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Andreas Kuhn *Editor*

Bacterial Cell Walls and Membranes

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Chapter 1
The Bacterial Cell Wall and Membrane—A Treasure Chest for Antibiotic Targets

Andreas Kuhn

Abstract Astonishing progress has been made in recent years to understand the structural complexity and functions of the biosynthetic pathways of the bacterial and archaeal envelopes. This progress has prompted me to assemble the present book that provides a detailed overview and the state-of-art of the respective research field. Ideally, the book will provide students and advanced scientists an up to date picture of the different parts of the bacterial and archaeal cell envelope and enable them to understand their functional roles.

Keywords Antibiotic targets · Biosynthetic pathways · Conformational cycles · Multicomponent complex assembly · Regulatory interactions · Substrate movement in transporters

Outer Membrane

We will start off in Chap. 2 with the outer membrane and its outer exposed layer, the lipopolysaccharide (LPS). Here, tremendous progress has been made in unraveling the biosynthetic pathway. In this pathway, Lipid A with core sugars is flipped across the inner membrane catalyzed by MsbA. The flipped lipid A then binds to $LptB_2FG$ and is transferred to LptA and LptC that together forms a bridge across the periplasm. At the outer membrane, LptED receives the LPS precursor and can implant it most likely through a lateral gate allowing it to integrate into the outer leaflet of the outer membrane. An atomic structure of all the participating proteins is now available. This sets the basis for the understanding the molecular events that occur along the pathway and for further investigating the required conformational movements in these proteins which are driven by ATP hydrolysis within LptB.

The next Chap. 3 is focusing on lipoproteins which are found mostly in the inner leaflet of the outer membrane but also in the outer leaflets of both membranes to fulfill their many functional roles. Lipoproteins are synthesized in the cytoplasm with

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a signal peptide that is cleaved off by a unique signal peptidase (termed SPase II) after its transport across the inner membrane by the Sec translocase. The cysteine residue at the N-terminus of the mature protein is modified by three fatty acids that are recognized by LolCDE complex. Lipoproteins destined to the outer membrane are released from LolCDE and transferred to LolA, a periplasmic carrier protein. LolA then moves across the periplasm and delivers the lipoprotein to LolB at the outer membrane for its insertion into the inner leaflet. Although there is a clear distinction between the biosynthetic pathways of lipoproteins and lipopolysaccharides, common principles of their transport are evident. In both pathways, ABC transporter components are involved in releasing the substrate from the inner membrane and bind their substrates in the periplasm for transfer onto carrier components.

Porins, the main proteins of the outer membrane, are discussed in Chap. 4. A major focus is on the unique regulation of the expression of the porins by stress response circuits involving two component systems and anti-sense RNA molecules. The biosynthesis and folding of the β-barrel porins by the BAM complex is discussed that is currently a topic of ongoing research. A further, very important aspect of the porins is their involvement in multidrug resistance. Interestingly, distinct transcriptional regulation systems offer possibilities to manipulate the sensitivity to antibiotics that can also lead to resistance.

Periplasm

We then move to the periplasm (Chap. 5), which is filled with a sponge-like structure of the peptidoglycan. Although the chemical structure and the major enzymes involved in peptidoglycan synthesis has been known for a long time, a more detailed picture has emerged in analyzing the peptidoglycan from different species, including Gram-positive bacteria. There is a variety among the cross-linking peptides and the modifications that occurs within the glycan strands. In addition, the usual 4-3 cross-linking can be replaced by 3-3 crosslinks. This diversity has a big impact on whether antibiotics kill certain bacteria or develop resistance. Moreover, it has been noticed that pathogenic strains are enriched in such modifications, which may contribute to the survival of the bacteria in host organisms.

The biosynthetic machineries for peptidoglycan synthesis are the elongasome and divisome that coordinate transpeptidase and glycosyltransferase activities but consist of specific components. The details of how septation is regulated and how the two new daughter sacculi that are formed are controlled by the temporal and spatial amidase (Ami ABC) activities is currently investigated.

The chaperones Skp and SurA play an essential role for escorting the outer membrane proteins to the outer membrane after they leave the Sec translocon at the periplasmic side of the inner membrane. In Chap. 6 the mechanistic details of Skp and how it binds various client proteins are discussed including the important role of the flexible arms of the Skp trimer that holds onto the client protein. SurA has, as some other periplasmic proteins do, a prolyl-isomerase activity domain. However,

the functional relevance of this activity is still unknown. The SurA domain that is involved in binding to the client proteins does this in a clamp-like fashion.

Preproteins and prolipoproteins, which are processed by signal peptidases, are discussed in Chap. 7. SPase II removes the signal peptide of prolipoproteins and was detected after the discovery of globomycin from *Actinomyces* that inhibits the peptidase. In contrast, SPase I cleaves the signal peptides of periplasmic and outer membrane proteins and is important for the release from the inner membrane allowing them to reach their destination. Recent research has led to the finding that arylomycins are potent inhibitors of signal peptidases. These arylomycins are promising candidates for a new class of antibiotics.

Inner Membrane

The many different proteins of the inner membrane are represented here by transporters, proteins translocases and respiratory complexes. We start in Chap. 8 with the phosphotransferase PTS systems which are central for sugar uptake in bacteria. The predominant function the PTS systems have in bacterial metabolism is reflected by the many regulatory interactions of their components. The phosphorylation cascade and network is a beautiful example of a simple signal transduction system in cells. The coupling of the phosphorylation cascade to transport sugar across the membrane is a mechanistically challenging problem but the novel structural information of the maltose and the diacetylchitobiose transporters from *Bacillus cereus* has now shed light on this. Intriguingly, the substrates are moved through the membrane by an "elevator mechanism" involving topological rearrangements of the transmembrane helices of the transporter. This mechanism of substrate movement is distinct from the rocking switch mechanism found in LacY.

The secondary active transporters are found with 3 different protein folds and are discussed in Chap. 9. LacY and other members of the major facilitator superfamily (MFS) are organized as two symmetric 6-helical bundles that bind the substrates in a central cavity between the two bundles. The rocking movement of the bundles switches LacY from an outwards open to inwards open state, which is used to transport the lactose. Only the protonated LacY can bind the substrate sugar at the periplasmic face and is released after deprotonation at the cytoplasmic face of the membrane. In contrast to MFS, the LeuT and NhaA folds are organized pseudo-symmetrically with interwound 5-helical bundles. In addition, structural flexibility has been observed for NhaA after substrate binding, which lead the authors to propose that transport occurs also here by an elevator transport mechanism.

A wide variety of respiratory complexes present in different bacteria is covered in Chap. 10. Starting with aerobic systems in *E. coli* that operate to establish a proton gradient across the inner membrane and following with respiration of mainly marine bacteria, that generate a sodium ion gradient are discussed. The most fascinating feature of the respiratory complexes is the electron transfer among the individual modules each containing electron binding cofactors. The aerobic and anaerobic res-

piratory chains act as redox loops and are coupled to proton (or sodium) pumps. Bacteria show a wide variety of different respiration systems. Nevertheless, some of these systems could serve as antibiotic targets depending on their essential function in specific environments.

Newly synthesized membrane proteins are inserted by the multi-subunit Sec translocase which operates to translocate unfolded protein chains. In Chap. 11 the details of targeting the ribosome nascent chain or the preprotein chaperone complex to the Sec translocase are presented as well what is known about the movement of the protein chain through SecY and how the movement is powered by conformational cycles of the SecA ATPase. The roles of the YidC insertase alone, or of YidC together with Sec to insert proteins are discussed. Folded proteins, in particular metallo-proteins are translocated by the TAT system which might operate in a concerted action of TatA oligomers surrounding and translocating the client protein in a yet unknown mechanism.

Pili

Since pili are exposed on the bacterial cell wall they are primary antigenic targets and are also involved in biofilm formation. In addition, pili are important for the adherence of bacteria to eukaryotic cells and often involved in the invasion into these cells. Because of their importance in pathogenic infections there is a major medical interest to control the invasion process by compounds that interact with pili or even inhibit pilus formation. Chapter 12 gives an overview on the different pili systems known, starting with the chaperone-usher (CU) system of Gram-negative bacteria. Their assembly pathway at the periplasmic face of the outer membrane is an impressive example of protein folding events leading to consecutive subunit interactions. The folding mechanisms of donor-strand-exchange and the donor-strand-complementation between the subunits lead to subunit pairing and filament formation, respectively. Different from the pili that are assembled by the CU system are the type IV pili, which are involved in the twitching motility but also in cell adhesion, phage and DNA uptake. They are anchored to the inner membrane and to the outer membrane as multi-component complexes that also control filament extension and retraction reactions. New cryo-EM and crystal structures of several components of the type IV pili provide now the first structural details.

The amyloid fibrillar structure of Curli and Fab pili is another fascinating and ongoing area in protein folding research. The pili subunits are transported from the periplasm through a β barrel in the outer membrane and presumably assemble into an amyloid cross- β architecture by a mechanism supported by a chaperone on the extracellular surface.

Different from the pili involved in adhesion are the conjugative pili of the secretion system T4SS. These pili support DNA uptake and DNA delivery into recipient cells in natural competence and bacterial conjugation. The T4SS conjugative pili consist of a multi-component structure that is anchored in the outer and inner membrane

forming a continuous structure from the cytoplasm to the extracellular surface. The conjugative pili are retractile and lead to an intimate donor-recipient cell contact before the DNA is transported through the membranes of the mating cells.

Actinobacteria and Archaea

The cell walls of the Gram-positive Actinobacteria and of archaea are of great interest because of their differences to the well-studied Gram-negative cell wall. In addition, research on the pathogenic Mycobacteria and Corynebacteria has made substantial advances in our knowledge of the actinobacterial cell walls during recent years. Although most of the Actinobacteria have the monodermic Gram-positive cell wall architecture, Mycobacteria and Corynebacteria have evolved a diderm cell envelope. Interestingly, the outer membrane of Mycobacteria, the mycomembrane, is rich in mycolic acids especially in the inner leaflet of the outer membrane bilayer. Another component is the mycocerosyl lipid also present in the outer leaflet which is presumably transported by the ß-barrel protein LppX during its biosynthesis. These components lead to the dense and waxy character of the mycomembrane. Together with a complex capsule structure, which is composed of secreted polysaccharides, a thick electron transparent zone surrounds the mycobacterial cells. The unique properties of the actinobacterial cell wall pose many evolutionary questions such as how they have been developed but they also provide a multitude of promising new chemotherapeutic targets.

Archaea have the capacity to develop an astonishing variety of cellular envelopes. For example, monodermic and didermic cell walls are found in archaea. The didermic cell walls are similar to those of Gram-negative although there are differences in their biochemical composition. Most common among most archaea is the existence of a surface layer (S-layer) consisting of highly glycosylated proteins arranged in 2-dimensional crystal layers. The S-layers contribute to the extreme heat and osmotic stability of the archaeal cells.

Part I
Outer Membranes

Chapter 2
Lipopolysaccharide Biosynthesis and Transport to the Outer Membrane of Gram-Negative Bacteria

Paola Sperandeo, Alessandra M. Martorana and Alessandra Polissi

Abstract Gram-negative bacteria have an outer membrane that is positioned at the frontline of the cell's interaction with the environment and that serves as a barrier against noxious molecules including many antibiotics. This protective function mainly relies on lipopolysaccharide, a complex glycolipid located in the outer leaflet of the outer membrane. In this chapter we will first summarize lipopolysaccharide structure, functions and biosynthetic pathway and then we will discuss how it is transported and assembled to the cell surface. This is a remarkably complex process, as amphipathic lipopolysaccharide molecules must traverse three different cellular compartments to reach their final destination.

