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Cracking outer membrane biogenesis

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ABSTRACT

The outer membrane is a distinguishing feature of the Gram-negative envelope. It lies on the external face of the peptidoglycan sacculus and forms a robust permeability barrier that protects extracytoplasmic structures from environmental insults. Overcoming the barrier imposed by the outer membrane presents a significant hurdle towards developing novel antibiotics that are effective against Gram-negative bacteria. As the outer membrane is an essential component of the cell, proteins involved in its biogenesis are themselves promising antibiotic targets. Here, we summarize key findings that have built our understanding of the outer membrane. Foundational studies describing the discovery and composition of the outer membrane as well as the pathways involved in its construction are discussed.

1. The Gram-negative cell envelope

One hundred and thirty-eight years ago, Hans Christian Gram developed a stain that distinguished two major classes of bacteria ([1]; for an English translation, see [2]). His eponymous stain is still in wide use today and it has as its basis fundamental differences in the cell envelopes of Gram-positive and Gram-negative bacteria. We now know that in addition to the cytoplasmic membrane, the former have a thick peptidoglycan cell wall, while the latter have a thin peptidoglycan and an additional membrane, the outer membrane (OM; Fig. 1; [3]), but it took more than a century and the development of novel methods and technologies to reveal these differences in detail.

Although eukaryotic organelles such as mitochondria and chloroplasts have an OM reflecting their evolution from bacterial ancestors, novel lipid composition and bilayer asymmetry distinguish the Gram-negative OM from all other biological membranes. For these organisms it is likely essential because it functions to contribute mechanical strength to compensate for the thin cell wall [4]. In addition, the OM functions as an effective permeability barrier allowing free passage of small molecule nutrients such as amino acids and sugars, but excluding large, hydrophobic and harmful compounds [5]. The OM permeability barrier explains why Gram-negative bacteria are innately more resistant to many antibiotics than Gram-positive bacteria, and with the ever-increasing threat of multi-drug-resistant bacteria there is growing interest in identifying ways to circumvent this obstacle.

Here we summarize major discoveries that led to our current understanding of the anatomy of the Gram-negative cell envelope and the composition, permeability properties and biogenesis of the OM in particular.

2. Anatomy

Not long after Gram's report, Richard Pfeiffer, a student of Robert Koch, made a remarkable discovery. He showed that heat-killed *Vibrio cholerae* retained their toxic potential; i.e., the guinea pigs still died. He realized that there must be a non-proteinaceous toxin associated with the insoluble part of the bacterial cell and this toxin became known as endotoxin [6]. His observations generated enormous interest because they seemed to violate one of his mentor's famous postulates, namely successful recovery of the infectious agent from the victim. Soon after, it became clear that many Gram-negative bacteria have essentially the same endotoxin [7]. The chemistry of endotoxin is complex, and it took decades and the work of many people to elucidate the structure, but an important advance came with the development of a method for extracting the molecule in a more purified form [8]. Because it contained both lipid and sugars, it became known as lipopolysaccharide (LPS). Indeed, the fact that antibodies against endotoxin recognize the O-antigen that formed the basis of the Kauffmann-White classification scheme for *Salmonella* led to the realization that LPS is located at the cell surface (for reviews see [9,10]). It is worth noting that the structure of LPS was confirmed by the co-crystallization of LPS with the iron transport protein FhuA [11].

The development of the electron microscope and techniques such as ultrathin sectioning allowed the first real glimpses of the bacterial cell

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envelope. With Gram-negatives such as *Escherichia coli*, a double-layered envelope was commonly observed. One of the layers corresponded to the cytoplasmic or inner membrane (IM), but the other, outer-most layer seemed to be a complex multidimensional cell wall that contained LPS (e.g., [12]). This likely reflects the fact that the peptidoglycan and OM in this bacterium are tightly associated (see below).

A major breakthrough came in 1964 with a paper published by Bladen and Mergenhagen in the *Journal of Bacteriology* [13]. The micrographs in this paper clearly show that the cell envelope contains three distinct layers: an OM that contained LPS, a cell wall that could be degraded by lysozyme, and a cytoplasmic or IM. Significantly, this paper is the first to use the term OM in the context that we understand it today.

What is notable about this Bladen and Mergenhagen paper is that they were studying the dental pathogen *Veillonella*. This is truly remarkable because DNA sequence analysis shows that this is a Gram-positive bacterium that also contains the genes required for OM biogenesis! It clearly belongs to the phyla Firmicutes, which includes Bacillus, Staphylococcus and Streptococcus, but to one of two different clades in this phyla that have two membranes; Veillonella is a Negativicute. The existence of these diderm Gram-positives argues strongly that monoderms evolved from diderms by the loss of the OM [14]. The ancestor of the diderms remains to be identified, but it is hard to imagine that there were two membranes before there was one.

The discovery of diderm bacteria provided a satisfying explanation for another enduring enigma. It had long been known that when Gram-positive bacteria are disrupted, degradative enzymes such as DNase and alkaline phosphatase pellet with the cell debris, and this was interpreted correctly to mean that these enzymes were attached to the cell wall. With Gram-negatives however, many of these degradative enzymes are soluble, and it seemed most unlikely that they would be cytoplasmic. Leon Heppel [15] developed methods, such as cold osmotic shock, to show that these enzymes were safely contained in the cellular compartment bounded by the IM and the OM, an extra-cytoplasmic, aqueous compartment called the periplasm (Fig. 1).

