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CHAPTER 12

# Molecular organization and structural role of outer membrane macromolecules

ROBERT E.W. HANCOCK, D. NEDRA KARUNARATNE and CHRISTINE BERNEGGER-EGLI

*Department of Microbiology, University of British Columbia, 300 – 6174 University Blvd., Vancouver, B.C., V6T 1Z3, Canada*

## *1. Introduction*

The outer membrane has been the subject of intensive research over the past two decades. During this time, our image of this layer has matured from one of a rather simple capsule-like girdle, the lipopolysaccharide layer, to that of a sophisticated, unique and multifunctional membrane. This evolution arose from the research of pioneers like Leive, Nikaido and Nakae who recognized the importance of the outer membrane as a semi-permeable barrier [1,2]. A representative molecular model of a section of the outer membrane based on the data presented by several researchers is shown in Fig. 1. The reader is referred to several recent reviews [3–6] and to other chapters in this book for specific discussions of outer membrane constituents and functions. In this review, we attempt to present an overview of how the individual constituents of outer membranes are integrated into a complex multifunctional unit. Discussion is based on the well studied *Escherichia coli* and *Pseudomonas aeruginosa* outer membranes, with exceptions presented where appropriate.

## *2. Lipopolysaccharides*

### *2.1. General principles*

Lipopolysaccharide (LPS) is a major constituent of the bacterial cell envelope accounting for 3–8% of the dry weight of the cell [7]. It is an amphipathic molecule consisting of a hydrophilic portion represented by the O-antigenic polysaccharide and core oligosaccharide linked to the glycolipidic Lipid A residue. The molecular weight of individual LPS molecules can vary from about 8000 to 54 000 according to the lack or presence of variable numbers of the repeating saccharide units that comprise the O-antigenic polysaccharide. However, certain bacterial species, including *Neisseria* sp., *Haemophilus influenzae* and *Bordetella pertussis* do not produce long O-polysaccharides. Instead the carbohydrate

