Bacterial Outer Membranes: Evolving Concepts

Specific structures provide gram-negative bacteria with several unique advantages

R. E. W. Hancock

The outer membrane of gram-negative bacteria is an interface between the environment and the interior of the cell. As such, it plays a major role in determining what enters a cell, which molecules are exported from the cell, and how the cell interacts with molecules, surfaces, and other cells in its environment. In the past 25 years of intense research on the bacterial outer membrane, models of outer membrane structure and our understanding of the diversity of outer membrane functions have rapidly evolved.

This rapid evolution has led to substantial confusion in interpreting the voluminous literature on outer membranes. Information about them, as presented in textbooks, often is incomplete. This brief overview of outer membrane structure and functions is intended to reduce some of that confusion. I will focus here mainly on membranes of *Escherichia coli* and *Pseudomonas aeruginosa*. Although outer membrane researchers have pioneered studies on protein secretion in bacteria, two-component regulatory systems, and macromolecule biosynthesis, no attempt will be made to discuss these areas of research.

Since 1965, the picture of the bacterial outer membranes has changed from one of a rather simple lipopolysaccharide (LPS) layer that was viewed as a capsulelike coat to that of a dynamic structure intimately involved in many important cell processes. One might ask, given that many gram-positive and other bacteria manage quite well without outer membranes, why do gram-negative bacteria have them? In many cases, the properties of outer membranes create selective advantages for gram-negative bacteria.

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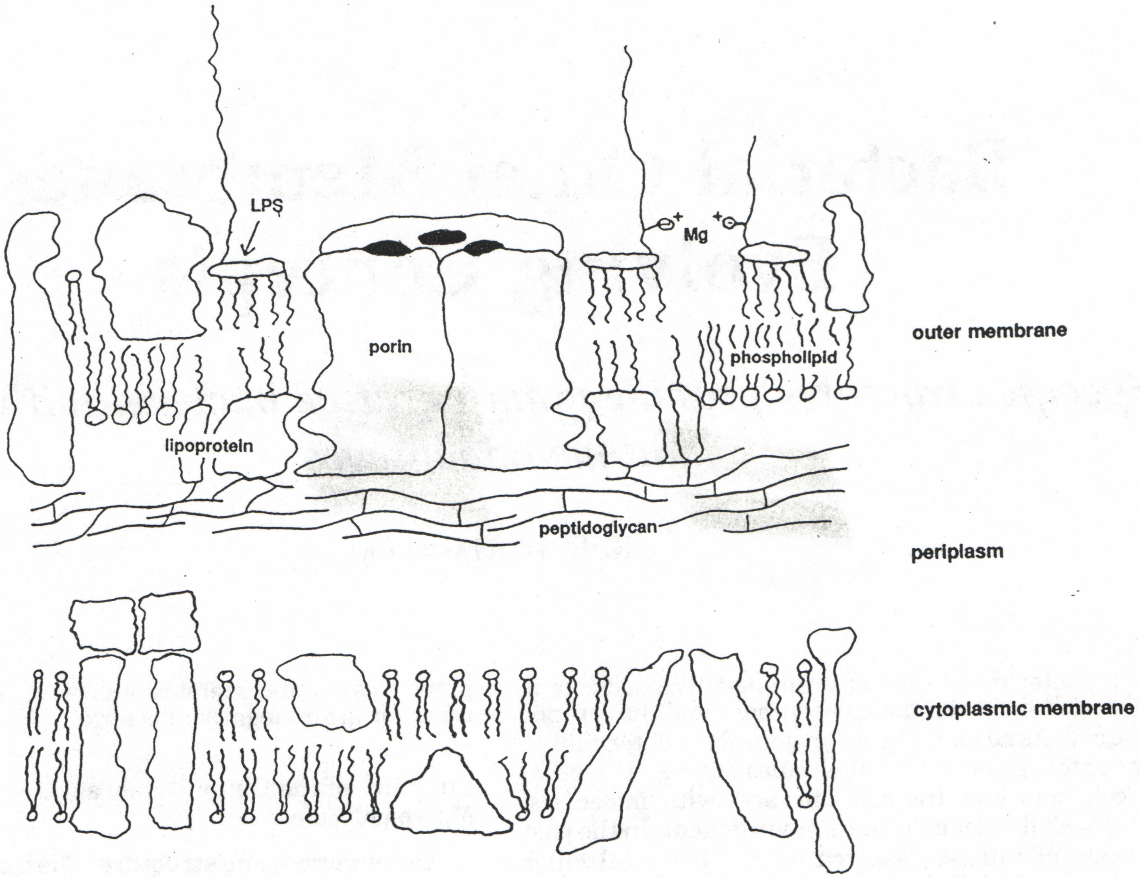
Structure of the Outer Membrane and Its Macromolecules

