Biogenesis of the Gram-Negative Bacterial Outer Membrane

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Abstract
The cell envelope of gram-negative bacteria consists of two membranes, the inner and the outer membrane, that are separated by the periplasm. The outer membrane consists of phospholipids, lipopolysaccharides, integral membrane proteins, and lipoproteins. These components are synthesized in the cytoplasm or at the inner leaflet of the inner membrane and have to be transported across the inner membrane and through the periplasm to assemble eventually in the correct membrane. Recent studies in Neisseria meningitidis and Escherichia coli have led to the identification of several machineries implicated in these transport and assembly processes.
INTRODUCTION

The cell envelope of gram-negative bacteria consists of two membranes, the inner membrane (IM) and the outer membrane (OM), that are separated by the periplasm containing the peptidoglycan layer. The two membranes have an entirely different structure and composition. Whereas the IM is a phospholipid bilayer, the OM is an asymmetrical bilayer, consisting of phospholipids and lipopolysaccharides (LPS) in the inner and outer leaflet, respectively. Additionally, these membranes differ with respect to the structure of the integral membrane proteins. Whereas integral IM proteins typically span the membrane in the form of hydrophobic α-helices, integral OM proteins (OMPs) generally consist of antiparallel amphipathic β-strands that fold into cylindrical β-barrels with a hydrophilic interior and hydrophobic residues pointing outward to face the membrane lipids (49). Both membranes also contain lipoproteins, which are anchored to the membranes via an N-terminal N-acyl-diacylglycerolcysteine, with the protein moiety usually facing the periplasm in the case of Escherichia coli. In other gram-negative bacteria, however, the protein moiety of OM lipoproteins may also extend into the extracellular medium; examples are the LhpB and TbpB components of the lactoferrin and transferrin receptor, respectively, of Neisseria meningitidis (70).

The OM functions as a selective barrier that protects the bacteria from harmful compounds, such as antibiotics, in the environment. Unlike the IM, the OM is not energized by a proton gradient and ATP is not available in the periplasm. In the absence of readily available energy sources, nutrients usually pass the OM by passive diffusion via an abundant class of trimeric OMPs called porins (66). Porins form water-filled channels that allow the passage of small hydrophilic solutes with molecular weights up to ~600 Da. Nevertheless, energy-requiring transport processes in the OM have also been described. Such processes are dependent on complex energy-coupling systems, such as the TonB system, which couples the proton-motive force of the IM to receptor-mediated uptake processes in the OM (110).

Whereas the composition, structure, and function of the OM have been known for decades, its assembly in the absence of energy sources has remained largely enigmatic. All the components of the OM are synthesized in the cytoplasm or at the cytoplasmic face of the IM, and they have to be transported across the IM and through the periplasm to reach their destination and to assemble into the OM. Recently, many new components involved in these processes have been described, and in this review we focus on these recent developments. Apart from studies in the classical model organisms, E. coli and Salmonella enterica, much progress in this field was reached by studying N. meningitidis, and a comparison between these systems reveals important differences in these fundamental processes. Hence, what is true for E. coli is not necessarily true for other bacteria.
INTEGRAL OUTER MEMBRANE PROTEINS

Passage Across the IM and Through the Periplasm

Integral OMPs are synthesized in the cytoplasm as precursors with an N-terminal signal sequence, which is required for translocation across the IM. Two translocation machineries have been identified: the Sec system for the translocation of unfolded proteins (23) and the Tat system, which transports proteins folded in the cytoplasm (57). However, to our knowledge, all OMPs studied to date are transported via the Sec system, indicating that they reach the periplasm in an unfolded state.

After transport across the IM, the nascent OMPs are accessible to periplasmic chaperones (30). These chaperones have been studied extensively, mainly in *E. coli*. One of these chaperones, Skp (seventeen-kilodalton-protein), immediately interacts with the OMPs as soon as they emerge from the Sec channel (37). The crystal structure of this homotrimeric protein resembles a three-pronged grasping forceps that could bind a nonnative OMP between the prongs and protect it and prevent it from aggregating during passage through the periplasm (52, 109).

An *skp* mutant of *E. coli* is viable but contains decreased amounts of OMPs (16). Another periplasmic chaperone, SurA, was initially identified as a protein required for survival during the stationary phase (102). Mutants in *surA* display an OMP assembly defect (56), and specifically the conversion of unfolded monomers of OMPs into folded monomers appears affected in such mutants (77). The protein has peptidyl-prolyl cis/trans isomerase (PPIase) activity (56, 62), which nevertheless appears to be dispensable for the chaperone function (5). Unlike most cytoplasmic chaperones, SurA is selective and preferentially binds nonnative OMPs over other proteins (5). By screening peptide libraries, Hennecke et al. (38) demonstrated that SurA binds peptides rich in aromatic residues and preferentially those containing Ar-Ar or Ar-X-Ar motifs (where Ar is an aromatic residue and X is any residue). Transmembrane β-strands of OMPs are particularly enriched with such motifs. Like *skp* mutants, *surA* mutants are viable, but the combination of an *skp* and a *surA* mutation results in a synthetically lethal phenotype (75). Therefore, it was suggested that Skp and SurA are functionally redundant and that they operate in parallel pathways for chaperone activity (75). However, this is not the only possible explanation for the synthetic phenotype.

