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Outer membrane biogenesis in *Escherichia coli*: genetic and physiological cell response to lipopolysaccharide transport defects

Federica Anna Falchi

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Scientific tutor: Gianni Dehò

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Federica Anna Falchi

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Abstract

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The outer membrane (OM) of Gram-negative bacteria is an asymmetric bilayer formed by phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, with a large number of embedded or associated proteins. The primary function of this structure essential for Gram-negative viability is to establish an additional selective permeability barrier that enables the cell to maintain favourable intracellular conditions even in harsh environments and the LPS layer greatly contributes to this peculiar property. The transport of LPS to the cell surface is an essential process for OM biogenesis; the LPS transport (Lpt) system, originally identified in *E. coli*, is the protein machine responsible for LPS delivery from the periplasmic side of the inner membrane (IM) to the OM. It is composed of seven proteins forming a complex which spans from IM to OM. At the IM the ABC transporter LptBFG, associated to the membrane-bound protein LptC interacts with the periplasmic protein LptA that connects, through structurally conserved domains, the IM ABC transporter with the OM translocon LptDE, responsible for LPS assembly at the cell surface.

In order to gain more insight in the mechanism of LPS transport and more in general in OM homeostasis we used both a genetic and a proteomic approach. The former was based on the selection of suppressors of LPS transport defects obtained with two different types of mutants. i) a quadruple non-lethal *lptA* mutant (*lptA41*) that displayed increased sensitivity to toxic compounds, and ii) a lethal deletion mutant of *lptC*.

Genome sequencing analysis of spontaneous suppressors of *lptA41* phenotype revealed two different mechanism of suppression: one mechanism involves the Mla system, a protein machinery which contributes to maintain OM asymmetry; the second mechanism involves both an intragenic mutation improving LptA41 protein stability and an extragenic mutation affecting osmoregulated periplasmic glucans (OPGs) synthesis.

Viable mutants lacking *lptC* were obtained using a plasmid shuffling technique. Genome sequencing of such mutants revealed single amino acid substitutions at the R212 residue of the IM component LptF (*lptF*^{Sup}mutants). Our results suggest that LptC may serve as a chaperon of the Lpt machine assembly and/or activity rather than an essential structural component and the periplasmic domain of LptF might be implicated in the formation of the Lpt bridge.

The latter approach consisted of the analysis of differential envelope proteins content of an *E. coli* *lptC* conditional expression mutant upon depletion of LptC and thus impairment of LPS transport. By

two-dimensional chromatography coupled to tandem mass spectrometry (Multidimensional Protein Identification Technology, MudPIT) we identified 123 proteins whose level is significantly modulated upon LptC depletion. Most of these proteins belong to pathways that contribute to repair OM and restore its permeability barrier properties, including protein involved in maintaining OM asymmetry, in the synthesis of phospholipids and exopolysaccharides as substrate for lipid A modification enzymes, and in peptidoglycan synthesis/remodelling.

Overall these data contribute to our understanding of the multiple strategies that *E. coli* cells may adopt to respond to perturbations of the OM permeability barrier and to restore OM functionality.

Abbreviations

ABC	ATP-binding cassette
ACP	Acyl carrier protein
ATP	Adenosine triphosphate
Bam	β-Barrel assembly machine
CAMP	Cationic antimicrobial peptide
GlcNAc	*N*-Acetyl-D-glucosamine
IM	Inner membrane
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
L-Ara4N	4-amino-4-deoxy-L-arabinose
Lol	Localization of lipoproteins
LPS	Lipopolysaccharide
Lpt	Lipopolysaccharide transport
Mla	Maintenance of OM lipid asymmetry
NBD	Nucleotide binding domain
OM	Outer membrane
OMP	Integral outer membrane protein
PEtN	phosphoethanolamine
PL	Phospholipid
SEC	General secretory pathway
TMD	Transmembrane domain

1.1

Outer membrane biogenesis in *Escherichia coli*:
genetic and physiological cell response to lipopolysaccharide transport defects

1.2 Introduction

All living cells are surrounded by the cytoplasmic membrane composed of a symmetric lipid bilayer and its associated proteins, whose architecture is conserved among *Bacteria, Eukarya*, and, although with relevant peculiarities, in *Archaea*. The cell envelope, however, may be a much more elaborated construction, as most organisms have developed diverse complex structures outside of the cytoplasmic membrane that provide additional properties to the cell, including increased mechanical strength, shape determination, selective permeability barrier, specific interactions with other cells, organisms, and environments.

Typically, Gram-negative *Bacteria* are surrounded by two biological membranes, the cytoplasmic (or inner) membrane (IM) and the outer membrane (OM). In such bacteria, which are thus more properly described as diderms (Gupta, 1998; Sutcliffe, 2010; Desvaux *et al.,* 2009) the OM is an essential structure that, together with the IM, delimits an aqueous compartment, the periplasm, which contains a cell-wall composed of peptidoglycan (reviewed by Silhavy *et al*., 2010). Different diderm phyla may have OM of different architectures (reviewed by Sutcliffe *et al*., 2010); needless to say that the OM has been most extensively studied in *Escherichia coli* and few other γ-P*roteobacteria*, although relevant information have been also obtained in other *Proteobacteria* such as *Neisseria meningitidis; Helicobacter pylori, Bordetella parapertussis.* Several structural and functional aspects differentiate the OM from the IM, the most striking structural difference being the asymmetry of the OM lipid bilayer.

1.3 The Outer Membrane is an asymmetric permeability barrier

The OM is an asymmetric bilayer consisting of phospholipids and glycolipids, principally lipopolysaccharide (LPS), in the inner and in the outer leaflet, respectively (Nikaido, 2003). Proteins are associated to the lipidic bilayer as either integral OM proteins (OMP) or lipoproteins (Bos *et al*., 2007). The primary function of the OM is to establish a permeability barrier that enables the cell to maintain favourable intracellular conditions even in harsh extracellular environments. While typical membrane bilayers are impermeable to polar solutes, the OM is impermeable also to lipophilic molecules (Nikaido, 2003). This property of the OM is attributed to LPS; in fact, the presence of LPS causes the OM to be approximately two orders of magnitude less permeable to

lipophilic substances than an equivalent phospholipid membrane bilayer (Plesiat and Nikaido, 1992).

LPS is an amphipathic molecule composed of lipid A, a core oligosaccharide, and a long polysaccharide called O-antigen (Raetz and Whitfield, 2002) (Fig. 1).

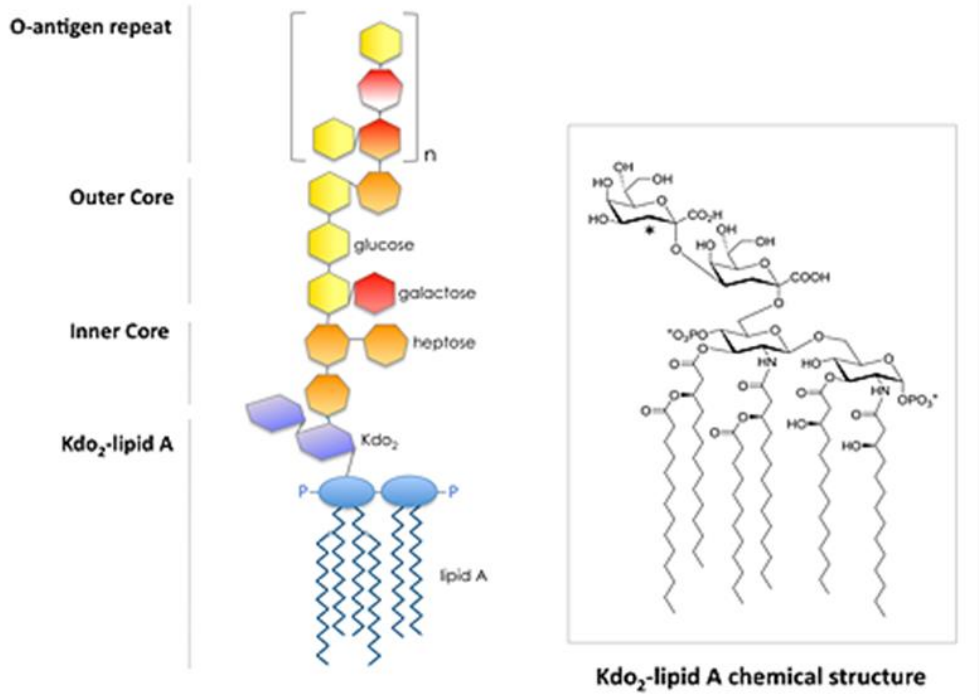

Figure 1. General structure of LPS. LPS is composed of lipidA, a core oligosaccharide and a highly variable O-antigen constituted of repeating oligosaccharide units. Lipid A is connected to a Kdo dimer to give the minimal form of LPS needed for viability. Chemical structure of Kdo_2-lipid A is shown on the right. From Sperandeo *et al*., 2014.

Lipid A is a unique glycolipid consisting of a phosphorylated β-(1→6)-glucosamine disaccharide backbone decorated with several acyl chains. The core is covalently linked to lipid A and can be divided into inner and outer core. The inner core composition is less variable and normally characteristic within a genus or a family; the first residue of the core region linked to lipid A is 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). Kdo is peculiar sugar acid present in the LPS core of *Enterobacteriaceae* and represent the chemical hallmark of LPS and a marker of Gram-negative bacteria (Holst, 2007). The O-antigen is the distal, surface exposed LPS moiety. It is responsible of the immunogenic properties of the molecule and is the most variable LPS portion (Raetz and Whitfield, 2002). The O-antigen moiety is not essential and is missing in common laboratory *E. coli* K12 strains due to inactivation of the *wbbL* gene (Reeves *et al*., 1996; Rubires *et al*., 1997); however, it is present in most wild type strains and clinical isolates where it contributes to virulence by protecting bacteria from phagocytosis and complement-mediated killing (Trent *et al*., 2006).

LPS is essential in most Gram-negative bacteria, a known exception being *Neisseria meningitidis* (Steeghs *et al*., 1998). The minimal LPS structure required for viability, however, varies among different species. In *E. coli* it has been defined as Kdo_2-lipidA (Raetz and Whitfield, 2002), whereas *Pseudomonas aeruginosa* requires to be viable the full inner core and at least part of the outer core (Rahim *et al*., 2000; Walsh *et al*., 2000).

Within the LPS layer, the negative charges of phosphate groups present on adjacent molecules are neutralized by the presence of cations like Mg^{2+} and Ca^{2+} and the acyl chains are widely saturated, thus facilitating tight packing. Moreover the porins limit diffusion of hydrophilic molecules larger than about 700 Daltons, contributing to create a very effective selective permeability barrier (Nikaido, 2003).

LPS organization can be disrupted by defects in OM components assembly (Ruiz *et al*., 2006), in mutant producing LPS truncated in sugar chains (Young and Silver, 1991) or by exposure to antimicrobial peptides and chelating agents such as EDTA, which displace divalent cations between LPS molecules (Nikaido, 2003). In all these cases the consequence is that much of the LPS layer is lost and PLs migrate from the inner to the outer leaflet, generating locally symmetric bilayer rafts that are more permeable to hydrophobic molecules (Nikaido, 2005). Cells have evolved systems to monitor the asymmetry of the OM and to respond either by removing PLs from the outer leaflet or by modifying LPS. Two main mechanisms have been described that restore OM asymmetry by acting on PLs: the phospholipase OmpLA and the Mla pathway.

OmpLA, encoded by *pldA*, is a phospholipase that normally resides as an inactive monomer at the OM; however, in the presence of PLs a catalytically active OmpLA dimer is formed. Activated OmpLA degrades PLs that have accumulated in the outer leaflet of the OM under stress conditions (Dekker, 2000).

The Mla (Maintenance of OM lipid asymmetry) proteins constitute a highly conserved ATP-binding cassette (ABC) transport system that prevents PLs accumulation in the outer leaflet of the OM under non-stress conditions. Mutations in the Mla system are not lethal but lead to PLs accumulation in the outer leaflet of the OM (Malinverni and Silhavy, 2009). It comprises at least six protein distributed across the cell envelope. MlaA (formerly VacJ) is a predicted OM lipoprotein, MlaC is a periplasmic protein, and MlaFEDB form a putative ABC transporter (Malinverni and Silhavy, 2009). Recently MlaA has been found to interact specifically with the OM β-barrel OmpC (Chong *et a*l., 2015) The evidence that cells lacking OmpC accumulate PLs in the outer leaflet of the OM in stationary phase indicate a role for OmpC in maintaining lipid asymmetry, thus suggesting OmpC to be an additional OM component of the Mla system (Fig. 2) (Chong *et a*l., 2015).

An alternative response to OM asymmetry perturbation consists in LPS modification. As will be discussed in paragraph 1.3.2.2, LPS can be palmitoylated at the position 2 of lipid A by PagP, an OM β-barrel acyltransferase that uses PLs flipped in the OM as substrates (Bishop *et al*., 2000). The product of the PagP reaction is a hepta-acylated LPS which possesses increased hydrophobicity (Bishop, 2008) thus contributing to better packing within the LPS layer.

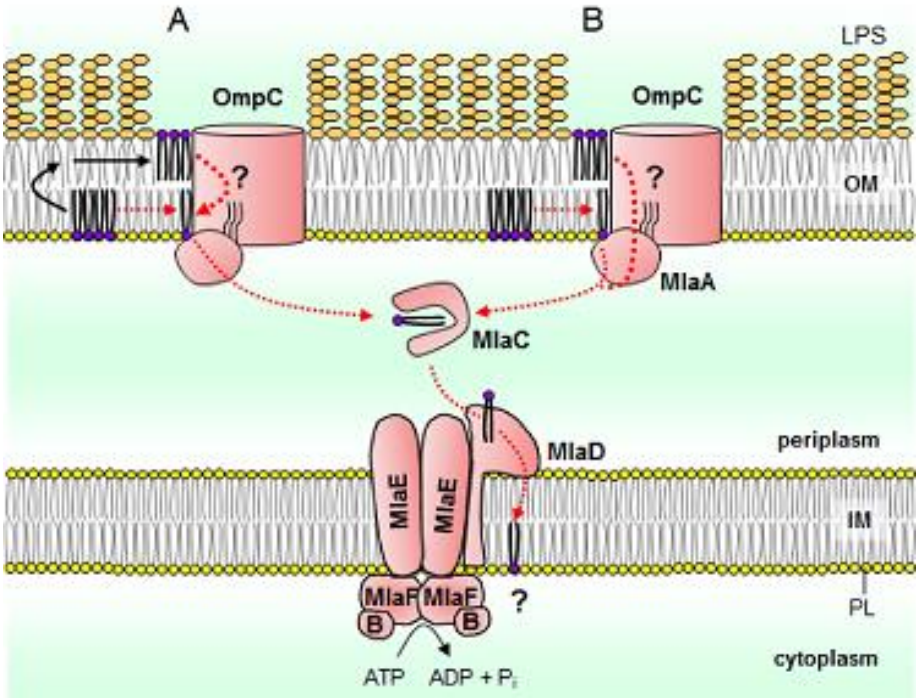

Figure 2. MlaA-OmpC working model. MlaA (VacJ) may extract PLs from the inner leaflet of the OM to prevent flipping into the OM. MlaA form a complex with OmpC that functions to remove PLs directly from the outer leaflet. (A) OmpC may allow PLs to be flipped back into the inner leaflet, where they are removed by MlaA or (B) OmpC may allow MlaA to become surface exposed, thus giving access to PLs accumulated in the outer leaflet. Removed PLs may be routed back to the IM by the rest of the Mla system. From Chong *et al*., 2015.

BOX 1. Antibiotics that affect cell envelope biogenesis: bacitracin action and resistance mechanisms.

Bacitracin is a cyclic polypeptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis* (Johnson *et al*., 1945) that specifically interferes with cell wall biosynthesis by interfering with generation of undecaprenyl phosphate (C_{55}-P) from its precursor undecaprenyl pyrophosphate (C_{55}-PP). C_{55}-P is an essential lipid carrier required for the synthesis of bacterial cell wall polysaccharides such as PG, LPS O-antigen, and teichoic acids (van Heijeenort, 2001; Raetz and Whitfield, 2002; Nauhaus and Baddiley, 2003). It is synthesized *de novo* as C_{55}-PP on the cytoplasmic side of the inner membrane and then dephosphorylated by an undecaprenyl pyrophosphatase prior to its use. C_{55}-P transports various hydrophilic precursors in the C_{55}-PP-substrate form across the hydrophobic inner membrane, transfers the oligosaccharide unit to the growing polymer on the periplasmic side, and is then released as C_{55}-PP. (Touzé *et al*, 2008). It thus requires a second dephosphorylation step for its recycling as a C_{55}-P carrier molecule.

Bacitracin prevents C_{55}-PP dephosphorylation, by specifically binding to a complex of C_{55}-PP and metal cations, thereby disrupting regeneration of C_{55}-P (Stone and Strominger, 1971; Storm and Strominger, 1973). As a result cell wall biosynthesis is inhibited and cell lysis occurs (Siewert and Strominger, 1967; Stone and Strominger, 1971; Storm and Strominger, 1973).

Several mutations leading to bacitracin resistance were identified in *E. coli* and other Gram-negative bacteria. Interestingly, some mutations interfere with the synthesis of non-essential cell envelope polymers such as osmoregulated periplasmic glucans (OPGs) and capsule polysaccharides that also require the C_{55}-P for their formation. It has been proposed that in these mutants the reduced requirement for C_{55}-P results in higher availability of the lipid carrier for the synthesis of essential polymers (PG and LPS) and, as a consequence, in increased tolerance to bacitracin (Pollock *et al* 1994; Fiedler and Rotering, 1998).

Overexpression of genes encoding proteins with a C_{55}-PP phosphatase activity has also been associated to bacitracin resistance (Cain *et al*., 1993¸ El Ghachi *et al*., 2005). In *E. coli* different proteins with C_{55}-PP phosphatase activity have been described: the membrane-bound BacA, and several members of a superfamily of phosphatase with a common phosphatase sequence motif, including YbjG, PgpB and LpxT. The latter, LpxT, transfers a phosphate group from the undecaprenyl pyrophosphate donor to lipid A to form lipid A 1-diphosphate (Touzé *et al*., 2008; Valvano, 2008). It has been proposed that the overexpression of phosphatase activity might accelerate the conversion of the pool of C_{55}-PP to C_{55}-P, thereby reducing the availability of the bacitracin specific target (Cain *et al*., 1993).

1.3 Overview of Outer Membrane biogenesis

OM components synthetized in the cytoplasm (proteins) or at the inner leaflet of the IM (LPS and PLs) must not only be translocated across the IM, but must also cross the aqueous periplasmic space and be assembled in the amphipathic bilayer. The compartments outside the IM are devoid of ATP or other high-energy carriers. Thus the energy required for OM biogenesis must be provided by exergonic reactions involving substrates energized in the cytoplasm or be transduced by devices connected to the IM. Commonly these devices are protein machines able to use the energy released by ATP hydrolysis in the cytoplasm or the proton motive force. Only in recent years the protein systems responsible for the transport of lipoproteins, OMPs, and LPS have been identified and aspects of their mechanisms have been elucidated.

1.3.1 OM proteins and Lipoproteins

Cell-environment exchanges across the OM are ensured by OM proteins, which are implicated in several functions: nutrients uptake, transport and secretion of various molecules (proteins, polysaccharides, drugs), assembly of proteins or proteinaceous structures at the OM.

Typical OM integral proteins (OMPs) are β-barrel proteins (Rigel *et al*., 2012), whereas OM-associated proteins are generally lipoproteins that are anchored to the periplasmic side of the OM via a lipid tail attached to an N-terminal *N*-acyldiacylglycerylcysteine residue (Sankaran and Wu, 1994).

OMPs and lipoproteins are synthesised as pre-proteins in the cytoplasm and then secreted across the IM by the SEC translocase, a universally conserved machine that transports unfolded proteins (Du Plessis *et al*., 2011). Lipoproteins are then processed into mature forms on the periplasmic side of cytoplasmic membrane where a lipid moiety is attached to the N terminus to anchor these proteins to the membrane (Tokuda, 2009). The Lol system, composed of five essential proteins, is responsible for the transport of lipoproteins with OM location (Fig. 3). LolCDE constitute an ABC transporter and mediate the detachment of lipoproteins from the IM and their transfer to the periplasmic chaperone LolA. The hydrophilic complex lipoprotein-LolA crosses the periplasm and LolA transfers its cargo to LolB at the OM, where lipoproteins are incorporated into the lipid bilayer (Okuda and Tokuda, 2011). Once secreted in the periplasm, misfolding of β-barrel OM proteins precursors is prevented by molecular chaperones, such as SurA and Skp (Sklar *et al.,*

2007) which deliver OMPs to the Bam complex (Fig. 3), a molecular machine driving β-barrel assembly (Ricci and Silhavy, 2012).

The Bam machinery consists of the OM β-barrel protein BamA and four lipoproteins BamB, BamC, BamD, and BamE. The Bam complex is a modular molecular machine in which BamA forms the protein-lipid interface at which OMP substrates enter into the lipid phase of the membrane. BamB interacts with BamA and is proposed to form a scaffold to assist β-barrel folding. BamB, BamC, and BamD interact and form a module suggested to drive a conformational switch in the Bam complex that enables β-barrel insertion into the OM (Ricci and Silhavy, 2012).

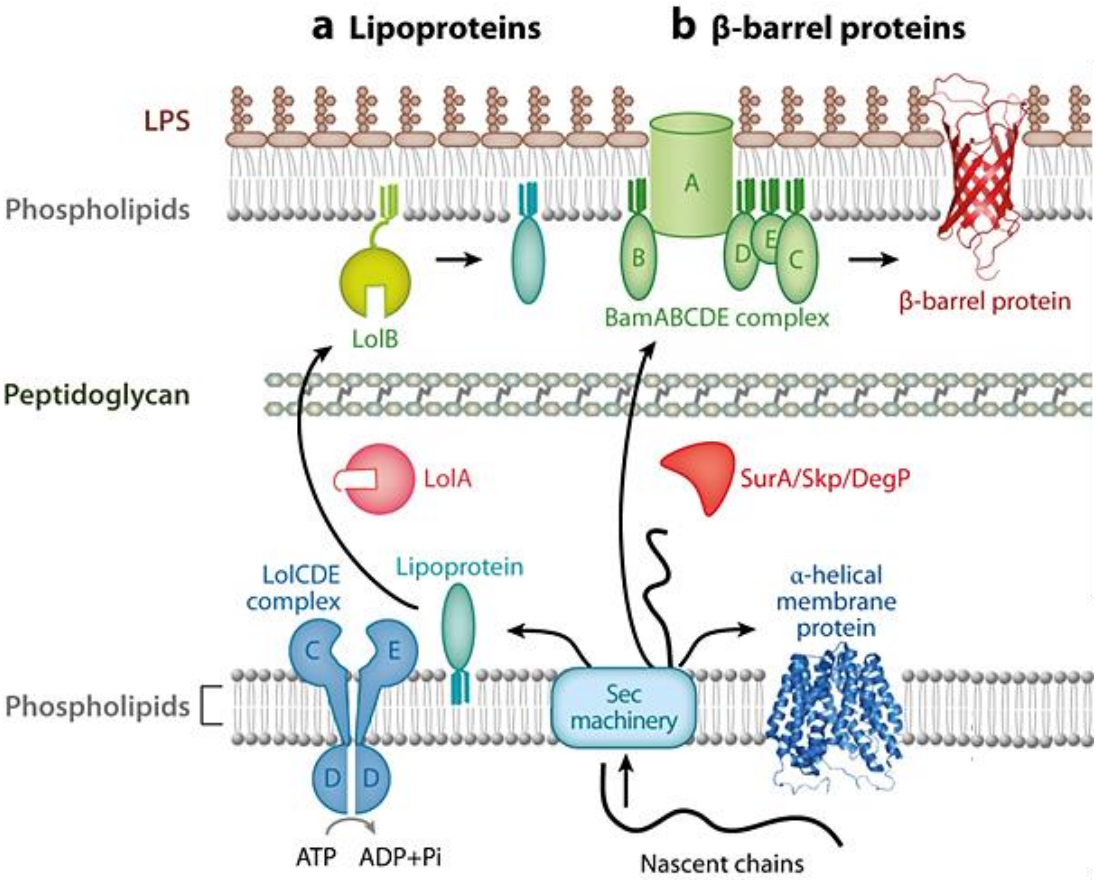

Figure 3. Lipoproteins and OMPs biogenesis. Periplasmic and OM proteins are synthesized as precursors with a signal peptide at their N termini in the cytoplasm and are then translocated across the IM by a Sec translocon. (a) OM lipoproteins are released from the IM in an ATP-dependent fashion and translocated to the OM by the Lol system. (b) OMPs are inserted into the OM from the periplasm by the Bam machine, consisting of the β-barrel protein, BamA, and four lipoproteins, BamB/C/D/E. Periplasmic chaperones, SurA, Skp, and DegP, are involved in the formation of the folded β-barrel structure. (Modified from Okuda and Tokuda 2011).

1.3.2 Lipopolysaccharide biogenesis in the Inner Membrane

The biosynthesis of LPS is a complex process that occurs in three different cellular compartments, cytoplasm, IM and periplasm, thus, requiring spatial and temporal coordination of several independent biosynthetic pathways (Raetz and Whitfield, 2002; Valvano, 2003; Samuel, 2003) (Fig. 4).

The first stage of the biosynthetic pathway is the synthesis of Kdo_2-lipid A. The pathway is mediated by nine enzymes and takes place in the cytoplasm and on the inner surface of inner membrane. The initial building block of lipid A is UDP-N-acetylglucosamine (UDP-GlcNAc). The first three reactions are catalyzed by soluble enzymes LpxA, LpxC and LpxD, resulting in the addition of two 3-OH fatty acid chains to the 2- and 3-positions of the UDP-GlcNAc to form UDP-diacyl-GlcN (Jackman, 1999). Next, reactions catalyzed by LpxH, LpxB, and LpxK result in the synthesis of the tetra-acylated lipid IV_A that, in *E. coli*, is the substrate of WaaA, the CMP-Kdo dependent transferase that catalyzes the sequential incorporation of two Kdo residues synthesized in a separate pathway (Raetz and Whitfield, 2002). Two further acylation reactions lead to the synthesis of the hexaacylated Kdo_2-lipid A (Clementz *et al*., 1996, Clementz *et al*., 1997). The additional sugars composing the oligosaccharide core are added to Kdo_2-lipid A by specific glycosyl-transferases to generate the core-lipid A structure.

The core-lipid A, anchored to the IM, is then flipped across the IM by the ABC transporter MsbA, thus becoming exposed to the periplasm (Polissi and Gergopoulos, 1998; Zhou *et al*., 1998). O-antigen repeat units are synthesized in the cytoplasm, flipped to the periplasmic face of the IM linked to the lipid carrier undecaprenyl diphosphate and then ligated to core-lipid A by the WaaL ligase, thus forming a mature LPS molecule (Perez *et al*., 2008).

The enzymes for lipid A and Kdo biosynthesis are constitutively expressed (Raetz and Whitfield, 2002; Raetz *et al*., 2009). However, in *E. coli* the synthesis of Kdo_2-lipid A is post-transcriptionally regulated by FtsH, an essential IM metalloprotease, in conjunction with the recently identified heat shock protein LapB (previously YciM) (Ogura *et al*., 1999; Klein *et al*., 2014). LpxC catalyzes the first committed step of the lipid A biosynthetic pathway (Sorensen *et al*., 1996) and FabZ is the enzyme that catalyzes the first key step of PLs biosynthesis, thus competing with LpxC for their common precursor molecule, R-3–hydroxymyristoyl ACP. Increased cellular levels of LpxC are lethal to the cells due to the excess of LPS over PLs (Ogura *et al*., 1999), thus regulation of LpxC by FtsH and LapB is crucial for this biosynthetic checkpoint. The absence of FtsH or LapB result in an increased LpxC level and consequently an increased LPS level and this phenomenon can be compensated by suppressor mutations in *fabZ* gene or by *fabZ* overexpression

(Ogura *et al*., 1999; Klein *et al*., 2014; Mahalakshmi *et al*., 2014). Recently, LapB was found to co-purify WaaC, the enzyme responsible for transfer of the first heptose sugar onto the Kdo_2 moiety, and LPS transport (Lpt) proteins, suggesting that LapB could serve as a docking site for the LPS assembly by various IM-associated or IM-anchored enzymes, ensuring that only completely synthesized LPS molecules are translocated (Klein *et al*., 2014). FtsH also controls the turnover of WaaA, the enzyme catalyzing incorporation of Kdo residues in lipid IV_A (Katz *et al*., 2008).

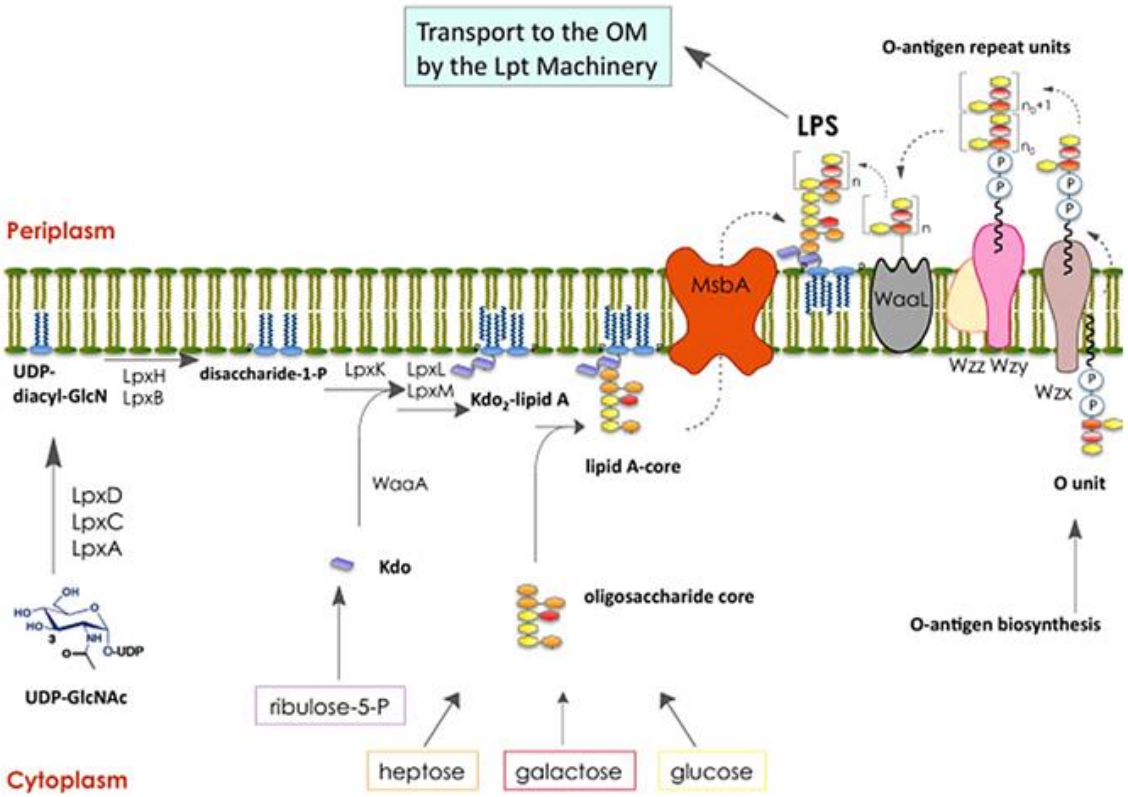

Figure 4. LPS biosynthesis in *E. coli*. UDP-diacyl-GlcN is synthesized in the cytoplasm by LpxA, LpxC and LpxD enzymes. The synthesis of β-(1→6) disaccharide (Disaccharide-1-P) requires LpxH and LpxB. LpxK synthetizes the tetra-acylated lipid IV_A (not shown) that is converted in Kdo_2-lipid A by the sequential action of WaaA (which transfers two molecules of Kdo) and the acyltransferases LpxL and LpxM. Core oligosaccharide is assembled on Kdo_2-lipid A via sequential glycosyl transfer of sugar precursors. The lipid A-core is then flipped across the IM by the ABC transporter MsbA. *O*-antigen repeat units are synthesized in the cytoplasm and at the IM; they are then transported and polymerized by a separated pathway (Wzx-Wzy dependent pathway). WaaL ligase catalyzes Lipid A-core ligation to O-antigen at the periplasmic face of the IM. LPS is then delivered to the Lpt machinery. From Sperandeo *et al*., 2014.