3. OM composition

Not long after the *Veillonella* paper appeared, three different labs published methods to separate the IM and the OM and determine their composition. Two of the methods, one from the lab of Shoji Mizushima [16] and the other from the lab of Mary Jane Osborn [17], used sucrose gradients to exploit the difference in density between the membranes. The third, from the lab of Carl Schnaitman [18] used differential detergent solubility. These methods demonstrated that the OM contains four major components: phospholipids (PLs), LPS, lipoproteins, and integral OM proteins (OMPs; Fig. 1). The Osborn method has proven the most reliable. Mary Jane did much to convince the world that the OM was a membrane and not some complex cell wall, and she was also an early role model for women in science [19].

As noted above, a unique feature of the OM is the asymmetric distribution of its lipids with LPS located in the outer and PLs located in the inner leaflet, a fact first demonstrated in the lab of Hiroshi Nikaido (Fig. 1) [20]. It is worth noting that in his extensive review of OM permeability [5], he notes that this 1976 paper "was rejected twice by *Biochemistry* because a reviewer insisted that such an asymmetric distribution of bilayer components was thermodynamically impossible." The content of OM PLs is similar to IM PLs [17]. Like PLs, LPS is synthesized in the inner leaflet of the IM, and we refer readers to reviews that summarize the LPS biosynthetic pathway [21,22].

The OM is unusually protein rich [23], but in *E. coli* K-12 the bulk of the protein is comprised of four extremely abundant proteins. The most abundant protein in *E. coli* is Lpp, a protein also known as Braun's lipoprotein. Volkmar Braun elucidated the structure of the lipid moiety attached to the amino terminus of this and all other bacterial lipoproteins and he demonstrated that the carboxy terminal lysine residue of Lpp is covalently attached to the peptidoglycan cell wall [24]. In a recent biography, he describes this work in a more personal tone [25], noting that he was simply trying to figure out why the protease trypsin in particular seemed to release the OM from the cell wall [26]. The reactions required to modify lipoproteins follow a specific pathway (for review see [27]) and will be discussed in more detail below.

In *E. coli* K-12, the three remaining major OMPs are now known as

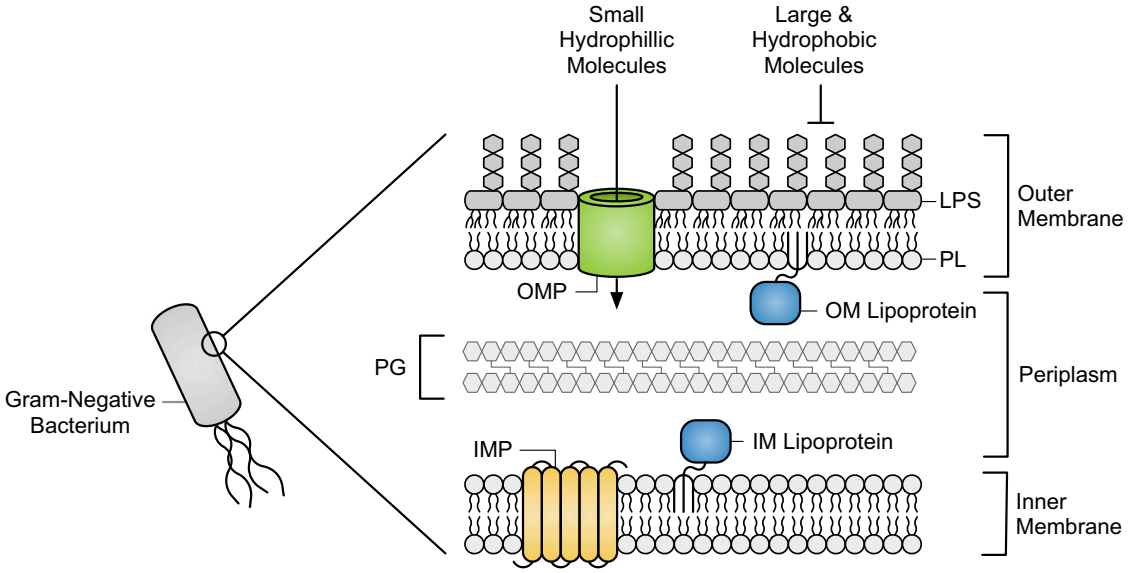

Fig. 1. Structure and composition of the Gram-negative envelope. The envelope of Gram-negative bacteria consists of an inner membrane, an outer membrane, and a peptidoglycan sacculus. The peptidoglycan sacculus lies within the aqueous compartment between the inner and outer membranes called the periplasm. While the inner membrane is a traditional glycerophospholipid bilayer, the outer membrane is a highly asymmetric lipid bilayer consisting of the glycolipid lipopolysaccharide in the outer leaflet and glycerophospholipids in the inner leaflet. Integral inner membrane proteins are alpha-helical while integral outer membrane proteins are β-barrels. Lipoproteins are located in the periplasmic leaflet both membranes. Small hydrophilic molecules can freely diffuse across the outer membrane while large and/or hydrophobic molecules cannot. IM, inner membrane; IMP, inner membrane protein; LPS, lipopolysaccharide; OM, outer membrane; OMP, outer membrane protein; PG, peptidoglycan; PL, phospholipid.

