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# THE OUTER MEMBRANES OF PSEUDOMONADS

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## 1. INTRODUCTION

The study of bacterial cell surfaces began in 1675 when Leeuwenhoek peering through his microscope wondered what "held [bacteria] together, or what contained them"[92]. Since that time, our knowledge of cell surfaces, namely the Gram-negative outer membrane, has grown considerably. In addition to containing the bacteria, the outer membrane mediates a myriad of functions including other structural roles such as maintaining bacterial shape and providing a scaffold for fimbriae and flagella. Another key function of the outer membrane is to mediate the interactions between Gram-negative bacteria and their environment, primarily by determining which compounds enter and exit the cell. This complex task involves the integration of lipidic components involved in the barrier function of the outer membrane and proteins involved in the uptake and efflux of the various compounds able to traverse this barrier.

Pseudomonads are intrinsically resistant to a large variety of toxic compounds including antibiotics, organic solvents, dyes, detergents, and heavy metals[40]. Yet, at the same time, these organisms are able to take up an astonishing array of metabolites. Therefore the *Pseudomonas* outer membrane has emerged as a prototype to study the intricacies of this dynamic organelle. This chapter covers the structural basis of *Pseudomonas* outer membrane impermeability. The current status of *Pseudomonas* porins (upto April 2003) involved in

*Pseudomonas, Volume 1*, edited by Juan-Luis Ramos
Kluwer Academic / Plenum Publishers, New York, 2004.

uptake is also discussed. We recently reviewed the structure, function, and genomics of the known and predicted *Pseudomonas aeruginosa* outer membrane proteins and refer the reader to this review for additional information[42].

## 2. STRUCTURE OF THE OUTER MEMBRANE

The outer membrane is an atypical bilayer (Figure 1). The inner leaflet is composed of phospholipids similar in composition to those of the cytoplasmic membrane while the outer leaflet contains few, if any, of these same phospholipids, but is primarily composed of the complex glycolipid lipopolysaccharide (LPS). Embedded in this bilayer are a series of around 160 transmembrane

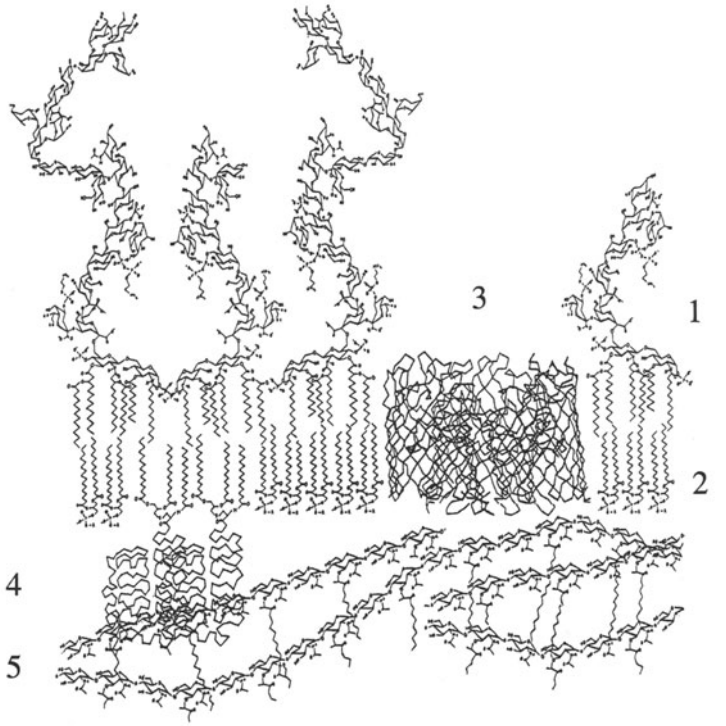


**Figure 1.** Representation of a typical *Pseudomonas* outer membrane. LPS molecules (1) comprise the majority of the outer leaflet. The inner leaflet is composed of phospholipids (2), mainly phosphotidylethanolamine. The outer membrane also contains proteins involved in transport, adhesion, membrane stability and recognition by the immune system. These proteins include porins (3) and lipoproteins (4). The lipoproteins anchor the outer membrane to the peptidoglycan (5) in the periplasm. This figure was originally published in ref. [43].

proteins, most of which have a β-barrel structure, and a few lipoproteins that are associated with one (usually the inner) monolayer. These components combine to confer on outer membranes their important properties that include the formation of a selective permeability barrier; participation in the relatively non-specific efflux, and specific export of molecules and proteins from cells; interaction with the immune system, immune cells, molecules and surfaces in the environment; an ability to exclude and resist enzyme attack; a structural role in cells; and the anchoring of structures like pili and flagella. We discuss below the specifics of the structure and functions of a subset of these outer membrane components. For a detailed description of the structure of outer membranes, we refer the reader to ref. [43].

One function of interest that is based on these components is antibiotic uptake. Some antibiotics pass across the outer membrane through the water-filled channels of porins (see below). In addition Gram-negative bacteria permit the passage of polycations across the outer membrane, using a system termed self-promoted uptake[38] that was first proposed based on studies of gentamicin and polymyxin B resistance in *P. aeruginosa* in 1981. In this hypothesis, polycationic compounds (i.e., those with two or more positive charges) such as trisaccharide aminoglycosides (the second most utilized antibiotics in hospitals), lipopeptide polymyxins (used intensively as topical agents), and cationic antimicrobial peptides, interact with bacterial cell surfaces at divalent cation binding sites on polyanionic LPS. These sites are normally involved in cross-bridging adjacent LPS molecules and stabilizing the outer membrane, but have 2–4 orders of magnitude higher affinity for the polycationic antimicrobials than for the native divalent cations (usually $Mg^{2+}$ or $Ca^{2+}$). Since the polycationic antimicrobials are far bulkier than the divalent cations they competitively displace, they lead to a disruption of the outer membrane through which probe molecules, and more importantly the disrupting polycations themselves, traverse the outer membrane. Thus the name "self-promoted uptake" stems from the concept that these polycationic antibiotics are promoting their own uptake (e.g., as opposed to diffusing through the channels of porins).