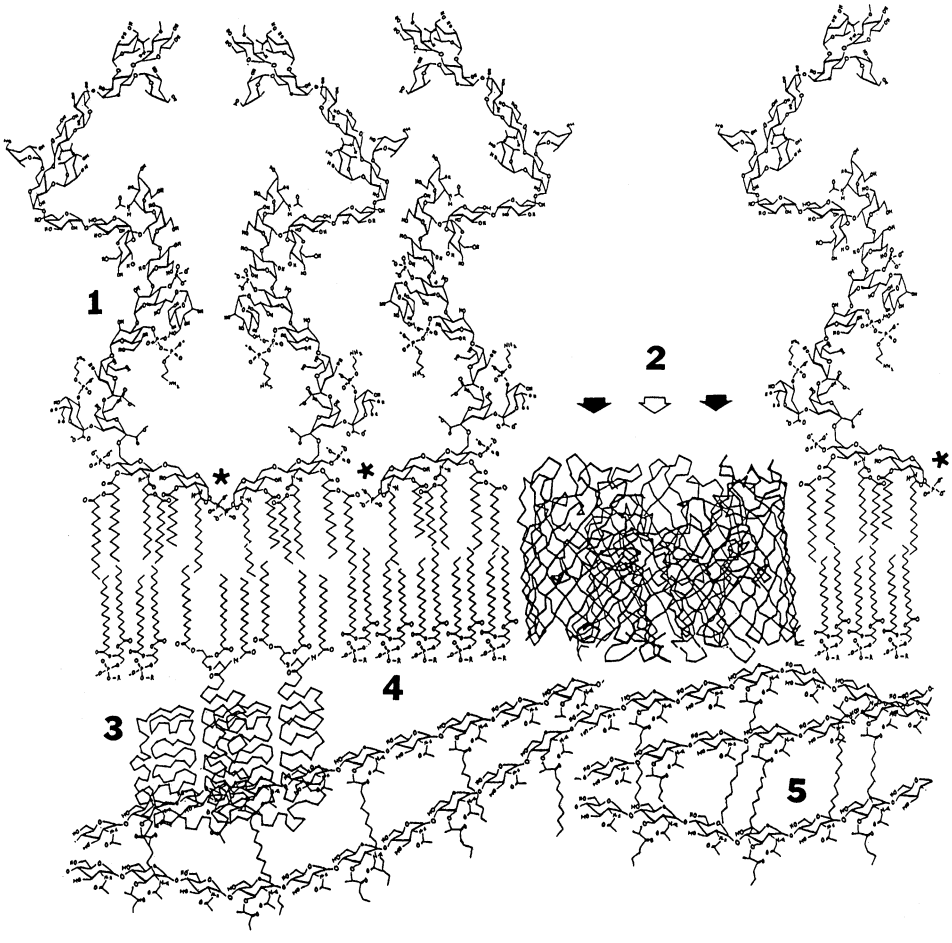


Fig. 1. Side view of a chemical model of part of the *E. coli* outer membrane. LPS (1), matrix porin OmpF (2), lipoprotein (3), phospholipids (4), peptidoglycan (5) and the proposed outer membrane stabilizing binding sites for divalent cations (*) are shown. The structure of LPS shows two O-polysaccharide units, however LPS can contain up to 40 of these pentasaccharides as indicated in Fig. 2. The structure of OmpF (kindly provided by S. Cowan) shows a section of the trimeric porin, having two channels in the front (solid arrows) and one in the back (open arrow). The lipid part of the two lipoproteins (that may be part of a trimeric arrangement) are inserted into the inner leaflet of the outer membrane. Their carboxy termini are linked (covalently or non covalently) to the peptidoglycan layer, which consists of crossbridged *N*-acetylmuramic acid-*N*-acetyl glucosamine-tetrapeptide units. For clarity only, the amino acid backbones of the crossbridging peptide chains of peptidoglycan, of OmpF, and of lipoprotein are shown. Phosphatidylethanolamine is the major lipid component in *E. coli* outer membrane, but other phospholipids such as phosphatidylglycerol and cardiolipin are also found.

portion attached to the Lipid A consists of about ten monosaccharides and hence these molecules are termed lipooligosaccharides (LOS; also termed R-type or rough LPS) [8].

The distribution of LPS in cells has been probed using immunoelectron microscopy, freeze fracture studies and enzyme accessibility studies [9,10]. LPS is exclusively localized in the outer leaflet of the outer membrane. Although the most predominant type of

LPS molecule in most Gram-negative bacteria is LPS that is unsubstituted with O-polysaccharide (i.e. rough LPS), the protruding O-polysaccharide chains of the remaining smooth LPS molecules form a capsule-like coating over the bacterium [11]. For example, in *P. aeruginosa*, where smooth LPS species comprise less than 10% of the total LPS molecules, a polysaccharide matrix extending 40 nm from the cell surface has been observed.

LPS is anchored in the outer membrane in part, by the fatty acyl chains of its Lipid A portion [12], and thus it contributes substantially to the formation of the outer monolayer of the outer membrane bilayer. In addition, interactions with divalent cations [13,14] and with proteins [4,15–17] are important in stabilizing LPS in the outer membrane. Indeed all major outer membrane proteins studied have been found to interact with LPS. It is generally accepted that LPS comprises by far most of the lipidic material in the outer monolayer of the outer membrane of wild type bacteria [18]. However, in mutants with altered LPS composition, the picture is not as clear with some authors suggesting similar levels of LPS and some suggesting lesser amounts and the presence of lipidic patches [16,19]. In addition, such mutants can demonstrate alterations in the protein constituents of the outer membrane [17], suggesting that the LPS can in some way influence the overall outer membrane composition. It is known that such mutants have an increase in outer membrane fluidity with decreasing polysaccharide chain length [13].

### *2.2. Chemistry*

As outlined above, changes in the structure of LPS lead to alterations in the structure and function of the outer membrane. Therefore, it is necessary to know the chemical structure of LPS to better understand the derivation of outer membrane functions. The LPS has been discussed in several reviews [7,20]. It is made of three general regions (Fig. 2): (a) O side chain polysaccharides, which are immunodominant, (b) a core oligosaccharide

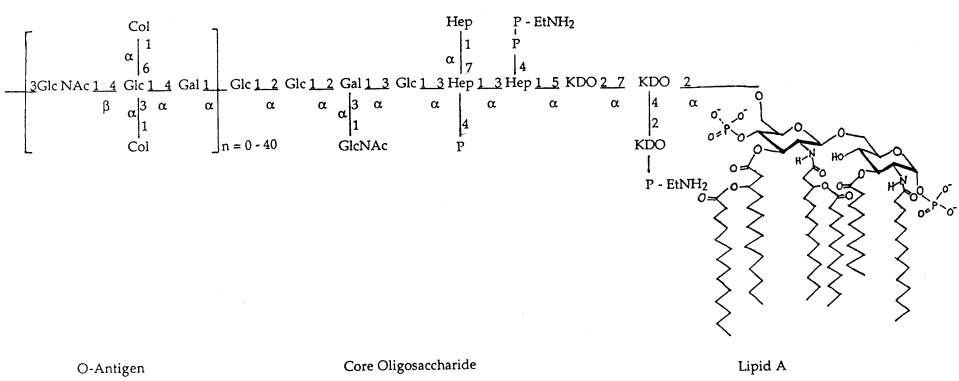


Fig. 2. The structure of LPS from *E. coli*. The three regions Lipid A, core and O-polysaccharide are shown here. The hexose region of the outer core is the R3 core structure found in *E. coli* 0111, whose O antigen contains the very labile sugar colitose. KDO, 3-deoxy-D-manno-2-octulosonic acid; Glc, glucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Col, colitose; Hep, heptose; $EtNH_2$, ethanol amine; P, phosphate.

usually containing heptose, 2-keto-3-deoxyoctulosonate (KDO), phosphate and hexose, and (c) the hydrophobic, biologically active endotoxin, Lipid A. Many publications on the chemical structures of these different regions of LPS, have appeared in recent years. From these studies, it is evident that the O side chains are highly variable in structure, composition and polymerization. The core oligosaccharide structure is conserved to a very high degree, only changing from species to species. Lipid A is even more highly conserved but can vary to some extent in different genera [21]. In addition to this heterogeneity, it is now clear that cells may contain more than one type of LPS [22]. For example, *Pseudomonas aeruginosa* cells are known to produce A band and B band LPS which are quite different [22]. Also *Bordetella pertussis* [23] and *Klebsiella* [24] cells can produce two major lipopolysaccharides LPS1 and LPS2 which differ in the side chain region. Structural microheterogeneity in the LPS of *Salmonella* and *E. coli*, and more recently in other species has been observed using SDS polyacrylamide gel electrophoresis to resolve the heterogeneous LPS fractions [25,26]. The capping frequency, or the extent of side chain length, introduces a heterogeneity in O side chain length visualized by a ladder pattern on polyacrylamide gel electrophoresis. Therefore, molecules of varying chain lengths from short chain LOS to long chain LPS may be seen in isolated LPS preparations [22].