OmpA, OmpF and OmpC. OmpA plays a structural role by interacting with the cell wall. Destroying both the covalent and non-covalent interactions between the OM and the cell wall by removing both Lpp and OmpA cause severe synthetic defects [28]. Studies in the Henning lab with OmpA also revealed the remarkable stability of OMPs. When run on an SDS gel without heating they remain folded. Heating disrupts the structure, and the unfolded protein runs more slowly through the gel, a property termed heat modifiability [29]. Since the final folded conformation of the OMP only occurs when it is in the membrane, heat modifiability serves as a simple assay for proper assembly of OMPs and it is used extensively today for that purpose. OmpF and OmpC are general porins as described in the following section. These and nearly all other OMPs adopt a β-barrel structure as first revealed in the crystal structure of the porin from *Rhodobacter capsulatus* [30] and shortly thereafter for OmpF and PhoE from *E. coli* [31]. The porins are 16-stranded barrels while the major form of OmpA has an 8-stranded barrel [32] and a carboxy-terminal periplasmic domain. There is also a form of OmpA with a larger barrel that has pore-forming activity [33], but the barrel structure of that form is unknown.

A recent study using atomic force microscopy revealed OM organization at cellular scale. It shows that the porins form a near-static network across the entire cell surface interspersed with phase separated patches of LPS. OmpA is embedded within the porin network. The lasting impression from these images is that the OM is wall-to-wall porins [34].

4. OM permeability

It is worth noting that at the time Watson and Crick [35] published their model for the structure of DNA, it was widely believed, because of their assumed simplicity, that bacteria lacked specific transport systems and were basically semipermeable bags of enzymes. This view was reinforced by the great success of biochemical genetics, an approach that analyzed metabolic pathways by isolating mutants and adding pathway intermediates to the media to rescue growth [36]. This changed with the demonstration that the "cryptic" *lacY* mutants, which could not grow on lactose despite having active β-galactosidase, lacked a "permease" that could actively transport galactosides ([37]; for a review in English, see [38]). In Monod's Nobel lecture he states: "Some of the researchers who did not really doubt its existence still reproached me from time to time for giving a name to a protein when it had not been isolated." We now know that 15 % of the *E. coli* genome is devoted to transport proteins [39]. Of course, most of these proteins reside in the IM.

Early hints of a molecular sieve-like function for the cell envelope came from the work of Payne and Gilvarg [40]. Using peptides to satisfy an amino acid auxotrophy, they could show that peptides greater than six amino acids were excluded from the oligopeptide transport system in the IM. They did this by showing that these larger peptides could not satisfy the auxotrophy and were not competitive inhibitors the IM transport system. To explain their results, they proposed a barrier-like function for the "cell wall." Similarly, Decad and Nikaido [41] found that only di- and trisaccharides could freely diffuse into the periplasm. These studies suggested a cutoff around 600 Da for peptides and sugars.

Kaspar von Meyenburg [42] selected a mutant *E. coli* B/r that required increased glucose concentrations for half-maximal growth rate. His method involved mutagenesis followed by a penicillin enrichment for non-growing cells in media with a very low glucose concentration and then screening this population for delayed coloring on MacConkey plates with very low glucose concentrations. This mutant, which he termed *kmt*, exhibited an approximately 100-fold increase in the K_m for every transport system that he measured, including not only sugars, but also amino acids and ions. However, at high nutrient concentration, the mutant was indistinguishable from the wild type.

Taiji Nakae [43] demonstrated that a heat modifiable oligomer of the major OM protein we now know as OmpF from *E. coli* B (the parent of B/r) produces transmembrane channels in reconstituted OM vesicles. These channels allowed rapid diffusion of saccharides less than 550 Da, and he proposed the name porins to describe this class of proteins.

Joe Lutkenhaus [44] isolated Cu^{2+}-resistant *E. coli* B/r mutants and showed that they had the same phenotypes of von Meyenburg's *kmt* mutant. Lutkenhaus found that these mutants lacked OmpF. The *kmt* mutant maps the *ompB* region which contains the genes for the two-component regulatory system (OmpR/EnvZ) that controls porin gene expression [45], and likely affects the positive regulatory protein OmpR. In contrast to *E. coli* B, *E. coli* K-12 has two very similar major OM proteins now called OmpF and OmpC. Lutkenhaus [44] showed that in *E. coli* K-12, Cu^{2+}-resistance and the *kmt*-like phenotypes require the loss of both of these porins. Loss of either individually have little effect on overall permeability properties.

A detailed discussion of porins in general can be found in the aforementioned review by Nikaido [5]. For hydrophilic molecules it is clear that their penetration across the OM depends on their size. As molecular mass increases above 600 g/mol, the rate of diffusion through the porins decreases substantially.

Owing to the classic work of Loretta Leive, we have long known that the OM is an effective barrier for hydrophobic compounds such as the actinomycin (molecular mass 1255), and that this barrier function is dependent upon LPS. She showed that brief treatment of cells with low concentrations of EDTA renders *E. coli* sensitive to this drug without altering viability [46], and that this treatment causes release of substantial amounts of LPS [47]. LPS is negatively charged and the strong lateral interactions between molecules are stabilized by divalent cations such as Mg^{2+}. Chelators like EDTA remove the cations and cause release of LPS. This loss is compensated by PLs flipping from the inner leaflet into the outer leaflet resulting in patches of PL bilayer through which lipophilic molecules like actinomycin freely pass [5].