## 2.1. Phospholipids

The phospholipids in the inner leaflet of the outer membrane are similar in composition to those of Gram-negative bacterial cytoplasmic membranes, consisting primarily of phosphotidylethanolamine, with smaller amounts of phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), and other acidic phospholipids[84]. Some *Pseudomonas* species may also have ornithine amine lipids in their outer membranes. These lipids are analogous to phospholipids and in *Pseudomonas fluorescens*, are generally synthesized in phosphate limiting conditions[68, 96].

The fatty acyl tails of the phospholipids are predominantly palmitate (C16:0), with lesser amounts of oleate (C18:1), and minor amounts of palmitoleate (C16:1). An important difference between the Pseudomonads and other Gram-negative organisms is that the predominant unsaturated fatty acid in Pseudomonads is C18:1 instead of C16:1[75]. The predominance of a longer fatty acid could result in Pseudomonad outer membranes being more rigid than those of their Gram-negative counterparts.

In addition to the fatty acids mentioned above, *Pseudomonas* sp. produce an assortment of other fatty acids[84]. The saturated species range from C10:0 to C17:0 and may be terminally branched. The unsaturated fatty acids range from 15 to 18 carbon atoms. These fatty acids are usually monounsaturated with *cis*-double bonds being predominant. As well, there are some species that produce cyclopropane fatty acids (either C17:Δ or C19:Δ). If isolates are grown in controlled conditions, this diversity in fatty acid profiles between species can be used to differentiate between them.

*2.1.1. Changes in Fatty Acid Profile.* Bacteria can alter their fatty acid profiles to make their membranes more resistant to environmental stresses such as exposure to organic solvents, temperature, heavy metals, and nutrient limitation[87]. There are two means for achieving this result. The first is a short-term response involving the modification of the *cis*-unsaturated fatty acids into their *trans*-isomers by a periplasmic *cis–trans* isomerase. The second is a longer term response involving the differential synthesis of saturated fatty acids to increase their proportion in the membrane. Both mechanisms result in elongated fatty acid tails, which pack tightly leading to a more rigid membrane able to withstand harsher conditions while still maintaining transport activity.

## 2.2. LPS

The biosynthesis of Pseudomonad LPS will be described in detail in Chapter 1 of Volume 3 of this series. *P. aeruginosa* has two varieties of LPS molecules, the more-typical O-antigenic LPS, also called B-band LPS, and a common LPS referred to as A-band LPS (discussed later). LPS is a complex glycolipid with a tripartite structure. The endotoxic Lipid A moiety is inserted into, and is the major component of, the outer (surface) monolayer of the outer membrane. The structure of Lipid A is highly conserved among the Pseudomonads, consisting of a di-phosphorylated diglucosamine inserted into the membrane via several attached fatty acids. The core oligosaccharide is covalently attached to the Lipid A. Within a given species this region is similar in composition, and in addition to containing various sugars, in *Pseudomonas* harbours several phosphate molecules that together with anionic sugars and the Lipid A phosphates are the divalent cation binding sites of LPS.

The O-polysaccharide caps off about 10% of LPS molecules[43]. This component, comprising a repeated tri- to tetra-saccharide is exposed to the external environment and is the most variable one, giving each *Pseudomonas* serotype its unique immunogenic properties[75].

*2.2.1. Lipid A.* The inner portion of LPS is Lipid A. In *Pseudomonas*, this moiety consists of a phosphorylated β-1,6-glucosamine disaccharide that is usually substituted with phosphate monoesters at the C1 and C4′ positions. The disaccharide is anchored to the outer membrane by 6 or 7 fatty acyl chains linked through either ester or amide linkages[75]. These fatty acids are predominantly hydroxyl fatty acids, although there may be small amounts of saturated fatty acids as well. The major hydroxyl fatty acyl chains in *Pseudomonas* are 3-hydroxydodecanoate (C3OH-12:0) followed by 2-hydroxydodecanoate (C2OH-10:0) and trace amounts of 3-hydroxydecanoate (C3OH-10:0); this is in contrast to the Enterobacteriaceae where 3-hydroxytetradecanoate (C3OH-14:0) predominates. The shorter hydroxyl fatty acyl chains in *Pseudomonas* may serve to make their outer membranes more fluid than those of their Gram-negative counterparts. Additionally, the hydroxyl group of these fatty acyl chains may be substituted with either a palmitate (C16:0) or another 2-hydroxydodecanoate (C2OH-12:0)[43]. These substituents may influence the packing of the fatty acids and thus contribute to the fluidity of this region of the outer membrane, and its ability to intermediate in self-promoted uptake.

In addition to the major fatty acids mentioned above, the LPS molecules from different *Pseudomonas* species can contain minor hydroxyl fatty acyl chains. These fatty acyl chains range from 10 to 18 carbon atoms in length and may be saturated or monounsaturated, although saturated forms predominate[84]. Terminally branched hydroxyl fatty acids have only been detected in one species, *Pseudomonas rubescens*, to date. As with the unsaturated fatty acyl chains, the diversity of hydroxyl fatty acyl chains can be used to classify *Pseudomonas* species. However, as fatty acyl profiles can change under different environmental conditions, the isolates must be grown under carefully controlled conditions.

*2.2.2. Biological Role of Lipid A.* Lipid A has an ability to induce the mammalian innate immune system by interacting with Toll-like receptor 4 on the surface of immune cells. Included in this is a pro-inflammatory response involving among other things the up-regulation, largely through transcription factor NFκB, of the production of specific cytokines. If too large an LPS stimulus occurs (e.g., when *Pseudomonas* is treated with antibiotics causing it to release large amounts of LPS) a septic response, also called endotoxaemia, occurs and can lead to reduced blood pressure, organ failure, and death from endotoxic shock. Studies with synthetic Lipid A molecules indicate that the disaccharide backbone with phosphates placed at the C1 and C4′ positions is

required for activation. The number and length of the acyl chains also influence the extent of the response. One of the most potent Lipid A moieties examined to date is that of *Escherichia coli*, which has 6 acyl chains that are 14 carbon atoms in length[28]. The shorter hydroxyl fatty acid tails of the Pseudomonads' Lipid A moiety[52] are probably responsible for the moderately decreased toxicity of their Lipid A cf. enterobacterial Lipid A[83]. Other structural features of LPS, such as the core region, can also modulate the effect of Lipid A on the immune response[59].