We favor the possibility that Skp and SurA act sequentially in the same pathway (Figure 1). Skp may act as a holding chaperone, preventing aggregation of nonnative OMPs in the periplasm, and SurA may act as a folding chaperone. The demand for the holding chaperone may be limited when the subsequent folding and assembly steps are efficient. Under those conditions, the OMPs have only little chance to aggregate and an *skp* null mutation

### Lipoprotein:
protein attached to the bacterial inner or outer membrane via an N-terminal lipid moiety

### Porin:
protein that forms water-filled channels in the outer membrane

### Sec system:
general protein export apparatus

### Chaperone:
protein that guides another molecule to its destination, being the folded state or the right cellular compartment

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**Figure 1**
Model for OMP biogenesis. As soon as an OMP emerges from the Sec translocon, it is bound by the chaperone Skp, which may prevent aggregation in the periplasm. The C-terminal signature sequence of the OMP functions as a targeting signal and binds the periplasmic domain of Omp85. Other chaperones and folding catalysts, such as SurA, may act on the OMP. After folding, the OMP inserts into the lipid bilayer, possibly in between the Omp85 subunits. Oligomerization of certain OMPs, such as porins, may occur after insertion. The exact function of the accessory lipoproteins YfgL, YfiO, and NlpB is not known. Another accessory lipoprotein recently identified, SmpA (84), is not indicated here.
PPIase: peptidyl-prolyl cis/trans isomerase

σ^E: alternative sigma factor that guides RNA polymerase to the promoters of genes involved in relieving periplasmic stress conditions

has only a limited effect. The demand for such a chaperone will increase when the subsequent folding and assembly processes are hampered, e.g., in a surA mutant. Hence, it is not possible to construct the skp surA double mutant.

Apart from SurA, the periplasm contains three other PPIases, PpiA (also known as RotA), PpiD, and FkpA. PpiA is by far the most active of them, because its inactivation leads to barely detectable residual PPIase activity (47). However, a ppiA null mutation had no detectable effect on OMP assembly (47). In contrast, a ppiD null mutation led to an overall reduction in the level and folding of OMPs, and a combination of ppiD and surA mutations was lethal (19), suggesting functional redundancy. However, PpiD is anchored in the IM, whereas OMP folding is presumably initiated after targeting to the OM, with which, indeed, a proportion of the SurA molecules cofractionated (38). Later, it was reported that ppiD mutants, like ppiA and fkpA mutants and even a ppiD ppiA fkpA triple mutant, did not show different overall OMP patterns or OM permeability compared with the wild type and that a ppiD surA double mutant could be constructed, which had the same phenotype as a surA single mutant (44). Overall, there is little evidence for a direct role of PpiA, PpiD, or FkpA in OMP biogenesis.

Other proteins that may play a role at the periplasmic stage of OMPs are DsbA and DsbC, two enzymes involved in the formation and isomerization, respectively, of disulfide bonds. DsbA catalyzed the formation of a disulfide bond between two cysteines engineered in OM porin PhoE at positions not accessible from the periplasm once the porin is inserted into the OM. This observation showed that the disulfide bond was formed during periplasmic transit and that at least partial folding occurs prior to OM insertion (30). DegP is a protease that degrades unfolded or misfolded proteins in the periplasm but also has chaperone activity, as was shown in vitro on the soluble substrates MalS and citrate synthase (87). A combination of surA and degP mutations was synthetically lethal, suggesting a role for DegP in OMP assembly (75). However, the role of DegP as a protease, removing misfolded or unfolded OMPs from the periplasm, may be more important than its function as a chaperone in this respect (13).

Assembly into the OM

The insertion of OMPs into the OM has long remained enigmatic and has been considered a spontaneous process (98). However, recent work has identified a proteinaceous machinery that is essential for this process.

Omp85, an essential component of the OMP assembly machinery. Recently, we (105) identified a first component required for OMP insertion, i.e., a protein designated Omp85 in N. meningitidis. Omp85 was found to be essential for the viability of the bacteria, and homologues of the omp85 gene were found in all gram-negative bacteria for which the genome sequence was available (106), suggesting its involvement in an important process. Moreover, in many of these genomes, including those of N. meningitidis and E. coli, the omp85 gene is flanked by the skp gene, which encodes the periplasmic chaperone involved in OMP biogenesis, and rseP (formally designated yaeL), which encodes a protease involved in the σ^E-dependent stress response of E. coli that is induced upon accumulation of unfolded OMPs in the periplasm (see below). All these features are consistent with a vital role of Omp85 in OMP assembly. Upon depletion of Omp85 in a genetically engineered strain, unfolded forms of all OMPs examined, including porins, a siderophore receptor, an enzyme, and a secretin involved in type IV pili assembly, accumulated as aggregates in the periplasm (105, 106). The role of Omp85 in OMP assembly was confirmed in E. coli, in which the corresponding gene, designated yaeT, was also an essential gene. Either depletion of Omp85 or growth of a temperature-sensitive mutant at the restrictive temperature resulted in severe OMP assembly defects (27, 112, 114). Even more interestingly, a
A homolog of Omp85 was essential for the assembly of β-barrel proteins into the outer membrane of mitochondria, a eukaryotic cell organelle of endosymbiont origin (33, 54, 69). Apparently, the OMP assembly pathway is highly conserved during evolution.

A difference was observed with respect to the fate of OMPs in Omp85-depleted cells of either *N. meningitidis* or *E. coli*. Whereas unfolded OMPs accumulated as aggregates in such cells of *N. meningitidis* (105), Omp85 depletion in *E. coli* primarily led to severely reduced amounts of detectable OMPs (112, 114). The difference is presumably caused by the absence of the σ^E^ -dependent periplasmic stress response in *N. meningitidis*. In *E. coli*, this stress response is induced upon accumulation of unfolded OMPs in the periplasm (for a review, see Reference 79). Such OMPs are sensed by the PDZ domain of the membrane-bound protease DegS, upon which a proteolytic cascade is initiated that involves the protease domain of DegS and RseP, resulting in the cleavage of the antisigma factor RseA and the release of σ^E^ in the cytoplasm (Figure 2). The released σ^E^ then binds the RNA polymerase core enzyme, resulting in the transcription of the genes for periplasmic