Outer membrane structure. The inner or cytoplasmic membrane of the bacterial cell envelope (Figure 1) is a typical lipid bilayer membrane containing a variety of polypeptides with functions in transport, cellular energization, and biosynthesis and excretion of external components. The region between the outer and inner membranes is called the periplasm. This region contains both proteins and anionic oligosaccharides (termed membrane-derived oligosaccharides) as well as peptidoglycan. The earlier concept of peptidoglycan as a single-layered girdle around the cell is incorrect. Instead, short pieces of peptidoglycan are cross-linked together into a gel. When cells are transferred to hypertonic solutions, the cytoplasmic membrane shrinks at all except a few places (termed Bayer adhesion zones), leaving the outer membrane closely associated with peptidoglycan. Thus, the periplasm is probably located primarily between the peptidoglycan gel and the cytoplasmic membrane. The periplasm functions in the traffic and processing of molecules entering or leaving the cell, while peptidoglycan is a major determinant of cell shape and osmotic stability.

The outer membrane is biologically unusual in that it apparently is a fully asymmetric bilayer. Thus, the inner monolayer is composed of lipids, often phospholipids, whereas the outer monolayer contains the unique lipid species LPS. The asymmetric distribution and the chemical characteristics of LPS give the outer membrane many of its unique barrier properties. The self-association of anionic LPS through divalent cation

Figure 1. Major Features of the Cell Envelope, Including the Outer Membrane, of a Gram-Negative Bacterium



Note the asymmetric distribution of lipid-containing species in the outer membrane, with a paucity of phospholipids in the outer leaflet. The outer membrane is associated through strong noncovalent or occasionally covalent interactions of proteins with the underlying peptidoglycan.

cross bridging and the strong association of LPS with proteins are critical in this regard.

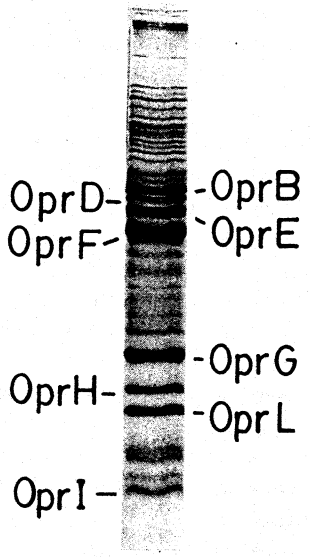
Outer membranes contain a limited number of (usually three to eight) major proteins present in very high copy number, 50,000 to 200,000 copies per cell, making these the most predominant proteins in bacterial cells (Figure 2). These proteins, in addition to their various functions, anchor the outer membrane to the underlying peptidoglycan through strong noncovalent or even covalent interactions. In addition to these major outer membrane proteins, as many as 50 to 100 minor species may be present.

LPS. The basic LPS structure comprises three regions, the lipid A, rough core, and O-antigen regions (Figure 3). The lipid A region, which is inserted into the outer membrane bilayer, usually contains diglucosamine (or diaminoglucose) phosphate substituted by fatty acids, the majority of which typically are unusual hydroxy fatty acids (that are useful for monitoring LPS in environmental samples). This region is antigenically and chemically conserved. It bears the alternative name endotoxin because of its involvement in causing septic shock in humans and animals.

The rough core is covalently bound to lipid A. It typically contains 10 to 15 heterogeneous sugar residues, often including unique octose (2-keto-3-deoxy-octulosonic acid [KDO]) and heptose sugars. The rough core may be capped by repeating tri- to pentasaccharide units termed the O antigen. This latter repeating saccharide portion is one of the most immunogenic antigens of smooth gram-negative bacteria and determines the O serotype of such bacteria.