The structure of Lipid A can change in response to environmental conditions. In *P. aeruginosa*[65] as with *Salmonella enterica* serovar Typhimurium[37], the PhoP–PhoQ two component regulatory system acts in response to low divalent cation ($Mg^{2+}$, $Ca^{2+}$) concentrations to promote *in vivo* survival of the bacteria. Under these conditions, in *Salmonella*, an operon encoding enzymes involved in adding 4-amino-4-deoxyarabinose residues to the Lipid A disaccharide is activated[103]. This modification renders the bacteria more resistant to cationic antimicrobial peptides, presumably by decreasing the net negative charge of LPS. *P. aeruginosa* LPS isolated from the lungs of cystic fibrosis patients or from strains grown under low $Mg^{2+}$ conditions is also modified with 4-amino-4-deoxyarabinose[27]. It is believed that the mechanism for this alteration is related to that of *Salmonella* as *P. aeruginosa* possesses a similar LPS modification operon (PA3552–3558, ranging from 61% to 81% similar to the equivalent genes in *S. typhimurium*) that is activated in response to cationic antimicrobial peptides (J.B. McPhee and R.E.W. Hancock, manuscript submitted).

*2.2.3. Core Oligosaccharide.* Attached to the Lipid A is the core oligosaccharide. The core components are common to all *Pseudomonas* species examined to date and consist of sugar molecules including galactosamine, rhamnose, glucose, L-glycero-D-manno-heptose and the unique octose 2-keto-3-deoxyoctulosonic acid (KDO). The core is also differentially substituted with alanine and phosphate molecules. The *Pseudomonas* core region is rich in phosphate molecules, containing approximately twice the amount found in the Enterobacteriaceae[75]. It has been suggested on immunological grounds that there are four core structures in *P. aeruginosa* but this may represent differential substitution or connectivity of the component sugars[82, 111]. A similar trend is seen in the Enterobacteriaceae[46].

The phosphate molecules in the core region contribute to the barrier function of the outer membrane. The phosphorylated LPS imparts a strong net negative surface charge to the bacterium, which is partly neutralized by $Mg^{2+}$ ions. This causes repulsion of strongly negatively charged substrates but permits the uptake of polycationic substances through the self-promoted uptake system. These sites are also presumably the sites for uptake of hydrophobic molecules, which can then be effluxed out of cells as described below.

*2.2.4. O-polysaccharide.* Capping off about 10% of LPS molecules and facing the external environment is the O-polysaccharide. This is the most diverse region of the LPS molecule and varies with respect to sugar composition, linkage, sequence, and branch length. A typical *Pseudomonas* O-polysaccharide chain is made up of repeating units of 3–5 sugars consisting of such sugars as glucosamine, glucose, rhamnose, fucosamine, and often amino hexuronic acids such as quinavosamine, 2-imidazolinomannuronic acid, and 2,3-diacetamido-2,3-dideoxyhexuronic acid[66].

*P. aeruginosa* is unusual among bacteria because most strains possess 2 forms of LPS, referred to as A-band and B-band LPS[60]. The B-band confers serotype specificity to each strain. The A-band is common to all strains (and has also been observed in other Pseudomonads) and is composed mainly of trisaccharide repeats of D-rhamnose, with minor amounts of 3-O-methyl rhamnose, glucose, ribose, and mannose. The chains of A-band LPS are considerably shorter than those of B-band LPS. Studies with monoclonal antibodies to the core region of B-band LPS suggest that these two species of LPS share a common core[90]. However, A-band chains contain sulphate instead of phosphate and have low levels of KDO and amino hexuronates. It is not clear whether there is a common Lipid A molecule for both LPS types.

The majority of LPS molecules on the cell surface are rough, in that the core is not capped with O-polysaccharide. Only 10% of the LPS molecules on a cell have an O-polysaccharide[43]. These smooth LPS molecules are of variable length and result in a characteristic ladder pattern when resolved on an SDS-PAGE gel[39].

The term smooth LPS is derived from smooth appearance that the O-polysaccharide confers on bacterial colonies. The LPS forms a capsule-like covering that can extend up to 40 nm from the cell surface into the environment[43]. Because of this exposure, the O-polysaccharide, specifically the B-band, is the major antigen recognized by the immune system. This antigenicity is the basis of the O-serotyping system for classifying Gram-negative bacteria. Currently, 20 O-serotypes in *P. aeruginosa* have been identified[82]. Smooth LPS is also involved in complement activation and inhibition by preventing the membrane attack complex from attaching to the cell. Rough mutants of *P. aeruginosa* are avirulent in animal models of infection indicating that the O-polysaccharide is required for pathogenicity[21]. Interestingly, strains isolated from chronically infected cystic fibrosis patients lack the O-polysaccharide, perhaps in an attempt to avoid detection by the immune system[14].

## 3. PROTEINS

The outer membrane has a myriad of other functions critical to bacterial physiology. These functions are mediated by different classes of outer

membrane proteins. The lipoproteins OprI, OprL, and the Tol system have structural and membrane stabilization roles, such as the maintenance of cell shape[63, 91]. Flagella and fimbriae are involved in cellular mobility and adherence. Porins mediate the selective uptake of a number of compounds ranging from small nutrient molecules to larger iron–siderophore complexes. Many antibiotics enter the cell through this route as well. There are also a large number of efflux and secretion systems responsible for exporting toxic compounds, proteins, DNA, virulence factors, and a variety of other substrates from the cell. Because of their location on the cell surface, these proteins, as well as many other outer membrane proteins, can act as adhesins, antigens, and receptors for phages and bacteriocins. We will only discuss the porins, as the other protein classes mentioned above are covered in other chapters. A more detailed discussion of the known outer membrane proteins of *P. aeruginosa* and especially the putative porins is found in Hancock and Brinkman[42].

## 3.1. Selective Permeability of the Outer Membrane

The outer membrane of *P. aeruginosa* is a formidable barrier for antibiotics and other large, hydrophobic molecules having a net permeability that is only 1–8% that of *E. coli*[38]. Only the water-filled channels of porins are available for the passage of small hydrophilic molecules, including substrates and antibiotics. In particular, the porin channels used by *P. aeruginosa* for nutrient uptake are either inefficient or highly specialized, and permit few substrates larger than a monosaccharide (~200 Da) into the cell[42].