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**Figure 2**

Periplasmic stress response. Upon accumulation of unfolded OMPs in the periplasm, a stress response is initiated that starts with the recognition of the C termini of the OMPs by the PDZ domain of the protease DegS. This recognition initiates a proteolytic cascade, resulting in the release of the alternative sigma factor σ^E^, which binds RNA polymerase and leads to enhanced transcription of genes encoding periplasmic chaperones, such as Skp and SurA, and the protease DegP. The σ^E^ response also leads to production of sRNAs that negatively regulate OMP expression.
chaperones, such as Skp and SurA, and for the potent protease DegP. Together these chaperones and protease relieve the periplasmic stress. Moreover, the $\sigma^E$ response results in the production of small regulatory RNAs (sRNAs) in Enterobacteriaceae that prevent OMP expression at the translational level (31, 43, 68). Thus, in E. coli, misfolded OMPs are rescued or degraded in the periplasm and OMP synthesis is inhibited during the stress period. Although chaperones such as Skp and SurA are present in N. meningitidis, many essential components of the signal transduction pathway are lacking. Whereas E. coli contains three genes for the related proteases DegP, DegS, and DegQ (107), only a homologue of DegQ can be found in N. meningitidis (encoded by the NMB0532 locus in the N. meningitidis strain MC58). Moreover, homologues of RseA and RseB appear to be absent. Although there is an alternative $\sigma$ factor belonging to the ECF (extracytoplasmic factor) family (to which $\sigma^E$ also belongs) encoded on the meningococcal chromosome (i.e., NMB2144 in strain MC58), it seems to have an entirely different function. Its inactivation in the related bacterium Neisseria gonorrhoeae did not affect global gene expression as analyzed by microarray analysis, whereas its overexpression affected only very few genes, including those for methionine sulfoxide reductase (35). Thus, the $\sigma^E$-dependent periplasmic stress response appears to be absent in the pathogenic Neisseriae. As a result, when there is an OMP assembly defect, OMPs will continue to be synthesized and they will accumulate in the periplasm, where they will form aggregates.

**Omp85 is part of a multisubunit complex.**
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) analysis under non-denaturing conditions revealed that Omp85 of N. meningitidis is present in a high-molecular-weight complex (105). The only other protein identified in this complex was the RmpM protein (J. Geurtsen, R. Voulhoux & J. Tommassen, unpublished results), which has a peptidoglycan-binding motif and largely resides in the periplasm but is firmly associated with many different integral OMPs via an N-terminal peptide (71). RmpM probably anchors the OM to the underlying peptidoglycan layer (88), and it has no particular function in OMP biogenesis. Cross-linking experiments revealed that the Omp85 homolog of E. coli forms a complex with three lipoproteins, YfgL, YiiO, and NlpB (114). A similar complex was identified upon a proteomic analysis of OMP complexes resolved by blue-native PAGE (93). Copurification experiments using strains with mutations in the genes for various components of the complex indicated that YfgL and YiiO directly interact with Omp85, whereas NlpB is associated with the complex via YiiO (58).

The $yfgL$ gene was originally identified in a screen for suppressors that restored the OM permeability defect of a partial loss-of-function mutation in the imp gene (29, 78), which encodes an OMP involved in LPS biogenesis (see below). Although $yfgL$ is not an essential gene, null mutations create an OM permeability defect and result in reduced amounts of OMPs (67, 78, 114), consistent with a role in OMP assembly. Remarkably, although the $yfgL$ gene is widely disseminated among gram-negative bacteria, we could not identify a homolog in the sequenced genomes of N. meningitidis and N. gonorrhoeae. The $yfiO$ gene is essential in E. coli (67), but a mutant with a transposon insertion near the 3’ end of the gene was viable (114). This mutant showed increased OM permeability and reduced amounts of OMPs, and a yfiO depletion strain showed a phenotype similar to that of the yaeT/omp85 depletion strain of E. coli (58). In N. gonorrhoeae, a transposon insertion in the middle of the yfiO homolog was described (32). This mutant was viable, showed a reduced cell size, and was transformation deficient; therefore, the gene was designated comL. However, attempts to introduce other comL truncations into the chromosome failed (32), and we have not yet succeeded to generate a complete comL deletion in the chromosome of N. meningitidis (E. Volokhina,
M.P. Bos & J. Tommassen, unpublished results). Hence, also in the Neisseriaceae, YfiO (ComL) appears essential for viability and the N-terminal half of the protein appears sufficient for partial functionality. The ComL protein is covalently linked to the peptidoglycan, both in *N. gonorrhoeae* and when expressed in *E. coli* (32). Furthermore, *yfgL* mutations affected peptidoglycan synthesis, possibly by regulating the activity of lytic transglycosylases (29). Thus, both YfgL and YfiO might have a role in modulating the peptidoglycan to facilitate the passage of OMPs through this layer.

Whereas *yfiO*, like *yfgL* and *omp85*, is widely disseminated among gram-negative bacteria, this is less so for *nlpB*. Null mutants in *nlpB* of *E. coli* are viable; they show only moderate OM permeability defects and no obvious defects in OMP assembly (67, 114). However, because an *nlpB surA* double knockout mutant has a synthetic lethal phenotype (67), it is clear that NlpB also has a direct role in OMP assembly, possibly redundant to that of the periplasmic chaperone SurA.

**Structure of Omp85.** On the basis of the sequence, we (105) have proposed that Omp85 consists of two domains, a C-terminal β-barrel embedded in the OM and an N-terminal domain extending into the periplasm. Support for this model was obtained in proteolytic digestion experiments, which resulted in the degradation of the N-terminal domain and left the predicted membrane-embedded β-barrel domain intact (76, 91). The periplasmic extension contains repeats of a conserved domain, POTRA (polypeptide transport associated), suggested to have chaperone-like qualities (81). Such POTRA domains were further identified in other members of the Omp85 superfamily, which includes the Omp85 homologs of mitochondria, the Toc75 OM component of the chloroplast protein import machinery, and the OM-localized TpsB component of the two-partner secretion (TPS) systems of gram-negative bacteria, and in members of the FtsQ/DivIB family of IM proteins involved in cell division. The bacterial Omp85 homologs are unique in having five of these POTRA domains, whereas the number of such domains in the other proteins with known functions is restricted to one or two. Another member of the Omp85 superfamily that is widely disseminated among gram-negative bacteria (encoded by the *ytfM* gene in *E. coli* and the NMB2134 locus in *N. meningitidis* strain MC58) contains three POTRA domains. The function of this protein is unknown (92).