As noted above, LPS is present in the OM of *E. coli* patches that behave as liquid phases [34]. Two different mechanisms operate in this bacterium to maintain OM asymmetry. One system, MlaABCDEF, removes PLs from the outer leaflet and transports them back to the IM. In the other system, the enzyme PldA destroys PLs in the outer leaflet. When both systems are removed, the amount of PLs in the outer leaflet increase 25-fold and this effectively destroys the hydrophobic barrier [48]. In such double mutants, patches of PL bilayer appear on the cell surface in pimple-like fashion [34].

5. OM biogenesis

All OM components are synthesized in the cytoplasm or cytoplasmic leaflet of the IM. Each component must cross both the IM and periplasm prior to being added to the OM. As this environment lacks conventional sources of energy, constructing the OM presents an engineering challenge. In this section, we describe how LPS, PLs, OMPs, and lipoproteins are transported and inserted into the OM.

5.1. Lipopolysaccharides

LPS consists of lipid A, a phosphorylated glucosamine disaccharide containing 6 or more acyl chains, ligated to a core oligosaccharide and a repeating polysaccharide known as O antigen [49]. Each component of LPS is synthesized in the cytoplasm and/or cytoplasmic leaflet of the IM [22,49–51]. Lipid A-core and the O antigen are synthesized separately and flipped into the periplasmic leaflet of the IM by the ABC transporter MsbA and the Wzx/Wzy system, respectively (for reviews on the Wzx/Wzy system, see [52–54]). MsbA was first identified as a multicopy suppressor of a temperature sensitive allele of *lpxL* (formerly *htrB*) [55]. While the function of LpxL at the time of this discovery was unknown, it was later shown that LpxL is an acyltransferase involved in lipid A biosynthesis [56]. A role for MsbA in LPS transport was proposed by Mdluli et al. [57], who found that *Francisella novocidia msbA* mutants have defects in the OM barrier. In support of this proposed function, Polissi and Georgopoulos [58] showed that LPS in *E. coli lpxL* mutants

accumulates at the IM and that accumulation is relieved upon MsbA overexpression. These authors also showed that LPS is trapped in the IM of mutants with decreased MsbA activity. Newly synthesized LPS is not modified by periplasmic enzymes in *msbA* mutants, indicating that the trapped LPS accumulates in the cytoplasmic leaflet of the IM [59].

Once in the periplasm, O antigen is attached to lipid A-core by the glycosyltransferase WaaL [60,61]. The complete LPS molecule is extracted from the IM and transported directly to the outer leaflet of the OM by the LPS transport pathway, an envelope spanning protein complex consisting of the OM channel LptDE, the IM pump LptBCFG, and the periplasmic protein LptA (Fig. 2) [22,62–67]. LPS is removed from the periplasmic leaflet of the IM by the ABC transporter LptBFG [68–70]. ATP binding and/or hydrolysis loads the LPS molecule onto a continuous bridge formed by LptA and the periplasmic domains of LptC and LptD [62,71–75]. LPS is pushed across the bridge and out the LptDE OM channel as each new molecule of LPS is loaded into the IM pump [62,68].

The first component of the LPS transport pathway to be identified was LptD (formerly Imp and OstA). To gain insight into the barrier function of the OM, Misra et al. [76] selected for mutants that would allow large polymers of maltose, called maltodextrins, to diffuse across the OM in the absence of the dedicated maltodextrin OM channel LamB. One of the mutations isolated, named *lptD*4213, not only allowed the *lamB* mutant to grow using maltodextrins as the sole carbon source, but also increased sensitivity to several dyes, detergents, and antibiotics that cannot normally penetrate the OM barrier [77]. Over a decade later, Braun and Silhavy [78] showed that LptD is an essential OM β-barrel and that depleting LptD alters the buoyant density of the OM by increasing the protein to lipid ratio. Taking advantage of the fact that LPS is not essential in *Neisseria meningitidis* [79], the Tommassen group [80] showed that deleting *lptD* prevents LPS from reaching the cell surface without stopping synthesis, solidifying a role for LptD in LPS transport to the OM.

Other components of the LPS transport pathway were identified using various approaches. The OM lipoprotein LptE (formerly RlpB) was isolated as an interacting partner of LptD [81], while LptA, LptB, and LptC (formerly YhbN, YhbG, and YrbK) were found in a screen for essential genes [82]. Depleting any of these proteins results in phenotypes that would be expected for defects in LPS transport, including changes in OM density, accumulation of newly synthesized LPS in the IM, and abnormal membrane structures in the periplasm [69,78,81–83]. Based on primary sequence analysis, LptB was predicted to be the cytoplasmic ATP-binding component of an ABC transporter [69,83]. However, as LptA, LptC, LptD, and LptE lacked homology to ABC transporters, additional components of the LPS transport pathway likely existed. To identify these components, Ruiz et al. [70] performed a comparative genomics study looking for envelope-localized proteins of unknown function that are essential in *E. coli* and present in the Gram-negative endosymbiont *Blochmannia floridanus*, which has one of the smallest genomes in the Enterobacteriaceae family [84]. This analysis yielded two proteins, LptF and LptG (formerly YjgP and YjgQ), whose predicted topology is similar to that of the transmembrane component of ABC transporters. Like other proteins of the LPS transport pathway, cells depleted of LptF or LptG have aberrant membranes and fail to transport newly synthesized LPS to the cell surface.