There are four classes of porins. General porins allow the diffusion of a wide range of structurally diverse compounds into the cell. Specific porins facilitate the uptake of unique substrates via stereo-specific binding sites (Figure 2). Gated porins selectively take up large molecules such as iron–siderophore complexes. Efflux porins, or channel tunnels, work in conjunction with inner membrane pump and periplasmic linker proteins to expel toxic molecules from the cell. These latter proteins, forming a family of 18 proteins related to OprM[42] are covered in more detail in Chapter 21 in this book.

It should be noted that the outer membrane does not completely exclude either small hydrophilic or hydrophobic compounds from the cell. Antibiotics eventually do equilibrate across the *P. aeruginosa* outer membrane, albeit at a much slower rate, than observed with other bacteria. The lower rate of entry ensures that secondary resistance mechanisms such as efflux pump systems (with the major efflux system involved in intrinsic antibiotic resistance being MexAB–OprM for many antibiotics and MexXY–OpmG for aminoglycosides) and β-lactamases work efficiently, and are not overwhelmed by high

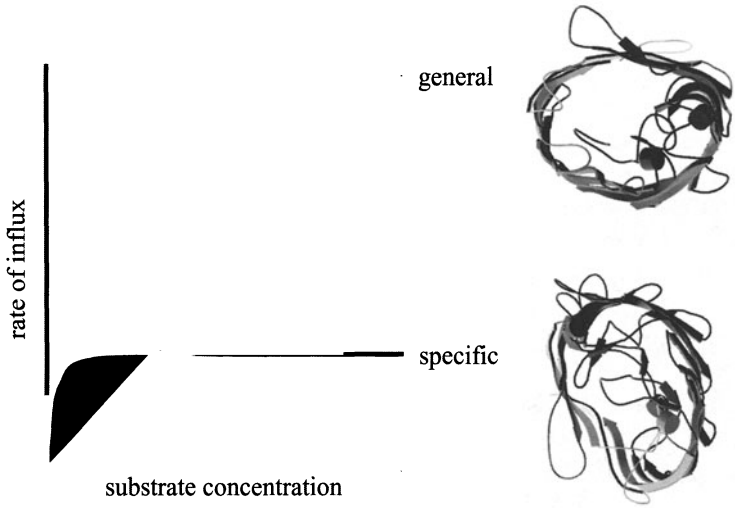


**Figure 2.** Uptake through general and specific porins. Potential substrates of a given size and charge are able to pass through general porins. The rate of uptake through these porins is proportional to the substrate concentration. Shown here is the top, extracellular view of a monomer of the trimeric PhoE anion selective porin of *E. coli* ref. [19]. Specific porins have substrate-specific binding sites that may be formed in part by extracellular loops that fold into the channel mouth as with the *E. coli* maltodextrins-specific LamB channel ref. [91]. Uptake through specific porins is accelerated at low substrate concentrations and plateaus at substrate concentrations high enough to saturate the binding sites of these channels.

concentrations of antibiotics[38]. This then provides the major basis for the high intrinsic resistance of *P. aeruginosa* to antibiotics, a major factor in the known clinical resistance to therapy of this organism.

## 3.2. General Porins

In Gram-negative bacteria, most hydrophilic compounds traverse the outer membrane via non-specific (general) porins. These channels are considered passive since transport is dependent on the solute's physicochemical properties. The diameter of the water-filled space in the most constricted part of the channel determines the size of molecule that can pass through, that is, the exclusion limit. In the prototypic bacterium, *E. coli*, the major porins are OmpF and OmpC and these permit molecules up to 600 Da to diffuse through the outer membrane. The external vestibules of non-specific porin channels are rich in charged amino acids, and there are also charges around the constriction zone[54], imparting some selectivity based on the charge of the solute. General porins are able to support the rapid influx of solutes in nutrient-rich

conditions. Under these conditions, the incoming molecules can be either rapidly metabolized by enzymes in the periplasm or transported to the cytoplasm via high affinity cytoplasmic membrane transporters, thus maintaining a large concentration differential between the two faces of the outer membrane.

The *Pseudomonads* are different from many Gram-negative organisms because of their lack of general porins with homology to the non-specific porin family. This results in a generally low outer membrane permeability, although in apparent contradiction to the exclusion limit of *P. aeruginosa* which is relatively high (in the order of 3,000 Da)[9]. In *P. aeruginosa*, whose porins have been the most extensively characterized, two porins apparently provide the ability to permit passage of general substrates. OprD, is a specific porin, but appears to be the major conduit for small molecules of less than 200 Da[48]. OprF, a member of the OmpA family of outer membrane proteins has an apparently large exclusion limit, but demonstrates functional heterogeneity with only a small proportion of the OprF molecules forming large channels. It is probably responsible for the permeation of molecules between 200 and 3,000 Da[9]. In addition, OprB appears to be the major conduit for saccharides[114]. The function of another putative porin, OprG, is not clear. The lack of members of the general porin family in *Pseudomonas* and the relatively limited expression of other porins, also contributes to the high intrinsic resistance these organisms display towards many toxic compounds.

*3.2.1. OprF.* OprF shares C-terminal homology with OmpA and thus is a member of the OmpA superfamily of porins[24]. It is one of the major *Pseudomonas* outer membrane proteins having a copy number of approximately 200,000 per cell[8]. Because it is so abundant, there has been considerable interest in developing OprF as a vaccine candidate[20, 86, 107]. In addition, OprF plays a number of roles important for cellular survival.

3.2.1.1. OprF Structure. Cross-linking studies indicate that OprF is an oligomer, possibly a trimer that is associated with both LPS and peptidoglycan[5]. The protein consists of three domains: the N-terminus, a hinge region, and the C-terminus. A three dimensional model of the N-terminus of OprF (the first 160 amino acids) was constructed based on the homologous regions of the N-terminus of OmpA, indicating that this region is an eight stranded β-barrel[13]. This model is in agreement with data obtained by circular dichroism spectroscopy.

A hinge region from amino acid 161 to amino acid 209 joins the C- and N-termini. This hinge and loop region contains multiple proline–alanine repeats and in *P. aeruginosa*, has two disulfide bonds, although this feature is not conserved in other Pseudomonads. We have previously suggested that alternative disulphide bonding of these four cysteine residues explains the two alternative forms and channel sizes observed in planar bilayer experiments with OprF[9].