To study its structure and function in more detail, we produced the Omp85 protein of *E. coli* in inclusion bodies, which were isolated, and refolded the protein into the native conformation in vitro (76). The refolded protein formed oligomers, presumably tetramers, similarly as reported for another member of the Omp85 superfamily, i.e., HMW1B, the TpsB component of a TPS system of *Haemophilus influenzae* (95). Of note, the interactions between the subunits in this homo-oligomeric complex are not stable; blue-native PAGE indicated equilibrium between monomeric and oligomeric forms (76). Consistently, the hetero-oligomeric Omp85/YfiO/YfgL/NlpB complex identified in vivo appeared to consist of one copy of each subunit (93).

**Interaction of Omp85 with its substrate OMPs.** When reconstituted in planar lipid bilayers, both in vitro refolded Omp85 and Omp85 extracted from *E. coli* cell envelopes formed narrow ion-conductive channels (76, 91). This property was used to study the interaction between Omp85 and its substrate OMPs, emanating from the idea that such an interaction would affect the channel activity. Indeed, nonnative OMPs drastically enhance Omp85 channel activities (76), showing that these substrates interact directly with Omp85. Furthermore, using mutant OMPs and synthetic oligopeptides in this assay, we demonstrated that Omp85 specifically recognizes a C-terminal motif in its substrates (76) that was shown to be required for efficient assembly...
of these proteins into the OM in vivo (94). This C-terminal signature motif, which consists of a phenylalanine (or tryptophan) at the C-terminal position, a tyrosine or hydrophobic residue at position 3 from the C terminus, and also hydrophobic residues at positions 5, 7, and 9 from the C terminus, is present in most bacterial OMPs, including porins, receptors, enzymes, and autotransporters. It is interesting to note that the same signature sequence is recognized by the PDZ domain of DegS when unfolded OMPs accumulate in the periplasm (108), thereby initiating the σE-dependent periplasmic stress response (see above).

In the planar lipid bilayer experiments an OMP of *N. meningitidis*, in contrast to *E. coli* OMPs, did not enhance the activity of the *E. coli* Omp85 channels (76). Consistently, high-level expression of neisserial OMPs in *E. coli* is often lethal and leads to their misassembly, suggesting that the *E. coli* OMP assembly machinery cannot deal efficiently with neisserial proteins. Indeed, expression of *E. coli omp85* cannot complement an *omp85* mutation in *N. meningitidis* and vice versa (E. Volokhina, V. Robert, M.P. Bos & J. Tommassen, unpublished observations). Although the C termini of neisserial OMPs do contain the signature sequence with the features outlined above, they are further characterized by the presence of a positively charged residue at the penultimate position, which could inhibit the interaction with *E. coli* Omp85. Indeed substitution of this positively charged residue in the *N. meningitidis* porin PorA drastically improved PorA assembly into the *E. coli* OM in vivo (76). Thus, although the OMP assembly machinery is highly conserved among gram-negative bacteria, species-specific adaptations seem to have occurred during evolution.

Of note, the C-terminal signature sequence of OMPs is not absolutely essential for assembly into the OM. Thus, whereas the high-level expression of a mutant form of the *E. coli* porin PhoE lacking the C-terminal phenylalanine was lethal and resulted in its periplasmic aggregation (21, 94), its low-level expression was tolerated and allowed for its assembly into the OM (21). Similarly, several studies reporting the assembly of neisserial OMPs into the *E. coli* OM in vivo (113) may be explained by low expression levels. Pulse-chase experiments in *E. coli* overexpressing PhoE revealed the existence of two assembly pathways: approximately half of the PhoE molecules assembled within the 30-s pulse period into their native conformation in the OM, whereas the other half of the molecules followed much slower kinetics and allowed for the detection of several assembly intermediates (42). In these assays, the mutant PhoE lacking the C-terminal phenylalanine followed only the slower kinetic pathway. This study underscores the role of the C-terminal signature sequence as a targeting signal and indicates that there must be alternative, less efficient targeting signals in OMPs. Furthermore, kinetic partitioning between OM incorporation and aggregation of periplasmic intermediates may explain the observation that OMPs with defective signature sequences are still assembled into the OM at low expression levels, when the kinetics of aggregation is low and hence the time span for assembly into the OM is elongated (76).

What could be the nature of the alternative targeting sequences in OMPs? Of note, the C-terminal signature sequence contributes an amphipathic β-strand to the β-barrel in the folded protein. In the absence of the C-terminal phenylalanine, Omp85 may recognize, though less efficiently, internal β-strands, many of which also end with an aromatic residue. In some classes of OMPs, the C-terminal signature motif could not be discerned (94). Perhaps, in these cases also, Omp85 recognizes an internal β-strand. One of the major OMPs of *E. coli*, OmpA, consists of two domains, an N-terminal OM-embedded β-barrel and a C-terminal periplasmic extension. The signature motif was found in this case at the end of the β-barrel domain (94) and its importance in OM targeting was demonstrated in a deletion analysis (48). Although this example demonstrates
that the targeting motif can be located internally in the primary structure of an OMP, this appears to be a rather exceptional case because the C-terminal extension of an OMP sequence with, for example, a His tag usually results in severe assembly defects (91).

Another class of OMPs that lack the C-terminal signature motif is constituted by TolC and its homologs, which are involved in type I protein secretion and drugs extrusion. In its native trimeric structure, TolC forms a \( \beta \)-barrel in the OM, to which each monomer contributes four \( \beta \)-strands, whereas the major portion of the protein extends as long \( \alpha \)-helices into the periplasm (53). TolC is dependent on Omp85 for its assembly (112); hence, possibly also in this case, an internal \( \beta \)-strand is recognized. In contrast, Wza, an OMP involved in the export of capsular polysaccharides, has an entirely different structure; the membrane-embedded portion of this octameric protein consists of a barrel of eight amphipathic \( \alpha \)-helices, to which each monomer contributes one helix (28). It is conceivable that this protein assembles entirely independently of the Omp85 machinery. The secretins form a class of OMPs involved in diverse processes, including type II and type III protein secretion, type IV pilus biogenesis, and the extrusion of filamentous phages (7). The structure of these large multimeric proteins is not known at atomic resolution, but they appear to be rather poor in \( \beta \)-sheet content (14), which could indicate that they also follow a different assembly pathway. However, at least one secretin, PilQ of \( N. \) meningitidis, depends on Omp85 for its assembly (105). Perhaps, the binding of these proteins and other OMPs that lack the C-terminal signature motif is indirect and requires accessory factors, such as the lipoproteins YfO, YfgL, and NlpB, or specific chaperones, such as the pilotins in the case of the secretins (3).

