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Biogenesis of the Gram-negative bacterial outer membrane

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Gram-negative bacteria are bounded by two membranes. The outer membrane consists of phospholipids, lipopolysaccharides, lipoproteins and integral outer membrane proteins, all of which are synthesized in the cytoplasm. Recently, much progress has been made in the elucidation of the mechanisms of transport of these molecules over the inner membrane, through the periplasm and into the outer membrane, in part by exploiting the extraordinary capacity of *Neisseria meningitidis* to survive without lipopolysaccharide.

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Abbreviations

ABC	ATP-binding cassette
IM	inner membrane
Imp	increased membrane permeability
LPS	lipopolysaccharide
OM	outer membrane
OMP	outer membrane protein
POTRA	polypeptide transport associated
PPIase	peptidyl-prolyl *cis–trans* isomerase
Skp	seventeen-kilodalton-protein

Introduction

Gram-negative bacteria are characteristically surrounded by a double membrane: the cytoplasmic or inner membrane (IM), which is a phospholipid bilayer, and the asymmetrical outer membrane (OM), which holds phospholipids and lipopolysaccharides (LPS) in its inner and outer leaflet, respectively. Both membranes contain numerous proteins that serve a diverse array of functions. These membranes, together with the enclosed peptidoglycan-containing periplasm comprise the bacterial cell envelope. *Escherichia coli* has long been the favored model system to study the molecular details of OM biogenesis. However, substantial progress in this field has been recently made using another bacterium, *Neisseria meningitidis*. The unexpected finding that *N. meningitidis* can survive without LPS, which was considered to be essential for Gram-negative bacteria [1], has opened up possibilities to identify components of individual OM biogenesis machineries by direct genetic approaches.

In this review, we discuss the biogenesis of the different constituents of the OM, with emphasis on recent discoveries made possible by the use of *N. meningitidis* as a model system.

Outer membrane proteins

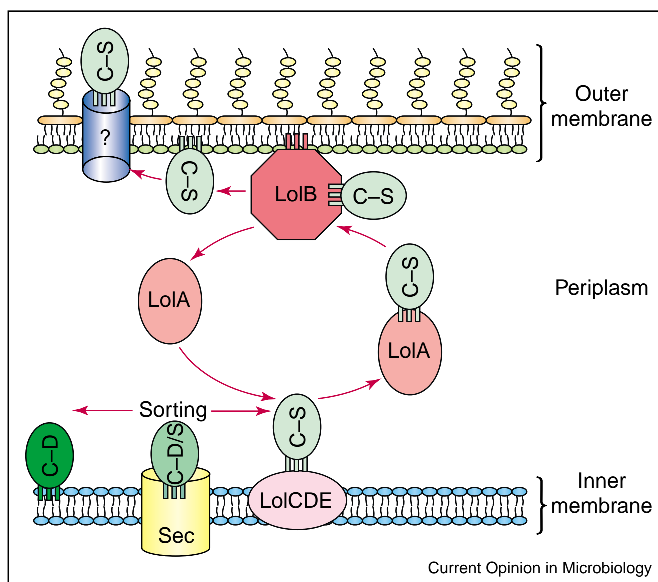
Proteins present in the OM are of either two classes: lipoproteins, which are anchored to the OM with a N-terminal lipid tail, and integral proteins that contain membrane-spanning regions. The latter proteins will be further referred to as outer membrane proteins (OMPs). All proteins destined for the OM are synthesized in the cytoplasm as precursors with N-terminal signal sequences, which are essential for translocation across the IM. Two translocation machineries have been identified: the Sec system, which translocates unfolded proteins [2], and the Tat-machinery, which moves folded proteins over the IM [3]. To our knowledge, no OM lipoproteins or OMPs have been demonstrated to use the Tat pathway.