O specific chains of LPS are made of repeating oligosaccharide units. The chemical structure of the side chains of several Gram-negative bacteria have been documented [27]. The sugars present in the repeating units may be a single sugar type with differences in linkage sequence resulting in a homopolymer, for example, a mannan in *E. coli* 09 [28] and a rhamnan in some *Pseudomonas* species [29]. In most cases, however, the repeating oligosaccharide contains units of 3–5 different sugars in specific linkages giving rise to a heteropolysaccharide. The O side chains of the *Enterobacteriaceae* have been extensively characterized. Classification into chemotypes according to the sugar composition, and into serotypes according to serological cross-reactivity has been performed [7,20]. A marked difference between O specific chains of pseudomonads and the enteric bacteria is the high content of amino sugars found in the former [30].

Chemical analysis of the core oligosaccharide has been greatly accelerated by the availability of mutants defective in LPS biosynthesis. In the case of *Salmonella*, mutants defective at each stage of biosynthesis of the core oligosaccharide have been used to study the core structure [7,25]. A similar study of the core structure of *E. coli* K12 using *rfa* gene deletions has been reported [31].

The inner core region contains 2–3 residues of the unique octulose, KDO, through which linkage to Lipid A occurs. The KDO residues are linked usually to two L-glycero-D-manno heptose residues. The outer core region consists of hexoses linked to the heptose in the inner core. Glucose, galactose, rhamnose and galactosamine are some of the common outer core hexoses found in the Enterobacteriaceae. Phosphate, pyrophosphate, and phosphoryl ethanolamine substituents may be attached to these sugars in varying degrees. Branching of the sugar core may diversify the structure of the outer core region. The inner core region of the Enterobacteriaceae seems fairly consistent, with variations being limited to the extent of phosphate, pyrophosphate and phosphoryl ethanolamine substitutions [25]. The presence of alanine amide linked to galactosamine in *Pseudomonas* [30] and the presence of galacturonic acid in *Proteus* sp. and *Morganella morganii* is indicative of the considerable variation in outer core structure between species [32].

A beta 1–6 linked diglucosamine disaccharide constitutes the backbone of most Enterobacteriaceae Lipid As. Fatty acids are attached as O- and N-acyl substituents to the glucosamine residues. Phosphates are usually attached to 4' and 1 position and may serve as linkage points for phosphoethanolamine, D-glucosamine, 4-amino-4-deoxy-L arabinose, ethanolamine or phosphate [21]. Variation of Lipid A structure from the regular backbone has been found in certain phototrophic bacteria as well as some non-phototrophic bacteria [33]. The fatty acids attached to the Lipid A disaccharide differ from species to species. The amide linked fatty acids are usually 3-hydroxy alkanoic acids. The number of carbon atoms in the fatty acids could vary from $C_{10}$ to $C_{21}$ [21,33]). In the *Salmonella* Lipid A, 3-hydroxy myristic acid is amide linked as well as ester linked [21].

### *2.3. Biophysics*

Three types of lipids are present in the envelope: phospholipid, LPS and lipoprotein. The phospholipid is distributed approximately equally between the inner and outer membranes, although the ratio of phospholipid/protein in the inner membrane is more than twice that of the outer membrane [34]. The distribution of phospholipid in the outer membrane is mostly in the inner leaflet, with LPS replacing the phospholipid in the outer leaflet of the bilayer [35].

Formation of a lipid bilayer is required for membrane fluidity. Fluid membrane bilayers are important for normal cell functions, e.g. transport across the membrane and excretion. The fluidity of the membrane undergoes major changes in state with temperature. The composition of the membrane constituents (ratios of protein/phospholipid/LPS), the nature of the lipid group (length of the fatty acyl chain, unsaturation) and association of lipids with membrane proteins (lipid–protein interactions) affect the temperature of the phase transition [36]. Usually a lower transition temperature arising from the melting of lipid, and a second transition due to protein is observed with membrane bilayers. Transitions have been monitored by X-ray diffraction, deuterium nuclear magnetic resonance spectroscopy, fluorescent probes, spin probes and scanning calorimetry [36,37]. A single transition was observed in wild type live *E. coli* cells. However, in whole cells and envelopes containing both inner and outer membranes, two reversible transitions have been observed. The first transition is characteristic of live cells, the second appears only after exposure to high temperature, prolonged storage, sonication or lysozyme-EDTA treatment. LPS from *E. coli* undergoes a broad thermal transition with a mid-point at 22°C well within the range of the first phase transition [38]. Probing the LPS domains in the outer membranes of *E. coli* by electron spin resonance spectroscopy confirmed these data [37]. Since the beginning of the phase transition indicates the melting of the 'frozen' membrane, there is no growth observed below this temperature. The end of the transition occurs at the temperature when the membrane is almost fluid and is usually around or above the physiological growth temperature. Thus, we may conclude that outer membranes have a fluid hydrophobic core.

