECIFICITY OF THE HIGH AFFINITY GLUCOSE TRANSPORT SYSTEM OF *PSEUDOMONAS AERUGINOSA*

4.1. Introduction[1]

Hancock and Carey (1980) proposed that OprB and GBP are components of the high affinity (phosphorylative) glucose transport pathway of *P. aeruginosa*. In reviewing the literature regarding carbohydrate transport by this pathway I noted an apparent discrepancy between the *in vivo* specificity of the intact transport system and the *in vitro* specificity of GBP. This was unexpected since previous analysis of an analogous transport system in *E. coli* (the maltodextrin transport system [Ferenci *et al.*, 1986]) showed that the specificities of LamB (maltodextrin selective pore) and the maltose binding protein were reflected in *in vivo* transport by the intact system. Generally, the specificity of a transport system as a whole should reflect the same specificity as the most selective individual component of the transport system. In the case of the high affinity glucose transport system of *P. aeruginosa*, *in vivo* studies by Midgley and Dawes (1973) and Guymon and Eagon (1974) demonstrated that substrates such as galactose and xylose are effective inhibitors of methyl α-

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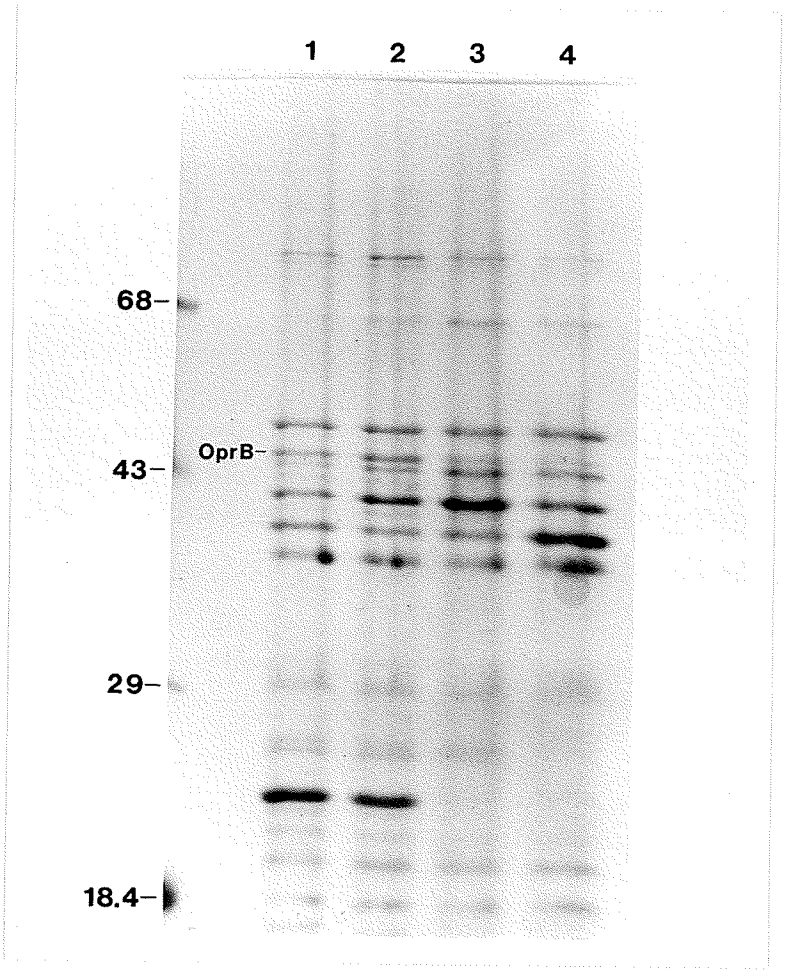
[1]Portions of this chapter are based on a previously published note (Wylie and Worobec. 1993. Can. J. Microbiol. 39:722-725). This analysis was conducted prior to the generation of an OprB-deficient strain of P. aeruginosa. Following cloning and mutagenesis of the oprB gene, substrate specificity of the glucose transport system was reexamined (Chapter 9). The results obtained using the OprB-deficient strain suggested that binding of substrate to OprB, as discussed in sec. 4.1, was not the cause of the observed inhibition of glucose transport. Although the exact cause of the inhibition is unknown, the two distinct sets of results obtained by analysis of inhibition at different concentrations still provides an explanation for the discrepancy between the in vitro and in vivo results reported in the earlier literature.

glucoside uptake, a glucose analogue recognized by the high affinity system. At approximately a two fold excess of galactose or xylose, methyl α-glucoside was inhibited by 73 and 79%, respectively (Midgley and Dawes 1973). Conversely, Stinson *et al.* (1977) found GBP to be highly selective for glucose in *in vitro* inhibition assays based on competition assays with 16 different carbohydrates. At a 10 and 100 fold excess of galactose to glucose the level of glucose binding inhibition was only 0 and 23%, respectively, as opposed to the 73% inhibition found for *in vivo* transport by Midgley and Dawes (1973) for a two fold excess of galactose.

To resolve these differences, I reexamined *in vivo* transport by this uptake pathway and compared the results with the specificity of GBP, *P. aeruginosa* OprB (Trias *et al.* 1988) and the OprB homologue of *P. putida* (Saravolac *et al.* 1991). Glucose inhibition was examined, rather than methyl α-glucoside, as the high concentrations needed to work with this analogue, due to its low affinity for the transport system, may play a role in the previous results obtained *in vivo*. I focused on carbohydrates with a known affinity for OprB (xylose, maltose, and galactose) and those which support the growth of *P. aeruginosa* (fructose, ribose, and mannitol). The latter 3 substrates induce the production of OprB (Fig. 4.1), suggesting OprB might play a role in the uptake of other carbohydrates.

Figure 4.1. Cell envelope proteins of *P. aeruginosa* separated by SDS-PAGE. Cells were grown on 1) glucose, 2) fructose, 3) mannitol, and 4) ribose. OprB was produced following growth on each of these substrates.


1
2
3
4
68–
43–
29–
18.4–
OprB–

4.2. Results and Discussion

4.2.1. Substrate specificity of glucose grown cells

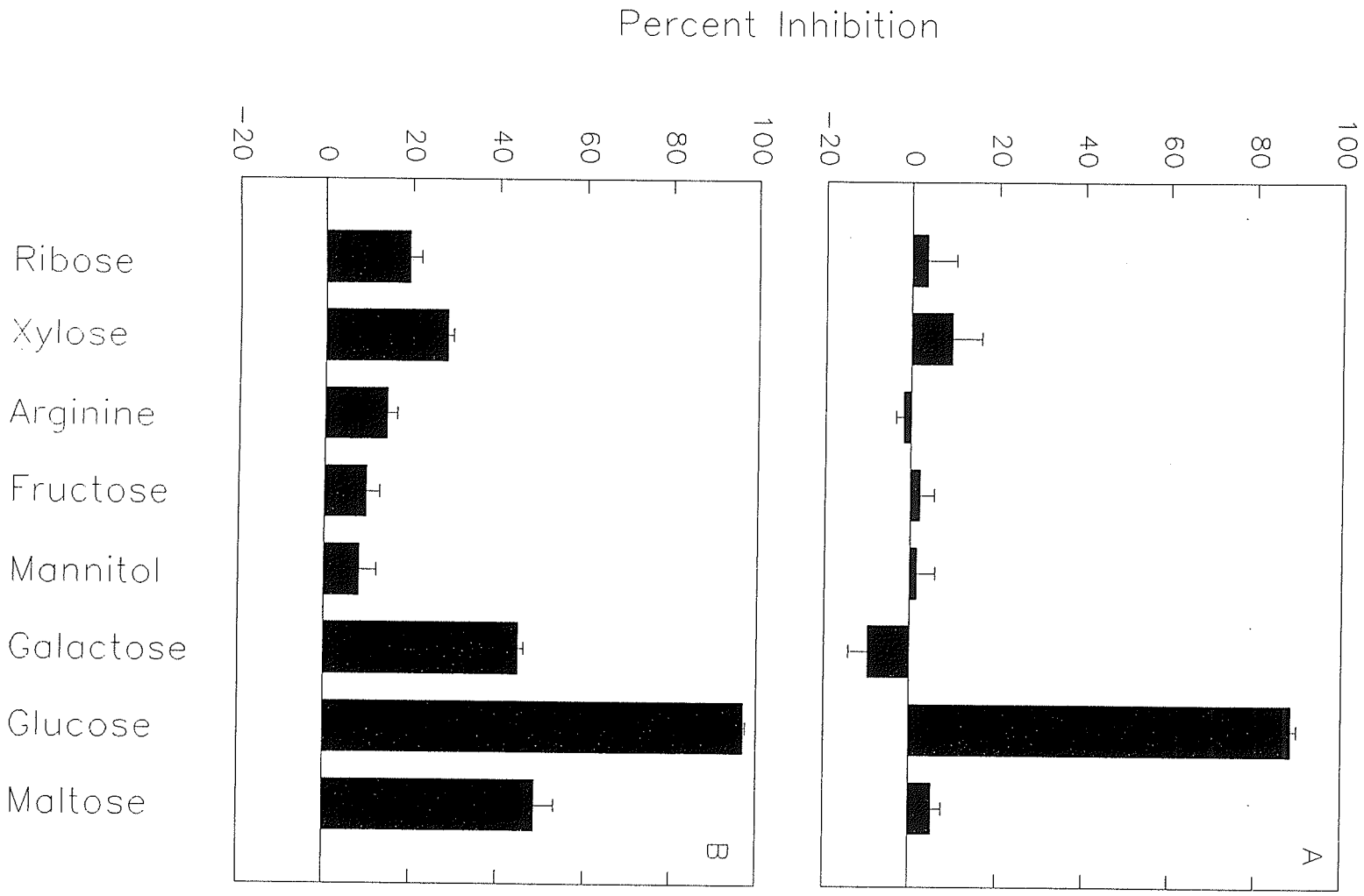
Inhibition of the high affinity glucose transport system was examined at two concentrations of [^{14}C] glucose (0.8 μM and 70 μM). At 0.8 μM glucose, transport would be near the lower limits of uptake by this transport system (Midgley and Dawes 1973). At 70 μM, uptake should be near the V_{max} of the high affinity system. Under these latter conditions a 50 fold excess of inhibitor ensured that inhibitor concentration (3.5 mM) was similar to that used in previous *in vivo* studies by Midgley and Dawes (1973) and Guymon and Eagon (1974). Inhibition was examined at the lower levels of labelled glucose and inhibitor (0.8 μM glucose and 40 μM inhibitor) as the high levels of inhibitor needed in the earlier *in vivo* studies may have contributed to the discrepancy between earlier *in vivo* and *in vitro* results. A 50 fold excess of inhibitor was used throughout to maintain a constant ratio of inhibitor to labelled substrate, for reasons discussed below.

As shown in Figure 4.2A, with 0.8 μM [^{14}C] glucose, only unlabelled glucose significantly inhibited transport; other substrates caused no significant inhibition (T-method for multiple comparison of means [Sokal and Rohlf 1981], p=0.01). When 70 μM [^{14}C] glucose was used (Fig. 4.2B), glucose, maltose, galactose, and xylose showed a significantly higher level of inhibition compared to other substrates (T-method, p=0.01) with glucose having the highest inhibitory capacity.

Galactose and maltose showed levels of inhibition statistically indistinguishable from each other. Xylose showed significantly lower levels of inhibition than galactose and maltose but higher than fructose, mannitol, ribose, or arginine. Fructose, mannitol, and ribose inhibited to the same extent as the non-carbohydrate control, arginine. Results obtained at 70 μM [^{14}C] glucose are similar to previously reported analyses of inhibition of methyl α-glucoside uptake by Guymon and Eagon (1974) and Midgley and Dawes (1973). The absence of inhibition by fructose, mannitol, or ribose suggested that the diffusion of these sugars was not facilitated by OprB.

Analysis of inhibition at different concentrations produced two distinct sets of results, providing an explanation for discrepancies between *in vitro* and *in vivo* results in the earlier literature. The *in vivo* results of Figure 4.2A are those expected based on the reported high degree of *in vitro* specificity of GBP for glucose (Stinson *et al.* 1977). In contrast, the results of Figure 4.2B are similar to earlier *in vivo* results and correlate with the substrate specificity of OprB. Trias *et al.* (1988) used the liposome swelling assay to demonstrate facilitated diffusion of xylose by OprB. In addition, black lipid bilayer studies have demonstrated an affinity for galactose and maltose by the OprB analogue of *P. putida* (Saravolac *et al.* 1991). This observed correlation suggests the pattern of inhibition seen

Figure 4.2. Inhibition of glucose uptake. Inhibiting substrates used are shown on the abscissa in increasing order of M_r. D-[^{14}C] glucose was used at a concentration of A) 0.8 μM, or B) 70 μM. Inhibiting substrates were used at a 50 fold excess of A) 40 μM, or B) 3.5 mM. Standard errors are calculated with n=3 except for B) where error bars for mannitol, fructose, ribose and arginine are calculated with n=4.


Percent Inhibition
−20
0
20
40
60
80
100
Ribose
Xylose
Arginine
Fructose
Mannitol
Galactose
Glucose
Maltose
A
B

in Figure 4.2B is due to recognition of substrate by OprB. Alternatively, it is conceivable that at the higher substrate levels used in Figure 4.2B, inhibition of glucose transport through the low affinity glucose transport pathway is beginning to occur. This pathway could have a different specificity than the high affinity pathway. However, this is unlikely since uptake of methyl-α-glucoside is restricted to the high affinity system (Midgley and Dawes 1973) and similar patterns of inhibition by xylose and galactose were observed when this substrate was used by Midgley and Dawes (1973) and Guymon and Eagon (1974). Additionally, it is possible that the specificity of GBP differs under the two sets of conditions used in the analysis described here, with some sugars beginning to inhibit glucose binding to GBP at the higher substrate levels used in Figure 4.2B. However, Kellerman and Szmelcman (1974) examined specificity of MBP at 5 μM maltose and 50 μM inhibitor. They later reexamined specificity using 100 μM and 1000 μM inhibitor and found their results unchanged (Szmelcman *et al*. 1976). Therefore, as long as the ratio of inhibitor to labelled substrate remains constant no change in specificity of a binding protein appears to occur. These observations suggest that the inhibition seen in Figure 4.2B and that previously reported by Midgley and Dawes (1973) and Guymon and Eagon (1974) is due to inhibition of glucose binding to OprB and not GBP.

Why the specificity of individual components of the high

affinity glucose transport system can be detected at different substrate levels is not clear. In analyzing the role of individual components of the maltodextrin transport system of *E. coli* on maltodextrin binding, Ferenci (1980) found that binding of large non-transportable maltodextrins by whole cells depended predominantly on a functional LamB protein (maltodextrin selective pore) and maltose binding protein. Inner membrane components had much less of an effect in comparison. The necessity of LamB is not unexpected given the inability of large dextrins to pass through other *E. coli* porins (Wandersman *et al.* 1979). However, the near elimination of maltodextrin binding in the absence of MBP is surprising since LamB can bind maltodextrins *in vitro* independently of MBP (Benz *et al.* 1987). Similarly, the preferential diffusion of phosphate through the PhoE porin *in vivo* appears to be correlated with the presence of the *E. coli* phosphate binding protein (Korteland *et al.*, 1982). These results indicate that uptake of nutrients through porins *in vivo* can depend on the action of binding proteins in the periplasm. Currently, the exact role of binding proteins in transport is not clear (Higgins *et al.* 1990). One proposal by Brass *et al.* (1986) is based on evidence that the periplasmic contents of *E. coli* are gel-like and capable of retarding the movement of both proteins and smaller substrates (Hobot *et al.* 1984; Brass *et al.* 1986). They proposed that binding proteins would facilitate diffusion across the periplasm by passing

substrate from protein to protein across the periplasm. This function would relate to results presented here and previously published results (Ferenci, 1980; Korteland *et al.*, 1982) if, in transporting substrate rapidly away from the outer membrane towards the inner membrane, binding proteins ensure continual binding and diffusion of substrate through porins by maintaining a concentration gradient across the outer membrane. In this way, diffusion across the outer membrane would indirectly depend on either binding of substrate by binding proteins, or more generally, transport of substrate from the periplasm to the cytoplasm.

If this process occurs in *P. aeruginosa*, diffusion through OprB at low substrate levels would depend on binding of substrate by GBP. Only substrates recognized by GBP (i.e. glucose) would significantly inhibit [^{14}C] glucose uptake (Fig. 4.2A). If the concentration of labelled glucose and inhibitors is raised (Fig. 4.2B) sufficient diffusion of substrates through the periplasm might occur to maintain a concentration gradient across the outer membrane independent of GBP. The extent of inhibition in this case would depend on the affinity of OprB for the inhibitor.

These results suggest that the high affinity glucose transport system of *P. aeruginosa* as a whole is highly specific for glucose when examined as an intact system *in vivo*. This is expected due to the specificity of GBP for glucose. OprB does have an affinity for other sugars in

addition to glucose (Saravolac *et al.* 1991; Trias *et al.* 1988) and does appear to recognize these substrates *in vivo* to some extent (Fig. 4.2B). The substrate level at which inhibition is examined determines the results obtained and clarifies the differences between earlier *in vitro* and *in vivo* studies.

4.2.2. Inhibition of glucose transport in cells co-induced for two transport systems

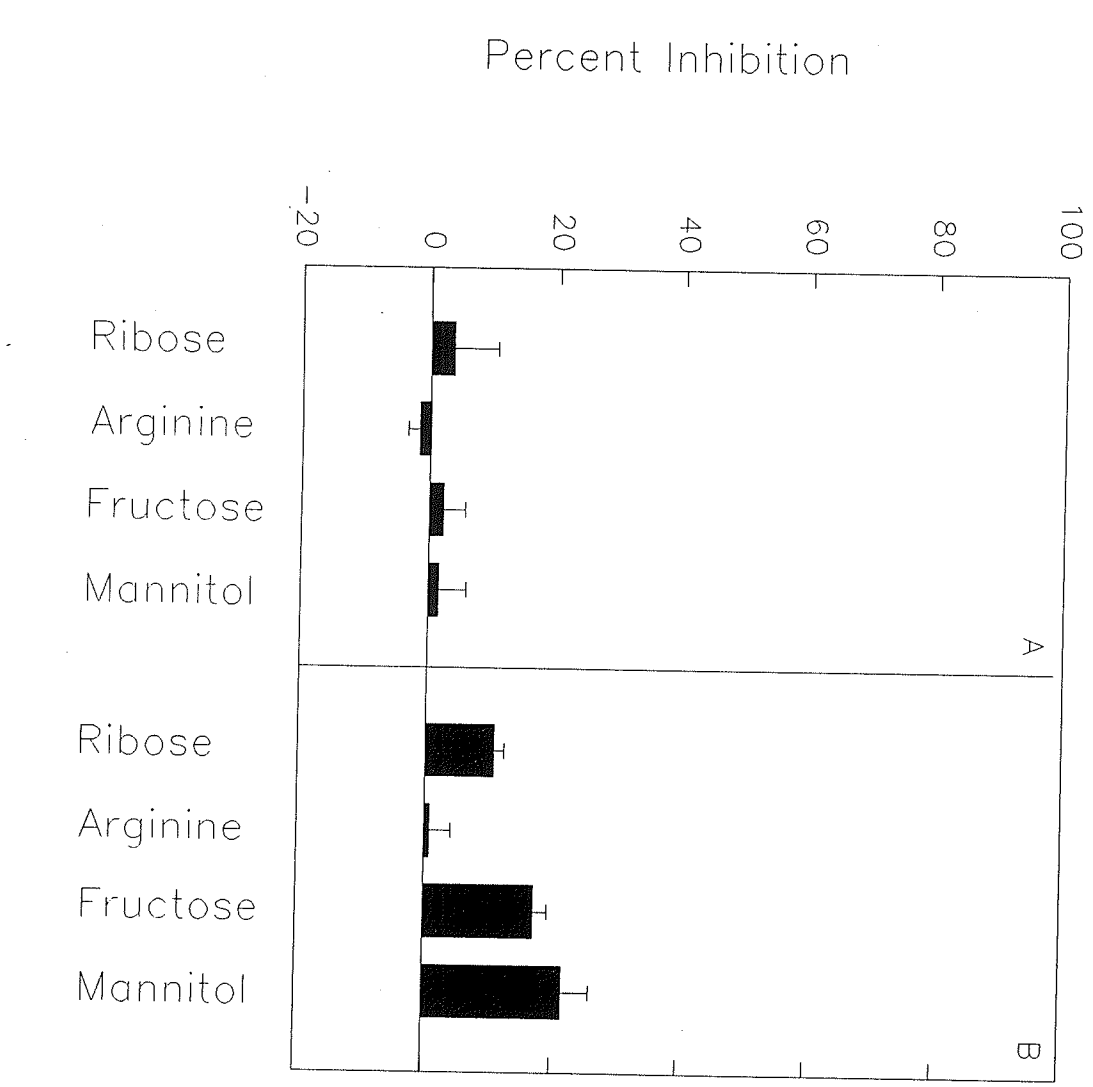
The absence of glucose transport inhibition by fructose, mannitol, and ribose suggests that these sugars do not bind to OprB and OprB does not specifically facilitate the transport of these sugars into the periplasm. However, as discussed above, in the absence of transport from the periplasm to the cytoplasm, diffusion of these substrates might be low. Therefore, inhibition of glucose diffusion through OprB may be difficult to observe unless the inhibiting substrates are specifically removed from the periplasm by a transport system. This may be especially relevant in assessing the potential of OprB to facilitate diffusion of fructose, mannitol, and ribose since the structures of these sugars, in comparison to glucose, are quite different and the binding affinity of these sugars to OprB might be low. I therefore repeated glucose uptake inhibition at 0.8 μM [^{14}C] glucose with cells co-induced for glucose and either mannitol, fructose or ribose. As a control, cells were co-induced for uptake of glucose and arginine. Arginine is specifically taken up by OprD, another

substrate-selective porin of *P. aeruginosa* (Trias and Nikaido, 1990b), and I reasoned that uptake of arginine would not interfere with glucose uptake through OprB. However, if OprB does facilitate the diffusion of mannitol, fructose or ribose to any extent, uptake of these substrates might interfere with glucose transport under these conditions.

Verification that cells were co-induced for transport of glucose and the substrate being examined, was conducted as follows. Cells were grown overnight in BM2 medium containing 2.5 mM of each substrate. In case diauxic growth occurred, use of these low substrate concentrations ensured that both substrates would be utilized before growth became limited by waste products in the media or by other factors. Cells were washed and the presence of a transport system verified by recording changes in A_{600} values in response to substrate addition. I had previously found that if cells were induced for growth on a specific substrate, growth was evident within 1 hr of addition of that substrate to the washed cells. If a transport system was not induced recordable growth did not occur for approximately 3 to 4 hr. Based on this criteria I found that it was possible to produce cells possessing two active transport systems.

When inhibition of glucose transport by fructose, mannitol, and ribose was reexamined in cells co-induced for two transport systems, inhibition increased in comparison to arginine (Fig. 4.3). These results suggest that when glucose

Figure 4.3. Inhibition of glucose uptake using A) glucose grown cells and B) cells co-induced for glucose and either mannitol, fructose, ribose or arginine. Concentration of labelled glucose was 0.8 μM. Inhibiting substrates were used at a 50 fold excess. For B) inhibition by a given substrate was examined when cells were co-induced for uptake of that substrate and glucose. Standard errors are calculated for n=4.


Percent Inhibition
-20
0
20
40
60
80
100
Ribose
Arginine
Fructose
Mannitol
A
Ribose
Arginine
Fructose
Mannitol
B

and a second carbohydrate are being transported simultaneously, some aspect of sugar transport is common to each uptake pathway. Since each has a different cytoplasmic membrane uptake system, the common factor may be diffusion through OprB.

It is also possible that the observed inhibition of glucose uptake could be due to competition for cytoplasmic catabolic enzymes. However, the short time scales involved make this unlikely. Eagon and Phibbs (1971) examined secondary metabolites present from 10 to 30 sec following sugar uptake and found only minimal conversion of substrate.

If facilitated diffusion by OprB is the cause of the observed changes in inhibition, it suggests that OprB does not act solely as a glucose pore, but rather could potentially act as a central component of carbohydrate uptake in *P. aeruginosa*. However, the low levels of inhibition by mannitol, fructose, and ribose suggest that OprB would be predominantly a glucose pore. Recognition of other sugars may be correlated to the degree of similarity of their stereochemistry to glucose and/or to their rate of transport into the cytoplasm (i.e. the extent to which a concentration gradient is maintained across the outer membrane). Generation of an OprB-deficient strain of *P. aeruginosa* should verify the conclusions suggested by these results.

5. BIOPHYSICAL CHARACTERIZATION OF OPRB

5.1. Introduction

This chapter describes the physical characterization of the OprB porin of *P. aeruginosa*. Saravolac *et al.* (1991) previously purified and characterized a protein from *P. putida* which they called OprB based on evidence suggesting it to be homologous to OprB of *P. aeruginosa* (both proteins were glucose-inducible, showed similar heat modifiability properties, and had similar amino acid compositions). However, the methodologies used to examine the functional properties of the two OprB proteins differed considerably and it is difficult to assess the extent of similarity between the two channels. *P. putida* OprB was examined using black lipid bilayer analysis and characterized as a glucose-specific porin possessing a small constricted channel (Saravolac *et al.* 1991). Trias *et al.* (1988) also proposed *P. aeruginosa* OprB to be a glucose specific pore but based their results on the liposome swelling assay. The results of their analysis, and a similar one by Hancock and Carey (1980), were consistent with the *P. aeruginosa* channel being quite large since substantial permeation of solutes larger than disaccharides was observed. This suggested the relationship between the two OprB proteins could be similar to that between the maltodextrin-selective porin LamB and the sucrose-selective porin ScrY (Hardesty *et al.*, 1991). Both are sugar-selective porins found in enteric bacteria, share some amino acid

homology, and possess a similarly constructed binding site with a length of 5 glucose residues, yet ScrY forms a larger channel and shows some characteristics of a general diffusion pore (Schülein *et al.*, 1991). These authors attributed the general diffusion properties of ScrY to an additional 70 amino acids at the N terminus of the protein. A similar situation could also occur with OprB of *P. aeruginosa* as the *P. aeruginosa* protein is 4,500 Da larger than *P. putida* OprB (Saravolac *et al.*, 1991).

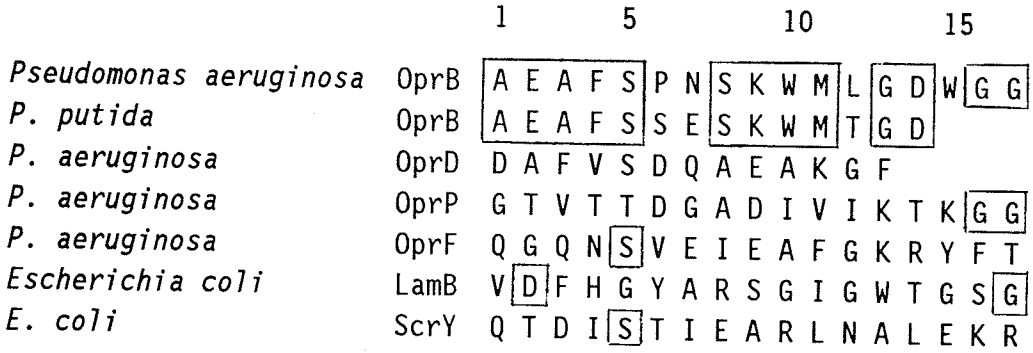
To resolve the potential differences between the two OprB proteins I have examined *P. aeruginosa* OprB using methodology identical to that used to characterize *P. putida* OprB. This provided a more detailed characterization of *P. aeruginosa* OprB and allowed a direct comparison between the channel-forming properties of the two proteins.

5.2. Results

5.2.1. Amino terminal amino acid sequence analysis

The amino acid sequence was determined for the first 17 and 14 residues of *P. aeruginosa* OprB and *P. putida* OprB, respectively. These sequences were compared to *P. aeruginosa* porins OprD, OprF, and OprP, and *E. coli* LamB and ScrY, as illustrated in Figure 5.1. OprF is believed to be the main non-selective diffusion pore of *P. aeruginosa* (Hancock *et al.*, 1979; Nikaido *et al.* 1991), whereas OprD and OprP are substrate specific pores recognizing basic amino acids (Trias

Figure 5.1. Amino terminal amino acid sequence comparison of various bacterial porins. Each sequence was individually aligned by eye with *P. aeruginosa* OprB. For a given protein identical or conserved amino acids in common with *P. aeruginosa* OprB are boxed. Conserved residues were considered to be I/L/V, S/T, and D/E. Sources for data are as follows: *P. aeruginosa* and *P. putida* OprB (this study); OprD (this study, Yoneyama *et al.*, 1992; Huang *et al.*, 1992); OprP (Worobec *et al.* 1988); OprF (Duchêne *et al.* 1988); LamB (Clément and Hofnung 1981); and ScrY (Hardesty *et al.* 1991).