1.3.2.1 LPS transport across the IM

After its biosynthesis, the lipid A-core is anchored to the IM with its hydrophilic moiety exposed to the cytoplasm and is then flipped across the IM by the essential ABC transporter MsbA. MsbA was originally identified in *E. coli* as a multicopy suppressor of the thermosensitive phenotype of a *lpxL* (formerly *htrB*) insertion mutant (Karow and Georgopoulos, 1992; see Box 2). LpxL is a lauroyl acyltransferase involved in LPS biosynthesis (Clementz *et al*., 1996). *lpxL* null mutants are not viable at temperatures above 33°C and when exposed to non-permissive temperature show cell morphology alterations (such as formation of bulges and filaments), accumulate PLs and tetra-acylated LPS precursor in the IM (Polissi and Georgopoulos, 1996; Zhou *et al*., 1998). In the *lpxL* null mutant, these phenotypes can be suppressed by the overexpression of *msbA* from a plasmid vector, thus suggesting that MsbA overexpression facilitates the transport of the immature LPS form to the OM, despite lipid IV_A acylation is not restored (Zhou *et al*., 1998). By contrast, MsbA depleted cells accumulate hexa-acylated lipid A at the IM (Zhou *et al*., 1998). The MsbA function as a flippase was finally verified by demonstrating that modification of LPS by aminoarabinose (L-Ara4N) and phosphoethanolamine (PEtN), which occurs at the outer leaflet of the IM, depends on MsbA (Doerrler *et al.,* 2004).

MsbA functions as a homodimer and each monomers is composed of a nucleotide binding and a transmembrane domains (NBD and TMD, respectively) (Doshi and van Veen, 2013). Furthermore, X-ray crystallography has revealed multiple conformations of MsbA including two inward-facing states where the NBDs undergo a large conformational change and fluorescence resonance energy transfer has revealed that binding of lipid A induces NBDs dimerization and stimulates ATP hydrolysis (Doshi and van Veen, 2013). Recently, these conformational changes have been studied in a membrane-mimicking environment by single particle electron microscopy and it has been demonstrated that most of MsbA molecules without ATP were in a closed or moderately open state (Moeller *et al*., 2015). This conformational change is thought to insert lipid A into the outer leaflet of the IM.

Interestingly, MsbA has also been implicated in multidrug transport (Reuter *et al*., 2003). Lipid A and amphipathic drugs can bind MsbA at the same time, suggesting that MsbA has separate binding site for these two types of substrates and may function as both a lipid flippase and a multidrug transporter (Siarheyeva and Sharom, 2009).

BOX 2. Genetic suppressors, the powerful tool

The identification of genetic suppressors is a commonly used strategy to identify functional relationships between genes. The strategy is to begin with a strain that already contains a mutation affecting the pathway of interest, and to select for mutations able to modify its phenotype. Those mutations that restore a wild-type phenotype despite the continued presence of the original mutation are termed suppressors. Two are the main reasons that make this strategy so powerful. First, a pre-existing mutation often sensitizes the pathway under study, and might thus allow the identification of additional components. Second, suppression of a pre-existing phenotype establishes a genetic relationship between genes that might not have been established by other methods (Prelich, 1999).

Among the described suppression mechanism, the simplest one is intragenic suppression, in which the primary mutation is compensated by a second mutation in the same gene affecting a different codon and causing an amino acid change in another position (Patterson, 1998; Nonet and Young, 1989).

A second class comprises mutations that alter the amount of the original mutant protein by affecting gene expression, transcription, mRNA processing (Hodgkin, 1989) or protein stability (Nouraini *et al*., 1997). Alternatively the suppressor mutation can lead to a modification of the activity of the original protein and particularly of its ability to interact with another protein, disrupting or restoring that kind of interaction.

Additional suppression mechanisms consist in the alteration of the mutant pathway or the alteration of a different pathway. The first class comprises mutations that affect one step of a multi-step pathway, often leading to the identification of other component of the pathway or facilitating ordering of the pathway (Huang and Sternberg, 1995; Avery and Wasserman, 1992). The second class comprises those suppressors that might affect the regulation of a pathway that has related or overlapping function or might alter the specificity of a functional unrelated pathway (Shuman and Beckwith, 1979).

1.3.2.2 Lipid A modification systems

Following MsbA mediated translocation, the core-lipid A moiety may be covalently modified during and after trafficking to the cell surface, resulting in the wide variety of lipid A structures that can be observed across species. Lipid A modifications are not essential, but are often necessary under specific conditions, such as host colonization (Raetz *et al*., 2007) and the involved enzymes

are subjected to both transcriptional and post-translational regulation (Fig. 5). To date, several two-component systems have been implicated in transcriptional control of lipid A modifications enzymes, including the PhoP/PhoQ and PmrA/PmrB systems in *E. coli* and *Salmonella* and the ParR/ParS, ColR/ColS and CprR/CprS systems in *Pseudomonas aeruginosa.* Furthermore, small RNAs, peptide feedback loop and substrate availability are all involved in directing the activity of the enzymes responsible for these modifications (Needham and Trent, 2013).

In *E. coli* and *Salmonella,* PmrA/PmrB regulon includes the enzymes ArnT and EptA, which catalyze the decoration of phosphate groups by addition of L-Ara4N and PEtN respectively (Trent *et al*., 2001; Lee *et al*., 2004) enzymes. The addition of these residues occurs at the IM before transport across the periplasm and, as a consequence, the overall negative charge of LPS decreases, thus improving resistance to positively charged antimicrobial peptides (CAMPs). PEtN can also be added to the outer core by EptB. EptB is not PmrA regulated, but its expression is repressed by a small RNA MgrR that is induced by PhoP/PhoQ (Moon and Gottesman, 2009; Moon *et al*., 2013). The PhoP/PhoQ system is itself regulated by another sRNA, MicA, whereas MicF negatively regulates *lpxR* transcript, which encodes in *Salmonella* a lipid A deacylase.

Post translational control of lipidA modification systems includes the inhibition of LpxT by the small peptide PmrR (Herrera *et al*., 2010). LpxT catalyzes the addition of a phosphate group from undecaprenyl-pyrophosphate to the position 1 phosphate to create lipid A 1-diphosphate, thus increasing the overall negative charge of the OM. Other enzymes modulate the number of acyl chains of lipid A; the OMP PagP (CrcA) catalyzes the addition of palmitate residue at position 2 of lipid A acyl chains (Hwang *et al*., 2002), LpxR and PagL, which are found in *Salmonella* but not in *E. coli* K-12, remove the 3-linked acyl chains (Reynolds *et al*., 2006; Kawasaki *et al*., 2005). Palmitate transfer by PagP occurs on the outer leaflet of the outer membrane where PLs are used as palmitoyl donors (Bishop *et al*., 2000). The active site of PagP faces the exterior (Hwang *et al*., 2002) suggesting that its activity may be regulated by substrate availability at the outer surface of the outer membrane.

Based on their sub-cellular localization and mechanism of action, lipid A modification enzymes have been extremely useful as reporters for LPS trafficking within the bacterial envelope.

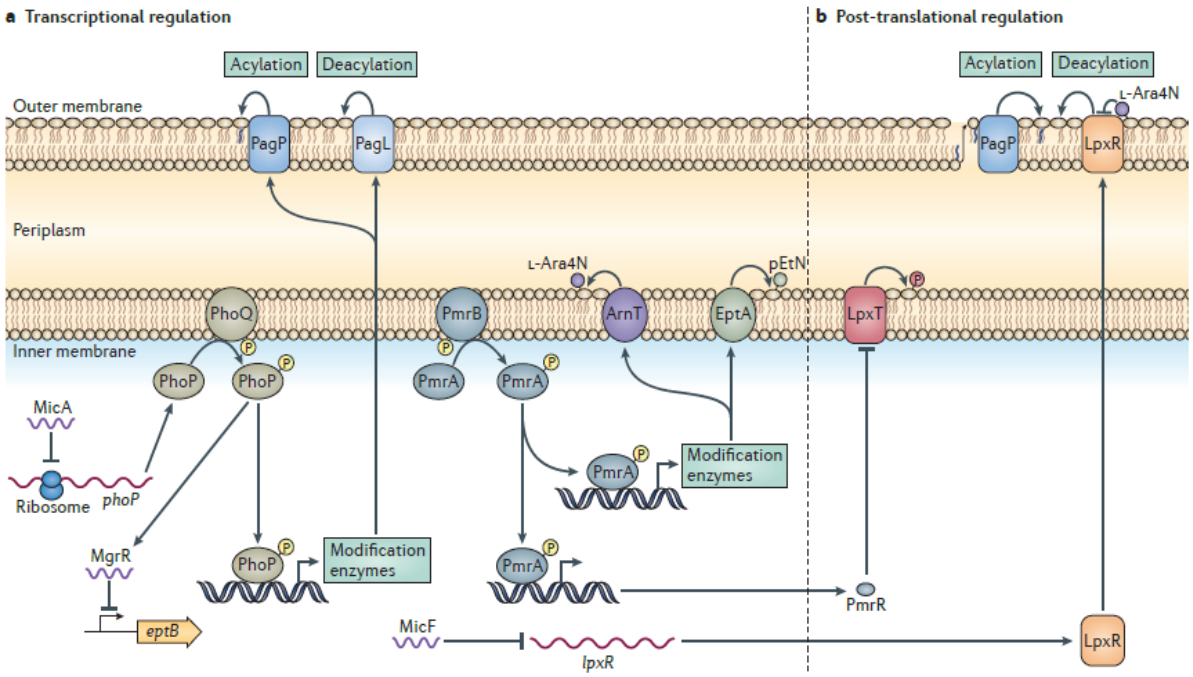

Figure 5 | (a) Transcriptional control of lipid A modification system. The two-component system PhoPQ upregulates transcription of the genes encoding PagP and PagL. The two-component system PmrAB upregulates transcription of ArnT and EptA, responsible for the addition of L-Ara4N or pEtN respectively. Expression of EptB is repressed by the sRNA MgrR, which is induced by PhoPQ. sRNA MicA represses the translation of the *phoP* mRNA. sRNA MicF increases degradation of the *lpxR* mRNA, which encodes a lipid A deacylase. **(b) Post-translational control of lipid A modification systems.** The kinase LpxT is inhibited by the small peptide PmrR, which is upregulated by the PmrAB system in response to high levels of Fe^{3+}. Membrane perturbation can lead to the displacement of PLs from the inner leaflet to the outer leaflet of the outer membrane. The presence of these donor substrates in proximity to PagP enhances enzyme activity. LpxR deacylates lipid A, but this activity is inhibited by the aminoarabinose lipid A modification. From Needham and Trent, 2013.

1.3.3 Lipopolysaccharide biogenesis: transport to the cell surface

1.3.3.1 The Lpt machinery

Mature LPS molecule assembled at the periplasmic face of the IM by WaaL ligase must be extracted from the IM and traverse the aqueous periplasmic space and be then inserted into the OM. Such functions are carried out by the Lpt (lipopolysaccharide transport) system (Silhavy *et al*., 2010, Sperandeo *et al*., 2009, Ruiz *et al*., 2009).

In *E. coli* the Lpt system is composed of seven essential proteins (LptABCDEFG) located in three distinct cellular compartments of the cell envelope (Fig. 7). The Lpt complex may be divided in three subassemblies: the IM sub-complex $LptB_2FGC$, the periplasmic LptA, and the OM sub-complex LptDE (Sperandeo *et al.,* 2006; Braun *et al*., 2002; Wu *et al*., 2006, Ruiz *et al*., 2008).

The IM sub-complex consists of $LptB_2CFG$ and is responsible for the extraction of LPS from the IM. LptB is a cytoplasmic protein belonging to the ABC transporter NBD family that associates as a dimer with IM proteins LptCFG (Chng *et al*., 2010, Narita and Tokuda 2009; Sherman *et al*., 2014). LptFG are integral IM proteins that constitute the TMD subunits of the $LptB_2FG$ ABC transporter (Ruiz *et al*., 2008). The bitopic protein LptC has a single transmembrane domain in the IM and a large periplasmic domain that is structurally homologous to both LptA and the periplasmic domain of LptD (Suits *et al*., 2008; Tran *et a*l., 2010), whereas LptA is the only component that completely resides in the periplasm (Tran *et al*., 2010). Finally, the LptDE OM sub-complex mediates LPS translocation across the OM and its assembly at the cell surface. The OM protein LptD has an N-terminal periplasmic domain and C-terminal β-barrel (Chng *et al*., 2010a), whereas LptE is a lipoprotein that resides within the lumen of the LptD barrel (Chng *et al*., 2010a; Freinkman *et al*., 2011).

1.3.3.2 Identification of the Lpt machinery components

LPS is an essential structural component of the OM in most Gram-negative bacteria (Raetz and Whitfield, 2002). *lptA*, *lptB* and *lptC* (formerly *yhbN*, *yhbG* and *yrbK* respectively) were the first genes implicated in LPS transport downstream MsbA to be discovered by Polissi and co-workers. They applied a genetic screen designed to identify novel essential functions in *E. coli* (Serina *et al*., 2004). In this work a Tn*5*-derived minitransposon carrying the inducible *araBp* arabinose promoter was used to generate mutants that were subsequently assayed for conditional lethal phenotypes. This genetic selection led to the identification of a chromosomal locus containing *lptA*, *lptB*, *lptC*,

and two LPS biosynthesis genes (*kdsD* and *kdsC*, which code for two enzymes involved in Kdo biosynthesis; Meredith and Woodard, 2003) (Fig. 6). The entire locus in *E. coli* is transcribed from a single upstream promoter, but at least two internal promoter regions may allow differential expression of the genes (Sperandeo *et al*., 2007; Martorana *et al*., 2011). In follow-up studies, Polissi and collaborators established the role of LptAB in LPS transport. Based on the observation that an altered expression of *lptAB* leads to increased sensitivity to hydrophobic toxic compounds or death, they proposed that these factors were essential proteins involved in OM biogenesis (Sperandeo *et al*., 2006) and demonstrated that depletion of either LptA or LptB causes the accumulation of newly synthesized LPS at the IM (Sperandeo *et al*., 2007).

A powerful genetic approach to identify genes involved in specific cellular functions is to develop screens for specific phenotypes. It is well known that viable mutants with an altered OM are more permeable to molecule that normally cannot cross the OM barrier, to hydrophobic compounds, detergents, bile salts, dyes and large hydrophilic antibiotics (Raetz and Whitfield, 2002; Nikaido, 2003), thus screening for increased permeability to such compounds may lead to the identification of mutants in OM-biogenesis factors.

LptD was first described as a determinant of membrane permeability, and thus designated *imp*, for increased OM permeability. In a genetic screen , Benson and co-workers (1989) found suppressors containing mutations in *imp* that enabled *E. coli* cells deficient in *lamB* (the beta-barrel required for maltodextrin import) to survive in media containing maltodextrins as the unique carbon source (Sampson *et al*., 1989). These *imp* mutants were also more permeable to certain antibiotics and other small molecules that normally cannot traverse the OM. Independently, *lptD* was also found to affect organic solvent tolerance and was thus designated *ostA* (Aono *et al*., 1994). Subsequently, *lptD* was demonstrated to be essential in *E. coli* by Braun and Silhavy (2002) and membrane fractionation experiments showed that LptD depletion generates a novel membrane fraction (Braun and Silhavy, 2002), further supporting its essential role in biogenesis of the cell envelope.

The role of LptD in LPS assembly was clearly established by Tommassen and co-workers. Exploiting the fact that in *Neisseria meningitidis*, unlike in *E. coli*, LPS is not essential (Steeghs *et al*., 1998; Zahng *et al*., 2013), they were able to obtain an *lptD* knockout mutant. The authors observed in *lptD* deleted cells a decrease of the LPS content, as expected for a gene involved in LPS biogenesis, and, most importantly, they showed that LPS is not accessible to extracellularly added neuraminidase (an enzyme that modifies LPS by adding sialic acid residues) and lipid A is not

deacylated by PagL, thus demonstrating that LPS is not transferred to the outer leaflet OM (Bos *et al*., 2004).

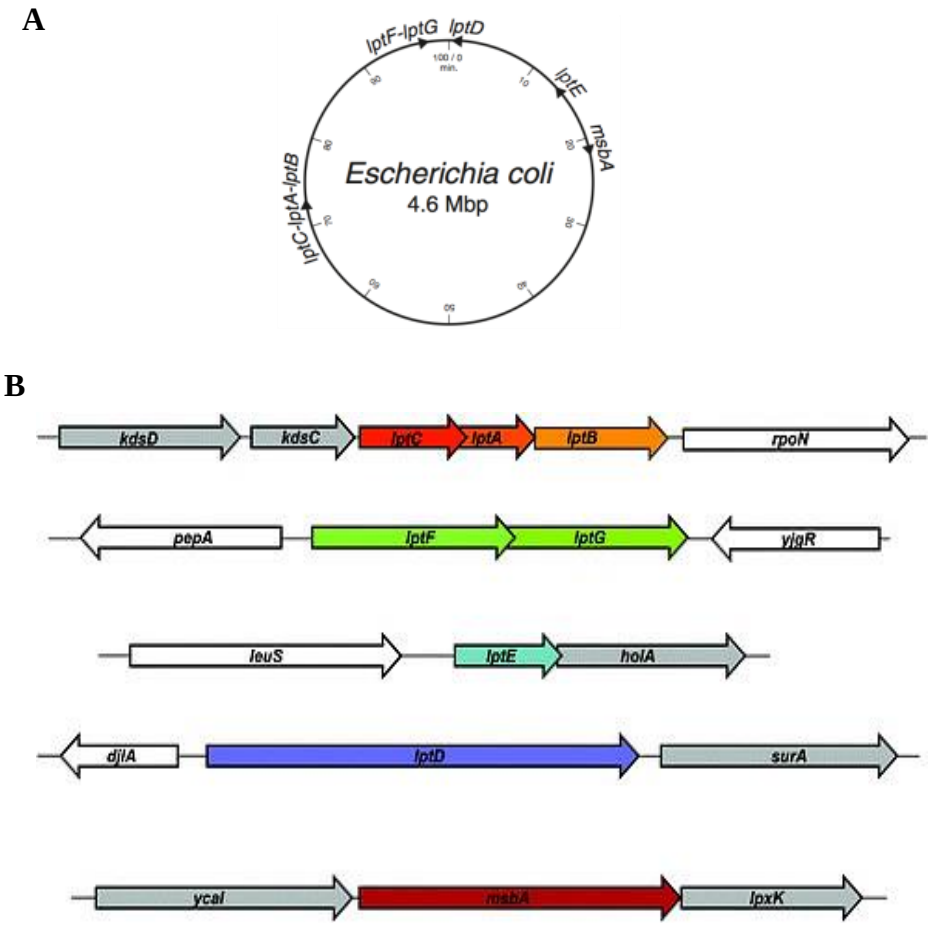

Figure 6. (A) Chromosomal localization of *lpt* genes and *msbA* in *E. coli* K-12 (From Narita, 2011). (B) Organization of genetic loci implicated in LPS transport in *E. coli*. Annotation is based on that of the *E. coli* strain MG1655 (http:// ecocyc. org/). Lpt ORF lengths are drawn to scale. In grey are represented neighbouring unrelated genes belonging to the same transcriptional units of Lpt genes. From Sperandeo *et al*., 2014.

Later, using affinity purification techniques with LptD as a bait, the Silhavy and Kahne laboratories discovered the other component of the OM translocon, the lipoprotein LptE (formerly known as RlpB) (Wu *et al*., 2006). Also LptE was found to be essential in *E. coli*; indeed LptE depletion strains show phenotypes similar to LptD depletions (Wu *et al*., 2006).

The identification of the Lpt proteins described so far, suggested a model where LptB is a cytoplasmic ATPase that provides the energy source for transport, LptA shuttles LPS across the periplasm, and the OM complex LptDE functions as a receptor/insertase for LPS (Sperandeo *et al*., 2006; Sperandeo *et al*., 2007; Sperandeo *et al*., 2008; Wu *et al*., 2006; Braun and Silhavy 2002; Bos *et al*., 2004). In Gram-negative bacteria transmembrane components of ABC transporters are constituted either by one protein with 12 transmembrane segments or two proteins with 6 transmembrane segments each (Davidson *et al*., 2008); for this reason it was clear that some component of the Lpt transporter were still missing. These transmembrane partners were identified by Ruiz and co-workers (2008) using a bioinformatic approach. They selected as a model organism an endosymbiont whit a reduced proteome (14% of *E. coli* proteome) but containing most of the OM biogenesis factors so far identified. This led to the discovery of two essential *E. coli* IM proteins of unknown function: YjgP and YjgQ (Ruiz *et al*., 2008). Each one was predicted to have a 6 transmembrane helical structure typical of ABC transporter TMDs; depletion of either YjgP or YjgQ resulted in phenotypes that resembled those reported for the depletion of other Lpt factors. YjgP and YjgQ were proposed to be the missing component of the Lpt ABC transporter and were thus renamed LptF and LptG, respectively (Ruiz *et al*., 2008). In *E. coli* the genes encoding LptF and LptG belong to an operon unlinked to *lptB* (Fig. 6). Their involvement in LPS transport was demonstrated using conditional expression mutants and analysing the PagP-mediated modification of *de novo* synthesized LPS. In LptF or/and LptG depleted cells the lack of LPS modification and its accumulation at the IM revealed that the two proteins are required for LPS transport downstream MsbA (Ruiz *et al*., 2008).

1.3.3.3 Model for LPS transport

The identification of the Lpt IM and OM complexes prompted a host of questions about the mechanism of transport to the OM. Two main transport models have been considered: the chaperone-mediated transit across the periplasm and the transport through a transenvelope proteinaceous bridge spanning IM and OM (Fig. 7).

The evidence that LptA is not a soluble periplasmic protein but fractionates with both IM and OM in sucrose density gradient centrifugation (Chng *et al*., 2010b) and its propensity to form oligomeric fibrils *in vitro* (Suits *et al*., 2008; Santambrogio *et al*., 2013) strongly suggest that the protein does not function as a soluble carrier but forms oligomeric structure spanning the width of the periplasm. However the most important evidence supporting the transenvelope model is that all the Lpt proteins co-fractionate in sucrose density centrifugation in a lighter IM fraction containing IM and OM components and that these proteins physically interact to form a transenvelope bridge (Chng *et al*., 2010b). Mutations impairing Lpt complex assembly result in degradation of the periplasmic component LptA, thus LptA abundance in the cell appears to be a marker of properly bridged IM and OM. (Sperandeo *et al*., 2011).

Recently, new insight about the mechanism by which the transenvelope protein machine physically favors LPS transit through the periplasm have been provided using photo-crosslinking *in vivo.* This chemical approach allows the identification of protein residues involved in protein-protein or protein-ligand interactions by labeling the proteins of interest *in vivo* with an UV reactive cross-linkable amino acid analog. LptE was previously shown to reside within the lumen of the β-barrel of LptD (Chng *et al*., 2010a) and to bind LPS *in vitro* (Chng *et al*., 2010a). Kahne and co-workers using photo-crosslinking demonstrated that LptE directly interacts with some residues of the predicted extracellular loop of LptD adopting a plug-and-barrell architecture (Freinkman *et al*., 2011), suggesting a dual role for this protein: a structural component of the LptDE complex and a recognition site for LPS at the OM (Freinkman *et al*., 2011; Chng *et al*., 2010a). The same approach has been used to identify the regions in LptA, LptC, and in the *N*-terminal domain of LptD that are implicated in protein contact and thus in the formation of the protein bridge. They showed that *in vivo* LptA interacts with LptC at the IM via its *N*-terminal region and with LptD at the OM via its *C*-terminal region thus creating a continuous bridge of antiparallel β-strands between IM and OM (Freinkman *et al*., 2012). Indeed, LptA and LptC belong to the same OstA superfamily of the *N*-terminal domain of LptD and share a very similar three dimensional structure (Suits *et al*., 2008; Tran *et al*., 2010). Interestingly, the periplasmic but not the transmembrane domain of LptC appears to be also required for interaction with the IM transporter LptBFG (Villa *et al.*, 2013). As the periplasmic loop of LptF and LptG are predicted to assume the β-jellyroll structure shared by LptA, LptC and LptD, it has been proposed that the transenvelope bridge is based on the conserved structurally homologous jellyroll domain shared by five out of the seven Lpt components (Villa *et al*., 2013).

It is not yet known how many LptA molecules compose the transenvelope bridges. The residues identified for LptA-LptA dimer interaction are the same involved in LptA-LptC interaction or in LptA-LptD interaction, but this has been proposed to be an artefact due to LptA overexpression (Freinkman *et al*., 2012). Structural data suggest that four OstA superfamily domains are necessary to span the width of the periplasm (Suits *et al*., 2008), as a consequence, taking into account LptC and LptD periplasmic domains, LptA would function as a dimer within the transenvelope bridge. Alternatively, in proximity of Lpt bridges the periplasm could be constricted and only one LptA molecule would be required.

Finally, Okuda and co-worker, using site-specific photoactivatable cross-linking in a right-side-out vesicle system, demonstrated that LPS interacts with specific residues within the hydrophobic grooves of LptC and LptA (Okuda *et al*., 2012). They also showed that cross-linking of LPS to LptC and subsequent transfer of LPS from LptC to LptA depends on ATP hydrolysis and that LptC cannot extract LPS on its own (Okuda *et al*., 2012).

How LPS is extracted from the IM and how it is transferred to the protein bridge are still open questions. Two main mechanisms have been proposed for LPS extraction and handing off. LptFG (both or just one of them) could interact directly with LPS and perform the extraction coupled to LptB's ATP hydrolysis; then they should somehow pass LPS to LptC. The alternative model proposes that, in an ATP-dependent manner, LptFG stimulate LptC to extract LPS from the IM (Simpson *et al*., 2015).

In conclusion, according to the current model of LPS transport, ATP hydrolysis is used to push a continuous stream of LPS through the transenvelope Lpt bridge in discrete steps against a concentration gradient (Sherman *et al*., 2012; Wang *et al*., 2014; Okuda *et al*., 2012), then LPS passes from LptA to the LptDE translocon which inserts it into the outer leaflet of the OM. The N-terminal domain of LptD comprises a hydrophobic slide that injects the acyl tails of LPS directly into the OM through an intramembrane hole, and the barrel domain, through a lumen gate, delivers LPS hydrophilic portions across the OM lipidic bilayer (Gu *et al.*, 2015).

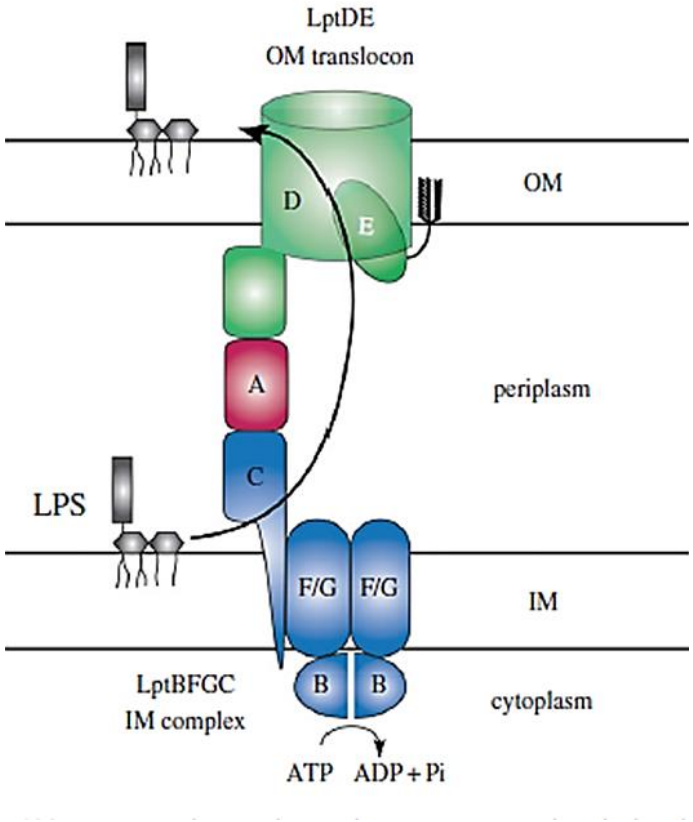

Figure 7. Transport of LPS across the cell envelope. After flipping across the IM LPS is transported across the periplasm and assembled at the cell surface. $LptB_2FG$ form an ABC transporter that uses ATP hydrolysis to extract LPS from the IM and push it along a periplasmic bridge built of homologous domains in LptCAD. At the OM, the LptDE form a plug-and-barrel translocon that inserts LPS into the outer leaflet of the OM. From May *et al*., 2015

2.1 The present investigation

This chapter is intended to discuss main results presented in the following two draft manuscripts to be submitted for publication and in one published paper.

DRAFT MANUSCRIPT 1

Falchi F. A., Maccagni E. A., Puccio S., Peano C., De Castro C., Polissi A., Dehò G and Sperandeo P. Mutational analysis of LptA, an essential LPS-transport protein, reveals strategies of outer membrane homeostasis in *Escherichia coli*. (To be submitted).

DRAFT MANUSCRIPT 2

Benedet, M., **Falchi, F.A**., Puccio, S., Di Benedetto, C., Peano, C., Polissi, A. and Dehò, G.
The lack of the essential LptC component in the *Escherichia coli* lipopolysaccharide transport machine can be circumvented by suppressor mutations in the inner membrane ABC transporter LptF. (To be submitted).

PUBLISHED PAPER

Martorana A M., Motta S., Di Silvestre D., **Falchi F**., Dehò G., Mauri P., Sperandeo P., Polissi A. Dissecting *Escherichia coli* outer membrane biogenesis using differential proteomics. *PLoS One*. 2014, 9(6):e100941. DOI: 10.1371/journal.pone.0100941

2.2 Aim of the project

The lipopolysaccharide (LPS)-rich OM is a unique feature of Gram-negative bacteria and LPS transport across the IM and through the periplasmic space is essential to the biogenesis and maintenance of the OM integrity. The Lpt protein machine, which in *E. coli* is composed of seven essential proteins (LptA through LptG), is responsible for LPS translocation across the periplasmic space to the outer leaflet of the OM.

LPS transport is a complex process that requires overcoming energetic, structural and physical barrier. In the last decade the Lpt system components (operating downstream of MsbA) have been discovered employing a combination of genetic, biochemical and bioinformatic approaches but, even though many structural and functional insights have been provided, to date the mechanism of LPS transport is not fully understood. The current model postulates that the Lpt proteins create a transenvelope bridge that connects IM and OM, by interacting via homologous domains. These domains exhibit high structural similarity, the β-jellyroll fold, despite scarce sequence conservation. At the IM the heteromeric ABC transporter, LptBFG, forms a complex with the membrane-bound protein, LptC; the C terminus of LptC interacts with the N terminus of LptA, and the N terminus of LptA interacts with the C-terminal periplasmic domain of LptD.

In this study, in order to gain more insight in the mechanism of LPS transport and its interactions with other cellular processes, we used both a genetic and a proteomic approach. The former was based on the selection of suppressors of LPS transport defects obtained with two different types of mutants: i) a quadruple non-lethal *lptA* mutant that displayed altered sensitivity to hydrophobic toxic compounds, and ii) a lethal deletion mutant of *lptC*. The latter approach consisted of the analysis of differential envelope proteins content upon impairment of LPS transport.