LPS molecules within a given bacterial cell can demonstrate enormous heterogeneity. This extends to the chain length of the LPS such that the majority species is usually the rough core uncapped by O-antigenic sugars, while substitutions with 1, 2, 3, 4, or more O-antigen units give rise to the typical ladder of LPS bands seen when LPS is extracted (Figure 3). Heterogeneity is also provided by variable substitutions of the basic structure, especially the lipid A-rough core portion (including fatty acid, phosphate, and side-chain sugar composition). In some cases, a single bacterium can produce two different LPS molecules or one LPS molecule plus an LPS-like molecule called enterobacterial common antigen.

Figure 2. Typical Appearance of Outer Membrane Proteins Separated by SDS-Polyacrylamide Gel Electrophoresis



The gel features a relatively small number of prominent major outer membrane proteins. The outer membrane shown is that of *P. aeruginosa* grown on minimal medium with glucose as a carbon source (which induces protein OprB and results in an increase in the levels of OprD).

Within a single species, the O-antigen sugar composition can vary substantially (e.g., *Salmonella enteritidis* comprises over 600 chemical types or serotypes); in contrast, the rough cores of such bacteria demonstrate substantially greater conservation. In addition to the so-called "rough" (because of colony morphology), O-antigen-lacking mutants of such organisms, many bacteria, including some important human pathogens (e.g., *Neisseria* and *Haemophilus* spp., etc.), produce an LPS that is uncapped by O-antigenic repeating sugar units. In such organisms, this molecule bears the name lipo-oligosaccharide (LOS).

Proteins. Outer membranes contain a restricted number of major proteins present in high copy number. Two structurally interesting classes have been identified. One class comprises lipoproteins. The best known of these, the Braun lipoprotein, can be found both covalently bound and noncovalently associated with peptidoglycan (although some bacterial species lack the former). This and other lipoproteins may be primarily associated, through their lipidic portion, with the inner monolayer of the outer membrane. The other, predominant class of outer membrane proteins contains a substantial portion (>60%) of β-sheet structure (Figure 4) that runs perpendicular to the plane of the membrane. This feature gives such proteins an unusual resistance to heating in strong detergents, such as sodium dodecyl sulfate (SDS). For example, as one extreme, protein OprF of *P. aeruginosa* can be boiled for 10 minutes in 2% SDS without substantial loss of β structure. This resistance to SDS denaturation in turn gives rise to the property of heat modifiability whereby the same polypeptide can appear in two different positions in an SDS-polyacrylamide gel electrophoretogram depending on the solubilization temperatures.

Functions

The outer membrane is a rich mosaic containing a half dozen or so major proteins and 50 or more minor ones, one or two very heterogeneous LPS molecules, and several other lipids. It is thus not surprising that the outer membrane has a variety of different functions (Table 1).

