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Review Article

REVIEW ARTICLE

Transport of lipopolysaccharide to the Gram-negative bacterial cell surface

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One sentence summary: The authors review the mechanism of transport of LPS to the Gram-negative bacterial cell surface, the distribution of the machinery involved even among organisms that don't produce LPS and the regulation of LPS synthesis.

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ABSTRACT

Lipopolysaccharides (LPS) are major lipidic components of the outer membrane of most Gram-negative bacteria. They form a permeability barrier that protects these bacteria from harmful compounds in the environment. In addition, they are important signaling molecules for the innate immune system. The mechanism of transport of these molecules to the bacterial cell surface has remained enigmatic for a long time. However, intense research during the last decade, particularly in *Escherichia coli* and *Neisseria meningitidis*, has led to the identification of the machinery that mediates LPS transport. In this review, we summarize the current knowledge of the LPS transport machinery and provide an overview of the distribution of the components of this machinery among diverse bacteria, even organisms that don't produce LPS. We also discuss the current insights in the regulation of LPS biosynthesis.

Keywords: ABC transporter; lipid A; lipopolysaccharide; Lpt machinery; outer membrane; translocon

INTRODUCTION

The cell envelope of Gram-negative bacteria is composed of two membranes, the inner membrane (IM) or cytoplasmic membrane and the outer membrane (OM). The compartment in between these membranes is called the periplasm and contains a layer of peptidoglycan. The IM is a bilayer of glycerophospholipids containing integral and associated membrane proteins. In contrast, the OM is highly asymmetrical, usually containing glycerophospholipids only in the inner monolayer, whilst the outer monolayer consists of lipopolysaccharides (LPS). Also with respect to the structure of its integral membrane proteins, the OM is very different from the IM: whilst integral IM proteins span the membrane as α-helices almost entirely composed of hydrophobic residues, the vast majority of integral OM proteins (OMPs) consists of amphipathic β-strands, which form a β-barrel structure (Fairman, Noinaj and Buchanan 2011). Besides integral OMPs, the OM also contains lipoproteins (Buddelmeijer 2015). These lipoproteins are anchored to the OM via an N-terminal *N*-acyl-diacylglycerylcysteine residue and can either extend into the periplasm or be exposed at the cell surface. Such lipoproteins are also found at the periplasmic side of the IM.

All the components of the OM are synthesized in the cytoplasm or at the cytoplasmic side of the IM. After their synthesis, they have to be transported across the IM and the aqueous periplasm to reach the OM in which they have to be assembled properly. Virtually nothing is known yet about the transport of glycerophospholipids to the OM. However, in the past two decades, we have witnessed an enormous progress in the understanding of the transport and assembly of the other OM components after the discovery of key machinery involved in these processes. We refer to recent reviews for the biogenesis of OMPs (Tommassen 2010; Ricci and Silhavy 2012; Selkrig *et al.*

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2014) and lipoproteins (Okuda and Tokuda 2011). Here, we focus on the biogenesis of LPS.

STRUCTURE AND BIOSYNTHESIS OF LPS

LPS consists of two or three moieties (Fig. 1): lipid A, which anchors the molecule in the membrane, an oligosaccharide known as the core, and a polysaccharide, called the O-antigen, which is absent in many bacteria, including, for example, the pathogens *Neisseria meningitidis* and *Bordetella pertussis*. LPS lacking O-antigen is sometimes referred to as lipooligosaccharide or LOS. Lipid A of *Escherichia coli* consists of a glucosamine disaccharide that is phosphorylated at the 1 and 4′ positions and contains fatty acyl chains connected via an amide linkage to the 2 and 2′ positions and via an ester bond to the 3 and 3′ positions. These primary fatty acids are hydroxylated and secondary fatty acids are esterified to the hydroxyl groups of the primary fatty acids bound to the 2′ and 3′ positions of the disaccharide backbone (Fig. 1) (Raetz and Whitfield 2002). The structure of the lipid A moiety of LPS is generally well conserved among Gram-negative bacteria although differences exist with respect to the length of the fatty acyl chains and the number and positions of the secondary fatty acids. In addition, various regulated modifications can occur (for a review, see Raetz *et al.* 2007; Needham and Trent 2013). For example, the secondary lauroyl chain in *E. coli* lipid A is replaced by palmitoleate when the bacteria are grown at low temperature, presumably to maintain optimal membrane fluidity (Carty, Sreekumar and Raetz 1999). Other modifications, such as the removal or substitution of the phosphate groups and the removal or addition of acyl chains, can occur during or after the transport of the LPS molecule to the OM and often serve to evade the host's defense systems (Raetz *et al.* 2007). The occurrence of such modifications has proven to be very useful tools in LPS transport studies as they can be used to determine at which stage transport is blocked in LPS transport mutants (Bos *et al.* 2004; Doerrler, Gibbons and Raetz 2004; Tefsen *et al.* 2005b).

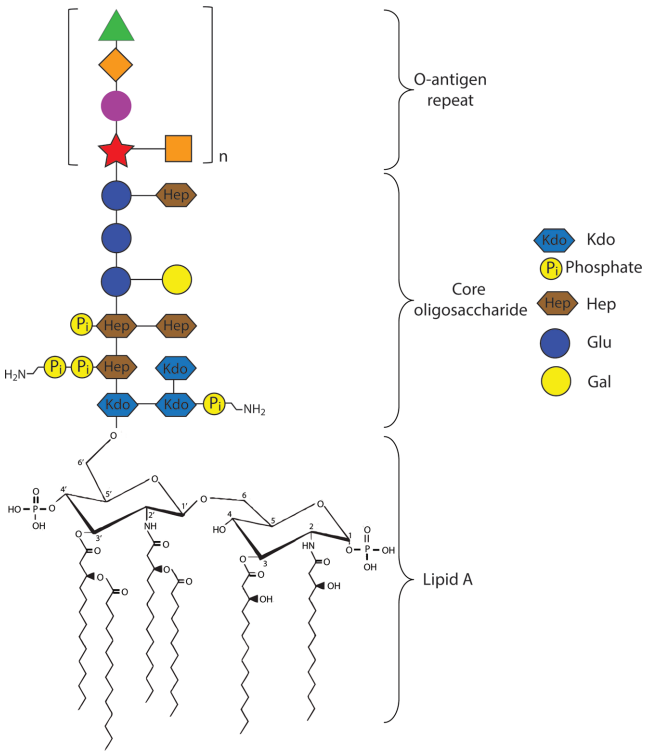

Figure 1. Structure of LPS consisting of lipid A, the core oligosaccharide and the O-antigen. The lipid A and core structures depicted correspond to those of *E. coli* K-12. The O-antigen, if present, is highly variable. Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Hep, heptose; Glu, glucose; Gal, galactose. The phosphoethanolamine residues and the third Kdo in the core are non-stoichiometric additions.

The core oligosaccharide is often divided in an inner core and an outer core. The inner core contains 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and usually also L-*glycero*-D-*manno*-heptose (heptose) residues and is rather conserved between different isolates of the same species, whilst the outer core is more variable. The O-antigen is composed of repeating units of one to six different sugar residues. It is highly variable; in *E. coli*, for example, ~170 different O-antigens have been described (Raetz and Whitfield 2002).

Lipid A substituted with the Kdo residues of the inner core is synthesized at the cytoplasmic side of the IM via a conserved pathway, the Raetz pathway, which requires nine enzymes (Fig. 2) (Raetz and Whitfield 2002). After the attachment of the other core residues by glycosyltransferases, the lipid A/core moiety is flipped across the IM to the periplasmic side (*vide infra*). The O-antigen is produced separately. The repeating units of the O-antigen are synthesized on the lipid carrier undecaprenyl phosphate and transported by the flippase Wzx to the periplasmic side of the IM, where polymerization takes place. Alternatively, polymerization already takes place at the cytoplasmic side of the IM, followed by transport to the periplasmic side by an ABC (ATP-binding cassette) transporter constituted by the Wzm and Wzt proteins. Attachment of the complete O-antigen to the lipid A/core moiety takes place in the periplasm and is mediated by the enzyme WaaL. The assembly of O-antigen was recently reviewed in this journal (Kalynych, Morona and Cygler 2014) and will not further be discussed here. Besides attachment of the O-antigen, several other modifications can occur at the periplasmic side of the IM, such as the removal of the phosphate groups of lipid A or their substitution with phosphoethanolamine or sugar residues, such as 4-amino-4-deoxy-L-arabinose (L-Ara4N). Also, various core sugars can non-stoichiometrically be substituted, e.g. with phosphate or phosphoethanolamine.

FUNCTION OF LPS

The OM protects bacteria by forming a permeability barrier for noxious compounds from the environment, including many antibiotics. Small hydrophilic compounds, including nutrients such as amino acids or simple sugars, can pass the membrane generally by passive diffusion via pore-forming proteins, known as porins, but larger and hydrophobic molecules are excluded (Nikaido 2003). LPS is responsible for the barrier function of the OM. The repulsive forces between the negative charges in the LPS are compensated by divalent cations. Together with the dense packing of the acyl chains, this creates a network that is barely permeable for hydrophobic compounds. Also the O-antigen, if present, has a protective role. It protects the bacteria from the lethal action of the complement system and macrophages (Murray, Attridge and Morona 2006). In addition, it forms a barrier for bacteriophages, bacteriocins and bactericidal antibodies targeting more conserved receptors located deeper in the OM (van der Ley, de Graaff and Tommassen 1986; van der Ley *et al.* 1986).

In spite of its important function, LPS is not an essential component of the OM of all Gram-negative bacteria. Some

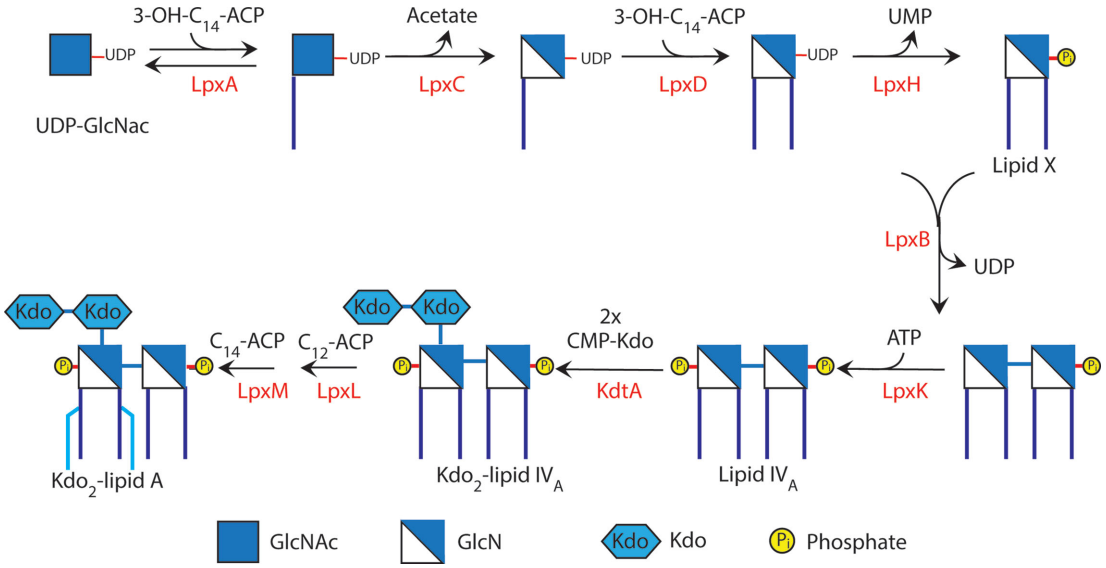

Figure 2. Schematic representation of the conserved Raetz pathway for the synthesis of Kdo_2-lipid A. The enzymes involved in each step are indicated in red. GlcNAc, N-acetylglucosamine; GlcN, glucosamine; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; ACP, acyl carrier protein. Primary and secondary acyl chains are indicated in blue and light blue, respectively. For a detailed structure of lipid A, refer to Fig. 1.