The C-terminus of OprF shares considerable similarity with that of OmpA (56%). By analogy with certain data for OmpA, it has been proposed that the OprF C-terminus (amino acids 210–326) may be a globular domain that lies in the periplasm since it is involved in peptidoglycan binding. However, there is considerable evidence suggesting that this region, or a portion of it, is surface exposed. First, there is strong bioinformatic evidence indicating that two regions in the C-terminus are β-strands[41]. Second, the C-terminus has been used to generate surface reactive monoclonal antibodies[50] and there have been numerous reports of surface reactive monoclonal antibodies recognizing this region[20, 108]. Indeed the C-terminal region of OprF is being considered as a component of a vaccine for *P. aeruginosa*. Third, the *P. fluorescens*[23] and *P. aeruginosa* OprF proteins[71] have surface accessible protease cleavage sites. One possibility is that this region can translocate to the surface and that this impacts on channel size. Interestingly OmpA also appears to demonstrate two protein forms with different channel diameters[100].

3.2.1.2. A Structural Role for OprF. *P. aeruginosa* mutants deficient in OprF synthesis have an almost spherical appearance, are shorter than wild-type cells and do not grow in low osmolarity medium[112] suggesting that like OmpA, OprF is involved in maintaining cell shape. Analysis of OprF mutants with truncated C-termini showed that it is this region that is involved both in binding to peptidoglycan and the above structural roles[88].

3.2.1.3. OprF as a General Porin. Planar bilayer methods have shown that *P. aeruginosa* OprF is a non-specific, weakly cation-selective channel with one of two channel sizes. The channels can be either small (0.36 ns) or relatively large (2–5 ns)[9]. This large channel size appeared to some researchers to be in contradiction with the measured low permeability of the *Pseudomonas* outer membrane, and was a major source of controversy. However, intact cell studies involving providing *P. aeruginosa* with a raffinose metabolism system, and measuring the ability to grow on large sugars, as well as plasmolysis experiments, confirmed a large exclusion limit and the role of OprF in determining this property. This dilemma was resolved by the demonstration that only a small proportion of OprF channels (approximately 400 out of 200,000)[13] form the large size channels and that outer membrane permeability of smaller molecules (the size of most *P. aeruginosa* substrates) is managed by low abundance porins such as OprD and OprB.

Interestingly, the full length OprF protein is required for large pore formation. Mutants with C-terminal truncations in OprF only form the smaller sized pores[13], suggesting that OprF adopts a different conformation for the larger channel size that probably would involve the participation of the C-terminal half[88].

3.2.1.4. Role of OprF in Pathogenesis. The OprF proteins from both *P. aeruginosa*[6] and *P. fluorescens*[23] have both been reported to be involved in adherence to surface receptors in their respective hosts. The homologue in *P. fluorescens* is a fibronectin-binding protein[89]. Recent work has suggested another role for OprF in human infection. Expression of this porin is substantially downregulated in aerobic biofilms compared to anaerobic biofilms, which are presumed to exist in the lungs of infected cystic fibrosis patients. Both OprF and OprF antibodies have been isolated from the mucus of chronically infected patients[115]. Additionally, OprF expression appears to be under the control of the AlgU regulator that is responsible for alginate production and conversion of *P. aeruginosa* strains to mucoidy[33], as well as the ECF sigma factor SigX[14]. OprF mutants form poor anaerobic biofilms. One reason for this defect is that these mutants also do not possess nitrate reductase activity, which is required to form anaerobic biofilms, therefore, it has been proposed that OprF may be required for the uptake of nitrate or nitrite into the cell[45]. Alternatively, the defect may reflect the structural defect in OprF null mutants. These results have reinforced an interest in developing OprF as a vaccine component.

It is not clear whether OprF plays other roles in infection. Clinical isolates that lack OprF and are resistant to multiple antibiotics have been isolated[14]. It is possible that OprF is downregulated in response to antibiotic treatment once an infection has been established. This strategy would enable the bacteria to evade the effects of both the immune system and antibiotics.

*3.2.2. OprG.* OprG was the last major *Pseudomonas* outer membrane protein to be identified. It first drew attention because of its complex regulation. An inverse relationship was noted when *P. aeruginosa* was grown in low iron conditions, therefore it was proposed that this porin functioned in iron uptake. Additionally, OprG expression was affected by entry into stationary phase, higher growth temperatures, low $Mg^{2+}$ concentrations, alterations in LPS structure, and the presence of various carbon sources[36]. Unpublished results from our laboratory have shown that OprG is regulated by ANR but not DNR suggesting that this porin functions during anaerobic conditions but independent of denitrification (J.B. McPhee and R.E.W. Hancock, unpublished results).

Indeed, attributing a function to this elusive protein has proven difficult. It was proposed that OprG may be involved in fluoroquinolone resistance as increased resistance to norfloxacin can be associated with a loss of OprG. However, these two phenotypes are not obligatorily linked as we have shown that OprG knockout mutants have no antibiotic resistance phenotype. Additionally, despite its similarity to other eight-stranded β-barrels, surface exposed proteins with functions in serum resistance and cell adherence[16, 109], no role for OprG in similar activities was detected.

## 3.3. Specific Porins

In contrast to other Gram-negative organisms that only use specific porins for the uptake of large, bulky substrates such as maltodextrins and nucleotides[10], Pseudomonads appear to almost exclusively use specific porins for the uptake of small molecules. There are a large number of specific *Pseudomonas* porins, with many of them having paralogues (between 1 and 18 sequence-related proteins) in the same organism[73, 99] (www.cmdr.ubc.ca/bobh/omps/). These paralogous families are a unique feature of the Pseudomonads and underlie the importance of this class of proteins to these organisms.

Specific porins have saturable stereo-specific binding sites for their substrates and uptake follows Michealis–Menten kinetics. Therefore, uptake is accelerated at low substrate concentrations and plateaus when the sufficient substrate is present to saturate the binding sites. If uptake under nutrient limiting conditions was solely mediated by diffusion, the high affinity active transport systems in the cytoplasmic membrane would be at most 5% saturated, making transport the rate-limiting factor for growth[47, 74]. Therefore, having specific porins gives organisms a competitive edge in environments deficient in nutrients. In addition, the specific porins of *Pseudomonas* can act as general selectivity filters by taking up low levels of structurally diverse compounds[48], thus complementing the low uptake activity of the OprF general porin.