Keywords Lipopolysaccharide · Outer membrane biogenesis · Lpt machinery · ABC transporters · β-Jellyroll

Introduction

Gram-negative bacteria have a multi-layered envelope consisting of two distinct membranes that define an aqueous space, termed periplasm, containing a thin layer of peptidoglycan (Silhavy et al. 2010). The cytoplasmic or inner membrane (IM) is a classical phospholipid bilayer whereas the external, outer membrane (OM) is highly asymmetric and contains phospholipids in the inner leaflet and lipopolysaccharides (LPS), in the outer leaflet (Kamio and Nikaido 1976). Because of the presence of two membranes, Gram negatives are also defined as "diderm" bacteria (Sutcliffe 2010) (Fig. 2.1a). IM and OM also differ with respect to their integral membrane proteins. Integral IM proteins span the membrane as α-helices, almost entirely composed of hydrophobic residues, while most integral outer membrane proteins (OMPs) are composed of amphipathic β-strands which adopt a β-barrel structure (Fairman et al. 2011; Schulz 2002). An important class of OMPs is constituted by porins, which form spe-

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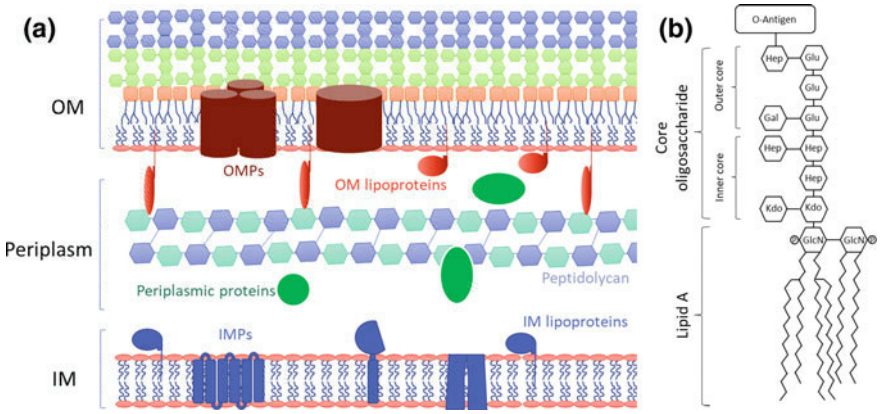

Fig. 2.1 The Gram-negative envelope and lipopolysaccharide structure. **a** The architecture of the Gram-negative envelope. The inner membrane (IM) and outer membrane (OM) are separated by an aqueous periplasm that contains the peptidoglycan cell wall. The IM is a symmetric phospholipid bilayer whereas the OM is asymmetric with LPS in the outer leaflet (for simplicity only inner and outer core are shown as colored light green and light blue hexagons, respectively). Major membrane proteins are shown and include: inner membrane proteins (IMP) and IM lipoproteins colored in blue; outer membrane proteins (OMP) colored in brown; OM lipoproteins colored in orange. Soluble proteins in the periplasm are colored in green. **b** The tripartite structure of LPS in Gram-negative bacteria. Lipid A, core oligosaccharide and O-antigen moieties are shown. Residues are indicated as hexagons: GlcN, glucosamine; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; Gal, Galactose; Glu, Glucose. Phosphate groups are indicated as circled P. See text for details

cific or non-specific channels that orchestrate the flux of hydrophilic molecules across the OM (Zeth and Thein 2010). IM and OM also contains lipoproteins anchored to the respective membranes via an N-terminal N-acyl-diacylglycerylcysteine residue (Sankaran and Wu 1994). Lipoproteins are located exclusively at the periplasmic side of the IM, whereas at the OM they can be anchored at the inner leaflet and thus extend into the periplasm (Okuda and Tokuda 2011) or be exposed at the cell surface (Konovalova and Silhavy 2015).

LPS, the major component of the OM outer leaflet, is a complex glycolipid that performs several functions. It is a fundamental structural component of the OM, essential in most Gram-negative bacteria (Moffatt et al. 2010; Peng et al. 2005; Steeghs et al. 1998). LPS is mainly responsible for the peculiar permeability barrier properties of the OM, which is effective against many toxic molecules and provides intrinsic resistance to a plethora of clinically useful antibiotics (Nikaido 2003). Finally, LPS is a major virulence factor and plays a crucial role in host-pathogen interaction by modulating the innate immune response (Kagan 2017).

In this chapter we will discuss several aspects of LPS biology. We will first review LPS structure, its barrier function and its ability to serve as signaling molecule for the immune system. Since most LPS functions stem from its localization at the OM outer leaflet, we will next revise the strategies bacteria have evolved to

maintain OM asymmetry. The LPS is a large glycolipid and its transport poses several challenges to the cell as this molecule needs to cross several compartments with different physicochemical characteristics. In the last part of this chapter we will describe how bacterial cells have solved the problem by using an intermembrane transport system.

LPS Structure and Function

LPS is a unique glycolipid whose chemical structure has been fully elucidated not only in *Enterobacteria* but also in an increasing number of *Proteobacteria*, the phylum containing the majority of known Gram-negative species. LPS is organized in three structural domains: lipid A, a core oligosaccharide and a highly variable O-antigen made of repeating oligosaccharide units (Raetz and Whitfield 2002) (Fig. 2.1b). Lipid A is the hydrophobic moiety that anchors LPS to the OM outer leaflet; in *Escherichia coli* and many Enterobacteriaceae it is a glucosamine disaccharide that is phosphorylated at the 1 and 4′ position and contains six acyl chains attached via an amide linkage (2 and 2′ positions) and via an ester bond (3 and 3′ positions) (Raetz and Whitfield 2002) (Fig. 2.1b). Lipid A is the most conserved portion of the LPS molecule, although variations exist across different species with respect to the length and number of acyl chains (Raetz et al. 2007). The core oligosaccharide is covalently linked to the lipid A and is often divided in an inner and outer core. The chemical structure of the outer core is variable whereas the inner core is more conserved between different isolates of the same species. The inner core contains at least one residue of 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), that links the inner core to lipid A, and usually also L-glycero-D-manno-heptose (heptose) (Holst 2007; Raetz and Whitfield 2002) (Fig. 2.1b). The O-antigen, composed of repeating units of one to six different residues, is the distal surface-exposed LPS moiety responsible for the immunogenic properties of this macromolecule; it is the most variable portion of LPS, a characteristic that has been used as tool for strains classification based on the different serological properties (Raetz and Whitfield 2002). Notably, the O-antigen is not synthesized in the *E. coli* K12 derivatives, which are mainly used as laboratory strains, due to a mutation in the *rfb* locus where the genes responsible for the O-antigen biosynthesis are clustered (Liu and Reeves 1994; Stevenson et al. 1994). *E. coli* strains unable to produce O-antigen are typically referred as "rough" strains, as opposed to the wild-type "smooth" strains. The O-antigen is also absent in several Gram-negative species including pathogens such as *Neisseria meningitidis* and *Bordetella pertussis* (Griffiss et al. 1987; Peppler and Schrumpf 1984), whose molecules composed of only lipid A and core oligosaccharide are typically referred as lipooligosaccharides (LOS).

The structural complexity of LPS reflects the many functions this molecule performs in the bacterial cell. LPS is the major component of the OM outer leaflet and its amphipathic nature contributes to a large extent to the permeability of the OM. The hydrophobic lipid A moiety provides a barrier to the flux of hydrophilic molecules

through the OM. The hydrophilic nature of the core oligosaccharide and O-antigen offers instead a barrier against the passage of hydrophobic compounds. Moreover, the chemical composition of the LPS aliphatic domain made of fully saturated fatty acyl chains is thought to create a gel-like lipid interior of very low fluidity further contributing to the low permeability of hydrophobic solutes across the OM (Carpenter et al. 2016; Nikaido 2003). The OM barrier properties are also the consequence of the very tightly packed layer of the LPS molecules bridged by the action of divalent Mg^{2+} and Ca^{2+} cations. The positive charges of Mg^{2+} and Ca^{2+} counteract repulsion between negatively charged phosphates in lipid A and in the core oligosaccharide (Nikaido 2003). Overall, the presence and the peculiar arrangement of LPS in the outer layer make the OM a formidable permeability barrier, a property that allows Gram-negative bacteria to colonize many different environments and survive in very harsh conditions.

LPS molecules cover the majority of the bacterial surface (Kamio and Nikaido 1976) and therefore play an important role in host-microbe interactions. The O-antigen, the outermost and variable portion of the molecule, protects the bacteria from phagocytosis and complement-mediated lysis (Lerouge and Vanderleyden 2002). Lipid A, the most conserved moiety of LPS and also known as endotoxin, is recognized by the innate immune system through the TLR4/MD2 (Toll-Like Receptor 4/Myeloid Differentiation factor 2) complex which initiates a robust signal cascade leading to cytokine production that is crucial for clearance of infection (Kagan 2017). Lipid A is therefore a pathogen associated molecular pattern (PAMP), namely a "pathogenic barcode", interpreted by the mammalian innate immune system as a sign of infection (Miyake 2004; Park and Lee 2013). The hexa-acylated lipid A from *E. coli* acts as a potent agonist of the TLR4/MD2 (Park et al. 2009) whereas variations in the lipid A acylation patterns alter the strength of TLR4/MD2 signaling (Needham et al. 2013). Many bacteria have evolved enzymes that alter their lipid A structure by modifying its number of acyl chains in response to environmental conditions or host interactions (Maldonado et al. 2016; Needham et al. 2013; Raetz et al. 2007). As an example, *Shigella flexneri* and *Pseudomonas aeruginosa* are able to modify the degree of acylation in their LPS during intracellular residence or in chronic lung infections, respectively (Ernst et al. 1999; Paciello et al. 2013). This is a powerful strategy to lower the sensing activity of the immune system and to escape from downstream effector mechanisms.

Maintaining the LPS Outer Layer

Maintenance of the LPS outer leaflet is critical to protect Gram-negative cells against bile salts, detergents, antimicrobial peptides and antibiotics, allowing bacterial cells to survive in harsh environments (Bishop 2008; Nikaido 2003). While OM asymmetry is primarily established by direct placement of LPS into the outer leaflet by the Lpt machinery (see section "Assembly of LPS at the OM Outer Leaflet"), several mechanisms are responsible for its disruption. Defects in LPS biosynthesis or transport,

exposure to chelating agents or antimicrobial peptides all lead to perturbation of the LPS layer and to a compensatory accumulation of phospholipids in the outer leaflet (Balibar and Grabowicz 2016; Bishop et al. 2000; Nikaido 2003). Phospholipids in the outer leaflet disrupt OM asymmetry and create patches in the membrane that are more susceptible to the influx of toxic molecules (Nikaido 2005).

In *E. coli* there are several systems that monitor the presence of phospholipids in the OM outer leaflet and act to restore OM asymmetry: the OM PagP and PldA enzymes act by degrading phospholipids in the outer leaflet of the OM, whereas the Maintenance of lipid asymmetry (Mla) system removes phospholipid from the OM via a retrograde transport system.

The OM palmitoyltransferase PagP catalyzes the transfer of a palmitate chain from the *sn*-1 position of a surface exposed phospholipid to the lipid A resulting in a *sn*-1-lyso phospholipid and a hepta-acylated lipid A (Bishop et al. 2000). It has been postulated that hepta-acylated LPS molecules reduce lipid fluidity and increase lateral interactions between LPS molecules thus stabilizing the OM (Bishop 2005). Notably, in *Salmonella*, *pagP* expression is induced via the PhoPQ two component system in response of low extracellular Mg^{2+}-ion concentration (Dalebroux et al. 2014), a condition known to induce migration of phospholipids in the OM outer leaflet.

The OM phospholipase PldA catalyzes the hydrolysis of acyl ester bonds in phospholipids. The PldA enzyme resides in the OM as an inactive monomer and the formation of a catalytically active PldA dimer occurs following phospholipids migration in the outer leaflet (Dekker 2000).

The MlaA-F system is a multiprotein complex whose components are located in every cellular compartment and functions as a retrograde phospholipid transport system. The MlaA-F proteins are not essential and deletion of any Mla component leads to phospholipids accumulations at the OM outer leaflet and, as a consequence, to OM permeability defects (Malinverni and Silhavy 2009). Retrograde transport is energized by the IM MlaBDEF ABC transporter in which the hexameric MlaD protein binds phospholipids, the proposed substrates of the Mla system, and modulate the ATPase activity of the complex (Thong et al. 2016). MlaC is the periplasmic component of the system which is thought to interact with MlaD. Direct transfer of phospholipids between these proteins has been recently shown (Ercan et al. 2018). At the OM the MlaA lipoprotein has been shown to interact with the OmpC porin (Chong et al. 2015) and the complex has been proposed to remove phospholipids directly from the outer leaflet to maintain OM asymmetry.