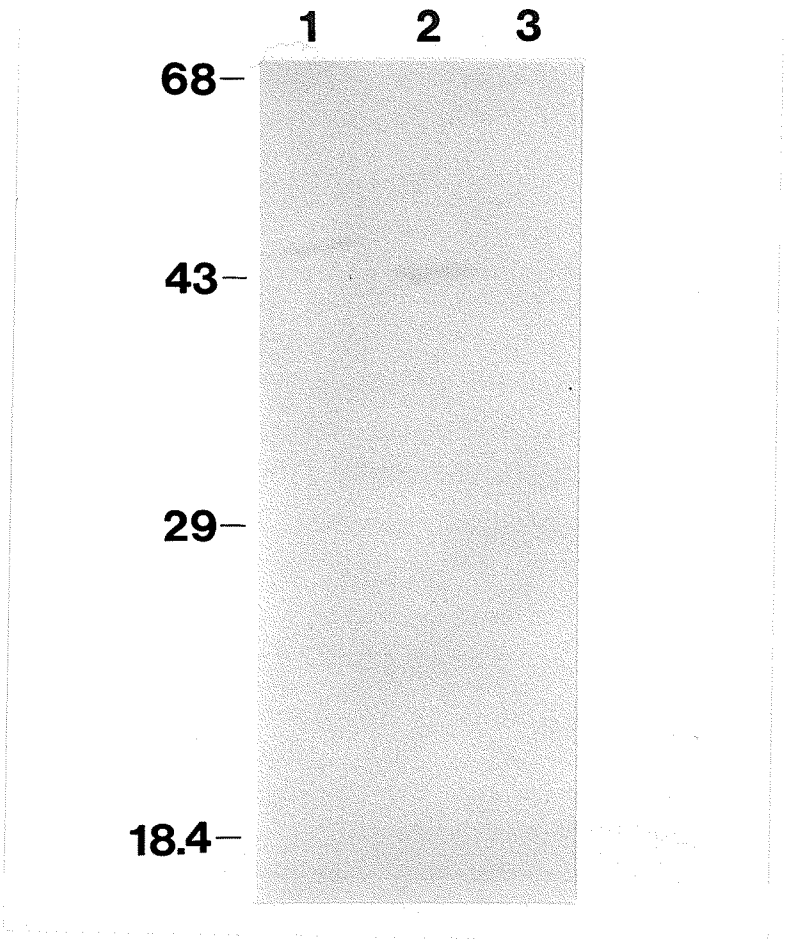

1 5 10 15
Pseudomonas aeruginosa OprB A E A F S P N S K W M L G D W G G
P. putida OprB A E A F S S E S K W M T G D
P. aeruginosa OprD D A F V S D Q A E A K G F
P. aeruginosa OprP G T V T T D G A D I V I K T K G G
P. aeruginosa OprF Q G Q N S V E I E A F G K R Y F T
Escherichia coli LamB V D F H G Y A R S G I G W T G S G
E. coli ScrY Q T D I S T I E A R L N A L E K R

and Nikaido, 1990b) and phosphate (Hancock *et al.*, 1982), respectively. LamB and ScrY are, respectively, maltodextrin-selective and sucrose-selective porins found in *E. coli* and several other enteric bacteria (Szmelcman and Hofnung, 1975; Palva, 1978; Hardesty *et al.*, 1991). A high degree of homology was evident for the first 14 residues (equal to the extent of information available for *P. putida* OprB) of the OprB proteins of *P. aeruginosa* and *P. putida,* with 78.5% of the residues being identical. In contrast, little sequence similarity was evident with other *P. aeruginosa* porins or LamB and ScrY of *E. coli*. This degree of homology is not unusual for closely related porins. Although many porins are known to show relatively little sequence similarity, homologous porins in closely related bacteria do show extensive homology (eg. LamB [Francoz, 1990]; OprF [Ullstrom *et al.*, 1991]).

5.2.2. Immunoblot analysis

To further examine the extent of homology between the OprB proteins, outer membrane proteins of glucose grown *P. aeruginosa* and *P. putida,* and maltose grown *E. coli* were electrophoretically transferred to nitrocellulose and exposed to antiserum specific for heat modified *P. aeruginosa* OprB (Fig. 5.2) No cross reaction was visible with heat modified LamB, whereas both *P. aeruginosa* and *P. putida* OprB were recognized by the antibodies under these conditions. The antibodies used were specific for heat-modified OprB. These

Figure 5.2. Immunodetection of outer membrane proteins with antiserum specific for heat modified *P. aeruginosa* OprB. Outer membrane proteins isolated from glucose grown *P. aeruginosa* (Lane 1), glucose grown *P. putida* (Lane 2) and maltose grown *E. coli* (Lane 3). Outer membrane protein samples were solubilized at 95°C for 10 minutes prior to electrophoresis. Molecular weight standards are indicated on the left ($x10^3$).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68
43
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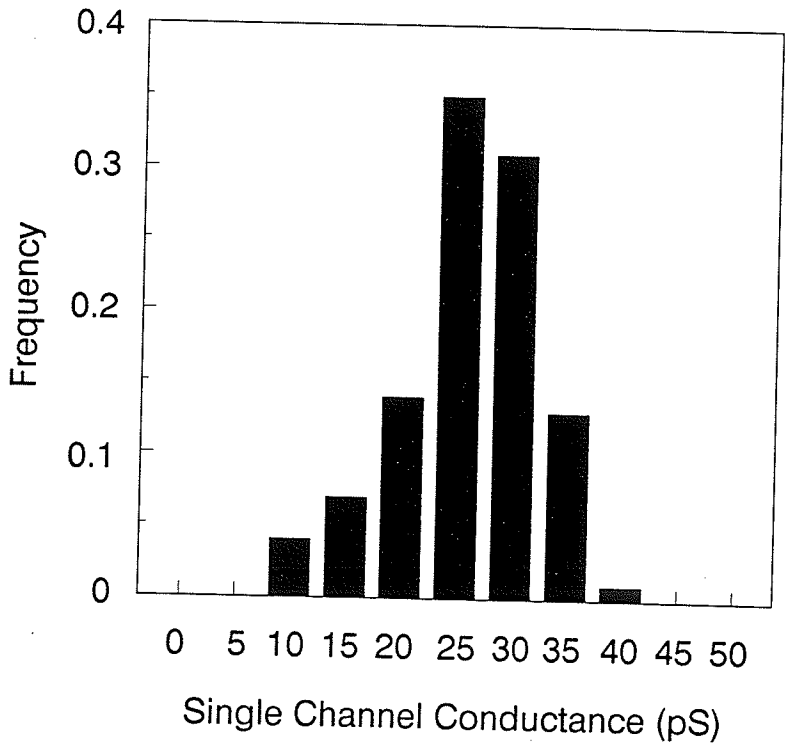
18.4

antibodies recognize predominantly linear epitopes rather than conformational epitopes and thus they provide a better indicator of amino acid homology. Poole and Hancock (1986b) demonstrated that antibodies specific for native OprP cross reacted with phosphate-selective porins from other Pseudomonads and *E. coli* whereas antibodies specific for heat modified OprP reacted only with *P. aeruginosa* OprP.

5.2.3. Single channel conductance measurements of *P. aeruginosa* OprB

P. aeruginosa OprB was added to the aqueous phase bathing black lipid bilayer membranes. Step-wise increases in conductance were observed with an average single channel conductance of 25 pS (Fig. 5.3). Values were concentrated within a narrow range indicating no significant contribution from contaminating porins. Single channel conductance for *P. aeruginosa* OprB was very close to that previously measured for *P. putida* (35 pS, [Saravolac *et al.*, 1991]). Significantly larger single channel conductance values have been previously measured for other carbohydrate-selective porins; 160 pS for LamB (Benz *et al.*, 1986) and 1400 pS for sucrose specific porin ScrY (Schülein *et al.*, 1991). Values for ScrY fall within the range characteristic of those for non-specific diffusion pores (Benz *et al.*, 1985). The similarity in single channel conductance between the OprB proteins, in comparison with the other carbohydrate-selective pores, suggests

Figure 5.3. Histogram of the conductance steps observed with diphytanoyl phosphatidylcholine-*n*-decane membranes in the presence of *P. aeruginosa* OprB. Average single channel conductance was 25 pS for 102 steps. Aqueous phase contained 1 M KCl and applied voltage was 20 mV.


0.4
0.3
0.2
0.1
0
Frequency
0 5 10 15 20 25 30 35 40 45 50
Single Channel Conductance (pS)

extensive conservation of the channel structure of these two proteins.

5.2.4. Glucose binding to *P. aeruginosa* OprB

Previously, *P. aeruginosa* OprB has been proposed to act as a glucose-selective porin based on results from liposome swelling assays (Trias *et al.*, 1988). This assay, however, does not specifically verify the presence of a glucose binding site (Saravolac *et al.*, 1991) nor was the binding affinity for glucose determined. In this study the potential presence of a glucose binding site in *P. aeruginosa* OprB was examined by means of macroscopic conductance black lipid bilayer analysis (Benz *et al.*, 1987). Sufficient *P. aeruginosa* OprB for incorporation of 100-1000 channels was added to the solutions bathing black lipid membranes and allowed to reach a maximum conductance (approximately 30 minutes). Addition of glucose led to a dose-dependent inhibition of conductance, consistent with the presence of a saturable binding site for glucose within the channel (data not shown).

Based on the two-barrier, one-site model proposed for transport of maltodextrins through LamB (Benz *et al.*, 1987) the sugar induced block of ion movement through OprB can be used to determine the stability constant, K, for sugar binding based on the equation

$$(X_{max}-X_{[c]})/X_{max} = K\cdot c/(K\cdot c+1)$$

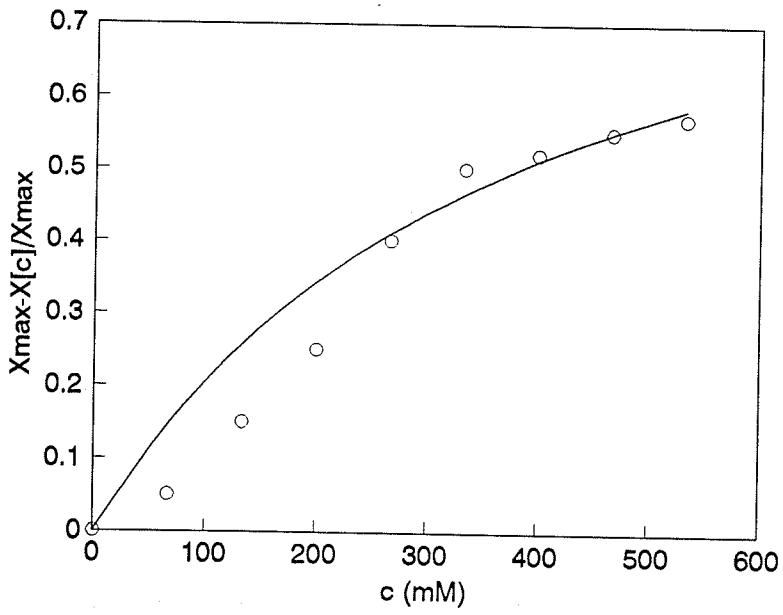
where X_{max} is the conductance before addition of sugar, $X_{[c]}$ is

conductance at a given sugar concentration, c is sugar concentration, and K is the stability constant (= $1/K_s$). K_s values for glucose binding to *P. aeruginosa* OprB are derived from a plot of the dose-dependent inhibition of conductance (Fig. 5.4) or by means of a Lineweaver-Burke plot. A value of 380 ± 40 mM (based on four experiments) was determined which is within the same order of magnitude as the K_s for glucose binding to *P. putida* OprB (110 mM; Saravolac *et al.*, 1991), LamB (110 mM; Benz *et al.*, 1987), and ScrY (120 ± 40 mM; Schülein *et al.*, 1991).

5.2.5. Ion selectivity

In order to determine the ion selectivity of *P. aeruginosa* OprB, zero current membrane potentials were determined in the presence of salt gradients of KCl, LiCl, or KCH_3COO. These salts are commonly used for black lipid bilayer experiments to assess ion selectivity due to the differing aqueous mobilities of the various ions. K^+ and Cl^- ions possess approximately the same aqueous mobilities (limiting molar conductivities of 73.5 and 76.4 S x cm^2 per mol, respectively [Castellan, 1983]). The mobilities of Li^+ and CH_3COO^- are nearly equal to each other (38.7 and 40.9 S x cm^2 per mol, respectively) but considerably smaller than K^+ or Cl^-. After insertion of approximately 100-1000 channels into the black lipid membranes, the salt concentration was raised in steps on one side of the membrane (from 100 mM to 500 mM).

Figure 5.4. Macroscopic conductance inhibition as a function of glucose concentration. OprB macroscopic conductance, X[c], was measured following titration with increasing concentration of glucose (c). Membranes were formed from diphytanoyl phosphatidylcholine-*n*-decane, aqueous phase contained 1 M KCl. The line was drawn from the equation, $X_{max}-X[c]/X_{max} = k \cdot c/(k \cdot c+1)$ using a value of $k_s = 380$.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Xmax-X[c]/Xmax
0
100
200
300
400
500
600
c (mM)

After each addition, the zero current membrane potential was measured and the ratio P_{cation}/P_{anion} was calculated according to the Goldman-Hodgkin-Katz equation (Benz *et al.*, 1979). Results for *P. aeruginosa* OprB, in comparison to other carbohydrate-selective porins, are presented in Table 5.1. In contrast to the cation selectivity of other carbohydrate-selective porins the results indicate that *P. aeruginosa* OprB is anion selective.

5.2.6. Circular dichroism

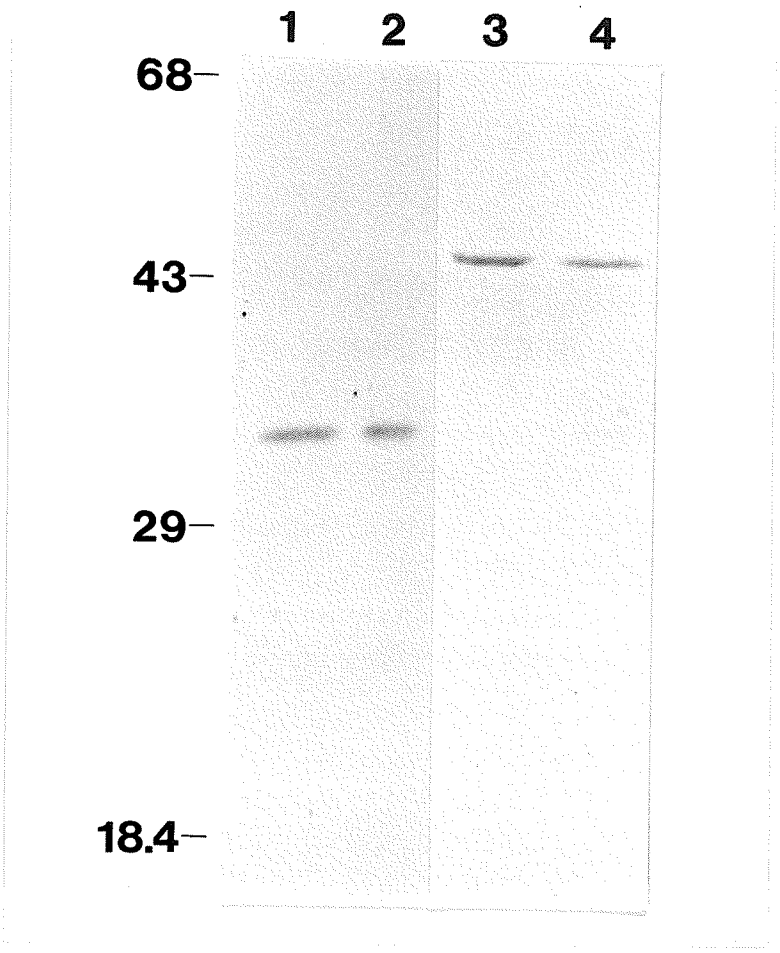
For circular dichroism analysis, *P. aeruginosa* OprB was transferred by acetone precipitation from a Triton X-100 buffer to one containing SDS. Triton X-100 absorbs strongly at several wavelengths and would have interfered with CD analysis. This treatment did not alter the native structure of OprB based on identical mobilities on SDS-PAGE gels before and after acetone treatment (Fig. 5.5, lanes 1 and 2). Furthermore, the acetone-treated protein was altered by heat treatment to the same extent as the triton-solubilized protein (Fig. 5.5, lanes 3 and 4). The property of heat modifiability is characteristic of porins and is related to their high ß sheet content. ß sheet structure is stable in the presence of SDS unless heated (Mizushima, 1974; Nakamura and Mizushima, 1976; Hancock and Carey, 1979). Heating these proteins in the presence of SDS results in a partial loss of secondary structure which alters their mobility on SDS-PAGE gels.

Table 5.1. Permeability ratios (P_{cation}/P_{anion}) for *P. aeruginosa* OprB, *P. putida* OprB, *E. coli* LamB and ScrY.

Salt	P_{cation}/P_{anion}			
	OprB[1]	OprB[2]	LamB[3]	ScrY[4]
KCl	0.64	17	28	8.6
LiCl	0.29	14	12	5.8
KCH_3COO	—[5]	19	50	16

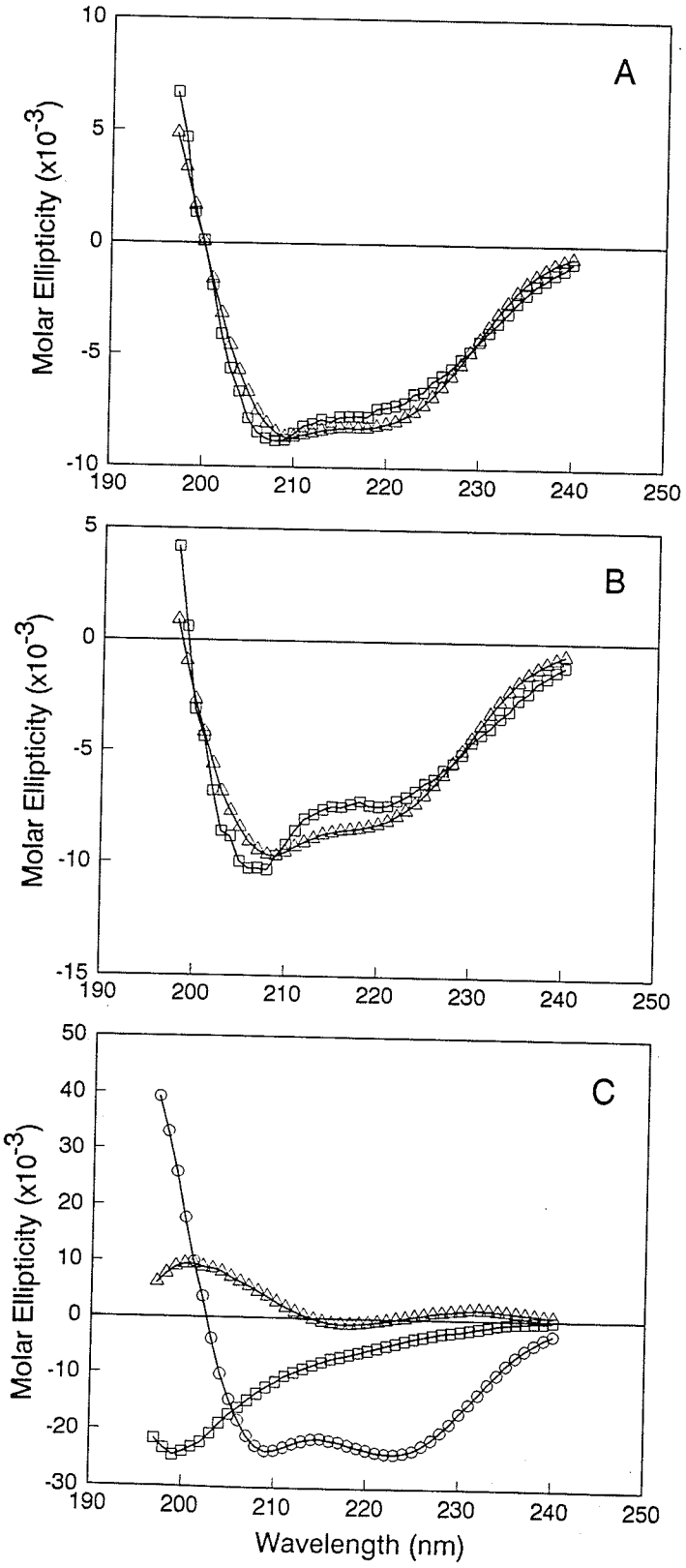
[1] *P. aeruginosa* OprB, zero current membrane potential measured using a fivefold gradient for each of the salts.
[2] *P. putida* OprB, data from Saravolac *et al.* (1991), salt gradient fivefold.
[3] Data from Benz *et al.* (1987), salt gradient tenfold.
[4] Data from Schülein *et al.* (1991), salt gradient tenfold.
[5] Due to the strong anion selectivity of *P. aeruginosa* OprB no activity could be detected when KCH_3COO was used as the salt.

Figure 5.5. SDS-polyacrylamide gel electrophoresis of purified OprB. Lanes 1 and 3, OprB in TTE buffer following purification by ion exchange chromatography; lanes 2 and 4, OprB in TNS buffer following acetone precipitation. Lanes 1 and 2, unheated, lanes 3 and 4 heated at 100°C for 10 min. Molecular weight standards are indicated on the left ($x10^3$).


1
2
3
4
68
43
29
18.4

The CD spectrum of native unheated OprB and heat modified OprB are presented in Figure 5.6A and B. The spectra of both forms of OprB show a broad minimum with a negative peak at 208 nm. The CD spectra of OprB were analyzed using the CCA algorithm of Perczel *et al.* (1992). Prior to analysis the number of pure component curves (P) comprising a given data set are unknown and must be determined *a priori* (Perczel *et al.*, 1992). I conducted four analyses of the OprB data using a value of either 2, 3, 4, or 5 for P. In the case of P = 2, one of the pure curves produced was flat and featureless, whereas for P = 4 or 5 some curves corresponding to specific secondary structures were duplicated (For P = 4 the random structure curve was duplicated while for P = 5 the α helical curve and the ß sheet curve were duplicated). As outlined by Perczel *et al.* (1992) these results are indicative of under and overestimation, respectively, of the information content of the data set. Figure 5.6C shows the pure curves generated for P = 3 for native OprB. In this case the curves generated were distinct and corresponded to α helix, ß sheet, and random structure (based on a comparison with spectra from Perczel *et al.* (1992), Park *et al.* (1992) [and references therein]). For heat modified OprB the curves generated for P = 3 were similar. The estimated amounts of these structures in native OprB were 25% α helix, 40% ß sheet, and 34% random structure. For heat modified OprB, random structure increased to 42% at the expense of ß sheet (34%) and α helix (24%).

Figure 5.6. Circular dichroism analyses of *P. aeruginosa* OprB. Circular dichroism spectra of native unheated OprB and heat modified OprB are shown in (A) and (B), respectively: □, experimentally measured; Δ , calculated from the weighted pure component curves produced by convex constraint algorithm (CCA) analysis (Perczel *et al.*, 1992). (C) Pure component curves generated by CCA analysis of native unheated OprB: ○ , α helix; Δ , antiparallel ß sheet; □ , random.


A
B
C
Molar Ellipticity (x10^{-3})
Wavelength (nm)
10
5
0
-5
-10
-15
-20
-30
40
30
20
190
200
210
220
230
240
250

5.3. Discussion

This chapter characterizes the OprB protein of *P. aeruginosa* and describes to a greater extent the functional and structural homology between *P. aeruginosa* and *P. putida* OprB suggested by Saravolac *et al.* (1991). Previously, the OprB proteins from both species of *Pseudomonas* were proposed to form glucose-selective pores (Trias *et al.*, 1988; Saravolac *et al.*, 1991). The methodology used to reach this conclusion for the two porins, however, differed greatly making direct comparisons of functional characteristics difficult. *P. aeruginosa* OprB was examined using the liposome swelling assay (Trias *et al.*, 1988) which is performed at very high substrate concentrations. Results of this analysis, and a similar one by Hancock and Carey (1980), were consistent with *P. aeruginosa* OprB forming pores of quite large diameter. In contrast, *P. putida* OprB was analyzed by black lipid bilayer analysis which indicated formation of very small constricted channels (Saravolac *et al.*, 1991). Given the possible difference between the two OprB pores in what is their basic functional property, this study was undertaken to more fully determine the extent of homology between these porins.

Results presented in this chapter indicated an extensive degree of homology in the primary structure and channel properties of the *P. aeruginosa* and *P. putida* OprB proteins. Evidence for primary structure homology is based on the N terminal sequences and the recognition of heat modified *P.*

putida OprB by antiserum specific for heat modified *P. aeruginosa* OprB. The N terminal sequence of *P. aeruginosa* OprB showed no obvious relationship to other *P. aeruginosa* porins or to other known carbohydrate specific porins. In addition, both OprB channels also possess a glucose binding site with a similar K_s. The single channel conductance measurements indicated that *P. aeruginosa* OprB formed a channel with a constriction very similar to that found in *P. putida* OprB and thus does not show the characteristics of general diffusion pores demonstrated in ScrY. Earlier evidence suggesting that the pore of *P. aeruginosa* OprB was quite large can be related to the requirements of the liposome assays used in previous studies. These analyses require either high substrate concentrations or lengthy incubation times which permits considerable diffusion of all solutes, including those with only a very low rate of permeation through a given pore.

Although many similarities exist between *P. aeruginosa* and *P. putida* OprB, a marked difference was noted in the ion selectivity, as determined by black lipid bilayer experiments. *P. aeruginosa* OprB was found to be anion selective, rather than cation selective as determined for *P. putida* OprB and other carbohydrate-selective porins. Variability in ion selectivity between related porins has previously been demonstrated for some non-specific porins by Benz *et al.* (1985). OmpF and OmpC of *E. coli*, although closely related to

E. coli PhoE, show cation selectivity as opposed to the anion selectivity of PhoE. Similarly, OmpD of *Salmonella typhimurium* is cation selective, whereas the related porin of *E. coli*, NmpC, is anion selective. However, in the case of OprB, this is the first reported instance of closely related substrate selective porins showing opposite ion selectivity. As such, future comparative studies may shed light on the mechanism of ion selectivity and substrate binding and if any relationship exists between the two.

Previously, the high cation selectivity of LamB was proposed to be linked to the presence of a carbohydrate binding site (Benz *et al.*, 1987) and was assumed to be due to carbonyl groups inside the channel which could form hydrogen bonds to the hydroxyl protons of sugars. The formation of cation-selective channels in ScrY and *P. putida* OprB (Săravolac *et al.*, 1991; Schülein *et al.*, 1991) reinforced the correlation between the presence of a carbohydrate binding site and cation selectivity. However, assuming a similar binding site is found in the two OprB proteins, the data presented here indicates that residues determining ion selectivity may be distinct from those forming the binding site of OprB. This may also be true of other carbohydrate-selective porins.

Chemical modification experiments have indicated that ion selectivity is determined by the number of positive vs. negative amino acid residues and their relative positions in

the channel (Benz *et al.*, 1984; Darveau *et al.*, 1984). In OprB most of the residues determining selectivity are presumably located at or near the exterior surface of the pore, away from the binding site. This location would be consistent with the difference in ion selectivity between the OprB proteins despite their structural and functional homology. Homologous porins are known to show variability in the residues exposed at the exterior of the outer membrane (Tommassen, 1988; Werts *et al.*, 1992). Relatively minor changes in the ratio of positive to negative residues at the mouth of the pore of the two OprB porins could be sufficient to alter the selectivity of the pore while not affecting the single channel conductances or binding sites. Future site-directed mutagenesis experiments on OprB will determine whether the residues determining ion selectivity are distinct from those involved in glucose binding.

The structure of *P. aeruginosa* OprB was analyzed by circular dichroism spectroscopy and the CCA algorithm of Perczel *et al.* (1992). To date this is the most appropriate algorithm developed for analysis of the CD spectra of membrane proteins. Other methods require a large data set consisting of the CD spectra of proteins and their known secondary structures, usually deduced from X-ray diffraction analysis. In comparison to soluble proteins, information of this kind is lacking for membrane proteins (Park *et al.* 1992). Additionally, errors in interpretation of the X-ray data

compound the errors associated with CD analysis. In contrast, the CCA algorithm extracts the common spectral components from a set of data without relying on previously determined secondary structures of the proteins comprising the data set.

Analysis of native OprB yielded a secondary structural content of 25% α helix, 40% ß sheet, and 34% random structure. These values fall within the range of those determined for other porins analyzed with the CCA algorithm. Park *et al.* (1992) examined *E. coli* OmpF, LamB, and PhoE porins and *Rhodobacter capsulatus* porin and found values ranging from 15-39% α helix (sum of transmembrane and peripheral α helical components [see Park *et al.* (1992) for details regarding these two types of α helical components], 30-54% ß sheet, and 1-35% random structure. The deduced ß sheet content seems lower than expected in comparison to the X-ray data of *R. capsulatus* (Weiss and Schulz 1992). These lower than expected values may be due to the inclusion of only a limited number of proteins with a high ß sheet content in the data set. As additional CD spectra of porins becomes available prediction of ß sheet content by CCA should improve. An underestimation of ß sheet would probably be reflected in an overestimation of random structure rather than an effect on α helical content. Hennessey and Johnson (1981) note that α helical content is accurately predicted using only the wavelengths above 200 nm. For OprB an α helical content of 25% is much higher than the α helical content previously determined for two other *P.*

aeruginosa porins, OprP (3%) and OprF (9%) (Siehnel *et al.* 1990a). Although some error may be inherent in the CD analyses for each of these proteins, a qualitative assessment of the CD spectra of OprB and OprP is consistent with the apparent differences in structural content. The CD spectrum of OprB shows a definite negative peak at 208 nm (Fig. 5.6A), characteristic of α helical structure, which is absent in the CD spectrum of OprP (Worobec *et al.*, 1988). Further analyses of *P. aeruginosa* porins are necessary to determine where these structural differences occur within the proteins and whether they are related to the differing functions of these porins.

The determination of the amino acid sequence of *P. aeruginosa* OprB is described in the following chapter. This information will serve as a basis for further studies centering on the identification of those residues which determine the ion selectivity and single channel conductance properties of OprB and those which form the substrate binding site of this porin.

6. CLONING, EXPRESSION, AND NUCLEOTIDE SEQUENCE OF THE *OPRB* GENE

6.1. Introduction

In comparison to the number of nonspecific porins which have been identified, substrate-selective porins are relatively rare with only 8 types having been identified. Of the substrate-selective porins identified, 3 are carbohydrate-selective, the maltodextrin-selective LamB porin and the sucrose-selective ScrY porin of enteric bacteria, and the OprB porin of *P. aeruginosa* and *P. putida*. The LamB and ScrY porins have been sequenced and found to be partially homologous to each other. Since both porins are found in enteric bacteria a common origin for these two porins is possible. This chapter describes the cloning, expression, and nucleotide sequence of the OprB porin of *P. aeruginosa* and compares the amino acid sequence to the LamB and ScrY amino acid sequences to identify any regions of homology shared between these proteins.