The size of the LPS molecule is dependent on the length of its O antigen. The protruding O antigens which will have the greatest interaction with the external environment of the cell are thus involved in the physiological properties of the outer membrane. On the basis of freeze fracture electron microscopy on *E. coli* K12, LPS was shown to occur in

three different structures in the outer membrane: in a lamellar orientation, as hemi-micelles complexed with proteins, and as hemi-micelles introduced by divalent cations and/or polyamines [39]. Ferritin-labelled antibodies to the O antigen were reacted with the ribbon-like structures formed by purified LPS and subjected to electron microscopy. The electron-dense ferritin lay external to the polysaccharide ribbon and pictures indicated that LPS could extend outwards up to 30 nm [40]. A study using intact cells of *Pseudomonas* labelled with anti O-specific monoclonal antibody and a protein A–dextran–gold conjugate showed that the gold particles were located 30–40 nm beyond the outer membrane [11].

The electrostatic charge of the cell surface is a net charge resulting from the combined charges of the molecules comprising the cell surface and their counterions. At neutral pH, the net charge of several bacterial strains was found to be negative [41]. The largest contribution to charge is from the enterobacterial polysaccharide capsule in encapsulated strains with anionic capsules; however, LPS is the major contributor in non-encapsulated bacterial species.

Neutralization in part of the negative charges of LPS by metal cations helps to stabilize the membrane by decreasing the strong electrostatic repulsion between the highly negatively charged LPS molecules. $Ca^{2+}$ and $Mg^{2+}$ ions are primarily essential for the existence of the membrane. The phosphoryl groups on the LPS as well as the carboxyl group on one of the KDO units were shown to be involved in binding $Ca^{2+}$ and $Mg^{2+}$. This was confirmed by metal binding studies conducted with heptoseless mutants of *E. coli* by $^{13}C$ and $^{31}P$ nuclear magnetic resonance [42].

## 3. *Chemistry and biophysics of membrane proteins*

### 3.1. *Porins*

The outer membrane of Gram-negative bacteria is perforated by a variety of different hydrophilic channels, that are formed by proteins called porins. The bacterial cell can express up to $10^5$ copies of each different channel [4,6]. While some are constitutively expressed, others are inducible under certain growth conditions. Porins from many Gram-negative bacteria have been isolated and characterized [5,6]. They fall into two functional classes, the general diffusion porins, which are chemically non-specific although they may be weakly ion selective, and the specific porins, which contain substrate specific, saturable binding sites [4,6; see also Chapter 19]. While varying substantially in sequence, their physical properties are highly conserved [43]. Their monomeric molecular weight usually varies between 28 000 and 48 000, and they form trimeric arrangements in vivo. Most bacterial porins characterized to date have an acidic pI and a high content of beta-sheet structure (for structural information see Chapter 15).

### 3.2. *Lipoproteins*

Two different types of lipoprotein have been found in the outer membranes of *E. coli*: the Braun lipoprotein [44] and the so-called peptidoglycan-associated lipoproteins (PAL)

[45; see also Chapter 14]. The Braun lipoprotein is a small 7.2 kDa protein, existing in high copy numbers ($7 \times 10^5$ per cell) in the outer membrane of *E. coli.* One-third of this protein is covalently linked to the peptidoglycan, while the remaining two-thirds are non-covalently associated [44,46]. The covalent linkage to the peptidoglycan occurs between the $\varepsilon$-amino group of the C-terminal lysine (or arginine) of the lipoprotein and every tenth to fifteenth carboxy group of diaminopimelic acid [44] of the peptidoglycan. The sulfhydryl group of the N-terminal cysteine is substituted with a diglyceride, while the amino group is substituted with a fatty acid through an amide linkage [44]. The polypeptide chain of both bound and free lipoprotein seems to be largely organized in $\alpha$-helices as shown by Braun et al. [47]. Crosslinking studies of a hybrid lipoprotein lacking the lipid moiety showed that it exists as trimers [48] which may reflect the aggregation stage of the free lipoprotein. No clear evidence exists indicating the exposure of the lipoprotein on the cell surface. The hydrophilic amino acid composition of the lipoprotein indicates that possibly only the lipid portion penetrates into the outer membrane. Mutants in the structural gene for the lipoprotein are quite viable [49] and show normal diffusion rates of small hydrophilic solutes [50]. However, the cell wall structure of these mutants appears to be unstable based on their increased production of outer membrane vesicles, leakage of periplasmic enzymes and increased sensitivity to EDTA [51] demonstrating a structural role for lipoprotein. One proposed model predicts the peptidoglycan bound lipoprotein as a periplasmic space keeper, linking the outer membrane and the peptidoglycan at a fixed distance of 4.8 nm [44].