5.2. Phospholipids

The first insights into the mechanism by which PLs are transported from their site of synthesis in the IM to the OM came, not surprisingly, from the lab of Mary Jane Osborn [85]. Using *Salmonella Typhimurium*, they developed a method of incorporating exogenous lipids into the OM. When phosphatidylserine was incorporated, she could show that it was rapidly converted to phosphatidylethanolamine (PE). Since the enzyme that catalyzes this decarboxylation in located in the IM, this indicated that PLs rapidly equilibrate between membranes. Rapid equilibration was also observed with other PLs, even cholesterol esters, a lipid foreign to the bacterium, but notably, not with LPS or with a LPS precursor. They suggested diffusional flow of PLs between membranes perhaps through what had been termed zones of adhesion by Manfred Bayer [86].

Results showing rapid equilibration of PLs were also obtained by

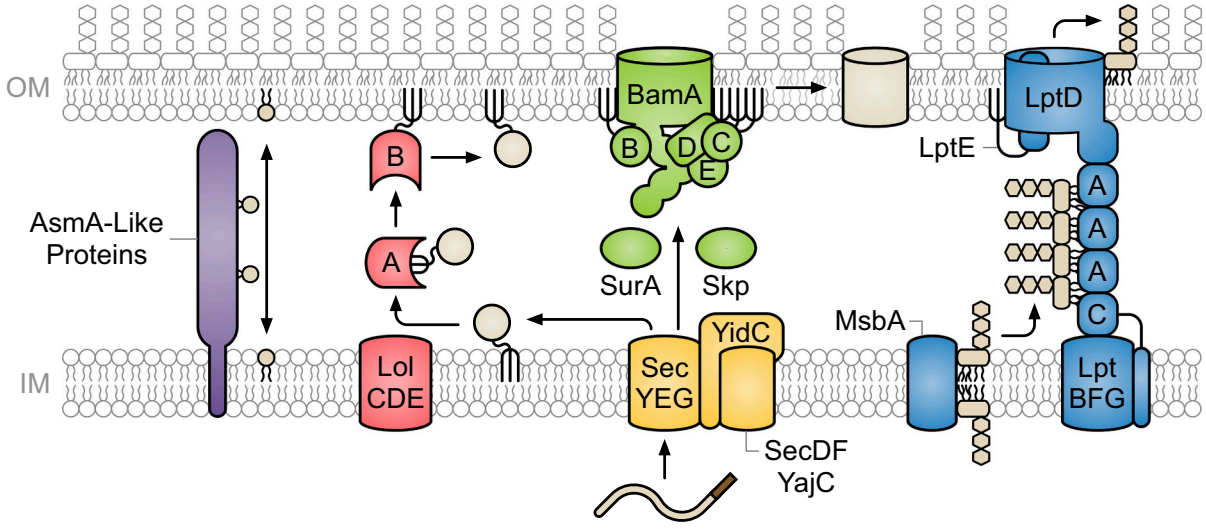

Fig. 2. Outer Membrane biogenesis pathways. All components of the outer membrane are synthesized in the cytoplasm and/or cytoplasmic leaflet of the inner membrane. Proteins destined for the outer membrane contain an amino-terminal signal sequence (brown) that directs them to the general secretion system (SecYEG-SecDF-YajC-YidC) for transport across the inner membrane. Secreted outer membrane proteins are bound by periplasmic chaperones (SurA and Skp) and delivered to the β-barrel assembly machine (BamABCDE), which folds and inserts them into the outer membrane. Lipoproteins are modified in the periplasmic leaflet of the inner membrane. They are extracted from the inner membrane, carried across the periplasm, and inserted into the periplasmic leaflet of the outer membrane by the localization of lipoprotein pathway (LolABCDE). Lipopolysaccharide is flipped into the periplasmic leaflet of the inner membrane by MsbA. It is then transported directly to the outer leaflet of the outer membrane by the lipopolysaccharide transport pathway (LptABCDEFG). Anterograde and retrograde phospholipid transport is carried out by proteins of the AsmA-like family, including YhdP, TamB, and YdbH. Proteins involved in secretion are shown in yellow, outer membrane protein assembly are in green, lipoprotein transport are in red, lipopolysaccharide transport are in blue, and phospholipid transport are in purple. Substrates are shown in tan. IM, inner membrane; OM, outer membrane.

Donohue-Rolfe and Schaechter [87] and Langley et al. [88] in *E. coli* using different pulse-labeling methods. Donohue-Rolfe and Schaechter [87] also showed that the translocation of newly synthesized PE to the OM could be inhibited by agents that deplete the proton motive force. Both studies also suggested that translocation may occur at zones of adhesion, but proposed that these sites may be hemifusions in which the outer leaflet of the IM is fused to the inner leaflet of the OM. It was suggested that the PMF could be required to establish these hemifusions, or to flip newly synthesized PLs from their site of synthesis in the inner leaflet of the IM to the outer leaflet. Despite the efforts of many, little progress was made for decades.

Studies with the aforementioned Mla retrograde PL transport system led to the identification of components involved in anterograde PL transport. The Mla system contains an OM lipoprotein MlaA, a periplasmic protein MlaC, and an ABC transporter complex in the IM MlaFEDB [48]. MlaA is a doughnut shaped molecule imbedded in the inner leaflet of the OM with a central amphipathic pore. The α-helices that form the doughnut prevent access of PLs from the inner leaflet to the pore but allow access to PLs in the outer leaflet [89]. The current model proposes that binding of PLs from the outer leaflet opens the pore allowing transfer of the PL to periplasmic MlaC, which binds PLs with high affinity. MlaC is then unloaded in an ATP-dependent manner by the Mla ABC transporter, which transfers the PL to the IM (for review see [67]).