6.2.1. Cloning strategy and expression in *E. coli*

The strains and plasmids used for cloning and sequencing the *oprB* gene are described in Table 6.1. *P. aeruginosa* PAO1 strain H103 was the source of the *oprB* gene. *Escherichia coli* strain LE392 containing the *P. aeruginosa* genomic library within the cosmid vector pLAFR1 was a gift from K. Piers (Dept. of Microbiology, U. of British Columbia, Vancouver,

Table 6.1. Bacterial strains and plasmids used for cloning and sequencing the P. aeruginosa oprB gene

Strain	Characteristics	Source/reference
Pseudomonas aeruginosa		
H103	wild-type PAO1 prototroph reference strain	R.E.W. Hancock/Hancock and Carey, 1979; Holloway *et al.*, 1979
Escherichia coli		
LE392	*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*	Promega
NM522	*supE thi Δ(lac-proAB) hsd5 F'[proAB⁺ lacI^q lacZΔM15]*	UMCC
Plasmids		
pLAFR1	Tc^R; cosmid vector	Friedman *et al.*, (1982)
pTZ19R	Ap^R	Mead *et al.*, (1986)
pE7(9)	pE7(9) to pF7(36) each contain approximately 25 kb of P. aeruginosa genomic DNA cloned in pLAFR1	This study
pD6(10)		This study
pF9(14)		This study
pH6(20)		This study
pE6(30)		This study
pF7(36)		This study
pJBB34	Ap^R; pTZ19R with 3.4 kb *Bgl*II/*Bgl*II fragment from pE7(9)	This study
pJES34	Ap^R; pTZ19R with 3.4 kb *Eco*RI/*Sph*I fragment from pJBB34	This study
pJHS6	Ap^R; pTZ19R with 0.6 kb *Hinc*II/*Sca*I fragment from pJBB34	This study

Abbreviations: ATCC, American Type Culture Collection, NCTC, National Collection of Type Cultures, UMCC, University of Manitoba Culture Collection.

[1] *opdE* is a putative regulatory gene controlling OprD porin production. H673 does not produce OprD.

[2] PFB311 contains mutations in glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gnd*). Therefore, of the two glucose transport pathways known in *P. aeruginosa*, only the high affinity pathway is functional.

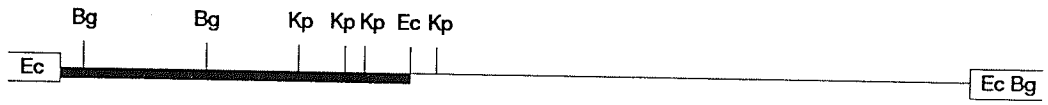
Canada). Subcloning was carried out in *E. coli* NM522 using pTZ19R.

Screening of the *P. aeruginosa* genomic library in *E. coli* LE392 was conducted using oligonucleotides derived from the N terminal sequence of purified OprB. Oligonucleotides were 30 mers consisting of 162 different sequences (GA[AG] GC[CGT] TTC TC[CGT] CC[CGT] AAC TC[CGT] AAG ACC ATG) corresponding to the amino acid sequence EAFSPNSKTM. Colony blots of recombinant *E. coli* LE392 were prepared and approximately 4000 colonies were screened. Six positive colonies were isolated, each of which contained inserts of approximately 25 kb (Fig. 6.1). Of these colonies, 4 showed distinct *Eco*RI restriction enzyme fragments, however, all contained a common 10.2 kb *Eco*RI fragment. Southern blot analysis demonstrated hybridization of this 10.2 kb fragment to the oligonucleotide pool used to screen the *P. aeruginosa* library. One recombinant plasmid, pJE79, was selected for further analysis. Hybridization of the probe was localized to a 3.4 kb *Bgl*II fragment within the 10.2 kb *Eco*RI fragment (Fig. 6.2). This 3.4 kb fragment was subcloned into pTZ19R to create pJBB34.

pJBB34 was large enough to contain the entire *oprB* gene therefore *E. coli* cells containing pJBB34 were examined for expression. Cell lysates were obtained from cells grown in LB media supplemented with 50 μg/ml ampicillin, either in the presence or absence of the *plac* inducer, isopropylthio-

Figure 6.1. Restriction enzyme map of 6 plasmids isolated from recombinant *E. coli* LE392. These plasmids were isolated from colonies which hybridized with an oligonucleotide corresponding to the N terminal sequence of the *oprB* gene. The bold lines correspond to a 10 kb *Eco*RI/*Eco*RI fragment common to all plasmids. Open boxes represent pLAFR1 DNA. Abbreviations: Ec, *Eco*RI; Bg, *Bgl*II; Kp, *Kpn*I.

pE7(9)
pD6(10)

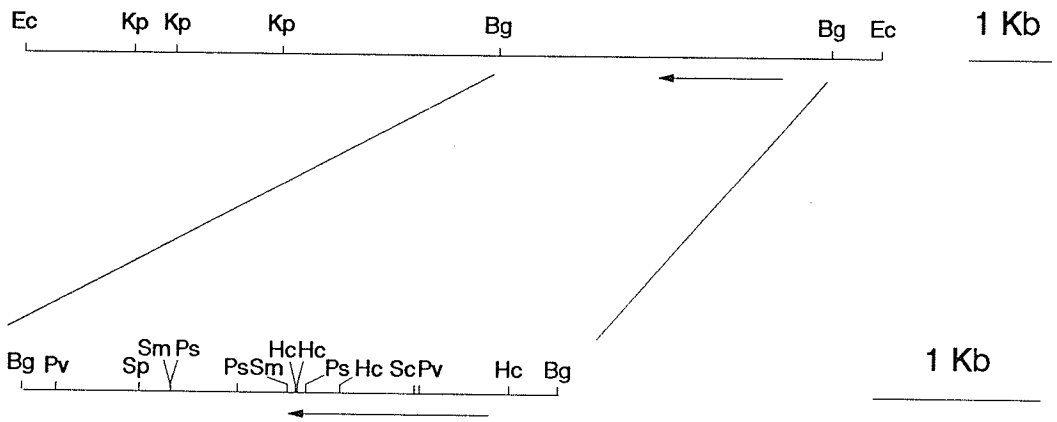


pE6(30)

pF7(36)

pF9(14)
pH6(20) - Not mapped

Figure 6.2. Restriction enzyme map of *P. aeruginosa* genomic DNA containing the *oprB* gene. Arrows indicate the location of the *oprB* gene. Upper map illustrates a 10.2 kb genomic DNA fragment from pJE79. Lower map illustrates the genomic DNA insert of pJBB34, containing a 3.4 kb *Bgl*II fragment from pJE79. Abbreviations: Ec, *Eco*RI; Bg, *Bgl*II; Pv, *Pvu*II; Sp, *Sph*I; Sm, *Sma*I; Ps, *Pst*I; Hc, *Hinc*II; Sc, *Sca*I.


Ec
Kp
Kp
Kp
Bg
Bg
Ec
1 Kb
Bg
Pv
Sp
Sm
Ps
Ps
Sm
Hc
Hc
Ps
Hc
Sc
Pv
Hc
Bg
1 Kb

galactoside (IPTG). Following SDS-PAGE, Western blotted cell lysate samples were probed with OprB specific polyclonal antiserum. OprB was expressed from pJBB34 verifying the identity of the *oprB* gene (Fig. 6.3). pJBB34 contains the *oprB* gene in the same orientation as the ß-galactoside gene of pTZ19R and in close proximity to *plac*, suggesting transcription originated from this promoter. pJES34, containing the *oprB* gene in opposite orientation to *plac*, did not produce OprB following growth in LB (Fig. 6.3) or BM2 supplemented with glucose (not shown). pJES34 contains approximately 1.2 kb upstream of the initiation codon of *oprB*. Since the region upstream of *oprB* is large enough to potentially carry the promoter for *oprB*, the lack of expression from this plasmid suggests the *oprB* promoter is not recognized by *E. coli*.

Having verified the presence of the *oprB* gene, Southern hybridization analysis localized the N terminal portion of the gene to a 0.6 kb *Hinc*II/*Sca*I fragment of pJBB34 (pJHS6). Subcloning and sequencing of pJHS6 identified a nucleotide sequence able to encode the N terminal portion of OprB.

6.2.2. Nucleotide sequence of *oprB*

The sequencing strategy for nucleotide sequence analysis of the *oprB* gene is shown in Figure 6.4 and the nucleotide sequence is illustrated in Figure 6.5. An open reading frame of 1362 bp was identified encoding a protein of 454 amino

Figure. 6.3. Expression of OprB in *E. coli* NM522. Cell lysates were screened with polyclonal antiserum specific for OprB. Media was BM2 supplemented with glucose for *P. aeruginosa*, and LB supplemented with 50 μg/ml ampicillin for *E. coli*. Lanes 1) *P. aeruginosa* H103; 2) *E. coli* NM522 (pJBB34), IPTG not present; 3) *E. coli* NM522 (pJBB34), IPTG added (0.4 mM); 4) E. coli NM522 (pJES34), IPTG not present; 5) E. coli NM522 (pJES34), IPTG added (0.4 mM). The low M_r bands in lanes 2 and 3 suggest that OprB is partially degraded when expressed in *E. coli*.

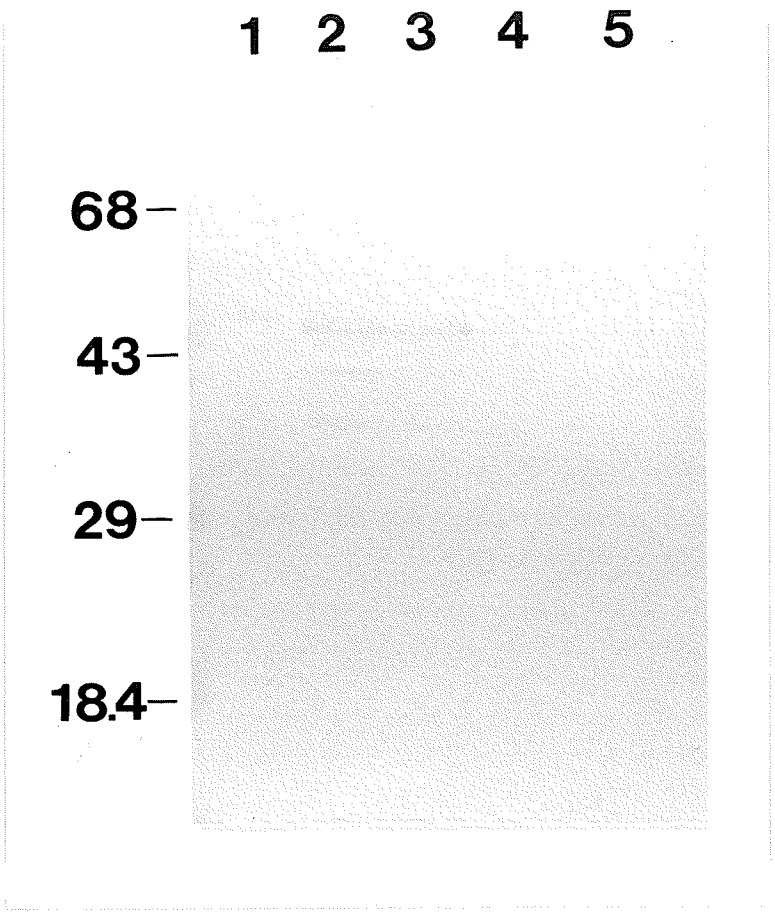

1 2 3 4 5
68
43
29
18.4

Figure 6.4. Sequencing strategy for the *P. aeruginosa oprB* gene.

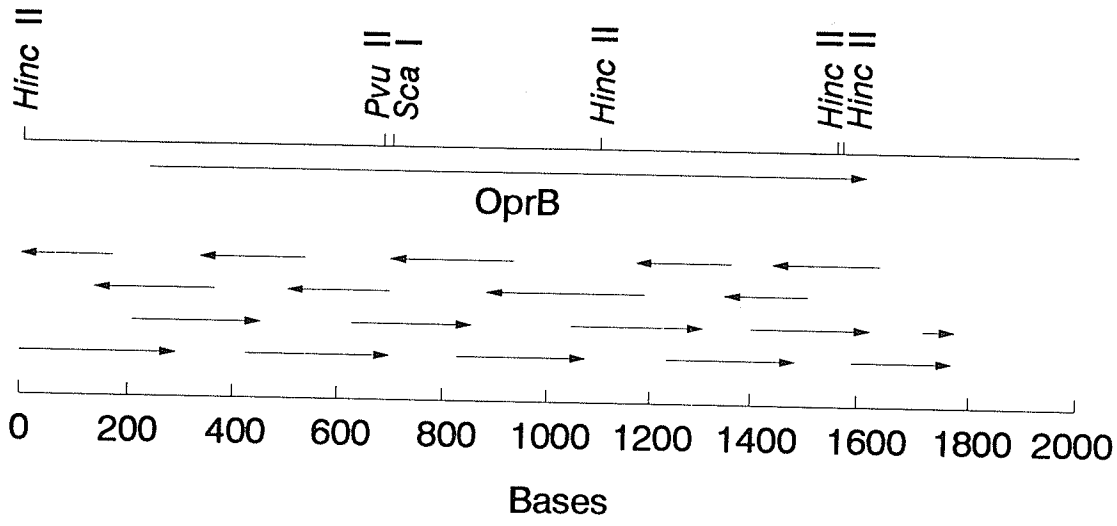

Hinc II
Pvu II
Sca I
Hinc II
Hinc II
Hinc II
OprB
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Bases

Figure 6.5. Nucleotide sequence of the *P. aeruginosa oprB* gene. The amino acid sequence of the signal sequence is indicated in italics. The putative Shine-Dalgarno sequence is bold and underlined. Two palindromes preceding the N terminal portion of *oprB* are bold and double-underlined. The potential rho-independent terminator is bold and dashed-underlined.

```
-237  ACC AGG TCA AGG TCT GTT GCC GCC TGG CGC CGG ACG TCG CGC CGG
-192  CCG CGG GCA GCG GCA TGA GCC TGC GCT TCG ATC CCG CGC GGG TCC
-147  TGC TGT TCG ACG CGC AGA GCG GCG AGC GCG TGG CCG TGC GCA CGG
-102  CGG GCG TGG CGG CGG AGA AGG TCA CCG CCT TGA ACG GCA ATC GCC
 -57  AGA AGA ACT CCT GAA TCC TTT CGT ACT GCC TAG ACC ACA ACC ACG
 -12  AGG ACG CTG GAA ATG TAC AAG AAC AAG AAA ACC AGA CCG GCC GCC
                      M   Y   K   N   K   K   T   R   P   A   A
  34  AGG ACC GTT GGC TGC CTG TTT GCC CTT GGA GCG CTG GGC TTG GGG
       R   T   V   G   C   L   F   A   L   G   A   L   G   L   G
  79  AGC GCG GCG CAC GCC GCC GAG GCG TTC TCC CCG AAC TCG AAA TGG
       S   A   A   H   A   A   E   A   F   S   P   N   S   K   W
 124  ATG CTC GGC GAC TGG GGC GGC AAG CGC ACC GAG CTG CTG GAG AAG
       M   L   G   D   W   G   G   K   R   T   E   L   L   E   K
 169  GGC TAC GAC TTC AAG CTG GAG TAC GTC GGC GAG GCG GCG GCC AAC
       G   Y   D   F   K   L   E   Y   V   G   E   A   A   A   N
 214  CTC GAC GGC GGC TAT GAC GAC GAC AAG ACA GGA CGC TAC ACC GAC
       L   D   G   G   Y   D   D   D   K   T   G   R   Y   T   D
 259  CAG TTC GCC CTG GGC GTG CAC ATG GAC CTG GAG AAG ATC CTC GGC
       Q   F   A   L   G   V   H   M   D   L   E   K   I   L   G
 304  TGG AAG GCT ACC GAG TTC CAG TTC ACC GTC ACC GAG CGC AAC GGC
       W   K   A   T   E   F   Q   F   T   V   T   E   R   N   G
 349  AAG AAC CTT TCC AAC GAC CGC ATC GGC GAC CCG CGT GCC GGG CAC
       K   N   L   S   N   D   R   I   G   D   P   R   A   G   H
 394  ATC AGC TCG GTG CAG GAG GTC TGG GGC CGC GGG CAG ACC TGG CGG
       I   S   S   V   Q   E   V   W   G   R   G   Q   T   W   R
 439  CTG ACC CAG CTG TGG CTC AAG CAG CAG TAC TTC GAC GGC GCG CTG
       L   T   Q   L   W   L   K   Q   Q   Y   F   D   G   A   L
 484  GAC GTG AAA TTC GGC CGT TTC GGC GAG GGC GAG GAC TTC AAC AGC
       D   V   K   F   G   R   F   G   E   G   E   D   F   N   S
 529  TTC CCC TGC GAT TTC CAG AAC CTG GCC TTC TGC GGC TCG CAG GTG
       F   P   C   D   F   Q   N   L   A   F   C   G   S   Q   V
 574  GGC AAC TGG GCG GGG AGC ATC TGG TAC AAC TGG CCG GTC AGC CAG
       G   N   W   A   G   S   I   W   Y   N   W   P   V   S   Q
 619  TGG GCG TTG CGG GTG AAG TAC AAC TTC GCG CCG GAC TGG TAC GTG
       W   A   L   R   V   K   Y   N   F   A   P   D   W   Y   V
 664  CAG GTC GGC GCC TAC GAG CAG AAC CCG TCG AAC CTG GAG ACC GGC
       Q   V   G   A   Y   E   Q   N   P   S   N   L   E   T   G
 709  AAC GGC TTC AAG ATG AGC GGC AGC GGG ACC AAG GGC GCG CTG CTG
       N   G   F   K   M   S   G   S   G   T   K   G   A   L   L
 754  CCG GTG GAG CTG ATC TGG CAG CCG AAG GTC GGC GCC GAG CAA CTG
       P   V   E   L   I   W   Q   P   K   V   G   A   E   Q   L
 799  CCG GGC GAG TAC CGG CTG GGC TAC TAC TAC AGC ACG GCG AAG GCC
       P   G   E   Y   R   L   G   Y   Y   Y   S   T   A   K   A
 844  GAT GAC GTC TAC GAC GAC GTC GAC GGC CAG CCG CAG GGG CTG ACC
       D   D   V   Y   D   D   V   D   G   Q   P   Q   G   L   T
 889  GGC AAC GAC TTC AAG TCG CGC GGC AGC AAG CAT GGC TGG TGG GTG
       G   N   D   F   K   S   R   G   S   K   H   G   W   W   V
 934  GTG GCG CAG CAG CAG GTC ACT TCG CAC AAC GGC GAT GCC TCG CGC
       V   A   Q   Q   Q   V   T   S   H   N   G   D   A   S   R
 979  GGG TTG AGC CTG TTC GCC AAC CTG ACG GTC CAC GAC AAG GCG ACC
       G   L   S   L   F   A   N   L   T   V   H   D   K   A   T
1024  AAC GTG GTG GAC AAC TAC CAG CAG CTC GGG GTG GTC TAC AAG GGG
       N   V   V   D   N   Y   Q   Q   L   G   V   V   Y   K   G
1069  CCG TTC GAC GCG CGG CCG AAG GAC GAC ATC GGC CTG GGC ATC GCG
```

```
      P   F   D   A   R   P   K   D   D   I   G   L   G   I   A
1114  CGC ATC CAT GTC AAC GAC GAT GTG AAG AAG CGC CAG CGC CTG GTG
      R   I   H   V   N   D   D   V   K   K   R   Q   R   L   V
1159  AAC CAG GTG AAC GGC ATC GAC GAC TAC GAC AAC CCG CTG TAC CAG
      N   Q   V   N   G   I   D   D   Y   D   N   P   L   Y   Q
1204  CCG CTG CAG GAC ACC GAG TAC AAC GCC GAG CTG TAC TAC GGG GTG
      P   L   Q   D   T   E   Y   N   A   E   L   Y   Y   G   V
1249  CAT GTG ACC GAC TGG CTG ACG GTG CGG CCG AAC CTG CAA TAC ATC
      H   V   T   D   W   L   T   V   R   P   N   L   Q   Y   I
1294  AAG CAG CCG GGC GGG GTC GAC GAG GTC GAC AAC GCG CTG GTG GCG
      K   Q   P   G   G   V   D   E   V   D   N   A   L   V   A
1339  GGG ATC AAG ATC CAG ACG GTG TTC TGA TCG TCG CGT TGC CTG CTC
      G   I   K   I   Q   T   V   F   -
1384  CGG TGT TGT GCC TGG GCC CGC CCG AGC GTT TCG GGC GGG CCG TTT
                      ---------------     --------------
1429  TTT TCC GGC GAC CGC TCT TCC CGG CTG CCT GTT ACA AAC CGA CTA
1474  CGC TTT TCC TGT GCC GAT CAT TTG GGG CTT GCG GCG AAA TGT AGT
1519  AAA ACT ACA CGG AT
```

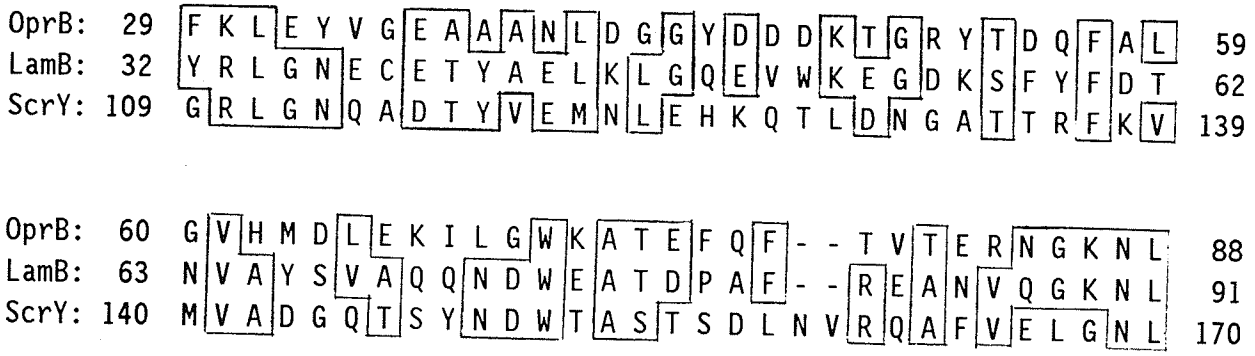
acids. The mature N terminus of the protein could be encoded by residues beginning at nucleotide 94. Codon usage and G/C composition (65.1%) was similar to that determined for other *P. aeruginosa* genes (West and Iglewski, 1988). Preceding the N terminus was a predicted 31 amino acid segment with features typical of procaryotic signal sequences. A potential Shine-Dalgarno sequence was found 10 nucleotides upstream of the initiation codon. A strong potential rho-independent transcription terminator site (ΔG° = -30.9 kcal/mol) was found 33 bp after the termination codon. Two palindromes of 10 and 14 bp were found 151 and 101 bp, respectively, in front of the initiation codon. Schmid *et al.* (1991) demonstrated that two palindromes preceding the sucrose porin *scrY* gene act as operator binding sites for control of induction of ScrY. The palindromes in *P. aeruginosa* may also act as operator binding sites for controlling carbohydrate induction of OprB.

The predicted molecular weight of the mature protein is 47,597, in close agreement with that predicted by SDS-PAGE. The amino acid composition derived from the nucleotide sequence is almost identical to that determined by Saravolac *et al.* (1991) for the purified protein. The isoelectric point of mature OprB is 4.73. Amino acid cluster analysis using PC/Gene revealed 4 significant clusters of specific amino acid residues: the two cysteine residues occur 7 residues apart (C148 and C156), 4 of 12 isoleucine residues occur over a 27 residue span (I335, I339, I342, and I361), 5 of 15 tryptophan

residues occur over 26 residues (W163, W168, W171, W176, W188), and 7 of 18 phenylalanine residues occur over 30 residues (F126, F134, F137, F143, F146, F150, F155). A phenylalanine residue also forms the C terminal residue of OprB which is highly conserved in porins and outer membrane proteins in general (Struyvé *et al.*, 1991).

Using the PCOMPARE and PALIGN programs of PC/Gene, I compared the predicted amino acid sequence of OprB to several porins of *E. coli* (OmpC, OmpF, LamB, ScrY, and Tsx) and *P. aeruginosa* (OprF, OprD, and OprP) to search for any potential homologies. The alignment scores indicated OprB was not strongly related to any of the porins tested. However, I did note, as shown in Figure 6.6, that for OprB and LamB many of the aligned residues identified by the PALIGN program were clustered over a 60 residue span in the N terminal portion of the proteins (OprB, F29 to L88; LamB Y32 to L91). For all other porins in the analysis aligned residues were randomly scattered throughout the sequence (not shown). Hardesty *et al.* (1991) has previously aligned the LamB and ScrY proteins and demonstrated some homology between these proteins. Using their alignment results, I visually aligned the region of ScrY corresponding to LamB Y32 to L91 and found several of the conserved residues in OprB and LamB were also present in ScrY, although to a lesser extent (Fig. 6.6). Alignment of the LamB and ScrY proteins by Hardesty *et al.* (1991) required introduction of a large N terminal gap in LamB to accommodate

Figure 6.6. Amino acid sequence comparison of an N terminal portion of OprB, LamB and ScrY. Identical or conserved amino acid residues identified by the PALIGN program of PC/GENE are boxed. Sequence information for LamB and ScrY from Clément and Hofnung (1981) and Hardesty *et al.* (1991), respectively.


OprB: 29 F K L E Y V G E A A A N L D G G Y D D D K T G R Y T D Q F A L 59
LamB: 32 Y R L G N E C E T Y A E L K L G Q E V W K E G D K S F Y F D T 62
ScrY: 109 G R L G N Q A D T Y V E M N L E H K Q T L D N G A T T R F K V 139
OprB: 60 G V H M D L E K I L G W K A T E F Q F - - T V T E R N G K N L 88
LamB: 63 N V A Y S V A Q Q N D W E A T D P A F - - R E A N V Q G K N L 91
ScrY: 140 M V A D G Q T S Y N D W T A S T S D L N V R Q A F V E L G N L 170

the presence of an additional 70 N terminal amino acid residues in ScrY. In my analysis, the PALIGN program did not introduce this gap in OprB to accommodate the extra residues in ScrY, so I initially did not detect any regional homology between OprB and ScrY.

6.3. Discussion

The glucose-inducible OprB porin of *P. aeruginosa* is one of a small group of porins known to contain a substrate specific binding site. Knowledge of its structure and function will provide information on the formation of the binding site of substrate-selective porins in comparison to the non-selective channels formed by most porins. Additionally, comparisons between OprB and the carbohydrate-selective porins of enteric bacteria (LamB and ScrY) will be of interest in determining any similarities or differences in the formation of the binding sites of these porins. This chapter describes the cloning and expression of the gene for *P. aeruginosa* OprB and the determination of its nucleotide sequence.

Amino acid analysis of OprB revealed several unusual features. OprB contains two cysteine residues (C148 and C156), unlike most porins which characteristically do not contain cysteine residues (Gerbl-Reiger *et al.*, 1991). Cysteine residues are known to occur in two other porins: the major nonspecific OprF porin of *P. aeruginosa* (Duchêne *et al.*,

1988), and the maltodextrin-selective LamB porin of *E. coli* (Clément and Hofnung, 1981). In OprF, 4 closely spaced cysteine residues (C176, C185, C191, C205) are predicted to form part of an external loop between two ß strands (Siehnel *et al.*, 1990a) and appear to play a role in OprF function. Hancock and Carey (1979) identified cystine disulfide bonds in OprF by demonstrating changes in mobility of OprF on SDS-PAGE gels as a result of modification by 2-mercaptoethanol. Hancock (1987) proposed that alternative cystine bridges may alter OprF pore size and structure. In contrast, the two cysteine residues of LamB, C22 and C38, apparently have no functional role. Ferenci and Stretton (1989) found that changing the cysteine residues to serine had no effect on maltodextrin binding although a slight decrease in trimer stability was noted. The role, if any, of the cysteine residues in OprB is unknown at this time. They are close enough to potentially interact, however, unlike OprF, 2-mercaptoethanol has no effect on OprB mobility on SDS-PAGE gels (Chapter 3). Therefore, it is unlikely that they form a cystine bond in native OprB.

Several other clusters of amino acid residues were found in OprB. A group of phenylalanine and tryptophan residues occur in the N terminal portion of OprB. Several investigations have consistently linked residues in the N terminal third of porins to channel size and ion selectivity (Benson *et al.*, 1988; Misra and Benson, 1988; Tommassen *et*

al., 1985) and formation of a binding site in substrate-selective porins (Werts *et al.*, 1992). In *R. capsulatus* porin, and OmpF and PhoE of *E. coli*, crystal structure analysis has demonstrated that the third external loop in the N terminal portion of these proteins folds into the ß barrel (Cowan *et al.*, 1992; Weiss and Schulz, 1992). Epitope insertion and deletion mutants demonstrate that this loop affects channel size in PhoE, OmpF, and OmpC and ion selectivity of PhoE (Struyvé *et al.* 1993, Benson *et al.*, 1988; Misra and Benson, 1988). Given the location of the phenylalanine and tryptophan clusters in OprB some of these residues could potentially be involved in formation of the constriction in the OprB channel and/or formation of the carbohydrate binding site. The aromatic ring of phenylalanine and tryptophan residues contributes to protein-sugar interactions in carbohydrate binding proteins (Quiocho, 1989). These aromatic residues stack on one or both sides of the pyranoside rings of sugars, contributing to Van der Waals contacts and conferring some specificity to the binding site. The concentration of phenylalanine and tryptophan residues in this particular region of OprB is suggestive of a potential role in formation of the binding site of this protein.

The predicted amino acid sequence of OprB from residue F29 to L88 shows several conserved and identical residues in common with LamB residues Y32 to L91 and ScrY G109 to L170. Residues 40 to 60 of LamB have previously been implicated in

LamB function and structure (refs. in Chan and Ferenci, 1993) and several of the residues within this region are conserved in LamB, OmpA and OmpF suggesting a general role in porin structure or function (Nikaido and Wu, 1984). Chan and Ferenci (1993) have performed a detailed mutagenic analysis of all of the residues within this region of LamB and identified those residues important in the structure and function of this protein. Of particular interest are residues 44 to 54. Seven of the 11 residues within this region accept a range of mutations indicating a noncritical role in LamB function. Exceptions were L44, E49, K52, and G54. Mutagenesis of these residues affected maltoporin structure and function. Surprisingly, each of these residues is conserved in the corresponding region of OprB. Three residues are identical (L41, K49, and G51) and one conservative substitution maintains the negative charge of LamB E49 (OprB D46). Conservation of these residues within OprB could be indicative of a specific role in carbohydrate binding or a general role in porin stability and structure. Neither the sucrose porin ScrY nor the nonspecific *E. coli* porins conserve these residues to the extent found in OprB suggesting a possible role in binding maltodextrins; a characteristic common to both OprB and LamB. Residue W74 in LamB and conserved in OprB and ScrY as W71 and W151, respectively, has also been specifically linked to maltodextrin binding (Charbit *et al.*, 1988; Francis *et al.*, 1991) suggesting some of the conserved

residues within this region may also be involved in the formation of a binding site.