How the OmpC-Mla pathway mediates retrograde phospholipids transport from the OM back to the IM is not fully clear yet. The architecture of the OmpC-MlaA complex has been determined by X-ray crystallography and UV-photocrosslinking experiments (Abellon-Ruiz et al. 2017; Yeow et al. 2018). Structural data suggest that a donut-shaped MlaA is embedded in the inner leaflet of the OM, where it forms an amphipathic channel to deliver the removed phospholipids from the OM outer leaflet to the periplasmic MlaC component. Although stably associated with MlaA, the OmpC porin does not appear to play an active role in phospholipid transport and is thought to serve as a scaffold for correct positioning of MlaA in the lipid bilayer

(Chong et al. 2015; Abellon-Ruiz et al. 2017; Yeow et al. 2018). A recent mutation reported in MlaA (designated *mlaA**) provided a more robust understanding of the Mla pathway. In MlaA* mutants phospholipids accumulates in the OM outer leaflet suggesting that the MlaA* variant may have the reverse activity of the wild-type protein (Sutterlin et al. 2016). The MlaA structure explains why the MlaA* variant accumulates phospholipids at the OM outer leaflet. Indeed, the *mlaA** mutation disrupts the donut shape and allows phospholipids from the inner leaflet to enter the amphipathic channel and flow in the outer leaflet in a process likely driven by mass action (Abellon-Ruiz et al. 2017).

A functional connection between the Mla pathway and the PldA system was initially proposed since PldA overexpression suppresses the OM permeability defects of Mla mutants (Malinverni and Silhavy 2009). The overlapping between the two systems has been further supported by the finding that *pldA* deletion suppresses the death phenotype of the gain-of-function *mlaA** allele in which accumulation of phospholipids at the OM outer leaflet is accompanied by a corresponding and detrimental increase in LPS production (Sutterlin et al. 2016). The *pldA* suppression phenotypes have been nicely explained by the finding that PldA, in addition to degrade mislocalized phospholipids, acts as a sensor signaling to cells that more LPS is needed to properly feed the OM outer layer (May and Silhavy 2018).

Overall, the MlaA-OmpC complex represents a novel class of lipid transport proteins and defines a novel mechanism in bacterial lipid homeostasis.

LPS Biosynthesis

The biosynthesis of LPS is a complex process that takes place in different cellular compartments and requires spatial and temporal coordination of several independent pathways that converge to produce the full-length LPS molecule. The synthesis of lipid A substituted with the Kdo residues of the inner core, the so called Kdo_2-lipid A moiety, occurs in the cytoplasm and at the inner leaflet of the IM. This pathway, which is well conserved across Gram-negative bacteria, has been extensively characterized in *E. coli* and *Salmonella* and it is referred to as the "Raetz pathway", since most of research work on it has been performed by Christian Raetz and his team (Raetz et al. 2009; Raetz and Whitfield 2002; Raetz et al. 2007).

The first step of Kdo_2-lipid A biosynthesis is the fatty acylation of *N*-acetylglucosamine linked to a nucleotide carrier (UDP-Glc*N*Ac) by LpxA (Fig. 2.2). In this reaction the thioester *R*-3-hydroxymyristoyl acyl carrier protein (ACP) is the donor substrate (Anderson et al. 1993; Anderson and Raetz 1987). The product of the reaction, UDP-3-*O*-(acyl)-Glc*N*Ac, is next deacetylated to UDP-3-*O*-(acyl)-GlcN by LpxC, a Zn^{2+}-dependent metalloenzyme (Young et al. 1995). Since the equilibrium constant for UDP-Glc*N*Ac acylation by LpxA is unfavorable (Anderson et al. 1993), the deacetylation of UDP-3-*O*-(acyl)-Glc*N*Ac by LpxC is the first committed step of Kdo_2-lipid A biosynthesis (Fig. 2.2). Indeed, LpxC represents the major control point of LPS synthesis whose turnover is controlled by the FtsH

protease (Fuhrer et al. 2006; Ogura et al. 1999), the YciM/LapB heat-shock proteins (Klein et al. 2014; Mahalakshmi et al. 2014) and a not yet identified function (Emiola et al. 2016). Following deacetylation, a second *R*-3-hydroxymyristate chain is added by LpxD to make UDP-2,3-diacyl-GlcN (Kelly et al. 1993). LpxH is the pyrophosphatase that cleaves the pyrophosphate linkage of UDP-2,3-diacyl-GlcN to form 2,3-diacyl-GlcN-1-phosphate (lipid X) (Babinski et al. 2002). Formation of the β,1′-6-linked lipid A disaccharide is generated by LpxB, which condenses UDP-2,3-diacyl-GlcN with lipid X and releases UDP (Fig. 2.2) (Radika and Raetz 1988). LpxK phosphorylates the 4′-position of the disaccharide 1-phosphate produced by LpxB to generate lipid IV_A (Ray and Raetz 1987). The reaction catalyzed by LpxK precedes the addition of the Kdo residues by the bifunctional enzyme WaaA (formerly KdtA) (Fig. 2.2) (Belunis and Raetz 1992). The Kdo residue is synthesized by a separate pathway that requires four sequentially acting enzymes (Cipolla et al. 2009). The sugar nucleotide CMP-Kdo is the donor substrate for WaaA that catalyzes the sequential incorporation of two Kdo residues in lipid IV_A to generate the Kdo_2-lipid IV_A moiety. Notably, the FtsH protease also controls the turnover of WaaA (Katz and Ron 2008) to effectively control the concentrations of both, the lipid A and the sugar moiety (Kdo) precursors. The two last steps of Kdo_2-lipid A biosynthesis in *E. coli* involve the addition of the secondary lauroyl and myristoyl residues to the distal glucosamine unit by LpxL and LpxM (previously designated HtrB and MsbB, respectively), which require the Kdo disaccharide moiety in their substrates for activity (Brozek and Raetz 1990; Clementz et al. 1996, 1997). Based on their activity in the last steps of the LPS biosynthetic pathway, LpxL and LpxM are sometimes referred to as "late" acyltransferases (Fig. 2.2). However, *P. aeruginosa* LPS biosynthesis differs since fully acylated lipid A is required before Kdo residue addition (King et al. 2009). Finally, the additional sugars composing the oligosaccharide core are added to the Kdo_2-lipid A moiety by specific glycosyl-transferases (Raetz and Whitfield 2002). The complete lipid A-core moiety anchored at the IM inner leaflet is now ready to be translocated across the IM by the MsbA ABC transporter (see section "Translocation Across the IM: The ABC Transporter MsbA").

In O-antigen producing strains, residues composing the repeat units are synthesized in the cytoplasm, flipped to the periplasmic face of the IM attached to the lipid carrier undecaprenyl diphosphate and extension of the O-polysaccharide chain may occur in the periplasm via wzy- or synthase-dependent pathways (Greenfield and Whitfield 2012).

LPS Export to the Cell Surface

After completing its synthesis at the cytoplasmic side of the IM, lipid A-core is translocated across the IM to gain the periplasmic orientation necessary for the WaaL ligase-mediated O-antigen decoration (Han et al. 2012; Ruan et al. 2012) and for the subsequent transport to the cell surface. The trans-bilayer movement ("flipping") of amphipathic molecules into phospholipid membranes is energetically unfavorable

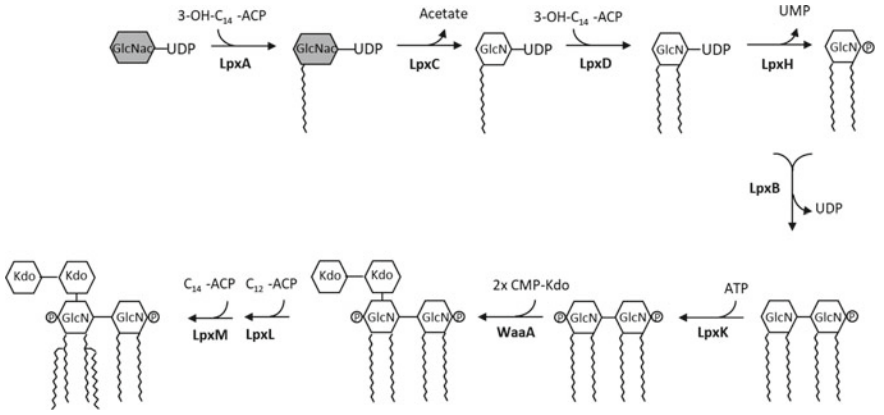

Fig. 2.2 Schematic representation of the Raetz pathway for the synthesis of Kdo_2-lipid A. Grey hexagons indicate: Glc*N*Ac, *N*-acetylglucosamine; white hexagons indicate: GlcN, glucosamine; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid. Phosphate groups are indicated as circled P. See text for details

and spontaneous translocation is very slow, therefore the cell needs a dedicated protein to carry out this function. The essential ABC transporter MsbA is the best-known bacterial lipid flippase among the ABC (ATP binding cassette) transporter superfamily and couples ATP hydrolysis to trafficking of lipid A-core across the IM (Doerrler et al. 2004).

Most of the heterogeneity displayed by the LPS molecules, not only among different Gram-negative bacteria but also within a single bacterial strain, is due to modifications of the conserved lipid A-core module (O-antigen ligation, chemical modifications on the glucosamines of lipid A, phosphate group decoration, etc.), that may take place at the periplasmic leaflet of the IM and, therefore, downstream of the MsbA-mediated lipid A-core flipping. These modifications will not be discussed in this chapter as they have been the topic of excellent reviews (Raetz et al. 2007; Whitfield 2006). The mature modified LPS molecules must then be escorted across the periplasm and assembled at the OM outer leaflet.

Due to the chemical nature of LPS and of the different cellular compartments it crosses during its journey to the OM, the transport of mature LPS molecules across the periplasm to the cell surface is a challenging process. LPS transport indeed implies the extraction of the lipid A acyl chains from the hydrophobic milieu of the IM, their shielding during transport across the aqueous environment of the periplasm and their insertion into the pre-constituted phospholipid/LPS layer of the OM. Accumulation of LPS in the IM is detrimental for the cells (Zhang et al. 2013), therefore such a demanding process must occur unidirectionally from IM and OM, against concentration gradient and without perturbing the integrity of the OM.

During the past two decades, the joint efforts of several research groups, mainly using *E. coli* as model organism, have led to the discovery of a multiprotein complex made of seven essential proteins located in every cellular compartment that assemble

to span the entire cell envelope. This trans-envelope complex harnesses the energy of ATP hydrolysis in the cytoplasm to transport LPS from the IM to the OM (Braun and Silhavy 2002; Chng et al. 2010a, b; Ruiz et al. 2008; Sperandeo et al. 2007, 2008; Wu et al. 2006). These proteins named LptABCDEFG from Lipopolysaccharide transport (Sperandeo et al. 2007), physically interact with each other employing a common structural domain and function in a concerted way to coordinate the chemical energy to the mechanical work through the transmission of conformational changes in their structures (Fig. 2.3). The Lpt machinery functions as a single device and when any component is depleted LPS accumulates at the IM outer leaflet (Ruiz et al. 2008; Sperandeo et al. 2008).