More in detail, in the first part of the work we investigated more closely on the role of LptA, the soluble periplasmic component of Lpt system. LptA has been demonstrated to bind LPS *in vitro* and *in vivo* (Tran *et al*., 2008; Okuda *et al*., 2012) and is believed to chaperon LPS through the periplasm. We generated an *lptA* mutant allele (*lptA41* quadruple mutant) by mutagenizing four LptA residues putatively involved in LPS or LptC binding and conserved among LptA homologues in most representative γ-Proteobacteria. The mutant was viable but displayed increased sensitivity to a panel of hydrophobic toxic compounds (Shc phenotype, for sensitivity to hydrophobic compounds) as compared to the wild-type strain, suggesting that *lptA41* is a partial loss-of-function allele of *lptA*. Biochemical characterization of the mutant showed that the LptA41 mutant protein

was impaired in the assembly of the Lpt complex but not in LPS binding. We selected and characterized three different classes of suppressor in which tolerance to bacitracin was restored but exhibited different profiles of tolerance to other hydrophobic toxic compound. In the first mutant suppression appeared to depend on overexpression of LptA41. Analysis of the second suppressor mutant implicated the pathway for the maintenance of lipid asymmetry (Mla system) in suppressing bacitracin sensitivity exerted by *lptA41*. Finally, suppression of the Shc phenotype in the third suppressor strain was ensured by an additional mutation in LptA41 (*lptA42* allele) that appeared to stabilize LptA protein and partial deletion in the *opgH* gene implicated in the synthesis of osmoregulated periplasmic glucans (Draft manuscript 1).

In the second part of this work we selected for *E. coli* mutants lacking the essential gene *lptC*. All the isolated mutants harbour a suppressor mutation in LptF leading to a unique amino acid substitution at position 212, within the predicted periplasmic domain of the protein. This strongly implies such LptF region in the formation of the periplasmic bridge between the IM and OM complexes, and suggests that LptC may have evolved as a chaperon of a six-component Lpt machine assembly and/or activity (Draft manuscript 2).

Finally, in the last part of this work we performed a proteomic analysis of *E. coli* cell envelope upon inhibition of LPS transport by LptC depletion. By this analysis 123 proteins were identified whose level is modulated in these conditions. Most such proteins belong to pathways implicated in cell envelope biogenesis, peptidoglycan remodelling, cell division and protein folding (Published paper Martorana *et al*., 2014). These data show that *E. coli* cells respond to severe OM biogenesis defect by modulating different pathways that acts integrating complementary functions to restore OM functionality.

2.3 Mutational analysis of LptA, an essential LPS-transport protein, reveals strategies of outer membrane homeostasis in *Escherichia coli* (Falchi *et al.*, to be submitted**).**

LptA is an essential periplasmic protein that has been implicated in LPS transport from the IM to the OM, thereby contributing to building the cell envelope and maintaining its integrity. According to the current model the Lpt proteins form a transenvelope bridge spanning IM and OM. The C-terminal domain of LptC interacts with the N-terminal domain of LptA and the C-terminus of LptA interacts with the N-terminal periplasmic domain of LptD, thus forming a continuous channel through which LPS is moved (Freinkman *et al*., 2012; Okuda *et al*, 2012; Villa *et al*., 2013).

The crystal structure of *E. coli* LptA has been solved by Suits and co-workers and it consists of 16 consecutive antiparallel β-strands, folded to resemble a slightly twisted β-jellyroll (Suits *et al*., 2008) Crystallographic data showed that LptA forms oligomers in a head-to-tail fashion designing a continuous cavity (Suits *et al*., 2008).

LptA has been demonstrated to interact with LPS *in vitro* (Tran *et al*., 2008) and some of the interaction sites with LptC in the IM and with the N-terminal domain of LptD in the OM, as well as with LPS have been recently identified using *in vivo* crosslinking experiments (Freinkman *et al*., 2012; Okuda *et al*. 2012).

In a previous work, several rationally designed *lptA* mutant alleles turned out to be able to complement LptA depleted strain for growth, although their overexpression was somewhat detrimental to LPS transport (Suits *et al*., 2008). Thus in the present study we tested whether multiple mutations (*lptA41* allele, encoding the amino acid substitutions I36A, I38A, R76D, and K83D) could impair LptA functionality. We generated an *E. coli* strain in which the deletion of *lptAB* operon is complemented for growth, under standard conditions, by a plasmid harboring *lptA41 lptB*. However, the mutant strain displayed increased sensitivity to anionic detergents (SDS), hydrophilic (bacitracin) and hydrophobic (rifampicin and novobiocin) toxic compounds as compared to a similarly generated strain expressing wild type LptA (p*lptA*$^{+}$ strain), suggesting that *lptA41* is a partial loss-of-function allele that impairs LPS transport thus causing severe OM defects.

To characterize the properties of the LptA41 mutant protein that could be correlated with the functional defects, we first assessed whether the amino acid substitutions in LptA41 could impair its affinity for LPS and/or its assembly into the Lpt complex. We performed an *in vitro* LPS binding assay using purified C-terminally His-tagged LptA and LptA41 proteins and smooth-type LPS.

Then we performed affinity co-purification experiments from solubilized membranes of wild type, *lptA*$^+$ and *lptA41* strains ectopically expressing C-terminally His tagged LptC (LptC-H). Overall, our results suggest that, at least *in vitro*, LptA41 retains the ability to interact with LPS whereas the phenotype associated to *lptA41* allele could be imputed to impairment in Lpt complex assembly.

In order to identify interactions between genes/protein involved in the same functional pathways, we sought to identify second-site mutations able to restore the integrity of OM permeability barrier and to overcome the increased susceptibility to toxic compound exhibited by the *lptA41* mutant. Spontaneous bacitracin resistant mutants were selected and found at a frequency of 1×10^{-8}.

To identify potential suppressor mutations we performed the genomic sequencing of three strains with different profile of tolerance to the other toxic compound.

Sequencing results revealed in PS102, in which only bacitracin sensitivity is suppressed, a deletion of 6 nucleotides in *vacJ* (*vacJ102* allele) that improves resistance of *lptA41* mutant to bacitracin. *vacJ* gene product, now renamed *mlaA*, is the OM lipoprotein component of the Mla system required in *E. coli* to maintain OM lipid asymmetry (Malinverni and Silhavy, 2009). To genetically characterize *vacJ102* we generated suitable strains ectopically expressing the wild type and the mutant *vacJ102* and confirmed that the mutant allele conferred bacitracin resistance.

In suppressor strain PS103 bacitracin, rifampicin and SDS tolerance have been restored, whereas novobiocin sensitivity was not fully suppressed. Genome sequencing revealed an additional amino acid substitution (M112I) in LptA (allele *lptA42*) and a nonsense mutation in *opgH* (formerly *mdoH*) gene resulting in protein truncation (allele *opgH103*). *opgH* encodes for a glycosyltransferase involved in the synthesis of membrane derived oligosaccharides (MDOs) (Weissborn and Kennedy, 1984) and in control of cell size *via* interaction with FtsZ in a nutrient-dependent manner (Hill *et al*., 2013). We thus analysed the possible contribution of each mutations to the suppressed phenotype exhibited by PS103 strain.

First, we tested Lpt complex assembly performing affinity purification experiments from solubilized membranes of a *lptA42* strain bearing *lptA42* allele and ectopically expressing LptC-H. M112I mutation, although improving protein structural stabilization, did not restore LptA interaction with LptC and, therefore, with the Lpt complex. However, as expected, LptA42 mutant retained the ability to co-purify LPS *in vitro*. Finally, we tested the effect of *opgH103* mutation alone or in combination with *lptA42* allele in OM integrity restoration. *lptA42* allele in *opgH*$^+$ background resulted in partial OM integrity restoration. However, *opgH* deletion did not restore OM permeability in *lptA41* mutant, suggesting that the increased bacitracin resistance is not solely

the result of OPGs synthesis inhibition. *lptA42* allele in *opgH*⁻ strain conferred partially OM integrity restoration, which was improved by expression of the truncated OpgH103 mutant.

2.4 The lack of the essential LptC component in the *Escherichia coli* lipopolysaccharide transport machine can be circumvented by suppressor mutations in the inner membrane ABC transporter LptF. (Benedet *et al.,* to be submitted).

LptC is an IM bitopic protein with a single trans-membrane helical domain and a large periplasmic region (Tran *et al.*, 2010) which stably associates to LptBFG (Narita and Tokuda, 2009) and to LptA (Sperandeo *et al*., 2011). LptA and the periplasmic domain of LptC share very little amino acid sequence conservation (about 13% identity); nevertheless, comparison of their 3D structures reveals a remarkably conserved fold based on a slightly twisted β-jellyroll, (Suits *et al.*, 2008; Tran *et al.*, 2010; Villa *et al.*, 2013). Like LptA, LptC binds lipopolysaccharide *in vitro*, and LptA can displace lipopolysaccharide from LptC, but not vice versa (Tran et al., 2010), consistent with their locations and their proposed placement in a unidirectional export pathway. However, LptC specific role in LPS transport remains unclear.

Point mutations in the N-terminal periplasmic region (G56V) or at the C-terminus (G153R) are unviable and neither mutant is able to assemble the transenvelope machinery (Sperandeo *et al.*, 2011; Villa et al., 2013). Moreover the transmembrane N-terminal domain of LptC is not required for proper assembly and functionality of the Lpt complex and that the periplasmic region of LptC is sufficient to promote binding to the the LptBFG IM complex (Villa et al., 2013).

Considering the dispensability of LptC transmembrane domain and the high structural similarity of LptC periplasmic domain and LptA, here we tested whether some functional redundancy could occur between these structurally analogous components of the Lpt machine and we selected for *lptC* deletion mutants.

By plasmid shuffling, we isolated *E. coli* Δ*lptCA* mutants complemented by plasmids harbouring *lptA* or *lptAB* genes and missing *lptC*. Whole genome sequence analysis of three mutants revealed that the *E. coli* lethal phenotype associated to the lack of LptC is suppressed by single amino acid substitutions at a unique position of the IM component LptF. All the independent viable clones obtained harbored, in addition to the Δ*lptC* deletion a single amino acid substitutions at arginine 212 (either R212C or R212S), a residue located in the predicted periplasmic domain of

the protein. Nine additional independent Δ*lptC* mutants obtained by plasmid shuffling exhibited a mutation in LptF R212 residues, including the new substitution R212G. These data strongly suggested that such *lptF* mutations suppress the lack of LptC.

Complementation assays in an *E. coli* strain harbouring *lptC* under the control of the inducible *araBp* promoter and the chromosomal wild *lptF* allele showed that *lptF*R212G and *lptF*R212S are able to suppress cell lethality of LptC-depleted cells. On the contrary, *lptF*R212C does not restore cell growth in this condition, suggesting that *lptF*R212C is a recessive allele.

Moreover, to address whether $lptF^{Sup}$ mutations in the haploid state are compatible with the presence of *lptC* we replaced by plasmid shuffling in each type of $lptF^{Sup}$ mutants the resident plasmid harboring either *lptA* or *lptAB* with an incompatible plasmid harboring a different antibiotic resistance marker and either *lptCA* or *lptCAB*. All the $lptF^{Sup}$ clones could be transformed by the plasmid carrying *lptCA* or *lptCAB* and lost the resident plasmid. On the contrary, none of the strains transformed, as a control, by a chasing plasmid without *lpt* genes lost the resident plasmid as it carried genes essential for viability and $lptF^{+}$ strains transformed by the chasing plasmid with *lptA* or *lptAB* but missing *lptC* did not lose the resident plasmid. By sequencing we assessed that the original *lptF* allele had been retained in each type of $lptF^{Sup}$ shuffled clones, thus suggesting that all the three haploid $lptF^{Sup}$ mutations are compatible with the presence of LptC.

Overall our data suggest that the periplasmic region of LptF might be implicated in the formation of the periplasmic bridge between the IM and OM complexes, and LptC might have evolved as a chaperon of a six-component Lpt machine assembly and/or activity.

Studies are in progress to understand the structure and functioning of the six-component Lpt machine in order to understand how the $LptF^{Sup}$ mutants overcome the lack of LptC.

2.5 Dissecting *Escherichia coli* outer membrane biogenesis using differential proteomics (Martorana *et al*. (2014) *PLoS One*. 9:e100941).

Gram-negative bacteria such as *Escherichia coli* have extracytoplasmic compartments, collectively known as the cell envelope, that play a variety of protective and adaptive roles. Three are the principal layers in the envelope: the OM, the peptidoglycan cell wall, and the IM. The two concentric membranes delimit an aqueous cellular compartment, the periplasm.

In *E. coli* at least five different pathways constitute complex signalling systems that monitor cell envelope stress. (Joly *et al*., 2010; Ades, 2008; Majdalani and Gottesmann, 2005; Raivio, 2005). σ^{E}, Bae, Psp, and Rcs appears to be systems specialized in assuring a specific aspect of

envelope biogenesis and maintenance, whereas CpxR might have a role as modulator of the response by integrating other endogenous signals (Bury-Monè *et al*., 2009). These pathways can be activated simultaneously in response to exogenous or endogenous stimulation and regulate mainly complementary functions whose contributions are integrated to mount a full adaptive response (Bury-Monè *et al*., 2009).

To investigate on the cell response to a severe OM defect, we performed a proteomic analysis of *E. coli* cell envelope upon inhibition of LPS transport obtained by LptC depletion. The entire protein content of the cell envelope fractions of an *E. coli lptC* conditional expression mutant grown in permissive and non-permissive conditions was analyzed by two-dimensional chromatography coupled to tandem mass spectrometry [(2DC-MS/MS or MudPIT (Multidimensional Protein Identification Technology)] (Link *et al*., 1999).

We identified 123 proteins whose level is significantly modulated upon LptC depletion. Most such proteins belong to pathways that may contribute to repair OM and restore its permeability barrier properties, including protein involved in maintaining OM asymmetry, in the synthesis of phospholipids and exopolysaccharides as substrate for lipid A modification enzymes. We found that the level of several enzymes implicated in peptidoglycan synthesis/remodelling changes in in LptC depleted cells, suggesting that the synthesis of peptidoglycan is inhibited and that the arrest of cell wall growth shifts bacteria to the cell division program. The level of ribosomal and transport proteins and of many folding factors decreases upon LptC depletion, conversely the level of several IM, periplasmic and OM proteases increases. These data are consistent with the notion that extracytoplasmic stress response is activated upon the block of LPS transport, as many functions modulated in LptC depleted cells are under the control of σ^E, Bae, Cpx, and Rcs signalling systems.

2.6 Conclusions

In this study, we focused on the mechanism of LPS transport, its interaction with other cellular processes and especially on the strategies adopted by the cells to face LPS transport defects. To this end we adopted both a genetic and a proteomic approach, the former based on the selection of suppressors of LPS transport defects obtained with two different types of mutants and the latter based on the analysis of differential envelope proteins content upon impairment of LPS transport.

We first generated a quadruple non-lethal *lptA* mutant (*lptA41*) that displays altered sensitivity to hydrophobic toxic compounds, a phenotype that is diagnostic of altered OM permeability. Biochemical data revealed that LptA41 ability to interact with LptC is impaired under our experimental conditions, whereas LPS binding seems not to be compromised. These observations suggested that in *lptA41* strain LPS transport, although not abolished, may not be as efficient as in a wild type strain. Genome sequencing analysis of two selected suppressor mutants (PS102 and PS103) implicated a small in-frame deletion in *vacJ* gene (*vacJ102* allele) and an intragenic mutation associated to a partial deletion in the *opgH* gene in the respective mechanism of suppression.

VacJ (MlaA) is the OM lipoprotein component of the Mla machinery, which has been proposed to contribute to maintain OM asymmetry. *vacJ102* mutant allele is able to suppress sensitivity to bacitracin (the antibiotic used for the suppressors selection), but it does not restore the OM permeability barrier. Moreover it appears to be a negative dominant mutation that impairs the Mla system. These observations suggest that a specific mechanism for bacitracin tolerance have been selected. We may speculate that the VacJ mutant interferes with bacitracin diffusion through the OM or, alternatively, with the bacitracin-C_{55}-PP interaction, in a direct or indirect way. However, it might be interesting to test whether the *vacJ102* mode of suppression is specific to *lptA41* mutant or rather it is able to increase bacitracin resistance in different *lpt* mutant backgrounds or to suppress mutations affecting other OM biogenesis pathway.

In suppressor strain PS103, *lptA41* phenotypic suppression appears to be ensured by the additive effects of an intragenic and an extragenic suppressor. The intragenic suppressor (*lptA42* allele) encodes an additional amino acid change (M112I) in LptA41. The LptA42 quintuple mutant protein appeared to be more stable than its parental LptA41, but did not seem to co-purify with LptC. LptA42 might ameliorate LPS transport and reduce OM alteration thus improving resistance to detergents and to the large hydrophilic antibiotic bacitracin, but not to small hydrophobic compounds. *opgH* encodes a glucosyltransferase implicated in the biosynthesis of the so called

osmoregulated periplasmic glucans (OPGs), a class of periplasmic glucans that function as osmoprotectant. The nonsense mutation *opgH103* restores resistance of *E. coli lptA42* to the lipophilic antibiotic rifampicin and, partially, to novobiocin. Thus we suggest that the lack of OPGs in the periplasm and/or of the OpgH in the IM make the cell envelope less permeable to these lipophilic compounds.

A second type of suppressors of LPS transport defects have been obtained using a plasmid shuffling technique. We selected for Δ*lptC* viable mutants and genome sequencing analysis revealed in all the isolated mutants a single amino acid substitutions at a unique position (R212) in the periplasmic domain of the IM component LptF ($LptF^{Sup}$). Then, by complementation assays we confirmed that $LptF^{Sup}$ are able to suppress the lethal effect associated to the lack of LptC. Such a complete association between lack of LptC and $LptF^{Sup}$ mutations strongly suggests that a specific suppressor is required for viability of the Δ*lptC* mutants and that LptF is the preferred suppressor gene. Interestingly, using the same technique, we did not obtain Δ*lptA* mutants, thus suggesting that LptA might have a more fundamental role in Lpt machine or more than one suppressor mutation might be required, thus reducing the chance of finding a mutant.

Finally we showed by plasmid shuffling that $LptF^{Sup}$ is compatible with the presence of LptC.

Overall our data highlights the relevance of the putative LptF periplasmic domain in LPS transport. $LptF^{Sup}$ could restore a functional hydrophobic groove by direct interacting with LptA or by recruiting LptA to replace LptC. LptC might serve as a chaperon of the Lpt machine assembly and/or activity rather than be an essential functional component. Affinity co-purification experiments will be performed to test whether $LptF^{Sup}$ can promote the assembly of a stable Lpt bridge in absence of LptC and to test whether LptC can be recruited by the Lpt system when a $LptF^{Sup}$ protein is present.

In the last part of this study, we performed a comparative analysis of envelope proteome in *E. coli* LptC depleted and not-depleted cells. We show that the block of LPS transport (upon LptC depletion) induces the modulation of pathways that collectively may contribute to repair the OM and restore its permeability barrier properties. Interestingly our results suggest a functional interconnection between LPS transport and PLs removal from the OM (as proteins of the Mla system are upregulated) and support the notion that peptidoglycan synthesis and OM biogenesis are tightly coordinated. Moreover our data point for the first time to a feed-back control on lipid A synthesis signalled from the external surface of the cell when LPS transport is compromised. This concept is supported by the finding that the IM enzyme FtsH increases in LptC depleted cells, as FtsH is known to control LPS biosynthesis. The recently identified LapB IM protein, that has been

recently suggested to act in concert with FtsH, could have a role in transducing the signal inside the cell.

In conclusion, these data contribute to our understanding of the multiple strategies that *E. coli* cells may adopt to respond to perturbations of the OM permeability barrier and to restore OM functionality.

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Manuscript and published paper

1. Draft manuscript

Falchi F. A., Maccagni E. A., Puccio S., Peano C., De Castro C., Polissi A., Dehò G and Sperandeo P. Mutational analysis of LptA, an essential LPS-transport protein, reveals strategies of outer membrane homeostasis in *Escherichia coli*. (To be submitted).

2. Draft manuscript

Benedet, M., **Falchi, F.A**., Puccio, S., Di Benedetto, C., Peano, C., Polissi, A. and Dehò, G.
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3. Published paper

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Mutational analysis of LptA, an essential LPS-transport protein, reveals strategies of outer membrane homeostasis in *Escherichia coli*

Federica A. Falchi[1], Elisa A. Maccagni[2], Simone Puccio[3], Clelia Peano[3], Cristina De Castro[4], Alessandra Polissi[2], Gianni Dehò[1], and Paola Sperandeo[2*]

[1] Dipartimento di Bioscienze, Università degli Studi di Milano, Milano, Italy;

[2] Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Milano, Italy;

[3] Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, Milano, Italy;

[4] Dipartimento di Agraria, Università degli studi di Napoli Federico II, Napoli, Italy.

* Corresponding author:

Paola Sperandeo, Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Piazza della Scienza 2, 20136 Milano, Italy.

Phone: +39 02 6448 3432; e-mail: paola.sperandeo@unimib.it

ABSTRACT

Lipopolysaccharide (LPS) is the major outer leaflet constituent of the Gram-negative bacteria outer membrane (OM). In *E. coli* LptA protein is a periplasmic component of the LPS transporter (Lpt) made of seven components (LptABCDEFG), which ferries LPS from the inner membrane (IM) to OM. LptA interacts with LptC and chaperons LPS through the periplasm. The crystal structure of LptA has been solved and some residues involved in binding LPS and other Lpt proteins have been identified. In order to characterize LptA structure-function we generated by site-directed mutagenesis *lptA41*, a quadruple mutant in four conserved amino acids potentially involved in LPS or LptC binding. Although viable, the mutant exhibited increased sensitivity to antibiotics (the large, hydrophilic bacitracin, and the hydrophobic rifampicin and novobiocin) and to a detergent (SDS). This suggests that *lptA41* affects LPS transport thus impairing OM permeability barrier. To identify interactions between genes implicated in OM functionality, we selected and characterized two phenotypic bacitracin resistant suppressors of *lptA41*. One mutant, in which bacitracin sensitivity had been suppressed, harbors a small in-frame deletion in *vacJ*, which codes for an OM lipoprotein involved in maintaining OM asymmetry by reducing accumulation of phospholipids in the outer leaflet. The other one, in which bacitracin, rifampicin and SDS sensitivity had been suppressed, harbors an additional amino-acid substitution in LptA41 (*lptA42* allele) and a nonsense mutation in *opgH*, a gene encoding a glycosyltransferase involved in periplasmic membrane-derived oligosaccharides synthesis. The former mutation is responsible for suppression of bacitracin and SDS sensitivity, whereas both mutations are required for restoring the permeability barrier against rifampicin. These results reveal different strategies adopted by the cell to overcome OM defects caused by defective LPS transport.

INTRODUCTION

The majority of Gram negative bacteria are characterized by a cell envelope composed of two concentric membranes, the inner (IM) and the outer membrane (OM), separated by a hydrophilic compartment, the periplasm, in which the murein wall is embedded reviewed by (Silhavy *et al*., 2010; Ruiz *et al*., 2006) The two membranes have different composition and permeability properties. While the IM is a typical phospholipid bilayer, the OM is an asymmetric bilayer with phospholipids (PLs) and lipopolysaccharides (LPS) in the inner and outer leaflet, respectively. LPS is a negatively charged amphipathic molecule composed of three covalently linked moieties: the lipid A, a core oligosaccharide and a long polysaccharide called O-antigen (Fig. 1) (Raetz and Whitfield, 2002). In the presence of divalent cations, LPS molecules pack tightly together to form an outer leaflet that reduces OM fluidity and permeability, selectively controlled by dedicated OM proteins (Nikaido, 2003).

LPS organization can be disrupted by defects in OM components assembly (Ruiz *et al*., 2006), in mutants producing LPS truncated in sugar chains (Young and Silver, 1991) or by exposure to antimicrobial peptides and chelating agents such as EDTA, which displace divalent cations between LPS molecules (Nikaido, 2003). Perturbation of the LPS layer induces PLs migration from the inner to the outer leaflet, generating locally symmetrical bilayer rafts that are more permeable to hydrophobic molecules (Nikaido, 2003). Cells have evolved systems to monitor the asymmetry of the OM and to respond either by removing PLs from the outer leaflet or by modifying LPS. Two main mechanisms have been described that restore OM asymmetry by acting on PLs: the phospholipase OmpLA and the Mla pathway. OmpLA, encoded by *pldA*, is a phospholipase that normally resides as an inactive monomer at the OM; however, in the presence of PLs a catalytically active OmpLA dimer is formed. Activated OmpLA degrades PLs that have accumulated in the outer leaflet of the OM under stress conditions (Dekker, 2000). The Mla (Maintenance of OM lipid asymmetry) proteins constitute a highly conserved ATP-binding cassette (ABC) transport system that prevents PLs accumulation in the outer leaflet of the OM under non-stress conditions. Mutations in the Mla system are not lethal but lead to PLs accumulation in the outer leaflet of the OM (Malinverni and Silhavy, 2009). It comprises at least six proteins distributed across the cell envelope. MlaA (formerly VacJ) is a predicted OM lipoprotein, MlaC is a periplasmic protein, and MlaFEDB form a putative ABC transporter (Malinverni and Silhavy, 2009). Recently, MlaA has been found to interact specifically with the OM β-barrel OmpC (Chong *et al.*, 2015). The evidence that cells lacking OmpC accumulate PLs in the outer leaflet of the OM

in stationary phase indicate a role for OmpC in maintaining lipid asymmetry, thus suggesting OmpC to be an additional OM component of the Mla system (Chong *et al*., 2015).

An alternative response to OM asymmetry perturbation consists in LPS modification. LPS can be decorated with the addition of a palmitoyl acid chain to lipid A by PagP, an OM β-barrel acyltransferase that uses PLs flipped in the OM as substrates (Bishop , 2000). The product of the PagP reaction is a hepta-acylated LPS which possesses increased hydrophobicity (Bishop , 2000) thus contributing to better packing within the LPS layer.

LPS biosynthesis takes place at the IM and, for the crucial role of this molecule in OM permeability, has been object of study since long time (Raetz and Whitfield, 2002). Conversely, LPS transport from its site of synthesis to the OM is not yet completely understood. The first step in LPS transport to the cell surface is its flipping across the IM performed by the essential ABC transporter MsbA (Doerrler *et al*., 2001; Doerrler *et al*., 2004; Doerrler and Raetz, 2002). Then, mature LPS molecule assembled at the periplasmic face of the IM by WaaL ligase must be extracted from the IM and translocated through the periplasmic space to the OM, where LPS is finally assembled at the cell surface. Such functions are carried out by the Lpt (lipopolysaccharide transport) system. In *E. coli* the Lpt system is composed of seven essential proteins (LptABCDEFG) located in three distinct cellular compartments of the cell envelope. (Chng *et al*., 2010; Ruiz *et al*., 2008; Sperandeo *et al*., 2007; Sperandeo *et al*., 2008; Wu *et al*., 2006). At the IM, the ABC transporter $LptB_2FG$, associated with the bitopic protein LptC, provides the energy to the system to extract LPS from the membrane and to release it from LptC to LptA (Narita and Tokuda, 2010; Okuda *et al*., 2012). In the periplasm, LptA provides the connection between the IM $LptB_2FGC$ and the OM translocon constituted by the β-barrel LptD protein and the lipoprotein LptE that are organized in the unique plug-and-barrel architecture (Chng *et al*., 2010; Freinkman *et al*., 2012). LptDE is responsible for the final stages of LPS assembly at the cell surface (Chimalakonda *et al*., 2011; Okuda *et al*., 2012; Qiao *et al*., 2014). Crystal structure determination of LptC, LptA and LptD from different organisms revealed that, despite very low sequence similarity, the periplasmic domains of these three proteins share a similar β-jellyroll fold made of juxtaposition of a variable number of antiparallel β-strands (Suits *et al*., 2008; Bollati *et al*., 2015; Tran *et al*., 2010; Qiao *et al*., 2014; Dong *et al*., 2014). The presence of such a fold (the "Lpt fold") is crucial for the assembly of a functional Lpt complex where the C-terminus of LptC interacts with the N-terminus of LptA and the C-terminus of LptA interacts with the N-terminus of another molecule of LptA or with the N- terminus of LptD (Freinkman *et al*., 2012; Villa *et al*., 2013) . The number of LptA molecules present in the functional Lpt complex is currently unknown. Some of the residues potentially involved in protein-protein interaction within the Lpt complex have been identified by *in*

vivo photo-crosslinking experiments that demonstrated also that the hydrophobic grooves of LptA and LptC interact with LPS (Freinkman *et al*., 2012; Okuda *et al*., 2012).

Although the overall architecture of the Lpt complex has been clarified, some questions remain open about the mechanism of LPS transport within the bridge build up by the Lpt proteins and in particular about the role of LptA.

In the past years, some studies made use of site-specific amino acid substitutions to gain insights into the role of LptA in *E. coli*. Suits and co-workers tested single site-specific substitutions in three conserved residues delimiting the N-terminal rim of the β-jellyroll of LptA (Ile 36, Ile 38 and Arg 76) and one in the interior of its cavity (Phe 95) for the ability to impair protein function. In that work, complementation analysis of an arabinose conditional *lptA* mutant revealed that physiologic expression of none of the LptA mutants impaired LPS transport (Suits *et al*., 2008). However, *in vivo* photo-crosslinking experiments performed in a following study revealed that Ile 36 and F95 are involved in LPS interaction together with residues Thr 32, Tyr 114 and Leu 116. Residue His 37 is involved in LptC interaction or LptA homodimerization whereas Val 163 is implicated in LptD interaction (Freinkman *et al*., 2012; Okuda *et al*., 2012).

Another couple of amino acid substitutions in LptA (Gln148Ala-Glu149Ala) have been reported, that prevents LptA oligomerization *in vitro* (Schultz *et al*., 2013), however, it was not tested for its ability to impair LptA function *in vivo*. The only inactive LptA mutant described so far is LptA Gln111Pro, which however is not informative for structure-function analysis (Ma *et al*., 2008).

To better understand the mechanism of LPS transport and of cell response to LPS stress, in this study we have isolated a viable quadruple *lptA* mutant that severely impair the OM permeability barrier and suppressor mutants that partially restore OM functionality. Characterization of the *lptA* and suppressor mutants provides insights into the strategies adopted by the cells to face LPS transport defects.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. *E. coli* bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively, with a brief outline of their construction by standard techniques. Oligonucleotides used in strain and plasmid constructions are listed in Table 3. All cloned DNA regions obtained by PCR were sequenced to rule out the presence of mutations. Site-directed λRed-mediated mutagenesis of *E. coli* was performed as described by (Datsenko and Wanner, 2000) and specified in Table 1. Δ*lptAB*::*kan* DNA was obtained by three-step PCR using the external primers AP54-AP267 and, as templates, the *kan* cassette PCR-amplified from pKD4 using AP79-AP80 primers and the two flanking homology regions obtained by PCR amplification of *E. coli* MG1655 DNA with oligonucleotide pairs AP54-AP268 and AP266-AP267. Transduction with P1 HFT (Wall and Harriman, 1974) was performed as (Miller, 1972). Unless otherwise stated, bacteria were grown at 30 °C in LD (Ghisotti *et al.*, 1992), or M9 minimal medium supplemented with 0.2% glycerol as a carbon source (Kunz and Chapman, 1981) and, when required, 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 25 μg/ml kanamycin. Solid media were as described above with 1% (w/v) agar.