In conclusion, nucleotide sequence analysis of *P. aeruginosa* OprB revealed several clusters of amino acid residues potentially involved in the structure and function of this protein. One of these regions showed a surprising degree of homology to *E. coli* LamB suggesting either a general role of these residues in porin structure or a specific role in maltodextrin binding. Site-directed mutagenesis of these residues in LamB and OprB may prove valuable for identifying the binding site of these porins.

7. TOPOLOGICAL MODELLING OF OPRB

7.1. Introduction

Topological models of proteins provide a framework for the identification and localization of residues potentially involved in protein function. Analysis of the primary structure of integral cytoplasmic membrane proteins often provides useful topological information, as these proteins are frequently characterized by the presence of long stretches of hydrophobic amino acids capable of forming membrane spanning α helices. An algorithm, developed by Kyte and Doolittle (1982), readily identifies these hydrophobic residues and is routinely applied to membrane proteins to identify potential membrane spanning domains. Unfortunately, the lack of extensive stretches of hydrophobic amino acid residues in porins prevents the application of this approach to these proteins. The use of other commonly applied secondary structure prediction techniques, such as that developed by Chou and Fasman (1978), have also been questioned since they are based on data derived from soluble globular proteins (Paul and Rosenbusch, 1985).

Given the inadequacy of these techniques for modelling porin structure, several groups have recently developed secondary structure prediction techniques designed specifically to take into account the unique folding patterns of porins. Paul and Rosenbusch (1985) developed an indirect approach, based on the identification of ß turns and external

loops, rather than the ß strands themselves. Vogel and Jähnig (1986), based their technique on the prevalence of amphipathic ß strands within porin structure, and attempted to identify patterns of alternating hydrophobic and hydrophilic residues. Schirmer and Cowan (1993) identified ß strands with an approach similar to that of Vogel and Jähnig, however, they concentrated on the identification of only the hydrophobic residues present in a ß strand. They also took into account the prevalence of aromatic residues at the membrane interface (Cowan *et al.*, 1992; Weiss and Schulz, 1992). Finally, Gromiha and Ponnuswamy (1993) also identified ß strands in porin by analyzing patterns of hydrophobicity, however, they used a hydrophobicity scale which incorporated the influence of the surrounding environment (i.e. neighbouring amino acids or membrane lipids) on the hydrophobic activity of a given residue.

This chapter describes the application of these techniques to OprB. By comparing the models produced using the individual methods, I was able to produce a final consensus model which potentially identifies 13 of the 16 ß strands assumed to occur in OprB.

7.2. Results and Discussion

7.2.1. Paul and Rosenbusch ß turn prediction

Paul and Rosenbusch (1985) developed an indirect approach to the prediction of porin structure based on ß turn

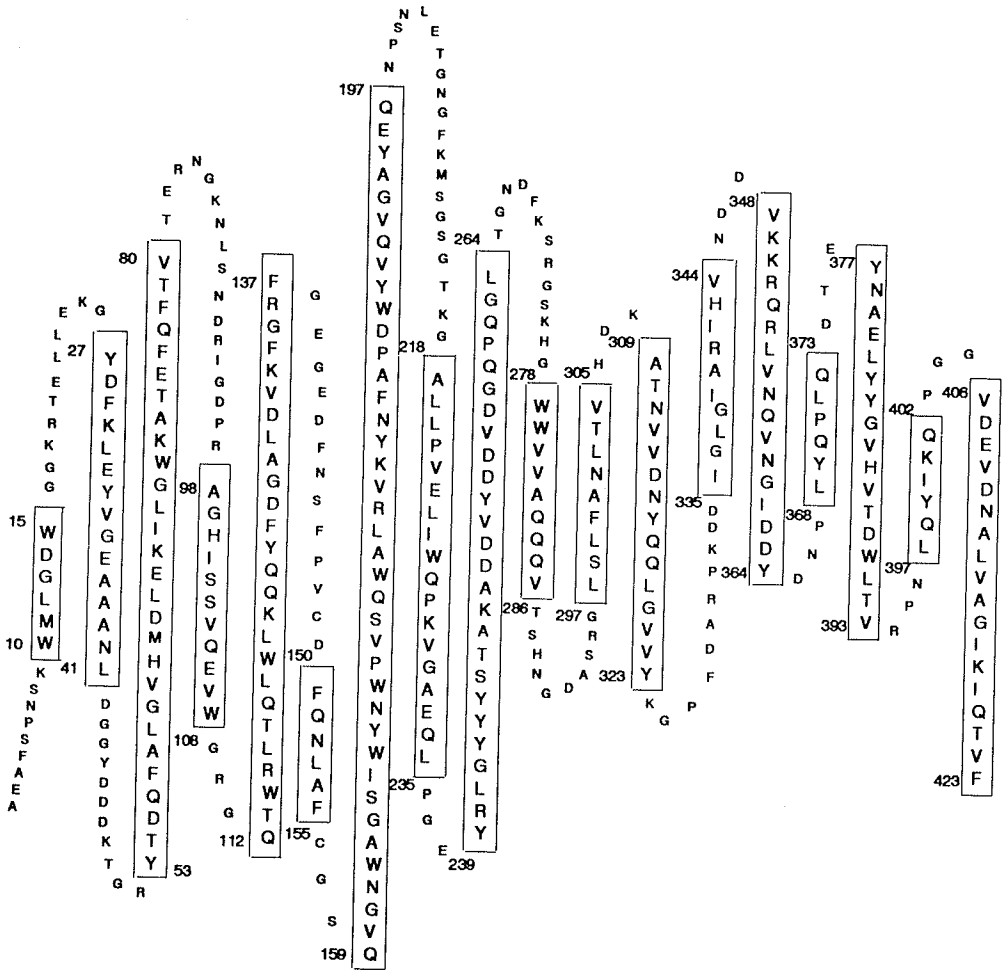
prediction. They argued that the large number of polar residues in porin, coupled with the lack of obvious hydrophobic domains, suggested that many of the polar residues must be buried in the hydrophobic environment of the membrane. Therefore, scanning porin primary sequences for hydrophobic stretches or applying prediction techniques based on data derived from soluble proteins would not be appropriate for predicting the folding patterns of porins. However, they felt that some of the data available from the analysis of soluble proteins may be applicable to porins. Since the ß turns of porins are exposed to an aqueous environment, the soluble protein data could be applied specifically to the prediction of these secondary structure components. Paul and Rosenbusch modified their approach slightly from that used for soluble proteins, since they felt that a tripeptide was sufficient for chain reversal in membrane proteins, as opposed to the tetrapeptide commonly observed in soluble proteins.

In the Paul and Rosenbusch (1985) approach, stretches of 3 or more amino acid residues containing at least one turn promoting residue (Asn, Asp, Gly, Glu, Pro, Ser) and no turn blocking residues (Ala, Gln, Ile, Leu, Met, Phe, Trp, Tyr, Val) are predicted to reverse the direction of the polypeptide. Segments of greater than 5 residues containing turn blocking residues are considered as potential membrane spanning segments. Turn clusters are classified as groups of turns linked by amino acid segments containing less than 6

residues. In applying this technique, and the other techniques described below, I placed the N terminus of OprB in the periplasm based on the crystal structures (Cowan *et al.*, 1992; Weiss and Schulz, 1992) and topological models of other porins (Charbit *et al.*, 1988; Tommassen, 1988; Siehnel *et al.*, 1990a; van der Ley *et al.*, 1991; Srikumar *et al.*, 1992).

Following these guidelines, 18 ß turns or turn clusters were predicted, resulting in the identification of 18 potential ß strands. The large number of ß strands predicted for OprB is similar to other porin models developed using Paul and Rosenbusch methodology (20 for S. typhimurium LamB [Francoz *et al.*, 1990], and 19 for *E. coli* LamB [Charbit *et al.*, 1988]). This is likely an overprediction since current evidence suggests porins consist of 16 stranded ß barrels (Jeanteur *et al.*, 1991; Cowan *et al.*, 1992; Weiss and Schulz, 1992). The large number of predicted ß strands for OprB and the LamB porins may be due to Paul and Rosenbusch reducing the minimum size of a ß turn from a tetrapeptide to a tripeptide. For the *R. capsulatus* porin some amino acid residues known to form part of a ß strand are predicted as ß turns following Paul and Rosenbusch methodology (Srikumar *et al.*, 1992). However, increasing the size of a ß turn to a tetrapeptide would have identified only 9 ß turns in OprB and made the model largely unworkable. The Paul and Rosenbusch model of OprB is illustrated in Figure 7.1.

Figure 7.1. Topological model of mature OprB based on Paul and Rosenbusch (1985) folding rules. ß strands are indicated by rectangles.


WDGLMW
YDFKLEYVGEAANL
VTFQFETAKWGLIKELDMHVGLAFQDTY
AGHISSVQEVW
FRGFKVDLAGDFYQQKLWLQTLRWTQ
FQNLAF
QEYAGVQVYWDPAFNYKVRLAWQSVPWNYWISGAWNGVQ
ALLPVELIWQPKVGAEQL
LGQPQGDVDDYVDDAKATSYYYGLRY
WWVVAQQQV
VTLNAFLSL
ATNVVDNYQQLGVVYY
VHIRAIGLGI
VKKRQRLVNQVNGIDDY
QLPQYL
YNAELYYGVHVTDWLTV
QKIYQL
VDEVDNALVAGIKIQTVF

7.2.2. Schirmer and Cowan ß strand prediction

Data derived from the crystal structures of the *E. coli* and *R. capsulatus* porins provided the foundation for the ß strand prediction technique developed by Schirmer and Cowan (1993). Specifically, they focused on 3 characteristic features of these porins. First, each of the porins folded into a 16 stranded antiparallel ß barrel. Second the ß strands were 7-9 residues long and the external facing residues were hydrophobic. Third, aromatic residues were prevalent in the flanking positions of the ß strands. Schirmer and Cowan placed no predictive value on the interior facing residues of a ß strand, since the crystal structures of the *E. coli* and *R. capsulatus* porins indicated that these residues could be either hydrophilic or hydrophobic depending on whether they faced the channel interior or were buried by internal loops.

The mean hydrophobicity of one side of a putative ß strand is determined by averaging the hydropathy values of every second residue within a sliding window of 4. The hydropathy value of an aromatic residue is increased to an arbitrarily chosen value of 1.6 if it occurs in the first or last position of a ß strand. The average hydropathy values for the odd and even numbered residues are plotted against residue number and high peaks (i.e. high hydrophobicity) represent potential ß strands. The ß strands are extended at both ends as long as the characteristic pattern of alternating

hydrophobic residues continues.

The hydrophobicity plot of OprB, calculated in this manner, is shown in Figure 7.2. Sixteen of the highest peaks were chosen as potential transmembrane ß strands. Many of the peaks appeared to be clustered in pairs (2 and 3, 6 and 7, 8 and 9, 12 and 13, 14 and 15). This pattern is found in other porins (Tommassen, 1988; Jeanteur *et al.*, 1991; Srikumar *et al.*, 1992) and results from the ß strands of a porin being connected by short ß turns on the periplasmic side of the protein and long extracellular loops on the extracellular side. Peaks corresponding to ß strands 4 and 10 were ambiguous, and could also have originated at residues 74 and 257, respectively. The respective locations used in the model were chosen to maintain the pairwise association of ß strands. The model of OprB, based on Schirmer and Cowan methodology, is shown in Figure 7.3.

7.2.3. Gromiha and Ponnuswamy ß strand prediction

The primary difference in the protein folding technique developed by Gromiha and Ponnuswamy (1993) is the development of a "surrounding hydrophobicity" scale for assessing the hydrophobicity and hydrophilicity of residues. They argue that the preference for a nonpolar environment by the 20 residues in a folded protein is not adequately reflected by indices derived from the behaviour of free amino acids in organic solvents. Additionally, since many of the hydrophobic

Figure 7.2. Hydropathy plot of OprB, calculated as outlined by Schirmer and Cowan (1993). The numbers indicate the predicted location of ß strands. Solid and dotted lines correspond to average hydropathy values of odd and even numbered residues, respectively.

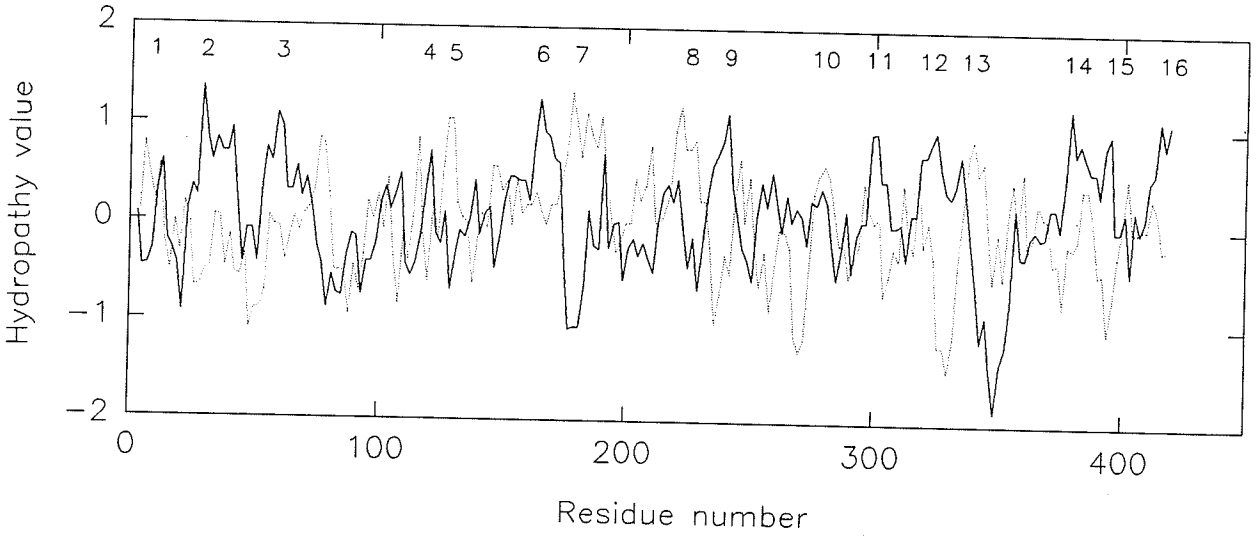
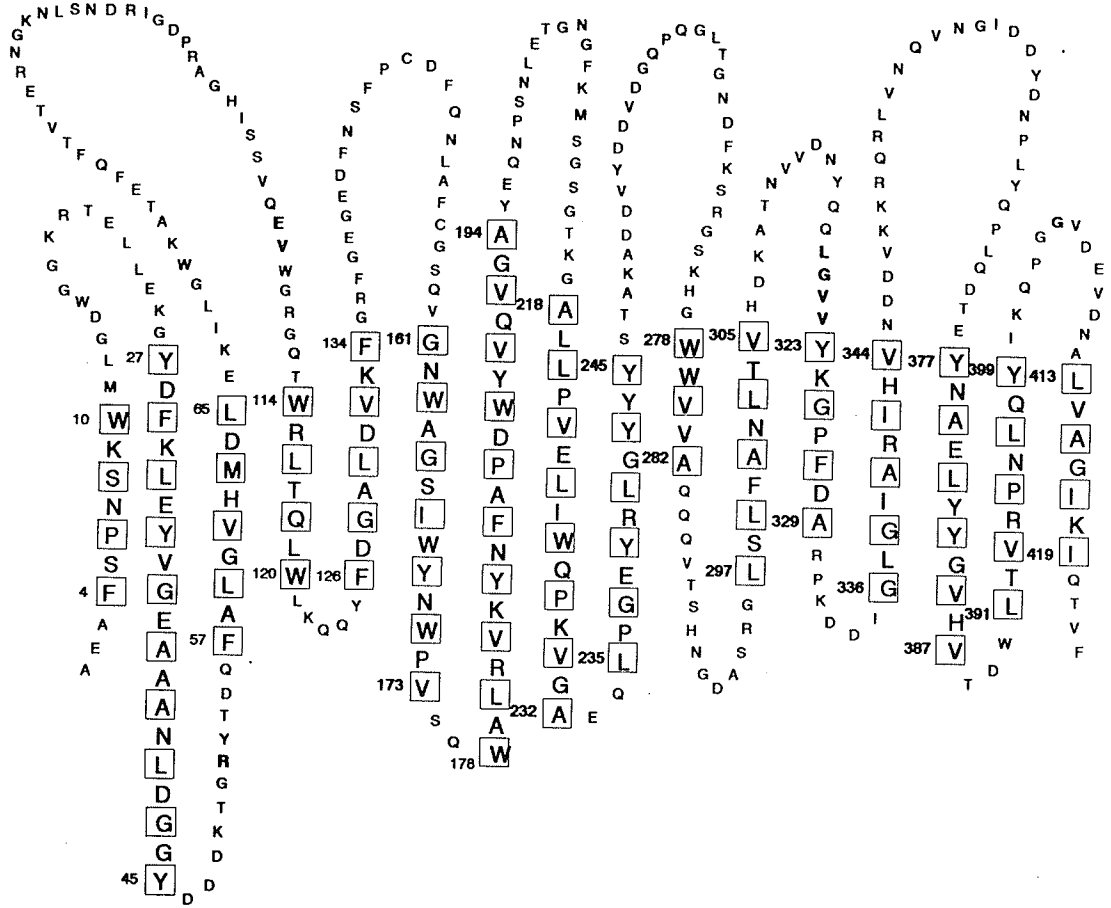

2
1
0
−1
−2
Hydropathy value
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
0
100
200
300
400
Residue number

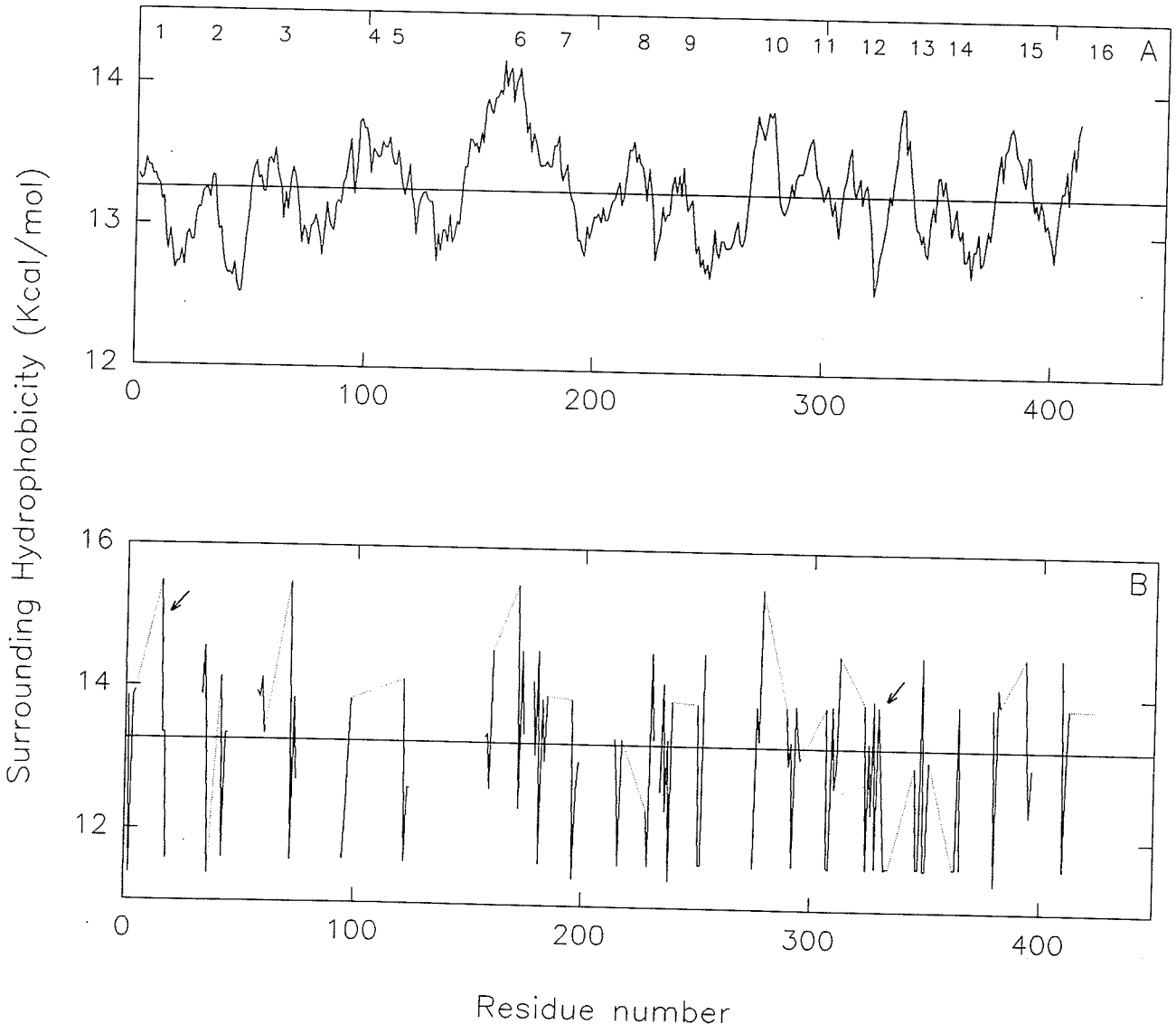
Figure 7.3. Topological model of mature OprB based on the prediction technique of Schirmer and Cowan (1993). Boxed residues indicate the residues forming the hydrophobic side of a putative ß strand.



residues of a membrane protein interact with the surrounding lipid environment, hydrophobicity scales developed from the examination of soluble proteins are inappropriate for examining the folding properties of membrane proteins. Gromiha and Ponnuswamy have combined information from the crystal structures of 64 soluble globular proteins and two membrane proteins, the photosynthetic reaction centre from *R. viridis* and the porin from *R. capsulatus*, to produce a common hydrophobicity scale (Column H_{gm}, from Table 1 in Ponnuswamy and Gromiha, 1993). They have used this scale to predict the secondary structure of soluble proteins and membrane proteins and have described prediction techniques for both transmembrane helices (Ponnuswamy and Gromiha, 1993) and transmembrane ß strands (Gromiha and Ponnuswamy, 1993).

In this technique, the surrounding hydrophobicity values of all of the residues within a segment of set length is summed and averaged. Segment lengths (m) of 6 or 12 are used. The average hydrophobicity of the protein as a whole is also calculated (sum of hydrophobicities of individual residues/number of residues) to act as a guide in identifying potential ß strands. The hydrophobicity plot for m = 12 is first examined (Fig. 7.4A) and the uppermost point in each peak is taken to correspond to the starting point of a ß strand. Peaks greater than 0.1 kcal above the average line are ß strands of 12 residues. Peaks less than 0.1 kcal above the average line (eg. peak numbered 2 in Fig. 7.4A) are ß

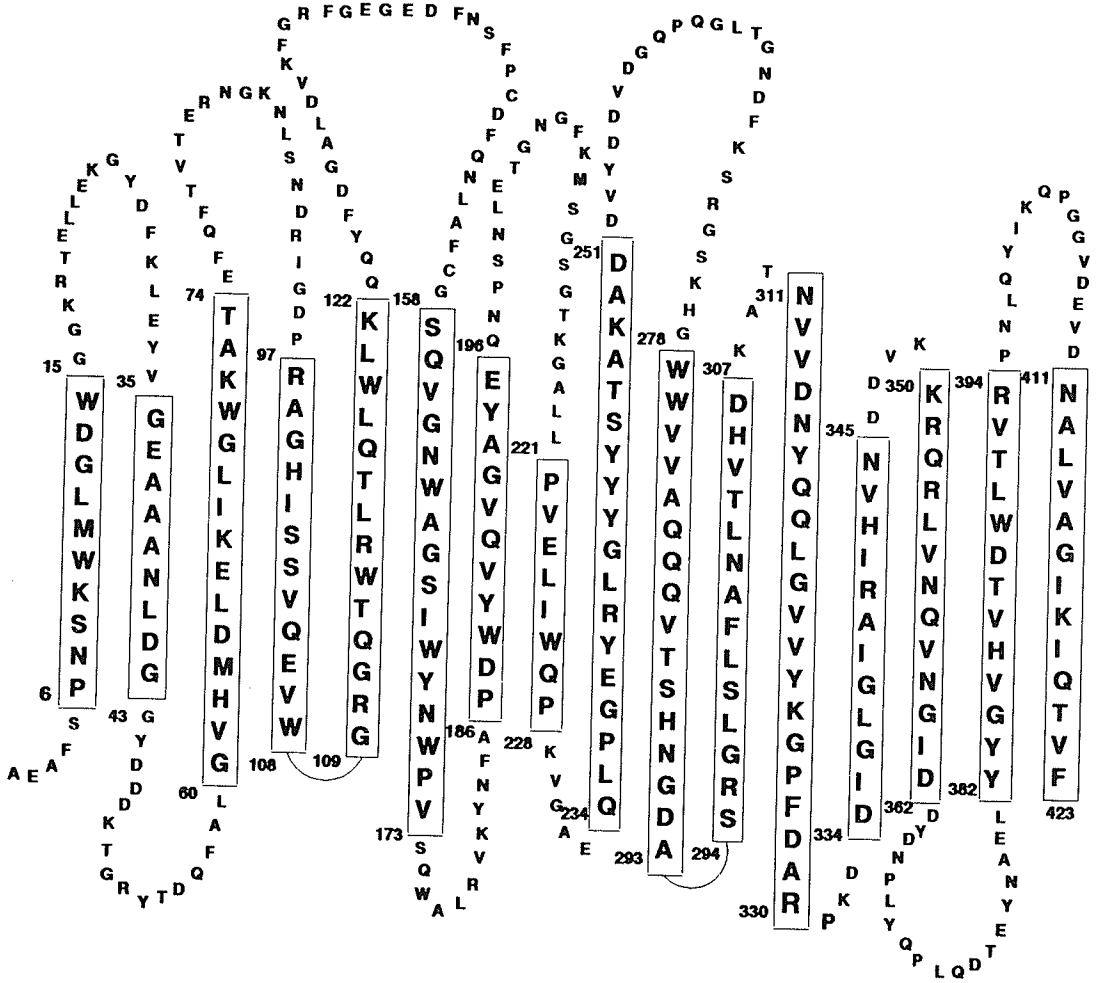
Figure 7.4. The surrounding hydrophobicity plot of OprB, calculated as outlined by Gromiha and Ponnuswamy (1993). The solid line crossing each graph is the average hydrophobicity of OprB. Graph (A) represents the hydrophobicity plot averaged over 12 residues. Numbers indicate the putative location of ß strands in OprB. Graph (B) represents the amphipathicity pattern of residues located at either end of a proposed ß strand. Solid lines indicate the hydrophobicity value of individual residues, dotted lines indicate the location of putative ß strands. The left most arrow indicates a series of residues where no amphipathic pattern is present. The right most arrow indicates a series of residues where a regular alternation of hydrophobic and hydrophilic residues occurs.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
A
14
13
12
0
100
200
300
400
16
14
12
B
0
100
200
300
400
Surrounding Hydrophobicity (Kcal/mol)
Residue number

strands of 6 residues. For peaks less than 0.1 kcal the starting residue is identified from either the m = 6 plot or by amphipathicity values as described by Gromiha and Ponnuswamy (1993). The ends of the ß strands are extended by examining the hydrophobicity values of each residue on either side of the predicted 6 or 12 residue ß strand (Fig. 7.4B). The ß strand is extended as long as the hydrophobicity values continue to alternate above and below the average hydrophobicity line. Finally, putative ß strands are examined for the presence of proline residues at or near the centre of a segment. If a proline residues occurs in this position, the strand is split and the longest resulting segment is used.

In applying this technique to OprB 14 peaks were clearly visible in the m = 12 plot (Fig. 7.4A). Since the fourth and fifth peaks were very broad, they were assumed to represent two paired ß strands and were therefore divided in two, bringing the total number of ß strands to 16. Only 1 ß strand (strand 2) was predicted to be 6 residues long, all others were 12 residues. With one exception, amphipathicity patterns extended all ß strands by at least one residue. Finally, ß strands 7 and 8 were reduced to 11 and 8 residues, respectively, due to the presence of proline residues near the centre of the original strands. The completed model is depicted in Figure 7.5.

Figure 7.5. Topological model of OprB based on the ß strand prediction technique of Gromiha and Ponnuswamy (1993). Rectangles indicate portions of the protein proposed to form ß strands.


WDGLMWKSNP
GEAANLDG
TAKWGLIKELDMHVG
RAGHISSVQEVW
KLWLQTLRWTQGRG
SQVGNWAGSIWYNWPV
EYAGVQVYWDP
PVELIWQP
DAKATSYYYGLRYEGPLQ
WWVVAQQQVTSHNGDA
DHVTLNAFLSLGRS
NVVDNYQQLGVVYKGPFDAR
NVHIRAIGLGID
KRQRLVNQVNGID
RVTLWDTVHVGYY
NALVAGIKIQTVF

7.2.4. Comparison of the 3 models

The ß strand assignment for each model was aligned against the amino acid sequence of OprB (Fig. 7.6). Since each model was similar in the placement of ß strands, an approximate consensus position for ß strands 1 - 3, 5 - 13, and 16 could be determined with some confidence. The remaining ß strands could not be unambiguously assigned because of disagreement between the various models on their location. However, experimental analysis of the topology of OprB should easily differentiate between the models at these specific points. The final consensus model is shown in Figure 7.7.

Although showing general agreement, the models differ slightly in the lengths of the predicted ß strands and the beginning and end points of the ß strands. In the case of the Paul and Rosenbusch model, given the different approach to ß strand prediction used by these authors in comparison to the other two groups, and the problems associated with turn prediction (Jeanteur *et al.*, 1991; Srikumar *et al.*, 1992), the differences are not surprising. Nevertheless, the overall agreement of the Paul and Rosenbusch model with the other two models suggests turn prediction still has some relevance to the problem of predicting porin folding patterns. For the Schirmer and Cowan model and the Gromiha and Ponnuswamy model, the differing lengths of the ß strands are largely due to the differences in size of the windows used to identify the ß

Figure 7.6. ß strand assignment from each model aligned with the amino acid sequence of OprB. The symbol, X, represents putative ß strands. First line, Paul and Rosenbusch (PR) model; second line, Schirmer and Cowan (SC) model; third line, Gromiha and Ponnuswamy (GP) model. Numbers below the sequence indicate the approximate location of ß strands 1 - 3, 5 - 13, and 16. ß strands 4, 14 and 15 are not assigned due to differences in the location assignments for these strands in the 3 models.