Lipoproteins

Bacterial lipoproteins are membrane-attached via an N-terminal N-acyl-diacylglycerylcysteine. Lipidation and folding of the lipoproteins take place after their translocation over the IM via the Sec machinery. A sorting signal of lipoproteins is comprised by the amino acids flanking the lipidated cysteine in the mature protein [4]. Lipoproteins lacking an IM retention signal are transported to the OM by the Lol system (Figure 1). LolC, LolD and LolE form an ATP-binding cassette (ABC) transporter in the IM. At the expense of ATP, this transporter releases lipoproteins into the periplasm where they are bound by the chaperone LolA. The LolA-lipoprotein complex crosses the periplasm and interacts with an OM receptor, LolB. LolB releases LolA from the complex owing to its higher affinity for the lipoprotein [5]. LolA and LolB are structurally strikingly similar, both containing a hydrophobic cavity. However, the LolA cavity is composed mostly of aromatic residues, whereas the cavity in LolB is made mostly of leucine and isoleucine residues, which is likely to explain the difference in lipoprotein-binding affinity [6••,7].

In *E. coli*, all lipoproteins face the periplasm, but in other bacteria, most notably in members of the spirochetes [8], also cell surface-exposed lipoproteins are present.

Figure 1



Lipoprotein transport through the bacterial cell envelope. Lipoproteins, after transport via the Sec system and subsequent modification, bind to the ABC-transporter LolCDE, provided they do not possess a Lol-avoidance motif. Energy from ATP hydrolysis by LolD is transferred to LolC and LolE, and then used to open the hydrophobic LolA cavity to accommodate the lipoprotein. When the LolA-lipoprotein complex interacts with the OM receptor LolB, the lipoprotein is transferred to LolB and then inserted into the OM. Further transport to the outer leaflet of the OM occurs through unknown mechanisms. The Lol-avoidance motif Asp at position 2 (C–D) causes the formation of a tight phosphatidylethanolamine-lipoprotein complex, resulting in retention of the lipoprotein in the IM. A serine in position 2 (C–S) results in OM localization [7].

Whether lipoprotein transport over the OM occurs through an extension of the Lol system, or by an unrelated transport system, is presently unclear.

Integral outer membrane proteins

Integral OMPs invariably comprise an even number of amphipathic antiparallel β-strands that fold into cylindrical β-barrels from which hydrophobic residues point outward [9]. *In vitro*, many denatured OMPs have been shown to fold correctly in the presence of detergents. Also, folding and insertion of OMPs into liposomes has been reported, suggesting that these processes take place spontaneously [10]. However, the much faster kinetics of these events *in vivo* and the specificity of OMP insertion exclusively in the OM argue that in the cell OMP insertion is assisted. Several periplasmic chaperones and folding catalysts involved in this process have been identified. Skp (seventeen-kilodalton-protein) binds OMPs directly after they emerge from the Sec channel [11]. The function of Skp is not known, but it might prevent premature folding and aggregation. The peptidyl-prolyl *cis–trans* isomerase (PPIase) SurA appears to act as a chaperone specifically for OMPs [12], as lack of SurA negatively affects expression of the major trimeric porins OmpC, OmpF and LamB, as well as of OmpA. Moreover, it preferentially binds unfolded OMPs over other similarly sized non-OMPs [13] or over folded OMPs [14]. Both PPIase domains of SurA are dispensable for chaperone activity [13], an observation that was at least partly explained by the crystal structure of SurA, which revealed that one PPIase domain did not participate in the formation of the putative peptide-binding cleft [15•]. Inactivation of both the *skp* and *surA* gene resulted in a synthetic lethal phenotype, suggesting that Skp and SurA share redundant chaperone activity [16]. Furthermore, disulfide bond formation and reshuffling are catalyzed in the periplasm by the DsbA and DsbC proteins [17], a process that precedes OM insertion, indicating that OMPs are at least partly folded before their OM insertion [18].

The exclusive insertion of OMPs in the OM could be related to the exclusive presence of LPS in this membrane. A role for LPS was indeed suggested in *in vitro* studies, showing a strong stimulatory effect of LPS on OMP folding [19]. However, a mutant of *N. meningitidis* that is completely devoid of LPS owing to disruption of the *lpxA* gene, encoding the first enzyme for LPS biosynthesis, correctly assembled all OMPs examined [20]. Thus, *in vivo*, LPS does not play an obligatory role in OMP sorting, folding or insertion.