In a search for suppressors of the LPS assembly defect caused by an *lptD* mutation, Sutterlin et al. [90] identified a mutation called *mlaA**, which causes dominant phenotypes in an otherwise wild-type strain that are independent of any of the other *mla* gene products. This mutation is a two-codon deletion that disrupts helix 1 allowing phospholipids to flow from the inner leaflet of the OM into the outer leaflet [89]. The excess PLs in the outer leaflet activate the phospholipase PldA and the fatty acids generated act as second messengers that signal the need for increased synthesis of LPS in an attempt to restore homeostasis. This demonstrates that PldA protects the OM not only by destroying PLs in the outer leaflet, but also by playing an important signaling role in regulating LPS synthesis [91]. This increase in LPS likely explains how it suppressed the *lptD* mutation.

Clearly, the most striking phenotype conferred by the *mlaA** mutation is lysis of stationary phase LB cultures [90]. The two-fold increase in LPS levels caused by *mlaA** destabilizes the OM and lipids are lost by vesiculation. The OM cannot shrink because of the PG wall, so to make up for the lost lipids, PLs flow from the IM to the OM. In starved cells these PLs cannot be replaced, and the cytoplasm shrinks until the IM ruptures. Data indicate that 1 % of the IM lipids can flow to the OM per minute in a starved cell [92]. This result is best explained by the existence of a diffusive flow mechanism, consistent with studies from the 70s and 80s noted above, although this diffusive flow was not inhibited by agents that disrupt the PMF. Does this diffusive flow occur at sites of hemifusion, or is there a protein bridge akin to the Lpt bridge that mediates LPS transport from the IM to the OM?

Following transposon mutagenesis, Grimm et al. [92] looked for mutations that would slow the rate of PL flow from the IM to the OM and thus the rate of *mlaA** lysis in spent LB media. They found that deleting *yhdP* delayed lysis by reducing the rate of PL transport between membranes by 50 %. Because removing YhdP slows PL flow, lysis takes longer, but now it occurs because it is the OM that ruptures, not the IM. Clearly YhdP is not essential, so if it does play a direct role in PL transport, then there must be multiple pathways.

Indeed, YhdP belongs to a family of six AsmA-like proteins [93] and members of this family share homology with eukaryotic PL transporters [94,95]. Ruiz et al. [96] systematically constructed mutants lacking all possible combinations of these six genes. Their results show that YhdP and the paralogs TamB and YdbH are functionally redundant. Similar results have also been obtained by Douglass et al. [97].

Strikingly, structural models released by Alphafold [98] show that YhdP, which contains 1266 amino acids, forms a long tube-like structure rich in β-strands that does actually resemble the LPS transport bridge formed by the Lpt proteins. Clearly many questions remain, but it seems quite likely that diffusive PL transport also occurs on protein bridges and the redundancy of these transporters explains why their identification was so difficult.

5.3. General secretion system

Most proteins destined for the IM or beyond are secreted from the cytoplasm by the general secretory (Sec) system (reviewed in [99–101]). Such proteins are flagged for secretion by an internal signal sequence, which is a short stretch of hydrophobic amino acids located at or near the amino terminus [102–104]. In Gram-negative bacteria, proteins are secreted either during or after translation. In the post-translational pathway, partially or completely translated proteins are bound and maintained in a secretion-competent state by the cytoplasmic chaperone SecB [105,106]. The presecretory proteins are then delivered to an IM translocon in a process facilitated by the ATPase SecA [107,108]. ATP hydrolysis by SecA and the proton motive force power transport of the presecretory protein across the IM through the translocon channel [109–111]. In the co-translational pathway, the signal sequence is bound by signal recognition particle (SRP) as it emerges from the ribosome [112]. SRP transfers the ribosome and nascent peptide to the IM translocon, and the protein is fed through the translocon channel as translation continues [113]. The signal sequence may be cleaved from the secreted protein on the periplasmic side of the IM by the signal peptidase LepB [114–117]. Notably, a different signal peptidase is used for lipoproteins and will be discussed below.

The translocon core consists of the protein conducting channel SecY and associated proteins SecE and SecG [109,118–120]. Additional IM proteins interact with the translocon core, including the SecDF-YajC subcomplex and YidC [121,122], and together these proteins comprise the holo-translocon (Fig. 2). SecD and SecF enhance secretion through SecYEG, most likely by coupling the proton motive force to the translocation process [101,123–127]. By contrast, the role of YajC in protein secretion remains completely unknown. While mutations in *secD* or *secF* greatly diminish secretion, deletion of *yajC* has little-to-no effect [123,124,126,127]. YidC facilitates insertion of proteins into the IM and, like SecY, SecE, and SecA, is essential for viability [128].