Gram-negative bacteria, including the pathogenic Spirochetes *Treponema pallidum* and *Borrelia burgdorferi*, do not produce LPS. These bacteria produce other glycolipids that may be functional substituents of LPS (Schröder *et al.* 2008). Similarly, glycosphingolipids appear to substitute for LPS in *Sphingomonas* spp. (Wiese and Seydel 1999). In addition, whilst LPS is essential for the viability of several Gram-negative model organisms, such as *E. coli* and *Salmonella*, LPS synthesis can be shut off genetically in others including *N. meningitidis* (Steeghs *et al.* 1998), *Moraxella catarrhalis* (Peng *et al.* 2005) and *Acinetobacter baumannii* (Moffatt *et al.* 2010). Such mutants were not only generated in the laboratory, but also clinical isolates without LPS have been reported (Moffatt *et al.* 2010; Piet *et al.* 2014). Furthermore, in several bacterial species, including, for example, *Helicobacter pylori* (Chiu, Lin and Wang 2007; Chiu *et al.* 2009), genes encoding crucial LPS transport proteins could be disrupted. Although the fate of LPS in these mutants was not investigated, the existence of these mutants suggests that also these bacterial species are viable without LPS in the OM. In *N. meningitidis*, the biogenesis of integral OMPs is unaffected in LPS-deficient mutants (Steeghs *et al.* 2001), and the absence of LPS appears to be compensated by increased amounts of glycerophospholipids (Tefsen *et al.* 2005a). *A. baumannii* responds to the loss of LPS by increased production of lipoproteins and the Lol system for the transport of lipoproteins to the OM, of poly-β-1,6-N-acetylglucosamine and of the Mla system that mediates the retrograde transport of glycerophospholipids from the outer leaflet of the OM to maintain OM asymmetry (Henry *et al.* 2012). The latter is difficult to understand if glycerophospholipids have to compensate for the loss of LPS in the outer leaflet of the OM. It is noteworthy, however, that also *E. coli* responds to a defect in LPS transport by overproduction of the Mla system (Martorana *et al.* 2014). The function of the OM as a permeability barrier is compromised in LPS-less mutants as evidenced by their increased sensitivity for antibiotics (Steeghs *et al.* 2001). It remains enigmatic why some bacteria are viable without LPS, in contrast to others. For example, whilst LPS synthesis can genetically be disrupted in *N. meningitidis* as described above, similar mutations cannot be introduced in its close relative *N. gonorrhoeae* (Bos and Tommassen 2005).

Besides its role in the OM, LPS, and in particular its lipid A moiety, is also an important signaling molecule for the innate immune system. Binding of LPS to a receptor constituted of TLR4 and MD2 on the plasma membrane of innate immune cells induces a signaling cascade resulting in the activation of the transcription factor NF-κB, which triggers the production of proinflammatory mediators, such as TNF-α and IL-6 (for reviews, see Miyake 2004; Maeshima and Fernandez 2013). This pathway also leads to the synthesis of costimulatory molecules that activate the adaptive immune response. Whilst the goal of the innate immune response is to eradicate the infection, the inflammation caused by overstimulation of the pathway is dangerous to the host and can result in septic shock. As a potent activator of the response, LPS is also known as endotoxin. Hexa-acylated and bis-phosphorylated lipid A is the strongest inducer of this response. Many pathogenic bacteria evade this immune response by modifying their LPS (for reviews, see Raetz *et al.* 2007; Needham and Trent 2013), e.g. removal of acyl chains by the enzymes PagL or LpxR, addition of an extra acyl chain by PagP, or removal of phosphate groups by LpxE or LpxF. The removal of the phosphate groups or their substitution with phosphoethanolamine or sugars, such as L-Ara4N, as well as the addition of an extra fatty acyl chain, also protects the bacteria from the activity of cationic antimicrobial peptides (CAMPs), another defense mechanism of the host that disrupts the OM barrier function by targeting the negatively charged LPS molecules.

TRANSPORT OF LPS TO THE CELL SURFACE

Transport across the IM

Upon its synthesis at the cytoplasmic side of the IM, the lipid A/core moiety of LPS is flipped to the periplasmic face of the membrane by a transporter called MsbA. The *msbA* gene was originally identified as a multicopy suppressor of an *lpxL* (*htrB*)

mutant (Karow and Georgopoulos 1993). The LpxL enzyme adds the first secondary acyl chain to lipid A (Fig. 2) and its inactivation results in the accumulation of tetra-acylated LPS forms in the cells and a temperature-sensitive phenotype. MsbA is an ABC transporter that is essential for viability in *E. coli* (Polissi and Georgopoulos 1996). The tetra-acylated LPS forms that accumulated in the IM of *lpxL* mutants were transported to the OM when MsbA was overproduced, indicating that MsbA is involved in LPS transport and that tetra-acylated LPS is a poor substrate for MsbA (Zhou *et al.* 1998), which makes sense as transport of incomplete, nascent LPS precursors should be avoided. Growth of a temperature-sensitive *msbA* mutant at the restrictive temperature led to the accumulation of nascent LPS at the cytoplasmic side of the IM (Doerrler, Gibbons and Raetz 2004) showing that MsbA is involved in flipping LPS from the cytoplasmic to the periplasmic side of the IM.

Interestingly, the temperature-sensitive *msbA* mutant of *E. coli* also appeared affected in the translocation of glycerophospholipids at the restrictive temperature, suggesting that MsbA might function as a general flippase for all major lipid species (Doerrler, Reedy and Raetz 2001; Doerrler, Gibbons and Raetz 2004). Indeed, purified MsbA reconstituted into proteoliposomes was shown to translocate fluorescently labeled phospholipids (Eckford and Sharom 2010). However, the *msbA* gene could genetically be disrupted in *N. meningitidis*, a bacterium that is viable without LPS (Tefsen *et al.* 2005a). The resulting mutant still produced an OM, where the absence of LPS appeared to be compensated by increased amounts of glycerophospholipids. These results demonstrated that MsbA is not essential for the translocation of glycerophospholipids, at least not in *N. meningitidis*.

The ATP hydrolysis rates of purified *E. coli* MsbA in the absence of substrate were found to be similar to those of other ABC transporters (Doerrler and Raetz 2002; Eckford and Sharom 2008; Doshi and van Veen 2013). The ATPase activity was stimulated in the presence of lipid A, phospholipids and also of some drugs that are substrates of drug-efflux pumps. However, the increase of ATPase activity was stimulated substantially stronger with lipid A than with phospholipids (Doerrler and Raetz 2002), suggesting a preference for lipid A as the substrate. Consistently, the MsbA-mediated translocation of fluorescently labeled phospholipids in proteoliposomes was inhibited by lipid A (Eckford and Sharom 2010), which also indicates that LPS is the preferred physiological substrate.

Crystal structures (Ward *et al.* 2007) showed that MsbA is a dimer with each subunit consisting of a transmembrane (TM) domain composed of six TM helices and a nucleotide-binding domain (NBD) extending into the cytoplasm (Fig. 3a). The structures showed two different orientations, one in which the TM helices are opened towards the cytoplasm, and the other where they are opened towards the periplasm (Ward *et al.* 2007). Biochemical studies (Doshi and van Veen 2013) indicated that the conversion between these states proceeds via an intermediate inward-closed conformation that is induced in the presence of the substrate lipid A and results in the dimerization of the NBDs (Fig. 3b). Dimerization of the NBDs stimulates ATP binding, which subsequently switches MsbA to the outward-facing conformation. ATP hydrolysis eventually returns MsbA to the inward-facing open orientation (Doshi and van Veen 2013). The exact path of the hydrophilic and hydrophobic moieties of the lipid A/core molecule during the MsbA transport cycle remains to be determined.

Besides MsbA, another IM protein, YhjD, has been implicated in LPS transport across the IM in *E. coli*. Lipid A substituted with two Kdo residues has long been considered the minimal LPS structure required for viability of *E. coli*. Strains that are unable to synthesize Kdo accumulate the tetra-acylated LPS precursor lipid IV_A (Fig. 2) and are non-viable. However, such strains can be rescued by overexpression of MsbA (Meredith *et al.* 2006) as well as by extragenic suppressor mutations introducing single amino-acid substitutions in the MsbA or YhjD proteins (Meredith *et al.* 2006; Mamat *et al.* 2008). These *msbA* mutations presumably relax the substrate selectivity of the MsbA protein enabling it to transport lipid IV_A. Accordingly, also overexpression of LpxL and LpxM rescued these strains by facilitating the addition of secondary acyl chains to lipid IV_A, which normally is a poor substrate for these enzymes (Reynolds and Raetz 2009). The resulting strains were viable and contained penta- or hexa-acylated lipid A in the OM. Interestingly, the *msbA* gene could be deleted in the selected *yhjD* mutant, indicating that lipid IV_A transport in this mutant is independent of MsbA and mediated by the mutant YhjD protein (Mamat *et al.* 2008). Whether YhjD has a role in LPS transport in a wild-type background is unclear. In wild-type *E. coli*, *yhjD* is classified as a non-essential gene (Baba *et al.* 2006). Inactivation of *brkB*, the *yhjD* homolog of *B. pertussis*, caused serum sensitivity (Fernandez and Weiss 1994), which could reflect an OM defect. The YhjD protein belongs to the major facilitator family implicated in small-molecule transport. It is widely disseminated among bacteria, but no homolog is present, for example, in *N. meningitidis*. Perhaps, wild-type YhjD is normally involved in the transport of a molecule that resembles lipid IV_A, such as cardiolipin, and the mutation that rescues strains unable to synthesize Kdo broadens the substrate specificity of this transporter. It is worth noting that *Neisseria* spp., which don't contain a *yhjD* homolog, contain only trace amounts of cardiolipin (Rahman *et al.* 2000).