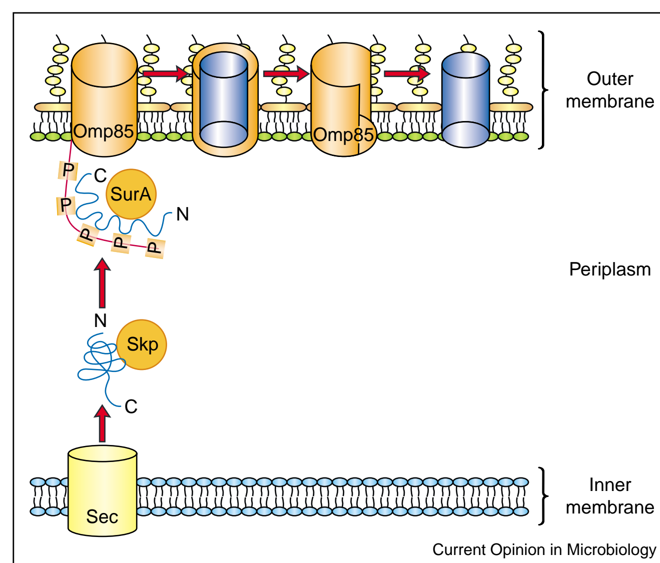
The insertion of OMPs into the OM has long remained an enigmatic process. However, recently, a component of a putative OMP insertion machinery was identified in

N. meningitidis. This component, an OMP called Omp85, was shown to be essential for viability. On depletion of Omp85 in a conditional mutant, OMPs were found to accumulate in an unfolded form and were not inserted in the OM. Moreover, overlay experiments showed that Omp85 bound non-native porin, indicative for a direct role of Omp85 in OMP assembly [21••] and arguing that no chaperone activity is required for targeting of the OMP to Omp85. An attractive motif for Omp85 recognition may be the C-terminal signature sequence in OMPs [22], which consists of alternating hydrophobic residues in the C-terminal nine residues, with almost invariably an aromatic ultimate residue. The same motif is recognized by the IM-bound protease DegS, thereby initiating periplasmic stress responses when unfolded OMPs accumulate in the periplasm [23••].

Omp85 is an evolutionary conserved protein that is present in all Gram-negative genomes sequenced to date [21••] as well as in the evolutionary related mitochondria. The mitochondrial Omp85 homolog in yeast was shown to be essential for cell viability and to function in OMP assembly [24••–26••]. Thus, the mitochondrial Omp85 homolog, also known as Tob55 or Sam50, appears to be functionally similar to Omp85. Both the bacterial Omp85 and the mitochondrial homolog reside in a complex [24••,27]. The identification of other components of these complexes will be essential to unravel the entire OMP assembly machinery. In mitochondria, two of those components, Mas37 and Sam35/Tob38, were found [28••,29•,30•], but as yet no function has been assigned to them. No bacterial homologs of these proteins could be identified ([28••], Bos MP, Tommassen J, unpublished). In the chloroplast OM, an Omp85 homolog, designated Toc75, is present. However, its function appears to have deviated from the bacterial homolog: Toc75 has an inverted topology compared with Omp85 and functions as a component of the chloroplast protein-import machinery [31] (see also Update).

Can we already envision how Omp85 might function in an OMP insertion machinery? The structure of Omp85 was predicted to be a 12-stranded β-barrel with a long N-terminal periplasmic extension that contains repeats of a conserved domain suggested to have chaperone-like qualities [21••,32]. These so-called POTRA (for polypeptide-transport-associated) domains are present in OM members of the Omp85 and two-partner secretion families [33] and in the FtsQ/DivIB family of IM proteins that act as chaperones in cell division processes [34]. The Omp85-POTRA domains may be involved in binding the unfolded OMP, whereupon folding could take place with the OMP still being accessible to different periplasmic folding catalysts. Subsequently, the OMP could insert in a channel formed inside the Omp85 complex. Electron microscopy studies of purified Tob55 complexes revealed the existence of channels with an inner diameter of 7 nm that should be large enough to accommodate a β-barrel of 16–22 β-strands [24••]. The complex should then laterally open to allow for the stable insertion of the OMP into the lipid bilayer of the membrane (Figure 2), resembling the insertion of IM proteins via the Sec translocon [2].