The engine of the system is constituted by $LptB_2FG$, the second ABC transporter involved in LPS biogenesis after MsbA (Dong et al. 2017; Luo et al. 2017; Ruiz et al. 2008). The energy obtained by the ATP hydrolysis is then used to push LPS into the periplasmic bridge formed by LptC, LptA and the N-terminal domain of LptD

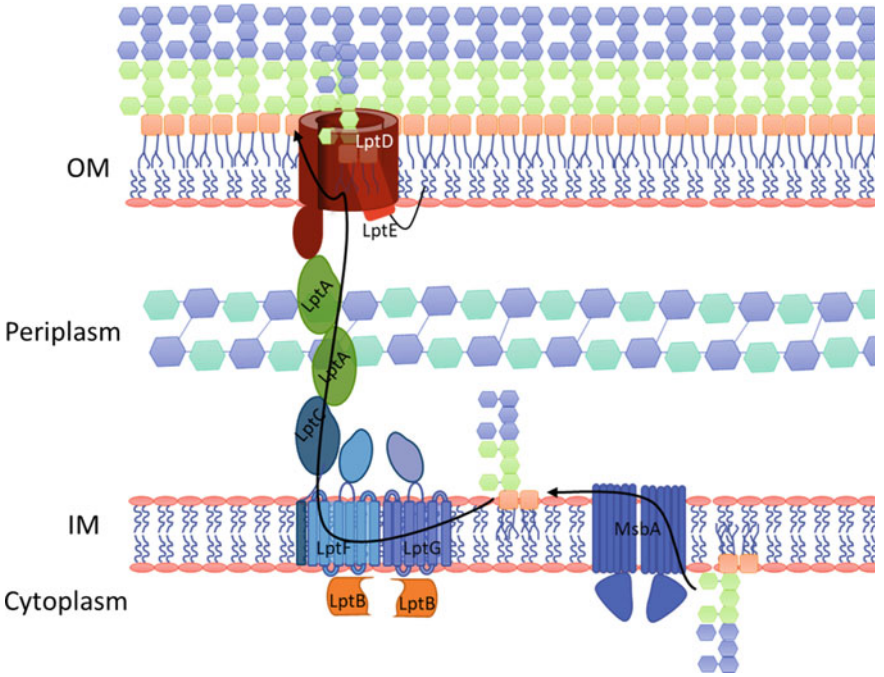

Fig. 2.3 The LPS export pathway in *E. coli*. The lipid A-core moiety synthesized at the inner leaflet of the IM is flipped across the IM by the MsbA ABC transporter. The flipped lipid A-core molecule is then extracted from the IM, escorted across the periplasm and assembled at the OM outer leaflet by the transenvelope Lpt machinery. LPS inner and outer core are shown as colored light green and light blue hexagons, respectively. See text for details. In O-antigen producing strains, the O-antigen polymer, linked to undecaprenyl phosphate carrier, is translocated across the IM by a MsbA-independent pathway and is ligated to the lipid A-core by the WaaL ligase. Here, the O-antigen domain is omitted

(Freinkman et al. 2012; Sperandeo et al. 2007, 2008, 2011). At the OM, the complex formed by the LptD β-barrel protein, and the tightly associated LptE lipoprotein, facilitates final LPS assembly at the cell surface (Chng et al. 2010a, b; Freinkman et al. 2011) (Fig. 2.3).

Translocation Across the IM: The ABC Transporter MsbA

The conserved IM protein MsbA is the first transporter working in the LPS export pathway and plays an essential role in building the LPS OM layer in LPS-producing bacteria. MsbA belongs to the ubiquitous ABC transporter superfamily (Locher 2016; Rees et al. 2009) and functions as a homodimer of two 64.3 kDa subunits, each containing one cytoplasmic nucleotide-binding domain (NBD) and one transmembrane domain (TMD), made by 6 transmembrane helices, which contains the LPS binding site (Fig. 2.4). The NBDs bind to ATP and catalyze its hydrolysis to provide the energy to flip the lipid A-core across the IM (Davidson et al. 2008).

MsbA involvement in LPS translocation across the IM was first inferred by the observation that depletion of MsbA, or its inactivation in a thermosensitive mutant carrying a single amino acid substitution (A270T) in the TMDs, led to the accumulation of LPS precursors in the IM, accompanied by extensive membrane invagination in the cytoplasm and, ultimately, cell death (Doerrler et al. 2001; Polissi and Georgopoulos 1996). The accumulated LPS precursor molecules in the *msbA*$_{A270T}$ mutant grown at the non-permissive temperature were later shown to be inaccessible by periplasmic lipid A modification enzymes, strongly suggesting that MsbA was indeed required for flipping of LPS across the IM (Doerrler et al. 2004). Initial attempts to directly demonstrate lipid flipping by MsbA in vitro failed (Kol et al. 2003) and only several years later the direct measurement of ATP-dependent translocation of phospholipids in proteoliposomes was reported (Eckford and Sharom 2010). However, neither the substrate specificity of MsbA, nor the molecular mechanism that couples energy production with LPS translocation have emerged clearly from these initial studies.

Several structural and biochemical data produced on *E. coli* MsbA and its homologs have been carried out during the last decade to shed light on these issues. A milestone in MsbA functional characterization is represented by the X-ray crystal structure determination of three MsbA orthologs from *E. coli*, *Vibrio cholerae* and *Salmonella enterica* (serovar Typhimurium) trapped in different conformations (Ward et al. 2007). The analysis of these crystal structures revealed the existence of two different inward-facing and one outward-facing conformation, suggesting that, at least under the experimental conditions used for crystal production, the protein undergoes a large range of motions as consequence of ATP hydrolysis. These conformational changes might be required for LPS flipping. Based on these evidences, the authors suggested an "inward-outward" model for MsbA function. According to this model, when MsbA is in the apo-form (without nucleotide bound), its TMDs open creating a cavity that faces the cytoplasmic side of the IM, possibly allowing

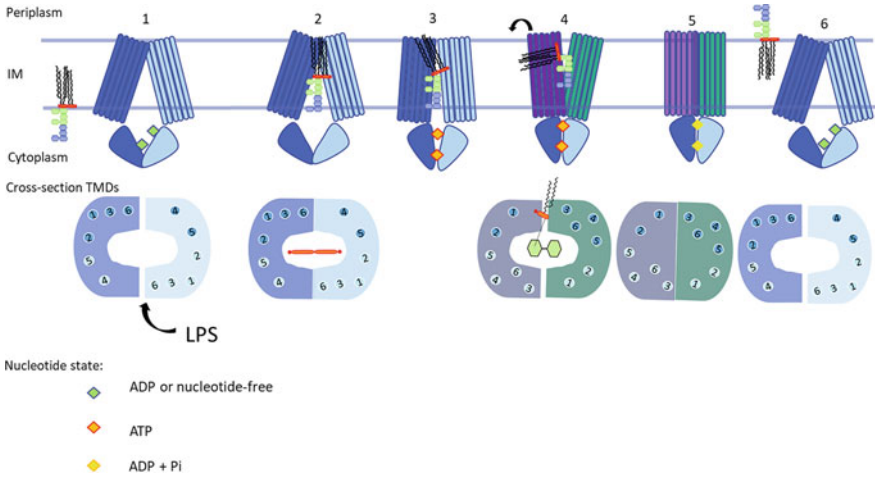

Fig. 2.4 "Trap and flip" model for MsbA-mediated lipid A-core flipping across the IM. MsbA catalyses lipid A-core flipping across the IM alternating between an inward (cytoplasmic)-facing and an outward (periplasmic)-facing conformation that allow binding and ATP hydrolysis-dependent release of lipid A-core through a six-step cycle. Upper panel: in the ADP or nucleotide-free state, MsbA dimer adopts the inward-facing conformation, opening the TMDs to allow LPS entry (step 1). LPS binding induces NBDs alignment for ATP binding (step 2). ATP binding triggers conformational changes in the TMDs that weaken lipid A-core interaction (step 3) and promote the release of the lipid A acyl chains into the periplasmic leaflet of the membrane (step 4). Lipid A-core translocation and MsbA rearrangement lead to ATP hydrolysis. After lipid A-core release, the TM helices adopt a compact assess (step 5). Finally, phosphate release induces MsbA to return in the inward-facing conformation (step 6). MsbA monomers in the resting state (inward-facing conformation) are shown in dark and pale blue. TMD rearrangement after ATP binding is represented by colour change of the monomers from dark and pale blue to violet and pale green, respectively. Lower panel: schematic representation of the cross-sections through the MsbA TMDs at the level of lipid A glucosamines for each step of the translocation process, as shown in the upper panel. Numbered circles represent the individual TM helices provided by each MsbA monomer, coloured as dark blue or pale blue according to the MsbA monomer they belong to. Glucosamine moiety of lipid A and phosphates are represented by orange ovals and red circles, respectively. The core oligosaccharide is depicted as in Fig. 2.1. Green, red and yellow diamonds represent ADP, ATP and ADP with phosphate, respectively. Adapted from Mi et al. (2017)

LPS binding from this side of the IM. On the contrary, upon ATP binding (mimicked by the binding of ADP-vanadate), MsbA TMDs open towards the periplasmic side of the IM, possibly leading to LPS release into the outer leaflet of the membrane (Ward et al. 2007). However, the major limitation of this work is that LPS was not co-crystallized with MsbA, undermining the reliability of the model.

The real turning point in the study of the molecular mechanisms of MsbA-mediated flipping of LPS across the IM has come very recently from the combined use of nanodiscs, instead of detergent, for MsbA solubilization, and cryo-electron microscopy (Cryo-EM) to image the protein in its native conformations (Mi et al. 2017; Voss and Trent 2018). Cryo-EM images of MsbA bound to LPS revealed that,

under native conditions, MsbA undergoes to a smaller range of motions compared to what observed in the crystal structures. Strikingly, the higher ATPase activity displayed by MsbA in nanodiscs confirmed that the protein was in a more native conformation than in detergent. The structure of MsbA in nanodiscs showed only one predominant inward-facing conformation with the two TMDs extending parallel from the membrane and the cytoplasmic NBDs close to each other. Interestingly, the hydrophobic cavity that was shown to accommodate LPS is localized in correspondence of the periplasmic leaflet of the IM, suggesting that the energy from ATP hydrolysis is required only for acyl chain flipping. This cavity is surrounded by a ring of charged residues required to position the glucosamine-linked phosphates into the TMDs. Upon nucleotide binding, the NBDs were shown to closely associate, determining a reorganization of the TM helices that block the LPS binding site and, probably, induce the opening of the hydrophobic pocket to allow the acyl chains to insert into the periplasmic side of the IM (Mi et al. 2017).

These evidences led the authors to propose the new "trap-and-flip" model for MsbA-mediated LPS flipping. According to this model, when MsbA is bound to ADP or is in a nucleotide-free state, LPS enters MsbA internal cavity exploiting the path formed by the TM4 and TM6 from two different monomers. LPS binding aligns the NBDs for ATP hydrolysis. This agrees with previous data from fluorescence resonance energy transfer (FRET) experiments, that showed NBDs dimerization and the stimulation of ATP hydrolysis upon lipid A binding (Doshi and van Veen 2013). Upon ATP binding and hydrolysis, conformational changes occur that close the cavity and expose the acyl chains of lipid A to the periplasm. This leads LPS to slip out the protein from the path formed by TM1 and TM3 from two different monomers. Upon release of LPS, MsbA resets to the inward-facing conformation (Mi et al. 2017) (Fig. 2.4).

From structural studies and inspection of the cavity in MsbA that hosts LPS, it can be speculated that both the strong interactions with lipid A and the dimension of the cavity itself might have a role in selecting the molecule that has to be accommodated (Ho et al. 2018). This is in line with the hypothesis that MsbA plays the role of "quality control" system for LPS export to the OM. Accordingly, although several lipid A precursor molecules, in addition to LPS, can stimulate MsbA ATPase activity in vitro (Doerrler and Raetz 2002; Eckford and Sharom 2008), *E. coli* MsbA is not able to transport lipid A precursor molecules missing Kdo residues and the lauryl and myristoyl acyl chains in vivo, resulting in death of the corresponding LPS biosynthesis mutants (see above, Mamat et al. 2008; Meredith et al. 2006). However, MsbA overexpression or mutations rescue the growth phenotype of these mutants. It is worth noting that MsbA was originally identified as a multicopy suppressor of defects resulting from a mutation in *lpxL*, that encodes for one of the "late" Kdo-dependent lipid A acyltransferases (Karow and Georgopoulos 1993). This suggests that an increase in the transport rate of poor substrates, due to protein overexpression or mutations that alter the substrate selectivity, may enable MsbA to transport under-acylated LPS precursors, even in vivo. Interestingly, in a screening for suppressor mutations of lipid A biosynthesis defects, a single amino acid substitution in an IM protein with unknown function, YhjD, has been shown to allow LPS transport in the

absence of MsbA (Mamat et al. 2008). The role of YhjD is still unknown and the reason why the identified mutation allows YhjD to functionally replace MsbA, albeit under extreme circumstances, is still an unsolved question.