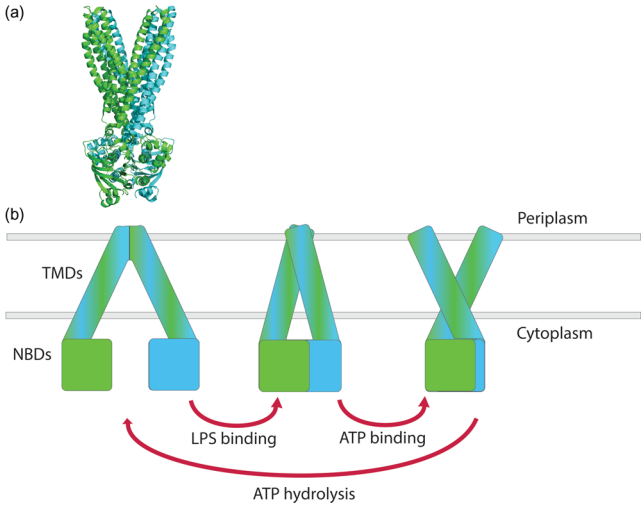

Figure 3. (a) Ribbon representation of the structure of an MsbA dimer in the nucleotide-bound state (Ward *et al.* 2007). The two subunits are indicated in blue and green, respectively. The figure was generated with PyMol (http://www.pymol.org) using PDB file 3B60 from the Protein Data Base (PDB). (b) Schematic presentation of the MsbA transport cycle as proposed by Doshi and van Veen (2013). MsbA shuttles from an inward-open conformation via an inward-closed and an outward-open conformation back to the inward-open conformation. Transitions are mediated by substrate binding, ATP binding and ATP hydrolysis as indicated. TMDs, transmembrane domains; NBDs, nucleotide-binding domains. The two subunits are colored as in panel a. Note that each of the arms in the TMDs is composed of elements of both subunits.

Transport from the IM to the cell surface

Identification of components of the LPS transport (Lpt) machinery

After the lipid A/core moiety of LPS is flipped to the periplasmic side of the IM, the O-antigen, if present, is added, and the mature LPS is transported to the cell surface by the Lpt machinery. The first component of this machinery identified was the OMP LptD in *N. meningitidis* (Bos *et al.* 2004). Earlier studies had demonstrated that the barrier function of the OM was compromised in *E. coli* strains expressing mutant forms of this protein, earlier designated Imp (increased membrane permeability) or OstA (organic solvent tolerance) (Sampson, Misra and Benson 1989; Aono, Negishi and Nakajima 1994). LptD was subsequently found to be essential for the viability of *E. coli*, and LptD depletion in a genetically engineered strain resulted in major membrane defects (Braun and Silhavy 2002). This observation triggered research into the exact role of the protein in *N. meningitidis*, where a knockout mutant in the corresponding gene appeared to be viable. It was demonstrated that this mutant produced drastically reduced amounts of LPS, and the residual LPS that was produced was not transported to the cell surface as it was neither modified by a lipid A deacylase present in the OM nor accessible to neuraminidase externally added to intact cells (Bos *et al.* 2004). Reduced amounts of LPS were not only detected in the *lptD* mutant, but also in an *msbA* mutant (Tefsen *et al.* 2005a) and other LPS transport mutants of *N. meningitidis* (Bos and Tommassen 2011) and are probably due to feedback inhibition on LPS synthesis when transport is blocked (*vide infra*).

In *E. coli*, LptD was found to exist in a hetero-oligomeric complex with a lipoprotein, LptE (formerly known as RlpB) (Wu *et al.* 2006). Like LptD, LptE was shown to be essential for the viability of *E. coli* and required for the transport of LPS to the cell surface. LptE is inserted as a plug inside the β-barrel of LptD (Freinkman, Chng and Kahne 2011; Dong *et al.* 2014; Qiao *et al.* 2014) and was shown to be required for the proper assembly of this protein into the OM (Ruiz *et al.* 2010; Chimalakonda *et al.* 2011). In addition, it was demonstrated that LptE interacts directly with LPS (Chng *et al.* 2010) suggesting that it has, besides its LptD-chaperone function, a direct role in LPS transport. However, an *lptE* knockout mutant in *N. meningitidis* showed decreased amounts of LptD, but LPS transport to the cell surface was unaffected (Bos and Tommassen 2011). Therefore, the LptD-chaperone function of LptE appears to be more prominent in *N. meningitidis*, and its direct involvement in LPS transport is questionable.

The *lptA* (*yhbN*), *lptB* (*yhbG*) and *lptC* (*yrbK*) genes of *E. coli* were discovered as essential genes in a well-conserved locus also containing genes encoding proteins involved in Kdo biosynthesis (Sperandeo *et al.* 2006). The essentiality of the *lptB* homolog of *Rhizobium* was already earlier demonstrated (van Slooten and Stanley 1991). Subsequently, it was demonstrated that *de novo*-synthesized radiolabeled LPS was not transported to the OM when cells were depleted of the corresponding proteins but accumulated in membrane fractions with lower density than the OM (Sperandeo *et al.* 2007, 2008). Other phenotypes of the depletion strains included the accumulation of multilayered membranous bodies in the periplasm, overproduction of LPS and the presence of LPS forms substituted with colanic acid (Meredith *et al.* 2007; Sperandeo *et al.* 2007, 2008). The latter can be explained by the residence of LPS at the periplasmic face of the IM allowing the WaaL ligase to substitute it with colanic acid. Similar phenotypes were reported for *E. coli* cells depleted for LptD and LptE (Sperandeo *et al.* 2008).

LptA is a periplasmic protein (Sperandeo *et al.* 2007), whilst LptC is an IM protein (Sperandeo *et al.* 2008) containing one N-terminal TM helix and a large soluble domain exposed to the periplasm (Tran, Dong and Whitfield 2010). Both LptA and LptC were reported to bind LPS (Tran, Trent and Whitfield 2008; Tran, Dong and Whitfield 2010; Sestito *et al.* 2014). LptA was actually able to bind different truncated forms of LPS including pure lipid A, but it was not able to bind glycerophospholipids indicating that LptA has a binding specificity for the lipid A moiety of LPS (Tran, Trent and Whitfield 2008). Based on its sequence, LptB was predicted to be the NBD of an ABC transporter (Sperandeo *et al.* 2007), but the corresponding TM domain (TMD) was missing. Nevertheless, LptB was found to fractionate with the IM as a part of a 140-kDa complex (Stenberg *et al.* 2005). The missing membrane subunits of the transporter were eventually identified in an elegant reductionist bioinformatic approach taking advantage of the small proteome size of a Gram-negative endosymbiont (Ruiz *et al.* 2008). This led to the identification of two new, essential IM proteins in *E. coli*, LptF (YjgP) and LptG (YjgQ), which are conserved and show mutual homology. Cells depleted of LptF and LptG were defective in the transport of LPS to the cell surface and showed phenotypes similar to those of cells missing other Lpt components (Ruiz *et al.* 2008). Furthermore, LptB, LptF and LptG could be purified as a complex with a 2:1:1 stoichiometry, which exhibited ATPase activity (Narita and Tokuda 2009). If cooverproduced, also LptC was part of this complex.

Structures of Lpt components

The first crystal structure solved was that of *E. coli* LptA (Suits *et al.* 2008). This structure showed a novel fold, consisting of 16 consecutive anti-parallel β-strands organized in a characteristic, slightly twisted β-jellyroll conformation (Fig. 4a). Interestingly, although it shows no sequence similarity to LptA, the periplasmic domain of LptC was found to have a strikingly similar structure (Fig. 4b) (Tran, Dong and Whitfield 2010). The structures of LptA and LptC did not immediately reveal a hydrophobic

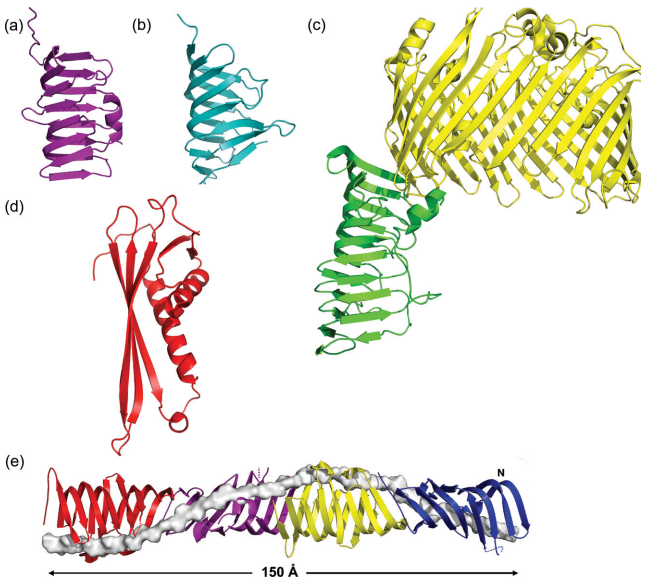

Figure 4. Ribbon representation of the crystal structures of LptA (**a**), LptC (**b**), LptD (**c**) and LptE (**d**). The figures were generated with PyMol using PDB files 2R19, 3MY2, 4Q35 and 4NHR, respectively. In panel c, the OM-embedded β-barrel of LptD and its periplasmic part are colored yellow and green, respectively. (**e**) Structure of an LptA tetramer consisting of head-to-tail stacked monomers, each represented in a different color. This figure is reproduced from Suits *et al.* (2008) by permission of the publisher. Highlighted in gray is a continuous β-sheet surface that spirals along the length of the LptA tetramer.

binding pocket that is deep enough to accommodate the fatty acyl chains of lipid A and co-crystals of either of these proteins with LPS have so far not been reported. However, *in vivo* cross-linking experiments revealed that the residues in LptA and LptC that were able to cross-link LPS are all located inside of the β-jellyroll indicating that this groove is important for LPS binding (Okuda, Freinkman and Kahne 2012).

Interestingly, the N-terminal part of LptD, which extends into the periplasm, shows sequence similarity to LptA, and the recently solved crystal structure of *Shigella flexneri* LptD indeed showed that this domain also has a similar β-jellyroll fold (Fig. 4c) (Qiao *et al.* 2014). The interior surface of this domain is highly hydrophobic and contained detergent molecules in the crystal structure, suggesting that it can bind the lipid A moiety of LPS. In agreement with this hypothesis is the observation that substitutions of aromatic residues involved in the binding of the detergent molecules in the crystal structure were lethal or impaired cell growth (Gu *et al.* 2015). The C-terminal domain of LptD from *S. flexneri* (Qiao *et al.* 2014) and *Salmonella enterica* serovar Typhimurium (Dong *et al.* 2014) forms a 26-stranded antiparallel β-barrel (Fig. 4c), the largest monomeric β-barrel of which the structure has been determined to date. All β-strands are connected via periplasmic and extracellular loops, the longer loops of which are on the extracellular side. These long loops close off most of the β-barrel pore from the outside. Extracellular loops 4 and 8 are located inside the barrel, where they bind the LptE protein which plugs the rest of the pore (Dong *et al.* 2014; Qiao *et al.* 2014). A large vestibule in the LptDE complex, which could transiently accommodate nascent LPS molecules, is accessible from the periplasmic side. Such LPS molecules could possibly leave the LptDE complex and be inserted in the OM via a lateral gate between the first and the last β-strands of the LptD barrel. These strands interact only weakly via three to five hydrogen bonds (Dong *et al.* 2014; Qiao *et al.* 2014), and molecular dynamics simulations suggested that they can separate (Dong *et al.* 2014). Moreover, mutations introducing disulfide bonds between β1 and β26 showed lethal phenotypes (Dong *et al.* 2014) indicating that the loose connection between these strands is essential for proper functioning of LptD.