Figure 2



Model for OMP assembly and insertion. As soon as OMPs emerge from the IM Sec translocon they are bound by the periplasmic chaperone Skp, which may prevent premature folding and aggregation. After transport of the complex through the periplasm, the OMP is bound by the POTRA domains (P) located in the amino-terminal part of Omp85. Other chaperones and folding catalysts, such as SurA, may act on the OMP. After folding, the OMP inserts into the channel formed by the Omp85 complex in the OM, which then laterally opens to allow for the stable insertion of the fully folded OMP into the lipid bilayer.

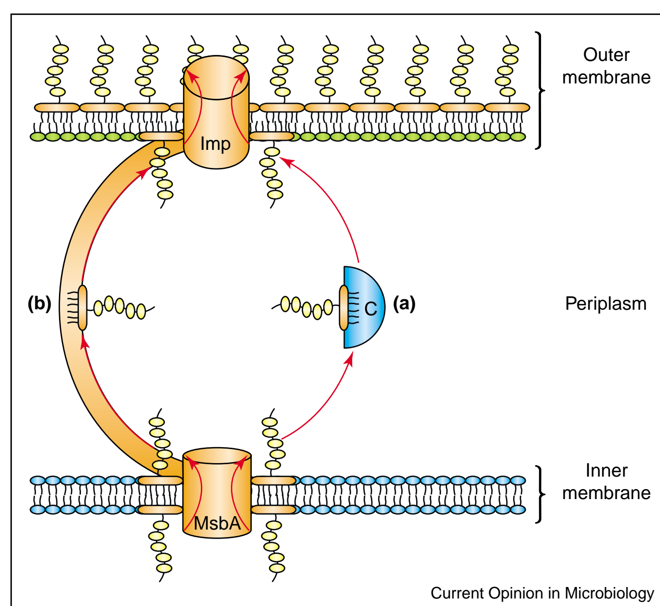
In another study, the meningococcal Omp85 protein was postulated to be involved in LPS and phospholipid transfer to the OM and not in OMP assembly [35•]. This conclusion was based on membrane separation experiments showing mislocalization (i.e. accumulation in the IM) of LPS and phospholipid in Omp85-depleted bacteria. However, an OM lacking inserted OMPs will have a decreased density and will probably fractionate aberrantly in sucrose gradients to fractions where IMs also end up. Moreover, as will be explained below, an OMP is required for LPS transport to the bacterial cell surface. Depletion of Omp85 will affect the correct assembly of this OMP and thereby the transport of LPS, which might then accumulate in the IM. Also, given the abundant evidence of an OMP assembly function of the Omp85 homolog in mitochondria, the data from Genevrois *et al.* [35•] should probably be reinterpreted.

Lipopolysaccharide

LPS consists of a hydrophobic membrane anchor, lipid A, substituted with an oligosaccharide core region that can be extended in some bacteria by a repeating oligosaccharide, the O-antigen. These different LPS constituents are synthesized at the cytoplasmic leaflet of the IM. The O-antigen is transported over the IM separately from the lipid A-core moiety by any of three different routes: the Wzy-, ABC-transporter- or synthase-dependent pathways [36]. The subsequent ligation of the O-antigen to the lipid A core at the periplasmic side of the IM is an incompletely understood process that involves at least the WaaL ligase [37].

The translocation of the lipid A core region over the IM is mediated by an ABC-family transporter, MsbA, as inferred from the accumulation of LPS in the IM in a temperature-sensitive *E. coli msbA* mutant at the restrictive temperature [38]. The function of MsbA is not confined to LPS transport, as suggested by the presence of MsbA homologs in Gram-positive bacteria and its capacity to act as a multidrug transporter [39]. The subsequent steps in LPS transport to the exterior of the bacterium have remained obscure for a long time. However, recently, an OM component required for the appearance of LPS at the bacterial cell surface was identified [40••]. This component is an OMP known as Imp (increased membrane permeability) or OstA (organic solvent tolerance) because *E. coli* strains expressing mutant versions of this protein showed altered membrane permeability [41]. Imp is an essential protein in *E. coli*. A conditional *imp* mutant produced membranes with an altered lipid:protein ratio demonstrating a role of Imp in cell envelope biogenesis [42••]. The precise role of Imp was demonstrated in *N. meningitidis*: Imp was not essential in this bacterial species, making it possible to obtain an *imp* deletion mutant. The phenotype of this mutant demonstrated a role for Imp in LPS biogenesis: it produced less than 10% of wild-type levels of LPS, which were not accessible to LPS-modifying enzymes present in