Isolation of bacitracin tolerant phenotypic revertants. Independent overnight cultures of PS003 were grown from single colonies in 10 ml of LD medium supplemented with ampicillin, pelleted and individually resuspended in100 μl of LD before plating onto LD-agar with bacitracin at the indicated concentration. Upon overnight incubation at 37 °C bacitracin resistant (Bct^R) phenotypic revertants were colony-purified in the presence of bacitracin.

Outer membrane permeability assay. OM sensitivity was evaluated by measuring efficiency of plating (e.o.p) on LD agar plates containing bacitracin, rifampicin, novobiocin, SDS-EDTA at non-inhibitory concentrations for the reference (wild type) strain, as indicated. Overnight cultures were grown from single colonies in 5 ml LD supplemented with the antibiotic required for the maintenance of the hosted plasmid. Cultures were serially diluted in LD in microtiter plates, replica plated on the selective LD agar plates and incubated overnight [or up to 24 h]. E.o.p. was estimated relative to plating on LD agar.

Genomic DNA sequencing and data analysis. The library for genomic DNA sequencing was prepared according to the TruSeq DNA Sample preparation protocol (Illumina). Briefly, 1 µg of genomic DNA was sonicated to fragments with a medium length of 400 bp; after end repair, indexed adapters were ligated at DNA fragment ends, libraries were quantified by quantitative Real Time PCR (qPCR) using KAPA Library Quant Kits (KAPA Biosystems). After a short amplification step the library was sequenced on Illumina GAIIX Sequence Analyzer to generate 85 bp paired-end reads. Raw reads were individually mapped to E. *coli* MC4100 genome (RefSeq accession number: HG738867) using the accurate alignment BWA mem algorithm (Li and Durbin, 2009) allowing 1% error; removal of duplicated reads was performed with SAMtools. A VCF file, containing all the variants for each sample relative to E. *coli* MC4100 was obtained by using SAMtools and Bcftools (Li *et al*., 2009) and filtered for low quality variants. Single nucleotide variations (SNVs) having a coverage of less than five high quality reads (Q>30) were discarded. Predicted insertion-deletion (indel) mutations having a coverage lower than six high quality reads (Q >30) were discarded. Then the VCF files were analysed by using SNPeff version 4.0 (De Baets *et al.*, 2012) and high quality SNVs and indels were subsequently annotated to determine their effect and impact on coding sequences.

Determination of LptA abundance. LptA abundance was assessed by Western blot analysis. Bacterial cultures were grown overnight at 30 °C in LD supplemented with 100 µg/ml of ampicillin. Samples for protein analysis were centrifuged 5 min at 16,000 *g* and pellets were resuspended in a volume (in ml) of SDSsample buffer equal to 1/24 of the total OD of the sample. Samples were boiled for 10 min, and equal volumes (20 µl) were fractionated by 12.5% polyacrylamide-SDS gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (GE Healthcare), and Western blot analysis was performed as previously described (Sperandeo et al., 2007) Polyclonal sera raised against LptA (GenScript Corporation) were used as primary antibody at a dilution of 1:1,000. As secondary antibodies, goat anti mouse immunoglobulins (LI-COR) were used at a dilution of 1:7,000 and bands were visualized by an Odyssey Fc Imaging System (LI-COR GmbH).

The Band Analysis tools of Image Studio Lite software version 5.0 (LI-COR GmbH) were used to select and determine the background-subtracted density of the bands in the blot.

Lipid A analysis by mass spectrometry. *E. coli* strains were grown in 1 l of LD supplemented with 100 µg/ml of ampicillin up to OD_{600} = 0.9, yielding to approx. 1 g of liophylized biomass, at 30°C under different conditions as specified. Cells were harvested by centrifugation at 4

°C (5000 g, 15 min). The cell pellets were washed once with 20 ml of phosphate-buffered saline (PBS), 0,1 mM, pH 7.4 and liophylized. Each pellet was treated twice with 10 ml of PCP solution (petroleum ether/chloroform/phenol 90% = 2/5/8 by vol.) (Galanos *et al*., 1969). For each strain, the two supernatants were removed at each passage by centrifugation (5000 rpm, 10 min.), pooled and concentrated in a rotary evaporator to a final volume of approximately 3 ml. LOS precipitation occurred by dropwise addition of water (ca. 0.35 ml). The solid phase was recovered by centrifugation, dissolved in water, dyalized (cut-off 12-14000 MW) against water for 2 days with five water changes, and freeze dried. On average, 5 mg of pure LOS was obtained for each strain. LOS (0.5-1.5 mg) was dissolved in 0.5 ml of 1% AcOH in water and heated at 100°C for 2 h to cleave Lipid A moiety, that was recovered as a precipitate after centrifugation (5000 rpm, 30 min.) in 20-25% yield. Reflectron MALDI TOF MS and MALDI TOF/TOF MS–MS of the lipid A fraction was performed, in negative ion mode, on a 4800 Proteomic analyzer (Applied Biosystems) supplied with a Nd:YAG laser (wavelength of 355 nm). Mass accuracy was better than 50 ppm in reflectron mode. The matrix solution was prepared by dissolving trihydroxyacetophenone (THAP) or 5-Chloro-2-mercaptobenzothiazole in CH_3OH/0.1 % TFA/CH_3CN (7/2/1 by vol) at a concentration of 75 mg/ ml. A sample/matrix solution mixture (1:1 v/v) was deposited (1 µl) onto a stain-less steel sample MALDI probe tip, and dried at room temperature.

Affinity purification of membrane Lpt complexes. Membrane Lpt complexes were affinity purified from strains expressing His-tagged LptC from pGS108 plasmids as previously described (Chng *et al*., 2010; Villa *et al*., 2013) with few modifications. Cells were lysed by a single cycle through a Cell Disrupter (One Shot Model by Constant Systems LTD) at a pressure of 22,000 psi and membranes were collected by ultracentrifugation of the supernatant at 100,000 *g* for 2 h. Membranes were extracted at 4 °C for 30 min with 5 ml of 50 mM Tris-HCl, pH 7.4, 10% glycerol, 1% dodecyl β-D-maltoside (DDM) (Anatrace), 5 mM $MgCl_2$. The mixture was centrifuged again at 100,000 *g* for 1 h and insoluble material was discarded. The supernatant was incubated with 0.5 ml TALON resin suspension for 20 min at 4 °C and the mixture was then loaded onto a column, washed with 10 ml of 50 mM Tris-HCl, pH 7.4, 10% glycerol, 0.05% dodecylmaltoside, 20 mM imidazole, and eluted with 5 ml of 50 mMTris-HCl, pH 7.4, 10% glycerol, 0.05% dodecylmaltoside, 200 mM imidazole. The eluate was concentrated using an ultrafiltration device (Vivaspin, GE Healthcare) by centrifugation at 7,000 x g to a final volume of 80 µl. Samples were mixed with 5x loading buffer, boiled and separated by 12,5% SDS-PAGE (Laemmli et al., 1970) electroblotted and immunodetected using the anti-His monoclonal antibodies (1:3,000) (Sigma Aldrich) to detect LptC-H, and anti-LptA (1:250), anti-LptD (1:500), and anti-

LptF (1:3,000) (GenScript Corporation). As secondary antibodies, goat anti rabbit or anti mouse immunoglobulin (LI-COR) were used at a dilution of 1:15,000.

Protein Expression and Purification. Cultures of BL21(DE3) strain carrying pET vector expressing full length LptA or LptA mutant proteins fused to a C-terminal tag ($SGRVEH_6$) (Suits et al., 2008) were grown up to OD_{600} 0.6 at 30 °C. Expression was induced by the addition of 0.5 mM IPTG (Sigma-Aldrich) and further incubation for 16–18 h at 20 °C. Cells were harvested by centrifugation at 4 °C (5000 *g*, 20 min). The cell pellets were resuspended in buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol), followed by 30 min incubation at 4 °C, shaking in the presence of lysozyme (1 mg/ml), DNase (100 μg/ml), 10mM MgCl2, and 1 mM phenylmethanesulfonylfluoride (PMSF, Sigma-Aldrich). Cells were disrupted as above. Unbroken cells and cell debris were removed by centrifugation at 4 °C (39,000 g, 30 min). The soluble proteins were purified from the supernatant by Ni-NTA affinity columns (Qiagen). The columns were washed with 10 column volumes (CV) of 4% buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 500 mM imidazole, 10% glycerol) in buffer A. Proteins were eluted by a 10%, 20%, 50%, 70%, and 100% buffer B stepwise gradient, 1 CV per step. Fractions were analyzed by 12.5% SDS-PAGE. Pooled fractions containing the purified protein were dialyzed against 20 mM TrisHCl, pH 7.4, 300 mM NaCl through cellulose membranes 10,000-Da cut-off (Sigma-Aldrich). Protein concentration was determined by a Coomassie (Bradford) assay kit (Thermo-Pierce), using bovine serum albumin as a standard.

LPS-Binding Assay. The in vitro LPS binding assay was based on the protocol described previously (Santambrogio *et al*., 2013), with minor modifications. Briefly, assays (500 μl) were carried out in buffer C (50 mM sodium phosphate, pH 8, 50 mM NaCl) containing 25 μM of purified His-tagged protein and a 5-fold molar excess of purified smooth LPS from *E. coli* serotype O55:B5 (Sigma-Aldrich; assumed mw 10,000 Da). The reactions were incubated at room temperature for 1 h on a rotary shaker to allow for the formation of LPS-protein complexes. Ni-NTA resin (200 μL, His-Select nickel affinity gel; Sigma-Aldrich), washed in 1 mL of buffer C, was added to the reaction mixtures and incubated for another hour to allow binding of LPS–protein complexes. The reaction mixture was centrifuged at 13,000 g for 1 min, and the supernatant (FT) was collected. The resin was then washed four times with buffer C and the protein–LPS complexes were eluted in two steps, with 500 μl of buffer C containing 300 mM imidazole (E1) or 500 mM imidazole (E2), respectively. To monitor LPS-LptA complex formation, equal volumes (20 μl) of the collected chromatographic fractions were analyzed by denaturing gel electrophoresis. For LPS

visualization, samples were fractionated by 18% tricine-SDS-PAGE (Lesse *et al*., 1990) and immunodetected using a 1:3,000 dilution of the anti-LPS WN1 222-5 monoclonal antibody (HyCult Biotechnology b.v.). LptA was analyzed by 12.5% SDS-PAGE and immunodetected using 1:3,000 dilution of the anti-His monoclonal antibodies (Sigma Aldrich).

RESULTS

Generation of a partial loss-of-function *lptA* allele. In a previous work (Suits *et al*., 2008) several rationally designed *lptA* mutant alleles turned out to be able to complement LptA-depleted strain for growth. We thus tested whether multiple mutations (*lptA41* allele, encoding the following amino acid substitutions: I36A, I38A, R76D, and K83D) could impair LptA functionality. The quadruple *lptA41* mutant allele was introduced in pWSK29-LptA LptB, a low copy number plasmid vector harbouring the wild type operon *lptAB,* and the construct transferred into the conditional expression *E. coli* mutant FL907, in which the *lptAB* operon is inducible by arabinose. In the absence of arabinose the LptA-depleted strain was complemented by *lptA41* for growth.

Impairment of LPS transport, albeit non-lethal, may be associated to increased OM permeability to hydrophobic toxic compounds and detergents (Ruiz *et al*., 2006). To test whether *lptA41* was a partial loss-of-function allele, plasmid pWSK29-LptA41 lptB was introduced into the reference wild-type strain AM604 and the chromosomal *lptAB* operon was replaced with the Δ*lptAB*::*kan* allele, generating the *lptA41* mutant strain PS003 in which deletion of *lptAB* operon is ectopically complemented. OM permeability of PS003 was probed by testing its sensitivity to a panel of antibiotics, namely bacitracin, novobiocin, and rifampicin. As a control, a similarly generated strain expressing wild type *lptA* (PS001) was used. As shown in Fig. 2B, the *lptA41* mutant displayed increased sensitivity to the antibiotics tested as compared to the corresponding wild-type *lptA* strain, suggesting that *lptA41* is a partial loss-of-function allele of *lptA*.

***lptA41* allele induces LPS modification by PagP.** Conditions that alter the level of LPS at the cell surface, such as defective LPS biogenesis or LPS release from the OM upon EDTA treatment, result in translocation of phospholipids (PLs) to the outer leaflet of the OM (Wu *et al*., 2006; Leive, 1965; Nikaido, 2003), activation of the enzyme PagP, which transfers a palmitate group from PLs to lipid A (Jia *et al*., 2004), and the consequent conversion of hexa-acyl lipid A into hepta-acyl lipid A (Bishop, 2000). We thus analysed by MALDI-TOF MS the composition of lipid A extracted from the ectopically complemented *lptA41* and *lptA* isogenic strains to detect the appearance of hepta-acyl lipid A species as a marker of LPS transport defects (Zhou *et al*., 1999); as controls we analysed the parental reference strain AM604 (wild type) untreated and treated with EDTA.

As shown in Fig. 3, the EDTA-untreated wild type sample and the wild type *lptA*-complemented strain (Fig. 3A and 4B, respectively) did not produce a detectable peak

corresponding to hepta-acyl lipid A, whereas it was present both in the EDTA-treated wild type and in the *lptA41*-complemented samples (Fig. 3C and D, respectively). In the latter sample, a peak corresponding to phosphoethanolamine (PEtN)-modified lipid A, a molecule that appears when OM is damaged (Raetz *et al*., 2007) was also present. These data suggest that the *lptA41* allele, although complementing the Δ*lptA* mutant for viability, induces the translocation of PLs in the outer leaflet of the OM, a diagnostic trait of LPS transport impairment.

LptA41 mutant fails to assemble the Lpt complex. To characterize properties of the LptA41 mutant protein that could be correlated with the functional defect we first assessed whether the amino acid substitutions in LptA impaired its expression level. As shown in Fig. 2C, the steady-state level of LptA41 was about three-fold lower than that of the wild-type LptA, as revealed by SDS/PAGE and immunoblotting with anti-LptA antibody, thus suggesting lower stability of the quadruple mutant protein. It should be noted, however, the mutant protein abundance in the ectopically complemented Δ*lptA* mutant was greater than that of the wild type LptA expressed from the chromosomal locus (undetectable under our experimental conditions; Fig. 2C). Thus, the phenotypic defects caused by *lptA41* allele could not be solely attributed to lower abundance of the mutant protein.

To determine whether LptA41 affects Lpt complex assembly, we performed affinity purification experiments from solubilized membranes of AM604, PS001 and PS003 strains ectopically expressing C-terminally His tagged LptC (LptC-H) from plasmid pGS108. Affinity purified samples were then analyzed by SDS-PAGE-immunoblotting with a panel of specific antibodies, as previously described (Villa *et al*., 2013). As shown in Fig. 4A, in the LptA41-complemented strain LptC-H co-purified LptF, suggesting that the IM $LptB_2FGC$ sub-complex was properly assembled; on the contrary, under these experimental conditions, the LptA41 signal could not be detected while the OM LptD signal was not detectable above the experimental background (no LptC-H). This result suggests that the LptA41 mutant protein is to some extent impaired in OM Lpt sub-complex assembly.

LptA and LptC directly interact with LPS and some residues involved in this interaction have been identified by photocrosslinking experiments (Okuda *et al*., 2012; Tran *et al*., 2008). One of the residues mutated in *lptA41*, I36, is among the LptA residues crosslinked with LPS (Okuda *et al*., 2012). Therefore, we also performed an *in vitro* LPS binding assay using purified C-terminally His-tagged LptA and LptA41 proteins (LptA-H and LptA41-H, respectively), as previously described (Santambrogio *et al*., 2013; Tran *et al*., 2008). Co-purification experiments with smooth-type LPS revealed that, at least *in vitro*, LptA41 retains the ability to interact with LPS (Fig. 4B). Overall,

these data suggest that the phenotype associated to *lptA41* allele could be imputed to impairment in Lpt complex assembly.

Screen for suppressors of *lptA41* increased antibiotic susceptibility phenotype. Analysis of suppressor mutants is a powerful tool to identify genetic and functional interactions between the gene of interest and other genes in the same or in different pathways (reviewed by (Prelich, 1999). We thus selected for spontaneous phenotypic revertants to bacitracin resistance by plating independent cultures of *lptA41* mutant on LD supplemented with 0.15 mg/ml bacitracin, a lethal concentration for the *lptA41* strain but not for the wild-type.

Fifteen independent bacitracin resistant mutants, which arose at a frequency of approximately 10^{-8}, were colony purified and tested for sensitivity to bacitracin, novobiocin, and rifampicin. Based on their sensitivity profile the mutants could be grouped in three classes and a representative of each class (strains PS101, PS102, and PS103) was chosen for subsequent analysis. None of the mutants fully reverted to the wild phenotype (Fig. 2B). PS102 retained sensitivity to both novobiocin and rifampicin, thus suggesting that suppression was acting on a pathway specific for bacitracin rather than alleviating the permeability barrier defect altered by *lptA41*. In addition to bacitracin resistance, PS101 exhibited increased tolerance to novobiocin but not to rifampicin whereas PS103 exhibited increased resistance to rifampicin and a slightly improved tolerance to novobiocin.

Sequencing the plasmid-encoded *lptA* gene of these three mutants revealed that PS101 and PS102 harboured the *lptA41* allele, whereas in PS103 an additional mutation causing the M112I amino acid substitution was present; such a quintuple mutant was designated *lptA42*.

Analysis of LptA steady-state level in the suppressor strains showed that in PS101 mutant the steady-state level of LptA was much higher than that of the parental PS003 (*lptA41*) strain and this appeared to correlate with a higher copy number of the complementing plasmid (Fig. 2C). In PS102 the LptA level resembled that of the parental *lptA41* strain whereas in PS103 the level of LptA appeared to be intermediate between the wild type and the *lptA41* mutant, suggesting that the additional M112I substitution may to some extent stabilize the mutant protein (Fig. 2C).

To identify potential chromosomally encoded suppressors of the increased antibiotic sensitivity, we performed the genomic sequencing of the three selected strains. The total number of reads obtained for each strain allowed us to reach a more than 360-fold mean coverage for the coding portion of each strain genome (see Supplementary Table 1). In order to identify single nucleotide variations (SNVs) or insertions/deletions (indels) in the coding sequences (CDS), which could be potential suppressors of the antibiotic sensitivity phenotype, a comparative analysis was performed by mapping all the reads obtained for each of the three suppressor strains and their

PS003 parental against the reference genome of strain MC4100. PS003 harboured several variations relative to MC4100 many of which were common to all the suppressor strains and thus may simply represent mutations accumulated in different laboratory lines. Table 4 reports the mutations in the suppressor strains not shared with the parental PS003. In our subsequent analysis we considered as relevant neither the variants identified in *lacZ*, a gene largely manipulated in MC4100 (Ferenci *et al*., 2009), nor the single nucleotide changes in intergenic regions.

Analysis of genomic sequencing results from PS101 did not reveal any mutation that could be correlated with the suppression and/or the increased complementing plasmid copy number and/or LptA41 abundance. It is possible that the increased copy number of the plasmid and consequently the higher expression level of LptA41 might contribute to the suppression of bacitracin and novobiocin susceptibility. Variations in non-sequenced regions (gaps) may contribute to suppression and/or increased plasmid copy number. No further analysis was performed on this suppressor strain.

A mutation in *vacJ* is implicated in suppression of PS102 bacitracin sensitivity. Strain PS102 harbours, in addition to mutations in *lacZ* and in intergenic regions, a nucleotide insertion that causes a frameshift mutation at codon 193 of *tus* (herewith named *tus-102* mutation), and a 6 nucleotides deletion in *vacJ* (herewith named *vacJ102* allele) that removes amino acids Asn41 and Phe42 of the encoded VacJ protein (Table 4).

Tus is a non-essential *E. coli* protein implicated in replication termination at *ter* sites (Hill *et al.*, 2013). VacJ, recently renamed MlaA, is the OM lipoprotein component of the Mla system, which is thought to maintain OM lipid asymmetry in *E. coli* (Malinverni and Silhavy, 2009). Therefore, we sought to determine whether *vacJ102* and/or *tus-102* mutations could be implicated in suppression of bacitracin sensitivity observed in PS102 strain.

First we sequenced the chromosomal *vacJ* gene from 5 independent members of this class of suppressor mutants and found that three of them harboured the same *vacJ102* allele; in another mutant an IS*1* element was inserted downstream *vacJ* codon 227, whereas in the last one no mutation was present in *vacJ*. None of the strains harbouring the *vacJ102* or the *vacJ*::IS1 alleles was mutated in *tus*, thus indicating that *tus-102* is not implicated in suppression.

To genetically characterize *vacJ102*, we cloned the wild and the mutant alleles in the plasmid vector pGS100 under the *ptac* promoter, transformed suitable strains, and tested the effects on cell permeability in the presence of *lptA41* allele. To this end we also constructed strains harbouring a deletion of the chromosomal *vacJ*.

It has been reported that an *E. coli* Δ*vacJ* mutant exhibits increased sensitivity at critical SDS-EDTA concentrations (SDS^S) (Malinverni and Silhavy, 2009), whereas no effect on sensitivity to other drugs, including bacitracin, was observed. Thus, we tested both bacitracin and SDS-EDTA sensitivity as diagnostic of OM defects in the complementation and suppression tests summarized in Table 5.

First of all, we observed that *lptA41* conferred increased sensitivity to SDS-EDTA relative to the isogenic wild type strain (compare rows 1-2 with 5-6) at SDS-EDTA concentrations (0.5% and 0.25 mM, respectively) partially tolerated even by the Δ*vacJ* strains (compare rows 13 with 1-3, and 15); the Sds^S phenotype was not suppressed in strain PS102 (rows 9-10). Second, in the suppressor strain PS102 SDS tolerance was not restored, thus further supporting the idea that suppression did not compensate for the OM permeability barrier defect. Ectopic expression of *vacJ102* was sufficient to restore, at least in part, tolerance to bacitracin in *lptA41* mutant strains, both in the haploid (row 20) and in the heterozygous state (row 8). Interestingly, in both conditions *vacJ102* also conferred sensitivity to SDS. Overall, these data suggest that *vacJ102* is an (at least partially) dominant negative mutant that suppresses bacitracin sensitivity exerted by *lptA41*.

LPS analysis of PS102 revealed a lipid A modification pattern similar to the one observed in the parental PS003 mutant (Fig. 3).

An additional amino acid substitution in LptA41and a missense mutation in *opgH* contribute to *lptA41* suppression in PS103. In the suppressor strain PS103 resistance to bacitracin and rifampicin and a slightly improved tolerance to novobiocin were restored. In addition, LPS analysis could detect only the PEtN modification of hexa-acylated lipid A but not the hepta-acylated forms detected in the parental mutant and in the suppressor strain PS102 (Fig. 3). Genomic sequencing of PS103 revealed that, besides the additional mutation in the plasmid encoded *lptA41* allele that generated *lptA42*, the strain harboured a Trp415Stop mutation in *opgH* (*opgH103* allele; Table4). *opgH* (formerly *mdoH*) encodes an IM glycosyltransferase of 847 amino acids implicated in both the synthesis of osmoregulated periplasmic glucans (OPGs), *alias* membrane derived oligosaccharides (MDOs) (reviewed by Kennedy, 1966; Bohin, 2000) and control of cell size *via* interaction with FtsZ in a nutrient-dependent manner (Hill *et al*., 2013). We thus analysed the possible contribution of each mutation to the suppressed phenotype exhibited by PS103 strain.

First, we tested Lpt complex assembly by performing affinity purification experiments from solubilized membranes of a strain bearing *lptA42* allele and ectopically expressing LptC-H. As shown in Fig. 2C, the steady state level of LptA42 appeared to be more abundant than LptA41, thus suggesting that the $LptA^{M112I}$ mutation, improved protein stability. Nevertheless, LptA42 did not

restore LptA interaction with LptC and, therefore, with the Lpt complex (Fig. 4C). As expected, LptA42 mutant retained the ability to co-purify LPS *in vitro*, as shown in Fig. 4B.

We then analyzed the contribution of *opgH103* and *lptA42* alleles alone or in combination, in the suppression of sensitivity to toxic compounds in a suitable set of strains harbouring ectopic and chromosomal *lptA* and *opgH* alleles in different combinations. It should be mentioned that *E. coli* Δ*opgH* strains have been reported to be sensitive to SDS ≥ 0.5% (Rajagopal, 2003). However, under our experimental conditions, the Δ*opgH* mutant was tolerant to 0.5% SDS 0.25 mM EDTA whereas, as shown above (Table 5), the *lptA41* mutant was sensitive.

Data presented in Table 6 indicate that i) in the PS103 suppressor strain (row 1) tolerance to bacitracin, SDS, and rifampicin has been restored, whereas sensitivity to novobiocin was only partially suppressed as compared with the parental strain (line 6); ii) *lptA42* allele was sufficient to restore bacitracin and SDS tolerance, but not rifampicin nor novobiocin tolerance in a wild type chromosomal *opgH* background (lines 10-11). On the other hand, *lptA42* fully restored rifampicin tolerance and only partially novobiocin tolerance only in the absence of a wild type *opgH* allele, irrespective of its chromosomal or plasmid location (compare lines 22, 23, and 25 with 10-12 and 24). The merodiploid *opgH*$^{+}$/*opgHJ103* heterozygous strain (ectopic *opgH103*) was also partially tolerant to novobiocin (line 13).

In *E. coli* inactivation of *opgH* leads to increased expression of colanic acid (CA) (Ebel, 1997). In fact, our Δ*opgH* and *opgH103* strains produced mucoid colonies. To test whether colanic acid production could play any role in the suppressed phenotype, we inactivated *wcaJ* gene, which codes for the UDP-glucose lipid carrier required for the biosynthesis of CA (Dumon *et al*., 2001; Patel *et al*., 2012). Deletion of *wcaJ* in PS103 and in Δ*opgH lptA42* mutant strains barely affected antibiotic resistance of the parental strains (Table 7), thus suggesting that CA overproduction in PS103 is not implicated in suppression of the increased antibiotic susceptibility. Interestingly, inactivation of *wcaJ* in *opgH* mutant expressing *lptA41* allele conferred some amelioration in OM permeability. Overall, these results suggest that suppression of *lptA41* Shc phenotype in PS103 is ensured by the combination of LptA structural stabilization and the production of a truncated OpgH protein and is independent from CA production.

DISCUSSION

LptA is the periplasmic component of the Lpt machine dedicated to ferry LPS across the periplasm to the cell surface. LptA connects, supposedly as a head-to-tail dimer (Santambrogio *et al.*, 2013) the inner membrane ABC transporter formed by the $LptB_2FGC$ sub complex with the OM LptDE translocon by contacting the C-terminal region of LptC at the IM and the N-terminal region of LptD at the OM *via* its N- and C-terminal domains, respectively (Freinkman *et al*., 2012; Polissi and Sperandeo, 2014). LptA also binds LPS and is thought to provide a hydrophobic environment for the passage of the amphipathic LPS through the periplasm (Okuda *et al*., 2012; Tran *et al*., 2008). LptA is the prototype of peculiar β-jellyroll fold (Suits *et al*., 2008) conserved in LptC and LptD, thought to be relevant for both protein-protein and protein-LPS interactions (Suits et al., 2008; Okuda *et al*., 2012; Qiao *et al*., 2014; Dong *et al*., 2014), in agreement with photo-crosslinking studies that identified specific residues implicated in LptA-LptC and LptA-LPS interaction (Freinkman *et al*., 2012; Okuda *et al*., 2012). Depletion experiments with conditional expression mutants have shown that LptA is essential for cell viability (Sperandeo *et al*., 2007; Ma *et al*., 2008). However, despite several attempts of gene-specific mutagenesis, the only negative mutants in *lptA* isolated so far have been Gln111Pro and Gly138Arg substitutions (Bollati *et al*., 2015; Ma *et al*., 2008). However, key LptA functional residues have never been yet identified as it is not known how the above-mentioned mutations affect LptA function.

In the *lptA41* quadruple mutant generated for this study four residues conserved in representative γ-Proteobacteria and lying at the N-terminal rim of LptA internal cavity were changed by site directed mutagenesis. Positively charged residues (R76 and K83) were substituted with the negatively charged residue Asp whereas apolar residues I36 and I38 were substituted with Ala to reduce the steric hindrance. Three such residues (I36, I38, and R76) had been previously mutated individually to both Asp and Glu without an appreciable phenotype (Suits *et al*., 2008). Since photocrosslinking experiments suggested that the region containing the isoleucines is also implicated in LptA-LptC and/or LptA-LptA interaction (Freinkman *et al*., 2012), the quadruple mutant LptA41 was designed so that it could have been impaired not only in LptA-LPS interaction but also in Lpt complex assembly.

Ectopic expression of LptA41 complemented for viability both LptA-depleted cells and *lptA* deletion mutants, and thus modification of the four selected amino acid residues, potentially involved either in interaction with other Lpt proteins or in substrate (lipid A) binding, does not abolish LPS transport to the OM. LPS binding does not seem to be compromised in LptA41,

whereas the mutant failed to interact with LptC in affinity purification experiments using his-tagged LptC as a bait. To our knowledge this is the first *lptA* mutant affected in LptC binding. We suggest that LptA41-LptC interaction is too weak to be detected by affinity purification under our experimental condition but sufficient to form a functional, albeit partially impaired, Lpt complex. Alternatively, LptA41-LptC interaction could be unstable and LptA41, like LolA in the lipoprotein transport system across the periplasm, might transport LPS by shuttling between the IM and OM Lpt sub-complexes (Narita and Tokuda, 2010). It should also be noted that in the *lptA41* mutant, LptC does not co-purify the OM sub-complex component LptD. This supports the model that the IM and OM Lpt sub-complexes are connected only by LptA.

Although *E. coli lptA41* is viable, the mutant exhibits increased sensitivity to three antibiotics (the large, hydrophilic bacitracin, and the hydrophobic rifampicin and novobiocin (Young and Silver, 1991) and to a detergent (SDS). This phenotype is diagnostic of an abnormal OM that is more accessible to the detergent, which may disrupt the OM itself, and more permeable to antibiotics, which have to diffuse through the OM to reach their targets either in the periplasm (bacitracin) or in the cytoplasm (rifampicin and novobiocin). Multiple mechanisms may contribute to the increased permeability of OM (see for reviews Vaara, 1993; Nikaido, 2003). Among these, defective LPS composition may interfere with OM proteins assembly and increase PLs concentration in the outer leaflet of the OM, which in turn would make the OM more permeable to lipophilic molecules such as rifampicin and novobiocin. On the other hand, bacitracin, a hydrophilic molecule too large to diffuse through porins, may exploit transient breaching of the OM to enter the periplasmic space or uncontrolled opening of large pores of OM plug-and-barrel protein such as LptDE (Ruiz *et al*., 2006) or the TonB-dependent receptor FhuA (reviewed by Koebnik *et al*., 2000). It thus appears that in the *lpt41* mutant OM biogenesis is severely impaired and that LPS transport by the mutant Lpt machinery may not be as efficient as with the wild type transporter. This is further supported by the observation that in the *lptA41* mutant lipid A could be detected in the hepta-acylated form (both mono- and di-phosphorylated) or decorated by PEtN. The former modification, catalysed by the lipid A palmitoyltransferase PagP, is induced by translocation of PLs to the outer leaflet of the OM (Jia *et al*., 2004; Bishop, 2000), where PLs are used as palmitoyl donors (Bishop, 2000), and is consistent with the hypothesis of inefficient transport, and consequently, sub-optimal concentration of LPS in the OM. On the other end, PEtN decoration, promoted by EptA (Raetz and Whitfield, 2002) and known to be induced by exposure of cells to mild acid (Gibbons *et al.*, 2005), occurs on the outer surface of the IM (Raetz *et al*., 2007); (Doerrler *et al*., 2004), where LPS might idle in conditions of inefficient transport. Although such

lipid A modifications may be part of the homeostatic cell response to diverse envelope stress conditions, they appear not be sufficient to fully restore the OM permeability barrier.