Role of LPS in OMP Biogenesis

Since the observation that the amounts of several OMPs are severely decreased in deep-rough mutants of \( E. \) coli and \( S. \) typhimurium, which contain truncated LPS molecules (2, 51), a role of LPS in OMP assembly has been suggested. Consistently, the OM porin PhoE of \( E. \) coli could be converted in vitro into a folded monomeric form in the presence of LPS, detergent, and divalent cations. This folded monomer appeared to be an assembly intermediate because it could subsequently be converted into its native trimeric OM-inserted form (22). Also for other OMPs, LPS-dependent folding in vitro has been reported (83). However, LPS-independent in vitro folding conditions were later established for many OMPs, including PhoE (42), arguing against a specific role of LPS in OMP folding. Moreover, an \( \text{lpxA} \) mutant of \( N. \) meningitidis, which is completely defective in LPS biosynthesis, appeared to be viable (90), and all OMPs examined, including porins, were correctly assembled in vivo into the LPS-free OM of this mutant (89). Nevertheless, species-specific differences with respect to the LPS dependency of OMP assembly cannot yet be excluded at this stage.

The crystal structure of \( E. \) coli Skp revealed a putative LPS-binding site (109), consistent with the earlier description of the protein in \( S. \) minnesota as an LPS-binding protein (34). Thus, LPS may exert its role in OMP biogenesis via Skp. Furthermore, it was demonstrated in protease-digestion assays that binding of LPS and phospholipids could inversely modulate the structure of Skp in vitro (20). Thus, after binding nonnative OMPs at the IM, a conformational change triggered by LPS binding may release the cargo at the OM. Note that LPS is present only in the outer leaflet of the OM. Hence, Skp should bind to LPS molecules that have not yet reached their destination. Consistently, OMP biogenesis is heavily affected by cerulin, a drug that inhibits lipid synthesis (8). However, the putative LPS-binding site in \( E. \) coli Skp is largely conserved in \( N. \) meningitidis Skp, where OMP biogenesis is independent of LPS. Hence, the role of this putative LPS-binding site remains to
be determined in site-directed mutagenesis experiments.

We speculate that the role of LPS in OMP biogenesis is restricted to late stages after the insertion into the OM, such as the stabilization of porin trimers (55), which presumably requires some LPS-mediated rearrangements in the cell surface-exposed loops of the proteins. The severely reduced amounts of OMPs in deep-rough mutants may be explained by the induction of the σE response in such mutants, resulting in the production of sRNAs that inhibit the synthesis of many OMPs (see above). Changes in LPS structure induce the σE response in E. coli by a hitherto unknown mechanism (97). At least one OMP of E. coli, TolC, appears totally unaffected by LPS structure (111). Presumably, the expression of TolC is unaffected by the sRNAs. The assembly of this lipid-independent OMP is independent of the accessory component YfgL of the Omp85 assembly machinery (15). Of note, a homolog of the yfgL gene is lacking in N. meningitidis, in which the assembly of all OMPs is independent of LPS. Hence, there may be an additional role for LPS in the assembly of lipid-dependent OMPs in E. coli, and YfgL may play a role specifically in the assembly of this class of OMPs. In this respect, it may indeed be relevant that the yfgL mutants were initially picked up as suppressors of a partial loss-of-function imp mutant (29), and the imp gene product is specifically involved in LPS biogenesis (9) (see below).

Model for OMP Biogenesis

We propose the following model for OMP biogenesis (Figure 1). After their transport through the Sec translocon, nascent OMPs are immediately bound by Skp, which may assist their release from the IM and prevent their aggregation in the periplasm. The Skp/OMP complex is targeted to the Omp85 complex in the OM, whereby the C-terminal signature motif of the OMPs functions as the primary targeting signal that binds directly to Omp85, presumably to its N-terminal POTRA domains. Binding initiates folding, which results in the release of Skp (20) and may be assisted by the presumed chaperone activities of the POTRA domains and by periplasmic proteins such as SurA and DsbA. Binding of an OMP also results in a conformational change in the C-terminal domain of Omp85, which is reflected by the increased pore activity observed in the planar lipid bilayer experiments (76). This conformational change allows the OMPs to insert into the OM, possibly in between the Omp85 subunits. Dissociation of the Omp85 subunits releases the assembled OMPs into the OM, where final conformation changes in the cell surface-exposed loops may be induced upon interaction with LPS.

The specific role of the accessory lipoproteins YfgL, YfO, and NlpB has not been addressed experimentally so far. Of note, no homologs of these proteins have been implicated in the assembly of β-barrel proteins into the mitochondrial OM, suggesting that their role is less crucial, although YfO is definitely essential, at least in E. coli (67). These proteins may play a role in the recognition of OMPs that do not display the C-terminal signature motif, which includes the essential protein Imp (increased membrane permeability), in modulating the peptidoglycan layer, and/or they may function as chaperones in the folding of OMPs. YfgL may have a role in coordinating the assembly of a subclass of OMPs that require LPS for their assembly, but such a role is difficult to assess in E. coli, in which assembly defects of OMPs are associated with a feedback inhibition on their synthesis via σE-induced sRNAs, whereas N. meningitidis, which does not display such a feedback inhibition, does not have a YfgL homolog. The absence of this feedback inhibition mechanism makes N. meningitidis a favorable organism to study the role of other assembly factors.