Another open question is whether MsbA can handle other lipophilic substrates besides LPS in vivo. In *E. coli*, the thermosensitive $msbA_{A270T}$ mutant was shown to be impaired not only in LPS but also in phospholipid translocation at the non-permissive temperature, suggesting that MsbA could be the transporter of the two major lipids in the cell envelope of Gram-negative bacteria (Doerrler et al. 2001). Accordingly, the ATPase activity of MsbA reconstituted in proteoliposomes is stimulated by different lipids (Eckford and Sharom 2008). On the contrary, the deletion of *msbA* can be obtained in *N. meningitidis* and the mutant is viable and produces an OM mainly made out of phospholipids (Tefsen et al. 2005a, b). Since this organism can survive without LPS (Steeghs et al. 1998), this suggests that MsbA might not be strictly required for phospholipid translocation. Nevertheless, this evidence can simply reflect a difference between the lipid transport systems of the two organisms and suggest the existence of another pathway for phospholipid flipping across the IM, at least in *N. meningitidis*.

Finally, it has been demonstrated that MsbA can bind simultaneously lipid A and amphipathic drugs, suggesting the presence of different binding sites in the protein (Siarheyeva and Sharom 2009). The relevance of such MsbA activity in drug resistance has still to be demonstrated.

Extraction from the IM: The Atypical $LptB_2FG$-C Transporter

After MsbA-dependent translocation, LPS molecules are anchored at the periplasmic leaflet of the IM. LPS accumulation at this location is toxic for the cell and LPS molecules must be efficiently removed from the IM to reach their final destination at the cell surface. The LPS export from the IM outer leaflet to the OM is accomplished by the Lpt complex, a specialized multiprotein machinery that uses energy to move millions of LPS molecules at each cell division, in a process that begins with its extraction from the IM. The energy for the process is provided by the $LptB_2FG$ transporter. $LptB_2FG$ is a non-conventional ABC transporter since, unlike MsbA, it does not translocate its substrate across the membrane, but rather it couples the energy of the ATP hydrolysis in the cytoplasm with the detachment of LPS from the outer leaflet of the IM. Moreover, $LptB_2FG$ is a tetramer of different subunits, comprising a dimer of LptB and two polytopic proteins, LptF and LptG (Narita and Tokuda 2009), thus sharing features with the class of the ABC importers. Another unconventional trait of $LptB_2FG$ is its stable association with LptC (Chng et al. 2010a, b; Villa et al. 2013), a single pass IM protein constituted by a transmembrane helix and a large periplasmic domain folded as a β-jellyroll (Fig. 2.5) (Tran et al. 2010), whose function within the ABC transporter is still not clear (see below). For its peculiar proprieties, the $LptB_2FG$ has been recently classified as a new type of ABC transporter, the type VI exporter (Thomas and Tampe 2018).

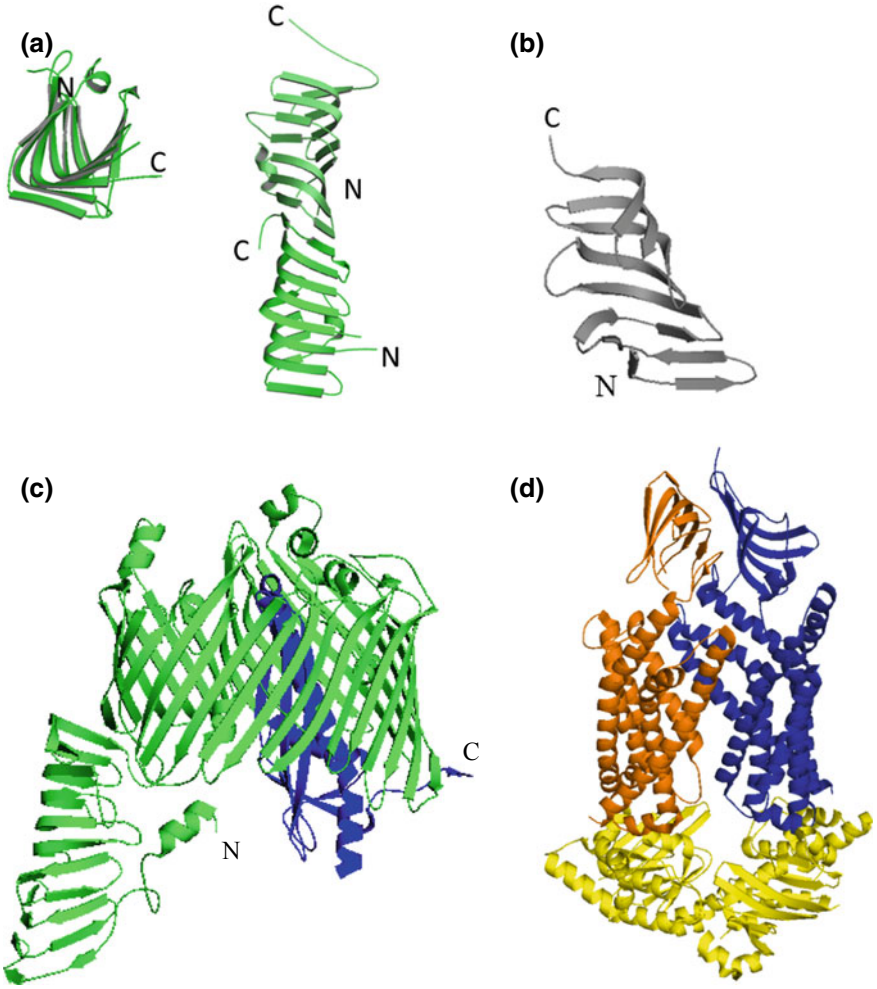

Fig. 2.5 Crystal structures of Lpt proteins. **a** Right: ribbon diagram of two molecules of *E. coli* LptA organized in head-to-tail fashion (PDB 2R19). Left: β-jellyroll structure of LptA, rotated by 90° to highlight the channel formed along the length of LptA. **b** The periplasmic domain of LptC from *E. coli* (gray, PDB 3M2Y). **c** LptDE complex from *K. pneumoniae* (PDB 5IV9). LptE, depicted in blue, resides within the LptD lumen (depicted in green). **d** $LptB_2FG$ complex from *P. aeruginosa* (PDB 5X5Y). LptB, LptF and LptG are represented in yellow, orange, and blue, respectively. N- and C-terminal end of LptA, LptC and LptD proteins are indicated

Clues on the molecular mechanism of LPS extraction from the IM by $LptB_2FG$ have recently come from the crystal structures of $LptB_2FG$ from *P. aeruginosa* and *Klebsiella pneumoniae*, obtained in the absence of nucleotides (Dong et al. 2017; Luo et al. 2017). Inspections of these structures revealed that the $LptB_2FG$ core transporter adopts a "milk jug" architecture, consisting of a pair of LptB that form the NBDs and two TM proteins, LptF and LptG, each containing 6 transmembrane helices and a large periplasmic domain between TM3 and TM4, folded as a β-jellyroll (Dong et al. 2017; Luo et al. 2017) (Fig. 2.5). LptF and LptG interact each other through their respective TM1 and TM5 helices and form an outward-facing V-shaped cavity that spans the IM and extends slightly into the periplasm. The internal surface of the IM cavity is hydrophobic, whereas the residues lining at the IM–periplasm interface are positively charged, suggesting that the region located deep inside the IM makes contact with the acyl chains of lipid A, whereas the region at the IM-periplasm interface interacts with the negatively charged phosphate groups. Interestingly, the periplasmic domains of LptF and LptG take opposite side-by-side orientations in both crystal structures and are directly connected to the hydrophobic cavity of $LptB_2FG$. This is in line with the hypothesis that LPS is accommodated within the cavity of $LptB_2FG$ before being pushed toward the OM through the periplasmic domains of LptF and/or LptG. Accordingly, in the crystal structure of $LptB_2FG$ from *K. pneumoniae*, a non-assigned extra electron density, which is compatible with a molecule of LPS, was observed within the central cavity (Dong et al. 2017). The structural data also revealed that the helices connecting TM2 and TM3 of LptF and LptG constitute the region of interaction between LptF/LptG and the LptB dimer (the so-called coupling helices), in agreement with previous predictions based on genetic and biochemical data. Indeed, residues within the coupling helices have been shown to be crucial for the assembly and the function of the IM complex by connecting LptF and LptG with a groove in LptB that experiences a large range of motions during ATP hydrolysis (Sherman et al. 2014; Simpson et al. 2016). It is likely that these interactions coordinate the ATPase activity of LptB with LPS extraction.

The interfaces between TM1 and TM5 from opposing LptF and LptG monomers have a limited number of interactions, suggesting that they may open as a consequence of the conformational changes triggered by ATP binding to LptB, thus leading to LPS entry into the cavity. Accordingly, a cluster of residues crucial for LPS interaction has been identified at the membrane-periplasm interface of the TM1 of LptG in *E. coli* and *Burkholderia coenocepacia* by a series of genetic and biochemical analyses (Bertani et al. 2018; Hamad et al. 2012). Interestingly, this cluster contains a residue (K43 in *E. coli* LptG) that is not conserved among LptG homologues and whose chemical properties correlate with the modifications occurring on the lipid A moiety in different bacterial species, suggesting that it might interact via electrostatic interactions with lipid A. The strong decrease in LPS release activity of mutant $LptG_{K34D}$ has been shown by UV-photocrosslinking assay, confirming the importance of the residue. The recognition between $LptB_2FG$ and lipid A might occur in an early step of the extraction process, driving the selection of the LPS variants to be transported to the cell surface under different conditions (Bertani et al. 2018).

The current model for $LptB_2FG$ function suggests that the transporter cycles between three conformational states: in the nucleotide-free state (represented by the published structures), LptB dimer adopts an open conformation. Upon ATP binding, LptB monomers move close together and the conformational changes are then transmitted from LptB, through the coupling helices, to LptF and LptG inducing the opening of the lateral gate between one of the TM1-TM5 interfaces of LptFG to allow the entry of LPS into the internal cavity of $LptB_2FG$. ATP hydrolysis and ADP release, then, induce the conformational switch back to the nucleotide-free state, closing the lateral gate and expelling LPS into the periplasmic domains of LptF and/or LptG (Fig. 2.6a) (Luo et al. 2017). Interestingly, the published structures of $LptB_2FG$ show a different orientation of the periplasmic domains of the transporter, with opposing opening states of the lateral gates. This suggests that $LptB_2FG$ might extract LPS from both the lateral gates, transferring the molecules alternately to the periplasmic domains of LptF or LptG.

The proposed model raises the question of whether the transporter might work in a symmetric way. It is worth noting that once extracted from the membrane by the $LptB_2FG$ complex, LPS is delivered to LptC (Okuda et al. 2012). However, since the interaction of $LptB_2FG$ with LptC has not been characterized yet, it is not possible to exclude that LptC could receive LPS alternately from LptF and LptG. Moreover, there are still no evidences of LPS interaction with the periplasmic domain of LptF and/or LptG. Nevertheless, two major observations argue against the hypothesis of a symmetric transport and suggest that LptF and LptG may have different functions within the transporter: (i) mutations in corresponding residues within the coupling helices of LptF and LptG lead to different degree of OM permeability defects (Simpson et al. 2016); (ii) mutations at a unique position in the periplasmic domain of LptF rescue the growth defect of a mutant lacking LptC, suggesting that LPS flow might have a preferential direction within the transporter (see below) (Benedet et al. 2016).