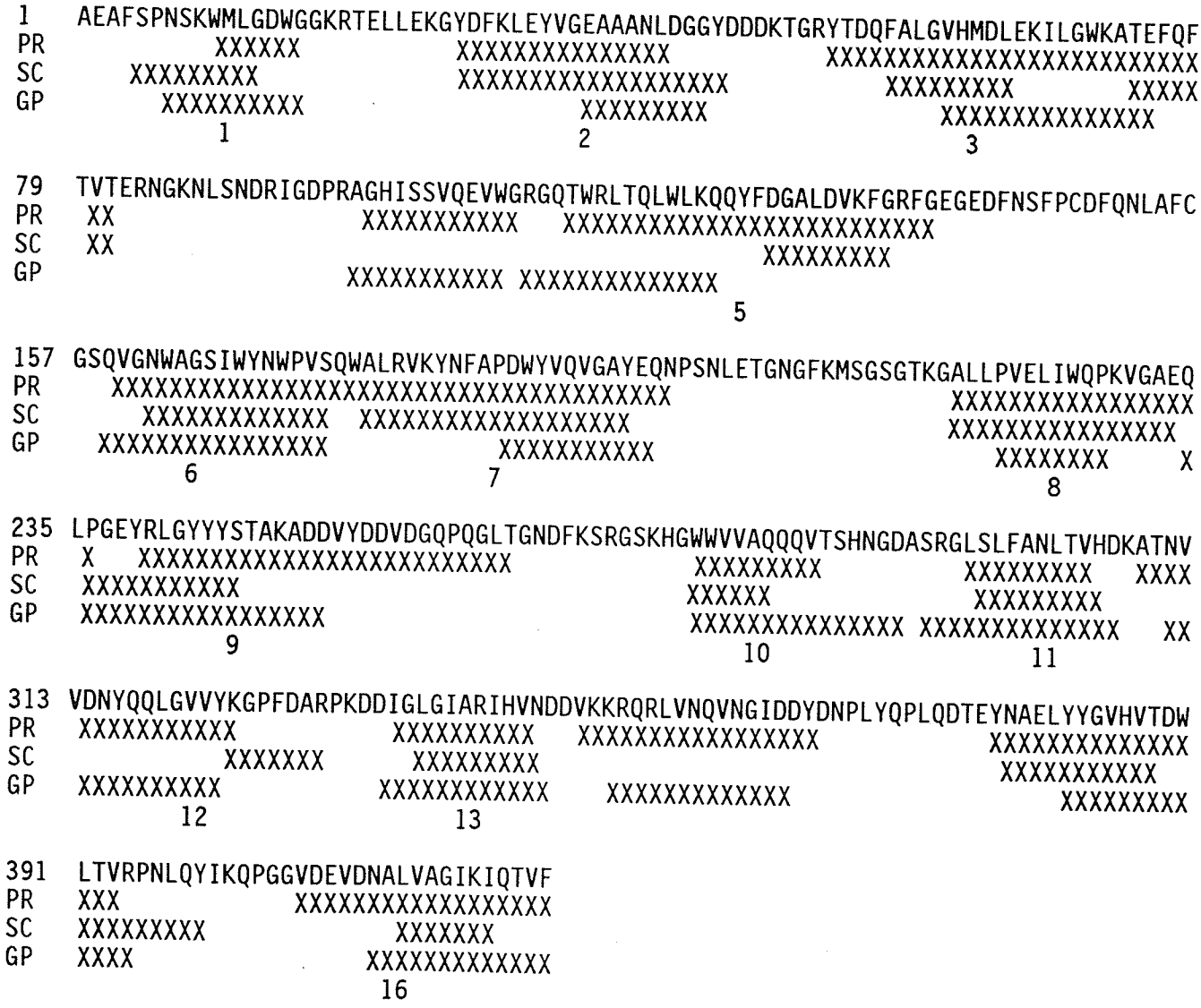
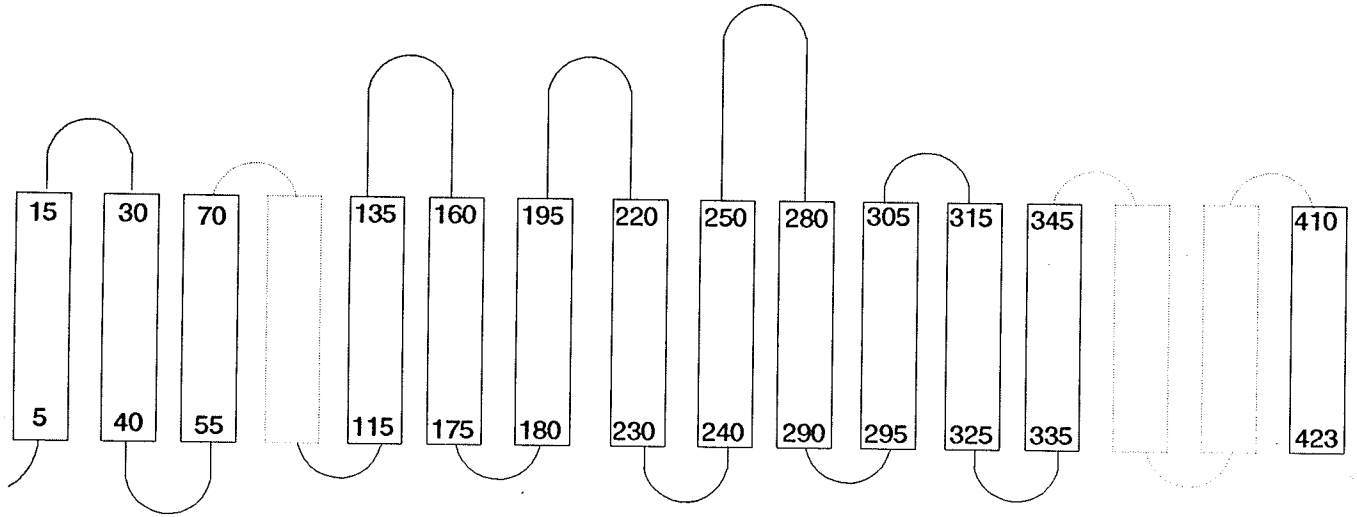

1 AEAFSPNSKWMLGDWGGKRTELLEKGYDFKLEYVGEAAANLDGGYDDDKTGRYTDQFALGVHMDLEKILGWKATEFQF
PR XXXXXX XXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXX
SC XXXXXXXXX XXXXXXXXXXXXXXXXXXX XXXXXXXXX XXXXX
GP XXXXXXXXXX XXXXXXXXX XXXXXXXXXXXXXXX
1 2 3
79 TVTERNGKNLSNDRIGDPRAGHISSVQEVWGRGQTWRLTQLWLKQQYFDGALDVKFGRFGEGEDFNSFPCDFQNLAFC
PR XX XXXXXXXXXXX XXXXXXXXXXXXXXXXXXXXXXXXXX
SC XX XXXXXXXXX
GP XXXXXXXXXXX XXXXXXXXXXXXXX
5
157 GSQVGNWAGSIWYNWPVSQWALRVKYNFAPDWYVQVGAYEQNPSNLETGNGFKMSGSGTKGALLPVELIWQPKVGAEQ
PR XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX
SC XXXXXXXXXXXXX XXXXXXXXXXXXXXXXXX XXXXXXXXXXXXXXXXX
GP XXXXXXXXXXXXXXXX XXXXXXXXXXX XXXXXXXX X
6 7 8
235 LPGEYRLGYYYSTAKADDVYDDVDGQPQGLTGNDFKSRGSKHGWWVVAQQQVTSHNGDASRGLSLFANLTVHDKATNV
PR X XXXXXXXXXXXXXXXXXXXXXXXXX XXXXXXXXX XXXXXXXXX XXXX
SC XXXXXXXXXXX XXXXX XXXXXXXXX
GP XXXXXXXXXXXXXXXX XXXXXXXXXXXXXXX XXXXXXXXXXXXXX XX
9 10 11
313 VDNYQQLGVVYKGPFDARPKDDIGLGIARIHVNDDVKKRQRLVNQVNGIDDYDNPLYQPLQDTEYNAELYYGVHVTDW
PR XXXXXXXXXXX XXXXXXXXXX XXXXXXXXXXXXXXXX XXXXXXXXXXXXXX
SC XXXXXX XXXXXXXXX XXXXXXXXXXX
GP XXXXXXXXXX XXXXXXXXXXXX XXXXXXXXXXXXX XXXXXXXXX
12 13
391 LTVRPNLQYIKQPGGVDEVDNALVAGIKIQTVF
PR XXX XXXXXXXXXXXXXXXXX
SC XXXXXXXXX XXXXXXX
GP XXXX XXXXXXXXXXXXX
16

Figure 7.7. Topological model of OprB based on a comparison of ß strand assignments by Paul and Rosenbusch, Schirmer and Cowan, and Gromiha and Ponnuswamy prediction techniques. Residue numbers indicate the approximate predicted locations of ß strands. ß strands 4, 14, and 15 are not specified, due to differences in location assignments for these strands in the 3 original models.


15
30
70
135
160
195
220
250
280
305
315
345
410
5
40
55
115
175
180
230
240
290
295
325
335
423

sheets. The differences in location of the ß strands predicted by these two techniques would be due mainly to the different hydrophobic indices used for ß strand assignment. Although each group identifies ß strands by hydrophobicity patterns, the indices used differ in the degree of hydrophobicity assigned to various residues.

At least one ß turn appeared to be missed in the Paul and Rosenbusch model, based on the length of one of the putative ß strands (39 residues from Q159 to Q197) and the prediction of two ß strands in this region by the Schirmer and Cowan and Gromiha and Ponnuswamy methods. Jeanteur *et al.* (1991) has previously noted that Paul and Rosenbusch rules do not accurately identify short periplasmic ß turns. Additionally, assuming that all porins consist of a 16 stranded ß barrel, Paul and Rosenbusch methodology appears to overpredict the number of ß turns, as previously noted by Srikumar *et al.* (1992). However, if future analysis of porins identifies ß barrels of more or less than 16 ß strands, the model of Paul and Rosenbusch could yet prove accurate.

Primary structure analysis of the amino acid sequence of OprB revealed 4 significant clusters of amino acids (chapter 6): 4 isoleucine residues (I335, I339, I342, I361), 5 tryptophan residues (W163, W168, W171, W176, W188), 7 phenylalanine residues (F126, F134, F137, F143, F146, F150, F155), and two cysteine residues (C148 and C156). Examination of the predicted secondary structure of OprB reveals possible

explanations for some of these clusters. Both the isoleucine and tryptophan clusters are predicted to occur in two closely adjoining ß strands. The prevalence of hydrophobic residues in porin ß strands coupled with the juxtaposition of two ß strands, could account for the clustering of these residues in these locations. Additionally, several of the tryptophan residues are predicted to occur near the ends of ß strands and could form part of an aromatic band around OprB. This may also be the case for at least two phenylalanine residues (F126 and F134), however, all of the models predict that most of the clustered phenylalanine residues form part of the third extracellular loop of OprB. Since this particular loop of PhoE, OmpF, and the *R. capsulatus* porin is critical for determining the channel characteristics of these porins and since phenylalanine residues are known to form part of the carbohydrate binding site of periplasmic carbohydrate binding proteins, the presence of phenylalanine residues in this region of OprB lends support to the hypothesis that phenylalanine residues may form part of the carbohydrate binding site of OprB.

Both the Schirmer and Cowan and Gromiha and Ponnuswamy models also place the two cysteine residues of OprB in this loop, suggesting that these residues may also be involved in formation of the binding site in OprB. Currently there is no evidence to suggest that disulfide bond formation occurs in OprB, since no change in the mobility of OprB was detected on

SDS-PAGE gels in the presence or absence of a reducing agent (chapter 3). The presence of disulfide bonds in *P. aeruginosa* OprF was evident under these conditions (Hancock and Carey, 1979). However, this does not completely rule out the possibility of disulfide bond formation in OprB, and additional analyses of a potential Cys-Cys bond and site directed mutagenesis of the Cys residues is warranted.

Several experimental techniques are available for testing topological models produced by secondary structure prediction techniques. Resolution of the crystal structure of the *E. coli* PhoE porin has revealed that insertion of extra amino acids in porins are permitted only within the loops connecting adjacent ß strands (Cowan *et al.*, 1992). Identification of permissive insertion sites in *P. aeruginosa* OprF (Wong *et al.*, 1993), *E. coli* PhoE (Struyvé *et al.*, 1993) and *E. coli* LamB (Charbit *et al.*, 1991) have proven useful for the identification of surface exposed loops of these porins. Additionally, monoclonal antibodies raised against peptide epitopes predicted to be located on either the extracellular or periplasmic side of a porin, would also be of use for testing topological models. If a model has accurately predicted the location of an extracellular or periplasmic ß turn or loop, monoclonal antibodies raised against corresponding epitopes would bind to whole cells or isolated outer membranes, respectively. Application of these techniques to OprB could be used to test the consensus model

shown in Figure 7.7, and for distinguishing between the various locations proposed for some ß strands (i.e. ß strands 4, 14 and 15).

8. CONSERVATION OF OPRB WITHIN THE FAMILY *Pseudomonadaceae*

8.1. Introduction

Analysis of homologous genes in related species of bacteria have proven useful as a means of predicting protein secondary structure. Jeanteur *et al.* (1991) and van der Ley *et al.* (1991) have generated topological models of enteric and neisserial porins by comparing the amino acid sequences of related porin proteins from species of bacteria within these families. In general, approaches of this type rely on the identification of conserved and variable regions of the proteins. Variable regions are taken to correspond to extracellular loops or periplasmic ß turns, while conserved portions of the proteins are believed to form the ß strands of porins.

This approach has not been applied to OprB or any other pseudomonad porins due to the lack of a sufficiently large data base of nucleotide sequence information. A necessary prerequisite for this type of analysis is the identification of homologous proteins in closely related species of bacteria, therefore, this chapter describes the identification of other species within the *Pseudomonadaceae* which carry the *oprB* gene. Future comparisons of sequence information from homologous *oprB* genes will assist in refining the topological model of OprB, and in the identification of conserved residues potentially involved in the structure and function of OprB.

Additionally, at the time I conducted these

investigations, I did not yet have available an OprB-deficient strain of *P. aeruginosa* for verifying the *in vivo* role of OprB. The substrate specificity data (chapter 4) suggested that OprB may facilitate the diffusion of other carbohydrates in addition to glucose, and act as a central component of carbohydrate uptake. However, since the structures of glucose and the other carbohydrates able to support the growth of *P. aeruginosa* were not similar, I felt that this hypothesis might be difficult to test in *P. aeruginosa*. The potentially very weak binding of fructose, mannitol or ribose to OprB could have made it difficult to detect differences in the uptake of these sugars as a result of the loss of OprB. In *P. aeruginosa*, the OprB protein could act primarily, if not wholly, as a glucose pore. Therefore, I wanted to identify other species of pseudomonad able to produce OprB while growing on xylose or maltose, sugars known to bind to the OprB porin (Trias *et al.*, 1988; Saravolac *et al.*, 1991). If necessary, mutagenesis of OprB in these species would be potentially more useful for testing the hypothesis that OprB acts to facilitate the diffusion of a wide range of carbohydrates.

8.2. Results

8.2.1. Biochemical and immunological analysis of carbohydrate-inducible proteins

Pseudomonads listed in Table 8.1 were screened for their

Table 8.1. Summary of analyses for detection of potential OprB homologues. Carbohydrate-inducible proteins identified by Coomassie Blue staining of SDS/PAGE gels. Apparent molecular mass of carbohydrate-inducible and cross-reactive proteins identified by SDS/PAGE analysis or immunodetection shown in brackets (kDa). Degree of cross-reactivity or strength of hybridization signal judged by eye from Figs. 8.3 and 8.4, respectively, +. weak reaction; ++, intermediate reaction; +++, strong reaction. rRNA group source: Palleroni et al. (1973).

Bacterial strains	rRNA group	Carbohydrate-inducible protein	Cross-reactivity with *P. aeruginosa* OprB antiserum	Hybridization with *P. aeruginosa oprB* gene probe
P. aeruginosa (PAO1 H103)	I	+ (49.6)	+++ (49.6)	+++
P. putida (ATCC 12633)	I	+ (44.9)	+++ (44.9)	++
P. chlororaphis (ATCC 9446)	I	+ (45.7)	++ (45.7)	++
P. fluorescens (UMCC)	I	+ (45.2)	-/+(63.4)[1]	+
P. stutzeri (ATCC 17588)	I	-	+ (71.0)	+
P. cepacia (NCTC 10661)	II	-	-	-
Comamonas acidovorans (NCTC 10683)	III	-	-	-
Xanthomonas maltophilia (ATCC 13633)	V	-	-	-

[1] No reaction was visible with the protein identified by SDS/PAGE analysis, however, a reaction did occur with a high-molecular-mass carbohydrate-inducible protein.

ability to grow on glucose, xylose, or maltose. Glucose supported the growth of all strains tested with the exception of *Comamonas acidovorans*. Xylose supported the growth of *P. fluorescens*, while maltose supported the growth of *P. stutzeri*, and *Xanthomonas maltophilia*. Sugar utilization by *C. acidovorans* was restricted to fructose, therefore, this sugar was used in place of glucose, xylose, or maltose when examining the production of carbohydrate-inducible proteins in this species.

Cell envelopes of the various pseudomonads were examined for the expression of novel membrane proteins following growth on various carbohydrates. Cell envelopes from succinate- or acetate-grown cells were used as a control to compare protein profiles (*P. aeruginosa* OprB is not produced under these growth conditions [Hancock and Carey, 1980]). Acetate was used for *P. stutzeri* as this species was unable to grow on succinate. In addition to *P. aeruginosa* OprB, new proteins induced by growth on carbohydrates were evident in *P. putida*, *P. chlororaphis*, and *P. fluorescens* (Fig. 8.1). The *P. fluorescens* protein was induced by growth on both glucose and xylose.

Amino terminal amino acid sequence analysis of the carbohydrate-inducible proteins verified homology to *P. aeruginosa* OprB (Fig. 8.2). The proteins expressed by *P. putida* and *P. chlororaphis* also demonstrated cross-reactivity with polyclonal antiserum raised against heat modified *P.*

Figure 8.1. SDS-polyacrylamide gel electrophoresis of pseudomonad cell envelopes. Species from which cell envelopes were isolated are indicated above gel. Abbreviations: succ, succinate; acet, acetate; glc, glucose; mal, maltose; xyl, xylose; fru, fructose. Samples were solubilized at 95°C prior to SDS-PAGE. Molecular weight standards are indicated on the left ($x10^3$). Arrows indicate proteins induced by growth on indicated carbohydrates. Fructose was used as the carbohydrate for *C. acidovorans* as this species was unable to utilize any of the other carbohydrates indicated.

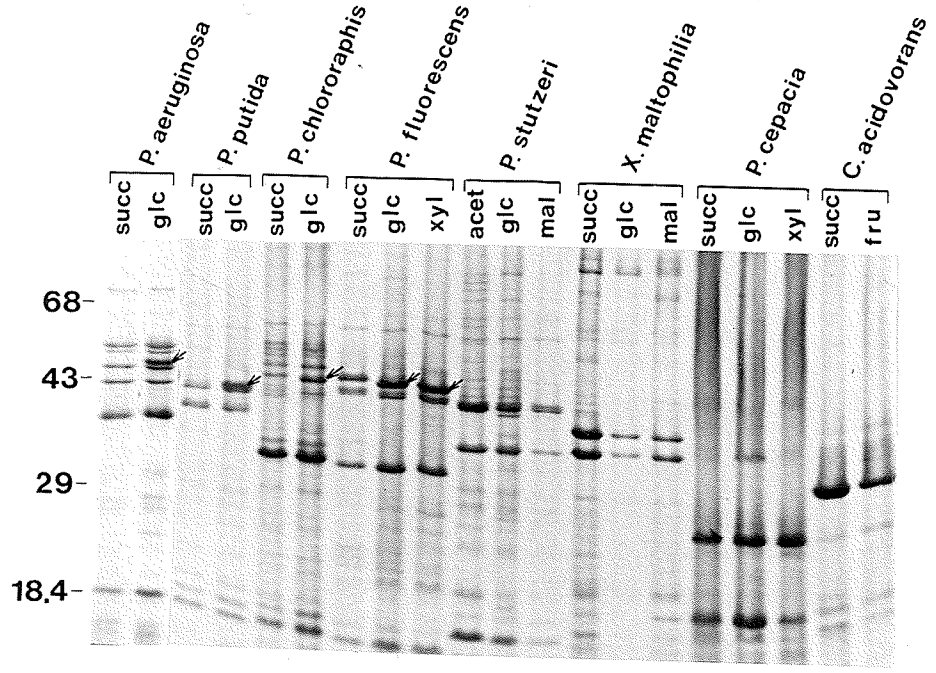
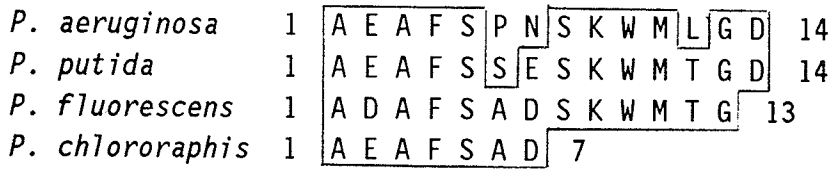

P. aeruginosa
succ
glc
P. putida
succ
glc
P. chlororaphis
succ
glc
P. fluorescens
succ
glc
xyl
P. stutzeri
acet
glc
mal
X. maltophilia
succ
glc
mal
P. cepacia
succ
glc
xyl
C. acidovorans
succ
fru
68
43
29
18.4

Figure 8.2. Amino terminal amino acid sequence comparison of pseudomonad carbohydrate-inducible proteins identified by SDS-PAGE analysis. Identical or conserved amino acid residues are boxed.


P. aeruginosa 1 A E A F S P N S K W M L G D 14
P. putida 1 A E A F S S E S K W M T G D 14
P. fluorescens 1 A D A F S A D S K W M T G 13
P. chlororaphis 1 A E A F S A D 7

aeruginosa OprB (Fig. 8.3). No cross-reaction was visible with the carbohydrate-inducible protein identified for *P. fluorescens*, however, in carbohydrate grown *P. fluorescens* and *P. stutzeri* a weak reaction was seen with a high molecular weight protein, possibly a porin dimer or trimer.

8.2.2. Pseudomonad genomic DNA homologous to *oprB*

To support the biochemical and immunological analyses, a 3.4 kb *Eco*RI-*Sph*I DNA fragment containing the *P. aeruginosa oprB* gene was used to probe *Hind*III digested genomic DNA. A strong hybridization signal was seen with *P. aeruginosa*, *P. putida*, and *P. chlororaphis* DNA while a weaker signal was seen with *P. fluorescens* and *P. stutzeri* DNA (Fig. 8.4). Repeating the hybridization with smaller probes consisting of portions of the *P. aeruginosa oprB* gene produced the same results (not shown). The above results are summarized in Table 8.1.

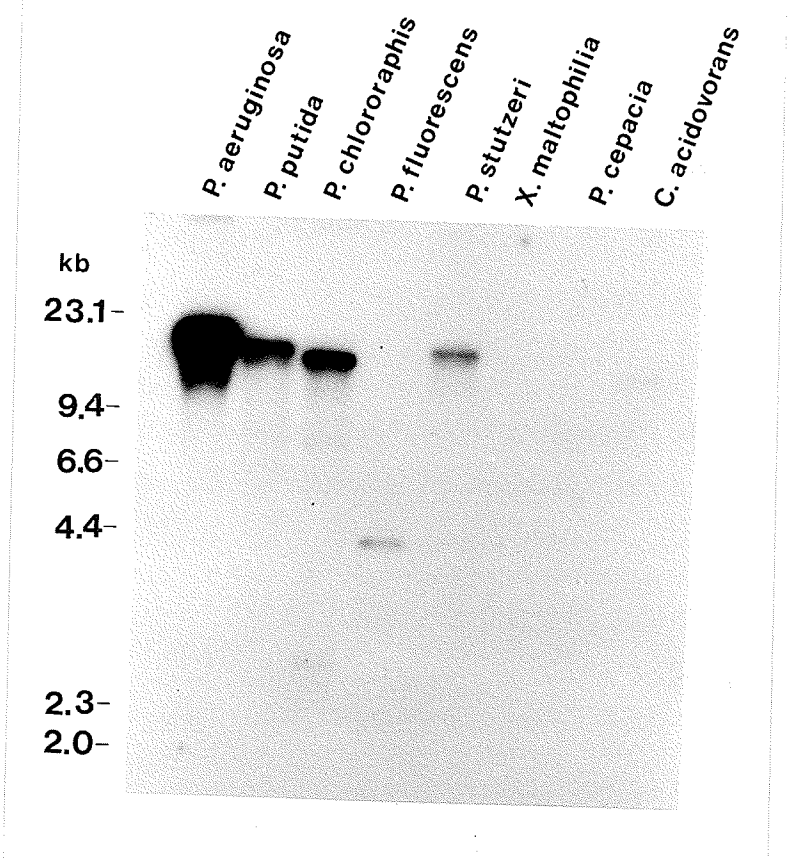
8.3. Discussion

Of the species examined only members of rRNA group I produced a protein similar to *P. aeruginosa* OprB. This is consistent with similar studies using the P. *aeruginosa oprF* and *oprP* porin genes (Siehnel *et al.*, 1990b; Ullstrom *et al.*, 1991) and the *P. aeruginosa* lipoprotein *oprI* gene (Saint-Onge *et al.*, 1992). *P. aeruginosa* genes involved in the production of the exopolysaccharide alginate were also well conserved in group I species (Fialho *et al.*, 1990; Fett *et al.*, 1992).

Figure 8.3. Western immunoblots of pseudomonad cell envelope proteins. Arrows indicate cross-reacting proteins identified by immunodetection with polyclonal antiserum raised against heat modified *P. aeruginosa* OprB. Species from which cell envelopes were isolated are indicated above gel. Abbreviations: succ, succinate; acet, acetate; glc, glucose; mal, maltose; xyl, xylose; fru, fructose. Samples were solubilized at 95°C prior to SDS-PAGE. Molecular weight standards are indicated on the left ($x10^3$).

P. aeruginosa
succ
glc
P. putida
succ
glc
P. chlororaphis
succ
glc
P. fluorescens
succ
glc
xyl
P. stutzeri
acet
glc
mal
X. maltophilia
succ
glc
mal
P. cepacia
succ
glc
xyl
C. acidovorans
succ
fru
68
43
29
18.4

Figure 8.4. Hybridization of *Hind*III digested pseudomonad genomic DNA with a *P. aeruginosa oprB* gene probe. *Hind*III digested λ DNA was used as size markers.


P. aeruginosa
P. putida
P. chlororaphis
P. fluorescens
P. stutzeri
X. maltophilia
P. cepacia
C. acidovorans
kb
23.1
9.4
6.6
4.4
2.3
2.0

Additionally, some of these genes were conserved in group V pseudomonads and to a lesser extent in some enteric bacteria (Fialho *et al.*, 1990). The distribution of specific genes within the *Pseudomonadaceae* is consistent with results obtained using whole genome hybridization analysis, which indicates that the 5 homology groups of the *Pseudomonadaceae* are only distantly related to each other (Palleroni *et al.*, 1973; Palleroni, 1983). Group I species are more closely related to group V species and the enterics than to groups II, III, and IV.

The cross-reactivity of the OprB proteins of *P. putida* and *P. chlororaphis*, coupled with the strength of the hybridization signal on Southern blots, suggests that of the OprB homologues identified, these proteins are most closely related to *P. aeruginosa* OprB. A carbohydrate-inducible protein, homologous to *P. aeruginosa*, *P. putida*, and *P. chlororaphis*, was identified in *P. fluorescens* by N terminal sequence analysis, although no cross-reactivity with *P. aeruginosa* OprB antiserum was detected. Hybridization of *P. stutzeri* genomic DNA with a *P. aeruginosa oprB* gene probe suggests that the *oprB* gene is present in this species, however, a monomeric form of OprB was not evident on SDS-PAGE gels. In this species, and also in *P. fluorescens*, a high molecular weight carbohydrate-inducible protein was identified by immunodetection and could represent OprB dimers or trimers resistant to heat denaturation. Although the *P. aeruginosa*

OprB antiserum was raised against heat modified OprB, considerable secondary structure remains in porin proteins treated in this manner (Worobec *et al.*, 1988; chapter 5) and some antibodies recognizing conformational epitopes may have been produced. If so, a weak reaction with *P. fluorescens* and *P. stutzeri* OprB trimers could occur. If the OprB monomers of *P. stutzeri* are resistant to dissociation, comparison of the various OprB proteins found in these species may be useful for analyzing the monomer/monomer interactions of porins.

Examination of OprB induction within the family *Pseudomonadaceae* identified species able to produce OprB while growing on carbohydrates other than glucose. *P. aeruginosa* and *P. putida* OprB are known to facilitate the diffusion of xylose and maltose in addition to glucose. However, of these carbohydrates, only glucose can support the growth of *P. aeruginosa* and *P. putida* and OprB function in these species may be restricted to glucose uptake. Some evidence exists to suggest that *P. aeruginosa* may be involved in facilitating the diffusion of other sugars (fructose, mannitol, ribose; chapter 4), however, this evidence is speculative without confirmation from analysis of an OprB-deficient mutant of *P. aeruginosa*. In contrast, *P. fluorescens* is able to grow on both xylose and glucose and produces OprB when either carbohydrate is present as carbon source. Assuming similar binding sites in the OprB porins, *P. fluorescens* OprB would not be restricted to glucose uptake but could serve as a multifunctional protein for

carbohydrate uptake. The same may hold true for uptake of glucose and maltose by *P. stutzeri* if the high molecular weight carbohydrate-inducible protein identified in this species by immunodetection analysis is OprB. In general, OprB could act in concert with several carbohydrate transport systems in those pseudomonads able to produce OprB and grow on sugars recognized by the binding site of OprB.

9. THE ROLE OF OPRB IN CARBOHYDRATE TRANSPORT

9.1. Introduction

Diffusion rates across the outer membrane of *P. aeruginosa* are 100 - 500 times lower than those measured for *E. coli* (Yoshimura and Nikaido, 1982). The low outer membrane permeability of *P. aeruginosa* has been attributed to the unusual channel forming properties of the outer membrane protein, OprF, considered to be the main nonspecific diffusion porin of this bacterium (Hancock *et al.*, 1979). OprF shows some homology to the OmpA protein of enteric bacteria (Woodruff and Hancock, 1989) and, like OmpA, shows only limited porin activity (Sugawara and Nikaido, 1992). In enteric bacteria, the low channel forming activity of OmpA does not have a major influence on outer membrane permeability as these bacteria also produce "classical" general diffusion porins (eg. OmpF and OmpC). The more efficient permeation of solutes through OmpF and OmpC would overshadow the diffusion properties of the OmpA protein. In contrast, the apparent lack of typical general diffusion porins in *P. aeruginosa* results in the diffusion properties of the outer membrane of this bacterium being determined largely by the relatively inefficient OprF porin.