Many components of the Sec system were identified by isolating mutants that fail to secrete proteins of the maltose transport system (reviewed in [129]), which consists of the OM channel LamB, the periplasmic carrier MalE, and the IM ABC transporter MalFGK. To track the location of the maltose transport components, each protein was fused to *β*-galactosidase. As with their wildtype counterparts, expression of the fusion proteins can be induced by adding maltose to the medium [130,131]. Unexpectedly, increased expression of the LamB-LacZ and MalE-LacZ fusion proteins causes lethal jamming of the secretion apparatus (maltose sensitive). The Beckwith [132] and Silhavy [133] groups took advantage of this lethality to isolate secretion-defective mutants that can grow in the presence of maltose (maltose resistant) without disrupting expression of the fusion proteins ($LacZ^+$). The mutations mapped to the amino terminus of the fusion proteins, providing strong support for the signal hypothesis proposed by Blobel and Dobberstein [102]. Indeed, otherwise wildtype LamB or MalE proteins containing the isolated mutations are retained in the cytoplasm and are larger than their secreted counterparts. This would be expected for presecretory proteins that still contain their signal sequence.

Though expressed at appreciable levels, the LacZ fusion proteins are not lethal in the absence of inducer. β-Galactosidase activity is unexpectedly low under these conditions as the fusion proteins are either secreted from the cytoplasm or caught in the secretion apparatus. Thus, mutants with impaired secretion can be selected for by demanding growth on lactose minimal medium. Genes encoding several proteins of the Sec system were identified using this strategy [123,134,135].

Another strategy used to identify components of the Sec machinery

made use of *malE* and *lamB* mutants with defective signal sequences. Since these mutant proteins are retained within the cytoplasm, *E. coli* expressing these mutant proteins cannot grow using maltose or maltodextrins, respectively, as the sole source of carbon. Dominant, gain-of-function mutations that secrete the mutant proteins despite their defective signal sequences were selected for by demanding growth on maltose or maltodextrin minimal medium [136–138]. This and the above selection isolated mutations that mapped to the same genetic loci [139,140].

5.4. β-Barrel OMPs

OMPs released from the Sec translocon are inserted into the OM by the β-barrel assembly machine (Bam) complex, which consists of the OMP BamA and the lipoproteins BamBCDE (Fig. 2) [141–143]. Their transport across the periplasm is facilitated by chaperones like SurA and Skp, which act as either soluble carriers or as part of a trans-envelope complex that connects the Sec translocon directly to the BAM complex [144–146]. However, as discussed below, the signal that initiates OMP assembly by the BAM complex is located at the carboxy-terminus of the substrate. Therefore, OMP assembly will not occur until translocation from the cytoplasm is complete. Although the mechanism by which the Bam complex folds and inserts β-barrel proteins into the OM bilayer is not yet fully understood, several features of this mechanism have been established. Unfolded OMP substrates are recognized by BamD [147]. The essential role of this lipoprotein is regulatory, likely functioning as a quality control checkpoint [148]. All of the reactions necessary to fold and insert OMPs in the membrane are carried out by BamA [148–150]. The carboxy-terminal β-strand of the OMP substrate interacts with the amino-terminal β-strand of the BamA barrel [151–158]. BamA then catalyzes the sequential addition of β-hairpins in a carboxy- to amino-terminal direction. Some folding can occur in the periplasm, some inside the BamA barrel, and some outside the opened BamA barrel [153,156,159,160]. Hydrogen bonding of the amino- and carboxy-terminal substrate β strands releases the folded OMP from the Bam complex.

A role for BamA (formerly Omp85 and YaeT) in OMP assembly was first proposed by the Tommassen group [141], who used a candidate approach to identify proteins involved in OM biogenesis. Such proteins were expected to be essential, located in the OM, and conserved among Gram-negative bacteria. Not only did BamA fit these parameters, but studies had also shown that eukaryotic homologues of BamA function as protein transporters [141,161–163]. The Tommassen group [141] showed that OMPs are expressed but not properly assembled in *N. meningitidis* depleted of BamA and that BamA directly interacts with an unfolded OMP substrate. However, a subsequent study from the van der Ley group [164] argued against a role for BamA in OMP assembly. These authors found that levels of LPS and PLs in the OM of *N. meningitidis* depleted of BamA are decreased while protein levels remain unchanged. As such, they concluded that BamA is involved in the assembly of OM lipids and that any effect on proteins is indirect.

The Kahne and Silhavy laboratories began working on the Bam complex in response to the unexpected findings of a study investigating the mechanism of action of chlorobiphenyl vancomycin and moenomycin [165]. These antibiotics are not effective against wildtype *E. coli* as they are unable to penetrate the OM. For this reason, resistant mutants were isolated in *E. coli* expressing *lptD*4213, which had previously been shown to increase OM permeability ([77]; see above). Nearly all the resistant mutants isolated had loss-of-function mutations in *bamB* (formerly *yfgL*). Surprisingly, loss of *bamB* in *lptD*4213 increased resistance to antibiotics that are structurally and functionally unrelated to chlorobiphenyl vancomycin and moenomycin, suggesting that the OM permeability barrier had been restored. Insight into the function of BamB was gained through protein interaction studies, which revealed that BamB is part of a multiprotein complex consisting of BamA, BamC (formerly NlpB), and BamD (formerly YfiO) [142]. As had been observed in *N. meningitidis* [141], depleting BamA in *E. coli* led to increased levels of unfolded and misassembled OMPs [142]. A study by Doerrler and Raetz [166] built upon these findings by showing that OMP assembly is affected by a temperature sensitive mutation in *bamA* while LPS and PL assembly are not. Together, these data support a role for BamA in OMP assembly as proposed by the Tommassen group [141].