LIPOPROTEINS

Bacterial lipoproteins are membrane attached via an N-terminal N-acyl-diacylglycercyleysteine. Lipidation and folding
of these proteins take place after their translocation over the IM via the Sec machinery. Prior to cleavage of the signal sequence, a diacylglycerol group is transferred by the enzyme Lgt from phosphatidylglycerol to the sulfhydryl group of the cysteine that is invariably present at the +1 position relative to the processing site (82). Subsequently, the diacylglycerol prolipoprotein is processed by a dedicated signal peptidase, signal peptidase II (24), after which the free α-amino group of the cysteine is acylated by the enzyme Lnt (36), yielding the mature lipoprotein.

Lipoproteins are sorted to the IM or OM according to a sorting signal that comprises the amino acids flanking the lipidated cysteine in the mature protein (101). Lipoproteins lacking an IM retention signal, usually an aspartate at the +2 position of the mature protein, are transported to the OM by the Lol system (Figure 3). The first component of the Lol system was identified in an in vitro system, whereby lipoproteins were synthesized in a radioactive form in spheroplasts of E. coli (60). The mature forms of the newly synthesized lipoproteins were retained at the surface of the spheroplasts, from which they could be released upon addition of a periplasmic extract. The active component of the periplasmic extract was identified and designated LolA. Furthermore, a complex of the three proteins LolC, LolD, and LolE, which constitutes an ATP-binding cassette (ABC) transporter in the IM, is required for lipoprotein transport (65). The integral membrane components of this transporter, LolC and LolE, show considerable sequence similarity and may function as a heterodimer. Of note, N. meningitidis contains only one of these proteins (encoded by locus NMB1235 in strain MC58), which may form a homodimer. The current model (Figure 3) states that lipoproteins destined for the OM are first bound by LolC/E in an ATP-independent manner. This binding results in an increase in the affinity of LolD for ATP. Subsequently, ATP binding to LolD causes a conformational change in the LolC/E moiety that results in a decrease in lipoprotein binding affinity. ATP hydrolysis is then required for transfer of the lipoprotein from LolC/E to the periplasmic chaperone LolA (41). The LolA-lipoprotein complex crosses the periplasm and interacts with an OM receptor, LolB (61). The lipoprotein is then transferred to LolB according to the affinity difference between LolA and LolB. LolA and LolB are structurally similar; both contain a hydrophobic cavity. 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 (64, 96). In E. coli, all known lipoproteins face the periplasm, but in other bacteria, most notably in members of the spirochetes (12), cell surface-exposed lipoproteins also are present.

**Spheroplasts:** bacterial cells of which the outer membrane and the peptidoglycan layer have been disrupted by treatment with EDTA and lysozyme

**ABC:** ATP-binding cassette
Whether lipoprotein transport over the OM occurs through an extension of the Lol system or by an unrelated transport system is presently unclear. The only cell surface-exposed lipoprotein studied in this respect is the starch-disbranching enzyme pullulanase of *Klebsiella oxytoca* (72). Pullulanase contains an aspartate at position +2, indicating that it is not a substrate for the Lol system. Indeed the transport of pullulanase to the cell surface requires a type II protein secretion apparatus. In the absence of such an apparatus, pullulanase is retained in the IM (72). Thus, in *K. oxytoca*, sorting between periplasmically and cell surface-exposed lipoproteins takes place at the IM level. However, this is not necessarily always the case.

*N. meningitidis* contains several cell surface-exposed lipoproteins, including LbpB and TbpB, but the sequenced genomes do not reveal the presence of a type II secretion apparatus (104). Furthermore, LbpB and TbpB contain an isoleucine and a leucine, respectively, at the +2 position, suggesting they are substrates for the Lol system. Thus, these lipoproteins may be transported to the cell surface via an extension of the Lol system, which remains to be identified.

**LIPOPOLYSACCHARIDE**

**Structure and Biosynthesis**

LPS is a complex glycolipid exclusively present in the outer leaflet of the OM of gram-negative bacteria. It consists of a hydrophobic membrane anchor, lipid A, substituted with an oligosaccharide core region, which in some bacteria (e.g., in most *E. coli* strains, but not in the laboratory strain *E. coli* K-12 and also not in *N. meningitidis*) is extended with a repeating oligosaccharide, the O-antigen. These different LPS constituents are synthesized at the cytoplasmic leaflet of the IM. The lipid A moiety of LPS is rather well conserved among gram-negative bacteria. It usually consists of a β-1,6-linked d-glucosamine disaccharide carrying ester- and amide-linked 3-hydroxy fatty acids at the 2, 3, 2’, and 3’ positions and phosphate groups at the 1 and 4’ positions. The primary 3-hydroxy fatty acids may be substituted with secondary fatty acids. The lipid A biosynthetic pathway, also known as the Raetz pathway (74), has been characterized in detail, mostly in *E. coli* and *Salmonella typhimurium*, but it appears to be highly conserved among other gram-negative bacteria (74). Remarkably, although LPS has so far only been detected in gram-negative bacteria, homologs of the genes encoding the lipid A biosynthetic enzymes have also been found in sequenced plant genomes (74).

The core oligosaccharide is much more variable between bacterial species. In addition, a huge amount of LPS heterogeneity is created by numerous modifications of both the lipid A part and the core part of LPS (73). The modifying enzymes, which are localized in different cellular compartments, are not generally conserved; so different species can express uniquely modified types of LPS (103). The O-antigen, if present, is the most variable part of LPS and shows even a high degree of variability between different strains of the same species.