It should be noted that increased sensitivity to detergents such as SDS may not be necessarily associated with [detectably] increased permeability to large hydrophilic and to hydrophobic compounds (see below, Mla system).

Bacitracin resistance in *E. coli* may occur through several indirect mechanisms, including amplification of the target gene product BacA and, unlike rifampicin and novobiocin, no chromosomal mutations in the antibiotic target gene have been isolated (Cain *et al.*, 1993). Selection for *lptA41* phenotypic revertants to bacitracin resistance was thus performed to avoid selecting mutants in *rpoB* and *gyrB*, which represent the majority of spontaneous Rif^R and Nov^R mutants, respectively. Bac^R mutants produced three different antibiotic resistance profiles, none of which fully reverted to the resistant phenotype of the *lptA* wild type parental. Mutations potentially implicated in suppression were searched by genomic sequencing. Among the variants with adequate coverage (greater than 5 and 6 high quality reads for SNV and indels, respectively), we considered as candidate only mutations in open reading frames, with the exception of *lacZ* in which, for unknown reasons, several mutations appeared to accumulate. As we could not rule out that in addition to the candidate genes other mutations in the non-sequenced gaps could be implicated in suppression, we reconstructed the suppression phenotype by deleting the candidate genes in the parental (non-suppressor) PS001 and PS003 strains and ectopically complementing them with the putative suppressor alleles.

The candidate suppressor mutation emerging from genomic sequencing analysis of strain PS102 was *vacJ102*, a two-codons in frame deletion in *vacJ*. The same mutation was found in additional three out of five independent mutants with similar phenotype, whereas in a fourth mutant an IS*1* element disrupted this gene at codon 227. Whereas the fully sequenced PS102 genome harboured an additional mutation in *tus*, none of these *vacJ* mutations was associated with mutations in *tus*, which therefore does not seem implicated in the suppressor phenotype.

In *E. coli* VacJ (MlaA) is the OM lipoprotein component of the Mla machinery, which is thought to contribute, together with other systems, to the maintenance of OM lipid asymmetry by removing PLs from its outer leaflet (Malinverni *et al*., 2009). It was suggested that upon disruption of the Mla system PLs raft accumulate in the OM outer leaflet (Carpenter *et al*., 2013; Malinverni *et al.*, 2006). *E. coli vacJ* deletion mutants exhibit increased sensitivity to SDS, but not to bacitracin, rifampicin, novobiocin, and erythromycin (Malinverni *et al*., 2006; see also Table 5); this suggests that the lesions inflicted by disruption of the Mla system facilitate the access of the detergent to the OM whereas do not significantly impair the OM permeability barrier. As the *vacJ102* allele confers

increased sensitivity to SDS but not to bacitracin in both haploid and merodiploid heterozygous conditions, we suggest that *vacJ102* is a negative dominant mutation that impairs the Mla system thus leading to accumulation of PLs in the outer leaflet of the OM.

Nevertheless, *vacJ102* suppresses sensitivity to bacitracin (the antibiotic used for the selection of suppressors) caused by *lptA41* whereas it does not restore tolerance to rifampicin and novobiocin. It could be suggested that *vacJ102* would partially restore selectivity of the OM permeability barrier disrupted by *lptA41* so as to reduce diffusion of large hydrophilic molecules such as bacitracin and not of the highly hydrophobic rifampicin and novobiocin. This would be in line with the persistence of phosphoethanolamine (PEtN) and hepta-acyl lipid A modification in LPS from PS102 mutant strain (Fig. 3E), suggestive of a partially impaired OM permeability barrier. However, other hypotheses implying bacitracin-specific mechanisms could be proposed. For example, the mutation in the OM-associated lipoprotein VacJ could interfere with bacitracin diffusion through the OM. This could require the presence of the lipoprotein, albeit inactive for the Mla function, in the OM, possibly in association with Lipid A modifications present in LPS of the *vacJ102* mutant. This model would be consistent with the fact that deletion of *vacJ* does not restore bacitracin tolerance in *lptA41* and with the negative dominant phenotype of the mutation. Alternatively, the *vacJ102* mutant could directly or indirectly (*e.g.* through the periplasmic and/or the IM components of the Mla machinery) interfere with the bacitracin-C_{55}-PP interaction.

In suppressor strain PS103 bacitracin, rifampicin, and SDS tolerance have been restored, whereas novobiocin sensitivity was not fully suppressed. The additive effects of an intragenic and an extragenic suppressor appear to contribute to this phenotype.

The intragenic suppressor (*lptA42* allele) encodes an additional amino acid change (M112I) in LptA41. The LptA42 quintuple mutant protein did not seem to co-purify with LptC any better than its parental LptA41, although it appeared to be more stable as judge by the higher steady state level of the protein. However, *lptA42* allele was sufficient to restore tolerance to bacitracin and SDS in a wild type chromosomal *opgH* background, but not to rifampicin and novobiocin. It thus appears that the intragenic suppressor in *lptA42* allele could fix major OM defects caused by *lptA41*, so as to prevent accessibility of the OM to the detergent and decrease permeability to large hydrophilic but not to hydrophobic molecules. It is conceivable that LptA42 could improve to some extent LPS transport efficiency so as to substantially restore OM asymmetry and prevent formation of PL rafts, which may favour access to detergents, and other structural defects that may allow diffusion of large molecules. In keeping with this hypothesis, it should be noted that in the PS103 strain hepta-acylation of lipid A, which is induced by translocation of PLs to the outer leaflet of the OM and

uses PLs as palmitoyl donors (Bishop, 2000), was not observed (Fig. 3F). Nevertheless, the LPS layer would remain sufficiently weakened so as to allow diffusion of hydrophobic compounds.

The extragenic suppressor associated with PS103 is an amber mutation about in the middle of *opgH*, which encodes a glucosyltransferase implicated in the biosynthesis of the so called osmoregulated periplasmic glucans (OPGs), a class of D-glucose oligosaccharides, heterogeneous in size and structure, found in the periplasm of all Proteobacteria (reviewed by Bohin, 2000). *E. coli* OPGs consist of a backbone of β-1,2,-linked glucose units to which β-1,6 linked branches are attached, ranging from 5 to 12 (typically 8-9) glucose residues. *E. coli* OPGs may be further decorated by *sn*-1-phosphoglycerol and phosphoethanolamine residues originating from the membrane phospholipids, hence the former name of membrane derived oligosaccharides. OPGs, whose synthesis is induced by low osmolarity (Kennedy and Rumley, 1988; Lacroix *et al*., 1991), have been implicated in several processes including chemotaxis, virulence, osmoregulation of OM proteins expression, synthesis of colanic acid, resistance to SDS (Ebel, 1997; Fiedler and Rotering, 1988; Rajagopal, 2003; Geiger *et al*., 1992; Bontemps-Gallo and Lacroix, 2015), but the underlying mechanisms are poorly understood.

OpgH is an integral membrane protein that contains 8 transmembrane domains, with the N and C-termini and an internal domain of 310 residues between the second and third transmembrane regions located in the cytoplasm (Debarbieux *et al*., 1997). In addition to its enzymatic role in OPGs biosynthesis, OpgH has been implicated in control of cell division as an UDP-glucose-activated inhibitor of FtsZ ring formation in *E. coli* (Hill *et al*., 2013).

Both deletion of *opgH* and the *opgH103* nonsense mutation restore resistance to rifampicin and, partially, to novobiocin in the *lptA42* mutant, thus recapitulating the phenotype of the PS103 suppressor strain. *opgH103* also appears to be recessive (strain PS111 with ectopic *opgH103* is Rif^S, albeit not fully Nov^S). It thus appears that the lack of OPGs in the periplasm and/or of the OpgH in the IM make the cell envelope of *E. coli lptA42* less permeable to the lipophilic antibiotic rifampicin and, to a lesser extent, to novobiocin, which is about three-fold more lipophilic than rifampicin (Young and Silver, 1991).

As the targets of both rifampicin and novobiocin are located in the periplasm and given the pleiotropic effects of *opgH*, we cannot tell from these data whether the increased selectivity of the envelope may depend on alterations of OM, periplasm, or IM composition. Although it is difficult to predict the effects in association with the *lptA42* allele, several known phenotypes of *opgH* mutants could impact on the envelope permeability. For example, OPGs affect osmoregulation of porins and other OM proteins (Fiedler and Rotering, 1988; Geiger *et al*., 1992); the same presence of the hydrophilic OPGs in the periplasm or of the Opg multiprotein complex in the IM might

modulate diffusion of some lipophilic molecules; recycling of phospholipids, the donors of *sn*-1-phosphoglycerol and phosphoethanolamine residues that decorate OPGs, may also affect the envelope permeability barrier. Finally, we showed that LptA42 does not co-purify with LptC, suggesting that assembly of the Lpt complex could be impaired to some extent. It could be proposed that the lack of OpgH in the IM or of OPGs in the periplasm somehow facilitate Lpt complex assembly with the LptA42 component, thus improving selectivity of the mutant OM.

In this work, we have identified two different mechanisms that fix, at least partially, the OM permeability barrier compromised in a mutant with defective LPS transport to. In the case of *vacJ102* mutant, the suppression is quite specific for the antibiotic used for the selection and unravels a possible new functional interaction of the Mla system, required in *E. coli* to maintain OM asymmetry. In the case of PS103 suppressor, on the contrary, we found a more global mechanism based on the synergistic effect of LptA structural stabilization and the production of molecules buffering OM permeability defects. Overall, this work reveals different strategies adopted by the cell to preserve OM barrier integrity.

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TABLES

Table 1. *E. coli* strains

Strain	Parental	Relevant Characters		Construction	Source or Reference
		Chromosomal	Plasmid		
AM604	MC4100	Ara^+		Spontaneous Ara^+ revertant	(Wu et al., 2006)
BL21(DE3)		F⁻ *ompT gal dcm lon hsdS$_B$(r_B^- m_B^-)* (λDE3 *[lacI lacUV5-T7 gene 1 ind1 Sam7 nin5])*			(Studier and Moffatt, 1986)
BW25113		*laclq rrnBT14 lacZWJ16 hsdR514 araBADAH33 rhaBADLD78*			(Datsenko and Wanner, 2000)
DH10B		*araD139Δ(ara,leu)7697 ΔlacX74 galU galK rpsL deoR Φ80*d*lacZΔM15 endA1 nupG recA1 mcrA(mrr hsdRMS mcrBC)*			(Grant *et al.*, 1990)
EM001	JW2343	Δ*vacJ*		By FLP-mediated excision of *kan* cassette	This work
EM004	BW25113/pACYC184-LptA LptB	Δ*lptAB*::*kan*	*plac-lptA lptB*; Amp^R	By by site-directed λRed-mediated mutagenesis with Δ*lptAB*::*kan* DNA	This work
FL907	AM604	AM604 Φ(*kan araC araBp-lptA*)1			(Sperandeo *et al*., 2008)
JW2343	BW25113	F⁻ Δ*(araD-araB)567 ΔlacZ4787*(::*rrnB-3*) *λ*⁻ Δ*vacJ754*::*kan rph-1 Δ(rhaD-rhaB)568 hsdR514*			(Baba *et al*., 2006)
PS001	AM604/pWSK29-LptA LptB	Δ*lptAB*::*kan*	*plac-lptA lptB*; Amp^R	By P1 HFT*EM004 transduction, selection for Kan^R	This work
PS003	AM604/pWSK29-LptA41 LptB	Δ*lptAB*::*kan*	*plac-lptA41 lptB*; Amp^R	By P1 HFT*EM004 transduction, selection for Kan^R	This work
PS101	PS003	Δ*lptAB*::*kan*	*plac-lptA41 lptB*; Amp^R	Bct^R, spontaneous suppressor mutant	This work
PS102	PS003	*vacJ102* Δ*lptAB*::*kan*	*plac-lptA41 lptB*; Amp^R	Bct^R, spontaneous suppressor mutant	This work

PS103	PS003	*mdoH103* Δ*lptAB*::*kan*	*plac-lptA42 lptB*; Amp^R	Bct^R, spontaneous suppressor mutant	This work
PS107	PS134/ pWSK29-LptA41LptB	Δ*vacJ* Δ*lptAB*::*kan*	*plac-lptA41 lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS109	PS132/pWSK29-LptA41 LptB	Δ*opgH* Δ*lptAB*::*kan*	*plac-lptA41 lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS110	PS132/pWSK29-LptA42 LptB	Δ*opgH* Δ*lptAB*::*kan*	*plac-lptA42 lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS111	AM604/pWSK29-LptA42 LptB	Δ*lptAB*::*kan*	*plac-lptA42 lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS112	PS132/pWSK29-LptA LptB	Δ*opgH* Δ*lptAB*::*kan*	*plac-lptA lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS113	PS103	*opgH103* Δ*lptAB*::*kan* Δ*wcaJ*::*cat*	*plac-lptA42 lptB*; Amp^R	By P1 HFT*PS135 transduction, selection for Cat^R	This work
PS114	PS109	Δ*opgH* Δ*lptAB*::*kan* Δ*wcaJ*::*cat*	*plac-lptA41 lptB*; Amp^R	By P1 HFT*PS135 transduction, selection for Cat^R	This work
PS115	PS110	Δ*opgH* Δ*lptAB*::*kan* Δ*wcaJ*::*cat*	*plac-lptA42 lptB*; Amp^R	By P1 HFT*PS135 transduction, selection for Cat^R	This work
PS116	PS112	Δ*opgH* Δ*lptAB*::*kan* Δ*wcaJ*::*cat*	*plac-lptA lptB*; Amp^R	By P1 HFT*PS135 transduction, selection for Cat^R	This work
PS130	PS134/pWSK29-LptA LptB	Δ*vacJ* Δ*lptAB*::*kan*	*plac-lptA lptB*; Amp^R	By P1 HFT*PS001 transduction, selection for Kan^R	This work
PS131	AM604	Δ*opgH*::*cat*		By site-directed λRed-mediated recombination; primers FG3116-FG3117, template pKD3	This work
PS132	PS131	Δ*opgH*		By FLP-mediated excision of *cat* cassette	This work
PS133	AM604	Δ*vacJ*::*kan*		By P1 HFT*JW2343 transduction (selection for Kan^R)	This work
PS134	PS133	Δ*vacJ*		By FLP-mediated excision of *kan* cassette	This work

PS135	AM604	Δ*wcaJ*::*cat*	By site-directed λRed-mediated recombination; primers FG3153-FG3154, template pKD3	This work
XL1-Blue		F^- λ^- *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* {F' *proAB, lacI*q*Z* Δ*M15* Tn*10*(Tet^R)}		Agilent Technologies

Table 2. Plasmids

Plasmid	Parental plasmid/replicon	Relevant characters	Construction/Origin
pACYC184		pSC101 *ori* p15A; Cam^R, Tet^R	(Bartolome *et al*., 1991)
pACYC184-LptA LptB	pACYC184	*plac-lptA lptB*; Cam^R	*lptAB* genes were excised from plasmid pWSK29-LptA LptB and subcloned into *BamHI- SalI* sites of pACYC184
pCP20		*bla cat;* thermosensitive replication	(Datsenko and Wanner, 2000)
pET-LptA41-H	pET21a	*pT7-lptA41*-His_6	*lptA41* allele was PCR amplified with AP182 and AP183 and cloned into *BamHI-NotI* sites of pET-LptA-H.
pET-LptA42-H	pET21a	*pT7-lptA42*-His_6; Amp^R	*lptA42* was obtained by site-directed mutagenesis with FG3191-FG3192 primers from pET-LptA41-H as template
pET-LptA-H	pET21a	*pT7-lptA*-His_6; Amp^R	Suits *et al*., 2008
pGS100	pGZ119EH	*ptac*-TIR *cat* $oriV_{ColD}$	(Sperandeo et al., 2006)
pGS100-OpgH	pGS100	*ptac-opgH*; Cam^R	*opgH* was PCR-amplified with FG3069-FG3070 primers from PS003 genomic DNA and cloned into *XbaI-PstI* sites of pGS100
pGS100-OpgH103	pGS100	*ptac-opgH103*; Cam^R	*opgH103* was PCR-amplified with FG3069-FG3070 primers from PS003 genomic DNA and cloned into *XbaI-PstI* sites of pGS100
pGS100-VacJ	pGS100	*ptac-vacJ*; Cam^R	*vacJ* was PCR-amplified with FG3067-FG3068 primers from PS003 genomic DNA and cloned into *XbaI-PstI* sites of pGS100
pGS100-VacJ102	pGS100	*ptac-vacJ102*; Cam^R	*vacJ102* was PCR-amplified with FG3067-FG3068 primers from PS102 genomic DNA and cloned into *XbaI-PstI* sites of pGS100
pGS108	pGS100	*ptac-lptC*-His_6 *cat* $oriV_{ColD}$	(Sperandeo et al., 2006)
pKD3		*oriRγ*; Amp^R Cam^R; source of *cat* cassette	(Datsenko and Wanner, 2000)
pKD4		*oriRγ*; Amp^R Kan^R; source of *kan* cassette	(Datsenko and Wanner, 2000)

pKD46		*oriR101 repA101ts araC araBp-λ red bla*	(Datsenko and Wanner, 2000)
pWSK29	pBSIISK	pSC101 *ori* f1 *ori lacZa*; Amp^R	(Wang and Kushner, 1991)
pWSK29-LptA LptB	pWSK29	*plac-lptA41 lptB*; Amp^R	(Santambrogio *et al*., 2013)
pWSK29-LptA36 LptB	pWSK29-LptA LptB	*plac-lptA*$^{I36A\ I38A}$ *lptB,* Amp^R	By site-directed mutagenesis with primers AP184-AP185
pWSK29-LptA37 LptB	pWSK29-LptA36 LptB	*plac-lptA36*^{R76D} *lptB,* Amp^R	By site-directed mutagenesis with primers AP112-AP113
pWSK29-LptA41 LptB	pWSK29-LptA37 LptB	*plac-lptA37*^{K83D} *lptB,* Amp^R	By site-directed mutagenesis with primers AP186-AP187
pWSK29-LptA42 LptB	pWSK29-LptA41 LptB	*plac-lptA41*^{M112I} *lptB*; Amp^R	Spontaneous mutant

Table3. Oligonucleotides

Name	Sequence [a]	Notes
AP54	cgagaggaattcaccATGAGTAAAGCCAGACGTTGGG	Δ*lptAB*::*kan* cassette for EM004 construction by three step PCR, with AP268; *EcoRI*
AP79	GTGTAGGCTGGAGCTGCTTCG	Amplification *kan* cassette from pKD4, with AP80
AP80	CATATGAATATCCTCCTTAG	Amplification *kan* cassette from pKD4, with, with AP79
AP112	CAAAGTGGTCGTTACC**GAT**CCGGGCGGCGAACAAGG	pWSK29-LptA41 LptB (R76D) construction with AP113
AP113	CCTTGTTCGCCGCCCGG**ATC**GGTAACGACCACTTTG	pWSK29-LptA41 LptB (R76D) construction with AP112
AP182	ctcgacgcggccgcTATATACCCTTCTTCTGTG	pET-LptA41-H construction with AP183, *NotI*
AP183	cgagatggatccATGAAATTCAAAACAAACAAAC	pET-LptA41-H construction with AP182, *BamHI*
AP184	CACTGATCAGCCG**GCC**CAC**GCT**GAATCGGACCAG	pWSK29-LptA41 LptB (I36A and I38A) construction with AP185
AP185	CTGGTCCGATTC**AGC**GTG**GGC**CGGCTGATCAGTG	pWSK29-LptA41 LptB (I36A and I38A) construction with AP184
AP186	GCGGCGAACAAGGT**GAT**GAAGTGATTGACGGC	pWSK29-LptA41 LptB (K83D) construction with AP187
AP187	GCCGTCAATCACTTC**ATC**ACCTTGTTCGCCGC	pWSK29-LptA41 LptB (K83D) construction with AP186
AP266	ctaaggaggatattcatatgGATAGGGTAGAAGTTTGCG	Δ*lptAB*::*kan* cassette construction, *kan* hybrid primer for EM004 construction by three step PCR, with AP267
AP267	CTAATGATCAGTCTGGCCTC	Δ*lptAB*::*kan* cassette for EM004 construction by three step PCR, with AP266
AP268	gaagcagctccagcctacacGATTAAGGCTGAGTTTG	Δ*lptAB*::*kan* cassette construction, *kan* hybrid primer for EM004 construction by three step PCR, with AP54
AP354	GTCATGGATGGCAAACTG	*vacJ102-SPA*::*kan* cassette construction, *SPA-kan* hybrid primer for EM002 construction by three step PCR, with AP403
AP387	TCCATGGAAAAGAGAAG	Amplification of *SPA*::*kan* cassette from CAG60297, with AP388
AP388	CATATGAATATCCTCCTTAG	Amplification of *SPA*::*kan* cassette from CAG60297, with AP387
AP400	GGTATCGACAACCAAGAACC	*vacJ102-SPA*::*kan* cassette construction for EM002 construction by three step PCR, with AP404
AP403	catcttctcttttccatggaTTCAGAATCAATATCTTTTAAATC	*vacJ102-SPA*::*kan* cassette construction, *SPA-kan* hybrid primer for EM002 construction by three step PCR, with AP354
AP404	ctaaggaggatattcatatgGAAACAAATAAAAAAGGTG	*vacJ102-SPA*::*kan* cassette construction, *SPA-kan* hybrid primer for EM002 construction by three step PCR, with AP400
FG3067	cgactagtctagaATGAAGCTTCGCCTGTC	pGS100-VacJ and pGS100-VacJ102 construction with FG3068; *XbaI*
FG3068	cgagatctgcagTTATTCAGAATCAATATC	pGS100-VacJ and pGS100-VacJ102 construction with FG3067; *PstI*

FG3069	cgactagtctagaATGAATAAGACAACTGAGTAC	pGS100-OpgH and pGS100-OpgH103 construction with FG3070; *XbaI*
FG3070	cgagatctgcagTTATTGCGAAGCCGCATC	pGS100-OpgH and pGS100-OpgH103 construction with FG3069; *PstI*
FG3116	gtgaaacctggagctaccagttacctgccaatgaataagGTGTAGGCTGGAGCTGCTTC	Δ*opgH*::*cat* cassette construction with FG3117
FG3117	gtaggcctgataagcgtagcgcatcaggcaactacgttttCATATGAATATCCTCCTTAG	Δ*opgH*::*cat* cassette construction with FG3116
FG3153	catcgttaatctctatggtgcaacgcttttcagatatcacGTGTAGGCTGGAGCTGCTTC	Δ*wcaJ*::*cat* cassette construction with FG3154
FG3154	caggaaaacgattttgatatcgaaccagacgctccattcgCATATGAATATCCTCCTTAG	Δ*wcaJ*::*cat* cassette construction with FG3153
FG3191	GTCACGCTTCCCAG**ATT**CACTACGAACTGGC	pET-LptA42-H construction with FG3192
FG3192	GCCAGTTCGTAGTG**AAT**CTGGGAAGCGTGAC	pET-LptA42-H construction with FG3191

[a] Upper case letters, sequence present in the template; lower case letters, additional/modified sequence not present in the template; restriction sites are underlined.

Table 4. Mutations in the suppressor strains

Strain	Genomic coordinate[a]	Mutation	Gene	Product[b]	Gene coordinate[c]	Amino acid change[d]
PS101	290676	T→G transversion	Intergenic	na	na	na
	4178702	G→C transversion	*lacZ*	β-galactosidase	246	Arg116Gly
	4178861	G insertion	*lacZ*	β-galactosidase	187	Ser63fs
	4178862	A→G transition	*lacZ*	β-galactosidase	186	none
	4178862	CGG insertion	*lacZ*	β-galactosidase	183	Asp62fs
PS102	290676	T→G transversion	Intergenic	na	na	na
	1574918	A insertion	*tus*	replication terminus site-binding protein	577	Ser193fs
	2348710	AAGTTG deletion	*vacJ*	putative lipoprotein	120	ΔAsn41-Phe42
	2613172	G→A transition	Intergenic	na	na	na
	4178702	G→C transversion	*lacZ*	β-galactosidase	346	Arg116Gly
	4178862	A→G transition	*lacZ*	β-galactosidase	186	none
	4178862	CG insertion	*lacZ*	β-galactosidase	184	Asp62fs
PS103	425601	A→G transition	*rhsD*	*rhsD* element	358	Ser120Gly
	1015255	G→A transition	*opgH*	glucosyltransferase	1245	Trp415Stop
	3667499	T→G transversion	Intergenic	na	na	na
	3228885[e]	G→T transversion	*lptA*	LPS transport protein	336	Met112Ile

[a] For deletions and insertions, the coordinate indicates the first deleted base and the base after which insertion occurred, respectively

[b] na, not applicable

[c] Coordinates from the first base of the ORF. For deletions and insertions, the coordinate indicates the first deleted amino acid and the amino acid after which insertion occurred, respectively

[d] fs, frame shift starting at the codon indicated

[e] this region is actually harboured by the complementing plasmid

Table 5. Phenotypic suppression pattern by *vacJ102* allele [a]

Strain	Chromosome		Plasmid		LD	Bct 50 µg/ml	Bct 150 µg/ml	SDS EDTA	Row
	lptA	***vacJ***	***lptA*[b]**	***vacJ*[c]**					
PS001	Δ*lptA*	*vacJ*$^+$	*lptA*$^+$	NP	+	+	+	+	1
				-	+	+	+	+	2
				vacJ$^+$	+	+	+	+	3
				vacJ102	+	+	+	–	4
PS003	Δ*lptA*	*vacJ*$^+$	*lptA41*	NP	+	–	–	–	5
				-	+	–	–	–	6
				vacJ$^+$	+	–	–	–	7
				vacJ102	+	+	-/+	–	8
PS102	Δ*lptA*	*vacJ102*	*lptA41*	NP	+	+	+	–	9
				-	+	+	+	–	10
				vacJ$^+$	+	+	-/+	–	11
				vacJ102	+	+	+	–	12
PS130	Δ*lptA*	Δ*vacJ*	*lptA*$^+$	NP	+	+	+	-/+	13
				-	+	+	+	-/+	14
				vacJ$^+$	+	+	+	+	15
				vacJ102	+	+	+	-/+	16
PS107	Δ*lptA*	Δ*vacJ*	*lptA41*	NP	+	-/+	–	–	17
				-	+	-/+	–	–	18
				vacJ$^+$	+	-/+	–	–	19
				vacJ102	+	+	-/+	–	20

[a] e.o.p. relative to growth on LD agar. +, e.o.p. >10^{-2}; -/+, between 10^{-2} and 10^{-3}; -, <10^{-3}

[b] *lptA*+, pWSK29-LptA LptB; *lptA41*, pWSK29-LptA41 LptB

[c] NP, no plasmid; -, empty vector pGS100; *vacJ*+, pGS100-VacJ; *vacJ102*, pGS100-VacJ102

Table 6. Phenotypic suppression pattern by *lptA42* and *opgH103* alleles [a]

Strain	Chromosome		Plasmid		LD	Bct 150 µg/ml	SDS-EDTA	Rif 2.5 µg/ml	Nov 10 µg/ml	row
	lptA	*opgH*	*lptA*[b]	*opgH*[c]						
PS103	Δ*lptA*	*opgH103*	*lptA42*	NP	+	+	+	+	-/+	1
PS001	Δ*lptA*	*opgH*+	*lptA*$^+$	NP	+	+	+	+	+	2
				-	+	+	+	+	+	3
				opgH$^+$	+	+	+	+	+	4
				opgH103	+	+	+	+	+	5
PS003	Δ*lptA*	*opgH*+	*lptA41*	NP	+	–	–	–	–	6
				-	+	–	–	–	–	7
				opgH$^+$	+	–	–	–	–	8
				opgH103	+	–	–	–	–	9
PS111	Δ*lptA*	*opgH*+	*lptA42*	NP	+	+	+	–	–	10
				-	+	+	+	–	–	11
				opgH$^+$	+	+	+	–	–	12
				opgH103	+	+	+	–	-/+	13
PS112	Δ*lptA*	Δ*opgH*	*lptA*$^+$	NP	+	+	+	+	+	14
				-	+	+	+	+	+	15
				opgH$^+$	+	+	+	+	+	16
				opgH103	+	+	+	+	+	17
PS109	Δ*lptA*	Δ*opgH*	*lptA41*	NP	+	–	–	–	–	18
				-	+	–	–	–	–	19
				opgH$^+$	+	–	–	–	–	20
				opgH103	+	–	–	–	–	21
PS110	Δ*lptA*	Δ*opgH*	*lptA42*	NP	+	+	+	+	-/+	22
				-	+	+	+	+	-/+	23
				opgH$^+$	+	+	+	–	–	24
				opgH103	+	+	+	+	-/+	25

[a] e.o.p. relative to growth on LD agar. +, e.o.p. >10^{-2}; -/+, between 10^{-2} and 10^{-3}; -, <10^{-3}

[b] [b] *lptA*+, pWSK29-LptA LptB; *lptA41*, pWSK29-LptA41 LptB; *lptA42*, pWSK29-LptA42 LptB

[c] NP, no plasmid; -, empty vector pGS100; *opgH*$^+$, pGS100-OpgH; *opgH103*, pGS100-OpgH103

Table 7. Colanic acid production does not affect PS103 suppressor phenotype [a]

Strain[b]	Chromosome		Plasmid[c]	LD	Bct 50 µg/ml	Bct 150 µg/ml	Rif 2.5 µg/ml	Nov 10 µg/ml	Row
	opgH	***wcaJ***	***lptA***						
PS103	*opgH103*	*wcaJ*$^{+}$	*lptA42*	+	+	+	+	-/+	1
PS113	*opgH103*	Δ*wcaJ*	*lptA42*	+	+	+	+	-/+	2
PS112	Δ*opgH*	*wcaJ*$^{+}$	*lptA+*	+	+	+	+	+	3
PS109	Δ*opgH*	*wcaJ*$^{+}$	*lptA41*	+	–	–	–	–	4
PS110	Δ*opgH*	*wcaJ*$^{+}$	*lptA42*	+	+	+	+	–	5
PS116	Δ*opgH*	Δ*wcaJ*	*lptA+*	+	+	+	+	+	6
PS114	Δ*opgH*	Δ*wcaJ*	*lptA41*	+	-/+	-/+	-/+	–	7
PS115	Δ*opgH*	Δ*wcaJ*	*lptA42*	+	+	+	+	–	8

[a] e.o.p. relative to growth on LD agar. +, e.o.p. >10^{-2}; -/+, between 10^{-2} and 10^{-3}; -, <10^{-3}

[b] All strains harbour the Δ*lptAB::kan* chromosomal deletion

[c] *lptA42*, pWSK29-LptA42 LptB; *lptA+*, pWSK29-LptA LptB; *lptA41*, pWSK29-LptA41 LptB

FIGURE LEGENDS

Fig. 1. The LPS transport system. (A) Transport of LPS from IM to OM. After flipping over the IM by MsbA protein, LPS is transported across the periplasm and assembled at the cell surface by the Lpt system. Modified from (Martorana et al., 2014). (B) Chemical structure of LPS from *E. coli* K12. Lipid A, core oligosaccharide and O-antigen are evidenced.Abbreviations: Gal, D-galactose; Glu, D-glucose; Hep, L-glycero-D-manno-heptose; KDO, 2-keto-3-deoxy-octonic acid; EtN, ethanolamine; P, phosphate. Modified from (Ruiz et al., 2009).