Evidence supporting a role for the Bam complex in OMP assembly continued to be gathered as the functions of BamB, BamC and BamD were investigated. Like BamA, BamD is essential [167,168]. *E. coli* depleted of BamD display similar phenotypes to those depleted of BamA and, consistent with the results of Doerrler and Raetz [166], depleting BamD has negligible effects on LPS assembly [167]. While *bamB* is dispensable, *E. coli* lacking *bamB* have clear defects in OMP assembly as indicated by increased detergent and antibiotic sensitivity and decreased levels of abundant OMPs [142,165]. Despite being part of the Bam complex, loss of *bamC* has no impact on OM permeability or steady-state OMP levels [142]. However, both parameters are greatly affected when *bamC* is deleted in *E. coli* lacking *bamB* or carrying a partial loss-of-function mutation in *bamD*. These synthetic phenotypes indicate that the function of BamC is partially redundant with BamB and BamD [149].

The final component of the Bam complex, BamE (formerly SmpA), was identified in a study looking for additional interacting partners of BamA [143]. *E. coli* lacking *bamE* displayed several phenotypes that had previously been associated with defects in OMP assembly, including decreased steady-state levels of abundant OMPs, increased levels of unfolded OMPs, and increased sensitivity to detergents and antibiotics. These phenotypes are exacerbated when *bamE* is deleted in *E. coli* lacking *bamC* [143], and a *bamB bamE* double mutant is viable only when grown on minimal medium [143,169].

5.5. Lipoproteins

Lipoproteins are anchored to the membrane by three acyl chains, which are attached to a conserved cysteine residue that immediately follows the signal sequence [24,170]. After secretion into the IM, diacylglyceryl from phosphatidylglycerol is transferred to the sulfhydryl group of the cysteine residue by Lgt [171,172]. Next, the lipoprotein-specific signal peptidase Lsp removes the signal sequence from the diacylated prolipoprotein [173–176]. The final acyl chain is then attached to the now exposed amino group of the diacylated cysteine by Lnt, which uses any phospholipid as an acyl donor [177–180].

Whether the mature lipoprotein remains in the IM or is translocated to the OM depends on the identity of the amino acids that immediately follow the acylated cysteine. The initial experiments leading to what is now known as the +2 rule, or the Lol (localization of lipoproteins) avoidance signal, were carried out by the Inouye group [181,182], who showed that a periplasmic protein containing the signal sequence and first nine amino acids of an OM lipoprotein localizes to the OM. Inspecting the amino-terminal sequence of several lipoproteins revealed that IM, but not OM, lipoproteins contain a negatively charged amino acid at position two of the mature protein sequence. Indeed, an IM lipoprotein can be targeted to the OM when the negatively charged aspartate at position two is replaced with a serine. Likewise, an OM lipoprotein can be targeted to the IM when the amino acid at position two is changed to aspartate. Together, these results showed that the second amino acid plays a critical role in determining lipoprotein localization. A clever selection by Seydel, Gounon, and Pugsley [183] revealed that amino acids at position two other than aspartate will target lipoproteins to the IM; however, these residues are rarely present in naturally occurring lipoproteins. Subsequent research found that the identity of the third amino acid can influence whether lipoproteins containing aspartate at position two are retained within the IM, demonstrating that the +2 rule is not absolute [184,185].

The proteins responsible for translocating lipoproteins to the OM were identified through a series of biochemical experiments performed by Tokuda and colleagues [186–189]. Their method involved

monitoring the movement of a model lipoprotein from spheroplasts or IM proteoliposomes to a periplasmic carrier and the periplasmic carrier to OM proteoliposomes using extracts containing different IM, periplasmic, or OM proteins. Proteins from extracts that catalyzed movement were identified by peptide sequencing. This work revealed that five proteins (LolA-E) are required to translocate lipoproteins to the OM in *E. coli*, each of them essential for viability. Lipoproteins are extracted from the IM and transferred to the periplasmic carrier LolA by the ABC transporter LolCDE. LolA delivers the lipoproteins to the OM acceptor LolB, which facilitates their insertion into the OM (Fig. 2).

6. Concluding remarks

The distinguishing feature of the Gram-negative cell envelope is the OM. Its asymmetric structure and unique composition bestow upon this membrane unusual mechanical strength and highly selective permeability properties. While other biological membranes, including the bacterial IM, are impermeable to hydrophilic molecules but easily penetrated by hydrophobic compounds, the OM is quite the opposite, being freely permeable to small hydrophilic molecules and impermeable to hydrophobic compounds. The widely contrasting properties of this double layered envelope offers considerable protection to all antibiotic targets that lie in the cytoplasm.

Since the turn of the century, we have had success identifying the cellular components that are required to transport the four essential constituents of the OM - lipoproteins, OMPs, LPS, and PLs - from the IM to the OM and these discoveries have established a number of new drug targets. Importantly, access to most of these targets requires that the drug only need cross the OM and rules for predicting molecule permeation through this membrane are improving [190]. Moreover, since some of these targets are surface exposed, it may be possible to develop drugs that avoid all membrane permeability problems and in addition, negate the activity of the efflux pumps, a common mechanism of drug resistance [191]. This volume provides a detailed examination of the targets revealed by studies of envelope biogenesis.

CRediT authorship contribution statement

Randi L. Guest: Writing – original draft, Writing – review & editing. **Thomas J. Silhavy:** Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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