*3.3.1. Structure of Specific Porins.* Despite very little sequence similarity, the primary sequences of porins share several conserved features that result in strikingly similar tertiary structures[19, 58, 95]. Overall, the sequences are rich in charged amino acids. There are no stretches of hydrophobic residues; instead there are several regions that have 12–25 alternating polar and non-polar amino acids flanked by aromatic residues[54]. In the porins with known structures, these amphipathic regions correspond to the β-strands that make up the walls of the channel, with the aromatic residues anchoring the porin into the membrane. The β-strands are connected at the periplasmic end by short periplasmic turns and at the extracellular surface by longer loops. These loops are the most variable regions among the porin superfamily and they largely determine the properties of the porin. The loops are involved in stabilizing the porin by interacting with LPS and the other porin monomers. As well, they modulate uptake activity by folding into the channel mouth to constrict the opening[94]. The C-terminal β-strand of these porins is the most conserved region among these proteins[51] with the terminal amino acid generally being an aromatic residue. Since these sequence characteristics appear to be shared among the specific porins of *Pseudomonas*, it is assumed that the tertiary structure will be conserved as well.

Cross-linking studies[5] suggest that *Pseudomonas* porins are composed of three subunits. Topological analysis of these porins, involving insertion and

deletion mutagenesis are consistent with the interpretation that each subunit comprises a β-barrel made up of 16 β-strands[49, 101]. This is reminiscent of the general porins of other Gram-negative organisms, but in contrast to the structure of the maltodextrins-specific porin LamB, which contains 18 β-strands per monomer.

The substrate-binding site is formed in part by some of the extracellular loops. In several porins, the longest loop, the third one, plays a critical role in substrate binding[49, 54, 102]. In addition the barrel walls may contain residues that are important for facilitating passage of the substrate, similar to the greasy slide motif of the LamB porin of *E. coli*[106].

*3.3.2. OprB.* In *P. aeruginosa*, glucose and other monosaccharides (xylose, mannitol, fructose, and glycerol) enter the cell via the OprB porin. These porins are also present in *Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas chloraphis*, and *Pseudomonas syringae* and for the first three organisms have been shown to facilitate the entry of glucose into cell, although their specificity for other carbohydrates differ[2]. OprB is the closest homologue the Pseudomonads have to the maltodextrins-specific LamB porin of *E. coli* and shares some structural features with the latter porin. Modelling of OprB suggests that like LamB, it has 18 β-strands and a cluster of aromatic residues resembling the greasy slide of LamB[41]. The aromatic rings in this motif provide Van der Waals contacts for the incoming pyranose rings and guide them through the channel. Circular dichroism spectropolarimetry of the functionally characterized OprB proteins has shown that the β-sheet content in these porins varies from 31% to 50%[2], suggesting that there may be slight structural differences in these channels that may account for the differences in secondary substrate and selectivity. The difference in ion selectivity among the *P. putida* (cation) and *P. aeruginosa* (anion) OprB channels also suggests that there may be structural variability among these proteins[113].

OprB is positively regulated in response to glucose[4]. In *P. aeruginosa*, the regulation is mediated by a two component regulatory system consisting of the GltR transcriptional activator[3] and an as of yet uncharacterized sensor kinase putatively encoded by PA3191. Also, OprB expression is under catabolite repression control and is downregulated in the presence of citric acid cycle intermediates[4].

Interestingly, both *P. aeruginosa* and *P. putida* have two OprB paralogues. In both organisms, the gene corresponding to the previously studied *oprB* is in an operon with genes encoding homologues of the high affinity glucose uptake system. The newly discovered *oprB* homologues (*opbA* in *P. aeruginosa*[99] and *oprB2* in *P. putida*[73]) are downstream of a glucose dehydrogenase gene. Glucose dehydrogenase plays a role in the low affinity uptake pathway for glucose[67]; whether the two OprB homologues are also involved in this pathway, remains to be determined.

*3.3.3. OprP and OprO.* OprP is the phosphate-specific porin of the Pseudomonads. It is induced under phosphate limiting conditions (i.e., 0.15 mM) by the PhoB regulator, which also controls the expression of a periplasmic phosphate binding protein[44]. This co-regulation, together with the fact that an OprP knockout mutant is deficient in high affinity phosphate-inducible transport[41] suggests that the transport systems in the outer and cytoplasmic membranes interact to bring phosphate into the cell, although there is no direct evidence supporting this assumption.

The molecular architecture of OprP has been probed using a variety of techniques. Predictions based on the OprP amino acid sequence as well as insertion mutagenesis suggest that OprP has 16 β-strands per monomer[101]. A lysine residue $K_{121}$ in the proposed loop 3 was shown by site-directed mutagenesis to be involved in phosphate binding and passage through the OprP channel. Two other lysine residues, one in loop 3, $K_{126}$, the other in transmembrane region 4, $K_{74}$, modulate rather than destroy phosphate binding and thus may represent secondary binding sites[102].

*P. aeruginosa* has an OprP homologue called OprO that is 76% similar to OprP[98]. The *oprO* gene is immediately upstream of *oprP*. Like OprP, OprO is induced during periods of phosphate starvation, and presumably regulated by PhoB, but it is only produced after growth into stationary phase. Unlike OprP, OprO preferentially binds pyrophosphate.

In addition to its OprP homologue, *P. fluorescens* possesses another phosphate starvation-inducible outer membrane protein called Ag1. Based on N-terminal protein sequencing results, this protein is not homologous to any known protein. When resolved on an SDS-PAGE gel, its apparent molecular weight, 55 kDa, is greater than that of OprP (48 kDa) and in contrast to OprP, its migration is not affected by different solubilization temperatures. Whether this protein is indeed an OprP homologue with an unusual N-terminal sequence, or a porin, remains to be determined[62].

*3.3.4. OprD Family.* The OprD porin has been studied quite extensively due to its specific role in antibiotic resistance[56, 81]. This porin facilitates the passage through the outer membrane of the basic amino acids, lysine and arginine, and small peptides containing these amino acids[105]. In addition it is also the major route of entry for the structurally related carbapenem antibiotics imipenem and meropenem. A variety of techniques have shown that these substrates compete for the same binding site in the channel[104]. In addition, OprD acts as a general porin by permitting the passage of structurally unrelated small molecules such as gluconate into the cell[48].