LptE is a lipoprotein that forms a sandwich of two α-helices packed against a sheet of four β-strands (Fig. 4d) (Malojčić *et al.* 2014). Even though sequence similarities are low, the structures of LptE proteins from different bacteria correspond very well with each other and with that of LptE in the LptDE complex (Dong *et al.* 2014; Malojčić *et al.* 2014; Qiao *et al.* 2014). In the LptDE complex, approximately 75% of LptE is located inside the β-barrel of LptD. Its N-terminal lipid moiety is located outside of the barrel and probably inserted in the inner leaflet of the OM. Interestingly, this lipid anchor is not essential for LptE function (Chng *et al.* 2010). LptE shows structural similarity with proteins binding negatively charged carbohydrates and with the anti-LPS factors (LALF) of black tiger shrimp and horseshoe crab, which bind negative charges in the lipid A moiety of LPS via basic residues (Malojčić *et al.* 2014). Accordingly, substitution of two basic residues in LptE impeded LPS binding (Malojčić *et al.* 2014).

Of the ABC transporter $LptB_2FG$, the structure of the NBD LptB has been resolved (Sherman *et al.* 2014; Wang *et al.* 2014), whilst those of the TMD components, LptF and LptG, remain unknown. The LptB sequence shows the characteristic motifs of the NBDs of ABC transporters, the Walker A and B boxes, the ABC signature motif and the Q-loop (Davidson *et al.* 2008). LptB was crystallized in its ATP-bound state and in its post-hydrolysis ADP-bound stage, revealing major structural changes, which could be transmitted to the TMD to power LPS transport (Sherman *et al.* 2014). Mutagenesis of the active-site E163 residue confirmed that ATP hydrolysis is essential for LPS transport, whereas also a mutation substituting F90, which affected the interaction with the TMD, was detrimental for function.

The Lpt proteins function as a transenvelope complex

Two models have been proposed for the functioning of the Lpt machinery (Bos, Robert and Tommassen 2007). In the first model, the Lpt system resembles the Lol system that is required for the transport of lipoproteins to the OM (Okuda and Tokuda 2011). According to this model, the ABC transporter $LptB_2FG$ expels LPS from the periplasmic side of the IM, LptA functions as a shuttle that shields the hydrophobic lipid A moiety during periplasmic transit, and LptDE functions as a receptor in the OM and flips the LPS from the periplasmic side to the cell surface. A major problem with this model is that the energy source required for flipping the LPS molecules across the OM is not directly obvious. In the alternative model, the Lpt components interact to form one transenvelope machine that transports LPS directly from the periplasmic side of the IM to the cell surface. The latter model is now strongly favored by recent as well as older data.

As early as in 1973, an electron microscopy study already suggested that newly synthesized LPS molecules appear at the cell surface at contact sites between IM and OM (Mühlradt *et al.* 1973). The nature of these contact sites, which were called zones of adhesion or Bayer's bridges, was unknown. Consistent with this notion was the observation that newly synthesized LPS was transported to the OM in spheroplasts of *E. coli* (Tefsen *et al.* 2005b). Spheroplasts are depleted of soluble periplasmic components, but OM fragments are still attached via adhesion sites. Thus, this study excluded a chaperone-bound soluble intermediate in the LPS transport pathway and suggested that transport takes place via contact sites between the membranes. Additional biochemical evidence for transport via contact sites was obtained in pulse-chase experiments combined with separation of IM and OM by sucrose density centrifugation. These experiments revealed that newly synthesized LPS passed through a membrane fraction with intermediate density between IM and OM (designated OM_L, for light OM fraction) and that contained components of both membranes (Ishidate *et al.* 1986). After the discovery of the Lpt proteins, it was reported that all of them cofractionate in a similar membrane fraction of intermediate density (Chng, Gronenberg and Kahne 2010). In the same study, it was demonstrated that all Lpt proteins can be copurified by affinity chromatography suggesting they can constitute a continuous connection between IM and OM.

How are the IM and OM components of the transport machinery interconnected? In crystallization experiments, it was found that LptA can oligomerize in a head-to-tail orientation (Fig. 4e) (Suits *et al.* 2008). The formation of such LptA oligomers *in vitro* was subsequently confirmed in various biochemical and biophysical experiments (Merten, Schultz and Klug 2012; Santambrogio *et al.* 2013). Oligomerization was concentration dependent and yielded fibrillar structures of up to 20 subunits in length. Head-to-tail stacked tetramers of LptA, as observed in the crystallographic study (Suits *et al.* 2008), are 150 Å long, which corresponds to the width of the periplasm of *E. coli* as observed in electron micrographs obtained after freeze-substitution of the bacteria (Hobot *et al.* 1984; Graham *et al.* 1991) or deduced from the dimensions of the structures of several transenvelope proteineous machines, such as the type III protein secretion system or a capsular polysaccharide exporter (Marlovits *et al.* 2004; Collins *et al.* 2007). Thus, a tetramer of LptA could span the periplasm

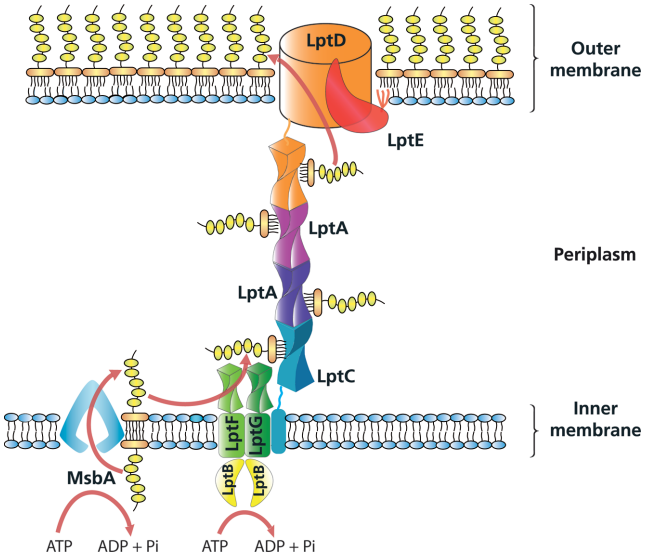

Figure 5. Model for LPS transport. LPS is flipped across the IM by the ABC transporter MsbA, expelled from the IM by the ABC transporter $LptB_2FG$, transported in a continuous stream across the periplasm via a bridge constituted of the structurally similar proteins LptC, LptA and the periplasmic domain of LptD, and eventually inserted into the outer leaflet of the OM via a translocon composed of the β-barrel of LptD and LptE. The entire transport process from the periplasmic side of the IM to the cell surface is powered by LptB-mediated ATP hydrolysis.

with a continuous groove spiraling along the oligomer (Fig. 4e) possibly providing the path for LPS transport. However, as already mentioned above, the periplasmic part of LptC has a similar structure as LptA and it has been shown to physically interact via its C-terminal edge with the N-terminal edge of LptA (Bowyer *et al.* 2011; Sperandeo *et al.* 2011), and the affinity of LptA for LptC is stronger than the affinity for LptA oligomerization (Schultz, Feix and Klug 2013). Similarly, cross-linking studies showed that LptA can interact via its C-terminal edge with the N-terminal edge of the periplasmic domain of LptD (Freinkman *et al.* 2012), which also has a similar structure (see above). Interestingly, three-dimensional structure predictions suggested that also the periplasmic domains of LptF and LptG, the integral membrane proteins of the ABC transporter, contain a similar β-jellyroll fold as LptC, LptA and the periplasmic domain of LptD (Villa *et al.* 2013). Moreover, the N-terminal membrane anchor of LptC appeared to be dispensable for interaction of LptC with the ABC transporter (Villa *et al.* 2013). Thus, a picture emerges of a periplasm-spanning fibrillar structure constituted of head-to-tail packed β-jellyrolls of LptF and/or LptG, LptC, one or two copies of LptA, and the periplasmic domain of LptD (Fig. 5). Moreover, whilst both LptC and LptA have been reported to bind LPS (see above), LptA could displace LPS from LptC but not vice versa (Tran, Dong and Whitfield 2010) indicating a unidirectional transport pathway (Fig. 5). In an *in vitro* reconstituted system with right-side-out vesicles, the transfer of LPS from the IM to LptC was found to require ATP (Okuda, Freinkman and Kahne 2012). Also its subsequent transfer from LptC to LptA in this system required ATP, which seems at odds with the experiments of Tran, Dong and Whitfield (2010), who reported spontaneous displacement of LPS from purified LptC when LptA was added. Probably, this discrepancy can be explained by a different binding of LPS to LptC in the two experimental systems (Okuda, Freinkman and Kahne 2012). The requirement for ATP in the transfer of LPS from LptC to LptA suggests that ATP powers a continuous stream of LPS through the periplasmic bridge to the OM.

After crossing the periplasm, LPS could be delivered into the vestibule in the LptDE translocon, where it could bind LptE. LptE binds various derivatives of LPS, including lipid A (Chng *et al.* 2010); the involvement of positively charged residues of LptE in LPS binding (Malojčić *et al.* 2014) suggests that the phosphate groups in the lipid A moiety could be involved. Where exactly the fatty acyl chains of lipid A are accommodated in the hydrophilic LptDE vestibule (Dong *et al.* 2014; Qiao *et al.* 2014) is not clear. Possibly, the hydrophobic moiety of the LPS never enters this hydrophilic vestibule. A recent molecular dynamics simulations study supported by site-directed mutagenesis data revealed that the C-terminal edge of the β-jellyroll of LptD penetrates into the lipid bilayer and forms a hydrophobic intramembrane hole through which the acyl chains of the LPS could be directly injected into the membrane (Gu *et al.* 2015). The core and the O-antigen would enter the barrel of LptD via a luminal gate, thereby triggering the opening of the lateral gate between the first and the last β-strands and allowing the translocation of the core to the cell surface. The importance of the luminal gate was confirmed by the generation of a disulfide bond, which prevented opening of the gate and which was lethal (Gu *et al.* 2015). Interestingly, it was reported that LptE disrupts LPS–LPS interactions *in vitro* (Malojčić *et al.* 2014). Thus, LptE may disaggregate LPS molecules in the transport pathway to allow their translocation via the lateral gate in the LptD β-barrel. This model may explain why LptE in *N. meningitidis* is not essential for the assembly of LPS in the outer leaflet of the OM (Bos and Tommassen 2011). The phosphoryl groups in the lipid A moiety of *N. meningitidis* LPS are, to a large extent, substituted with phosphoethanolamine (Cox *et al.* 2003), which protects these bacteria against polymyxin and other CAMPs (Tzeng *et al.* 2005). This substitution probably reduces the aggregation of periplasmic LPS transport intermediates in the presence of divalent cations. Thus, the major role of LptE in *N. meningitidis* is to assist the assembly of LptD (Bos and Tommassen 2011).