LptC stably associates to the $LptB_2FG$ complex, although its role in LPS transport is not completely clear. Several lines of evidence indicate that LptC is strictly required to funnel LPS within the periplasmic bridge towards the OM. UV-photocrosslinking experiments showed that LPS association to LptA in spheroplasts overexpressing the $LptB_2FG$ transporter depends on the ATP hydrolysis but cannot proceed in the absence of LptC (Okuda et al. 2012). Moreover, LptC itself has been demonstrated to be part of the periplasmic bridge, interacting directly with LptA (Bowyer et al. 2011; Freinkman et al. 2012; Sperandeo et al. 2011), and binding LPS (Okuda et al. 2012; Tran et al. 2010). These findings strengthen the idea that LptC might have a direct role in LPS transport. However, LptC can tolerate several mutations. A soluble version of LptC missing its hydrophobic anchor is still functional and can assemble into the complex via the N-terminal region of its soluble domain (Villa et al. 2013). Moreover, the C-terminal region of the periplasmic domain can be deleted upon overexpression of LptB, suggesting that also a truncated LptC variant can fulfill its role in the Lpt complex, albeit with less efficiency (Martorana et al. 2016). Strikingly, the complete lack of LptC can be overcome by a single amino acid substitution at residue 212 in the periplasmic domain of LptF (Benedet et al. 2016). Taken together, these observations implicate the periplasmic domain of LptF into the

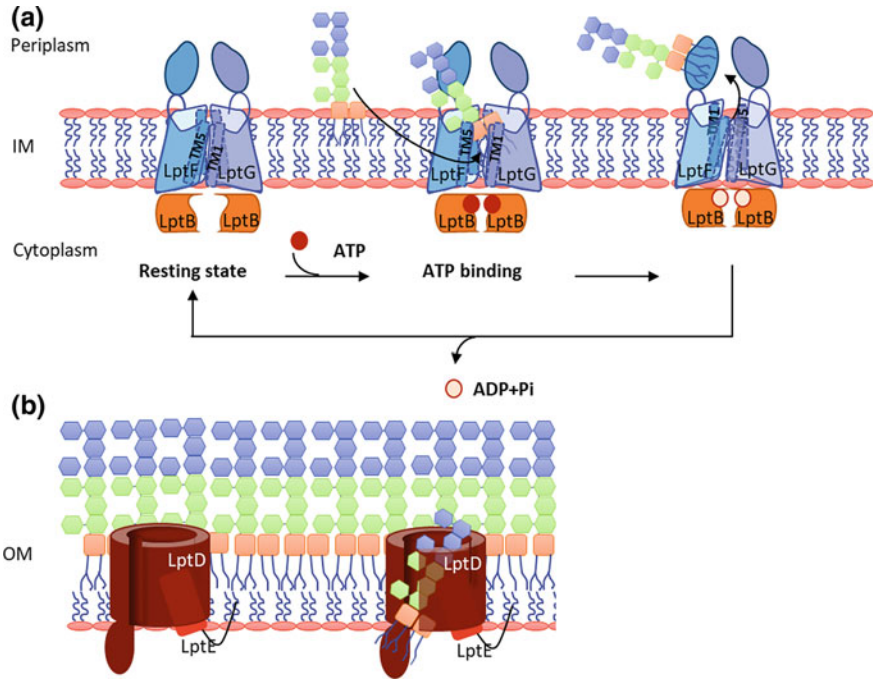

Fig. 2.6 Models for LPS extraction from the IM and assembly at the OM. **a** The $LptB_2FG$ transporter extracts and transports LPS through discrete steps of ATP hydrolysis, cycling from to the ATP-free state (resting state) to the ATP-binding state and finally ATP hydrolyzed state. Binding of LptB dimer to ATP triggers the conformational change of LptF-TM5 and LptG-TM1 resulting in the opening of a lateral gate between these two helices and the subsequent capture of LPS in the $LptB_2FG$ internal hydrophobic cavity. Upon ATP hydrolysis the lipid A is extracted from the IM and pushed to the periplasmic domain of LptF (or LptG). Finally, release of ADP induces $LptB_2FG$ to return to the ATP-free state. **b** The LPS molecule is delivered from the LptA to LptDE complex for insertion into the OM. The Lipid A portion of LPS passes from the N-terminal domain of LptD into the core of the membrane. The lateral gate formed by β1/β26 strands opens and allows the hydrophilic portion of LPS to pass through the lumen of LptD

periplasmic bridge and point to a regulative role of LptC within the transporter. The initial biochemical characterization of $LptB_2FG$-C purified in detergent showed that the ATPase activity of the transporter was not altered by the presence of LptC (Narita and Tokuda 2009), as it would be expected by a regulative subunit. However, it has been recently demonstrated that the association of LptC led to almost 40% decrease of the ATPase activity when the transporter was reconstituted in proteoliposomes, a condition that better mimics the membrane environment (Luo et al. 2017; Sherman et al. 2018).

Transport Across the Periplasm

Once LPS has been extracted from the IM by the $LptB_2FG$ complex it is transferred to LptC, and then to LptA, at the expense of ATP hydrolysis (Okuda et al. 2012). LptA is a soluble protein (Sperandeo et al. 2007) and initially proposed to function as a chaperone shuttling LPS between IM and OM (Ruiz et al. 2009) in analogy with the Lol pathway for lipoprotein transport (Okuda and Tokuda 2011). However, several lines of evidence argue against the chaperon model and support instead the view of LPS transported across the periplasm via a protein bridge connecting IM and OM: (i) LPS is not released into the medium after addition of periplasmic extract to *E. coli* spheroplasts as observed for lipoproteins, and in spheroplasts newly synthesized LPS is still transported to the OM, strongly suggesting that LPS transport could take place via contact sites between the membranes (Tefsen et al. 2005a, b); (ii) LptA fractionates with both IM and OM in sucrose density gradient centrifugation despite being a soluble periplasmic protein (Chng et al. 2010a, b); (iii) the seven Lpt proteins can be copurified as a complex by affinity chromatography, supporting the idea that they form a continuous protein bridge connecting IM and OM (Chng et al. 2010a, b); (iv) mutations impairing Lpt complex assembly lead to degradation of the periplasmic LptA component (Sperandeo et al. 2011) further supporting the biological significance of the transenvelope architecture.

LptA connects IM and OM by binding to LptC and LptD, respectively. The architecture of the protein bridge has been defined by co-purification and UV-photocrosslinking experiments. Whereas the N-terminal end of LptA interacts with the C-terminal end of LptC, the C-terminal end of LptA interacts with the N-terminal periplasmic region of LptD (Sperandeo et al. 2011; Freinkman et al. 2012). These intermolecular contacts are crucial for LPS transport as genetic disruption of these edge regions compromises the assembly and function of the Lpt bridge (Falchi et al. 2018; Freinkman et al. 2012; Sperandeo et al. 2011; Villa et al. 2013). Notably, the formation of LptC-LptA-LptD protein bridge occurs via the structurally homologous β-jellyroll domain, also termed "Lpt fold", shared by the three proteins (Fig. 2.5) (Botos et al. 2016; Qiao et al. 2014; Suits et al. 2008; Tran et al. 2010). The β-jellyroll fold represents a unique protein architecture initially identified in LptA, the first protein of the Lpt system for which a crystal structure was obtained (Suits et al. 2008), and afterwards found in all Lpt proteins with a periplasmic domain (Dong et al. 2017; Luo et al. 2017; Qiao et al. 2014; Tran et al. 2010). It consists of a variable number of consecutive, antiparallel β-strands arranged to form a semi-opened and slightly twisted structure whose interior is covered by hydrophobic residues (Fig. 2.5). The arrangement of the β-jellyroll domains of LptC, LptA and the N-terminal periplasmic region of LptD therefore forms a hydrophobic groove which is thought to accommodate the acyl chains of the lipid A moiety during LPS transport along the protein bridge, while the hydrophilic oligosaccharide portion remains exposed in the periplasm. Indeed, residues of LptA and LptC that crosslink to LPS are located in the β-jellyroll interior (Okuda et al. 2012).

LptA has a strong tendency to oligomerize in a head-to-tail orientation forming long fibrils in crystals obtained in the presence of LPS (Suits et al. 2008) and in solution in a concentration-dependent manner (Merten et al. 2012; Santambrogio et al. 2013). However, the affinity of LptA for LptC seems stronger than of LptA for itself in the oligomerization reaction (Bowyer et al. 2011; Schultz et al. 2013). The stoichiometry of LptA in vivo in the periplasmic protein bridge is still an open question. It has been recently demonstrated that an oligomeric-deficient form of LptA, lacking the last C-terminal β-sheet, partially fulfills its role in the transport of LPS to the OM (Laguri et al. 2017), suggesting that Lpt machinery with a single LptA molecule can be functional. However, it is more likely that LptA oligomerization imparts to the Lpt machinery the required flexibility to respond to variations in the periplasm width as a consequence of envelope stress or other growth conditions. This hypothesis is in line with the presence of an additional σ^E stress response promoter upstream the *lptA* gene (Martorana et al. 2011) and the recent finding that Enterobacteria are able to respond to changes of periplasm width by activating the Rcs stress response system (Asmar et al. 2017).

Assembly of LPS at the OM Outer Leaflet

The efficient insertion of LPS into the outer leaflet of the OM, avoiding a premature stop at inner leaflet, is essential to maintain the asymmetry of the OM. The step of LPS delivery and assembly at the OM surface is mediated by the OM translocon constituted by the β-barrel protein LptD and the lipoprotein LptE (Braun and Silhavy 2002; Wu et al. 2006). LptD consists of a C-terminal transmembrane β-barrel domain and a periplasmic N-terminal domain connected by two disulfide bonds between two non-consecutive cysteine residues (Botos et al. 2016; Dong et al. 2014; Qiao et al. 2014). The OM lipoprotein LptE is buried in the lumen of the barrel domain of LptD adopting a unique plug-and-barrel architecture (Fig. 2.5) (Freinkman et al. 2011).

The assembly and correct maturation of a functional LptDE complex is crucial to avoid LPS mistargeting and LptE accomplishes a non-obvious function within the translocon, assisting LptD in its folding and its assembly into the Lpt complex.

First evidence of LptE involvement in LptD assembly/maturation came from a screening for mutants able to suppress permeability defects of a two-codon *lptE* deletion that altered LptE interaction with LptD. In this screening, suppressor mutants were mapped not only in *lptD* but also in *bamA* (β-barrel protein essential for OMP assembly at the OM) (for a review see Konovalova et al. 2017; Noinaj et al. 2017), revealing that LptE association has a fundamental role in LptD assembly by the Bam (β-barrel assembly machinery) complex (Chimalakonda et al. 2011). Moreover, LptE is crucial for the functionality of LptD, since it helps the formation of the mature LptD form containing two disulfide bonds between non-consecutive cysteines (Chimalakonda et al. 2011). The plug function of LptE is critical to control the accessibility of the otherwise too large LptD lumen, as demonstrated by the isolation

of a mutation in LptE that affects its interaction with LptD, altering OM permeability without impairing LptD assembly or LPS export (Freinkman et al. 2011).

In *E. coli*, LptE does not have solely a structural role in LPS biogenesis, as it is able to specifically bind LPS (Chng et al. 2010a, b) and, in vitro, to disaggregate LPS molecules through electrostatic interactions between the lipid A moiety of LPS and a patch of positively-charged residues in the exposed loop connecting the β-strands 2 and 3 of the protein. Accordingly, disruption of this LPS-binding patch increases membrane permeability but does not affect LptD assembly (Malojčić et al. 2014). Based on these observations, it has been proposed that LptE facilitates LPS transfer into the outer leaflet while preventing its insertion into the inner leaflet of the OM. Overall these evidences point toward a triple role of LptE in LPS transport: to manage the assembly of a functional LptD, to preserve membrane permeability and to assist LPS insertion into the cell surface.

The crystal structure of the LptDE complexes from several microorganisms have been recently reported (Botos et al. 2016; Dong et al. 2014; Qiao et al. 2014). Notably, the basic plug and barrel architecture of the LptDE protein complex is maintained in all solved structures. LptD consists of a large C-terminal β-barrel domain, made by 26 antiparallel β-strands embedded in the OM, and a N-terminal periplasmic β-jellyroll domain made by 11 antiparallel β-strands arranged in two sheets. The LptD β-strands are connected via periplasmic and extracellular loops; the longer loops face at the extracellular side closing off most of the β-barrel pore. Extracellular loops 4 and 8 insert inside the barrel, binding to the LptE protein which plugs the pore thus preserving membrane permeability (Botos et al. 2016; Dong et al. 2014; Qiao et al. 2014). Hydrogen bonding between the first and last β-strands of LptD β-barrel is disrupted creating a crenellation, or small gap, essential for the lateral migration of the LPS in the OM. Indeed, mutations that introduce disulfide bonds between the first and last β-strands results in lethal phenotypes (Dong et al. 2014).

A significant rotation of the N-terminal domain with respect to the β–barrel domain is also observed in the available crystal structures. The configuration of the LptD N-terminal domain with respect to the C-terminal barrel depends on the correct formation of disulfide bonds in LptD and has a fundamental role to allow the insertion of the lipid A moiety of LPS into the membrane (Dong et al. 2014), and the interaction with LptA (Okuda et al. 2016).