**Transport to the Cell Surface**

In contrast to the understanding of its biosynthesis, the mechanism of the transport of LPS from its site of synthesis to its final destination forms a much less complete picture (25). It is clear that the lipid A–core moiety and the O-antigen subunits, if present, are transported separately over the IM. The O-antigen subunits are transferred over the IM by one of three different routes: the Wzy-, ABC-transporter-, or synthase-dependent pathway (74). After polymerization, the subsequent ligation of the O-antigen to the lipid A–core moiety at the periplasmic side of the IM is an incompletely understood process that involves at least the WaaL ligase (45). The translocation of the lipid A–core moiety over the IM is mediated by an ABC family transporter called MsbA, as inferred from
the accumulation of LPS in the IM of a temperature-sensitive E. coli msbA mutant at the restrictive temperature (116). The LPS accumulated was not modified by periplasmic enzymes, demonstrating that it was not transported to the periplasmic leaflet of the IM (26). More evidence for a role of MsbA in LPS transport came from a study of an msbA mutant of N. meningitidis. The viability of LPS-deficient N. meningitidis mutants makes this organism well suited for the study of LPS biogenesis, because clean knockouts of genes involved in this process can be constructed. Indeed, MsbA is a nonessential protein in N. meningitidis (99). A neisserial msbA mutant produced only low amounts of LPS, a feature indicative of a defect in LPS transport in this species. In N. meningitidis, biosynthesis and transport of LPS are coupled in such a way that synthesis is reduced under conditions in which transport is halted (9, 99).

The subsequent steps in LPS transport to the exterior of the bacterium have long remained obscure. However, an OM component required for the appearance of LPS at the bacterial cell surface was identified recently. This component is an OMP known as Imp or OstA (organic solvent tolerance), because E. coli strains expressing mutant versions of this protein showed altered membrane permeability (1, 80). Imp is an essential protein in E. coli. In a conditional imp mutant, correctly folded OMPs accumulate in aberrant membranes with an increased density, indicative of an altered lipid/protein ratio (11). The precise role of Imp was demonstrated in N. meningitidis. Imp was not essential in this bacterial species, allowing the construction of 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 recombinantly expressed in the OM or added to the extracellular medium (9). Therefore, Imp appears to function in the transport of LPS over the OM to the cell surface. This role of Imp was confirmed in an E. coli imp depletion strain. Upon depletion of Imp, newly synthesized LPS was not accessible to LPS-modifying enzymes in the OM, showing that it did not reach the outer leaflet of the OM (115). In E. coli, another OM component was identified to play a role in LPS transport, i.e., the essential lipoprotein RlpB. Depletion of this lipoprotein resulted in a phenotype similar to that expressed upon depletion of Imp. Moreover, Imp and RlpB exist in a complex. Depletion of Imp or RlpB resulted in an increased total cellular LPS content (115), indicating that in E. coli, in contrast to N. meningitidis, defective transport does not lead to feedback inhibition on LPS biosynthesis.

Imp and RlpB homologs are widely disseminated among gram-negative bacteria, suggesting that the mechanism of LPS transport is highly conserved. Imp is predicted to contain a β-barrel domain embedded in the OM, with a long N-terminal domain and a short C-terminal domain extending into the periplasm (Figure 4). In the conserved domain database at the National Center for Biotechnology Information (59), the β-barrel domain is recognized as a conserved domain, designated OstA-C, in all Imp homologs. Another conserved domain, COG1934, is recognized in the N-terminal periplasmic extension of many but not all Imp homologs (Figure 4). Nevertheless, all Imp homologs show sequence similarity over the entire length, and also when the COG1934 domain is not recognized.

Additional components putatively involved in LPS translocation were identified by Sperandeo et al. (86), who discovered several new essential genes in E. coli, some of which appeared to play a role in cell envelope biogenesis. Depletion of recombinant bacteria for the proteins encoded by two of these genes resulted in similar phenotypes as described for Imp- and RlpB-depleted cells: The bacteria exhibited an altered OM density and an increased cellular LPS content. The genes, which form an operon, were designated lptA and lptB (LPS transport) (85). Unfortunately, we cannot use the same designations in
N. meningitidis, for which the acronym lptA is used for a gene encoding an LPS phosphoethanolamine transferase (17). Also the neisserial homolog of E. coli LptA, encoded by the NMB0355 locus in N. meningitidis strain MC58, plays a role in LPS transport: In these bacteria, the corresponding gene is not essential, and its deletion results in severely decreased levels of LPS (10). The LPS is accessible to periplasmic LPS-modifying enzymes in this mutant, indicating that the LptA/NMB0355 protein acts at a step after translocation by MsbA (10). The LptA protein of E. coli was found in the soluble periplasmic fraction (85), but we found the majority of the corresponding neisserial protein (NMB0355) in the membrane fraction, although it has no obvious membrane-spanning segments (M.P. Bos & J. Tommassen, unpublished observations). LptA largely consists of the conserved domain COG1934, the same domain found in the N-terminal periplasmic domain of Imp (Figure 4), indicative of a common function.

LptB is a 27-kDa protein present in a 140-kDa IM complex; unfortunately, no interacting partners were identified (93). The protein possesses the typical features of an ABC protein but has no obvious membrane-spanning segments.

Thus, the current data suggest the involvement of a novel ABC transporter in LPS transport. LptB is the ABC component of this transporter, but the cognate integral membrane component remains to be identified. The protein encoded by the yrbK gene, which is located immediately upstream of the lptAB operon and which is also essential for viability in E. coli (86), may be a part of the transporter. The genetic organization of the yrbK-lptA-lptB locus is highly conserved among gram-negative bacteria, and the observation that a conserved protein domain present in YrbK is sometimes present in one polypeptide together with conserved domains from Imp or
LptA (Figure 4) is suggestive for a role of YrbK in LPS transport. However, secondary structure predictions of YrbK show only one putative transmembrane helix, making it unlikely that this protein functions as the integral membrane component of the ABC transporter, as such components of ABC transporters usually contain multiple transmembrane helices (6).