A broadly analogous lipoprotein in *P. aeruginosa* is the highly abundant low molecular-weight 9 kDa lipoprotein OprI, which shows 23–30% alignment with the *E. coli* major lipoprotein sequence [52]. Some strains of *Pseudomonas* apparently contain both a covalently bound and a free form of lipoprotein [53] whereas OprI from *P. aeruginosa* PA01 is entirely non-covalently peptidoglycan associated [54]. Lipoproteins analogous to OprI have been found in other *Pseudomonas* species [55].

Protein 21K from *E. coli* and OprL (21 kDa) from *P. aeruginosa* are also lipoproteins. However, they are larger than Braun lipoprotein and are exclusively non-covalently associated with the peptidoglycan. They thus belong to the class of the so-called peptidoglycan associated lipoproteins (PAL) [45,56]. While OprL is a major protein in *P. aeruginosa*, the 21K protein is of low abundance in the cell wall of *E. coli* [45]. Similar lipoproteins are found among many Gram-negative [45,57] bacteria.

### *3.3. Protein/peptidoglycan association*

Outer membrane proteins can be associated with the peptidoglycan either covalently or non-covalently. Examples of covalent interactions include the well known Braun lipoprotein of *E. coli* [44] and the major outer membrane protein (MOMP) of *Legionella* [58]. Alternatively certain proteins including porins and OmpA-like proteins have strong non-covalent associations with the peptidoglycan. The operational definition of non-covalent association is usually resistance to SDS solubilization at low to moderate temperatures, whereas covalently associated proteins resist extraction by boiling in SDS. Clearly even non-covalently associated proteins demonstrate strong associations with the peptidoglycan, and the necessity for heating in SDS (often to 56°C or greater) to release such pro-

teins indicates that localized denaturation of the part of the protein in contact with the peptidoglycan may be required to free them.

These strong associations are probably important. In plasmolyzed cells, the peptidoglycan is aligned along the bottom of the outer membrane rather than shrinking with the cytoplasmic membrane. This association is partially uncoupled during septum formation and cell division, but it nevertheless appears to be important during cell division. Thus, *lkyD* mutants of *Salmonella*, lacking covalently bound lipoprotein, show outer membrane blebbing, particularly at the position of the division septum [59]. Another role of such associations may be in cell shape and osmotic stability determination (see below).

Porin OmpF associated with the peptidoglycan has been visualized as being arranged in a mosaic crystalline (hexagonal) array [60]. As described elsewhere in this book, OmpF trimers contain a triplet of water-filled channels [61]. While visualization of the hexagonal array of OmpF porin requires selective solubilization techniques, the native surface of *B. pertussis* has been shown to be completely covered with a crystalline structure resulting from the 40 kDa porin [62].

### *3.4. Multifunctional, structural proteins*

OmpA is one of the most abundant and most widely studied outer membrane proteins in *E. coli* and many functions have been attributed to this 35 000 molecular weight, heat-modifiable protein (for review, see [4]). In addition to its role as a phage and colicin receptor, OmpA functions in stabilizing mating aggregates with $F^+$ donor cells [63] and in formation of a non-specific diffusion channel [64]. The heat- and 2-mercaptoethanol-modifiable 35 000 molecular weight porin OprF from *P. aeruginosa* shows high homology to OmpA. The variant N-terminal domains of both OmpA and OprF have both been proposed to cross the membrane eight times in antiparallel beta-sheets [65,66], while the highly homologous C-terminal domains have been proposed to be periplasmic for OmpA [40] and transmembrane for OprF [65]. The two largest gaps in sequence alignment in the C-terminal domain are in the regions encompassing the four cysteines of OprF and near the region of the two cysteines of OmpA [67] with these cysteines forming disulphide bonded 'cysteine loops'. An important function of OmpA is in stabilizing the outer membrane and the cell wall. This was evident from studies using a *lpp ompA* double mutant of *E. coli* lacking Braun lipoprotein and OmpA. Such cells grow in an almost spherical form instead of the normal rod form, require high concentrations of divalent cations for growth, and show frequent blebbing. These properties were ascribed to the observed defect whereby peptidoglycan was no longer connected with the outer membrane [68]. OprF deficient mutants of *P. aeruginosa* showed similar defects [49,68]. The elongated morphology of the above *E. coli lpp ompA* mutant could partially be reconstituted by the cloned *P. aeruginosa oprF* gene [67].

OmpA and OprF both have an Ala-Pro rich region at residues 176–187 and 163–174, respectively, that separates the N- and C-terminal domains and resembles the trypsin sensitive 'hinge' regions of the IgG light chain [69] . However, there is as yet no proof that this region functions as a hinge. The demonstration of immunological cross-reactivity of OmpA with many other Enterobacteriaceae [70,71] and with *Haemophilus influenzae* and *Aeromonas salmonicida* [35] indicates that OmpA has been strongly conserved through

evolution. In addition to the above-mentioned protein, protein PIII (the serum blocking protein) of *Neisseria gonorrhoeae* has significant sequence homology and cross-reacts immunologically with both OmpA [72] and protein OprF from *P. aeruginosa* (W.A. Woodruff and R.E.W. Hancock, unpublished data). It seems likely that in Gram-negative bacteria, there is a family of OmpA-like proteins, all of which have receptor and/or porin functions but which in addition have a major function in outer membrane stabilization through interactions with peptidoglycan.