OprD shares 15% amino acid sequence similarity with its closest *E. coli* homologue OmpF. Alignment of these two proteins suggested that the OprD monomer consisted of a 16-stranded β-barrel. This model was tested and refined by constructing OprD variants with short 4–8 amino acid deletions in

the putative loop regions[49, 76]. Further analysis of the deletion mutants has shown that both loops 2 and 3 are involved in substrate binding[76]. Loop 7 may also have a role in substrate binding since the OprD proteins of clinical isolates resistant to meropenem have several amino acid substitutions in this region as well as a two amino acid deletion[26]. Mutants with deletions in loops 5, 7, and 8 have larger channels and permit the passage of multiple antibiotics[49]. Therefore, these regions are thought to fold into or over the channel and thereby constrict the opening. In contrast to most other porins, the last OprD amino acid residue is a leucine rather than an aromatic residue, but how this contributes to the overall tertiary structure or folding remains to be determined.

The importance of OprD to the cell is highlighted by its complex regulation. It is induced by its substrate arginine via the ArgR regulator. Alanine and glutamate induce *oprD* independently of ArgR through an as of yet uncharacterized regulator[77]. OprD is also under the control of catabolite repression[78]. Additionally, imipenem stimulates the MexT regulator to repress *oprD* levels and induce the *mexEF–oprN* efflux operon, which extrudes carbapenems and quinolones[55]. Salicylate, a compound released by plants upon infection, represses OprD via an unidentified regulator[78].

The release of relevant genome sequences lead to the finding that OprD is the prototype of a large family of porins. There are 19 OprD homologues in *P. aeruginosa*[99], 21 in *P. putida*[73], 10 in *P. syringae*, and 10 in *P. fluorescens*, plus many more in Pseudomonads whose genomes have yet to be sequenced. The closely related soil bacterium *Azotobacter vinelandii* also boasts a large number of OprD homologues. The genomic context of these homologues indicates their involvement in the uptake of a variety of metabolites. The *P. putida* family has a large number of OprD homologues predicted to take up aromatic compounds. Phylogenetic analysis of the *P. aeruginosa* OprD family shows that members of this family belong to one of two subfamilies[42]. One subfamily contains OprD and the homologues in this group that have been studied are involved in the uptake of amino acids and peptides. The members of other subfamily are most similar to the PhaK, phenyl acetic acid uptake porin of *P. putida*[80]. It was originally predicted that members of this cluster would take up aromatic hydrocarbons. However, subsequent analysis of some of these homologues has shown that they are involved in the uptake of a variety of organic compounds, such as dicarboxylic acids, that better reflect the metabolic capabilities of *P. aeruginosa* (S. Tamber and R.E.W. Hancock, unpublished results).

## 3.4. Gated Porins: TonB-Dependent Receptors

Iron, an essential cofactor of many proteins involved in aerobic respiration, is an indispensable micronutrient for virtually all life forms. However, this

mineral is often limiting in environmental niches due to the low solubility of the ferric ion, and the high degree of competition from other organisms. The importance of iron to the microbial lifestyle is highlighted by the diversity of iron acquisition strategies they have evolved[12, 29]. *Pseudomonas* species produce a variety of siderophores, which are large molecules that bind strongly to $Fe^{3+}$ (see refs [18], [72]). These molecules have different affinities for the ferric ion and bind to specific surface receptors/uptake porins, thus allowing organisms to tailor their iron uptake capabilities to their particular niches. In addition, to their own siderophores, Pseudomonads can take up heterologous siderophores produced by other bacteria and fungi[85]. For example, *P. aeruginosa* also has the ability to acquire iron directly from haem and haem-containing proteins such as haemoglobin[79], as well as the *E. coli* siderophore enterobactin.

Siderophore–iron complexes enter the cell through specialized receptor/porins termed gated porins, or IROMPs (iron repressible outer membrane proteins). These gated porins comprise a large family with 35 homologues in *P. aeruginosa*[99], 29 in *P. putida*[73], 23 in *P. syringae*, 26 in *P. fluorescens*, and countless others from *Pseudomonas* species whose genomes have not been sequenced yet. Due to their demonstrated or assumed dependence on a particular energy-transducing system they are called the TonB-dependent family. Generally speaking, it is assumed that each channel is specific for a particular siderophore, explaining the great diversity of gated porins in *Pseudomonas*. This chapter will only cover those TonB-dependent receptors that have been functionally characterized.

*3.4.1. Structure of TonB-Dependent Receptors.* The crystal structures of two *E. coli* TonB-dependent receptors, FhuA and FecA show that these proteins are monomers consisting of 22-stranded β-barrels[30, 32]. Near the N-terminus of these channels is a globular domain, termed the plug, which contains a four-stranded β-sheet and four α-helices that form part of the gating mechanism. The plug is held in the opening of the channel by 9 salt bridges and more than 60 hydrogen bonds, and is thought to undergo a conformational change upon substrate and TonB engagement that leads to the opening of the channel[31].

The crystal structures of the above TonB-dependent receptors both with and without their substrates have been solved and suggest a functional uptake mechanism for this class of proteins. First, the siderophore docks into a binding pocket rich in aromatic residues on top of the plug domain. An arginine residue in the pocket then shifts towards the substrate. This shift results in a greater conformational change throughout the protein culminating in the unwinding of an α-helix at the periplasmic side of the channel and the movement of a peptide segment from the opposite barrel wall. Presumably, these changes serve as a signal to the TonB protein in the periplasm. Once the TonB protein recognizes that the channel is loaded with substrate, it is then proposed to utilize energy to induce

another series of conformational changes that both release the substrate from the binding pocket and produce a translocation pathway[30, 32, 61].

*3.4.2. Pyoverdin Receptors.* The pyoverdins comprise a large family of siderophores. The basic features of these molecules include a dihydroxyquinolone moiety linked by either a carboxylic acid or a carboxy-amide to a peptide region that varies among different *Pseudomonas* species. Pyoverdin has a very high binding affinity for the ferric ion. It is the predominant siderophore of the fluorescent Pseudomonads and it is this molecule that gives them their fluorescent green colour[1, 85].