LptE forms a sandwich of two α-helices packed against a sheet of four β-strands. Its N-terminal lipid moiety is located outside of the barrel and inserted in the inner leaflet of the OM. In the LptDE complex, approximately 75% of LptE is located inside the β-barrel of LptD (Botos et al. 2016; Dong et al. 2014; Malojčić et al. 2014; Qiao et al. 2014).

Based on genetic and structural data, a two-portal mechanism has been proposed. According to this model the lipid A moiety of LPS moves from LptA directly into the β-jellyroll domain of LptD allowing its insertion into the membrane through a hole formed between the periplasmic and the β-barrel domain of LptD, while the hydrophilic domain of LPS flows through the lumen of the β-barrel. Then, the lateral gap of LptD may open enough to allow the passage of the hydrophilic portion of LPS through the lumen up to the OM (Gu et al. 2015; Li et al. 2015) (Fig. 2.6b). In

this model, LptE helps LPS movement into the OM by providing more favourable interactions between itself and LPS rather than between aggregated LPS molecules. Finally, LptE pumps the LPS out of the LptD lumen towards its final destination (Malojčić et al. 2014).

LPS molecules must be transported to the OM to ensure cell growth and division and this process needs to be finely regulated to avoid the mislocalization of an otherwise toxic molecule. Early investigations on the LptDE complex suggested that only a correctly folded OM translocon allows the building of a functional Lpt system, enlightening a first step of regulation in the formation of the protein bridge between IM and OM, as failure of LptD folding makes impossible to form a transenvelope bridge (Chng et al. 2012; Freinkman et al. 2012; Okuda et al. 2016). As mentioned before, LptD has four cysteine residues, two located in the N-terminal periplasmic domain and the remaining two in the C-terminal domain (Ruiz et al. 2010). Functional LptD requires the formation of disulfide bonds between non-consecutive cysteines thus allowing the correct configuration of the N-terminal domain with respect to the C-terminal β-barrel (Freinkman et al. 2012; Ruiz et al. 2010). Notably, LptD mutant proteins that lack native disulfide bonds do not interact with LptA preventing the formation of the transenvelope bridge. It is worth to note that for LptD binding to LptA the formation of at least one of the two non-consecutive disulfide bonds is sufficient. These findings suggest a mechanism in which the C-terminal β-barrel domain of LptD hinders the interaction of the N-terminal periplasmic domain with LptA when LptE-plug is not properly inserted (Freinkman et al. 2012) disrupting the β-jellyroll oligomerization process.

Altogether, the previous functional data and the available crystal structures of the Lpt proteins support the so-called PEZ model, in which the energy released from ATP hydrolysis in the cytoplasm would be used to push a continuous stream of LPS along the periplasmic bridge to the cell surface (Okuda et al. 2016). Indeed, by using an in vitro system based on *right-side-out vesicles* it has been shown that LPS transits along Lpt components trough discrete steps that require energy of ATP hydrolysis to transfer LPS from $LptB_2FG$ to LptC, then from $LptB_2FG$-C to LptA (Okuda et al. 2012) and from LptA to OM-proteoliposomes containing LptDE (Sherman et al. 2018). Notably, the transfer of LPS from IM to OM proteoliposomes only occurs when LptA is added to the system further supporting the key role of LptA in connecting IM and OM (Sherman et al. 2018).

The PEZ model postulates that the transport through the channel formed by LptCAD functions as a "candy dispenser" in which the "candies" LPS molecules are pushed by ATP hydrolysis in a continuous flow up to the OM LptDE translocon driven by a constant pressure from the IM $LptB_2FG$ complex. However, the recent finding that the OM translocon is able to communicate with the IM complex to arrest the LPS transport, opens new questions about the regulation and the functioning of the Lpt machinery. Using a fluorescent assay that allows to follow the fate of LPS in the passage from the IM to OM proteoliposomes, Khane and co-workers showed that when the OM proteoliposomes reach a saturating quantity of LPS, the IM complex stops to hydrolyze ATP to avoid an otherwise useless consumption of energy (Xie et al. 2018). This finding unveils a negative feedback in which the OM LptDE translo-

con can control the ATPase activity of the IM $LptB_2GF$ transporter. This mechanism adds a second step of regulation within the Lpt system explaining why LPS stacks at the IM when transport is impaired at the OM. How the LPS delivery at the OM and the LPS extraction from the IM could be coupled will be the next fascinating question to answer about the Lpt system.

Conclusions and Perspectives

The OM is a fundamental organelle of the Gram-negative cell envelope that protects the cell from the entry of many noxious compounds. The LPS asymmetric distribution largely contributes to the peculiar properties of the OM. Much progress has been made in the last years in understanding how LPS amphipathic molecules can be efficiently synthesized, transported and assembled at the cell surface. However, the regulation mechanisms of LPS transport during cell growth remain virtually unknown.

LPS biogenesis has been successfully exploited as a target with the potential to develop compounds acting either as new drugs or as potentiator molecules (Zabawa et al. 2016). Despite there are no molecules that have been approved for clinical use yet, there are some promising candidates under validation and development. A novel class of inhibitors of LpxC deacetylase have been recently identified and proved capable of curing infections in animal models (Lee et al. 2016; Lemaitre et al. 2017). Another promising candidate is the peptidomimetic L27-11 which specifically targets LptD from *P. aeruginosa* (Srinivas et al. 2010; Werneburg et al. 2012). Notably, POL7080, a derivative of L27-11 with improved drug properties, is currently under clinical development. These findings highlight the potential of LPS biogenesis inhibitors as novel therapeutics to treat Gram-negative infections.

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Chapter 3
Lipoproteins: Structure, Function, Biosynthesis

Volkmar Braun and Klaus Hantke

Abstract The Lpp lipoprotein of *Escherichia coli* is the first identified protein with a covalently linked lipid. It is chemically bound by its C-terminus to murein (peptidoglycan) and inserts by the lipid at the N-terminus into the outer membrane. As the most abundant protein in *E. coli* (10^6 molecules per cell) it plays an important role for the integrity of the cell envelope. Lpp represents the type protein of a large variety of lipoproteins found in Gram-negative and Gram-positive bacteria and in archaea that have in common the lipid structure for anchoring the proteins to membranes but otherwise strongly vary in sequence, structure, and function. Predicted lipoproteins in known prokaryotic genomes comprise 2.7% of all proteins. Lipoproteins are modified by a unique phospholipid pathway and transferred from the cytoplasmic membrane into the outer membrane by a special system. They are involved in protein incorporation into the outer membrane, protein secretion across the cytoplasmic membrane, periplasm and outer membrane, signal transduction, conjugation, cell wall metabolism, antibiotic resistance, biofilm formation, and adhesion to host tissues. They are only found in bacteria and function as signal molecules for the innate immune system of vertebrates, where they cause inflammation and elicit innate and adaptive immune response through Toll-like receptors. This review discusses various aspects of Lpp and other lipoproteins of Gram-negative and Gram-positive bacteria and archaea.

Keywords Lipoprotein · Structure · Function · Biosynthesis · Immune stimulation · Bacteria

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Introduction

The first identified and fully characterized protein with a covalently linked lipid was the murein lipoprotein Lpp of *Escherichia coli*, also called the major lipoprotein or Braun's lipoprotein. It is the type protein of a large variety of proteins in Gram-negative and Gram-positive bacteria and in archaea. These proteins are abundant in the cells and all have in common a lipid structure through which they anchor the proteins to a membrane outside of the cytoplasm. However, their sequence, structure, and function strongly vary. They are involved in membrane protein incorporation, protein secretion, signal transduction, conjugation, cell wall metabolism, antibiotic resistance, biofilm formation, adhesion to host tissues, and signaling for the innate immune system of vertebrates. This review will discuss various aspects of *E. coli* Lpp and highlight features of other lipoproteins from other bacterial and archaeal species. The early work on Lpp has been summarized in (Braun 1975; Braun and Hantke 1974; Braun and Wu 1994).

E. coli Lpp

Discovery and Components of Lpp

The Lpp lipoprotein covalently bound to murein (peptidoglycan) of *Escherichia coli* was discovered in a search for a bond that upon cleavage with trypsin decreased the absorbance of the isolated cell envelope much more rapidly than when any other protease was used (Braun and Rehn 1969). Cell envelopes treated with trypsin separated into two membranes, namely the outer membrane and cytoplasmic membrane, as observed by electron microscopy of ultra-thin sections. Samples not treated with trypsin were closely attached to each other. Since trypsin released only small amounts of protein into the medium, the trypsin cleavage site was sought in the insoluble part.

It had been shown earlier that protein was associated with isolated murein, but the protein material was not investigated further because the focus was on the chemical composition of murein (Weidel and Pelzer 1964). Treatment of cell envelopes with a hot sodium dodecyl sulfate solution left murein as the sole insoluble component. It became morphologically thinner when treated with proteases, but kept the size and rod form of the exponentially growing cells from which it was isolated. Acid hydrolysis of murein of exponentially growing cells yielded the constituents of murein and a mixture of all but six common amino acids (Cys, His, Pro, Gly, Phe, and Trp; by contrast, in stationary phase cells, the proteins associated with murein consist of all amino acids). The specific lack of these common amino acids suggested that a defined protein was associated with murein. After trypsin treatment, only lysine remained associated with murein, which indicated that it linked the protein and murein. Further analysis showed that the lysine is bound through the ε-amino group to the optical L-center of diaminopimelic acid of the murein peptide side chain, where it replaces

D-alanine (Braun and Bosch 1972). The lysine residue is at the C-terminal end of Lpp (Fig. 3.1a).

The mature Lpp protein consists of 57 residues and an additional N-terminal modified cysteine. The amino acid sequence reveals 14 repeats composed of seven residues, of which every third or fourth residue is hydrophobic (Fig. 3.1a). Such a periodicity is typical for coiled-coils of α-helices in which seven residues (heptad repeats) form two turns of a helix and that the side chains of parallel helices interlock systematically. Lpp was the first coiled-coil protein to be sequenced (Lupas et al. 2017). Its high α-helical content was deduced from circular dichroism spectra (Braun et al. 1976b). Recombinant Lpp devoid of the lipid forms a long parallel α-helical trimer. The X-ray crystal structure reveals a three-stranded coiled-coil domain

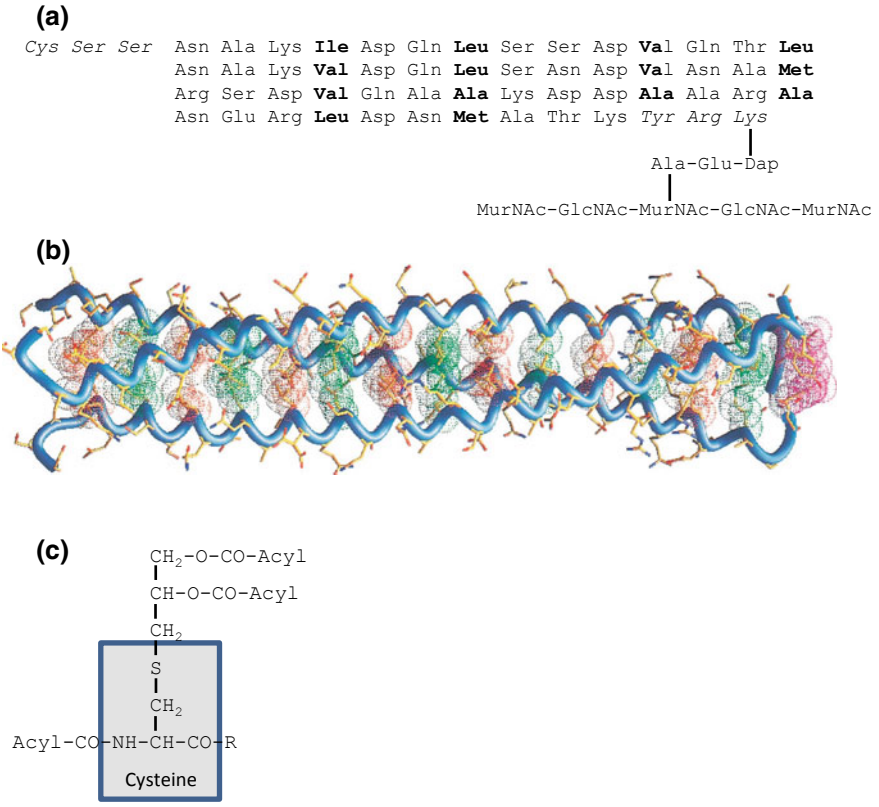

Fig. 3.1 **a** Amino acid sequence of the *E. coli* murein lipoprotein (Lpp) with bound murein (peptidoglycan) subunits. The heptad repeats (3 + 4) are in boldface. The N-terminal lipid attachment site and the C-terminal murein attachment site (in italics) are outside the heptad register. **b** High-resolution crystal structure of the Lpp trimer (residues 2–56) comprising a parallel three-stranded coiled coil and two helix capping motifs (Shu et al. 2000). **c** Chemical structure of glycerylcysteine with three bound fatty acids (mostly palmitic acid)

from residue 5 to 53, with the heptad repeat in register throughout the entire region (Fig. 3.1b) (Shu et al. 2000). The exceedingly high trypsin cleavage rate is explained by the accumulation of three trypsin cleavage sites at the very C-terminus and their exposure outside the triple helix. The sequences Tyr-Arg-Lys and Tyr-Lys-Lys are most frequently found in lipoproteins linked to murein.