*Chlamydia* uses a special strategy in outer membrane structural organization and stabilization. At all stages of the developmental cycle, the bacterium is surrounded by a double membrane; however, no significant amounts of peptidoglycan have been observed in *Chlamydia* at any stage of this cycle [73]. It is therefore apparent that *Chlamydia* requires a substitute to fulfil the role of peptidoglycan. The outer membrane of *Chlamydia* consist of up to 60% of a MOMP of approximate molecular weight 40 000. It acts as a trypsin-sensitive adhesin during infection [74], but also shows a porin function with an estimated pore radius of 0.65–0.9 nm. MOMP, however, is 200 times less efficient in porin formation than *E. coli* porins, and it becomes active only when treated with reducing agents [75]. MOMP contains at least three cysteines which are linked by disulphide bonds to other MOMP molecules and to two other cysteine-rich outer membrane proteins (12 kDa and 60 kDa), to form large aggregates [76]. These bonds play an important role in maintaining the structural integrity of the outer membrane of the infectious elementary body (EB) and they seem to replace the function of the missing peptidoglycan. The outer membrane of *Chlamydia* forms a hexagonal mesh [77] with depressions that can be seen by electron microscopy. A structural model proposed a hexagonal arrangement of six dimers of the MOMP arranged around the central depression [78] and freeze fracture experiments indicated a transmembrane channel [79] which has been proposed to correspond to the porin function of MOMP. However, given the evolutionary conservation of porins, it seems possible that the hexagonal arrangements instead reflect a trimeric porin unit. During the extracellular, inert stage, chlamydial elementary bodies (EB) are comparable to spores since they are resistant to osmotic pressure and sonication. After the EB is phagocytosed, it becomes exposed to the intraphagosomal reducing conditions. The cells subsequently change into reticulate bodies (RBs) which do not synthesize and are thus deficient in the cysteine-rich 12 kDa and 60 kDa outer membrane proteins and in which the intermolecular disulfide bonds of the MOMP are reduced. Under these conditions, the outer membrane becomes structurally pleiomorphic, and MOMP would open its pores, allowing uptake of ATP and other required nutrients. Lacking crosslinking by disulfide bonds, the RB becomes osmotically fragile. However, this is not a disadvantage in the high osmolarity intracellular environment.

Another special case is provided by spirochetes which contain outer membranes, called outer sheaths, which contain the periplasmic flagella that runs up the longitudinal axis of the cell giving these spirochetes their classical corkscrew motion. In one case, *Spirochaeta aurantia*, this outer sheath has no easily recognizable equivalent of an LPS molecule [80]. Two spirochetes examined to date [80,81] contain, as their predominant outer sheath proteins, porins which have by far the largest channel diameters of any porins observed to date. This has led to the suggestion that spirochetes are filter feeders and that the large porins ensure a continuous flow of nutrients through the periplasm during

movement. One of these spirochete porins, the 53 kDa protein of *Treponema denticola* [81] joins the MOMPs from *Legionella pneumophila* [82], the *Chlamydia,* to form a selected group of porins which have a dual adhesin/porin function.

### *3.5. Stability of outer membrane proteins*

Outer membrane proteins demonstrate remarkable stability to proteases and to detergent treatment [4]. Protease resistance is probably an appropriate feature given the surface localization of these proteins and the existence of some Gram-negative bacteria in environments in which they are often exposed to proteolytic attack (e.g. during infections). In the newly published *E. coli* OmpF structure [61], this protease resistance has been ascribed to the tight packing of the surface loop regions that separate adjacent transmembrane $\beta$-sheets. Presumably evolutionary selection based on deletion or alteration of susceptible amino acids has decreed such arrangements. Indeed this, together with antigenic drift, may be one of the driving forces that has led to sequence shuffling over evolution, thus limiting our ability to align the sequences of porins from different bacteria (see Chapter 17).

Detergent stability, on the other hand, would appear to be a property related to the predominant $\beta$-barrel structure of porins [61,83]. Thus, the ability of SDS to be inserted into this structure even after heating must be quite limited. As a result, we have proteins with amazing detergent stability. For example, OprF of *P. aeruginosa* retains substantial $\beta$-structure even after boiling in SDS [65], whereas most porins can reconstitute channels in lipid membranes even after SDS treatment at room temperature or greater.