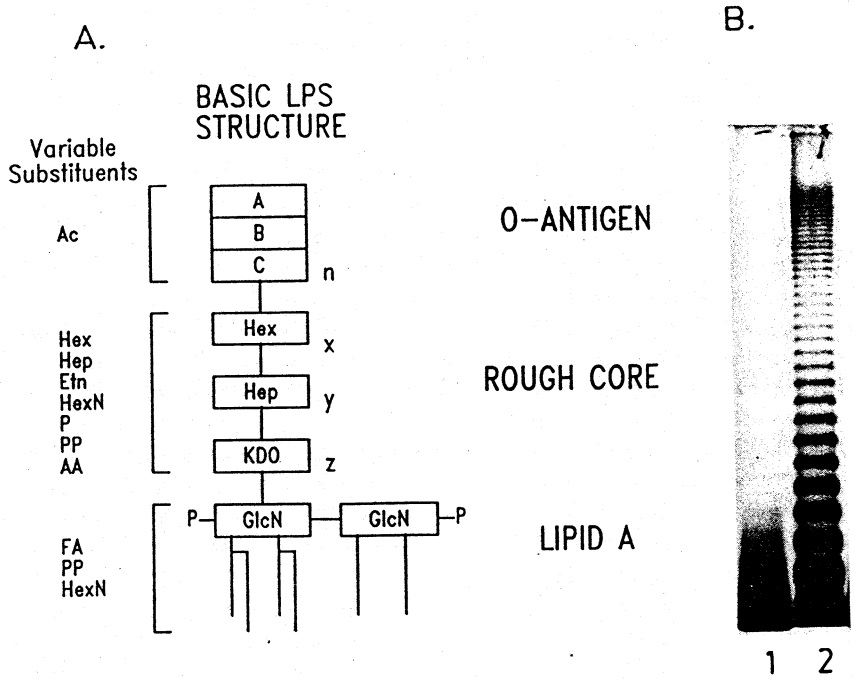
Exclusion barrier function. One of the most significant functions of outer membranes is to exclude a variety of environmental molecules but to permit selective uptake of others. In particular, outer membranes exclude a variety of proteins, including proteases, lipases, phospholipases, peptidases, saccharide hydrolases, deoxyribonucleases, and channel-forming toxins, all of which can attack bacterial constituents. Indeed, with some exceptions, all hydrophilic molecules above a given size limit, which varies from species to species, are excluded from gram-negative bacteria. Many gram-negative bacteria also exclude hydrophobic and amphiphilic molecules, as indicated by the high resistance of such cells to hydrophobic antibiotics, such as fusidic acid and erythromycin, and to detergents. Notable exceptions include several intracellular pathogens, in which LPS is substituted by LOS. They tend to be quite susceptible to hydrophobic antibiotics and detergents. *P. aeruginosa*, which normally excludes hydrophobic compounds, can lower this barrier when it adapts to grow on hexadecane as a carbon source.

The primary basis for this exclusion ability is the strong association of adjacent negatively charged LPS molecules due to divalent cation cross bridging. Thus, treating such bacteria with EDTA to remove divalent cations by chelation, or with polycations to competitively displace the cations, results in enhanced uptake of hydrophobic molecules, antibiotics, and enzymes such as lysozyme. A role for LPS in exclusion is also indicated by the increased susceptibility to hydrophobic antibiotics of deep-rough, LPS-altered mutants. Associations between LPS and proteins and the high negative surface charge of the outer membranes due to negatively charged LPS phosphates and KDO residues may also play a contributory role in barrier function.

The surface of the outer membrane also assists in preventing attack by enzymes. For example, virtually none of the phospholipid of the outer membranes is exposed, and the outer membrane proteins and LPS are quite resistant to digestion by proteases and sugar hydrolases, respectively.

Selective permeability. Two classes of uptake pathways determine the selective permeation of molecules across the outer membrane, namely, porin and

Figure 3. Schematic Representation and SDS-Polyacrylamide Gel Electrophoretogram of Typical LPS Species



(A) Basic LPS substituents and variable substituents of the three regions of LPS. n indicates that the number of O-antigen repeats varies from 0 to approximately 50 for individual LPS molecules. Abbreviations: A, B, and C, individual O-antigen saccharides which may be hexoses, methyl pentoses, hexuronic acids, or hexosamines (note that between three and five individual saccharides usually constitute one O-antigen repeat unit); Hex, hexose (or hexosamine or hexuronic acid)-containing outer core portion of the LPS rough core (x indicates that the number of saccharides varies, usually being around five per LPS molecule); Hep, heptose present in y (two to three) molecules per LPS; KDO, 2-keto-3-deoxyoctanate present in z (two to four) copies per LPS, GlcN, glucosamine in lipid A (the straight and branched lines pointing down from the GlcN residues are fatty acids, typically a majority of hydroxy fatty acids, which are inserted into and compose the outer part of the outer membrane bilayer); P, phosphate; Ac, acetyl; EtN, ethanolamine; HexN, hexosamine (or methyl pentosamine); PP, pyrophosphate; AA, amino acid; FA, fatty acid. (B) SDS-polyacrylamide gel of *Salmonella typhimurium*. Lane 1, Rough LPS comprising the rough core (or part thereof) plus lipid A; lane 2, smooth LPS comprising lipid A plus rough core plus O antigen. The ladder of LPS bands is created by substitution of LPS molecules with (from bottom of gel) 0, 1, 2, 3 . . . etc., O-antigen repeats.