Regulated assembly of the Lpt machinery

Obviously, to prevent mistargeting, LPS transport should not be initiated before all components of the Lpt machinery are properly assembled. LptD of *E. coli* is a large OMP with four cysteine residues (C1–C4) that form two non-consecutive disulfide bridges (Ruiz *et al.* 2008), which complicates its assembly. Upon its transport across the IM, LptD is escorted in the periplasm by the chaperone SurA (Vertommen *et al.* 2009). Actually, the adjacent *lptD* and *surA* genes on the chromosome constitute an operon and, indeed, LptD appears to be the primary substrate for SurA (Vertommen *et al.* 2009). Curiously, however, SurA has no role in LptD biogenesis in *N. meningitidis* (Volokhina *et al.* 2011). Also Skp, another prominent periplasmic chaperone for OMPs, has been shown to function in the biogenesis of *E. coli* LptD (Schwalm *et al.* 2013), confirming the hypothesis that SurA and Skp act in the same, rather than in parallel pathways (Bos, Robert and Tommassen 2007), at least for the LptD substrate. In the periplasm, the oxidase DsbA introduces a disulfide bond between the first two cysteine residues, C1 and C2, both located in the N-terminal periplasmic domain of LptD and possibly also between C3 and C4, both located in the C-terminal β-barrel domain (Chng *et al.* 2012). Subsequently, LptD is folded and inserted into the OM via the Bam complex (Wu *et al.* 2006; Chng *et al.* 2012), the machinery that inserts β-barrel OMPs into this membrane (for reviews, see Tommassen 2010; Ricci and Silhavy 2012; Selkrig *et al.* 2014). This

is the rate-limiting step in LptD biogenesis and is remarkably slow, taking ~20 min (Chng *et al.* 2012). At this stage, LptE, which is targeted to the OM via a different pathway, i.e. the Lol pathway for lipoproteins (for a review, see Okuda and Tokuda 2011), is required for proper folding of the β-barrel (Ruiz *et al.* 2010; Chimalakonda *et al.* 2011) and the subsequent reshuffling of the disulfide bonds in LptD, generating non-consecutive bonds, first between C2 and C4 and then between C1 and C3 (Chng *et al.* 2012). Normally, the disulfide isomerase DsbC would be expected to be required for reshuffling of the disulfide bonds (Berkmen, Boyd and Beckwith 2005). However, its involvement in LptD biogenesis is unclear as opposing results in this respect have been reported (Denoncin *et al.* 2010; Ruiz *et al.* 2010). The detection of a trapped mixed disulfide intermediate that covalently linked DsbA to C1 of LptD already containing the non-consecutive disulfide bond between C2 and C4 (Chng *et al.* 2012) suggests a role for DsbA in the reshuffling of the disulfide bonds. Besides LptE, and DsbA and/or DsbC, also a protease, dubbed BepA, was reported to stimulate disulfide bond reshuffling in LptD (Narita *et al.* 2013). How this activity is achieved is unclear, but the proteolytic activity of the enzyme was dispensable for this function. Since overexpression of LptE compensated for a *bepA* deletion and BepA was reported to interact with the Bam machinery, BepA may somehow facilitate the assembly of the LptDE complex. Only upon formation of the non-consecutive disulfide bonds, LptD is able to bind LptA and a complete and functional LPS transport complex is formed (Freinkman *et al.* 2012). Thus, reshuffling of initially incorrect disulfide bonds forms a quality-control mechanism that guarantees that the periplasmic Lpt bridge is only formed when the LptDE complex is completely matured and prevents possible mistargeting of LPS to an incomplete translocon in the OM. Accordingly, in the absence of LptD or LptE, i.e. if a complete Lpt machinery cannot be formed, LptA was found to be degraded (Sperandeo *et al.* 2011) and LPS was found to accumulate in the IM (Sperandeo *et al.* 2008). Actually, either one of the two non-consecutive disulfide bonds is sufficient for proper functioning of LptD in *E. coli* (Ruiz *et al.* 2010). A comparison of >1000 LptD sequences showed that C2 and C4 are conserved in 95% of the cases whilst C1 and C3 were much less conserved (Chng *et al.* 2012), suggesting that the C2–C4 bond is critical for the proper structure and function of the Lpt machines. It should be noted, however, that some LptD proteins, such as that of *Anabaena* sp. PCC 7120 (locus tag: alr1278) (Haarmann *et al.* 2010), do not contain any cysteine residues. Hence, the assembly of the Lpt machinery must proceed differently in these organisms.

REGULATION OF LPS SYNTHESIS

It has been reported that *E. coli* responds to defects in LPS transport by increasing the production of LPS (Sperandeo *et al.* 2007) apparently in an attempt to compensate for decreased amounts of LPS in the OM. In contrast, LPS production is strongly reduced under such conditions in *N. meningitidis* (Bos *et al.* 2004; Tefsen *et al.* 2005a; Bos and Tommassen 2011) possibly to prevent toxic accumulation of LPS in the IM. In both cases, an IM-anchored cytoplasmic protein, called YciM in *E. coli* or Ght in *N. meningitidis*, appears to be involved in this response. Whereas disruption of the *yciM* gene in *E. coli* led to toxic overproduction of LPS (Mahalakshmi *et al.* 2014), the deletion of its homolog *ght* diminished LPS production in *N. meningitidis* (Putker *et al.* 2014). The YciM/Ght protein consists of an N-terminal membrane-spanning fragment, six TPR motifs, which are usually involved in protein–protein interactions, and a C-terminal C4-type zinc finger, which was indeed demonstrated to bind zinc, but also iron (Nicolaes *et al.* 2014). The latter was considered more important and this domain was classified as a rubredoxin-type iron center. Both in *E. coli* and in *N. meningitidis*, the protein appears to exert its control on LPS production via the LPS biosynthetic enzyme LpxC. The deacetylase LpxC catalyzes the first committed step in lipid A synthesis (Fig. 2). This is also the rate-limiting step and the target for regulation of lipid A synthesis. In *E. coli*, the amount of LpxC is tightly controlled by the essential IM protease FtsH (Ogura *et al.* 1999), an enzyme that also controls the levels of another LPS biosynthetic enzyme, i.e. KdtA (WaaA), the enzyme that attaches Kdo residues to the lipid IV_A precursor (Fig. 2) (Katz and Ron 2008). Like inactivation of *yciM* (Mahalakshmi *et al.* 2014), inactivation of *ftsH* leads to increased levels of LpxC and, consequently, of LPS and is lethal (Ogura *et al.* 1999). Lethality appears to be caused by reduced synthesis of glycerophospholipids as a consequence of the depletion of the pool of *R*-3-hydroxymyristoyl-ACP, a common precursor of both LPS and glycerophospholipids, as overproduction of FabZ, the enzyme that shuttles this precursor into the glycerophospholipid-synthesis pathway, rescued both mutants (Ogura *et al.* 1999; Mahalakshmi *et al.* 2014). Thus, all evidence suggests that YciM works in concert with FtsH in controlling LpxC levels (Mahalakshmi *et al.* 2014). In agreement with this hypothesis, YciM, also designated LapB, and FtsH were found to copurify (Klein *et al.* 2014). In the same study, it was reported that also LPS and Lpt components copurified with YciM, suggesting that YciM might signal LPS accumulating at the cytoplasmic side of the IM and/or the activity of the Lpt system. The reported accumulation of LPS precursors in a *yciM* mutant (Klein *et al.* 2014) seems a logical consequence of the upregulation of *lpxC* expression without concomitant upregulation of genes encoding enzymes for downstream steps in the LPS biosynthesis pathway.

It is not known whether LPS levels in *N. meningitidis* are controlled by FtsH-mediated degradation of LpxC. Also other regulatory mechanisms have been described. In *E. coli* and other *Enterobacteriaceae*, FtsH-mediated proteolysis of LpxC requires a C-terminal targeting signal (Führer *et al.* 2007), which is lacking in LpxC of *N. meningitidis*. The LpxC proteins from *Agrobacterium tumefaciens* and *Rhodobacter capsulatus* are targets for the cytoplasmic protease Lon, whilst LpxC levels in *Pseudomonas aeruginosa* are not controlled by proteolytic degradation (Langklotz, Schäkermann and Narberhaus 2011) but at the transcriptional level with the involvement of a small non-coding RNA (Tomaras *et al.* 2014). Thus, also in *N. meningitidis*, the control of LpxC levels may be different from that in *E. coli*, although the YciM homolog Ght is involved. It was reported that LpxC is sequestered to the membrane when Ght is expressed, whilst it was found in the soluble fraction in a *ght* mutant (Putker *et al.* 2014). Possibly, LpxC is more active in the membrane-bound state or it is protected against FtsH or other proteases when it is bound to the IM via Ght. It should be noted that both deletion of *msbA*, which is expected to lead to accumulation of LPS at the cytoplasmic side of the IM (Tefsen *et al.* 2005a), and mutations in Lpt proteins, which should lead to accumulation of LPS at the periplasmic side of the IM (Bos *et al.* 2004; Bos and Tommassen 2011), result in the feedback inhibition of LPS synthesis. How both of these signals are picked up via Ght is unclear and requires further investigation.

Upstream of the *ght* and *yciM* genes, an open-reading frame encoding a small integral IM protein is found. Inactivation of this gene in *E. coli* (Klein *et al.* 2014; Mahalakshmi *et al.* 2014) or *N. meningitidis* (Rasmussen *et al.* 2005) had minor effects on cell viability and LPS production, which could be due to polar effects

on the downstream *ght/yciM* gene. Nevertheless, since this gene is always found upstream of *ght/yciM* in the same operon and is in some organisms, including *Nitrospina gracilis*, even fused with *ght/yciM* to form a single gene (Putker *et al.* 2014), it likely has a role in the same process.

Apart from the FtsH-mediated control of LPS levels via proteolysis of LpxC and KdtA described above, LPS synthesis in *E. coli* is also transcriptionally controlled as several genes encoding enzymes involved in lipid A synthesis (Dartigalongue, Missiakas and Raina 2001) or LPS transport (Dartigalongue, Missiakas and Raina 2001; Sperandeo *et al.* 2006, 2007) are part of the σ^E regulon. The σ^E regulon is activated as a stress response upon defects in OM biogenesis (for a review, see Ades 2008). Activation is dependent on an alternative σ factor, σ^E, which, under normal growth conditions, is kept inactive by the IM-spanning anti-σ factor RseA. The periplasmic part of RseA is a target for the protease DegS, which is normally inactive. DegS is activated when its PDZ domain binds the C-termini of unfolded OMPs accumulating in the periplasm (Walsh *et al.* 2003). However, RseA is protected from proteolysis by RseB. When LPS accumulates in the periplasm, it is bound by RseB, which is then released from RseA, thereby permitting cleavage of RseA by DegS (Lima *et al.* 2013). This is followed by a proteolytic cascade, eventually resulting in the release of σ^E in the cytoplasm and the activation of the transcription of target genes. Thus, a dual signal is needed for activation of this response, i.e. the accumulation of both unfolded OMPs and of LPS in the periplasm. This entire regulatory system is lacking in *N. meningitidis* (Bos, Robert and Tommassen 2007).

LPS TRANSPORT COMPONENTS IN OTHER GRAM-NEGATIVE BACTERIA

Lpt machinery in other LPS-producing bacteria

LPS transport has so far most extensively been studied in the β- and γ-proteobacteria *N. meningitidis* and *E. coli*, respectively. Is LPS transported by similar machinery to the cell surface in other bacteria? Homologs of LptA, B, D, F and G were readily identified in other proteobacteria (Haarmann *et al.* 2010; Sutcliffe 2010). LptC and LptE appear to be much less conserved, as their homologs are difficult to find in many proteobacteria (Silander and Ackermann 2009; Haarmann *et al.* 2010; Sutcliffe 2010). Silander and Ackermann (2009) determined changes in the levels of gene conservation in 448 bacterial genomes. The presence of orthologs for each protein-encoding gene of *E. coli* strain W3110 was determined and, together with information on phylogenetic relationships, used to calculate a rate of ortholog loss (ROL) value. Among the genes encoding proteins involved in LPS transport, the LptC and LptE proteins displayed much higher ROL values than any of the other Lpt proteins. However, taking into account other criteria, such as genomic context and secondary structure predictions, we were able to find likely LptE orthologs in the vast majority of proteobacteria; many of them remained undetected in BlastP searches or by searching for the conserved COG2980 domain of LptE in the CDD database (Bos and Tommassen 2011). Indeed, in α-proteobacteria, these putative LptE orthologs contained another conserved domain, i.e. COG5468, which is now recognized as a conserved domain belonging to the LptE superfamily (Fig. 6). Expanding on this approach, Malojčić *et al.* (2014) identified LptE homologs also in several other bacterial phyla.