The low outer membrane permeability of *P. aeruginosa* is compensated by the presence of several substrate-selective porins (Trias and Nikaido, 1990b; Huang and Hancock, 1993; Nikaido, 1994). Porins of this type are also present in

enteric bacteria, but, in these species, substrate-selective porins have generally been associated with the diffusion of relatively large substrates (eg. LamB, maltodextrins [Wandersman *et al.*, 1979]; ScrY, sucrose [Schülein *et al.*, 1991]; TolC, peptides [Benz *et al.*, 1993a]). In the absence of their respective porins, these substrates would show only limited or no diffusion through nonspecific channels. In contrast, the substrate-selective porins of *P. aeruginosa* facilitate the diffusion of much smaller substrates. In *E. coli* diffusion of small substrates, such as glucose or glycerol, is generally believed to occur via nonspecific channels. In *P. aeruginosa*, if facilitated diffusion across the outer membrane did not occur, the low outer membrane permeability would potentially limit the transport of almost all substrates, regardless of size.

Four substrate-selective porins have been identified in *P. aeruginosa*. The OprD porin facilitates the diffusion of basic amino acids and peptides containing these residues (Trias and Nikaido, 1990b). This porin has also been recently linked with gluconate transport across the outer membrane (Huang and Hancock, 1993). The OprP and OprO porins facilitate the diffusion of phosphate and polyphosphate, respectively (Poole and Hancock, 1986a; Siehnel *et al.*, 1992). The OprB porin has been associated with the diffusion of glucose (Trias *et al.*, 1988).

OprB was first identified by Hancock and Carey (1980),

following growth of cells in minimal medium supplemented with glucosé as the sole carbon source. Trias *et al.* (1988), using the liposome swelling assay, demonstrated the selectivity of this porin for glucose, leading to the designation of OprB as a glucose-selective porin. The OprB protein of *P. aeruginosa* is co-induced with a periplasmic glucose binding protein. These two proteins are believed to be components of a high affinity glucose transport pathway in *P. aeruginosa* (Hancock and Carey, 1980), one of two glucose transport pathways found in this bacterium. The high affinity pathway possesses a K_m for glucose of 8 μM, while a second, lower affinity pathway possesses a K_m of approximately 2 mM (Midgley and Dawes, 1973). The presence of a binding protein suggests that the high affinity pathway of *P. aeruginosa* is a member of a related family of transport systems, formerly named binding protein dependent transport systems, but more appropriately termed ABC-transporters, given their presence in gram-positive bacteria and eukaryotic cells (Higgins *et al.*, 1990). The low affinity glucose pathway of *P. aeruginosa* involves the extracellular conversion of glucose to gluconate and 2 ketogluconate by glucose dehydrogenase and gluconate dehydrogenase, respectively (Midgley and Dawes, 1973; Lessie and Phibbs, 1984).

Although well characterized *in vitro*, the *in vivo* role of OprB in glucose transport has not yet been demonstrated. This chapter describes the construction of mutant strains of

P. aeruginosa deficient in OprB production. I demonstrate that OprB does facilitate the diffusion of glucose across the outer membrane, in agreement with the *in vitro* data. However, rather than acting strictly as a glucose-selective pore, I also demonstrate that OprB actually facilitates the diffusion of a wide range of carbohydrates. OprB is, therefore, more appropriately described as a carbohydrate-selective porin.

9.2. Results

9.2.1. Construction of OprB-deficient strains of *P. aeruginosa*

The bacterial strains and plasmids used to generate OprB-deficient mutants of *P. aeruginosa* are listed in Tables 9.1 and 9.2, respectively. To construct a defined OprB-lacking mutant, I used the allele replacement technique developed by Schweizer (1992). This particular mutagenesis technique is advantageous in that it simplifies both the construction and selection of mutants. The cloning vectors are based on pUC19 and pBR322. These vectors have been modified to contain a unique *Not*I site (pNOT19 and pNOT322), designed to allow the insertion of "mobilization" and "counter-selection" cassettes. The mobilization cassette consists of *oriT* from plasmid RP4. Insertion of *oriT* into the *Not*I site of either pNOT19 or pNOT322 allows these plasmids to be transferred to *P. aeruginosa* from *E. coli* by triparental mating. The counter-selection cassette consists of the *Bacillus subtilis sacB* gene encoding levansucrase. Production of levansucrase, in the

Table 9.1. Bacterial strains used to generate OprB-deficient mutants of *P. aeruginosa*.

Strain	Characteristics	Source/reference
Pseudomonas aeruginosa		
H103	wild-type PAO1 prototroph reference strain	R.E.W. Hancock/Hancock and Carey, 1979; Holloway *et al.*, 1979
PFB311	PAO1 *gcd gnd*[1]	P.V. Phibbs/Cuskey and Phibbs, 1985
WW100	H103 *oprB*::Ω (Sm^r)	This study
WW151	PFB311 *oprB*::Ω (Sm^r)	This study
Escherichia coli		
MM294	*supE44 hsdR endA1 thi*; carries helper plasmid pRK2013 for triparental mating	R.E.W. Hancock
NM522	*supE thi* Δ*(lac-proAB) hsd5 F'[proAB$^+$ lacI^q lacZΔM15]*	

Abbreviations: ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; UMCC, University of Manitoba Culture Collection; Sm, Streptomycin.

[1] PFB311 contains mutations in glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gnd*). Therefore, of the two glucose transport pathways known in *P. aeruginosa*, only the high affinity pathway is functional.

Table 9.2. Plasmids used to generate OprB-deficient strains of *P. aeruginosa*.

Plasmid	Characteristics	Reference/Source
pTZ19R	Ap^R	Mead et al., (1986)
pJBB34	Ap^R; pTZ19R with 3.4 kb *Bgl*II/*Bgl*II fragment from pE7(9)	This study
pJSB18Δ1	Ap^R; pTZ19R with 1.2 kb *Sph*I/*Hinc*II and 0.6 kb *Sca*I/*Hind*III fragments from pJBB34	This study
pJSS14	Ap^R; pTZ19R with 1.4 kb *Sal*I/*Sal*I fragment from pJBB34	This study
pBR322	Ap^R Tc^R	Bolivar (1979)
pHP45ΩSm/Spc	Ap^R Sm^R Spc^R	Fellay et al. (1987)
pMOB3	Km^R Cm^R *sacBR oriT*	Schweizer (1992)
pNOT19	Ap^R; pUC19 with *Not*I site	Schweizer (1992)
pNOT322	Ap^R Tc^R; pBR322 with *Not*I site	Schweizer (1992)
pNOTSB18	Ap^R; pNOT19 with 1.8 kb *Sph*I/*Hind*III fragment from pJSB18Δ1	This study
pMOBSB18	Ap^R Cm^R *sacBR oriT*; pNOTSB18 with 5.0 kb *Not*I/*Not*I fragment from pMOB3	This study
pBR322Δ	Ap^R Tc^R; Deletion of 0.64 kb *Ava*I/*Pvu*II fragment from pBR322	This study
pBRSS14	Tc^R; pBR322Δ with 1.4 kb *Eco*RI/*Pst*I fragment from pJSS14	This study
pBRSS14::Sm	Tc^R Sm^R; pBRSS14 with 1.4 *Sma*I/*Sma*I fragment from pHP45ΩSm/Spc	This study
pNOTSS14::Sm	Tc^R Sm^R; pNOT322 with 3.2 kb *Eco*RI/*Pst*I site from pBRSS14::Sm	This study
pMOBSS14::Sm	Tc^R Sm^R Cm^R *sacBR oriT*; pNOTSS14::Sm with 5.0 kb *Not*I/*Not*I fragment from pMOB3	This study
pPZ375	Ap^R; Broad host range deriative of pGEM-3Z(+)	P.V. Phibbs
pPZXS27	Ap^R; pPZ375 with 2.7 kb *Xba*I/*Sph*I fragment from pJBB34	This study
pPZSH27	Ap^R; pPZ375 with 2.7 kb *Sph*I/*Hind*III fragment from pJBB34	This study

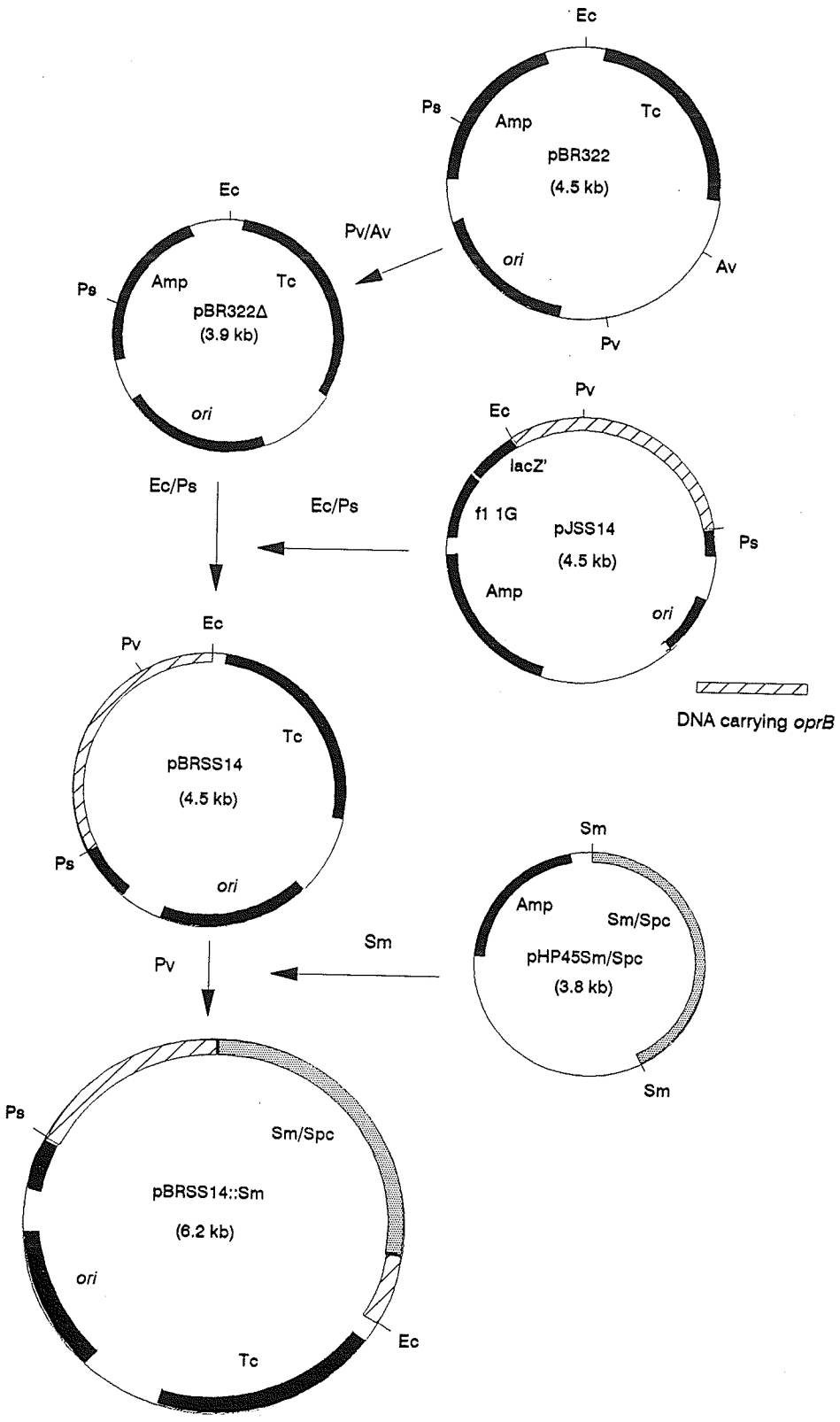
Abbreviations: Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Ap, ampicillin; Km, kanamycin; Cm, chloramphenicol; *sacBR*, levansucrase gene and its regulatory region; *oriT*, origin of transfer from RP4.

presence of sucrose, is lethal to all gram-negative bacteria so far tested (Gay *et al.*, 1985; Schweizer, 1992). This characteristic allows for the positive selection (sucrose resistance) of colonies in which excision of plasmid integrates has occurred.

The construction of vectors used for generating OprB-deficient strains of *P. aeruginosa* are shown in Figures 9.1 and 9.2. The *oprB* gene was inactivated in two ways: first, by insertion of an antibiotic (Sm) resistance cassette (Fig. 9.1) and second, by deleting a portion of the *oprB* gene from amino acids G109 to L391 (Fig. 9.2). My intention was to generate an OprB-deficient mutant using the *oprB* gene inactivated by insertion of the Sm cassette. Although the presence of the antibiotic cassette would facilitate selection of OprB mutants, the Sm cassette could also potentially produce polar effects on downstream genes once transferred onto the chromosome. Therefore, I also constructed a second OprB mutagenesis vector by deleting a portion of the *oprB* gene. Although more difficult to select OprB-deficient mutants, this approach would be less likely to produce polar effects on genes downstream of *oprB*. As demonstrated below, this approach was unnecessary as there was no evidence of polar effects, however, the construction of this plasmid is outlined here, as it may yet prove useful for future analyses.

Mutagenesis of OprB using the Sm Ω interposon (Fellay *et al.*, 1987) was conducted by inserting the cassette into a 1.4

Figure 9.1. Construction of an OprB mutagenesis vector by insertion of a streptomycin resistance cassette. Abbreviations: Tc, tetracycline; Amp, Ampicillin; Km, Kanamycin; Cm, chloroamphenicol; Sm, streptomycin; Spc, Spectinomycin; *ori*, origin of replication; *oriT*, origin of transfer from RP4; *lacZ'*, ß-galactoside gene; *sacR/sacB*, levansucrase gene and its regulatory region; f1 1G, origin of replication from phage f1; Ec, *Eco*RI; Ps, *Pst*I; Av, *Ava*I; Pv, *Pvu*II; No, *Not*I.


Ec
Ps
Amp
Tc
pBR322
(4.5 kb)
ori
Av
Pv
Pv/Av
Ec
Ps
Amp
Tc
pBR322Δ
(3.9 kb)
ori
Pv
Ec
lacZ'
f1 1G
pJSS14
(4.5 kb)
Ps
Amp
ori
Ec/Ps
Ec/Ps
DNA carrying oprB
Ec
Pv
Tc
pBRSS14
(4.5 kb)
Ps
ori
Sm
Amp
Sm/Spc
pHP45Sm/Spc
(3.8 kb)
Sm
Pv
Sm
Ps
Sm/Spc
pBRSS14::Sm
(6.2 kb)
ori
Tc
Ec

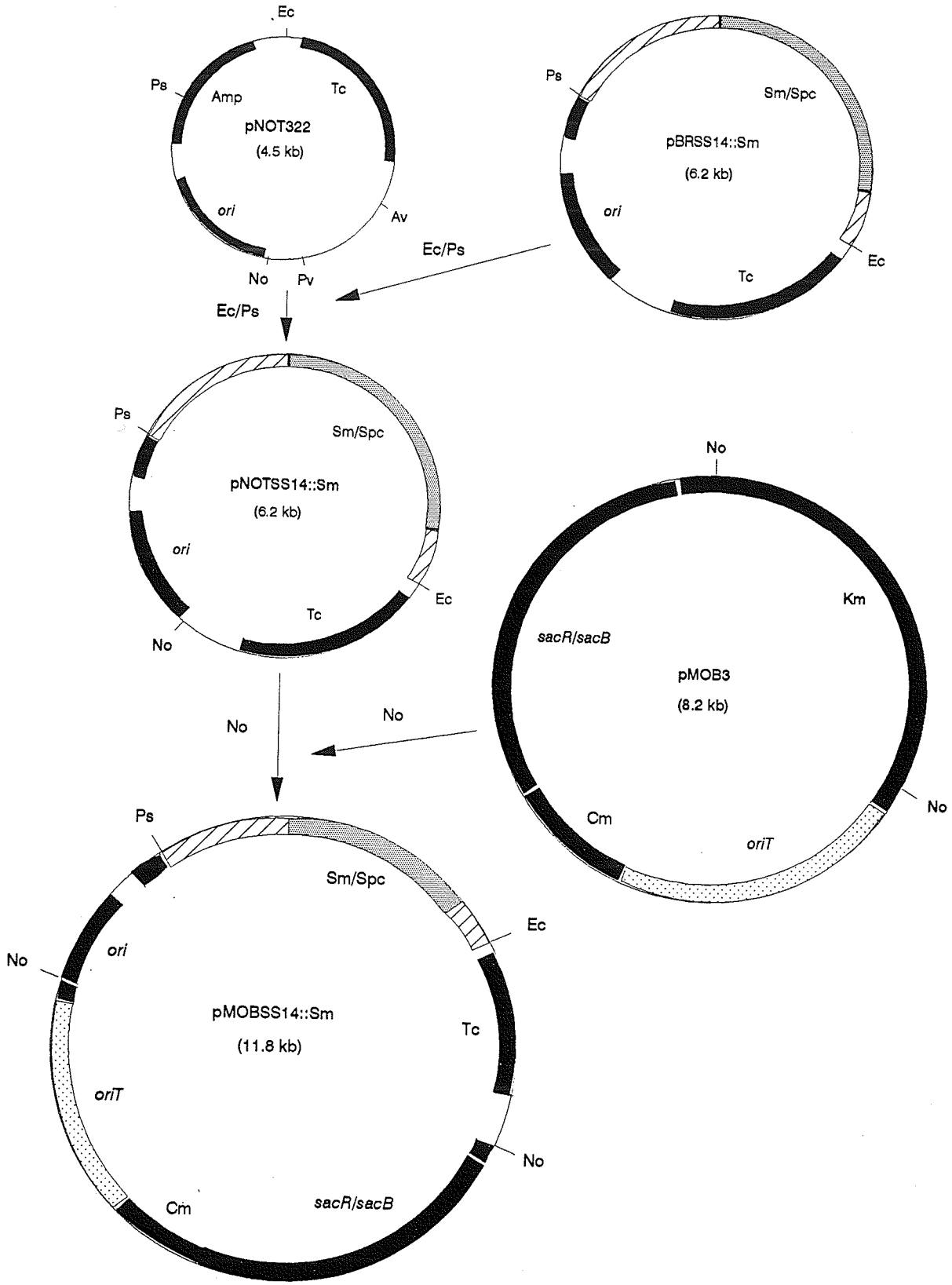
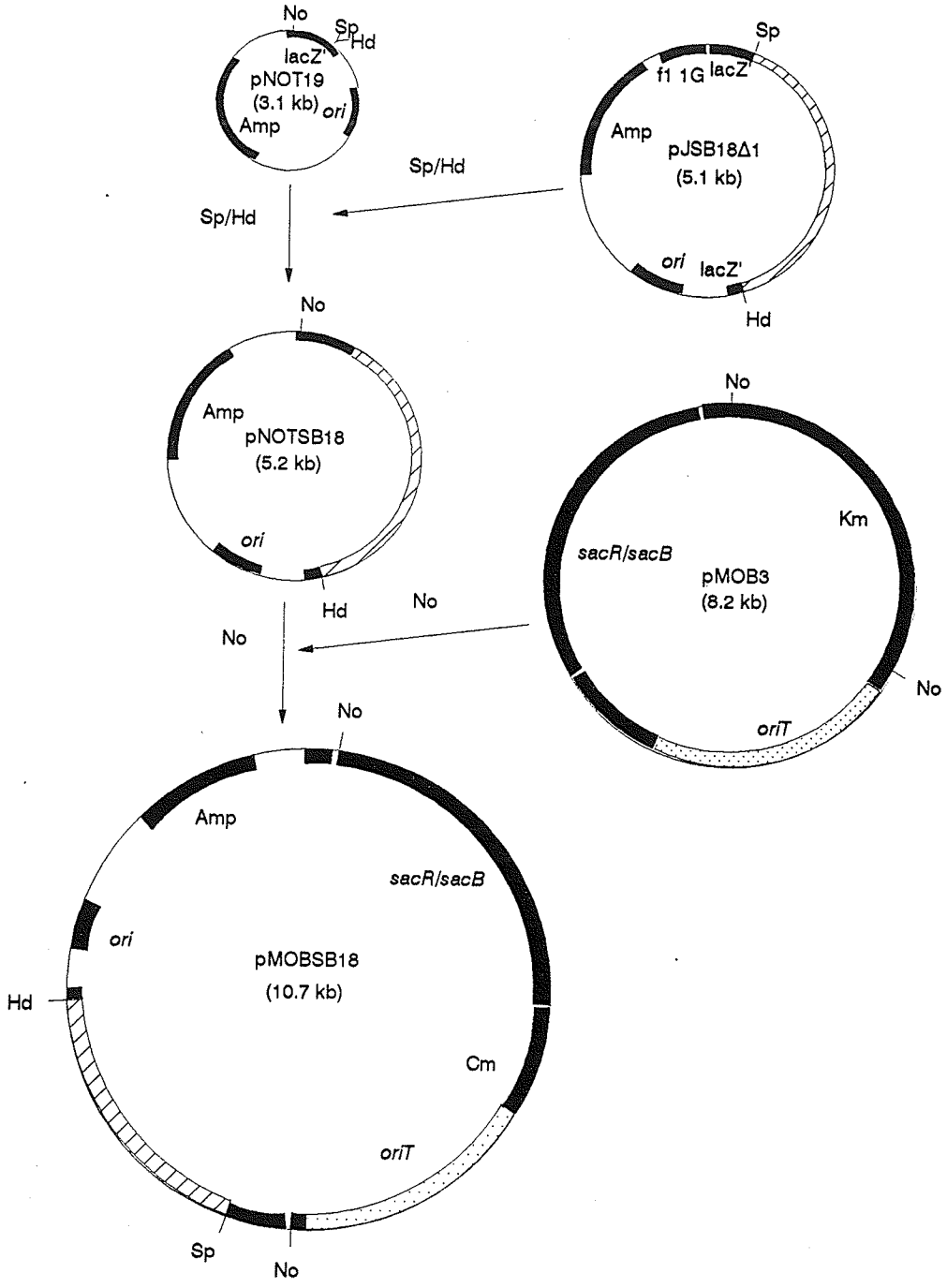

Ec
Ps
Amp
Tc
pNOT322
(4.5 kb)
ori
Av
No
Pv
Ps
Sm/Spc
pBRSS14::Sm
(6.2 kb)
ori
Ec
Tc
Ec/Ps
Ec/Ps
Ps
Sm/Spc
pNOTSS14::Sm
(6.2 kb)
ori
Ec
Tc
No
No
Km
sacR/sacB
pMOB3
(8.2 kb)
Cm
oriT
No
No
No
Ps
Sm/Spc
Ec
No
ori
pMOBSS14::Sm
(11.8 kb)
Tc
oriT
No
Cm
sacR/sacB

Figure 9.2. Construction of an OprB mutagenesis vector by deletion of a portion of the *oprB* gene. Abbreviations: Sp, *Sph*I; Hd, *Hind*III, other abbreviations as in Fig. 9.1.


No
Sp
Hd
lacZ'
pNOT19
(3.1 kb)
ori
Amp
Sp/Hd
Sp/Hd
Sp
f1 1G
lacZ'
Amp
pJSB18Δ1
(5.1 kb)
ori
lacZ'
Hd
No
Amp
pNOTSB18
(5.2 kb)
ori
Hd
No
No
Km
sacR/sacB
pMOB3
(8.2 kb)
No
oriT
No
No
Amp
sacR/sacB
ori
pMOBSB18
(10.7 kb)
Hd
Cm
oriT
Sp
No

kb fragment of *P. aeruginosa* genomic DNA carrying a portion of the *oprB* gene (pBRSS14::Sm). pNOTSS14::Sm was created by ligating the mutagenized *oprB* fragment into pNOT322. pMOBSS14::Sm resulted from the ligation of a 5.5 kb fragment from pMOB3, containing *oriT* and *sacB*, into the unique *Not*I site of pNOTSS14::Sm. pMOBSB18 (Fig. 9.2) was created in a similar way, except, as indicated above, *oprB* was mutagenized by deletion of a 0.85 kb fragment from the centre of the gene, rather than insertion of an antibiotic cassette.

Two strains of *P. aeruginosa* were selected for mutagenesis. H103 is a wild-type PAO1 reference strain (Hancock and Carey, 1979; Holloway *et al.*, 1979). PFB311 is also derived from a PAO1 wild-type but the low affinity glucose transport pathway (glucose dehydrogenase) of this strain has been inactivated by chemical mutagenesis (Cuskey and Phibbs, 1985). Examination of glucose transport in this strain allows the role of OprB to be examined in the absence of the low affinity glucose transport pathway.

pMOBSS14::Sm was transferred from *E. coli* LE392 to either H103 or PFB311 by triparental mating using the helper *E. coli* strain MM294. *P. aeruginosa* cells containing plasmid integrates were selected by growth on either VB or BM2 plates, supplemented with Sm or Tc. Both VB and BM2 media contained citrate which *P. aeruginosa*, but not *E. coli*, is able to utilize. The original protocol developed by Schweizer (1992) used VB medium, however, I found that BM2 medium supplemented

with citrate worked equally well. The presence of Sm and Tc in the medium selected cells which had integrated pMOBSS14::Sm into the chromosome. Plasmids derived from pUC or pBR322 are unable to replicate in *P. aeruginosa*, therefore colonies showing the proper combination of antibiotic resistant traits would contain an integrated plasmid at the site of the wild-type gene. Selection with both antibiotics reduces the likelihood of obtaining spontaneous antibiotic resistant colonies. Sm^R Tc^R colonies were visible on these plates after 48 - 72 hours.

Sm^R Tc^R colonies were selected and streaked on LB plates supplemented with sucrose (5% w/v) and Sm. From each streaked colony, several hundred $sucrose^R$ Sm^R colonies appeared after 24 hr growth at 37°C. For both H103 and PFB311, four $sucrose^R$ Sm^R colonies were selected. Each was Tc^S, suggesting that excision of the integrated plasmid had occurred in each case. One potential OprB-deficient mutant originating from H103 or PFB311 was selected and designated WW100 and WW151, respectively.

9.2.2 Characterization of OprB-deficient strains

Southern blots of *Bgl*II digested genomic DNA from H103 and PFB311 and their potential isogenic OprB mutants WW100 and WW151 were prepared and probed with a 3.4 kb fragment of DNA containing the *oprB* gene (Fig. 9.3). The hybridizing DNA fragment from both mutants increased in size from 3.4 kb to

Figure 9.3. Southern blots of *Bgl*II digested genomic DNA from wild-type and OprB-lacking strains of *P. aeruginosa*. Genomic DNA was probed with a 3.4 kb fragment of DNA containing *oprB*. Lane 1, H103; 2, WW100; 3, PFB311; 4, WW151. *Hind*III digested λ DNA was used as size markers.


1 2 3 4
23.1
6.6
4.4
2.3
2.0

6.0 kb, corresponding to insertion of the 1.6 kb Sm cassette. Examination of the cell envelopes from both mutant strains revealed that the OprB protein no longer appeared in the cell envelope (Fig. 9.4, lanes 2 and 6). Immunoblot analysis of whole cell proteins from these strains indicated that OprB was not present in any of the cell fractions (not shown).

9.2.3 Restoration of OprB expression in *P. aeruginosa*

OprB expression was restored in both of the OprB-lacking strains WW100 and WW151. Two plasmids were constructed, using the vector pPZ375. This vector was developed by P.V. Phibbs (East Carolina University, Greenville, NC) by insertion of a 1.8 kb fragment of DNA, containing *oriV*, into pGEM-3Z(+) (Promega). *oriV* is a broad host range origin of replication from pRP1, allowing replication of pPZ375 in both *P. aeruginosa* and *E. coli*. pPZSH27 and pPZXS27 were constructed by insertion of the *oprB* gene into pPZ375 (Fig. 9.5). pPZSH27 contains the *oprB* gene in the same orientation as the *plac* promoter on pPZ375 with the initiation codon located approximately 0.5 kb downstream from *plac*. pPZXS27 contains the same DNA fragment in the opposite orientation to *plac*. Both plasmids were transformed into WW100 and WW151. Examination of cell envelopes by SDS/PAGE analysis, and whole cell proteins by immunodetection, indicated that the H103 isogenic mutant, WW100, constitutively overexpressed OprB following transformation with pPZSH27 (Fig. 9.6 and Fig. 9.4,

Figure 9.4. SDS-PAGE analysis of cell envelope proteins from wild-type, OprB-deficient, and OprB-overexpressing strains of *P. aeruginosa*. Samples were heated at 95°C prior to loading on the gel. Lanes 1, H103; 2, WW100; 3, WW100 (pPZXS27); 4, WW100 (pPZSH27); 5, PFB311; 6, WW151; 7, WW151 (pPZXS27); 8, WW151 (pPZSH27).

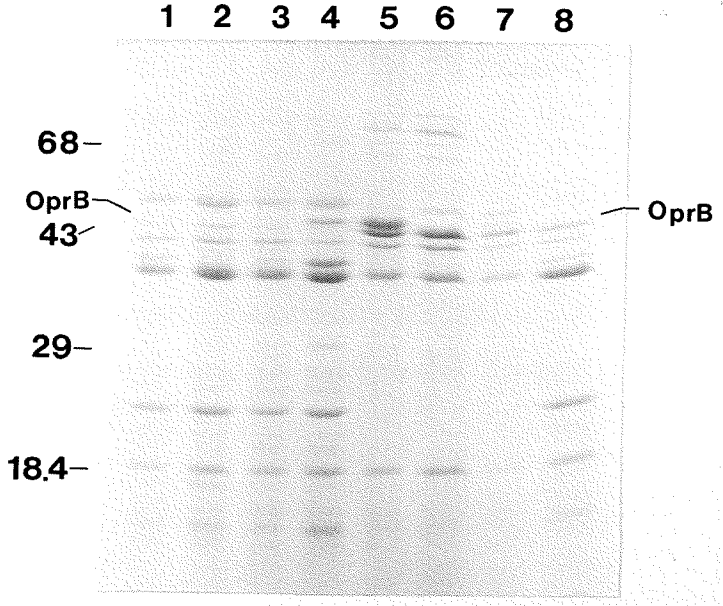

1 2 3 4 5 6 7 8
68
OprB
43
29
18.4
OprB

Figure 9.5. Plasmid pPZSH27 used for reconstitution of OprB production in OprB-lacking strains of *P. aeruginosa*. The *plac* promoter is located upstream of the *Hind*III site. pPZXS27 contains the same size insert except the *oprB* gene is oriented in the opposite orientation. Abbreviations as in Figs. 9.1 and 9.2.