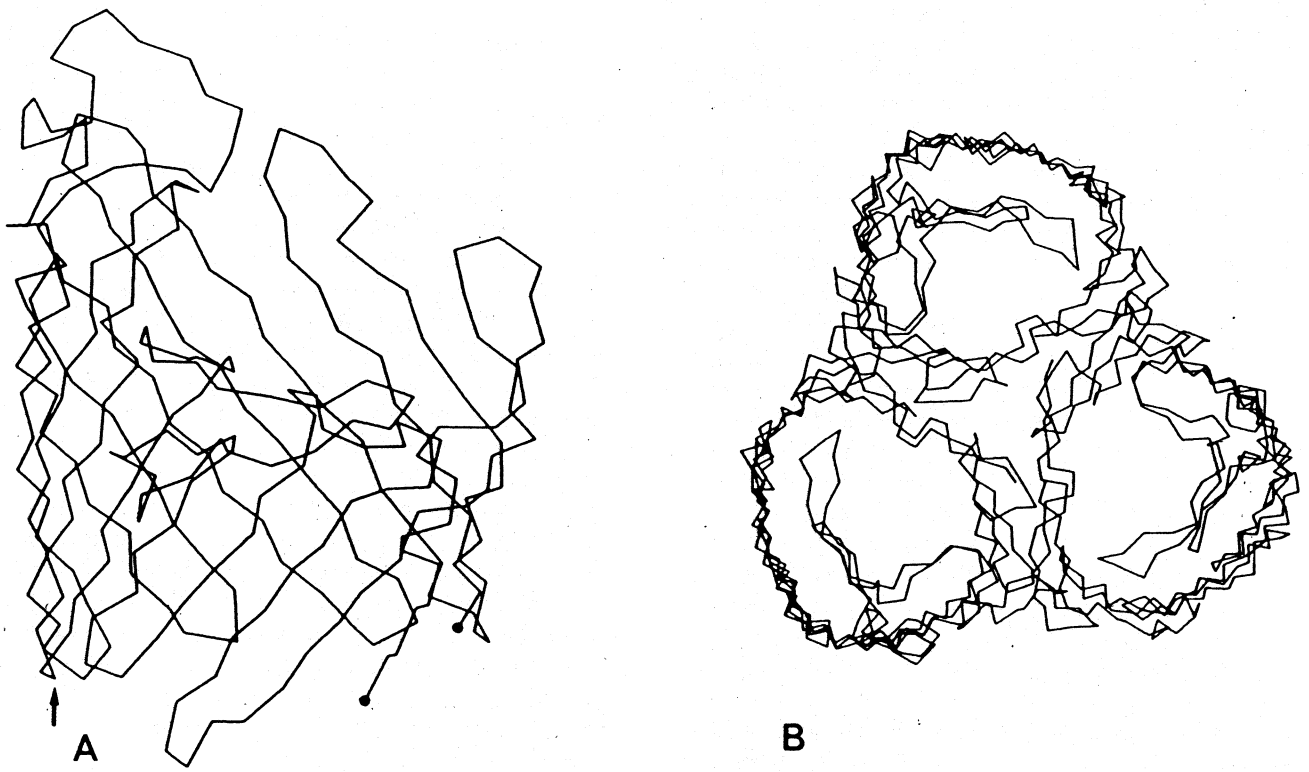
nonporin pathways. The porins mediate uptake of small hydrophilic molecules. Porins themselves belong to a class of apparently ubiquitous proteins in gram-negative bacteria that form water-filled channels across the outer membrane. Porins have remarkably conserved physical properties. Most form SDS-resistant trimers with monomer molecular weights of between 30,000 and 48,000, contain a majority of β-sheet structure, are associated with both LPS and peptidoglycan, and have an acidic pI. Despite this, those porins that have been sequenced from different species have at best localized homology, and their alignment requires insertion of many gaps. This variability may arise because they contain many β strands that zigzag across the membrane such that one surface of the β strand contacts the hydrophobic interior of the membrane and the other makes up part of the water-filled porin channel, possibly allowing for substantial evolutionary amino acid substitutions without the loss of porin function.

There are two classes of porin channels, nonspecific and specific. The nonspecific porins contain simple water-filled channels through which hydrophilic molecules smaller than a given size limit can travel. Thus, they determine the exclusion limit of the outer membrane and the cell. Model membrane techniques have shown that their channels are only weakly ion selective, because of charged amino acids at the mouth of the channel, and are chemically nonselective. Investigation of mutants lacking specific porins has demonstrated a key role of porins in the permeation of β-lactams and other specific antibiotics across the outer membrane. Uptake of molecules through porins occurs by diffusion and can be described by Fick's law.