Also similarity searches using LptC of *E. coli* or *N. meningitidis* often fail to yield homologs in the proteobacteria (Tran,

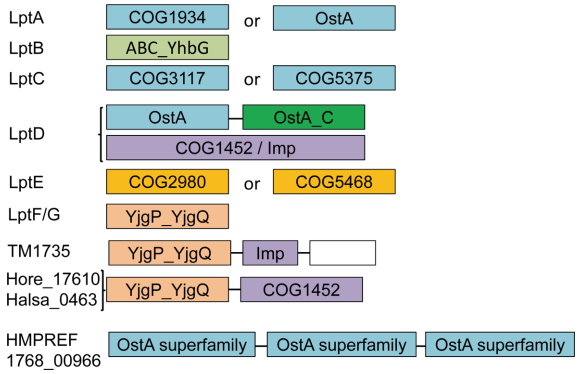

Figure 6. Conserved domain designations as indicated in the CDD present in selected Lpt proteins. COG1934, COG3117, COG5375 and OstA all belong to the OstA superfamily and are indicated in blue. COG2980 and COG5468 belong to the LptE superfamily and are indicated in orange. White square in TM1735: no domain recognized.

Dong and Whitfield 2010). Thus, we evaluated whether synteny and secondary structure prediction could help also to identify additional LptC homologs among proteobacteria. In representative genomes of β-, γ- and δ-proteobacteria, we found LptC homologs, classified in the COG3117 family (Fig. 6), in an identical genetic organization as in *E. coli*, i.e. *kdsC-lptC-lptA-lptB* (Fig. 7). In contrast, we found no such LptC homologs in the α-proteobacteria, but we did find hypothetical proteins encoded by genes positioned upstream of *lptA-lptB* (Fig. 7) with similar secondary structures predicted via the PsiPred server (Buchan *et al.* 2013) as LptC (see Fig. S1, Supporting Information). These proteins, for example CC_3602 from *Caulobacter crescentus* CB15 or RP506 from *Rickettsia prowazekii*, might represent functional LptC homologs, evolved to such an extent that they now are classified in a different COG family, i.e. COG5375, which, like COG3117, belongs to the OstA superfamily (Fig. 6). In the ϵ-proteobacteria, comprising amongst others *Campylobacter* and *Helicobacter* spp., we did not find LptC homologs or any proteins belonging to the COG3117 or COG5375 families in initial searches. However, in these genomes we found the genetic organization *kdsC-orfX-lptA* (Fig. 7). OrfX, e.g. HP1569 from *H. pylori*, is of similar size as LptC from *E. coli*, and secondary structure prediction suggests a

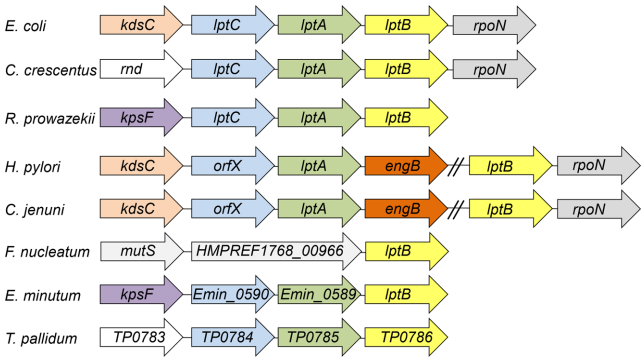

Figure 7. Organization of the (putative) *lptC-lptA-lptB* locus in different organisms. The *lptC*, *lptA*, and *lptB* genes and their proposed homologs in different bacteria are indicated in blue, green and yellow, respectively. HMPREF1768_00966 of *F. nucleatum* contains three OstA-superfamily domains; the gene may represent a chimera of one *lptC*- and two *lptA*-related domains. The *lptB* genes of *Cam. jejuni* (N196_08535) and *H. pylori* (HP0715) are present in a locus on another genomic location that, like in *E. coli* and *Ca. crescentus*, also contains the *rpoN* gene, encoding the alternative σ factor σ^{54}, immediately downstream of *lptB*.

similar structure as LptC consisting of an N-terminal α-helix with the rest of the protein consisting of β-sheet; only, a small α-helix was additionally predicted with low confidence level at the C-terminus of HP1569 (see Fig. S1, Supporting Information). Indeed, the structure of an N-terminal portion of HP1569 could be modeled using *E. coli* LptC as a template (Liechti and Goldberg 2012). Thus, HP1569 could be the functional homolog of LptC in *H. pylori*. None of the HP1569 homologs present in *Helicobacter* spp. was recognized in the CDD database as having any conserved domain. In contrast, many of the proteins encoded by the *Campylobacter* spp. *orfX* genes, e.g. N196_08635 of *Campylobacter jejuni* strain 30286, were, like COG5375 and COG3117, recognized as members of the OstA superfamily.

In many non-proteobacterial phyla of LPS-producing bacteria, Lpt components were not found even if BlastP searches were performed at low stringency (*E*-value 0.01) (Sutcliffe 2010) suggesting that alternative transport machinery may exist in these organisms. Alternatively, these Lpt proteins may not be highly conserved at the sequence level and, therefore, go unrecognized using limited searches with for example only the *E. coli* Lpt sequences as queries (Sutcliffe 2010). Indeed, homologs of these proteins were identified in representatives of a variety of bacterial phyla using alternative strategies, e.g. by using weak hits in a first BlastP search as baits for new searches (Hu and Saier 2006; Haarmann *et al.* 2010; Chng *et al.* 2012; Campbell, Sutcliffe and Gupta 2014). We further analyzed a number of species previously reported to lack Lpt proteins by searching for conserved domains defining the Lpt proteins (Fig. 6) at the NCBI protein and CDD databases or by using BlastP searches with several different baits and by taking *lptC-lptA-lptB* linkage into account. This exercise yielded at least four different Lpt components in all classes of LPS-producing bacteria examined (Table 1). In some instances, we could not clearly assign a protein containing an OstA superfamily domain to either LptA or LptC, as LptA- or LptC-specific conserved domains were not recognized. An example is the 931 amino-acid residues large protein HMPREF1768_00966 of *Fusobacterium nucleatum*, which contains three OstA superfamily domains (Fig. 6); its encoding gene is located upstream of LptB (Fig. 7). Possibly, this protein represents a fusion of LptC at the N-terminus followed by two copies of LptA, a hypothesis that is corroborated by the presence in the protein of an N-terminal hydrophobic α-helix which is not followed by a signal-peptide cleavage site according to SignalP 4.1 prediction (Petersen *et al.* 2011); hence, this predicted protein is likely anchored to the IM like LptC. *Elusimicrobium minutum* Pei192 contains two genes each with a single OstA superfamily domain, emin_0589 and emin_0590 (Table 1). In this case, the gene order *kpsF*-emin_0590-emin_0589-*lptB* (Fig. 7) clearly suggests that the proteins encoded by emin_0590 and emin_0589 represent the LptC and LptA homologs, respectively. Also DICTH_1877 from *Dictyoglomus thermophilum* H-6–12 contains a single OstA superfamily domain but no LptA- or LptC-specific conserved domain according to the CDD (Table 1). SignalP prediction provides weak evidence below the cutoff value for the presence of a signal-peptide cleavage site, making it hard to decide whether this protein represents an LptA or an LptC protein. However, in a close homolog of DICTH_1877, i.e. Dtur_0149 of *D. turgidum*, which shows 90% sequence identity, a cleavable signal sequence was clearly predicted, suggesting that both Dtur_0149 and DICTH_1877 represent LptA proteins. Alternative genomic organizations were found, such as in *D. thermophilum* H-6–12, where the gene for this putative LptA protein is clustered in a locus also containing an *lptD* and an *lptF* or *G* homolog (DICTH_1875–1877) (Table 1). The genes flanking the *lptB* gene, DICTH_0670, did not contain any Lpt-related domain. For the representative strain of the Chlamydiae, *Waddlia chondrophila* WSU86–1044, no other OstA domain-containing proteins were found besides LptD, and also inspection of the genomic environment of the *Waddlia* LptB homolog, Wcw_1548, yielded no putative LptA or LptC homologs.

Thus, it appears that all LPS-producing bacteria transport LPS to the OM via similar Lpt machinery, although variations on the *E. coli* theme may exist.

Lpt proteins in bacteria that don't produce LPS

Previously, the presence of a gene encoding the hypothetical protein TM1735 with a conserved YjgP_YjgQ domain, which signifies LptF and LptG, was noted in *Thermotoga maritima* (Ruiz *et al.* 2008). Curiously, this hyperthermophilic bacterium, although possessing a double membrane, does not produce LPS as it doesn't possess *lpxA* or *lpxC* homologs (Opiyo *et al.* 2010; Sutcliffe 2010). Thus, the TM1735 protein does not function in LPS transport in its host organism. To further investigate whether LPS transport-related proteins are restricted to LPS-producing bacteria or not, we searched for the presence of these proteins in representative strains of diderm bacteria that do not produce LPS, such as the Spirochetes *Bo. burgdorferi* and *T. pallidum*, members of the Sphingomonadales, such as *Novosphingobium aromaticivorans*, *Deinococcus radiodurans*, *Thermus thermophilus* and *Th. maritima* (Raetz and Whitfield 2002; Silipo *et al.* 2004; Opiyo *et al.* 2010). As controls, we included searches for orthologs of LpxA and of the central component of the OMP assembly machinery BamA (Voulhoux *et al.* 2003) to demonstrate the absence of LPS synthesis and the presence of an OM, respectively. We performed similar searches as explained above to find Lpt members (Table 2). Each identified protein was submitted to the CDD to inspect the presence and organization of Lpt-related domains (Fig. 6). Also their genomic context was inspected. All bacteria shown in Table 2 indeed lack *lpxA* but contain *bamA*, confirming them as diderm bacteria lacking LPS. They all encode LptA and LptB homologs of similar small sizes as in *N. meningitidis* and *E. coli*, i.e. $<$300 amino-acid residues, in the case of the *T. pallidum* even in a similar genetic organization as in *E. coli* (Fig. 7). *Deinococcus radiodurans* contains a second LptA homolog (DR_2320, which comprises 366 residues) just as *The. thermophilus* (TTHA1100, comprising 309 residues). Also, all inspected strains contained LptF and LptG homologs of similar length (350–400 amino acids) and in a similar tandem genetic organization as in *E. coli* and each carrying a characteristic YjgP_YjgQ domain. An exception was *Th. maritima* where the 1074 amino-acid residues large protein TM1735 is the only protein with a YjgP_YjgQ domain, present in its N-terminus. In *De. radiodurans* and *The. thermophilus*, we found a third LptF/G homolog, i.e. DR_1075 and TTHA1108, respectively (Table 2). DR_1075 is located in between the *fabZ* and *murG* genes, indicative for a role in cell envelope biogenesis. No LptE homologs were found except for the typical putative α-proteobacterial LptE (COG5468) in *No. aromaticivorans*. LptC homologs were found in *T. pallidum* and in *No. aromaticivorans*. Possibly, one of the two putative LptA homologs found in *De. radiodurans* and *The. thermophilus* (Table 2) may also be regarded as LptC variants.