*P. aeruginosa* strains synthesize three different pyoverdin types, each type having its own receptor[22]. The type I pyoverdin receptor, FpvA, has been the most extensively characterized and serves as the prototype for this subfamily. An alignment of the FpvA amino acid sequence against TonB-dependent receptors of other Gram-negative organisms predicts that the FpvA monomer has 26 β-strands[35]. Two residues in the sixth loop, Y350 and A402, have been implicated in ferric-pyoverdin binding and uptake[53]. Also, FpvA possesses a 70 amino acid N-terminal extension that is required for the induction of its own expression as well as that of the pyoverdin biosynthetic operon[97].

The uptake mechanism through FpvA differs significantly from the TonB-dependent receptors of other Gram-negative organisms[93]. In its resting state, FpvA is bound to iron-free pyoverdin[34]. The iron-free pyoverdin is displaced by ferric-pyoverdin, which then enters the cell. Since FpvA binds both iron-free and iron-loaded pyoverdin with equivalent affinities, it is believed that TonB mediates the displacement.

The pyoverdin receptors of *P. putida*, PupA[11] and PupB[57], and of *P. fluorescens*, PbuA[69] have been cloned and characterized. A pyoverdin receptor from *P. syringae* has also been described but has not yet been cloned[17].

*3.4.3. Other TonB-Dependent Receptors.* Generally one TonB-dependent receptor is specific for the uptake of one type of siderophore. Given the large number of siderophores, both endogenous and heterologous, utilized by *Pseudomonas* species, the equivalently large number of TonB-dependent receptors in these organisms comes as no surprise. Some members of the TonB-dependent family are conserved among the Pseudomonads, while others are species specific and thus may be involved in the adaptation of these organisms to their particular niches[18].

Many *Pseudomonas* species produce secondary siderophores that have considerably lower affinities for $Fe^{3+}$ than pyoverdin. Pyochelin, produced by *P. aeruginosa* and *P. fluorescens*[15], binds to iron, cobalt, and molybdenum. This siderophore is taken up by the FptA receptor. In *P. aeruginosa*, pyochelin is involved in the acquisition of iron from transferrin, and FptA has been

implicated in virulence[110]. Quinolobactin[70], another siderophore produced by *P. fluorescens* is taken up by a 75-kDa outer membrane protein that is induced by quinolobactin and repressed by pyoverdin, illustrating the siderophore hierarchy in this organism.

In addition to their own siderophores, Pseudomonads can use siderophores produced by other Pseudomonads, other bacteria, and fungi[64]. Again there is a specific receptor that is induced by and takes up a particular siderophore. Since the energy expenditure required to produce many different siderophores is avoided, the ability to use heterologous siderophores gives bacteria a competitive edge in the environment. However, TonB-dependent receptors can also serve as a point of entry for bacteriocins produced by closely related organisms[7].

*P. aeruginosa* can take up enterobactin, the major *E. coli* siderophore, via the PfeA receptor[25]. PfeA shares 60% sequence identity with the *E. coli* enterobactin receptor, FepA and can complement an *E. coli* FepA mutant. PfeA is unusual among the characterized *Pseudomonas* TonB-dependent receptors because it contains a TonB box. Whether this motif is important for uptake activity has not been determined. *P. aeruginosa pfeA* mutants can still take up reduced levels of enterobactin, indicating that a second enterobactin uptake system exists in this organism[85]. Indeed, there is a candidate protein for this role, PirA (PA0931), which shares 72% sequence similarity with PfeA.

In addition to PfeA and PirA, there are several other paired members of the TonB family in *P. aeruginosa, P. putida*, and *P. fluorescens* that exhibit greater than 50% sequence similarity to each other. This redundancy may contribute to the overall flexibility of these organisms, allowing them to optimize uptake in a variety of different niches or take up sequence-related variants of a given siderophore. *P. syringae* also has pairs of redundant TonB-dependent receptors, but to a much lesser degree than the three organisms mentioned above.

Iron can also be acquired directly from haem and haem-containing proteins such as haemoglobin. Two receptors in *P. aeruginosa*, HasR and PhuR, are involved in this process[79]. In addition, the genome encodes two as of yet uncharacterized haem receptors, a HasR homologue, OptI (PA4897, 52% similarity), and PA1302 (57% similar to a haem-utilization protein in *Haemophilus influenzae*). PhuR homologues are found in *P. putida, P. fluorescens*, and *P. syringae*.

It is important to note that not all TonB-dependent receptors will be involved in siderophore uptake. For example, the gene for the SftP TonB-dependent receptor of *P. putida* lies in a sulphate ester utilization operon. Therefore this protein is predicted to take up sulphate esters. An *sftP* knockout mutant is able to grow on sulphate esters however; this is presumably due to the expression of a redundant TonB-dependent receptor[79]. Other unusual TonB-dependent receptor proteins in *P. aeruginosa* and their putative substrates include OprC that is specific for $Cu^{2+}$ (see ref. [42]) and PA1271 that is homologous to the BtuB vitamin B12-uptake porin.

## 4. CONCLUSION AND FUTURE DIRECTIONS

Several features of the *Pseudomonas* outer membrane have contributed to the success of this genus in inhabiting a wide variety of environmental niches. The negatively charged, gel-like matrix of LPS in the outer leaflet of the membrane presents a considerable barrier for large, hydrophobic compounds entering the cell. However, far from being a static structure, the barrier properties of the outer membrane can be modulated, for example, by altering the structure of LPS in the lungs of cystic fibrosis patients. Lipid modifications are also important for maintaining outer membrane integrity and transport capabilities in various conditions. Characterizing additional LPS and lipid modifications and how these changes contribute to the physiology of *Pseudomonas* will continue to be an important area of future research.

Multiple transport systems highlight the inherent flexibility of Pseudomonads to permit growth on a variety of substrates over a range of concentrations and physiological conditions. The challenge in this area will be to assign functions to the large number of newly discovered *Pseudomonas* transport porins. In addition, elucidating the regulatory networks controlling the expression of these proteins will contribute greatly to understanding how they contribute to the fitness of this diverse group of organisms.

## 5. ACKNOWLEDGEMENTS

ST is supported by a Canadian Cystic Fibrosis Studentship. REWH holds a Canada Research Chair in Microbiology. The laboratory's outer membrane research is supported by grants from the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research.

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