Some investigators, but not I, feel that the nonspecific porins are regulated by voltage and/or pressure. This controversy will probably be resolved as more details of the three-dimensional structure of porins are understood. The crystal structure of *Rhodobacter* porin was recently published (Figure 4).

The second class of porins involves the specific porins, of which only two—the phosphate-specific porin OprP of *P. aeruginosa* and the maltodextrin-specific porin LamB of *E. coli*—have been well studied and only four others have been described. Nevertheless, they may be more common than these few examples suggest. For example, the iron-regulated outer membrane proteins that are assumed to be important in bacterial pathogenesis and are present in most bacterial species have characteristics that are consistent with their functioning as specific porins for iron-siderophore or iron-transferrin complexes. In the well-studied cases, specificity is mediated by a specific

Figure 4. Membrane-Associated Portion of the *Rhodobacter capsulatus* Porin



At the time of publication of this diagram, a first for outer membrane research, the sequence of amino acids was not known for this porin. Therefore, the electron density map, solved by X-ray crystallography of isomorphous heavy metal derivatives of this porin, was best fitted with a polyglycine chain. Note the extensive β-sheet structure running through the membrane and perpendicular and at a slight angle to the plane of the membrane (arrow). (A) Side view of one monomer of the porin trimer; (B) top view of the porin trimer. (M. W. Weiss, T. Wacker, J. Weckesser, W. Welte, and G. E. Schulz, FEBS Lett. 267:268–272, 1990. Reprinted with permission.)

binding site, with a binding affinity (K_s) of approximately 10^{-2} to 10^{-4} molar within the channel. However, the binding sites of OprP and LamB vary substantially in their molecular characteristics.

A second class of permeation routes is provided by the nonporin pathways. Several intracellular pathogens as well as deep-rough LPS-altered mutants of other bacteria have a pathway for uptake of hydrophobic agents. Since these intracellular pathogens also contain LOS rather than LPS, one might infer that LPS is the primary determinant of a hydrophobic uptake pathway.

An apparently more widespread nonporin pathway is self-promoted uptake (Table 1). Polycations, such as polymyxin B, interact at the divalent cation cross-bridging sites between adjacent LPS molecules in the outer membrane (Figure 1). The polycations have much higher affinity for these sites than do divalent cations, which thus are competitively displaced. Being bulkier than the cations they displace, the polycations change the packing order of LPS and increase the permeability of the outer membrane to a variety of molecules. On the basis of such experiments, we proposed that the polycations increased their own uptake (hence the name, self-promoted uptake).

The relevance of this pathway to cell killing by such "permeabilizers" was revealed by mutant studies. Thus, mutants with alterations affecting the interaction of the permeabilizers with the outer membrane are either resistant or supersusceptible to killing by these compounds. The use of polycations in antibacterial defense systems appears repeatedly among a variety of organisms: defensins from the neutrophils of animals and humans, insect immunity peptides including cecropins, magainins from the skin of frogs, and seminal plasmin from the semen of bulls, as well as several antibiotics produced by microbes (Table 2).

Investigation of specific outer membrane mutants leads us to suspect other nonporin pathways of uptake. However, no definitive descriptions of such a mechanism(s) of uptake yet exist.

Export. Gram-negative bacteria can export (i.e., excrete into the surrounding medium) several different classes of molecules, including secondary metabolites, siderophores, the building blocks of carbohydrate capsules or exopolysaccharides and protein