Proteobacterial LptD proteins generally have two domains: an OstA domain near the N-terminus, which corresponds to the β-jellyroll and is also found in LptA proteins, and an OstA_C domain at the C-terminus, which corresponds to the β-barrel (Bos, Robert and Tommassen 2007). Together they constitute an Imp domain (COG1452). Saro_0889 from *No. aromaticivorans* and TP0515 from *T. pallidum* belong to the COG1452 family; they

Table 1. Presence of orthologs of Lpt-related proteins in non-proteobacterial Gram-negative bacteria.

Class Species and strain	LptA COG1934 OstA	LptB YhbG	LptC COG3117 COG5375	LptD COG1452 OstA_C	LptE COG2980 COG5468	LptF/LptG YjgP/YjgQ
Bacteroidetes						
Porphyromonas gingivalis ATCC33277	PGN_1553	PGN_0669	PGN_1512	PGN_0884	n.d.	PGN_1471 PGN_0642
Chlamydiae						
Waddlia chondrophila WSU86–1044	n.d.	Wcw_1548	n.d.	Wcw_1167	Wcw_1521	Wcw_0681 Wcw_0682
Fusobacteria						
Fusobacterium nucleatum CTI-7	HMPREF1768_00966	HMPREF1768_00967	HMPREF1768_00966	n.d.	n.d.	HMPREF1768_01286 HMPREF1768_01287
Verrucomicrobiae						
Akkermansia muciniphila ATCC BAA-835	Amuc_0678	Amuc_0679	Amuc_0677	Amuc_1439	Amuc_0811	Amuc_0318 Amuc_1510
Dictyoglomi						
Dictyoglomus thermophilum H-6–12	DICTH_1877	DICTH_0670	n.d.	DICTH_1875	n.d.	DICTH_1191 DICTH_1876
Elusimicrobia						
Elusimicrobium minutum Pei191	Emin_0589	Emin_0588	Emin_0590	n.d.	Emin_0202	Emin_0111 Emin_0112
Fibrobacteria						
Fibrobacter succinogenes subsp. Succinogenes S85	n.d.	FSU_1701	FSU_1703	FSU_3094	FSU_0791	FSU_2690 FSU_2691
Gemmatimonadetes						
Gemmatimonas aurantiaca T-27	GAU_1619	GAU_1618	GAU_1620	GAU_1938	n.d.	GAU_2572 GAU_2573
Synergistetes						
Thermanaerovibrio acidaminovorans DSM6589	Taci_1237	Taci_1236	n.d.	Taci_1245	n.d.	Taci_1288

The Protein and CDD databases at NCBI were searched with the queries shown in the top row. Hits in the analyzed strains are indicated by locus tags. n.d., not detected.

Table 2. Presence of orthologs of Lpt-related proteins in Gram-negative bacteria that don't produce LPS.

Species and strain	LptF/LptG COG0795 YjgP/YjgQ	LptA COG1934 OstA	LptB COG1137 YhbG	LptC COG3117	LptD COG1452 Imp OstA_C	LptE COG2980 COG5468	BamA YaeT D15
Borrelia burgdorferi B31	BB_0807 BB_0808	BB_0465	BB_0466	n.d.	BB_0838	n.d.	BB_0795
Treponema pallidum subsp. Pallidum str. Nichols	TP0883 TP0884	TP0785	TP0786	TP0784	TP0515	n.d.	TP0326
Thermotoga maritima MSB8	TM1735	TM0714	TM1054	n.d.	n.d.	n.d.	TM0448
Novosphingobium aromaticivorans DSM 12444	Saro_0495 Saro_0496	Saro_3249	Saro_0296	Saro_3250	Saro_0889	Saro_3216	Saro_1377
Deinococcus radiodurans R1 chromosome I	DR_2430 DR_2431 DR_1075	DR_2319 DR_2320	DR_2134	n.d.	DR_0573	n.d.	DR_0379
Thermus thermophilus HB8	TTHA0397 TTHA0398 TTHA1108	TTHA1100 TTHA1101	TTHA0439	n.d.	TTHA0396	n.d.	TTHA0561

The Protein and CDD databases at NCBI were searched with the queries shown in the top row. Hits in the analyzed strains are indicated by locus tags. n.d., not detected. None of the strains showed hits for LpxA or its signature family COG1043.

comprise 756 and 991 amino-acid residues, sizes comparable to *E. coli* and *N. meningitidis* LptD, and both contain an OstA and an OstA_C domain; therefore, we classified them as LptD homologs (Table 2). Moreover, located immediately downstream of Saro_0889 is a homolog of *surA*, similar to the *lptD-surA* genetic organization in *E. coli*. Because of its size (1146 amino acids), its inclusion in the COG1452 family, and its similar genomic context as TP0515, we also classified BB_0838 from *Bo. burgdorferi* as an LptD homolog, although no significant OstA and OstA_C domains are recognized in the CDD. In *The. thermophilus*, TTHA0396 contains a C-terminally truncated COG1452 domain, but its size of 824 amino acids makes it a probable LptD homolog. This possibility is underscored by psipred secondary structure prediction, which showed β-sheets over the entire protein (see Fig. S2A, Supporting Information). Also, its location next to the LptF and LptG homologs (TTHA0397 and TTHA0398, Table 2) suggests its involvement in the same pathway. Using TTHA0396 as bait, we also identified a homolog in *De. radiodurans*, i.e. DR_0573, in a BlastP search. It should be noted that LptD proteins may contain very divergent C-terminal domains, such as the characterized LptD of the cyanobacterium *Anabaena* sp. PCC 7120, which contains a DUF3769 domain instead of the OstA_C domain (Haarmann *et al.* 2010).

The TM1735 protein of *Th. maritima* contains a YjgP_YjgQ domain of 364 amino-acid residues, characteristic for LptF and LptG proteins, at the N-terminus but consists in total 1076 residues. Interestingly, when searching for LptD homologs in this organism, we noticed that residues 398–567 of this protein show homology (E-value 4×10^{-4}) with residues 52–223 of *E. coli* LptD and are recognized in the CDD as a partial Imp domain (Fig. 6). Furthermore, secondary structure predictions showed that its C-terminal part consists entirely of β-strands, potentially indicating an OM location (see Fig. S2B, Supporting Information). Hence, TM1735 may represent a chimera containing both IM- and OM-located parts of the Lpt machinery in a single protein. Clear homologs of similar size and domain organization were found in all seven members of the *Thermotogaceae* family for which sufficient sequence data were available, i.e. *Thermotoga*, *Petrotoga*, *Thermosipho*, *Fervidobacterium*, *Marinitoga* and *Kosmotoga* spp. Further BlastP searches with this protein revealed the presence of homologs of similar size in *Halothermothrix orenii* (Hore_17610) and in *Halanaerobium hydrogeniformans* (Halsa_0463). Both of these proteins have, besides the YjgP_YjgQ domain, a complete COG1452 domain, signifying LptD (Fig. 6). Although belonging to the Firmicutes, *Ha. orenii* possesses a complete lipid A biosynthetic pathway (Mavromatis *et al.* 2009), and we confirmed the presence of *lpxA* and *lpxC* genes in *Hy. hydrogeniformans* by BlastP searches. Both belong to the family *Halanaerobiaceae*. Other members of this family do not possess this TM1735-like fusion protein. The existence of such chimeric genes encoding large proteins with both IM and OM domains substantiates the notion that the Lpt system functions as a transenvelope machine.

Substrate specificity of the Lpt machinery

Clearly from the analysis above, the presence of Lpt-related proteins is not restricted to LPS-producing bacteria, suggesting that the Lpt system may not be exclusively devoted to LPS transport. What could be the function of the system in these unusual Gram-negatives? Several of these organisms have been shown to produce other types of glycolipids, such as glycosphingolipids in the case of *Sphingomonadales* spp. The Spirochetes *Treponema* spp. and *Borrelia* spp. produce glycolipids with diacylglycerol as

the hydrophobic backbone with often a simple head group in the form of just one glycosyl residue (Schröder et al. 2008). Also *The. thermophilus*, *De. radiodurans* and *Th. maritima* produce different types of diacylglycerol-based glycolipids (Leone et al. 2006; Hölzl and Dörmann 2007). So, possibly, the Lpt system in these bacteria functions in the transport of alternative glycolipids. In this respect, it is perhaps relevant that, for example, the gene for the LptF/G homolog TTHA1108 of *The. thermophilus* (Table 2) is located in a cluster including genes putatively encoding a glycosyltransferase (TTHA1109) and a homolog of O-antigen ligases (TTHA1106), which are characterized by a conserved Wzy_C domain; note, however, that such a Wzy_C domain can also be found in proteins involved in the *O*-glycosylation of proteins (Schulz et al. 2013). Although the commonalities between lipid A and other glycolipids that might be the basis of their recognition by a similar transport system are not immediately apparent, the Lpt system may be adjusted to the type of glycolipid produced. In this respect, it would be very interesting to test whether the Lpt proteins of LPS producers and non-producers could substitute each other. It is noteworthy that MsbA homologs are also widely distributed among bacteria and are even found in Gram-positive bacteria where they obviously have other functions. Intriguingly, one of the Gram-positive MsbA homologs, the multidrug transporter LmrA from *Lactococcus lactis*, has been reported to be able to complement an *msbA* mutant of *E. coli* (Reuter et al. 2003).

At least in *E. coli*, the Lpt machinery appears to be rather promiscuous with respect to substrate recognition, as penta- and hexa-acylated lipid A and even lipid IV_A reach the OM in strains that contain appropriate adaptations to facilitate transport of these substrates across the IM (see above), although it has not been directly proven that these substrates indeed utilize the Lpt machinery. However, in several other bacteria, the machinery appears to be more selective. In *P. aeruginosa*, the *waaP* gene, encoding an inner-core kinase, is essential for viability. In a strain depleted for WaaP, truncated LPS forms were found to accumulate in the IM, demonstrating that core phosphorylation is essential for LPS transport (Delucia et al. 2011). Since the lipid A moiety of these halted LPS species was substituted with L-Ara4N, a modification that occurs at the periplasmic side of the IM, the transport defect was apparently not in the MsbA-mediated flipping across the IM but in the subsequent Lpt-mediated transport to the OM. In *Burkholderia cenocepacia*, mutations that prevent substitution of the lipid A and core moieties of LPS with L-Ara4N were shown to be lethal, and strains depleted for L-Ara4N synthesis showed membrane invaginations and other morphological deviations indicative of defects in OM assembly. Viability was restored by extragenic suppressor mutations, which were located in the *lptG* gene (Hamad et al. 2012). Thus, LPS molecules that lack the L-Ara4N substitutions appear to be poor Lpt substrates in this organism. These examples demonstrate that the Lpt machinery in different organisms varies with respect to the specificities in substrate recognition.