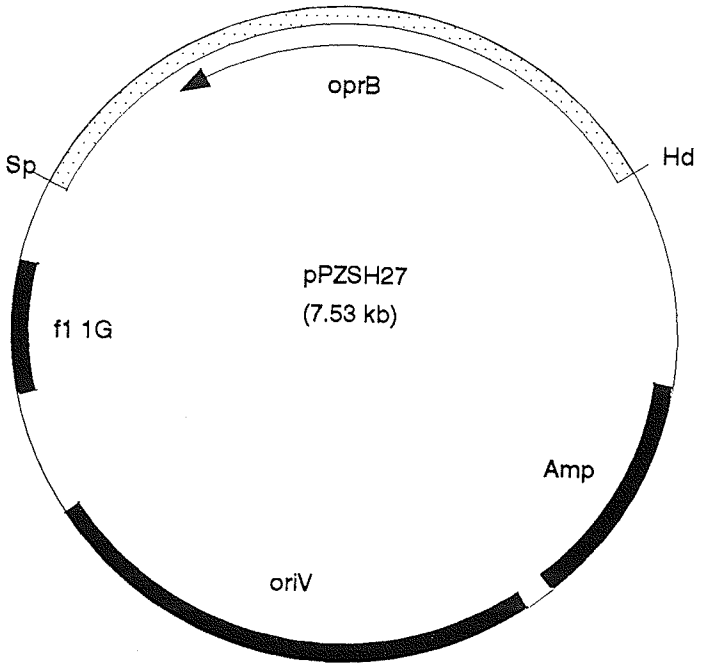
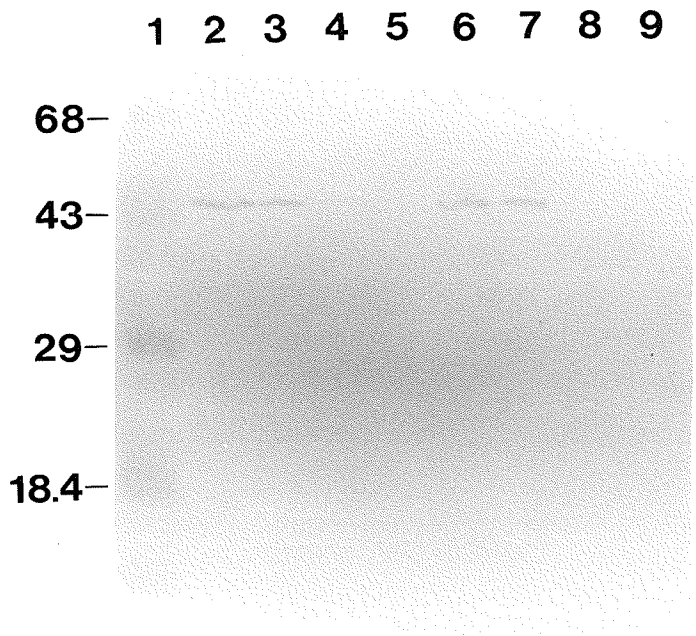

oprB
Sp
Hd
pPZSH27
(7.53 kb)
f1 1G
Amp
oriV

Figure 9.6. Western immunoblots of whole cell proteins of OprB-deficient and OprB-overexpressing strains of *P. aeruginosa*. Whole cell proteins were exposed to polyclonal antiserum raised against *P. aeruginosa* OprB. Lane 1, M_r standards; Lanes 2, 3, WW100 (pPZSH27); Lanes 4,5, WW100 (pPZXS27); Lanes 8,9, WW100 (pPZ375). Samples for lanes 3, 5, and 9 were prepared from cells grown in LB, while samples for lanes 2, 4, and 8 were prepared from glucose-grown cells. Lanes 6 and 7 show samples from another plasmid in which *oprB* is oriented in the same direction as in pPZSH27, but which contains a larger fragment of DNA downstream of *oprB*.


1 2 3 4 5 6 7 8 9
68
43
29
18.4

lane 4). Expression of OprB from this plasmid presumably originates from *plac*, which in *P. aeruginosa* is not regulated since the *lac* repressor is not produced by *P. aeruginosa*. In contrast, WW100 (pPZXS27) produced OprB only when cells were grown in BM2 minimal media supplemented with glucose (Fig. 9.6). However, OprB was not produced to wild-type levels (Fig. 9.4, compare lanes 1 and 3). This result indicates that in WW100 (pPZXS27), OprB is produced from its own promoter, however, given the lower levels of expression, some of the operator sequences necessary for OprB regulation may be absent.

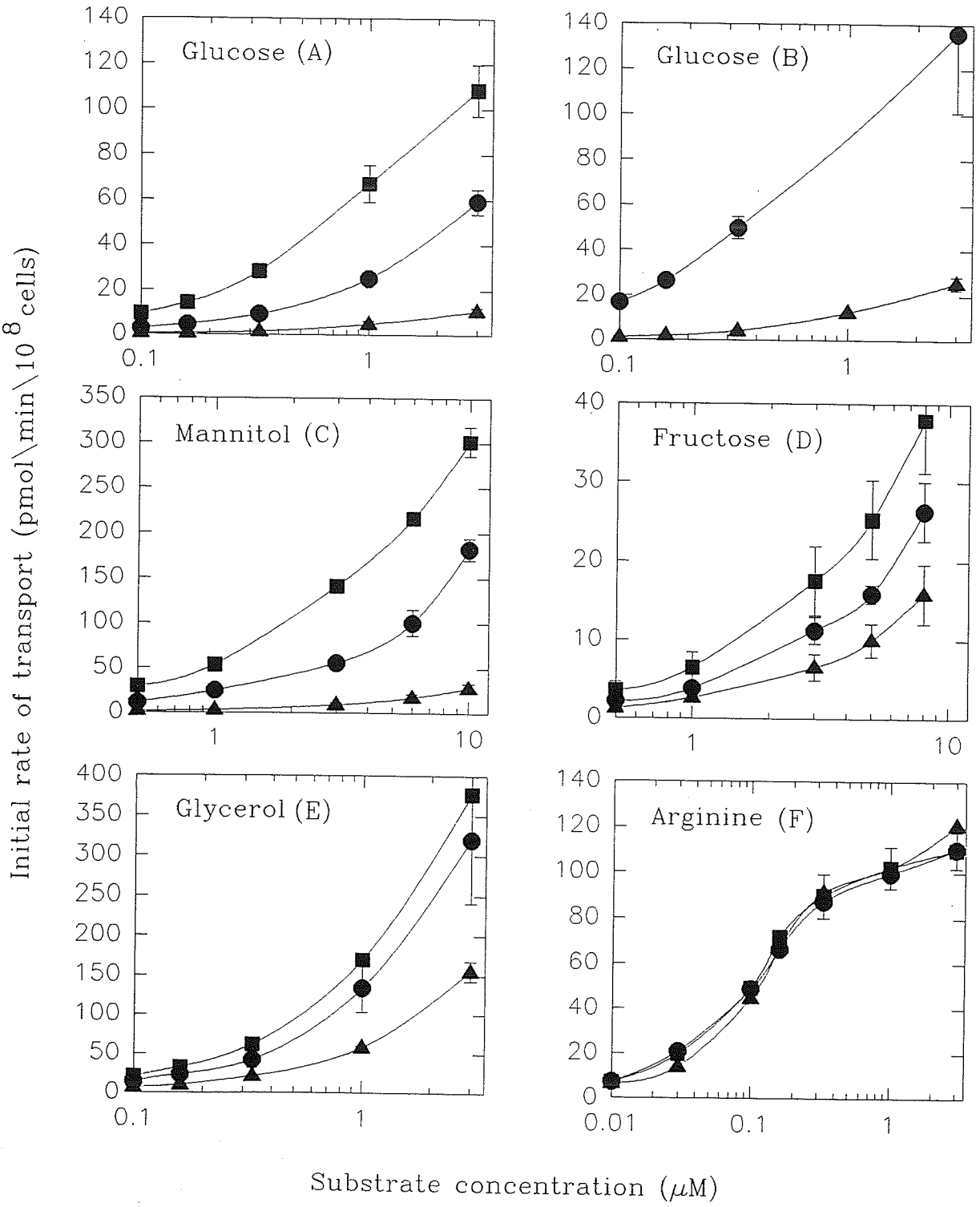
Identical analyses of the PFB311 OprB-deficient mutant, WW151, produced similar results. OprB was constitutively expressed by WW151 (pPZSH27), while WW151 (pPXS27) produced OprB only following growth on glucose (not shown). However, in contrast to WW100, OprB expression by WW151 was very low and did not approach the levels of OprB production seen in the parent strain PFB311 (Fig. 9.4, compare lanes 7 and 8 with lane 5). PFB311 overexpresses OprB (Fig. 9.4, lane 5), presumably in response to inactivation of the low affinity glucose transport pathway. The mechanism behind overexpression of OprB in PFB311 is currently unknown. Whatever factors are involved, they may preclude proper expression of the wild type gene of H103 when transferred to the isogenic mutant of PFB311, WW151.

9.2.4. The role of OprB in glucose transport

Previous *in vitro* analysis suggested that OprB was a glucose porin (Trias *et al.*, 1988), therefore, the effect of OprB loss on glucose transport was examined. Comparison of the initial rates of glucose uptake in H103 and WW100, demonstrated that loss of OprB resulted in a decrease in glucose uptake (Fig. 9.7A). At an external glucose concentration of 1 μM, glucose uptake decreased approximately 5 fold. Restoration and overexpression of OprB in WW100 (pPZSH27) increased the rate of glucose uptake to levels greater than that seen in the wild-type H103 strain. These results are consistent with the proposed role of OprB in facilitating the diffusion of glucose across the outer membrane.

An alternative possibility for the decreased transport of glucose in the absence of OprB is that interposon-mutagenesis of OprB produced a polar effect on some other component of the high affinity glucose transport system of this bacterium. This could potentially eliminate or reduce the activity of the high affinity pathway, leaving the low affinity glucose pathway as the sole or primary means of taking up glucose. Several lines of evidence suggest polar effects did not occur. First, mutagenesis of OprB in PFB311 reduced, but did not eliminate, glucose transport (Fig. 9.7B). Since PFB311 produces only the high affinity glucose transport pathway, inactivation of a gene downstream of *oprB* encoding

Figure 9.7. Initial rates of substrate transport by wild-type, OprB-deficient, and OprB-overexpressing strains of *P. aeruginosa*. Strains in A and C - F are H103, ● ; WW100, ▲ ; WW100 (pPZSH27), ■ . Strains in B are PFB311, ● ; WW151, ▲ .


Glucose (A)
Glucose (B)
Mannitol (C)
Fructose (D)
Glycerol (E)
Arginine (F)
Initial rate of transport (pmol\min\10 8 cells)
Substrate concentration (μM)

some other component of the high affinity transport system, would be manifested as an inability to transport glucose into the cell. Second, recovery of glucose transport occurred following expression of OprB from a plasmid-encoded *oprB* gene, suggesting that the effects on glucose transport were due specifically to OprB. Third, at high external glucose concentrations (eg. 100 μM), transport rates of PFB311 and WW151 are the same (243 ± 49 vs 231 ± 17 pmol/min/10^8 cells, t-test, $p>.08$). If the number of functional inner membrane transport complexes had been lowered by reduced transcription rates of a corresponding gene, glucose uptake by WW151 would not be expected to be able to attain a wild-type V_{max}.

Although V_{max} values for wild-type and mutant strains can be estimated by measuring glucose transport rates at high levels of external substrate, accurate determination of K_m values, by mean of Lineweaver-Burke plots, is difficult for porin-deficient mutants. As previously outlined by several authors (Klein and Boos, 1993; Brass *et al.*, 1984) biphasic (concave upward) curves are obtained when transport data from porin-deficient mutants are plotted on a Lineweaver-Burke plot. In a porin-deficient mutant, transport across the outer membrane is rate limiting at low substrate concentrations. When plotted on a Lineweaver-Burke plot, transport data obtained at low substrate concentrations extrapolates to the origin, reflecting the nonsaturable kinetics of nonspecific diffusion through porins. As substrate concentration

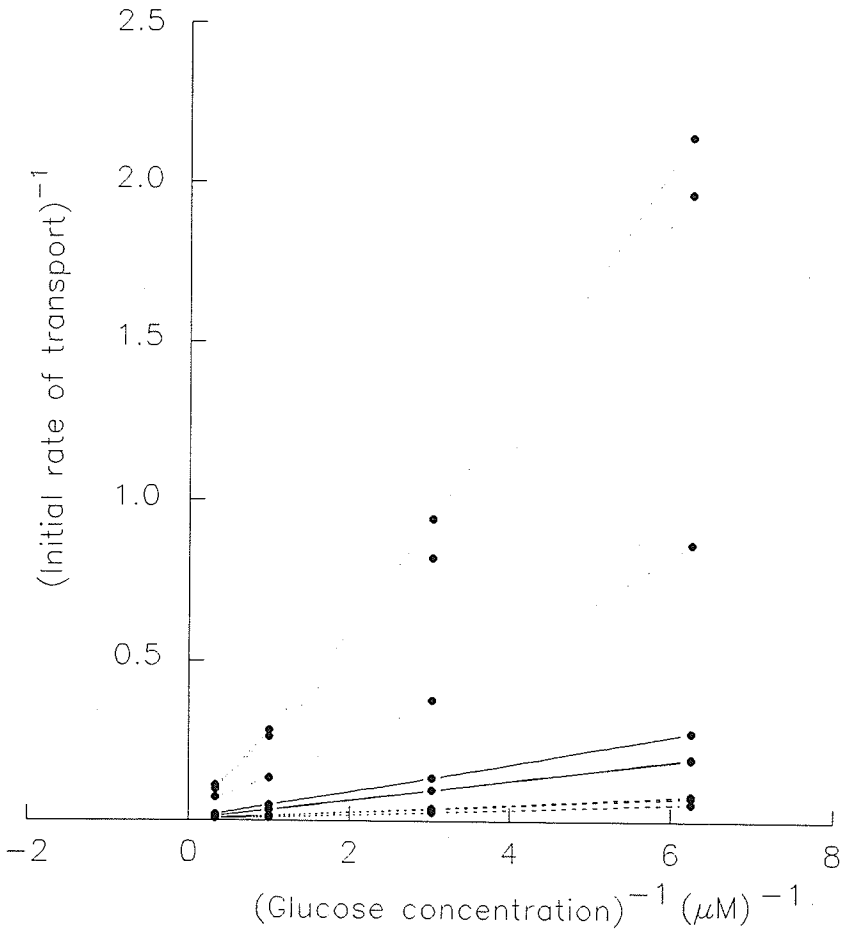
increases, transport by the inner membrane components becomes rate limiting. The plot is deflected towards the y-axis and intersects the axis at the characteristic $1/V_{max}$ value of the transport system.

Kinetics of this type are illustrated in Figure 9.8. In the wild-type (H103) and OprB overexpressing (WW100 [pPZSH27]) strains of *P. aeruginosa*, the Lineweaver-Burke plots of the transport data are linear, indicating that OprB scavenges sufficient glucose at low substrate concentrations to prevent transport across the outer membrane from becoming rate limiting. V_{max} values remain unaltered, while K_m values decrease in the overexpressing strain. The plot for WW100, however, is nonlinear. In the absence of OprB, glucose diffuses nonspecifically across the outer membrane, and transport across the outer membrane becomes rate limiting at low substrate concentrations.

9.2.5. Effect of OprB loss on the transport of other sugars

The effect of OprB loss on the transport of several other sugars able to support the growth of *P. aeruginosa* (fructose, mannitol, glycerol) was examined. Strain PFB311 and the OprB-deficient mutant WW151 were not analyzed since the transport systems for these sugars should be identical in PFB311 and H103. Figure 9.7 (C - E) indicates that the loss or overexpression of OprB resulted in a decrease or increase,

Figure 9.8. Lineweaver-Burke plot of the initial rates of glucose uptake. Strains are H103, solid line; WW100, dotted line; WW100 (pPZSH27) dashed line. Three independent assays are shown for each strain.


2.5
2.0
1.5
1.0
0.5
(Initial rate of transport)$^{-1}$
−2
0
2
4
6
8
(Glucose concentration)$^{-1}$ (μM)$^{-1}$

respectively, of the initial rates of transport of each sugar tested.

To rule out the possibility that OprB mutagenesis had a pleiotropic effect on transport in general, arginine transport by the various strains was examined. Arginine diffuses across the outer membrane through the OprD porin of *P. aeruginosa*. Loss of OprB should therefore have no effect on arginine transport. OprB is not normally induced by growth on arginine, therefore, cells were grown in minimal media containing both arginine and glucose[2]. Figure 9.7F demonstrates that the loss or overexpression of OprB had no effect on the transport of arginine, verifying that the differences in transport observed in OprB-deficient strains were specific for carbohydrates.

9.2.6. Inhibition of glucose transport

In Chapter 4, I outlined several experiments designed to resolve conflicts in the literature pertaining to the specificity of glucose transport. The results indicated that the different results reported in the literature were related to the substrate levels at which inhibition was examined. The patterns of inhibition suggested that the binding specificities of either the glucose binding protein or OprB

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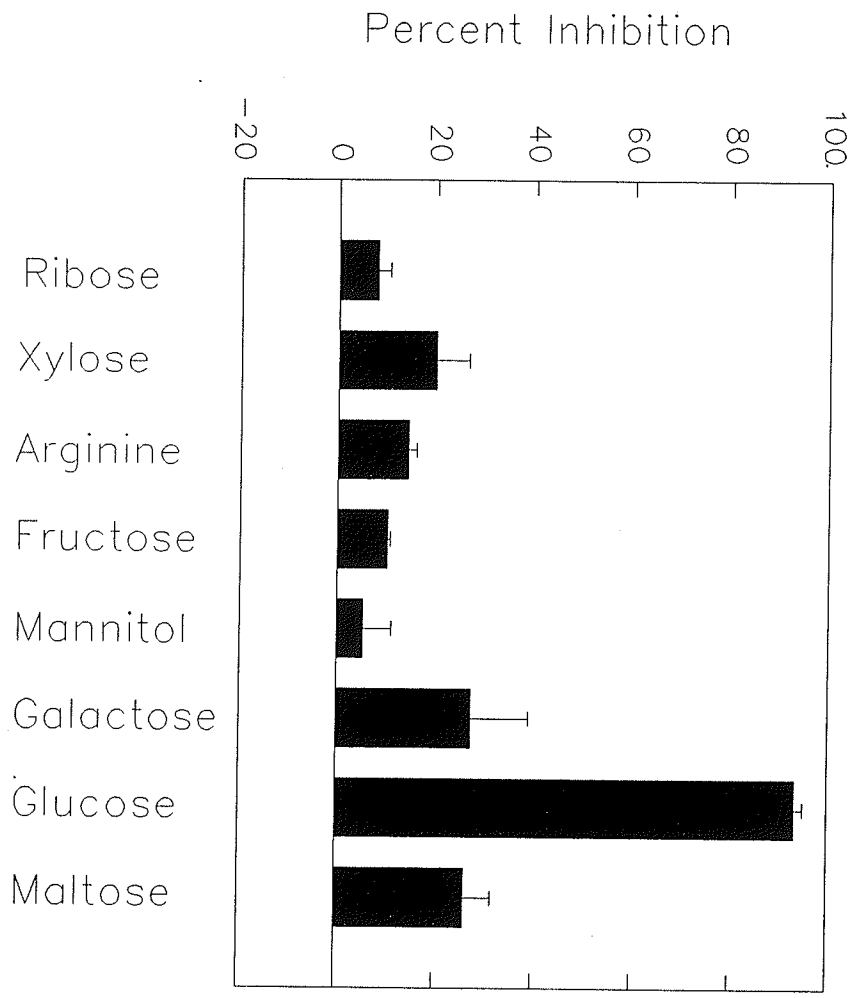
[2]Prior to conducting the transport assays I verified that OprB was induced under these conditions (by examination of cell envelope proteins from glucose/arginine-grown cells) and that the arginine uptake system was induced to the same extent as that seen in cells grown on arginine as the sole carbon source (by examination of uptake of radiolabelled arginine). I found that OprB was produced under these conditions and there was no apparent difference in arginine uptake between arginine-grown cells and those grown on a combination of arginine and glucose (data not shown).

could be detected, depending on the substrate level used. The generation of an OprB-deficient strain of *P. aeruginosa* allowed this hypothesis to be tested.

The inhibition of glucose transport by fructose, mannitol, ribose, xylose, galactose, maltose, and arginine was reexamined at an external glucose concentration of 70 μM and an inhibitor concentration of 3.5 mM, as described in chapter 4. As previously illustrated (Fig. 4.2B, page 121) in wild-type *P. aeruginosa*, galactose, maltose, and to a lesser extent xylose partially inhibited glucose transport. Given the known binding affinity of OprB for these substrates (Trias *et al.*, 1988; Saravolac *et al.*, 1991) this inhibition appeared to result from the binding of these substrates to OprB. However, in the OprB lacking strain WW100, inhibition of glucose transport by galactose, maltose, and xylose did not decrease to the same level shown by other substrates (Fig. 9.9), as would be expected if these substrates were binding to OprB. Given that the observed inhibition in wild-type strains does not appear to be due to binding of substrate to OprB, it is unclear at what point the inhibitors are interfering with glucose transport. It is possible that as substrate concentration increases the specificity of the glucose binding protein changes, however, this contradicts results obtained from the maltose binding protein of *E. coli* (Szmelcman *et al.*, 1976). Generation of defined mutants of glucose binding protein may shed some light on this question.

Figure 9.9. Inhibition of glucose uptake in P. aeruginosa strain WW100. Inhibitors (final concentration 3.5 mM) were added 20 sec prior to addition of labelled glucose (70 μM). Standard errors are calculated with n = 3.


Percent Inhibition
-20
0
20
40
60
80
100
Ribose
Xylose
Arginine
Fructose
Mannitol
Galactose
Glucose
Maltose

9.3. Discussion

This chapter describes the construction of defined OprB-deficient strains of *P. aeruginosa* and the demonstration of the *in vivo* role of OprB. Previous analyses, including the induction of OprB by growth on glucose (Hancock and Carey, 1980), the demonstration of facilitated diffusion of glucose into liposomes containing OprB (Trias *et al.*, 1988), and the verification of a glucose binding site in OprB (chapter 5), suggested that OprB would form a glucose-selective porin in the outer membrane of *P. aeruginosa*. The results presented here verify that, *in vivo*, OprB does facilitate the diffusion of glucose into the cell.

Although previous *in vitro* analyses suggested OprB would form a glucose porin, the role OprB might play in the diffusion of other sugars was more difficult to predict. Previously (chapter 4) I had demonstrated that fructose, ribose, and mannitol could inhibit glucose transport if the respective transport system for the inhibiting sugar was also induced. Although these results could be an indication of competition for an OprB binding site, competition for intracellular enzymes, common to the metabolism of the various sugars, could not be ruled out. Data obtained from liposome swelling assays also did not give a definite answer on OprB specificity. Results from this assay suggested OprB specifically facilitated the diffusion of glucose and xylose (Trias *et al.*, 1988). Other substrates diffused through OprB

nonspecifically. However, the high substrate concentrations used in the liposome swelling assay make it difficult to predict the transport rates which would be seen at substrate levels normally encountered by a cell.

The results presented in this report verify that, *in vivo*, OprB acts as a substrate-selective porin for a variety of different sugars. The decrease in transport seen in OprB-deficient strains is such that it is clear that OprB is not acting simply as a nonspecific channel for these sugars. If OprB was acting as a nonspecific porin, its absence would not be expected to have such a dramatic effect on sugar transport, since several other porins are present in the *P. aeruginosa* outer membrane which could permit the nonspecific diffusion of these sugars (eg. OprF, OprD, OprE, etc.). Additionally, the lack of an effect on arginine transport suggests the loss of OprB specifically affects carbohydrates. It is also unlikely that mutagenesis of OprB had a pleiotropic effect on carbohydrate transport, since the transport systems for these sugars are regulated independently of each other (Eagon and Phibbs, 1971; McCowen *et al.*, 1981).

The lack of any great structural similarity between the various sugars examined in this report suggests that the presence of a hydroxyl group may be sufficient to permit some degree of binding to OprB to occur. Previously, Trias *et al.* (1988) demonstrated the importance of hydroxyl groups in determining rates of diffusion through OprB. These same

authors also found that serine diffused faster into OprB-containing liposomes than alanine. The hydroxyl group on the side chain of serine may have permitted some facilitated diffusion of serine to occur through the OprB channel, despite the smaller size of alanine. Although OprB could potentially facilitate the diffusion of a diverse assemblage of compounds, dependent on the presence of a hydroxyl group, the inducible nature of OprB would presumably restrict its role to carbohydrate transport.

Recent analysis of other substrate-selective porins suggests that the broad specificity observed for OprB is not unusual. Huang and Hancock (1993) demonstrated that OprD, generally considered to be a basic amino acid porin, also plays a role in the diffusion of gluconate into the cell. In this case, the necessary determinant for binding may be a carboxylic acid group. The LamB porin of *E. coli*, long considered to be a maltodextrin-selective porin, has recently been shown to play a role in the transport of a diverse assemblage of sugars including, glucose, arabinose, glycerol, lactose, and trehalose (Death *et al.*, 1993; Klein and Boos, 1993). In general, the broad specificity of substrate-selective porins may be advantageous to the cell, as it need not produce a separate type of porin for each substrate encountered.

Although OprB and LamB are similar with respect to facilitating the diffusion of a wide range of sugars across

the outer membrane into the periplasm, some aspects of their regulation differ. In batch culture, only maltodextrins induce the production of LamB (Death *et al.*, 1993), with maltotriose being the actual inducer (Raibaud and Ricket, 1987). Induction of LamB by maltodextrins under these conditions was the primary reason for the designation of LamB as a maltodextrin porin. However, Death *et al.* (1993) have recently shown that other sugars, such as glucose, arabinose, trehalose, also induce LamB, but only under chemostat conditions. In contrast, batch growth of *P. aeruginosa* on all of the sugars analyzed in this report, induces production of OprB (Hancock and Carey, 1980; and Fig. 4.1, page 117).

These differences in the regulation of OprB and LamB may relate to the low outer membrane permeability of the *P. aeruginosa* outer membrane. *E. coli* possesses typical nonspecific diffusion porins (OmpF and OmpC). These porins may permit sufficient nonspecific diffusion of a monosaccharide if it is present at high external concentrations. Therefore, LamB induction during batch growth on monosaccharides would be unnecessary. Only when substrate must be continually scavenged from the media (i.e. chemostat growth) would facilitated diffusion of monosaccharides by LamB be necessary. In contrast, the lack of typical general diffusion porins in *P. aeruginosa* may have made it advantageous for OprB to be induced whenever a sugar is detected in the external environment, regardless of the

substrate concentration.

Facilitated diffusion of small substrates by LamB makes it necessary to revise the view that the substrate-selective porins of *P. aeruginosa* are present to compensate for the low outer membrane permeability of this bacterium. Although, as outlined above, the regulation of a porin may differ depending on outer membrane permeability, the facilitated diffusion of small substrates by substrate-selective porins may be a general rule in most bacteria, rather than an exception. Chemostat growth of other species of bacteria may yet reveal a range of substrate-selective porins not normally induced under batch growth conditions.

10. SUMMARY AND FUTURE RESEARCH

10.1. Summary

Prior to conducting the research reported in this study, three principle observations regarding OprB had been made. First, OprB was strongly induced by growth of *P. aeruginosa* in minimal media supplemented with glucose. Second, OprB allowed the diffusion of solutes into liposomes. Third, glucose diffused into OprB-containing liposomes faster than expected based on the M_r of glucose. Together, these observations suggested OprB was a substrate-selective porin facilitating the diffusion of glucose across the outer membrane of *P. aeruginosa*.

The results presented in this thesis extend these observations to include the characterization of the purified protein, the nucleotide sequence of the gene encoding OprB, and the demonstration of the *in vivo* role of OprB. The protein was characterized by black lipid bilayer analysis and circular dichroism. These analyses revealed that the OprB porin formed small, anion selective channels containing a glucose binding site. Circular dichroism analysis revealed that ß sheet was a primary component of the secondary structure of this protein.

Nucleotide sequence analysis of the cloned *oprB* gene revealed that *oprB* encoded a protein of 454 amino acids. Several clusters of amino acids, potentially involved in formation of the binding site of OprB, were identified. An

area of regional homology with *E. coli* LamB was also found, and could potentially form part of a carbohydrate-binding site.

Several secondary structure prediction techniques were applied to the amino acid sequence of OprB to generate a working model of the protein. This model should be amenable to testing by various techniques designed to assess the secondary structure of porins. OprB homologues in several other rRNA group I pseudomonads were identified which may also prove useful for testing the model of OprB.

Finally, OprB-deficient mutants of *P. aeruginosa* were constructed which allowed the *in vivo* role of OprB to be determined. The OprB porin was shown to facilitate the diffusion of glucose, as expected based on previous *in vitro* analyses. However, the porin was also shown to facilitate the diffusion of several other sugars able to support the growth of *P. aeruginosa*. These observations indicated that the OprB porin is more appropriately designated as a carbohydrate-selective porin, rather than a glucose-selective porin.

10.2. Future Research

High resolution structural information is now available for 3 nonspecific porins (Cowan *et al.*, 1992; Weiss and Schulz, 1992), however, information of this kind is not yet available for any substrate-selective porin. Of particular interest is the structure and location of the binding site of

these porins. The third extracellular loop of nonspecific diffusion porins is critical for determining channel properties (Cowan et al., 1992; Weiss and Schulz, 1992), however, it is not known if this loop performs a similar function in substrate-selective porins or whether the residues located on this loop contribute to the binding site of these proteins. Identification of the residues involved in the binding site of OprB will provide some of the information needed to answer this question. The following section outlines several approaches which could be used to study various aspects of OprB structure and function.