**Models for LPS Transport**

Two models for LPS transport through the cell envelope have been considered, and with the current situation, no model can be considered definitively proven or discounted (Figure 5). One possibility is that LPS passes through the periplasm in a soluble complex with a chaperone that shields its hydrophobic moiety, similar to the Lol system for lipoprotein transport (Figure 3). Indeed, the recent identification of a novel ABC transporter involved in LPS transport may suggest similarities with the lipoprotein transport system. The LptA protein, which is a soluble periplasmic protein in *E. coli* (86), may function as the LPS chaperone, as LolA does for lipoproteins. Furthermore, while LolA passes its cargo to the structurally related OM receptor LolB, LptA may pass the LPS molecules to the periplasmic N-terminal domain of Imp, which shows sequence similarity to LptA. The β-barrel of Imp may form a channel for further transport to the cell surface. Secondary
structure predictions for LptA show many β-strands, possibly forming a soluble beta-barrel, resembling LloA (10, 96). The LptB protein could be the functional LolD homolog of the LPS transport system. As explained above, the putative LolC/E components of such an LPS transport system remain to be found.

The other model postulates that LPS actually never leaves its membranous environment and that it is transported at contact sites between the IM and OM (Figure 5). The first indication for the existence of such sites, known as zones of adhesion or Bayer junctions, came from electron microscopy studies (4), although fixation procedures may have affected the results (46). Later, Mühlradt et al. (63) reported that newly synthesized LPS appears in patches in the OM, close to the membrane contact sites. Membrane fractionation studies showed the existence of a minor fraction, designated OM₁, that contains IM, peptidoglycan, and OM. This membrane fraction, which contained peptidoglycan biosynthesis activity, may represent membrane contact sites. Pulse-chase experiments combined with fractionation procedures showed that newly synthesized LPS transiently passed through this fraction on its way to the OM (40). Furthermore, when a similar approach was used that led to the identification of LoLA (see above), newly synthesized LPS could not be released from spheroplasts upon addition of periplasmic extracts. Rather, LPS transport from IM to OM continued in the spheroplasts, suggesting that this process does not involve a soluble periplasmic component and proceeds via contact sites (100). In this model, the LptA, LptB, and YrbK components may have a role in the formation of these contact sites (Figure 5).

PHOSPHOLIPIDS

The major OM phospholipids of E. coli are phosphatidylethanolamine and phosphatidylglycerol. Phospholipids are synthesized at the cytoplasmic side of the IM (18, 39). Then, in order 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 because the conditional E. coli msbA mutant accumulated both LPS and phospholipids in the IM under restrictive conditions (116). 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 (99). Another distant msbA homolog in N. meningitidis, i.e., the NMB0264 locus in strain MC58, could be disrupted without causing any obvious phenotype (M.P. Bos, unpublished observations). Moreover, various α-helical membrane-spanning peptides, but curiously not MsbA, induced phospholipid translocation in synthetic lipid bilayers (50). Thus, flip-flop of phospholipids may not require a specific transporter but merely the presence of the typical α-helical membrane-spanning segments of some IM proteins. The next steps in phospholipid biogenesis, i.e., transport through the periplasm and incorporation into the inner leaflet of the OM, remain obscure. Unlike LPS transport, the transport of phospholipids was halted in spheroplasts, and unlike lipoproteins, newly synthesized phospholipids could not be released from the spheroplasts upon addition of a periplasmic extract (100). Thus, the transport mechanism appears different from those of LPS and lipoproteins. Any components involved in phospholipid transport remain to be identified.

PERSPECTIVES

In the past few years, much progress has been made in the field of OM biogenesis with the identification of many new components involved in the process. The field will rapidly move forward, gaining mechanistic insights to which structural analysis of the newly identified components by X-ray crystallography will make important contributions.
The major players required for OMP assembly have likely been identified. Provided that no energy-coupling system is required and that protein folding and partitioning into the hydrophobic environment of the membrane are the driving forces, it may be possible to set up an in vitro system for OMP assembly with purified components. For LPS, a major issue remains how it is transported through the periplasm. Studying the binding of LPS to the components together with immunogold electron microscopy studies to determine whether these components are associated with the contact sites between IM and OM will help to address these questions. For lipoproteins, an important issue is how such molecules are transported to the cell surface. Research on the transport of phospholipids to the OM has to start more or less from the beginning.

Importantly, much progress in the field has been reached by studies in two model organisms, *E. coli* and *N. meningitidis*. These studies have revealed similarities as well as differences. For example, whereas an LPS transport defect in *N. meningitidis* results in feedback inhibition of its synthesis, this is not the case in *E. coli*. For OMP assembly, the reverse is true: An OMP assembly defect leads to feedback inhibition in *E. coli*, but not in *N. meningitidis*. Such differences make it attractive to study specific aspects of OM biogenesis in different organisms. In addition, considering the differences already observed between these two model organisms, it is likely that studies in other bacteria will uncover new, unanticipated features.

Further studies in this field will remain important, because they will uncover fundamental biological processes. In addition, the knowledge gained from these studies may be useful for medical applications: The essential nature of the bacterial machineries involved and their surface localization make them attractive targets for the development of new antimicrobial drugs and vaccines.

**SUMMARY POINTS**

1. OMPs and lipoproteins are transported across the IM via the Sec system.
2. Assembly of bacterial outer membrane proteins requires the outer membrane protein Omp85, which is evolutionarily conserved and found even in the OM of mitochondria.
3. Omp85 recognizes its substrate OMPs by virtue of their C-terminal signature sequences.
4. Other proteins involved in OMP transport and assembly are the periplasmic chaperones Skp and SurA, and the OM-associated lipoproteins YfO, YfgL, NlpB, and SmpA, the function of which remains to be determined.
5. Transport of lipoproteins to the OM depends on the Lol system, which consists of an ABC transporter in the IM, a soluble periplasmic chaperone, and an OM-attached receptor.
6. MsbA is an ABC transporter required for the transport of LPS across the IM.
7. Further transport of LPS to the cell surface requires, at least, the ABC protein LptB, the periplasmic protein LptA, the OM-attached lipoprotein RlpB, and the integral OMP Imp.
8. Nothing is known regarding the transport of phospholipids to the OM.
DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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105. First demonstration that an integral OMP, Omp85, is required for the assembly of OMPs.

106. Demonstrates that the C-terminal signature sequence of OMPs triggers the σE-dependent periplasmic stress response when unfolded OMPs accumulate in the periplasm.