## *4. Consequences of these properties*

### *4.1. Exclusion properties of the outer membrane*

The concept of the outer membrane as a molecular sieve provides a descriptive overview of its exclusion properties [4,6,84]. In general, one can state that the 'holes' of the sieve (i.e. the channels of porin proteins) define the size exclusion limit for most hydrophilic molecules (and ions) by limiting the size of molecules that can pass through these channels and by restricting the rate of passage of molecules of sizes approaching the diameters of the porin channels (as described by the Renkin correction, see Chapter 27 and [6]). Another generalization that would follow from this concept of a molecular sieve would be that the fabric of the sieve, in this case comprising LPS and various proteins, would be nearly impermeable to various molecules. As discussed below, this is a gross oversimplification. Several classes of molecules can pass across the outer membrane without accessing the channels of porins. These include polycations varying in size from trisaccharides through 30 amino acid peptides to polycationic proteins, under some circumstances DNA, certain classes of zwitterionic or uncharged antibiotics, and specific proteins, including antibacterial bacteriocins and hydrophobic compounds in some bacterial species. This is not to say that such molecules pass rapidly across the outer membrane. For example, black lipid bilayer experiments have indicated that small ions like $K^+$ and $Na^+$ can pass

through the OmpF porin channel under an applied voltage of 10 mV (i.e. much less than the existing Donnan potential across the outer membrane [85]), at a rate exceeding $10^5$ ions per channel per second. Given $10^5$ OmpF channels per *E. coli* cell, the flux of $Na^+$ or $K^+$ across the outer membrane can exceed $10^{10}$ ions per second per cell. However, a far slower rate of passage for, for example, an antibacterial compound would suffice to give rise to physiological effects such as cell death. A rate of passage of only one molecule per second could build up a periplasmic concentration of the antibiotic of 2.4 $\mu$M within one (40-min) generation time. Thus, one must be cautious when applying the terms 'exclusion' or 'impermeability' with regards to outer membranes. Although a wide variety of molecules can pass across the outer membrane at slow rates, we use these terms here in the physiological sense. As discussed repeatedly in earlier reviews [4,35,86,87], the outer membranes of many bacteria are considered to exclude most hydrophobic substances, including detergents and hydrophobic antibiotics, as well as proteinaceous enzymes, including nucleases, phosphatases, kinases, proteases, peptidases, etc. This concept of 'exclusion' reflects the probability that these substances are not taken up across the outer membrane at a rate sufficient to give rise to physiological effects on cells (i.e. solubilization, inhibition of function or modification of bacterial macromolecules). This resultant barrier function, involving semi-selective exclusion of potentially harmful environmental molecules, is one of the most critical roles of outer membranes in Gram-negative bacteria, and affords these bacteria a generalized advantage in surviving in many ecological niches which contain high concentrations of potentially lethal substances.

### *4.2. LPS/LPS interactions: antibiotic uptake and interaction pathways.*

As described in Section 2.3, a variety of data indicate that adjacent LPS molecules interact with one another. This is due to the partial neutralization of negative charges by monovalent, but more importantly divalent, cations. Removal of the divalent metal cations by chelators like EDTA [13] results in increased outer membrane permeability [88], structural perturbations [18,35] and, at higher concentrations, extraction of LPS and/or LPS–protein complexes from the cell surface [13]. Similar effects may be observed with various polycations [89] including polymyxins, aminoglycosides, etc. (see below) which competitively displace rather than chelate divalent cations and due to their bulky nature cause similar disruptions. Utilization of a polycationic fluorescent probe, dansyl polymyxin [90] has indicated that parallel interactions occur between the probe and purified LPS or the probe and intact cells [91]. However, certain important concepts must be recognized when one considers the nature of the cell surface. First, bacterial cell surfaces are highly negatively charged [41]. Thus, neutralization of LPS charges by divalent cations must be incomplete. In addition, the general concept of negatively charged LPS molecules bridged by divalent cations is an oversimplification. Indeed, the surface of bacterial cells can be best described as a Guoy–Chapman–Stern [92] layer with high negative electrostatic potential and with divalent and monovalent cations diffusing quite rapidly across this surface. Thus, the interaction of a large polycation with such a surface will first involve a localized neutralization of such a surface layer, including charge displacement or localized exclusion of cations, followed by integration of the polycation into the outer

surface of the outer membrane bilayer. Probe displacement experiments have indicated that such polycations have a very high affinity for LPS (e.g. around 0.3–3 $\mu$M for polymyxin B) [90,93] although the affinity tends to decrease with decreasing cationic nature [93]. Evaluation of binding kinetics using both the fluorescent probe dansyl polymyxin [90], for intact cells and purified LPS, or the spin label probe $CAT_{12}$ (4-dodecyldimethylammonium-1-oxyl-2,2,6,6-tetramethylpiperidine bromide) for purified LPS [93] have indicated that such interaction is cooperative. Thus, the interaction of one molecule of polycation with the outer membrane promotes the interaction of subsequent molecules.

Such interactions have substantial physiological importance since they explain two key properties of the outer membranes of bacteria such as *E. coli* and *P. aeruginosa*, namely their ability to exclude or resist attack by hydrophobic molecules and the existence of a specific pathway of uptake termed self-promoted uptake. Thus, exclusion of hydrophobic molecules including antibiotics, bile salts and anionic or neutral detergents reflects the inability of these molecules to pass across the area of strong negative electrostatic potential at the surface of the outer membrane. Consistent with this, disruption of this surface potential by treatment with polycations, or removal of divalent cations with EDTA causing charge repulsion amongst adjacent LPS molecules, causes enhanced susceptibility to hydrophobic probes [88] and antibiotics [94]. Bacteria that do not have such a strong electrostatic potential would presumably be more susceptible to such agents. For example, deep rough mutants of *Salmonella* and *E. coli* demonstrate enhanced susceptibility to such agents because of decreased surface potential due either to the abnormal presence of phospholipids in the outer monolayer of the outer membrane (in the view of [19] but not [16]), or the reduction of negatively charged groups on the LPS molecules of such bacteria [95] or both. Similarly, we hypothesize that other bacteria such as *Haemophilus influenzae*, *Neisseria* sp. etc. which contain a unique LOS species, instead of conventional LPS, could demonstrate a reduced surface potential (perhaps due to a requirement to interact with the negatively charged surfaces of eukaryotic cells). This would then explain their known increased susceptibility to hydrophobic agents [87].