Lipid Covalently Bound to the Protein

When structural work on Lpp began, lipoproteins were not defined molecules but rather mixtures of proteins with non-covalently linked mixtures of lipids. But in Lpp, lipid was associated with a protein that could not be removed with detergents or organic solvents and was therefore considered to be covalently bound. The chemical structure of the protein lipid attachment site was determined by chemically degrading isolated Lpp, incorporation of radioactively labeled suspected precursors, and finally by chemical synthesis of *S*-glycerylcysteine thioether (Hantke and Braun 1973). The structure was shown to be a glyceryl group with a fatty acid composition similar to that of phospholipids and bound as a thioether to the sulfhydryl group of cysteine, and was named glycerylcysteine (Fig. 3.1c). The amino group of the N-terminal cysteine is bound to a fatty acid, mainly palmitic acid. This unique lipid structure was later shown to be part of all bacterial lipoproteins with a covalently bound lipid. The first three-dimensional structure of triacylated cysteine in a lipid bilayer was recently found in the electron cryomicroscopy structure of a cytochrome oxidase (Sun et al. 2018). In this structure, the lipid anchors at the ActB and ActE proteins are tilted with respect to the plane of the lipid bilayer, thereby restricting the ability of other lipids to pack around them. The lipids are localized close to the entry point of menaquinol in the complex.

Linkage of Lpp to Murein (Peptidoglycan)

In *E. coli*, one-third of Lpp is covalently bound to murein (Braun and Rehn 1969; Inouye et al. 1972). The linkage is formed between the carboxyl group of the optical L-center of meso-diaminopimelate (Dpm) of the murein tetrapeptide and the ε-amino group of the carboxyl-terminal lysine (Lys) of lipoprotein (Fig. 3.1a) (Braun and Bosch 1972). The reaction is catalyzed by three very similar L-D-transpeptidases, namely ErfK, YcfS, and YbiS (Magnet et al. 2007), whereby the D-Ala linked to Dpm is replaced by Lys and the peptide energy of the Dpm-Ala bond is preserved to form the Dpm-Lys bond. The genes encoding all three enzymes must be deleted to obtain murein free of Lpp, but YbiS plays the major transpeptidation because the deletion of its gene largely prevents binding of Lpp to murein. The presence of several transpeptidases is not surprising because redundant enzymes in murein synthesis and murein modification have been frequently observed (Pazos et al. 2017). Murein is

essential for the structure of cells that they cannot afford a defective synthesis. In addition, the structure of murein changes under different growth condition which requires different enzyme activities (Vollmer et al. 2008).

The C-terminal Lys in the Tyr-Arg-Lys triad plays a critical role in linkage of Lpp to murein (Zhang and Wu 1992; Zhang et al. 1992). By contrast, alterations at the N-terminus, such as lack of lipid modification, addition of a signal peptide, and fusion of the outer membrane protein OmpF, reduce but do not abolish covalent attachment of Lpp to murein (Zhang et al. 1992). The amount of Lpp bound to murein can vary; for example, in stationary phase cells, Lpp binding increases by 70% (Glauner et al. 1988).

Free Lpp

Free Lpp not attached to murein was detected when proteins of cell envelopes labeled with radioactive arginine or histidine were separated by SDS-polyacrylamide gel electrophoresis (Inouye et al. 1972). A fast migrating band, i.e., a small protein, contained labeled arginine but not histidine. The only protein known to be devoid of histidine in *E. coli* was the small protein Lpp. Its identity was confirmed by double labeling with other amino acids present and absent in Lpp. The electrophoretic mobility of free Lpp was faster than that of Lpp released from murein by lysozyme; the latter preparation also contains muropeptides. The amount of free Lpp was twice as high as that of murein-bound Lpp.

Distinct Arrangement of Bound and Free Lpp in the Outer Membrane

The early proposal (Braun and Rehn 1969) that Lpp extends from murein across the periplasm into the outer membrane was supported by the immunological determination of Lpp in separated outer and cytoplasmic membranes (Bosch and Braun 1973). Lpp is bound by the C-terminal lysine to diaminopimelic acid of murein and is fixed to the outer membrane by insertion of the lipid part into the inner lipid leaflet of the outer membrane. It was assumed that free lipoprotein occupies the same location as the bound form in the cell envelope. This assumption was supported by cross-linking the free form with the bound form and non-covalent binding of the free form by the C-terminal lysine to murein (Choi et al. 1987). However, distinct cellular locations of bound and free Lpp were discovered over two decades later (Cowles et al. 2011). Free Lpp spans the outer membrane and its C-terminus is surface-exposed, whereas bound Lpp resides in the periplasm. This conclusion was drawn from differential labeling with membrane-impermeable biotin reagents and proteolytic removal of the labels at the cell surface. The biotin label was attached to the C-terminal Lys55

and Lys58 residues. In addition, the C-terminal FLAG epitope (DYKDDDDK) of a constructed Lpp derivative could be proteolytically removed by treating cells with trypsin, which showed that the C-terminus of free Lpp is exposed at the cell surface.

The question arises how the free form inserts into and is partially translocated across the outer membrane. The parallel three-stranded coiled-coil structure of Lpp is too hydrophilic to insert spontaneously into a lipid bilayer. Since the free form is first synthesized and then converted to the bound form (Inouye et al. 1972), the two forms might transiently associate with each other, which would explain their cross-linkage. The free form is either translocated by the Bam complex, or by a specific translocase. Specific translocases were identified for various proteins, e.g., NalP or RcsF with the Bam complex, pullulanase with the type II secretion system, and in *Neisseria* Slam1 with TbPB, LbpB, fHb1, and Slam2 with HpuA (see section "Lipoproteins in Other Gram-Negative Bacteria"). The translocation of lipoproteins to the cell surface has recently been reviewed (Hooda and Moraes 2018).

Biosynthesis of Lpp

E. coli Lpp is synthesized in the cytoplasm, modified with lipids at the outer leaflet of the cytoplasmic membrane, and translocated to and inserted into the outer membrane. All these steps are mediated by dedicated proteins.

Unmodified lipoprotein can be synthesized in vitro with an mRNA of about 250 nucleotides (Hirashima et al. 1974). The mRNA has an unusually long average half-life of 11.5 min, in contrast to the half-life of 2.1 min of cytoplasmic protein mRNAs (Hirashima and Inouye 1973). The long half-life is suitable for allowing synthesis of this most abundant protein in *E. coli* (about 10^6 molecules per cell).

Lpp is synthesized in the cytoplasm with a signal sequence that serves to transfer the protein across the cytoplasmic membrane by the Sec translocon. The cysteine residue located at the border between the signal sequence and the mature protein is strictly conserved in all lipoproteins. It is surrounded by a partially conserved sequence in lipoproteins—Leu(Ala/Val)-Leu-Ala(Ser)-Gly(Ala)-Cys. This so-called lipobox is recognized by lipid-modifying enzymes. Lipid modification takes place at the periplasmic side of the cytoplasmic membrane. First, diacylglycerol is added to the sulfhydryl group of the cysteine by phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt) (Fig. 3.2). Then, a special lipoprotein signal peptidase (Lsp or signal peptidase II) cleaves the signal peptide in front of the Cys residue (Innis et al. 1984). Signal peptidase II preferentially recognizes didecanoyl glycerol as the optimal lipid length and exclusively the enantio (*R*) form of the diacylglycerol (Kitamura and Wolan 2018). The protein remains attached to the cytoplasmic membrane via the diacylglycerol. Finally, a fatty acid is transferred from phospholipids to the amino group of the Cys residue by membrane-bound phospholipid/apolipoprotein transacylase (Lnt) (Tokunaga et al. 1982; Noland et al. 2017).

Genes encoding the three enzymes Lgt, Lsp, and Lnt are essential for *E. coli* and most other Gram-negative bacteria. The crystal structure of the diacylglyceryl

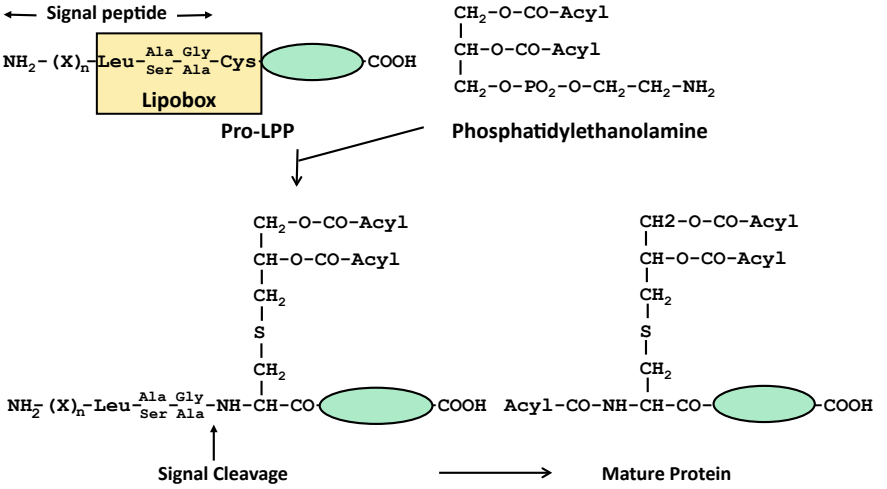

Fig. 3.2 Biosynthesis of Lpp. Refer to the text for details. The polypeptide chain of LPP is indicated in green

transferase Lgt shows a central cavity about 20 Å deep, with a major opening to the periplasmic side and two clefts exposed to the membrane lipid (Mao et al. 2016). Phosphatidylglycerol is positioned with the two acyl chains in the lower hydrophobic part of the cleft and the hydrophilic head protruding into the interface between the outer leaflet of the cytoplasmic membrane and the periplasm. It is predicted that the two clefts (6 and 10 Å wide) are entrances for phosphatidylglycerol and the lipobox of the lipoprotein. Substrates and product enter and leave laterally relative to the lipid bilayer.

The crystal structure of the Lsp signal peptidase II from *Pseudomonas aeruginosa* complexed with the inhibitor globomycin has been determined (Vogeley et al. 2016). The enzyme consists of a periplasmic domain fixed to the cytoplasmic membrane by a domain of four transmembrane helices. Globomycin acts as a noncleavable peptide that sterically blocks the active site. It mimics diglyceride Lpp in that it is composed of a 19-member cyclic depsipeptide that includes an α-methyl-β-hydroxy fatty acid. It is first partitioned in the cytoplasmic membrane and then diffuses laterally into the active site of the enzyme. The lipopeptide helix of Lpp fits into the space between membrane helices 12 and 14, which positions the Cys residue of the lipobox into the active site while leaving the protein in the periplasm. It is predicted that Lsp is an aspartyl endopeptidase with two Asp residues suitably positioned to form the catalytic diad. After release of the signal peptide, lipoprotein moves out of the active site but remains bound to the cytoplasmic membrane by the diacylglyceride moiety.

The crystal structures of the *N*-acylase Lnt of *E. coli* (Noland et al. 2017) and *P. aeruginosa* (Wiktor et al. 2017) have been determined. Both structures are composed of a large periplasmic domain that is fixed to the cytoplasmic membrane by eight transmembrane helices. The active site with the catalytic Cys387 is positioned at