Table 1. Summary of Outer Membrane Functions

Function	Molecules or agents affected	Major determinant
Exclusion	Enzymes in the environment, hydrophobic molecules, large hydrophilic molecules	Strong association of LPS with itself and with proteins
Selective permeability	Small hydrophilic molecules (porin pathway)	Nonspecific or specific porins
	Large polycations, organic cations (self-promoted uptake pathway)	Divalent cation-binding sites on LPS
	Hydrophobic molecules	Specific types of LPS
Export	Excreted proteins	Specific export mechanisms
	Proteins in periplasm	Anchoring or lack of access to specific export mechanisms
Receptors	Phages, bacteriocins, conjugative pili, iron-siderophore or iron-transferrin complexes, vitamin B_{12}, etc.	Specific proteins or LPS
Interaction with environment	Antibodies	Specific proteins or LPS
	Complement	LPS
	Surfaces, cells, metals	Uncertain
Anchoring of external structures	Flagellin	Ring proteins of basal body
	Pilin	Proteins
	Capsules	Some have lipid attachment
Structural role	Cell shape	OmpA-like proteins and/or covalently bound Braun lipoprotein

S layers, and protein enzymes and toxins. The outer membrane maintains its barrier function and selective permeability during the operation of such export functions. For example, switching *P. aeruginosa* from a phosphate-rich to a phosphate-deficient medium leads to excretion of most of the phospholipase C and about half of the alkaline phosphatase produced by induction of the Pho regulon. At the same time, the coregulated phosphate-binding protein and a β-lactamase remain within the periplasm, and outer membrane permeability to β-lactam antibiotics does not increase. Such selective behavior implies that export pathways across the outer membrane are very specific. Although none is well understood, several different pathways are under study, including one involving a protease-processing step (which may be outer membrane associated), ones involving the participation of several accessory proteins, and others in which the exported proteins may be present transiently or in precursor form in the outer membrane.

Table 2. Molecules Which Interact with and/or Are Taken Up by the Self-Promoted Pathway

Polycation(s) (class; origin)	Monovalent or divalent cation	Divalent cation chelators[a]
Polymyxins (antibiotic; bacteria)	Gramicidin S	EDTA
Aminoglycosides (antibiotic; bacteria)	Azithromycin	NTA
Defensins (peptide; various)[b]	Tris	HMP
Magainins (peptide; frog skin)[c]	48/80	
Cecropins (peptide; moths)[c]		
Sarcotoxin I (peptide; flesh fly larvae)[c]		Ascorbate[d]
Eosinophil cationic proteins (proteins; humans)[c]		Salicylate[d]
Bactenicin (peptide; cow neutrophils)[c]		
Seminal plasmin (peptide; bull semen)		
BPI/CAP_{57} (protein; human, rabbit neutrophils)		
Polylysine (peptide; synthetic)		
Protamine (protein; fish sperm)		

[a]NTA, Nitrilotriacetate; HMP, hexametaphosphate.
[b]See *ASM News* (June 1990, p. 315–318); defensins are found in many animal and insect species and in several types of monocytic cells.
[c]Interaction with self-promoted uptake pathway inferred from literature data.
[d]Identity as chelators is uncertain; both compounds are reducing agents.

There is one other implicit export-related role, namely, the ability of the outer membrane to contain the periplasm by preventing excretion of periplasmic proteins. Presumably this process could involve two factors, the anchoring of periplasmic proteins within the periplasm (e.g., by association with the cytoplasmic membrane as peripheral membrane proteins or with peptidoglycan) and a lack of access to the specific export mechanisms, perhaps due to a lack of specific amino acid sequence signals.

Receptors. Being relatively exposed to the environment, the outer membrane is one of the major cellular structures containing receptors for binding specific nutrients and

proteins. Such receptors fit into three classes. The first class comprises nutrient receptors. For example, specific outer membrane proteins from many gram-negative bacteria bind the complexes of excreted siderophore (i.e., iron-chelating) compounds with environmental iron or, in the case of some pathogens (e.g., *Neisseria*, *Haemophilus*, and *Pasteurella* spp.), complexes of transferrin or lactoferrin with iron (Fe^{3+}). This class concentrates this relatively insoluble and hence scarce, but important, nutrient on the surface of the cell, permitting subsequent uptake. Another such protein receptor binds vitamin B_{12}. Some of the specific porins contain substrate-binding sites, allowing them to be considered as specific receptors, too.

A second class of receptors is involved in binding pili to permit conjugations between strains of a given species or, in the case of broad-host-range plasmids, between species. The specific nature of any receptor is not precisely known, although OmpA protein and LPS are implicated in the case of *E. coli* F-factor conjugal transfer and there is some evidence of LPS involvement in binding other types of pili.