Fig. 2. LptA41 mutations and *lptA41* suppressors phenotypes. (A) Ribbon diagram of *E. coli* LptA. Residues mutated by site-directed mutagenesis (I36, I38, R76, K83) are indicated in red. The intragenic suppressor (*lptA42* quintuple mutant allele) encodes an additional amino acid change at the position indicated in green (M112). (B) OM permeability assay of *lptA41* strain and suppressor mutants. Serial ten-fold dilutions of stationary phase cultures of AM604 (wild type reference strain), PS001 (wild type *lptA* control strain ectopically expressing *lptAB*), PS003 (ectopically expressing *lptA41 lptB*), PS101, PS102, PS103 (PS003 derivative suppressor mutants) were replicated on LD (for AM604) or LD-ampicillin agar plates supplemented with bacitracin (50 μg/ml), novobiocin (10 μg/ml), rifampicin (2.5 μg/ml)as indicated. (C) Cell lysates from AM604, PS001 , PS003, PS101, PS102, PS103 were analyzed by Western blotting with anti-LptA antibody as described in Materials and Methods. Culture samples of equal OD_{600} were processed and loaded into each lane. An aspecific band was used as loading control (LC). Plasmid DNA form normalized amount of PS003, PS101, PS102; PS103 cells were prepared and analyzed by agarose gel electrophoresis.

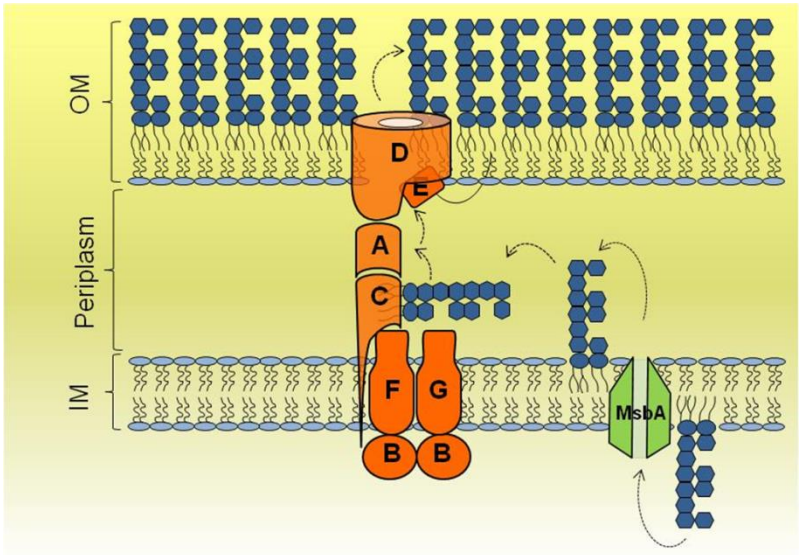
Fig. 3. Lipid A modifications in *lptA41* suppressor strains. MALDI-TOF profiles of lipid A isolated from wild type strain AM604 treated (B) or not (A) with EDTA, PS001 (C), PS003 (D) and suppressor strains PS102 (E) and PS103 (F). For details see Materials and Methods.

Fig. 4. Affinity purification of membrane Lpt complexes and LPS binding by LptA mutants. (A) Total membranes were collected from strains AM604 [expressing chromosomal LptA, LptA (c)], PS001 [ectopically expressing LptA, LptA (p)], PS003 [ectopically expressing LptA41, LptA41 (p)], harboring pGS108 expressing His-tagged LptC (LptC-H) or pGS100 expressing the His tag (none) as a negative control. Samples were solubilized with dodecyl β-D-maltoside (DDM) and affinity purified using a Talon metal affinity resin. Proteins were then fractionated by SDS-PAGE and immunoblotting was performed with antibodies anti-LptD, anti-LptA, anti-LptF and anti-His (to detect LptC-H). (B) The ability of His-tagged LptA, LptA41, and LptA42 to bind purified LPS was assessed by their co-elution from Ni-NTA chromatography resin. LPS and purified His-tagged proteins were incubated and affinity purified on Ni-NTA resin as described in Materials and Methods. As a negative control LPS was incubated without any added protein (none). FT, flow-through; E1–E2, elutions. To monitor LPS-LptA complex formation, equal volumes of the collected chromatographic fractions were analyzed by denaturing gel electrophoresis. LptA-H protein was detected by SDS-PAGE and Western blotting with anti-His antibodies; for LPS visualization, samples were analyzed by Tricine-SDS-PAGE and Western blotting was performed with anti-lipid A-core antibodies.(C) The ability of LptA42 to assembly the Lpt complex was evaluated as described in panel A. Samples were prepared from PS001 [LptA (p)], PS003 [LptA41 (p)], PS111 [LptA42 (p)] strains harboring pGS108 expressing His-tagged LptC (LptC-H).

Supplementary Table 1. Sequencing metrics

Strain	Total reads	Mapped reads	% mapped reads	Mapped reads on CDS	% mapped reads in CDS	Mapped bases	Mean coverage
PS101	20.200.414	19.490.163	96	17.283.373	89	1.388.740.785	360.72
PS102	30.486.926	30.110.142	99	26.598.378	88	2.134.108.807	554.33
PS103	22.546.138	21.942.298	97	19.477.080	89	1.566.968.775	407.01

A



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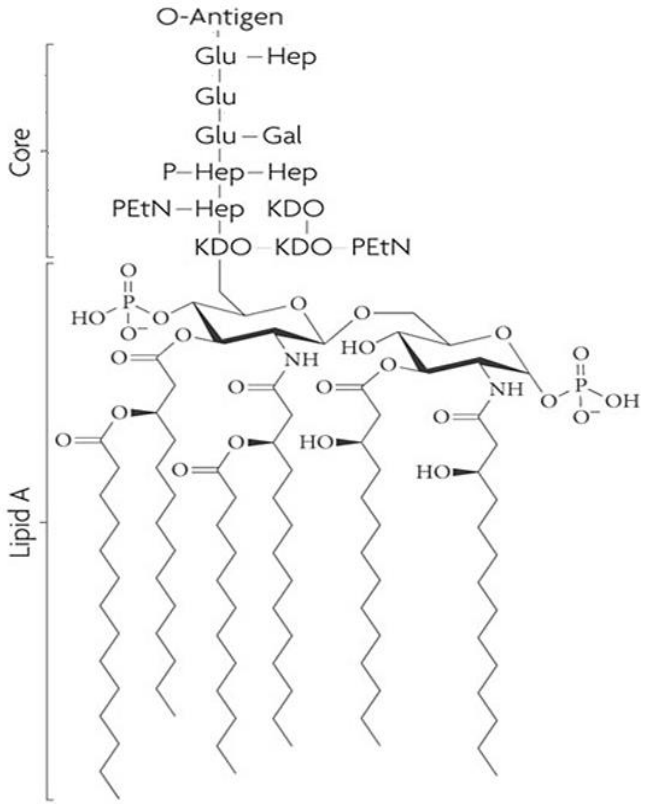
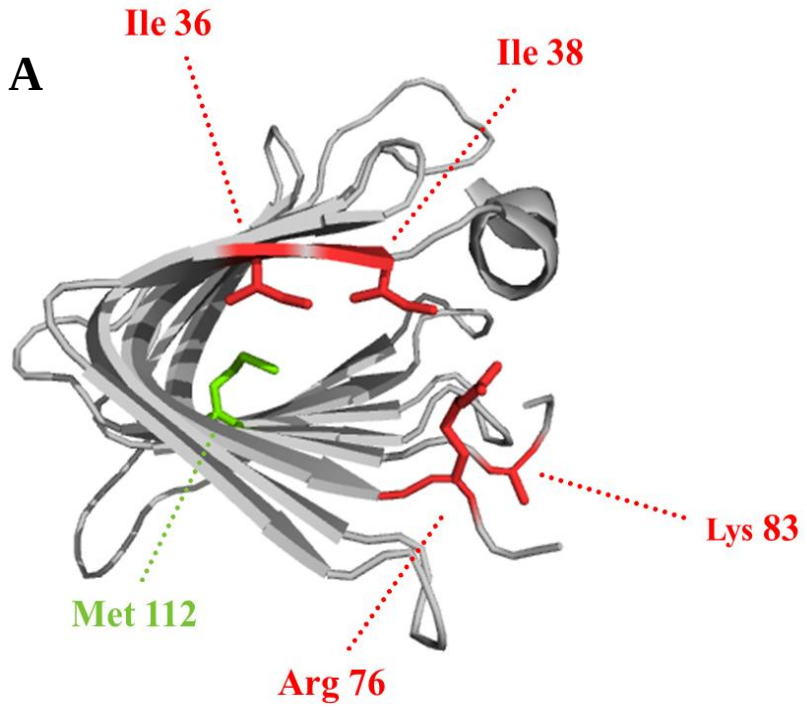
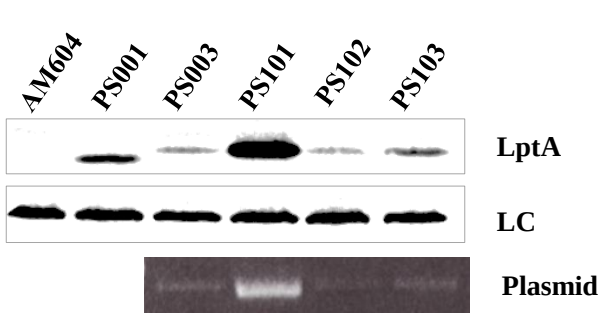
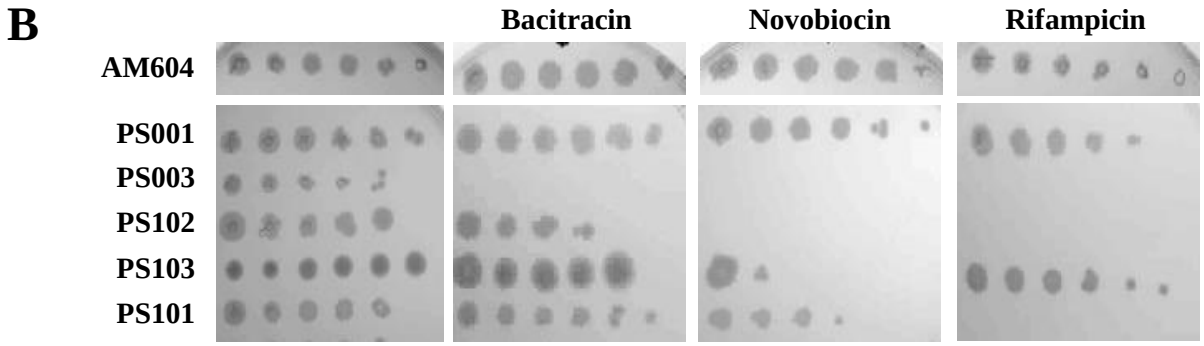


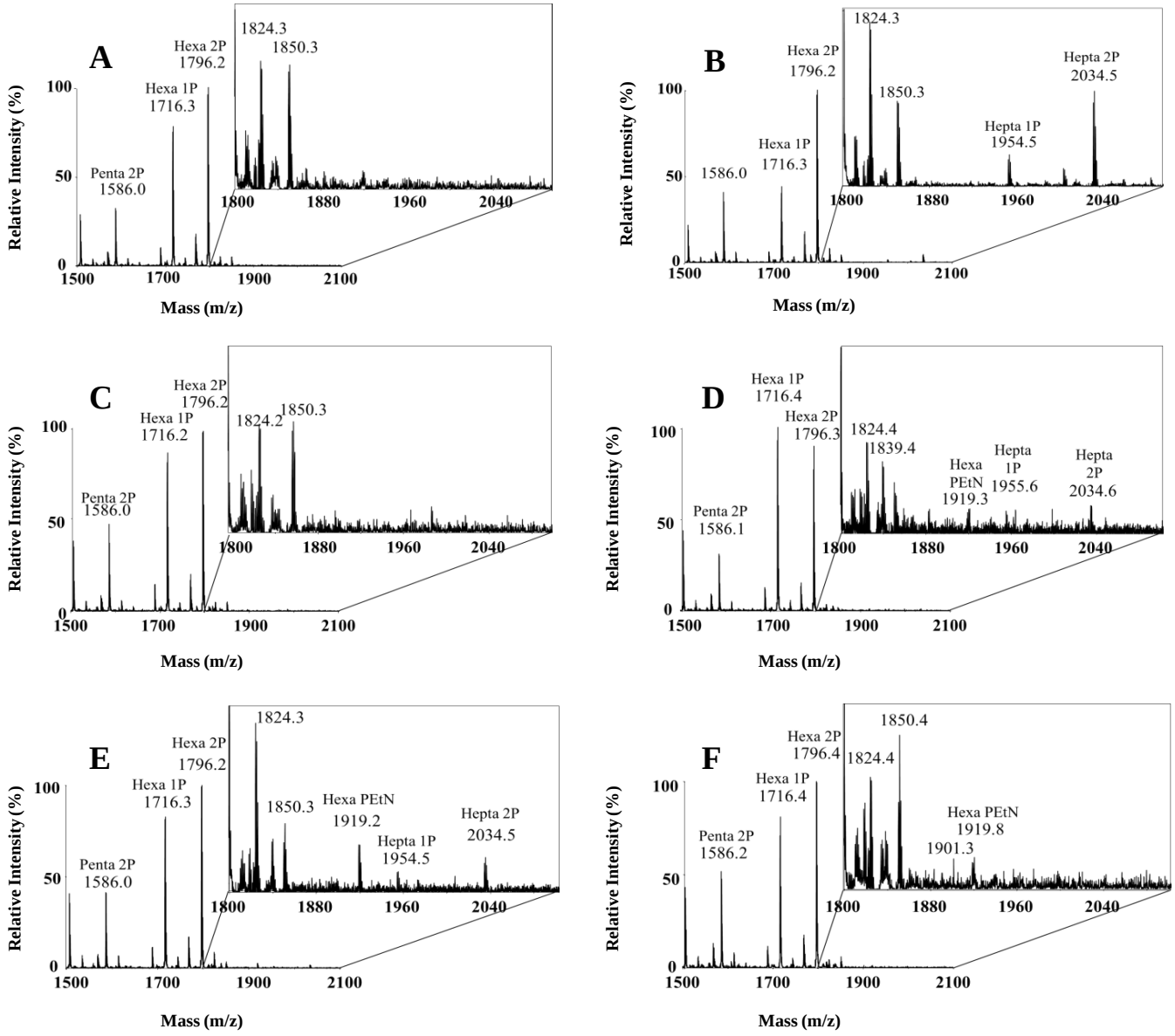
Falchi *et al.* - Fig. 1



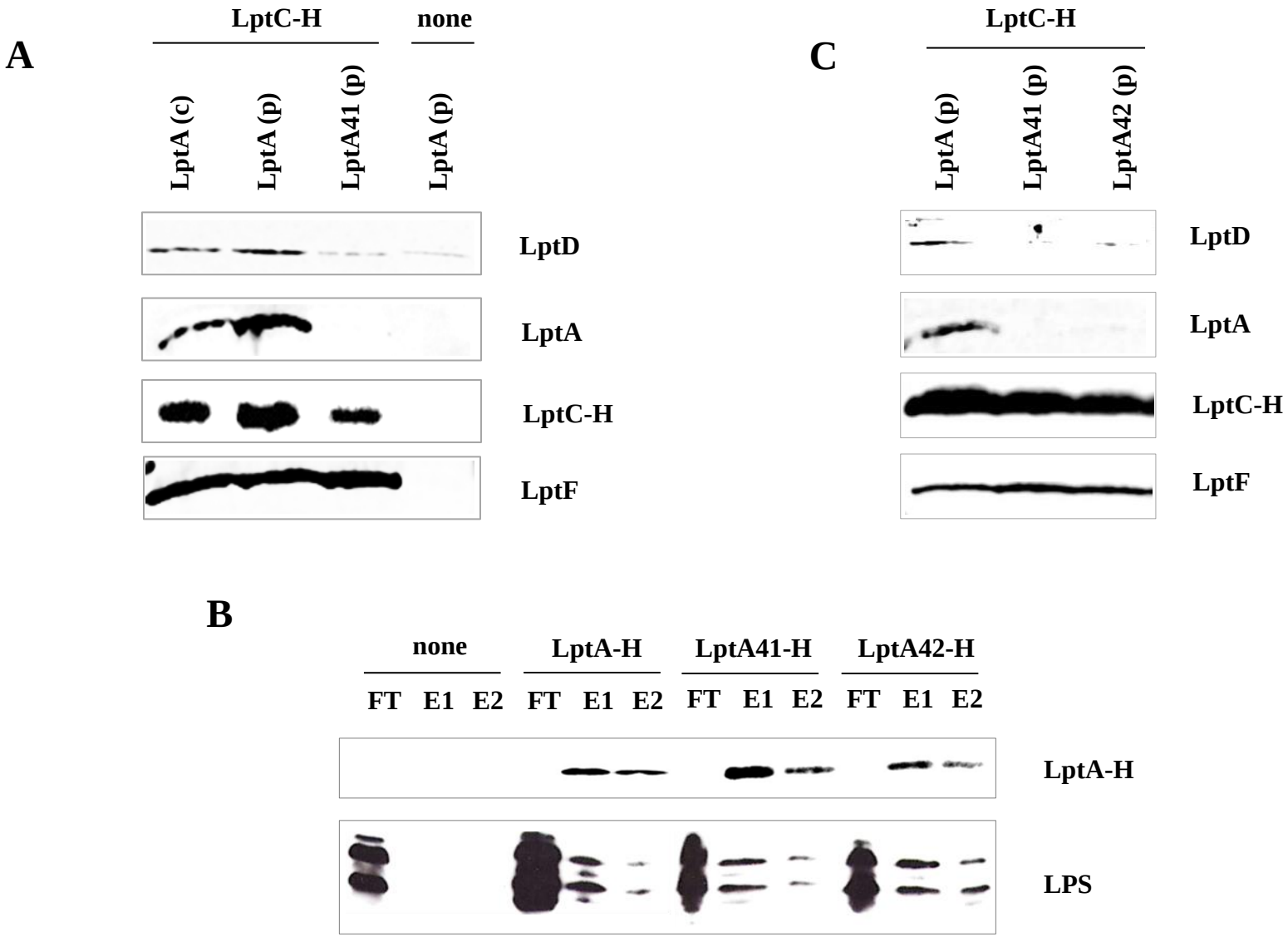




Falchi *et al.* - Fig. 2



Falchi *et al*. - Fig. 3



Falchi *et al*. - Fig. 4

2. Draft Manuscript

The lack of the essential LptC component in the *Escherichia coli* lipopolysaccharide transport machine can be circumvented by suppressor mutations in the inner membrane ABC transporter LptF

Mattia Benedet[1], Federica Falchi[1], Simone Puccio[2], Cristiano Di Benedetto[1], Clelia Peano[2], Alessandra Polissi[3], and Gianni Dehò[1*]

[1] Dipartimento di Bioscienze, Università degli Studi di Milano, Milan, Italy

[2] Istituto di Tecnologie Biomediche, Consiglio Nazionale delle Ricerche, Milan, Italy

[3] Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Milan, Italy

* Corresponding author:
Gianni Dehò, Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy. Phone +39 02 5031 5019; e-mail: gianni.deho@unimi.it

SUMMARY

The lipopolysaccharide (LPS) transport (Lpt) system is responsible for transferring LPS from the periplasmic surface of the inner membrane (IM) to the outer leaflet of the outer membrane (OM), where it plays a crucial role in OM selective permeability. In *E. coli* seven essential proteins form the Lpt trans-envelope complex: LptBFG form the IM ABC transporter; LptDE form the OM translocon for final LPs delivery; LptC, an IM-anchored protein with a periplasmic domain, interacts with the IM ABC transporter, the periplasmic protein LptA, and LPS. Although essential, LptC can tolerate several mutations and its role in LPS transport is unclear. To clarify the functional role of LptC in the Lpt machine we selected, using a double selection plasmid shuffling approach, viable mutants lacking *lptC*. Genome sequencing of Δ*lptC* mutants revealed single amino acid substitutions at a unique position in the periplasmic domain of the IM component LptF ($LptF^{Sup}$). In complementation tests, $lptF^{Sup}$ mutants suppress lethality of both Δ*lptC* and *lptC* conditional expression mutants. Our data show that mutation in a specific residue of the large LptF periplasmic domain can compensate the lack of the essential protein LptC, implicate this LptF region in the formation of the periplasmic bridge between the IM and OM complexes, and suggest that LptC may have evolved as a chaperon of a six-component Lpt machine assembly and/or activity.

INTRODUCTION

Lipopolysaccharide (LPS), the major glycolipid in the outer layer of Gram-negative bacteria outer membrane (OM), is synthesized at the level of the inner membrane (IM) to be then transported to its final destination (reviewed by (Nikaido, 2003; Sperandeo *et al.*, 2014)). In *Escherichia coli*, where this process has been best characterized, the LPS transporter (Lpt) exhibits the overall organization of a trans-envelope ATP-binding cassette (ABC) transporter (Davidson *et al.*, 2008) composed by seven proteins, LptA through LptG, which co-sediment in a membrane fraction that contains both IM and OM (OM_L fraction) and co-purify as a single complex spanning the cytoplasmic, IM, periplasmic and OM cell compartments (Chng *et al.*, 2010a).

LptC, LptA, and LptB are encoded, in this order, as the three promoter-distal genes of the six-cistrons *yrbG* operon, in which *lptC* and *lptA* overlap for 32 nucleotides (Fig. 1A). In addition to the strong *yrbGp* promoter, two minor promoters (*lptAp1-p2*) are located upstream of *lptA* within *lptC* (Sperandeo *et al.*, 2006; Martorana *et al.*, 2011). Although *lptAp1* requires σ^E, it is not activated by several extracytoplasmic stress conditions known to induce the σ^E-dependent promoters, whereas it responds to conditions affecting lipopolysaccharide biogenesis such as depletion of LptC or LptAB, thus implying a specialized σ^E-dependent LPS stress signaling pathway (Martorana *et al.*, 2011; Lima *et al.*, 2013). A bicistronic operon encodes *lptF* and *lptG* (Ruiz *et al.*, 2008), whereas *lptD* and *lptE* map at unlinked loci (Sampson *et al.*, 1989; Takase *et al.*, 1987; Bos *et al.*, 2004; Wu *et al.*, 2006). Genetic evidence indicates that *in vivo* each of the proteins composing the transenvelope complex is essential and that the LPS transporter operates as a single device. In fact, depletion of any Lpt protein using arabinose dependent conditional expression mutants leads to similar phenotypes, namely cell letality, LPS accumulation in the periplasmic leaflet of the IM, and abnormal envelope morphology (Sperandeo *et al.*, 2008; Ruiz *et al.*, 2008; Ma *et al.*, 2008).

The seven Lpt components form the IM ABC transporter (LptBFGC) and the OM translocon (LptDE) sub-complexes, connected with each other across the periplasm by LptA. LptF and LptG (formerly YjgP and YjgQ, respectively) (Ruiz *et al.*, 2008) are IM proteins with six predicted transmembrane segments and a C-terminus located in the cytoplasm (Daley *et al.*, 2005). Unlike the other components of the Lpt complex, structural information

for these two proteins is still lacking. LptF and LptG are thought to form the dimeric IM core of the ABC transporter and have been shown to form a complex with a dimer of the ABC protein LptB, which binds and hydrolyzes ATP, at the cytoplasmic side (Stenberg *et al.*, 2005; Ruiz *et al.*, 2008; Narita, and Tokuda, 2009). LptB 3D structure exhibits an overall fold resembling the NBD (nucleotide binding domain) proteins, with a RecA-like and an α-helical domain (Sherman *et al.*, 2014; Wang *et al.*, 2014).

The $LptB_2FG$ IM sub complex, which provides energy to LPS transport system through the LptB ATPase activity (Okuda *et al.*, 2012), is connected to the LptDE OM sub complex across the periplasm through LptC and LptA proteins (Sperandeo *et al.*, 2008; Sperandeo *et al.*, 2011; Freinkman *et al.*, 2012). LptC is an IM bitopic protein with a predicted trans-membrane helical domain and a periplasmic region of about 175 amino acids (Tran *et al.*, 2010), whereas LptA is a periplasmic protein of about 150 residues (Sperandeo *et al.*, 2007; Tran *et al.*, 2008). LptA and the LptC periplasmic domain share very little amino acid sequence conservation (about 13% identity); nevertheless, comparison of their 3D structures reveals a remarkably conserved fold based on a slightly twisted β-jellyroll, composed of 16 (LptA) or 15 (LptC) antiparallel β-strands (Suits *et al.*, 2008; Tran *et al.*, 2010; Villa *et al.*, 2013). Likewise, although sharing modest sequence identity (~24%), LptA of *E. coli* and *Pseudomonas aeruginosa* 3D structures are largely superimposable and the latter can functionally complement *E. coli* Δ*lptA* mutants, thus indicating that, despite the scanty sequence homology, the xenogeneic protein properly interacts with the other components in an Lpt hybrid machine (Bollati *et al.*, 2015).

Concentration-dependent LptA oligomerization has been observed in solution (Merten *et al.*, 2012; Schultz *et al.*, 2013; Santambrogio *et al.*, 2013) and, in the crystal, the C-terminal β-strand of one protomer interacts with the N-terminal β-strand of an adjacent molecule (Suits *et al.*, 2008). LptA-LptC interactions have also been shown to occur *in vitro* (Sperandeo *et al.*, 2011) and *in vivo* (Freinkman *et al.*, 2012), where the C-terminal β-strand of LptC is predicted to form an interface with the N-terminal β-strand of LptA (Freinkman *et al.*, 2012). Both LptC and LptA have been shown to bind LPS, with LptC that binds with lower affinity than LptA. This is consistent with the idea that LPS transits across the periplasm, passing from the β-jellyroll fold of LptC to that of LptA (Tran *et al.*, 2008; Tran *et al.*, 2010; Okuda *et al.*, 2012; Sestito *et al.*, 2014).

Interestingly, the twisted β-jellyroll conformation of LptA and LptC is also conserved by the N-terminal region of LptD (Qiao *et al.*, 2014; Suits *et al.*, 2008; Tran *et al.*, 2010; Bollati *et al.*, 2015). The β-barrel protein LptD and the associated lipoprotein LptE form the

OM subcomplex of the LPS transporter (Chng *et al.*, 2010b; Chimalakonda *et al.*, 2011; Freinkman *et al.*, 2011). The solved crystal structures of the LptD/E complex from *Salmonella enterica* sv. Typhimurium and *Shigella flexneri* revealed an unprecedented β-barrel and plug architecture, in which LptD forms a 26-stranded β-barrel that surrounds the LptE plug (Dong *et al.*, 2014; Qiao *et al.*, 2014). It has been suggested that the N-terminal domain of LptD provides a hydrophobic intramembrane hole for the transit of the lipid A moiety of the LPS, whereas the hydrophilic polysaccharide moiety is translocated through the luminal gate and a lateral opening of the LptD β-barrel with the assistance of LptE (Gu *et al.*, 2015). The structure similarity between the LptD N-terminal domain and LptA and LptC (Qiao *et al.*, 2014; Suits *et al.*, 2008; Tran *et al.*, 2010; Villa *et al.*, 2013; Bollati *et al.*, 2015) suggests that these proteins, by interacting with each other, may form a hydrophobic groove that accommodates the lipid moiety of LPS for its transport from the inner membrane to the outer membrane (Gu *et al.*, 2015; Freinkman *et al.*, 2012; Okuda *et al.*, 2012; Tran *et al.*, 2010).

Modeling the proposed large periplasmic domains of LptF and LptG predicted the β-jellyroll structure (Sperandeo *et al.*, 2011; Villa *et al.*, 2013) similar to that of LptA, the periplasmic domain of LptC and the N-terminal region of LptD. It could thus be envisaged that a hydrophobic groove formed by the β-jellyroll domains of five different Lpt components accompanies LPS in his way from IM to OM. It thus appears that the β-jellyroll fold could provide both the hydrophobic environment for the LPS lipid moiety and the interfaces for the interactions of different Lpt components.

LptC specific role and mechanism in LPS transport, however, remain unclear. Deletion of its transmembrane N-terminal domain is viable and does not impair LPS transport and LptC assembly with the LptBFG IM complex, although the LptC periplasmic domain lacking the TM domain seems to interact with the IM complex less efficiently than the wild type protein or a chimera with a heterologous TM domain. Point mutations in the N-terminal periplasmic region (G56V) or at the C-terminus (G153R) are unviable (Sperandeo *et al.*, 2011; Villa *et al.*, 2013). The latter observation apparently contrasts with the fact that deletion of LptC C-terminus is not lethal, although the level of LptB required for the viability of the deletion mutant appears to be higher than that required for the wild type *lptC* (Serina *et al.*, 2004; Martorana *et al.*, accompanying manuscript).

Considering the dispensability of LptC transmembrane domain and the high structural similarity of its periplasmic domain and LptA, we tested whether some functional redundancy could occur between these structurally analogous components of the Lpt machine

by selecting for *lptC* deletion mutants. All the independent viable clones we obtained harbored, in addition to the Δ*lptC* deletion, a suppressor mutation in LptF Arg212, a residue in the predicted periplasmic domain of this IM protein. This finding implies that, with a very specific modification in the putative periplasmic domain of the IM complex, a six-components Lpt machine may be functional, and opens new scenarios for the understanding of the mechanism and evolution of the LPS transport system.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Tables 1 and 2, respectively, with a brief outline of their construction by standard genetic and cloning techniques. Oligonucleotides used in strain and plasmid constructions are listed in Table 3. All plasmid-cloned DNA regions obtained by PCR were sequenced to rule out the presence of mutations. KG-286/pGS104 harbors a chromosomal deletion of *lptC lptA* genes (Δ*lptCA*) and the *lptCAB* genes ectopically expressed from plasmid pGS104 under the *ptac* promoter (Bollati *et al.*, 2015). In this strain chromosomal *lptB* expression is driven by the main *yrbGp* promoter. KG-286 derivatives harboring plasmids other than pGS104 were obtained by plasmid shuffling (see below and Table 1).

Unless otherwise stated, bacterial cultures were grown at 37 °C in LB (Bertani, 1951) or LD (Ghisotti *et al.*, 1992) medium, supplemented, as required, with 0.2% arabinose, 0.2% glucose, 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, 50 μg/ml kanamycin, 50 μg/ml streptomycin, and 0.1 mM IPTG. Genomic and plasmid DNA were extracted using commercial DNA extraction kits.

Plasmid shuffling and strain characterization

Plasmid shuffling experiments were performed according to two different approaches. The first one is based on double selection against the resident plasmid and for a compatible chasing plasmid (Fig. 1B). The bacterial host (KG-286, an MC4100 derivative with the chromosomal deletion of *lptCA*) harbors on the chromosome the recessive *rpsL150* allele (which confers streptomycin resistance, Str^R) whereas the resident plasmid pMBM07, an *oriR101* replicon unable to replicate at temperatures ≥37 °C due to the *repA101*^{ts} mutation, harbors the dominant *rpsL*$^+$ allele, a selectable Amp^R marker (*bla*), and the *araBp-lptCA* cassette for arabinose-dependent complementation of the chromosomal deletion. The parental strain was thus grown at 28 °C in LB supplemented with arabinose and ampicillin; electrocompetent cells were prepared and transformed by electroporation with the chasing plasmid, a compatible plasmid (*oriV*$_{ColD}$) harboring the selectable Cam^R marker *cat* and different combinations of *lptC, lptA*, and *lptB* genes. After 1.5 h incubation at 37 °C in LB to prevent replication of the resident plasmid and allow expression of the incoming plasmid markers, the culture was plated and incubated at either 37 or 42 °C in LB plates supplemented with glucose (to fully repress the *araBp-lptCA* cassette expression),

chloramphenicol and streptomycin (to select for transformants by the chasing plasmid that had lost pMBM07). Transformants were then screened for Amp^S by replica plating on LB glucose supplemented with ampicillin and chloramphenicol. The second plasmid shuffling procedure is based on the spontaneous segregation of incompatible resident and chasing plasmid, both harbouring the $oriV_{ColD}$ replication origin and each expressing a different antibiotic resistance marker (*e. g.* Cam^R and Kan^R, respectively) with selection for the chasing plasmid. The parental strain was grown in LB with chloramphenicol at 37 °C, electroporated with the chasing plasmid, incubated 1.5 h in LB and plated on LB plates with kanamycin. Loss of Cam^R was screened by replica plating. Screening for the presence/absence of *lptC* and *lptA* were performed by PCR with primers FG2760- FG2761 and FG2762- FG2763, respectively. To assess the *lptF* allele harbored by individual clones, sequencing of PCR amplicons obtained with primers AP313- AP316 was performed.