Figure 3



Model for LPS transport through the bacterial cell envelope. LPS is synthesized at the inner leaflet of the IM. It is then transported over the IM by the ABC transporter MsbA. **(a)** LPS then travels through the periplasm by an unknown mechanism, which could involve a soluble periplasmic chaperone (C). **(b)** Alternatively, transport may take place at contact sites between the two membranes. These contact sites may implicate direct interactions between MsbA and Imp, or additional components bridging the periplasm. Imp is required to transfer LPS to its final destination, the outer leaflet of the OM, perhaps by acting as a flippase.

the OM or added to the extracellular medium [40••]. Therefore, Imp functions in LPS transport over the OM to the cell surface. The reduced LPS levels produced by the *imp* mutant are likely to be a consequence of feedback inhibition owing to LPS stalled in the transport pathway. A vital role for Imp in LPS biogenesis is further supported by its high conservation among Gram-negatives, and its absence from Gram-positives [40••,42••].

A remaining unsolved issue in LPS transport is the passage through the periplasm (Figure 3). LPS could be transported at membrane contact sites known as Bayer junctions [43,44]. Possibly, MsbA together with Imp, constitute the complete LPS translocon. Alternatively, a periplasmic component could be involved. In that respect, the homology of the MsbA protein to HlyB, an ABC-transporter that is part of the type I protein secretion system, is interesting. Type I secretion systems consist of an OMP, an IM ABC-transporter, and a so-called membrane fusion protein, which connects the IM and OM components [45]. Possibly, the LPS translocon is similarly organized (Figure 3), although such models should take into account that the lipid A core moiety must be accessible at the periplasmic side of the IM to the WaaL ligase for O-antigen attachment. Conversely, periplasmic transport may require a soluble component resembling the lipoprotein chaperone LolA.

Phospholipids

The major OM phospholipids of *E. coli* are phosphatidylethanolamine and phosphatidylglycerol. Phospholipids are synthesized at the cytoplasmic side of the IM [46,47]. To reach the OM, they first need to rotate ('flip-flop') over the membrane. It is not clear whether a dedicated flippase is necessary for this process. The LPS transporter MsbA was also implicated in phospholipid transport, as the conditional *E. coli msbA* mutant accumulated both LPS and phospholipids in its IM under restrictive conditions [38]. However, an *msbA* mutant of *N. meningitidis* appeared viable and still made a double membrane, showing that at least in this bacterium, MsbA is not required for phospholipid transport (Tefsen B, Bos MP, Tommassen J, de Cock H, unpublished). Moreover, various α–helical membrane-spanning peptides, but, curiously, not MsbA, induced phospholipid translocation when incorporated in synthetic lipid bilayers [48•]. Thus, flip-flop of phospholipids may not require a specific transporter, but merely the presence of the typical α-helical membrane-spanning regions of some IM proteins. The next steps in phospholipid biogenesis (i.e. transport through the periplasm and incorporation and confinement to the inner leaflet of the OM) remain obscure and no components involved in these steps have been identified.

Conclusions

In the past couple of years, the transport mechanism of lipoproteins was unraveled in considerable detail. Additionally, the first components have been identified of the OMP and LPS insertion machineries in the OM. These were much awaited achievements in the field of bacterial OM biogenesis. These discoveries will undoubtedly be instigators of further research to finally unravel the exact mechanisms of different OM assembly machineries, as they open up the possibility to identify other components of the machineries and to reconstitute them *in vitro*. Furthermore, bacterial OMPs have proven to be amenable to structural analysis by X-ray crystallography, and the resolution of the crystal structures of Omp85 and Imp, which may be achieved in the near future, will undoubtedly provide mechanistic insight in the processes they are involved in. Studies on the mitochondrial OMP insertion machineries will also, by comparison, contribute to understanding the bacterial machinery, and provide new insights into the evolutionary processes that have occurred since mitochondria developed from a prokaryotic endosymbiont [49]. Additionally, the essential nature of the bacterial machineries discussed and their surface localization make them attractive targets for the development of new antimicrobial drugs and vaccines.

Update

Recent work has demonstrated that Toc75 is also involved in the assembly of OMPs into the chloroplast OM [50••].

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