Lpt proteins in plants

Genes encoding the enzymes required for the lipid A biosynthesis pathway are not only found in Gram-negative bacteria, but also in plants (Raetz and Whitfield 2002). Although lipid A has never been detected in plants, these enzymes were found to be active in *Arabidopsis thaliana* as the expected precursors were found to accumulate when the individual genes were knocked out (Li et al. 2011). The mutants had no apparent phenotype, thus the function of lipid A synthesis in *A. thaliana* remains obscure. The enzymes are targeted to the mitochondria, but the precursor that accumulated in one of the mutants was found in chloroplast (Li et al. 2011), suggesting that it is transported from the mitochondria to chloroplasts. Intriguingly, Haarmann et al. (2010) identified two homologs of cyanobacterial LptD in *A. thaliana* (genes at2g44640 and at3g06960). When we performed BlastP searches with LptD of *Anabaena* sp. PCC 7120 as bait, we found no hit with at2g44640, but we picked up at3g06960 with very low sequence similarity (*E*-values 1.5 in a forward BLAST and 0.081 in a reverse BLAST, with 26% identity and 42% similarity over 109 amino-acid residues), although both plant proteins are mutually clearly homologous (*E*-value 2×10^{-80}). Neither of them was recognized in the CDD database as having a DUF3769 domain, which signifies cyanobacterial LptDs, but several homologs in other plants did contain this domain, possibly reflecting the endosymbiont origin of the plant protein family. These proteins are located in the chloroplasts and, hence, might be involved in the uptake of lipid A derivatives into the chloroplasts. Alternatively, since Lpt systems may not be exclusively dedicated to the transport of LPS as described above, these LptD homologs in chloroplasts might be involved in the transport of other lipids. Indeed, at3g06960 (a.k.a. TGD4) was recently identified as a phosphatidic acid-binding protein in the OM of chloroplasts involved in lipid transport from endoplasmic reticulum to chloroplast (Wang, Xu and Benning 2012; Wang, Anderson and Benning 2013). Thus, although perhaps evolutionary related to cyanobacterial LptD, the function and substrate specificity of this protein have obviously changed.

As already noted above, the C-terminal domain of cyanobacterial LptD is different from proteobacterial LptD, since it contains a DUF3769 domain instead of an OstA_C domain. Remarkably, using *E. coli* LptD as a query in BlastP searches, we found two proteins in the oilseed *Ricinus communis* (RCOM_1843130 and RCOM_1796170) that comprise an OstA_C domain, as recognized in the CDD database, and one protein containing both the OstA and the OstA_C domain in the cucumber family member *Cucumis sativis* (LOC101208768). Considering the phylogenetic relationship between proteobacteria and mitochondria, these LptD homologs might be located in the mitochondria and involved in the export of lipid A derivatives. However, since further BlastP searches using these plant proteins as baits only yielded significant sequence homologies with bacterial proteins and not with any other plant proteins, it is questionable whether these genes are really present in *R. communis* and *C. sativis* or whether they represent unfortunate incidents of sequence contamination (Merchant, Wood and Salzberg 2014).

CONCLUSIONS AND PERSPECTIVES

The pathway for transport of LPS to the OM has long remained enigmatic. Based on electron microscopic studies, it was suggested already in 1973 that newly synthesized LPS molecules might reach the OM via contact sites between IM and OM (Mühlradt et al. 1973), but the nature of these contact sites was unknown. In the past decade, we have witnessed an enormous progress in the field by the discovery of the Lpt proteins, which indeed appear to form a machine that spans the entire cell envelope. The involvement of this transenvelope machinery solves the question which energy source is used for flipping the LPS molecules across the OM to the cell surface: the entire pathway can be powered by ATP hydrolysis in the cytoplasm. Also the structures of most Lpt components have been solved, but still, much remains to be learned. First of all, the stoichiometry of the entire complex is still unclear. As discussed above, one or two

copies of LptA would suffice to close the bridge between LptC and the periplasmic domain of LptD. Protein abundance estimates based on ribosomal profiling (Li *et al.* 2014) indicated LptA levels are three to five times (dependent on the medium conditions) as high as those of LptC, suggesting the presence of perhaps four copies of LptA in the machinery. However, the levels of LptD were even higher than those of LptA suggesting that LptD also functions as a homo-oligomer in the complex. There are some indications that, indeed, LptD forms oligomers. First, LptD of *N. meningitidis* was reported to form SDS-resistant oligomers that did not dissociate into monomers when protein samples were left unheated before SDS-polyacrylamide gel electrophoresis (Bos and Tommassen 2011). Second, in electrophysiological experiments, the channel recordings of LptD of *Anabaena* spp. PCC 7120 showed evidence for trimers (Haarmann *et al.* 2010), whereas its distant chloroplast homolog TGD4 was shown to form dimers *in vivo* and *in vitro* (Wang, Anderson and Benning 2013). Third, the integral IM proteins LptF and LptG, which show considerable mutual homology, supposedly function as a heterodimer in the $LptB_2FG$ complex. In several bacteria, including *Thermanaerovibrio acidaminovorans* (Table 1), only one of these proteins was detected, which then presumably functions as a homodimer within the complex. In all cases where we detected large genes encoding a protein with an LptF/G-like domain at the N-terminus and an LptD-like domain at the C-terminus, such as Hore_17610 from *Ha. orenii* and Halsa_0463 from *Hy. hydrogeniformans* (Fig. 6), we did not detect another gene encoding an LptF/G-like protein on the chromosome. Thus, if in these cases the LptF/G domain of the chimeric protein dimerizes, the LptD part will obviously also be a dimer. Clearly, the stoichiometry of the machinery requires further study, and it may be different between different bacterial species.

Particularly for the LptDE translocon many details remain obscure regarding, for example, its assembly at the Bam complex, the precise path of LPS in the translocon and the nature of its interaction with LptE, and the dynamics of the translocon when inserting the LPS into the OM. Since the Lpt machinery spans the entire cell envelope, it will be difficult to reconstitute the entire system from purified components *in vitro* in two membranes. Cellular solid-state nuclear magnetic resonance spectroscopy (Renault *et al.* 2012) could be a promising approach to study the dynamics of the translocon but will certainly first require further technological development.

Also with respect to the regulation of LPS synthesis, much remains to be learned. In all organisms studied, LpxC appears to be a key regulatory target to control LPS production, but its levels are controlled in many different ways. It is particularly intriguing that inactivation of the same gene, *yciM* in *E. coli* and *ght* in *N. meningitidis*, leads to opposing effects on LPS synthesis in these organisms. The protein encoded by this gene appears to be involved in a pathway that signals the proper functioning of the LPS transport machinery and controls LpxC levels, but the molecular mechanisms are not clear yet. Another intriguing unsolved question is why some bacteria, including *N. meningitidis*, survive without LPS, whereas others, such as *E. coli*, don't.

Although we seem to have a more or less complete picture of proteins involved in LPS biogenesis, several additional proteins have been suggested to play a role in this process in *E. coli*, including TolA (Gaspar *et al.* 2000), MacA (Lu and Zgurskaya 2013), AsmA (Deng and Misra 1996), YfgH, YceK and YtfN (Babu *et al.* 2011). It should be realized that the workhorse *E. coli* K-12 does not produce LPS with an O-antigen and, thus, like *N. meningitidis*, in fact produces LOS rather than LPS. The presence of O-antigen could possibly complicate transport. It has been reported that the IM-anchored periplasmic protein TolA is somehow involved in the surface expression of O-antigen-containing LPS in *E. coli* (Gaspar *et al.* 2000). Probably, the effect of a *tolA* mutation on the surface presentation of O-antigen is at the level of polymerization, as it was demonstrated that such a mutation induces the σ^E-dependent envelope stress response, which was shown to reduce the *wzy*-dependent O-antigen polymerization (Vinés *et al.* 2005). MacA is the periplasmic membrane-fusion protein of the MacAB-TolC transporter involved in the efflux of macrolide antibiotics. It was demonstrated that MacA binds LPS specifically and with high affinity, and it was suggested that the MacAB-TolC transporter may have some role in LPS transport, perhaps under specific conditions (Lu and Zgurskaya 2013). AsmA is an IM protein and its inactivation was reported to lead to decreased LPS synthesis (Deng and Misra 1996), suggesting the protein might have a role in the regulation of LPS synthesis. In a large screen for genetic interaction maps, strong aggravating interactions between *lptD* and *yfgH*, *yceK* and *ytfN* were reported, suggesting that YfgH, YceK and YtfN might be implicated in the transport of LPS to the cell surface (Babu *et al.* 2011). YfgH and YceK are uncharacterized lipoproteins, whilst YtfN was suggested to be an integral OMP (Babu *et al.* 2011) but was later shown to be an IM protein (TamB) involved in the secretion of some autotransporters (Selkrig *et al.* 2012). It is noteworthy that YtfN/TamB has an AsmA_2 superfamily domain at the C-terminus like AsmA, suggesting some functional relationship between these proteins. The possible role of all these additional factors in LPS transport requires further investigation.

To cope with the increasing problem of multidrug resistance, novel targets for antibiotics are urgently needed. Since LPS is an essential component of the OM for many Gram-negative bacteria, its biogenesis pathway is an interesting target for the development of novel antibiotics. It is worth noting that such antibiotics may even be useful in the combat against Gram-negative bacteria that do not depend on LPS for viability, such as *N. meningitidis*. By inhibiting LPS biogenesis in these bacteria, such drugs would breach the OM barrier function, thereby sensitizing the bacteria for other drugs (Bos and Tommassen 2011). Similarly, such antibiotics could already be useful as codrugs at sublethal concentrations against bacteria like *E. coli* that are dependent on LPS. The Raetz pathway for lipid A synthesis is highly conserved and its enzymes, particularly LpxC, which exerts the first committed step in lipid A synthesis (Fig. 2), are already extensively being investigated in this respect (for reviews, see Zhang *et al.* 2012; Lee *et al.* 2013). Also the LPS transport pathway may represent an attractive novel target. The Gram-negative bacterial OM represents an effective barrier that prevents access of many potential antimicrobial compounds to their targets. Since the LptDE complex is an essential component of the transport machinery and is exposed at the cell surface, it represents a particularly attractive target as potential inhibitors may not need to cross the OM to reach it. Indeed, novel antimicrobials that target LptD of *P. aeruginosa* have already been described (Srinivas *et al.* 2010). The β-hairpin structure of these peptidomimetic derivatives of protegrin I was found to be important for their activity, and it was suggested their β-strands might interact with the edges of antiparallel β-strands of the β-barrel of LptD (Schmidt *et al.* 2013). Perhaps, the putative lateral gate between β1 and β26 represents the binding side. These antimicrobial compounds were specific for *Pseudomonas* spp., and did not inhibit other Gram-negative or Gram-positive bacteria. Further research will be required to select related compounds with a broader spectrum and/or that target other Gram-negatives. Although the LptDE complex is a prime candidate because of its surface

accessibility, also other components of the LPS transport machinery remain potential targets. Lead compounds that inhibit the ATPase activity of LptB have been described. However, these compounds only inhibited the growth of an *E. coli* strain with a leaky OM and not a wild-type strain (Gronenberg and Kahne 2010; Sherman *et al.* 2013). Also lead compounds that target LptC have been described (Sestito *et al.* 2014). Obviously, further fundamental insight into the structure and the molecular mechanism of the LPS transport machinery will be very valuable in the development of inhibitors of this promising drug target.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSRE online.

Conflict of interest. None declared.

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