Self-promoted uptake has been described in detail previously [87] and is only described in overview here. Basically, it represents a system by which bactericidal polycations and organic monovalent and divalent cations can interact with LPS binding sites, and cause permeabilization of the outer membrane to promote uptake of the permeabilizing antibiotic. Attack of Gram-negative bacterial cells by such compounds represents a conserved evolutionary theme (for review, see [88,96]) utilized by antibiotics such as aminoglycosides and polymyxins from certain microorganisms, and by peptides from insects or animal semen or the intracellular contents of eukaryotic cells, including phagocyte 'defensins'. It is known that the interaction of such compounds with cell surface LPS molecules (see above) is followed by structural perturbations to outer membranes and their increased permeability to probes including the $\beta$-lactam nitrocefin, the peptidoglycan-degrading enzyme lysozyme and hydrophobic compounds including the fluorescent probe 1-*N*-phenyl naphthylamine [97] and antibiotics. The relevance of such interactions to actual killing of cells has been demonstrated using mutants with increased susceptibility or resistance to such agents due to outer membrane alterations which influence the interaction of these compounds with the cell surface.

### *4.3. LPS–protein interactions*

An area about which far less is known is the association of LPS with outer membrane proteins. It is well known that outer membranes upon purification are often associated with molar or greater quantities of LPS, as demonstrated by co-purification [15,98,99] and, in *P. aeruginosa*, by crosslinking [100] and crossed immunoelectrophoresis [101] experiments. These associations probably involve both ionic and hydrophobic interactions since procedures that disrupt both interactions must usually be applied to obtain LPS-free outer membrane proteins [e.g. 61]. In one case, OmpF porin of *E. coli*, the influence of cations on intrinsic tryptophan fluorescence, was interpreted as evidence for the presence of a divalent cation binding site on this protein that could be involved in interaction with LPS [102].

The relevance of such interactions is currently somewhat obscure. Data with phages that utilize outer membrane protein receptors have demonstrated that the presence of a normal LPS seems important for interaction of the phages with their receptors [103]. Conversely, Parr et al. [99] isolated a monoclonal antibody specific for LPS which preferentially recognized LPS in complex with OmpF or OmpC porin. Thus, we may assume that LPS stabilizes, anchors and/or orients proteins at the surface of the outer membrane.

Recently, Young and Hancock [104] demonstrated that overproduction of an outer membrane protein OprH in *P. aeruginosa* led to an 8–16-fold enhancement in supersusceptibility to quinolones, including ciprofloxacin and nalidixic acid, chloramphenicol and trimethoprim, whereas susceptibility to $\beta$-lactams and rifampicin were unaffected. Since data were presented that OprH was not functioning as a porin, we are left with the conclusion that either enhanced uptake of specific antibiotics occurs via sites created by LPS–OprH interactions, or that OprH somehow neutralizes the surface electrostatic potential. This indicates the possibility that protein–LPS interactions can mediate in antibiotic permeation pathways.

### *4.4. Fluidity, energization and hydrophobic permeability*

Outer membranes have been traditionally viewed as quite rigid membranes due to their frequent intimate association with the underlying peptidoglycan and the bulky nature of LPS. However, this would appear to be an oversimplification. Two types of data indicate the fluidity of outer membranes. First, measurement of phase transitions in *E. coli* has indicated that when cells are grown at 37°C, the outer membranes are fluid above a transition temperature of 25°C [105]. Second, spin-label experiments and fluorine nuclear magnetic resonance spectroscopy data on *E. coli* vesicles demonstrated that the diffusion rate of lipids in the *E. coli* cell envelope is in the order of $10^{-8}$ cm/s which indicates that a given lipid molecule could move from one end of a bacterium to the other in less than a second (for review, see [106]).

The fluidity of outer membranes permits the passage of hydrophobic molecules under appropriate circumstances. However, one as yet unexplained phenomenon has been observed using hydrophobic fluorescent probes. When the outer membranes of *E. coli* and *P. aeruginosa* are permeabilized, they take up such probes transiently and then secrete them [97,107]. Administration of an inhibitor of cell energization prevents secretion such

that net uptake is observed. This implies that cells contain an energized secretion system for hydrophobic compounds, perhaps one analogous to the tetracycline and quinolone secretion systems [108].

## 5. Conclusions

Understanding how even a single macromolecule achieves its function is quite difficult, as discussed elsewhere in this book. However, understanding how a large number of molecules integrate to give rise to a variety of general properties is far more complex. Nevertheless, studies of outer membranes have progressed to a stage where we can start to make educated guesses about the relationships between outer membrane organization and outer membrane function. Much has been learnt but much remains to be learnt.

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