Southern blotting and LPS extraction and analysis were performed as previously described (Sambrook *et al.*, 1989; Sperandeo *et al.*, 2007). The DNA probe for Southern blotting, which covered *lptC* from nucleotide 4to 532, was obtained by PCR amplification with primers FG3129-FG3130.

Electron microscopy

Bacterial samples obtained as described above were pelleted in Eppendorf tubes, washed with cacodylate buffer 0.2 M (pH 7.4) and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. Samples were then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series and embedded in an Epon-Araldite mixture according to standard TEM methods (19). Ultrathin sections (~50 nm) were cut with a Reichert-Jung ULTRACUT E using diamond knives (DIATOME Ultra 45°). Ultrathin sections, collected on 300 mesh cupper grids, were stained with aqueous uranyl acetate and lead citrate (31), carbon coated under a EMITECH K400X carbon coater and observed with a Jeol 100 SX electron microscope. Micrographs were taken directly under the microscope by Kodak 4489 photographic films for TEM.

Genomic DNA sequencing and data analysis

The library for genomic DNA sequencing was prepared according to the TruSeq DNA Sample preparation protocol (Illumina). Briefly, 1 µg of genomic DNA was sonicated to fragments with a medium length of 400 bp; after end repair, indexed adapters were ligated at DNA fragment ends, libraries were quantified using a quantification Real Time PCR (qPCR)

by KAPA Library Quant Kits (KAPA Biosystems). After a short amplification step the library was sequenced on an Illumina MiSeq Desktop Sequencer sequencer to generate 300bp paired-end reads. Raw reads were individually mapped to E. *coli* BW2952 genome (Ferenci *et al.*, 2009) (NC_012759.1) using the accurate alignment BWA mem algorithm (Li, and Durbin, 2009) allowing 5% error; removal of duplicated reads was performed with SAMtools. SNV and indels detection was performed with SAMtools and Bcftools (Li *et al.*, 2009). A VCF file, containing all the variants for each sample relative to E. *coli* BW2952 was obtained and filtered for low quality variants. SNV having a coverage of less than five high quality reads (Q>30) were discarded. Predicted indels having a coverage lower than six high quality reads (Q >30) were discarded. Both high quality SNVs and indels were subsequently annotated using SNPeff version 4.0 (De Baets *et al.*, 2012) to determine their effect on coding sequences. The assembly of genomic sequences was performed using Velvet 1.2.10 (Zerbino, and Birney, 2008) by running the command with 20 different *k*-mers lengths (*k*) using VelvetOptimiser (Zerbino, 2010) and setting up the following parameters: minimum contig length 500bp, expected coverage automatic. The assembly metrics were obtained from the Velvet output.

RESULTS

Isolation of *E. coli* Δ*lptC* mutants

In *E. coli lptC* is an essential gene, as LptC-depleted cells in arabinose-dependent *lptC* conditional expression mutants are unviable (Sperandeo *et al.*, 2008). However, *E. coli* tolerates large variations of this protein, as i) *lptC*-deletion mutants lacking the N-terminal transmembrane domain can ectopically complement *lptC* conditional mutant in non-permissive conditions (Villa *et al.*, 2013); ii) some *lptC* point and C- terminal deletion mutants, the highly divergent *P. aeruginosa lptC* gene and several *E. coli-P. aeruginosa* chimeric genes can complement LptC-depleted *E. coli* cells under particular conditions of *lptB* expression (Martorana *et al.*, accompanying manuscript). Actually, upon LptC depletion in arabinose-dependent conditional expression mutants, *lptAB* expression is only driven by the ancillary promoters *lptAp1* and *lptAp2* (Martorana *et al.*, 2011), as transcription from the main strong promoter *yrbGp* (Sperandeo *et al.*, 2006) is interrupted by the *araBp* cassette. In such condition, complementation by *Pa-lptC* only occurs if a level of *lptB* expression higher than that granted by the ancillary promoters *lptAp1-p2* is ectopically provided (Martorana *et al.*, accompanying manuscript).

To stringently assess whether LptC or any LptC domain is strictly essential for *E. coli* viability, we selected for *lptC* deletion mutants using a previously described (Bollati *et al.*, 2015) plasmid shuffling approach in strain KG-286/pMBM07, outlined in Fig. 1B. This mutant harbors on the chromosome the *rpsL150* allele (which confers streptomycin resistance, Str^R) and the deletion of the overlapping *lptC* and *lptA* genes (Δ*lptCA*); the downstream *lptB* gene expression is thus driven by the principal *yrbGp* promoter whereas the Δ*lptCA* deletion is ectopically complemented by the *lptCA* genes on pMBM07, a thermosensitive-replication plasmid which cannot be maintained at temperatures ≥ 37 °C. This plasmid also carries a selectable ampicillin resistance (Amp^R) marker and the dominant $rpsL^+$ allele, which confers streptomycin sensitivity (Str^S) to the otherwise Str^R host. This strain was transformed with derivatives of the non-thermosensitive, compatible plasmid pGS100, which confers chloramphenicol resistance (Cam^R), harboring *lptA* (pGS321) or *lptAB* (pGS416), to provide different levels of *lptB* expression. We also tested whether strains missing *lptA* could be obtained by transforming KG-286/pMBM07 with plasmids harboring either *lptC* (pGS402) or *malE-lptC* (pGS420), an *lptC* derivative lacking the transmembrane domain (Villa *et al.*, 2013). Plasmid harboring *lptCA* (pGS404) was used as a positive

control, whereas the empty vector (pGS401) was used as a negative control. Loss of the resident plasmid was promoted by incubating the transformed cultures at 37 °C and selection for clones harboring the transforming plasmid (Cam^R) and missing the resident plasmid (Str^R) was performed by plating aliquots of the transformants at 37 and 42 °C on LD agar supplemented with the two antibiotics. Screening for the loss of Amp^R, the selective marker of the resident plasmid pMBM07, was then performed by replica plating.

Cam^R Str^R Ts^+ transformants were obtained, as detailed in Materials and Methods, with plasmids pGS321 (*lptA*) and pGS416 (*lptAB*) at frequencies between 0.2 and 0.7 per ng of plasmid DNA (Table 4). Transformation frequency with pGS404 (*lptCA*) was >3000 fold higher, whereas no transformants (<0.25/ng plasmid DNA) were obtained with the empty vector pGS401. Four out of four and five out of five clones obtained by transformation with pGS321 and pGS416, respectively, were found to be Amp^S. To rule out the presence of a displaced *lptC* gene in such mutants putatively lacking *lptC*, we performed both a PCR analysis on the plasmid shuffled clones with *lptC*-specific pairs of primers and Southern blot analysis with an *lptC*-specific probe covering the *lptC* region 4-532. No signal of *lptC* presence could be detected by either approach in these clones (Fig. 2), which thus represent *bona fide* viable mutants lacking LptC.

Cam^R Str^R Ts^+ transformants were also obtained at 37 and 42 °C at frequencies between 0.7 and 1.8 transformants/ng of plasmids DNA with pGS402 (*lptC*) and pGS420 (*malE-lptC*). However, PCR analysis with primers FG2762-FG2673 revealed the presence of *lptA* in all fifteen clones tested (Fig. 2A), including six Amp^S, thus suggesting that variable portions of pMBM07 containing *lptA* were integrated in the bacterial genome of the selected transformants. Thus viable Δ*lptA* mutants could not be obtained.

Phenotypic characterization of *E. coli* Δ*lptC* mutants

Impaired LPS transport may lead to growth defects (such as lower growth rate, cold- and/or thermosensitivity), LPS modifications (such as colanic acid decoration, which can be detected by LPS gel electrophoresis), increased sensitivity to toxic chemicals, and/or structural abnormalities of the cell envelope (Sperandeo *et al.*, 2006; Sperandeo *et al.*, 2007; Sperandeo *et al.*, 2008; Chimalakonda *et al.*, 2011). As shown in Fig. 3, different Δ*lptC* isolates exhibited variable degrees of sensitivity to a set of toxic compounds and two clones could not grow at 15 °C, whereas neither altered LPS electrophoretic mobility nor gross ultrastructural alterations could be detected (Fig. 04). Generation times of LB-glucose cultures at 37 °C of three strains complemented by *lptA* (pGS321) and three by *lptAB*

(pGS416) scattered, without any apparent correlation, between 28 min (like the parental AM604/pGS401) and 33 min.

E. coli Δ*lptC* mutation is suppressed by amino acid substitutions at a unique residue of LptF

The phenotypic variability exhibited by the different Δ*lptC* isolates suggests that different compensatory suppressor mutations could have been selected during the plasmid shuffling procedure; alternatively, different adaptive regulatory systems could have been activated to overcome the lack of LptC. To identify potential Δ*lptC* suppressor mutations, we sequenced the genome of the parental (KG-286/pMBM07) and three Δ*lptC* mutants that exhibited different phenotypes and had been obtained upon shuffling with the plasmid harboring *lptA* (strains KG-292/pGS321and KG-293/pGS321) or *lptAB* (KG-294/pGS416). The reads were mapped to the reference strain E.*coli* BW2952 complete genome (Accession number NC_012759.1) (Ferenci *et al.*, 2009) giving >99% coverage. Sequence variations between these four strains and the reference BW2952 that mapped in open reading frames are reported in Table 5. Comparison between the Δ*lptC* mutants and their parental KG-286/pMBM07 genomic sequences revealed the presence, in all three mutants, of a single nucleotide substitution at base 634 of *lptF* (either C→A transversion or C→T transition) that caused a single amino acid substitutions at arginine 212 of the encoded LptF protein (R212C in strains KG-292, and R212S in KG-293 and KG-294; Table 5). Strain KG-293/pGS321 harbored an additional missense mutation in a small ORF of unknown function (BWG_3693) and a synonymous mutation in the minor tail protein M (BWG_3735), both in the λp*lac*Mu harbored by the parental strain (Ferenci *et al.*, 2009). All remaining variations within protein coding sequences from the reference genome (4 mutations) were shared both by the parental and the three mutants. Variations between the four sequenced strains in intergenic regions, listed in Table 6, were clustered in regions harboring rRNA and/or tRNA genes or within pseudogenes. Additional 35 variations (not listed) from the reference sequence in intergenic regions were shared both by the parental and the mutant strains.

Although harboring a different chasing plasmid, the two *lptC*^{R212C} mutants derive from samples of the same culture and thus cannot be considered to bear independent mutations. Anyway, one of the sequenced mutants harbored a different mutation in the same residue (R212S) of LptF, thus being an independent mutant from the same culture. These data

strongly suggest that the change of a specific residue in LptF suppresses the lethal phenotype associated with the lack of the essential protein LptC.

To better support this hypothesis and identify other potential Δ*lptC* suppressor alleles of *lptF*, we selected for new, independent Δ*lptC* mutants from single-colony cultures of KG-286/pMBM07 by plasmid shuffling with pGS321 or pGS416, as described above, obtaining Cam^R Str^R Ts^+ transformants at 42 °C in nine out of ten cultures tested, four of which harboring *lptA* and five harboring *lptAB*. All nine independent isolates, upon screening for the Amp^R (by replica plating) and *lptC* (by PCR) markers of the resident pMBM07 plasmid, turned out to be ampicillin sensitive and *lptC*-negative. Sequencing of the *lptF* gene showed that all nine independent transformants harbored a single amino acid substitution at residue R212 (Table 7). In addition to the mutations found in the first round of selection (in two new independent R212C and three new independent R212S mutants), three R212G independent mutants were also obtained. Combining data of the first and second round of selection, however, no specific association of a given type of mutant with a specific transforming plasmid (harboring either *lptA* or *lptAB*) could be observed.

Characterization of LptF suppressors of Δ*lptC*

The presence of specific LptF R212 residue substitutions in all the eleven independent Δ*lptC* mutants isolated strongly indicates that such *lptF* mutations (henceforth collectively designated as $lptF^{Sup}$) suppress the lethal phenotype associated to the the lack of LptC. However, we could not rule out that additional mutations in the <1% genome not covered by sequencing or any of the mutations detected in non-coding regions could contribute to the Δ*lptC* suppressor phenotype selected by plasmid shuffling. We thus addressed whether the different mutations in LptF R212 residue could be sufficient to support the growth of *E. coli* upon LptC depletion in the *lptC* conditional expression mutant FL905. Since, as explained above, in the absence of the arabinose inducer the downstream *lptAB* operon can only be transcribed from the ancillary promoters *lptAp1-p2*, and *lptAB* expression from *lptAp1-p2* might not be sufficient to grant cell viability (Martorana *et al.*, 2011; Martorana *et al.*, accompanying manuscript), we ectopically expressed in FL905 either the $lptF^{Sup}G$ operon alone or both $lptF^{Sup}G$ and *lptAB*. As shown in Fig. 5A, depletion of LptC (no arabinose) i) was complemented, in the positive control strain, by ectopically expressed *lptC* both with and without *lptAB* coexpression; ii) was suppressed by $lptF^{R212G}$ and $lptF^{R212S}$ when coexpressed with *lptAB* but not when expressed alone; and iii) was suppressed by $lptF^{R212C}$ in neither

condition. As in FL905 a wild copy of *lptF* gene is harbored on the chromosome, the lack of suppression by *lptF*^{R212C} could be a consequence of the recessive nature of the mutant allele, although in higher copy number than the wild type. Alternatively, suppression of Δ*lptC* by *lptF*^{R212C} may require additional mutations that are present in the original mutant but not in the *lptC* depletion strain.

We also addressed whether *lptF*^{Sup} mutations in the haploid state are compatible with the presence of *lptC*. To this end we replaced by plasmid shuffling in each type of *lptF*^{Sup} mutants the resident plasmid harboring Cam^{R} and either *lptA* or *lptAB* with an incompatible plasmid harboring a different antibiotic resistance marker (Kan^{R}) and either *lptCA* or *lptCAB*. Selection of Kan^{R} transformants was done in the absence of chloramphenicol so as to allow segregation of the resident plasmid. Six Kan^{R} transformants for each strain were then colony purified and tested for the presence of the resident plasmid (Cam^{R}). As shown in Table 8, none of the strains transformed, as a control, by the chasing plasmid vector without *lpt* genes lost the resident plasmid (0/6 Cam^{S}) as it carried genes essential for viability; likewise, the *lptF*$^{+}$ strains transformed by the chasing plasmid with *lptA* or *lptAB* but missing *lptC* did not lose the resident plasmid, whereas all the *lptF*^{Sup} clones segregated a Cam^{S} progeny. Finally, both *lptF*$^{+}$ and *lptF*^{Sup} strains could be transformed, albeit at different efficiencies, by the plasmid carrying *lptCA* or *lptCAB*. We then assessed by sequencing for each type of *lptF*^{Sup} shuffled clones that the original *lptF* allele had been retained. Therefore, all the three haploid *lptF*^{Sup} mutations are compatible with the presence of LptC. To rule out that this effect could depend on additional mutations originated upon selection of the *lptF*^{Sup} strains, we transformed with plasmids harboring the *lptFG* operon with the mutant *lptF*^{Sup} alleles the NR1113 strain (Ruiz *et al.*, 2008), which expresses the wild type *lptFG* operon under the arabinose inducible promoter *araBp*. As shown in Fig. 05B, each of the three *lptF*^{Sup} was able to complement wild type LptF-depleted cells. Overall these data indicate that the *lptF*^{Sup} alleles, which suppress the lack of LptC, are not incompatible with the presence of LptC.

DISCUSSION

Genetic and biochemical evidence indicate that LptC is an essential component of the LPS transport machinery and is required for *E. coli* viability (Sperandeo *et al.*, 2006; Sperandeo *et al.*, 2008; Sperandeo *et al.*, 2011). LptC has been thought to connect the IM ABC transporter LptBFG with the periplasmic LptA that, in turn, would interact with the periplasmic N-terminal domain of LptD. LptA, the periplasmic C-terminal domain of the bitopic LptC, and the periplasmic LptD N-terminal domain exhibit high structural similarity, the β-jellyroll fold, despite the scarce sequence conservation (Qiao *et al.*, 2014; Suits *et al.*, 2008; Tran *et al.*, 2010; Villa *et al.*, 2013; Bollati *et al.*, 2015). It has been suggested that these three elements, by interacting with each other, form a hydrophobic groove that accommodates the lipid moiety of LPS for its transport from the inner membrane to the outer membrane (Gu *et al.*, 2015; Freinkman *et al.*, 2012; Okuda *et al.*, 2012; Tran *et al.*, 2010).

In this model, however, the connection between the LptC-A-DE complex and the IM LptBFG transporter, which provides energy to the entire Lpt system by ATP hydrolysis, has not been clarified. The transmembrane domain of LptC does not seem to be implicated in the interaction with LptBFG, as deletion of this domain impairs neither LPS biogenesis nor LptC binding to the IM complex (Villa *et al.*, 2013); therefore, LptF/G periplasmic domains might be implicated in forming the periplasmic bridge of the Lpt machine. Moreover, the role of LptC is still elusive. Its C-terminal end, which is thought to interact with the N-terminus of LptA, appears to be dispensable, at least under conditions of non-limiting LptB expression; on the contrary, point mutations in the same region are lethal (Sperandeo *et al.*, 2011; Villa *et al.*, 2013; Martorana *et al.*, accompanying manuscript).

It is also remarkable that *E. coli*-*P. aeruginosa* hybrid Lpt machines are functional. The *lptA* homologue from *P. aeruginosa*, *lptH*, complements *E. coli* Δ*lptA* mutants (Bollati *et al.*, 2015); likewise, *P. aeruginosa lptC* complements *E. coli* Δ*lptC*, although under conditions of increased *lptB* expression levels (Martorana *et al.*, accompanying manuscript). Although both homologous couples of proteins exhibit the β-jellyroll structure, their amino acid sequence identity is scanty, thus suggesting that interactions between structural features, rather than specific amino acids, play a predominant role in the interactions between the periplasmic protein domains of the complex (Villa *et al.*, 2013; Bollati *et al.*, 2015).

Given that i) *Ec*-LptA and *Ec*-LptC can be functionally replaced by the structurally similar but scarcely conserved (as far as the primary sequence is concerned) *Pa*-LptH and *Pa*-LptC, respectively, and ii) the LptC N-terminal transmembrane domain is dispensable (and thus the periplasmic β-jellyroll of LptC appears to be sufficient to carry out LptC function), we addressed whether LptA and LptC could replace each other by testing complementation of *E. coli* bearing a chromosomal deletion of both genes (Δ*lptCA*) with plasmids expressing *lptC* or *lptA* alone. Overall the genetic data we have obtained indicate that *E. coli* Δ*lptC* mutant is viable only in the presence of specific suppressor mutations in *lptF*, whereas we did not obtain Δ*lptA* mutants.

Construction of Δ*lptC* and Δ*lptA* mutants was attempted by replacing, in an ectopically complemented Δ*lptCA* mutant, the resident plasmid harboring *lptCA* with a chasing plasmid harboring either *lptA* or *lptC* alone (plasmid shuffling). Preliminary experiments using incompatible (*i.e.* with the same origin of replication) resident and chasing plasmids with different antibiotic resistance markers were unsuccessful, as the resident plasmid or large portions of it did not segregate for several rounds of growth of the transformed cultures selected for the presence of the chasing plasmid, eventually giving rise to rearrangements. However, using a strong double selection against the resident plasmid (high temperature to rapidly stop replication of the replication thermosensitive resident plasmid and streptomycin to select for the loss of the resident plasmid harboring the dominant $rpsL^{+}$ allele), we obtained clones lacking *lptC*.

In keeping with the low frequency of such Δ*lptC* mutants, eleven out of eleven independent mutants thus obtained were associated with an additional mutation of LptF arginine 212, being cysteine, serine, or glycine the substituting residues. Such a complete association between lack of LptC and $LptF^{R212}$ mutations strongly suggests that a specific suppressor is required for viability of the Δ*lptC* mutant and that LptF is the preferred (if not the only) suppressor gene.

Genomic sequencing was performed on three non-independent mutants obtained in a first round of screening. Two of them bore different amino acid substitution (R212C and R212S, thus *a posteriori* resulting independent mutants) without any other point mutation in ORFs, relative to the parental strain; the third one bore the same $lptF^{R212S}$ allele and two additional single nucleotide substitution (one of which leading to a synonymous codon) whithin the λp*lac*Mu insertion harbored by the parental strain. Mutations in non-coding regions were clustered within spacers of rRNA and/or tRNA operons and within

pseudogenes. It is thus very unlikely that these additional variations relative to the parental strain may significantly contribute to suppress the Δ*lptC* mutation.

Although the sequencing coverage was >99%, these observation do not completely rule out that additional mutations in regions not covered by sequencing may contribute to suppress the lethal phenotype of Δ*lptC*. However, the *lptF*^{R212G} and *lptF*^{R212S} alleles were capable to suppress lethality of conditional expression *lptC* mutants in nonpermissive conditions, thus demonstrating that i) a suppressor is necessary to overcome lethality caused by LptC depletion and ii) *lptF*^{R212G} and *lptF*^{R212S} alleles are sufficient for such suppression without the additional mutations associated with the plasmid-shuffled isolate. It remains to be elucidated whether the inability of *lptF*^{R212C} allele to suppress the lethal phenotype of conditional expression *lptC* mutants depends on the recessive nature of this mutation or by the need of an as yet discovered co-suppressor. The latter hypothesis, however, seems less likely, as suppressor mutants with the *lptF*^{R212C} allele were obtained at comparable frequency (3/11) as the other two suppressors.

The LptF R212 residue is located in a predicted large periplasmic domain (residues 122-269) connecting the 3rd and 4th transmembrane helices and a similar organization is predicted for LptG (Daley *et al.*, 2005). A β-jellyroll structure has been suggested for these two periplasmic domains (Villa *et al.*, 2013), but their structure has not yet been solved. The suppressor phenotype exhibited by three specific mutations of LptF R212 residue, however, highlights the relevance of this specific amino acid and of the periplasmic loop in the interaction with LptC.

We propose, as a working model, that in the wild type seven-component Lpt machine of *E. coli* the β-jellyroll domains of five different Lpt components, namely the central periplasmic domains of LptF and LptG, the C-terminal periplasmic domain of LptC, LptA, and the periplasmic N-terminal domain of LptD form a hydrophobic groove that accompanies LPS in his way from IM to OM. LptF, in its wild type form, specifically interacts with LptC and, in the absence of LptC, the continuity of the hydrophobic groove is compromised. The suppressor mutations in LptF^{R212} could restore a functional hydrophobic groove, for example by directly binding LptA without the mediation of LptC or by recruiting LptA to replace LptC, thus allowing a six-component Lpt machine to be functional. We also have shown that the presence of LptC is compatible with the LptF^{Sup} proteins. It remains to be assessed, however, whether LptC can be recruited by the Lpt machine when a LptF^{Sup} protein is present or whether the suppressors can only assemble a six-component Lpt machine. Clarifying this

point will shed light on the reciprocal interactions of the Lpt components and on the structure and mechanism of the LPS transporter.

Using the powerful plasmid shuffling technique that led to the selection of Δ*lptC* suppressors LptF^{Sup} we did not obtain Δ*lptA* clones, as all the clones selected upon shuffling with the chasing plasmid harboring *lptC* still bore a displaced *lptA* allele. It is possible that LptA plays a more fundamental role in the Lpt machine; for example, the interaction of LptA with OM complex might require specific function that cannot be fulfilled by LptC even with a suppressor mutation. Alternatively, more than one suppressor mutations would be required, thus decreasing the chance of finding a mutant.

The functionality of a six-component Lpt machine suggests a modular evolution of the LPS transport system in which a β-jellyroll module evolved by subsequent module duplications which diverged to form more specialized and efficient hydrophobic grooves for the periplasmic passage of LPS.

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TABLES

Table 1. Bacterial strains

Strain	Parental strain	Relevant characters		Features/construction	Origin
		Chromosomal	Plasmid		
AM604	MC4100	*rpsL150*			(Wu *et al.*, 2006)
AM604/pKD46	AM604	*rpsL150*	*bla*		(Bollati *et al.*, 2015)
AM604/pKD46/pGS104	AM604/pKD46	*rpsL150*	*ptac-lptCAB cat; bla*		(Bollati *et al.*, 2015)
FL905	AM604	Φ(*kan araC araBp-lptC*)*1*			Sperandeo et al., 2008
FL905/pGS442	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptFG cat*	by transformation	this work
FL905/pGS443	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212C}*G cat*	by transformation	this work
FL905/pGS444	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212S}*G cat*	by transformation	this work
FL905/pGS445	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptFG_lptAB cat*	by transformation	this work
FL905/pGS446	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212C}*G_lptAB cat*	by transformation	this work
FL905/pGS447	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212S}*G_lptAB cat*	by transformation	this work

FL905/pGS450	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212G}*G cat*	by transformation	this work
FL905/pGS451	FL905	Φ(*kan araC araBp-lptC*)*1*	*ptac-lptF*^{R212G}*G_lptAB cat*	by transformation	this work
KG-280/pGS104	AM604/pGS104/pKD46	Δ*lptCA*::*kan*	*ptac-lptCAB cat*	by gene specific mutagenesis	(Bollati *et al.*, 2015)
KG-286/pGS104	KG-280/pGS104/pCP20	Δ*lptCA*	*ptac-lptCAB cat*	by FLP-mediated kan cassette excision	(Bollati *et al.*, 2015)
KG-286/pGS308	KG-286/pGS315	Δ*lptCA*	*ptac-lptCA-kan*	by plasmid shuffling; selection for KanR StrR, screening for CamS	(Bollati *et al.*, 2015)
KG-286/pMBM07	KG-286/pGS308	Δ*lptCA*	*araBp-lptCA amp rpsL*$^{+}$ *repA101*^{ts}	by plasmid shuffling; selection for Amp^{R}, screening for Str^{S}, 28 °C	(Bollati *et al.*, 2015)
KG-286/pGS404	KG-286/pMBM07	Δ*lptCA*	*ptac-lptC_lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS	(Bollati *et al.*, 2015)
KG-286.10/pGS321	KG-286/pMBM07	Δ*lptCA*	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-286.12/pGS321	KG-286/pMBM07	Δ*lptCA*	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-286.13/pGS416	KG-286/pMBM07	Δ*lptCA*	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-286.14/pGS416	KG-286/pMBM07	Δ*lptCA*	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-286.15/pGS416	KG-286/pMBM07	Δ*lptCA*	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 37 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work

KG-286.17/pGS416	KG-286/pMBM07	Δ*lptCA*	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-292/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212C}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-293/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212S}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-294/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212S}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 37 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-295.01/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212G}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-295.02/pGS308	KG-295.01/pGS321	Δ*lptCA; lptF*^{R212G}	*ptac-lptCA kan*	by plasmid shuffling; selection for KanR, screening for CamS *lptC*$^{+}$	this work
KG-296/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212G}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-297.01/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212C}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-297.02/pGS308	KG-297.01/pGS416	Δ*lptCA; lptF*^{R212C}	*ptac-lptCA kan*	by plasmid shuffling; selection for KanR, screening for CamS *lptC*$^{+}$	this work
KG-298.01/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212S}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-298.02/pGS308	KG-298.01/pGS416	Δ*lptCA; lptF*^{R212S}	*ptac-lptCA kan*	by plasmid shuffling; selection for KanR, screening for CamS *lptC*$^{+}$	this work
KG-299/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212G}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work

KG-300/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212G}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-301/pGS321	KG-286/pMBM07	Δ*lptCA; lptF*^{R212S}	*ptac-lptA cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-302/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212S}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
KG-303/pGS416	KG-286/pMBM07	Δ*lptCA; lptF*^{R212C}	*ptac-lptAB cat*	by plasmid shuffling; selection for CamR StrR at 42 °C, screening for AmpS *lptA*$^{+}$ Δ*lptC*	this work
NR1113	NR754	Δ(λatt-lom)::*bla araBp-lptFG* Δ*lptFG*			(Ruiz *et al.*, 2008)
NR1113/pGS401	NR1113		*ptac*-void		this work
NR1113/pGS442	NR1113		*ptac-lptFG cat*		this work
NR1113/pGS443	NR1113		*ptac-lptF*^{R212C}*G cat*		this work
NR1113/pGS444	NR1113		*ptac-lptF*^{R212S}*G cat*		this work
NR1113/pGS450	NR1113		*ptac-lptF*^{R212G}*G cat*		this work

Table 2. Plasmids

Plasmids	Parental / replicon	Relevant characters	Construction/Origin
pCP20		*bla, cat,* thermosensitive replication	(Datsenko, and Wanner, 2000)
pGS100	pGZ119EH (*oriV*$_{ColD}$)	*ptac*-TIR, *cat, oriV*$_{ColD}$	(Sperandeo *et al.*, 2006)
pGS104	pGS100	*ptac-lptCAB, cat, oriV*$_{ColD}$	(Sperandeo *et al.*, 2006)
pGS105	pGS100	*ptac-lptAB, cat, oriV*$_{ColD}$	(Sperandeo et al., 2006)
pGS303	pGS100	*kan*	(Bollati *et al.*, 2015)
pGS305	pGS303	*ptac-lptCAB, kan, oriV*$_{ColD}$	*lptCAB* was obtained by *Eco*RI-*Xba*I digestion of pGS104 and cloned into pGS303 *Eco*RI-*Xba*I sites
pGS306	pGS100	*ptac-lptCA, cat, oriV*$_{ColD}$	*lptCA* was PCR-amplified with AP54-FG2723 primers from pgs104 and cloned into *Eco*RI-*Xba*I sites of pGS100
pGS308	pGS303	*ptac-lptCA, kan, oriV*$_{ColD}$	(Bollati *et al.*, 2015)
pGS321	pGS100	*ptac-lptA, cat, oriV*$_{ColD}$	*lptA* was PCR-amplified with AP55-FG2723 primers from pGS104 and cloned into *Eco*RI-*Xba*I sites of pGS100
pGS323	pGS303	*ptac-lptA, kan, oriV*$_{ColD}$	*lptA* was obtained by *Eco*RI-*Xba*I digestion of pGS321 and cloned into pGS303 *Eco*RI-*Xba*I sites
pGS324	pGS303	*ptac-lptAB, kan, oriV*$_{ColD}$	*lptAB* was obtained by *Eco*RI-*Xba*I digestion of pGS105 and cloned into pGS303 *Eco*RI-*Xba*I sites
pGS401	pGS100	*ptac*-SD1-*Eco*RI-*XbaI*-SD2-*SalI*-*Hin*dIII, *cat, oriV*$_{ColD}$	(Bollati *et al.*, 2015)
pGS402	pGS401	*ptac-lptC, cat, oriV*$_{ColD}$	(Bollati *et al.*, 2015)
pGS404	pGS402	*ptac-lptC-lptA, cat, oriV*$_{ColD}$	(Bollati *et al.*, 2015)
pGS416	pGS401	*ptac-lptAB, cat, oriV*$_{ColD}$	(Bollati *et al.*, 2015)

pGS420	pGS401	*ptac- malE$_{SS}$lptC$^{\Delta 1-23}$, cat, oriV$_{ColD}$*	*malE$_{SS}$lptC$^{\Delta 1-23}$* was amplified by three step PCR with FG3089, AP211, AP212 and FG3090 primers from AM604 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS401
pGS442	pGS401	ptac-*lptFG, cat, oriV$_{ColD}$*	*lptFG* genes were PCR-amplified with FG3195-FG3196 primers from KG-286/pMBM07 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS401 downstream of SD1
pGS443	pGS401	*ptac-lptF^{R212C}G, cat, oriV$_{ColD}$*	*lptF^{R212C}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-292/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS401 downstream of SD1
pGS444	pGS401	*ptac-lptF^{R212S}G, cat, oriV$_{ColD}$*	*lptF^{R212S}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-293/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS401 downstream of SD1
pGS445	pGS416	*ptac-lptFG_lptAB, cat, oriV$_{ColD}$*	*lptFG* genes were PCR-amplified with FG3195-FG3196 primers from KG-286/pMBM07 (2) genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS416 downstream of SD1
pGS446	pGS416	*ptac-lptF^{R212C}G_lptAB, cat, oriV$_{ColD}$*	*lptF^{R212C}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-292/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS416 downstream of SD1
pGS447	pGS416	*ptac-lptF^{R212S}G_lptAB, cat, oriV$_{ColD}$*	*lptF^{R212S}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-293/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS416 downstream of SD1
pGS450	pGS401	ptac-*lptF^{R212G}G, cat, oriV$_{ColD}$*	*lptF^{R212G}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-293/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS401 downstream of SD1
pGS451	pGS416	*ptac-lptF^{R212G}G_lptAB, cat, oriV$_{ColD}$*	*lptF^{R212G}G* genes were PCR-amplified with FG3195-FG3196 primers from KG-295/pGS321 genomic DNA and cloned into *Eco*RI-*Xba*I sites of pGS416 downstream of SD1
pKD46		*oriR101, repA101ts, araC, araBp-λ red, bla*	(Datsenko, and Wanner, 2000)

pMBM07	pKD46	*araBp-lptCA*, *rpsL*$^{+}$, *bla*, *oriR101*, *repA101ts*	(Bollati *et al.*, 2015)

Table 3. Oligonucleotides

Name	Sequence[a]	Notes
AP54	cgagaggaattcaccATGAGTAAAGCCAGACGTTGGG	pGS306 construction with FG2723; *Eco*RI
AP55	cgagagaggaattcaacATGAAATTCAAAACAAACAAACTC	pGS321 construction with FG2723; *Eco*RI
AP211	GTATCGTCTTTTTCGGCCATGGCGAGAGCCGAGGCGGAAAAC	pGS420 construction with AP212, FG3089 and FG3090
AP212	GTTTTCCGCCTCGGCTCTCGCCATGGCCGAAAAAGACGATAC	pGS420 construction with AP211, FG3089 and FG3090
FG2723	gactagtctagaTTAATTACCCTTCTTCTGTGCCGGGG	pGS306 and pGS321 construction with AP54 and AP55; *Xba*I
FG3089	catattcgtctcgaattcaccATGAAAATAAAAACAGGTGCACGC	pGS420 construction with AP211, AP212 and FG3090; *Esp*3I-*Eco*RI
FG3090	caggttcgtctctctagaTTAAGGCTGAGTTTGTTTGTTTTG	pGS420 construction with AP211, AP212 and FG3089; *Esp*3I-*Xba*I
FG3129	AGTAAAGCCAGACGTTGGG	Southern blotting *lptC* probe amplification by PCR
FG3130	CCTTTTCAATCAGCTCGGC	Southern blotting *lptC* probe amplification by PCR
FG3195	gataggaattcaccGTGATAATCATAAGATATCTGG	pGS442, pGS443, pGS444, pGS445, pGS446, pGS447, pGS450 and pGS451 construction with FG3196; *Eco*RI
FG3196	ggctagtctagaTTACGATTTTCTCATTAACAGC	pGS442, pGS443, pGS444, pGS445, pGS446, pGS447, pGS450 and pGS451 construction with FG3195; *Xba*RI

[a] Upper case letters, sequence present in the template; lower case letters, additional/modified sequence not present in the template; restriction sites are underlined.