A third class of receptors—that is, receptors for bacteriophages or bacteriocins—definitely puts cells at a disadvantage. Binding of these agents leads eventually to cell killing. In all cases studied in detail, the receptors play other roles in the outer membrane. As two examples, the T-even phage T6 and phage λ bind to the outer membrane proteins Tsx and LamB, respectively (although in the case of T6, LPS is a codeterminant of binding). Tsx serves as a specific porin for nucleosides, whereas LamB is a maltodextrin-specific porin.

Interaction with the environment. Outer membranes are in close contact with the environment, although in some gram-negative bacteria they may be surrounded by an S layer or capsule. Nevertheless, in many species, outer membrane components are sufficiently immunogenic and exposed to elicit a humoral and/or cellular immune response. For example, LPS, which contains the major type-specific O antigen, and several outer membrane proteins elicit strong antibody responses.

Such antibodies can interact with surface antigens and opsonize bacterial cells for phagocytosis or complement-dependent bacterial killing. If complement is activated in an appropriate spatial setting, the terminal components can insert into and permeabilize the outer membrane, leading to an eventual attack on the cytoplasmic membrane by the C9 channel-forming component. However, such an event usually occurs only for specific rough-LPS-containing bacteria. Indeed, many gram-negative pathogens are quite resistant to serum bacterial killing. Complement can also be activated by the alternate pathway which may be set in motion by interaction of serum factors with smooth, O-antigen-containing LPS. However, possibly the major function of complement pathway activation is to enhance chemoattraction of phagocytes and to opsonize bacteria for phagocytosis. There are several outer membrane components which, when released, may stimulate mitosis and factor secretion in eukaryotic cells.

Several researchers suggest a role for one or another outer membrane protein in interacting with cells or various environmental surfaces. However, there is little agreement about such interactions, although the *Chlamydia* porin (called MOMP) may play a role in attachment to cells.

Anchoring external structures. Outer membranes are the anchoring points for certain external structures. For example, flagella have a specific proteinaceous ring structure to allow the flagellar filament to pass through the outer membrane without stirring it during flagellar rotation. The S layer of *Aeromonas hydrophila* may be associated with the LPS O-side chains, since rough mutants cannot assemble an S-layer in this organism. Some capsules contain a lipid tail that anchors them to the outer membrane. In some instances, pili may be assembled from outer membrane pools of pilin protein.

Structural role. Upon plasmolysis of bacterial cells, outer membranes and peptidoglycan remain close together while the cytoplasmic membrane shrinks away at all except a few points. The peptidoglycan and several proteins of the outer membrane are bonded by strong noncovalent and, in some instances, covalent interactions. Such interactions resist attack by 2% SDS at 50°C or higher. Two classes of proteins are especially strongly attached to peptidoglycan. One is the Braun lipoprotein, a short polypeptide containing three amino terminus-attached fatty acid residues that insert into the outer membrane. In *E. coli*, approximately one-third of the Braun lipoprotein molecules are covalently attached to the peptidoglycan. Such molecules apparently help to hold the outer membrane to peptidoglycan during cell division, enabling the outer membrane to follow the ingrowth of the bacterial septum prior to cell separation. Another class of proteins that are strongly but noncovalently associated with peptidoglycan is the class of OmpA-like proteins. Included are immunologically cross-reacting proteins from several diverse species. In *E. coli*, loss by double mutation of the OmpA protein and the Braun lipoprotein causes cells to become rounded rather than rod shaped and to require osmotic stabilization for growth. In *P. aeruginosa* strains that lack a lipoprotein that is covalently bound to peptidoglycan, loss of the OmpA equivalent, OprF, has the same effect. Thus, in addition to cytoplasmic membrane penicillin-binding protein 2, outer membrane proteins help determine the shape of gram-negative bacteria.

One very specific example of a structural role for an outer membrane protein is provided by the *Chlamydia trachomatis* MOMP. During its extracellular phase, this organism exists as a nonmetabolizing, rigid-structured elementary body. Upon entering eukaryotic cells, *C. trachomatis* transforms into a more loosely structured, metabolizing reticulate body. During this transformation, disulfide bridges between adjacent MOMP molecules are reduced. Presumably, such cross-linking is a primary determinant of the rigidity of the elementary body.

Acknowledgments

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Suggested Reading

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