10.2.1. NMR studies

Analysis of the binding of substrates to proteins using NMR spectroscopy (Hibler et al., 1989) is a novel approach to the study of porin function. By growing cells in the presence of ^{15}N- or ^{13}C-labelled amino acids, specific residues within a protein can be labelled. OprB, labelled in this manner, could be examined for structural changes in the presence and absence of substrate. Information obtained from these analyses may provide an indication of which residues form the binding site of this protein.

NMR analysis requires large amounts of purified protein, therefore, the generation of *P. aeruginosa* strains overexpressing OprB would be useful. In reconstituting OprB into OprB-deficient strains of *P. aeruginosa*, overexpression

of the protein was obtained from strains carrying the *oprB*-containing plasmid pPZSH27. Transfer of this plasmid to H673 (the OprD-lacking strain used to purify OprB) should increase the levels of OprB produced in H673 and significantly improve yields of purified protein. Alternatively, PFB311 (*P. aeruginosa* strain lacking the low affinity glucose transport pathway) naturally overexpresses OprB to an even greater extent than pPZSH27-containing strains. Generating an OprD-deficient strain of PFB311 (R.E.W. Hancock, UBC, has available an OprD-mutagenesis vector) should allow even larger amounts of OprB to be purified.

10.2.2. Site-directed mutagenesis

During the course of analyzing the nucleotide sequence of *oprB*, several amino acid residues potentially involved in formation of the binding site of OprB were identified. Site-directed mutagenesis of these residues would be appropriate for testing the role these residues play in OprB function. Residues which could be examined in this way include: 1) C148 and C156. Cysteine residues are rare in porins and the proximity of these residues to the third extracellular loop of OprB (chapter 7) suggests that they may play a role in OprB function. 2) Phe and Trp clusters. Like C148 and C156, these residues are also predicted to occur near the third extracellular loop of OprB (chapter 7). Since aromatic residues are known to be involved in formation of the binding

site of periplasmic carbohydrate binding proteins (Quiocho, 1989), these residues may play a role in formation of the binding site of OprB. 3) L41, K49, G51, and D46. These four residues occur in a region of OprB which showed some homology with LamB. The four residues listed are conserved in LamB and OprB and are known to be involved in LamB function. Their presence in both proteins suggests that they may also play an important role in OprB function.

The wild type amino acid residues could be changed to smaller residues, such as Gly or Ala, and the effects of these changes assayed *in vitro* or *in vivo*. *In vitro* analysis would require purification of the mutant protein, followed by examination of the protein by black lipid bilayer analysis, circular dichroism, or NMR. *In vivo* analysis would require repeating the uptake assays described in chapter 9 and comparing uptake to that seen in a wild-type strain.

10.2.3. Topological analysis of OprB

Topological models of porins are useful for visualizing the location of residues and may provide clues as to which residues could potentially form the binding site of substrate-selective porins. A model of this type has been generated for OprB (chapter 7), however, it has not been verified by experimental analysis. Random linker insertion has proven useful for the topological analysis of other porins (Charbit et al., 1991; Struyvé et al., 1993; Wong et al., 1993) and

could be applied to OprB to identify extracellular or periplasmic regions of OprB. Analysis of other porins has demonstrated that permissive insertions (ie. production and localization of the porin to the outer membrane) occurs only in the extracellular or periplasmic loops of porins. Identifying permissive insertion sites allows specific regions of the protein to be assigned as either extra- or intramembranous. Deletion of potential extracellular loops would supply similar information.

The cloning and determination of the nucleotide sequence of *oprB* genes from other species of Pseudomonads would also prove useful for topological analysis. The amino acid residues within the extracellular loops of related porins generally show more variability than the residues found within ß strands (Gerbl-Rieger *et al.*, 1991; Jeanteur *et al.*, 1991). Comparison of homolgous *oprB* genes and identification of the conserved and variable regions within these proteins would help in the identification of the transmembrane portions of OprB.

10.2.4. Chemostat growth

Recent analysis of LamB by Death et al. (1993) has revealed that porin induction following chemostat growth can differ from that observed following batch growth. This is a novel approach to the investigation of porin regulation and the induction of *P. aeruginosa* porins has not been examined in

this way. Chemostat growth of this bacterium on various substrates may reveal changes in the induction of known porins or reveal novel porins, not previously identified during batch growth of cells.

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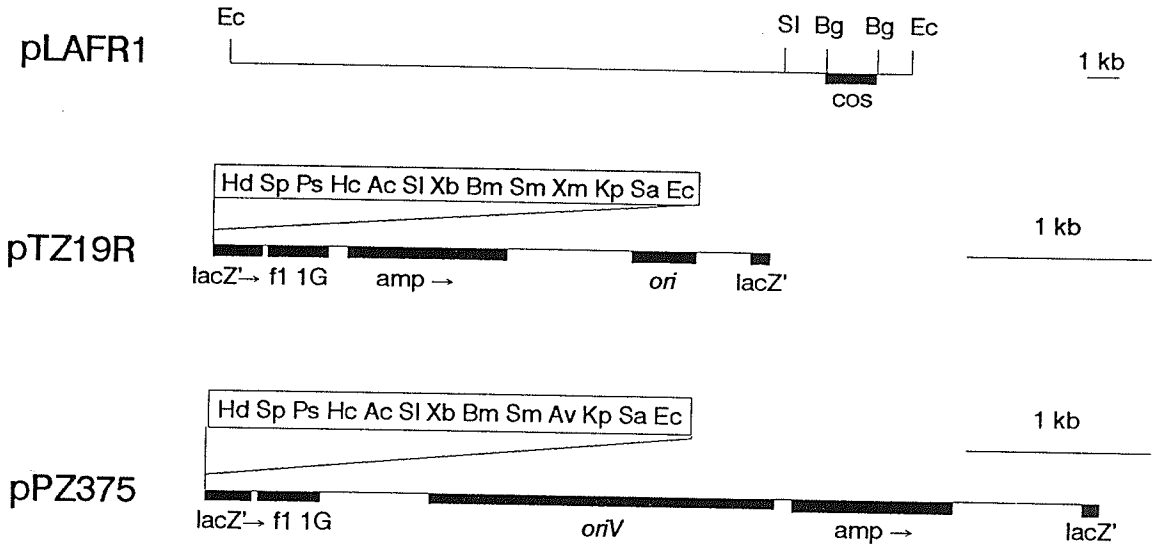
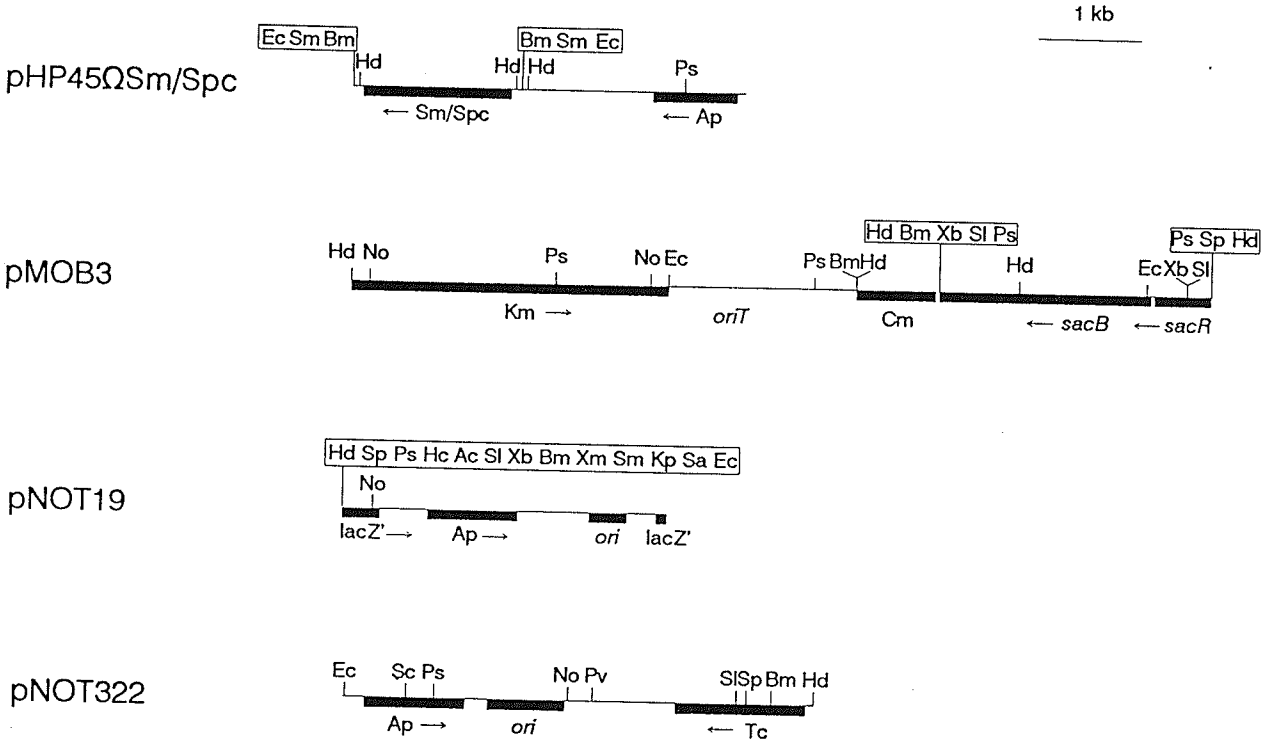
Appendix 1. RESTRICTION ENZYME MAPS OF PLASMIDS USED IN THIS STUDY

This appendix illustrates the restriction enzyme sites of all of the plasmids used in the course of this study. Open ended boxes placed at the ends of the illustrated DNA fragments represent vector DNA. The restriction enzyme sites shown in these boxes are those remaining in the multiple cloning site following the insertion of a DNA fragment into the vector. References/sources of cloning and mutagenesis vectors are as follows: pLAFR1, Friedman et al. (1982); pTZ19R, Mead et al. (1986); pPZ375, P.V. Phibbs (East Carolina University, Greenville, N.C.); pHP45ΩSm/Spc, Fellay et al. (1987); pMOB3, pNOT19, pNOT322, Schweizer (1992). Abbreviations used: Tc, tetracycline; Sm, streptomycin; Spc, Spectinomycin; Km, Kanamycin; Cm, chloramphenicol; *sacB/sacR*, gene from *Bacillus subtilis* encoding levansucrase and its regulatory region; f1 1G, origin of replication from phage f1; *lacZ'*, complementation fragment of ß-galactosidase gene; amp, ampicillin; *ori*, origin of replication; *oriV*, broad host range origin of replication; *oriT*, origin of transfer; Ec, *Eco*RI; Sl, *Sal*I; Bg, *Bgl*II; Sa, *Sac*I; Kp, *Kpn*I; Sm, *Sma*I; Bm, *Bam*HI; Sc, *Sca*I; Xb, *Xba*I, Hc, *Hinc*II; Ps, *Pst*I; Sp, *Sph*I; Hd, *Hind*III; Av, *Ava*I; Ac, *Acc*I; Xm, *Xma*I; No, *Not*I; Pv, *Pvu*II.

A1.1. Vectors obtained from other sources

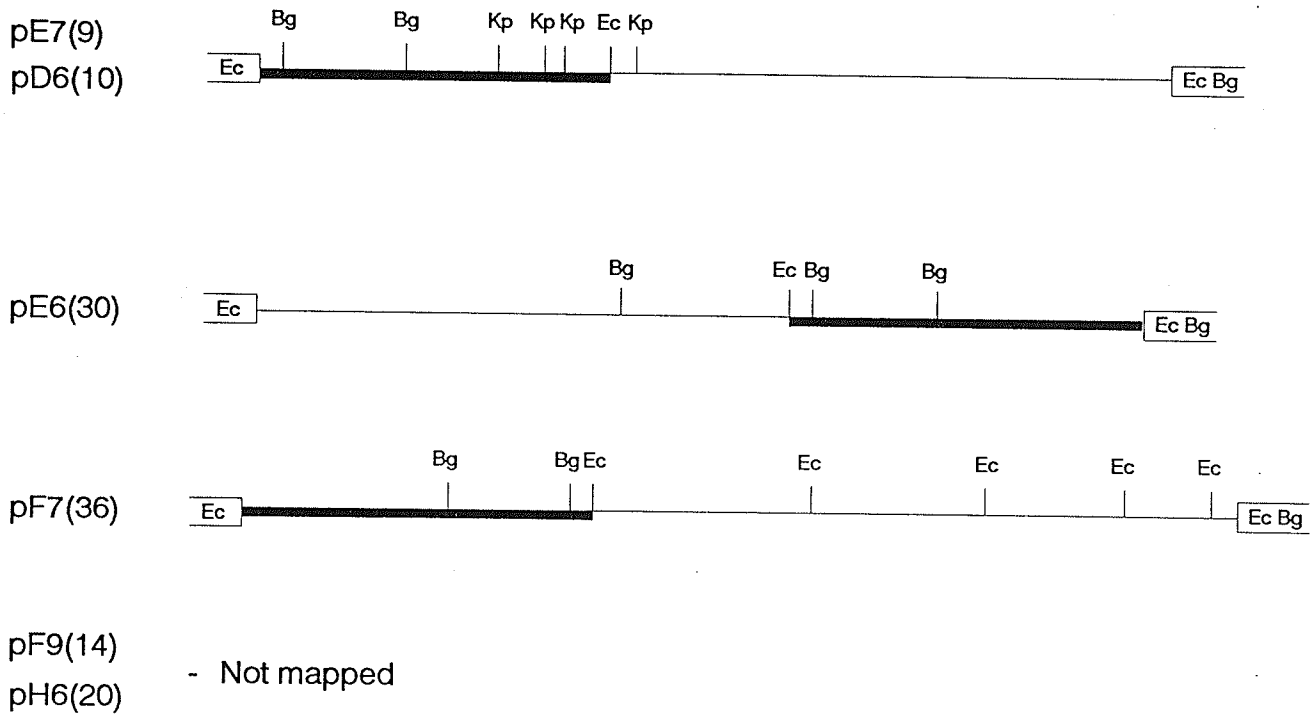


A1.1.1. Cloning and expression vectors



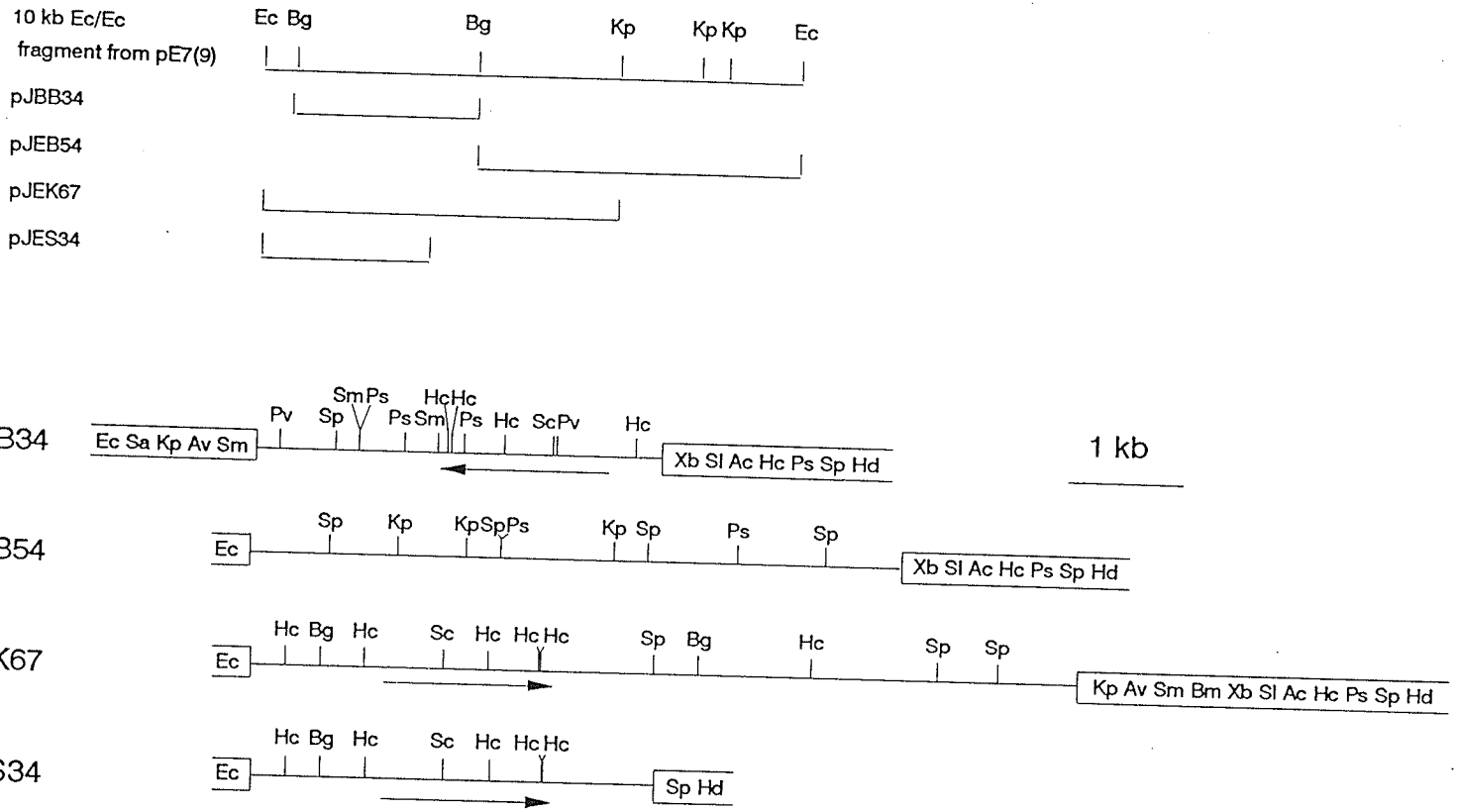
A1.1.2. Plasmids used for mutagenesis of OprB

A1.2. Plasmids constructed for this study

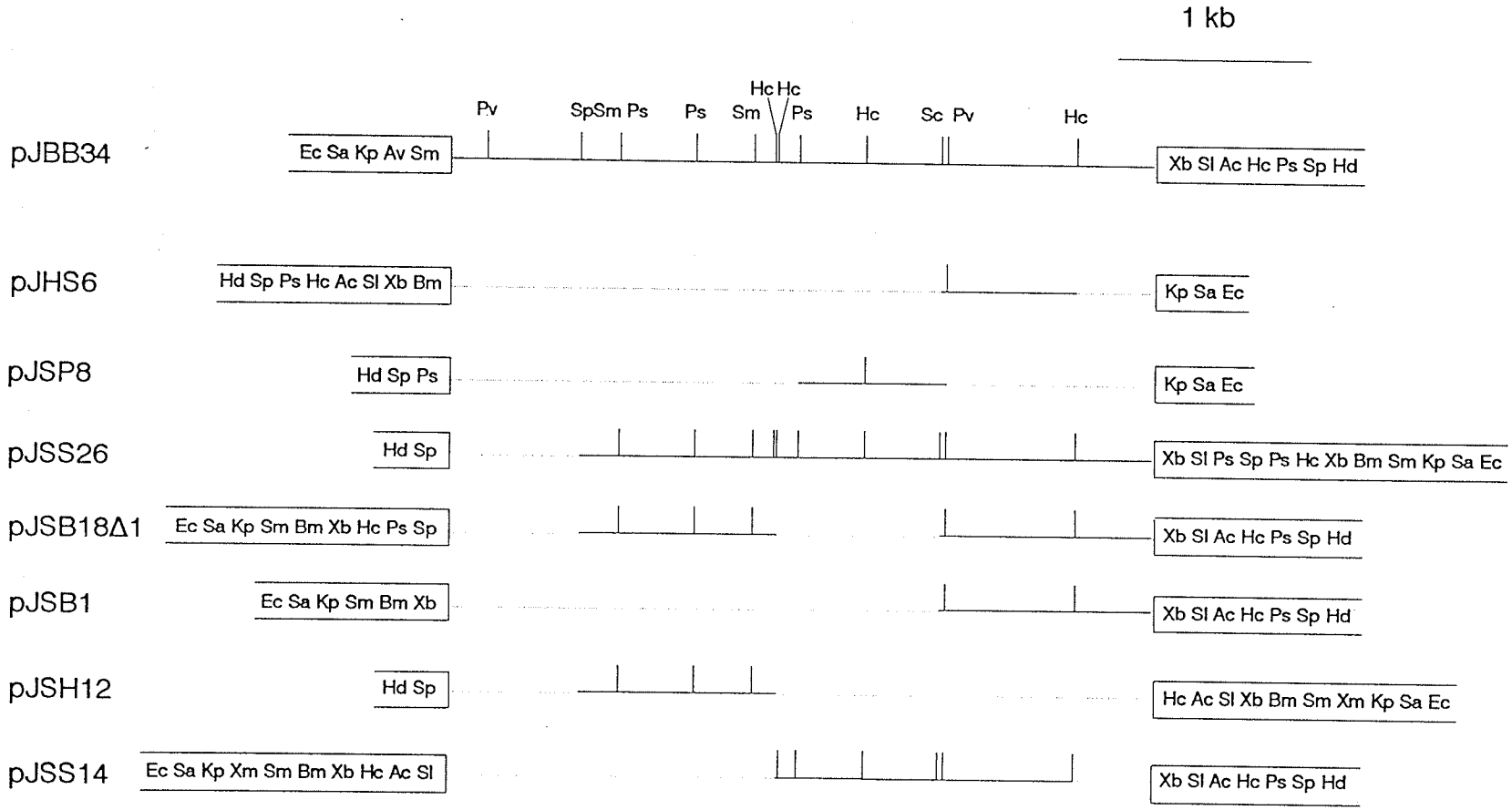


A1.2.1. P. aeruginosa genomic DNA subcloned in pLAFR1

One BglII site is indicated on the pLAFRI vector to indicate the orientation of the cloned fragment. The 10 kb EcoRI/EcoRI fragment, common to all plasmids, is in bold. The EcoRI sites of pF7(36) were not mapped and are ordered according to size.



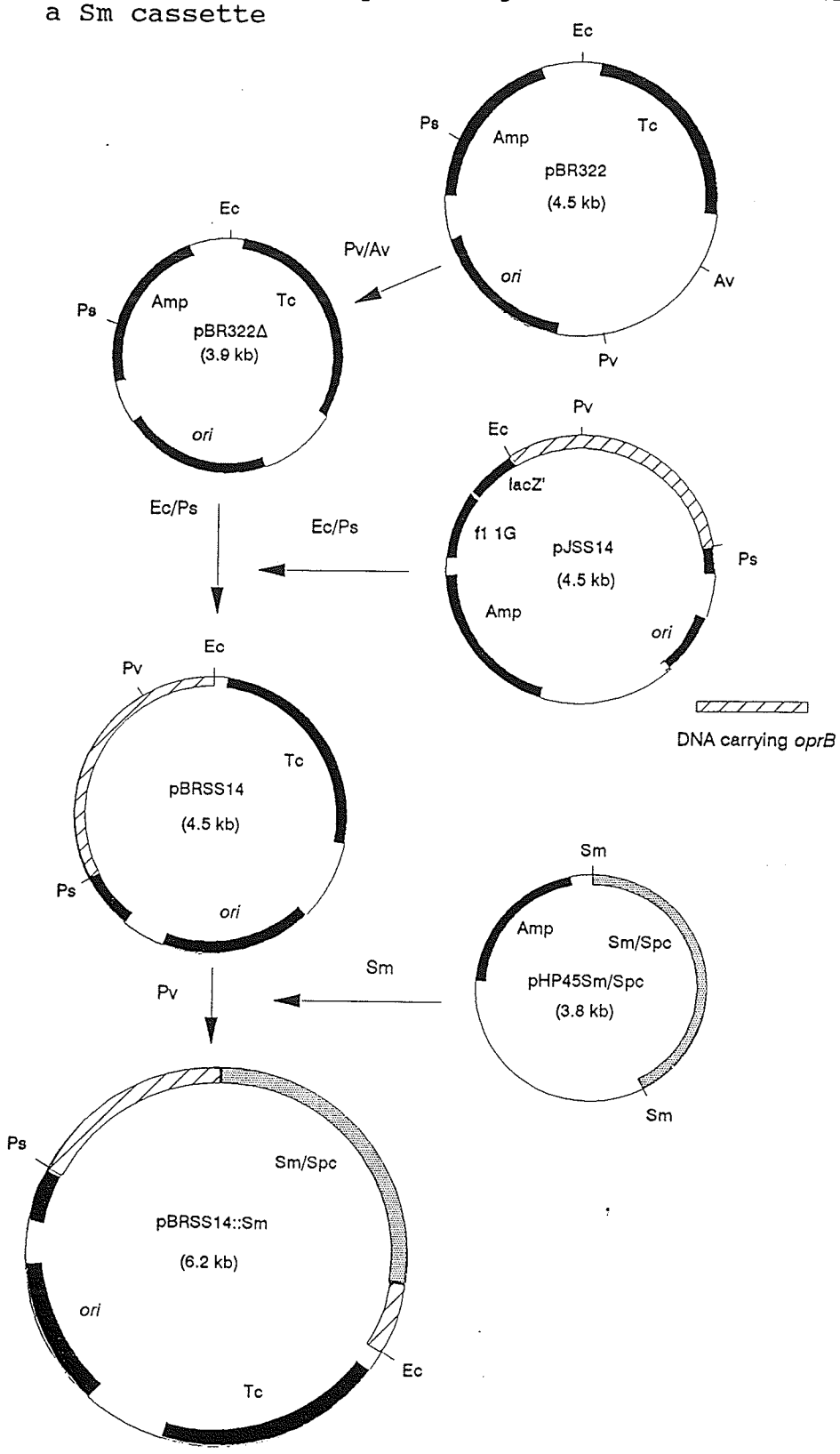
A1.2.2. Subclones of the 10 kb EcoRI/EcoRI fragment from pE7(9)

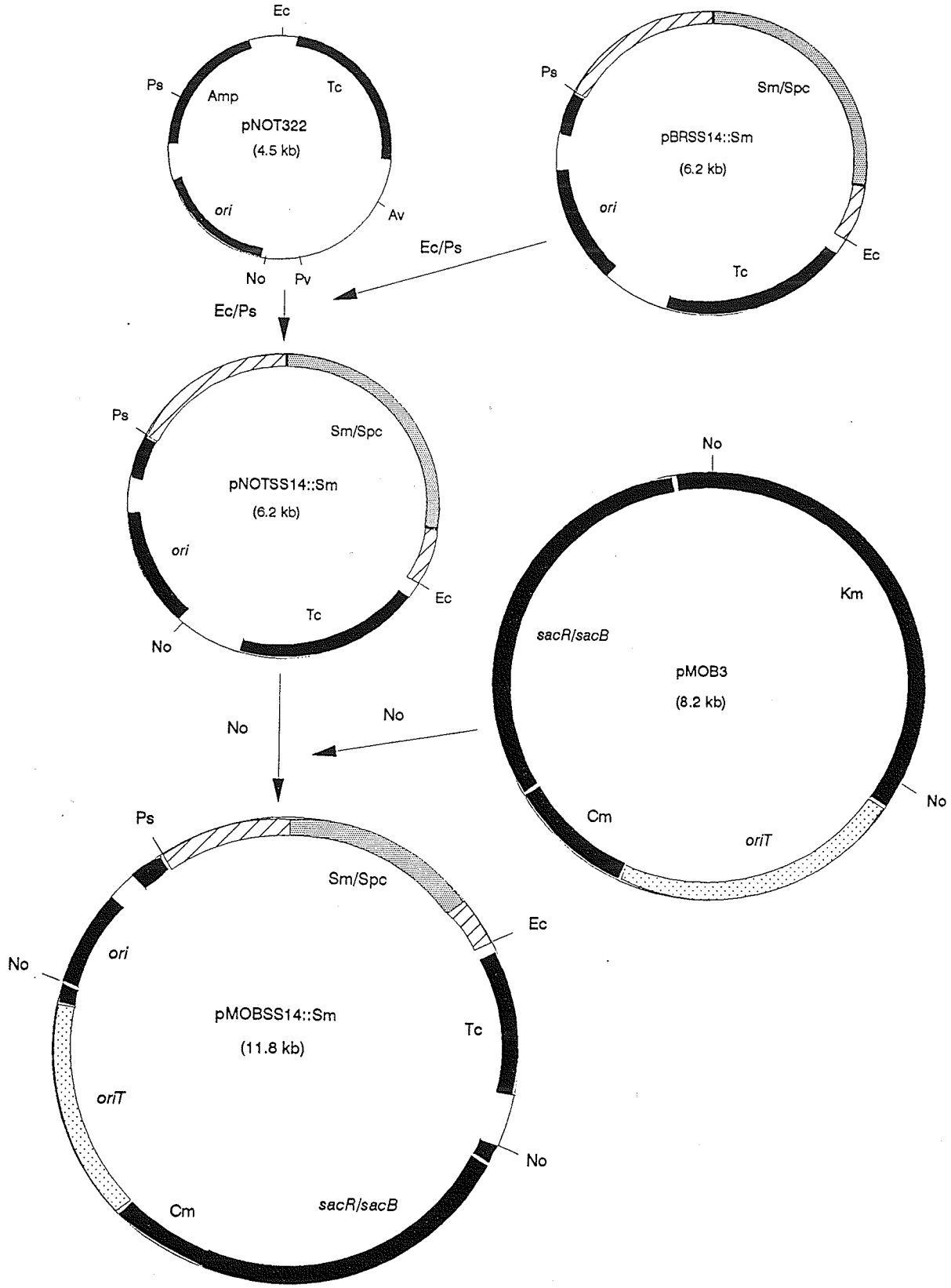


A1.2.3. Subclones of pJBB34

Fragments are shown in relation to their original position in pJBB34. Dashed lines indicate deletion of a fragment.

A1.2.4. Construction of an OprB-mutagenesis vector containing a Sm cassette




Ec
Ps
Amp
Tc
pNOT322
(4.5 kb)
ori
Av
No
Pv
Ec/Ps
Ps
Sm/Spc
pBRSS14::Sm
(6.2 kb)
ori
Ec
Tc
Ec/Ps
Ps
Sm/Spc
pNOTSS14::Sm
(6.2 kb)
ori
Ec
Tc
No
No
No
No
Km
sacR/sacB
pMOB3
(8.2 kb)
Cm
oriT
No
Ps
Sm/Spc
Ec
No
ori
pMOBSS14::Sm
(11.8 kb)
Tc
oriT
No
Cm
sacR/sacB

A1.2.5. Construction of an OprB-mutagenesis vector containing an unmarked deletion of *oprB*