Table 4. Frequency of transformants[a] upon selection for the chasing plasmid at non-permissive temperature for the resident plasmid complementing Δ*lptCA* mutant[b]

Chasing Plasmid	Genes[c]	Selection	
		42 °C Str^R Cam^R	37 °C Str^R Cam^R
pGS401	none	<0.25	<0.25
pGS404	*lptC-lptA*	>2000	>2000
pGS321	*lptA*	0.2	0.5
pGS416	*lptAB*	0.7	0.7
pGS402	*lptC*	0.7	1.1
pGS420	*malE-lptC*	1.8	0.9

[a] n. of transformants per ng of plasmid DNA in the indicated selective conditions

[b] recipient strain KG-286/pMBM07

Table 5. Point mutations in ORFs of parental and Δ*lptC* viable mutants as compared with *E. coli* BW2952 sequence

STRAIN[a]	ORF	ID	N[b]	Mutation type	Position in		Change in		Description
					CDS[c]	Protein	Codon	aa	
A, B, C, D	BWG_0606	*aroG-1*	A	missense	655	219	Gcg→Acg	A→T	Phosphoglyceromutase 1
A, B, C, D	BWG_1070	*orf*	C	frameshift	156-157	52-53	-	-	Predicted divalent heavy-metal cations transporter
A, B, C, D	BWG_1086	*yciE*	A	missense	388	130	Atc→Ttc	I→F	Conserved protein
A, B, C, D	BWG_3107	*insD*	C	frameshift	346-347	116	-	-	IS2 transposase
C	BWG_3693	*orf*	T	missense_	103	35	Gac→Aac	D→N	Protein of unknown function
C	BWG_3735	λ M	C	synonymous	153	51	ccT→ccC	P	Polypeptide: Minor tail protein M
B	BWG_3967	*yjgP*	T	missense	634	212	Cgc→Tgc	R→C	LptF
C, D	BWG_3967	*yjgP*	A	missense	634	212	Cgc→Agc	R→S	LptF

[a] A, KG-286/pMBM07 (parental); B, KG-292/pGS321; C, KG-293/pGS321; D, KG-294/pGS416

[b] Base substitution

[c] CDS, coding sequence;

Table 6. Point mutations in intergenic regions of parental or Δ*lptC* viable mutants[a]

STRAIN[b]	Position[c]		Nucleotide change	Left gene[d]	Right gene[d]	Notes
B	683,472		A	*lysZ*	*lysQ**	
B	683,474		G	*lysZ*	*lysQ**	
B	683,636		G	*lysQ**	*lysQ*	
BCD	683,681		C	*lysQ**	*lysQ*	
BCD	683,682		C	*lysQ**	*lysQ*	
BCD	683,695		T	*lysQ**	*lysQ*	
BCD	683,735		A	*lysQ**	*lysQ*	
BCD	683,736		T	*lysQ**	*lysQ*	
BCD	683,739		T	*lysQ**	*lysQ*	
BCD	683,742		T	*lysQ**	*lysQ*	
A	683,764		-	*lysQ**	*lysQ*	
B	683,765	683,766	GGTAACACCCGT	*lysQ**	*lysQ*	
D	683,767		T	*lysQ**	*lysQ*	
C	1,288,805		-	*fnr*	*ogt*	within *insH**
D	1,288,862	1,288,864	C	*fnr*	*ogt*	within *insH**
BCD	1,289,316	1,289,318	A	*fnr*	*ogt*	within *insH**
A	4,056,058	4,056,066	TTT	*rrsB*	*rrlB*	within *gltT**
A	4,056,061		-	*rrsB*	*rrlB*	within *gltT**
D	4,056,210		A	*gltT**	*rrlB*	

[a] 35 mutations shared by parental and mutants relative to the reference BW2952 sequence are not reported.

[b] A, KG-286/pMBM07; B, KG-292/pGS321; C, KG-293/pGS321; D, KG-294/pGS416

[c] Mutation occurred at the given coordinate or within the range indicated

[d] Genes delimiting at the left and right the intergenic region are reported; asterisk (*) denotes pseudogenes

Table 7. Independent[a] *lptF*^{Sup} mutants

Suppressor strain	Genes on plasmid[b]	Transform. efficiency	LptF aa change
KG-292/pGS321	*lptA*	0.5	R212C
KG-297/pGS416	*lptAB*	0.3	R212C
KG-303/pGS416	*lptAB*	0.7	R212C
KG-295/pGS321	*lptA*	1.5	R212G
KG-299/pGS321	*lptA*	0.03	R212G
KG-300/pGS321	*lptA*	0.3	R212G
KG-296/pGS416	*lptAB*	0.1	R212G
KG-293/pGS321*	*lptA*	0.5	R212S
KG-301/pGS321	*lptA*	0.3	R212S
KG-294/pGS416*	*lptAB*	0.7	R212S
KG-298/pGS416	*lptAB*	0.3	R212S
KG-302/pGS416	*lptAB*	0.7	R212S

[a] The two sequenced non-independent mutants are marked by an asterisk (*)

[b] *lptA*, pGS321; *lptAB*, pGS416

Table 8. *lptF*^{Sup} mutants are compatible with lptC

N	Strain	*lptF*[a]	Resident plasmid[b]	Chasing plasmid[c]					
				none		*lptA(B)*[d]		*lptCA(B)*[d]	
				n/µg	Cam^S	n/µg	Cam^S	n/µg	Cam^S
1	KG-286/pGS404	wt	*lptCA*	7.03E+03	0/6	1.77E+04	0/6	1.09E+02	6/6
2	KG-292/pGS321	R212C	*lptA*	5.73E+03	0/6	9.37E+03	6/6	8.00E+02	6/6
3	KG-293/pGS321	R212S	*lptA*	7.67E+03	0/6	2.87E+04	6/6	6.33E+02	6/6
4	KG-295/pGS321	R212G	*lptA*	2.42E+04	0/6	2.47E+04	6/6	1.50E+03	6/6
5	KG-286/pGS104	wt	*lptCAB*	5.17E+04	0/6	5.92E+04	0/6	6.05E+02	6/6
6	KG-297/pGS416	R212C	*lptAB*	5.87E+03	0/6	6.33E+03	6/6	1.00E+03	6/6
7	KG-294/pGS416	R212S	*lptAB*	1.01E+04	0/6	1.74E+04	6/6	2.00E+02	6/6
8	KG-296/pGS416	R212G	*lptAB*	1.54E+04	0/6	3.02E+04	6/6	4.67E+02	6/6

[a] *lptF* allele of the host strain

[b] *lpt* genes of the resident plasmid

[c] *lpt* genes of the chasing plasmid; none, pGS303; *lptA*, pGS323; *lptAB*, pGS324; *lptCA*, pGS308; *lptCAB*, pGS305;

[d] pGS323 and pGS308 were used for transformations n. 1-4, pGS324 and pGS305 for transformations n. 5-8

FIGURE LEGENDS

Fig. 1. Map of the *lpt* locus and schematics of the Δ*lptC* mutant selection by plasmid shuffling. A. Map of the *E. coli yrbG-lptB* locus. The ORFs (open large arrows) are drawn to scale. Promoters are indicated by bent arrows. B. Plasmid shuffling by double selection against the resident plasmid. The relevant chromosomal (linear drawings) and plasmid (circles) genotypes are depicted. See text for details.

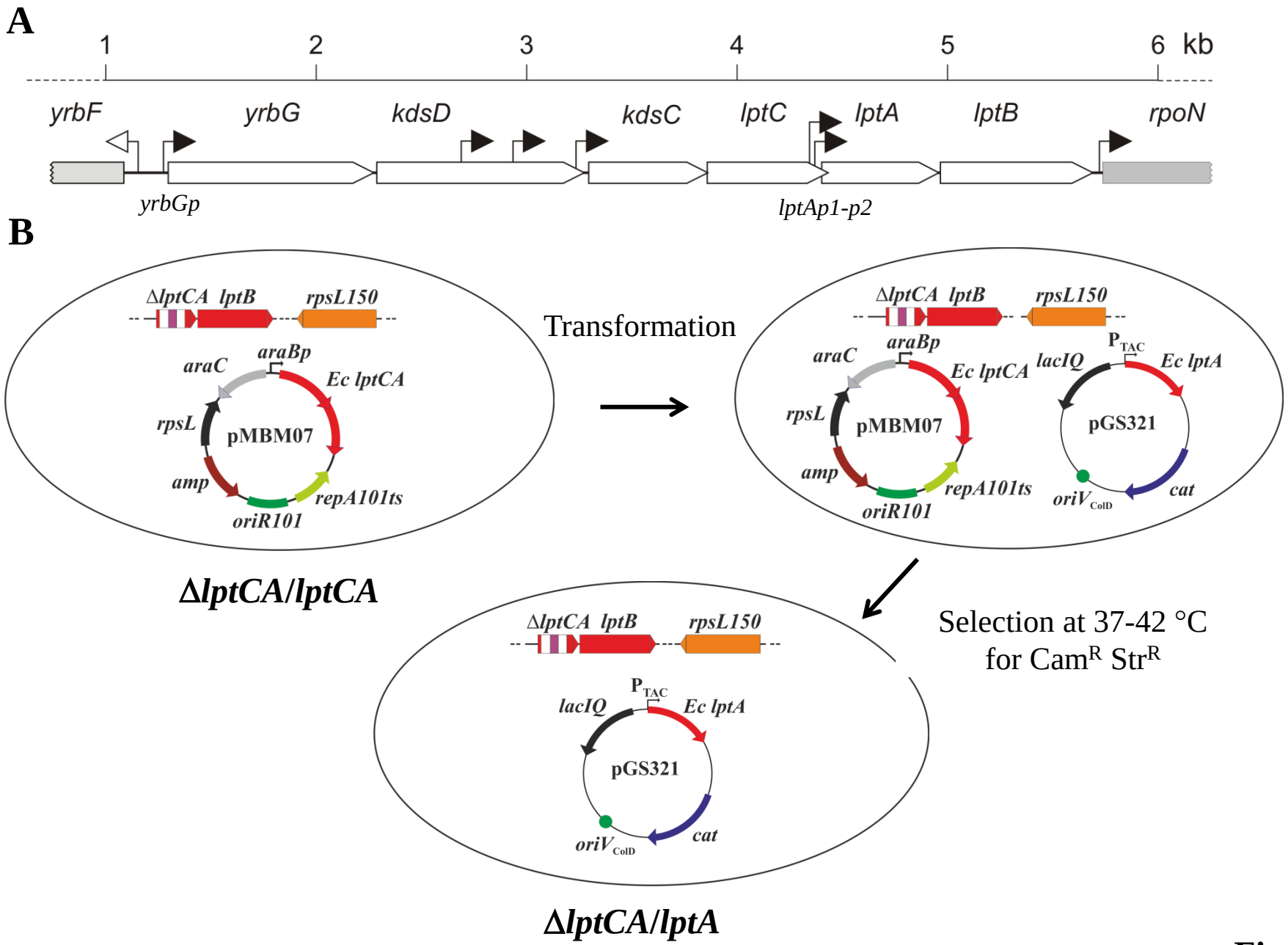
Fig. 2. Screening for the presence of *lptA* and *lptC* in plasmid shuffled clones by PCR and Southern blotting analysis. A. Electrophoretic analysis of amplicons obtained with *lptC*- and *lptA*-specific primers, as indicated on the bottom of the panel, from plasmid shuffled and control strains. On top of the lanes the *lpt* genes harbored by the plasmid used for transformation of the parental KG-286/pMBM07 are indicated: *lptC-lptA*, lanes 1-2, pGS404; *lptC*, lanes 1-7, pGS402; *malE-lptC*, lanes 1-8, pGS420; *lptA*, lanes 1-4, pGS321; *lptAB*, lanes 1-5, pGS416; M, molecular weight markers (100 bp ladder). See text for details. B. Southern blotting of DNA from plasmid shuffled transformant clones. Total DNA from strains indicated on top of the panel was digested with *Sal*I (odd lane numbers) or *Hind*III (even lane numbers), Southern blotted and hybridized with a radioactive DNA probe obtained with primers FG3129-FG3130 and covering the *lptC* region 4-532. Δ*lptCA*/*lptA*, KG-286/pGS321; Δ*lptCA*/*lptAB*, KG-286/pGS416; wt, AM604; Δ*lptCA*/*lptCA*, KG-286/pMBM07; M, molecular weight markers (1 kb ladder). The fainter bands in lanes M and Δ*lptCA*/*lptA* and Δ*lptCA*/*lptAB* is due to non-specific hybridization of the probe with the DNA marker (M) and with the chasing plasmids (pGS321 and pGS416), both of which are linearized by *Sal*I and *Hind*III.

Fig. 03. Phenotypic analysis of Δ*lptC* mutants. Cultures strains indicated on the left of the panels (*lptCA*/-, AM604/pGS401; Δ*lptCA*/*lptA* 1, KG-292/pGS321; Δ*lptCA*/*lptA* 2, KG-286.10/pGS321; Δ*lptCA*/*lptA* 3, KG-293/pGS321; Δ*lptCA*/*lptAB* 1, KG-286.13/pGS416; Δ*lptCA*/*lptAB* 2, KG-286.14/pGS416; Δ*lptCA*/*lptAB* 4, KG-294/pGS416) grown in LB-glucose-chloramphenicol at 37 °C were serially diluted 1:10 in microtiter wells and replica plated in LB agar plates supplemented with glucose and chloramphenicol alone or containing bacitracin (50 μg/ml), novobiocin (10 μg/ml), rifampicin (2.5 μg/ml) or SDS-EDTA (0.5%-0.25mM). MacConkey agar plate was supplemented with glucose and chloramphenicol. The plates were incubated

overnight at 37 °C (or 42 °C, where indicated) or 3 d at 15 °C, as indicated on top of the pictures. The log of the serial dilutions is indicated on the bottom.

Fig. 04. LPS analysis in Δ*lptC* mutants and electron microscopy. A. LPS extracted from the strains indicated on top (Left panel: *lptCA lptF*/pvoid, AM604/pGS401; Δ*lptCA lptF*^{R212C}/p*lptA*, KG-292/pGS321;Δ*lptCA*/p*lptA*, KG-286.10/pGS321; Δ*lptCA lptF*^{R212S}/p*lptA*, KG-293/pGS321; Δ*lptCA*/p*lptAB*, KG-286.13/pGS416; Δ*lptCA*/p*lptAB*, KG-286.14/pGS416; Δ*lptCA lptF*^{R212S}/p*lptAB*, KG-294/pGS416. Right panel: Δ*lptCA lptF*/p*lptCA*, KG-286/pMBM07; Δ*lptCA lptF*^{R212C}/p*lptAB*, KG-297.01/pGS416; Δ*lptCA lptF*^{R212G}/p*lptA*, KG295.01/pGS321; Δ*lptCA lptF*^{R212S}/p*lptAB*, KG-298.01/pGS416; Δ*lptCA lptF*^{R212C}/p*lptCA*, KG-297.02/pGS308; Δ*lptCA lptF*^{R212G}/p*lptCA*, KG-295.02/pGS308; Δ*lptCA lptF*^{R212S}/p*lptCA*, KG-298.02/pGS308) was fractionated by gel electrophoresis and silver stained (upper panels) or western blotted (lower panels) as described in Materials and methods. B. Electron micrographs of AM604/pGS401 (+LptC +LptA), KG286/pMBM07 in depletion of the ectopically expressed *lptCA* (-LptC -LptA), and KG293/pGS321 (-LptC +LptA).

Fig. 5.Suppression of LptC depletion and LptC compatibility by *lptF*^{Sup} alleles. A. Cultures of FL905 (*araBp-lptC*) strains transformed with pGS401 derivatives expressing the *lpt* genes listed on the left grown in LB-arabinose-chloramphenicol were serially diluted 1:10 in microtiter wells and replica plated in agar plates with arabinose (+ ara) or with glucose (+ glu) to fully repress the *araBp* promoter. The log of the serial dilutions is indicated on the right of the panel. B. The same procedure was applied to strain NR1113 (*araBp-lptFG*) transformed with pGS401 derivatives expressing the *lpt* genes listed on the left. The log of the serial dilutions is indicated on the bottom.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amp
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Transformation
ΔlptCA/lptCA
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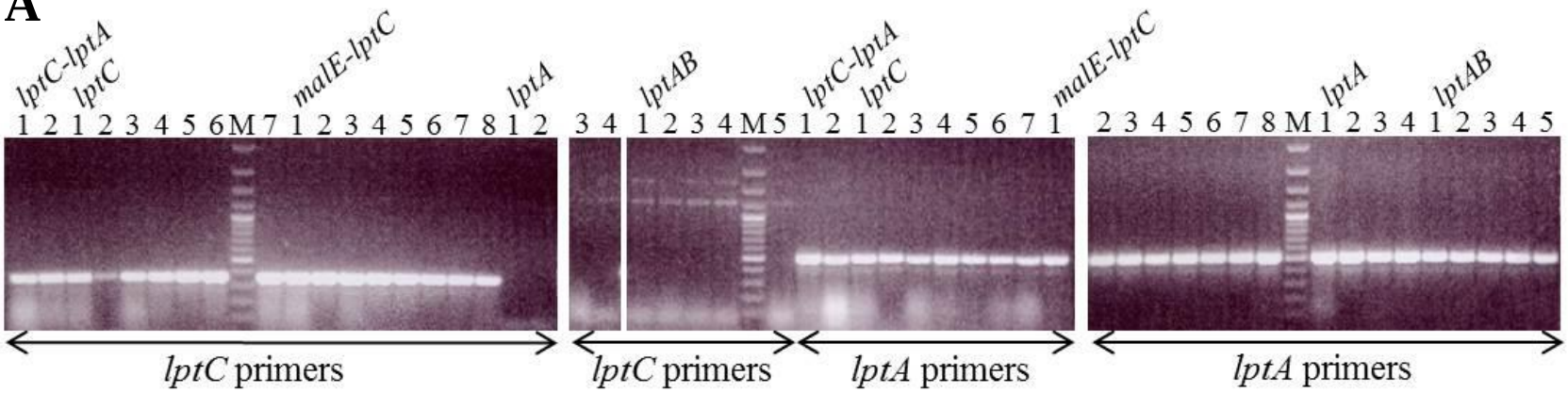
cat
oriV ColD
Selection at 37-42 °C
for Cam R Str R
ΔlptCA/lptA

Fig. 1

A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lptA primers

B

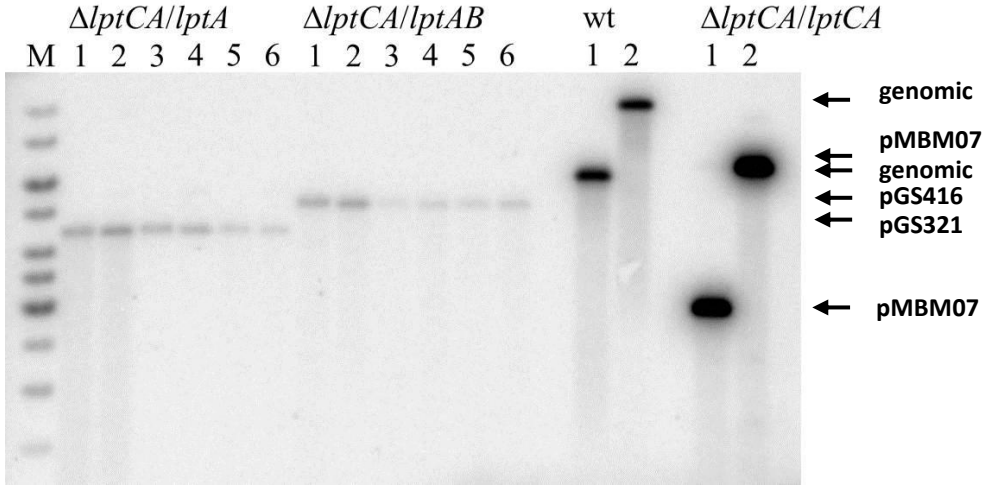
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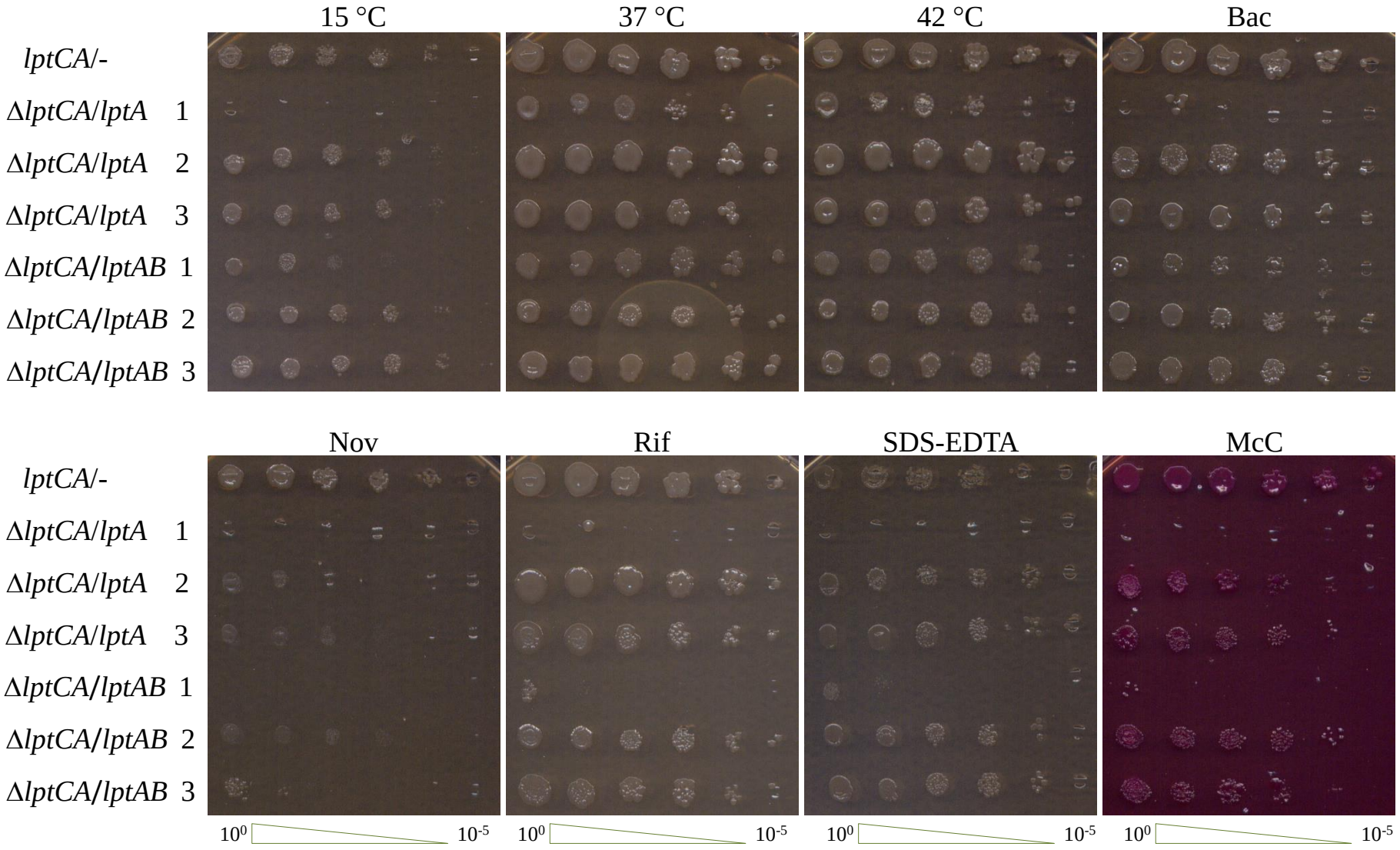
Fig. 2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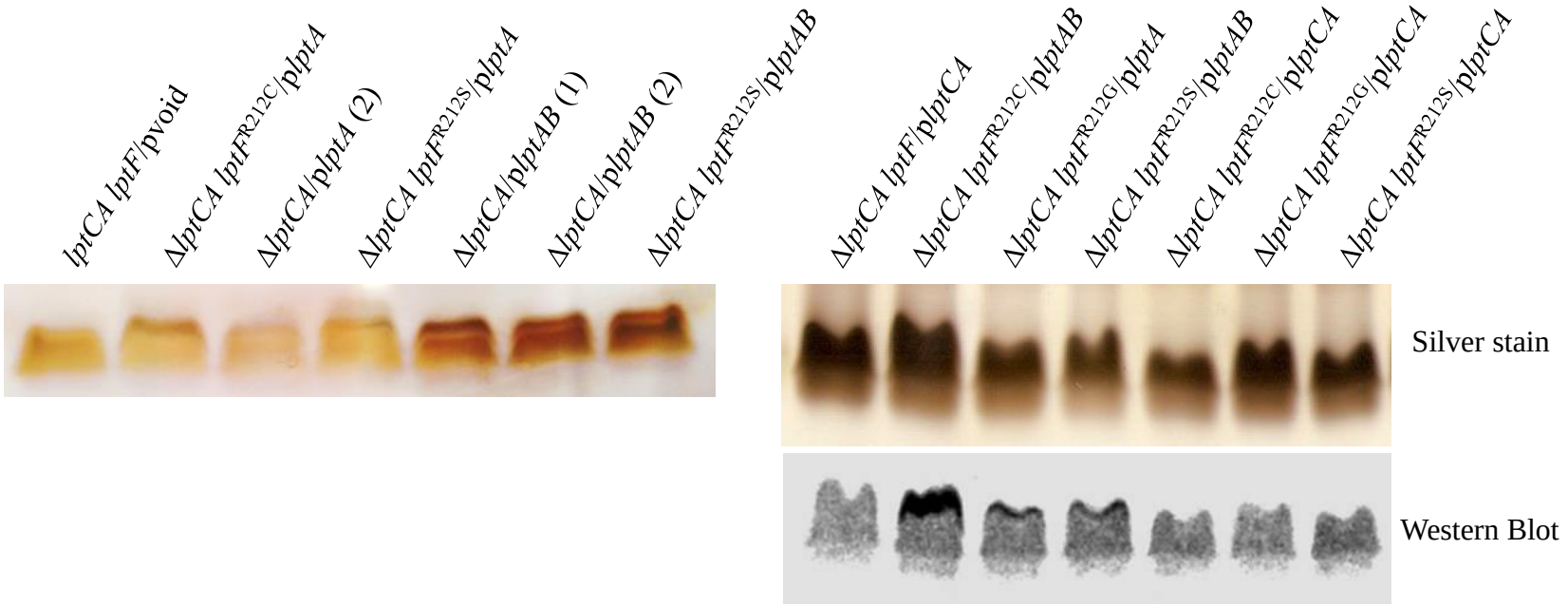
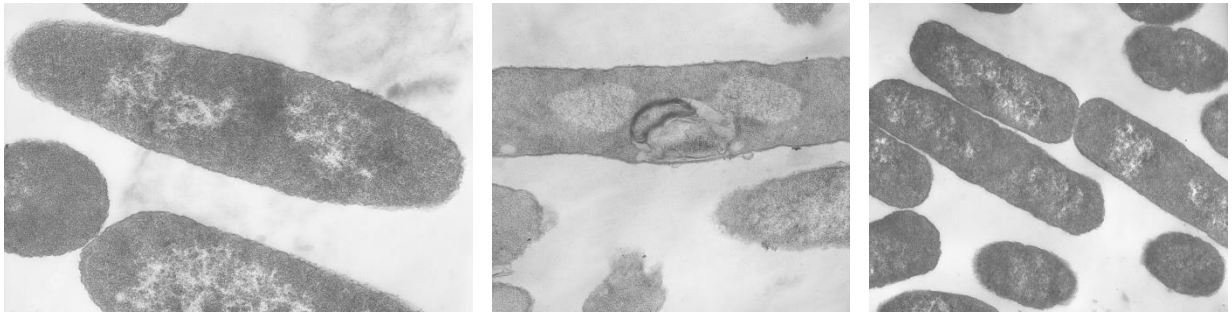
ΔlptCA/lptAB 1
ΔlptCA/lptAB 2
ΔlptCA/lptAB 3
10^0 10^-5
10^0 10^-5
10^0 10^-5
10^0 10^-5

Fig. 3

A

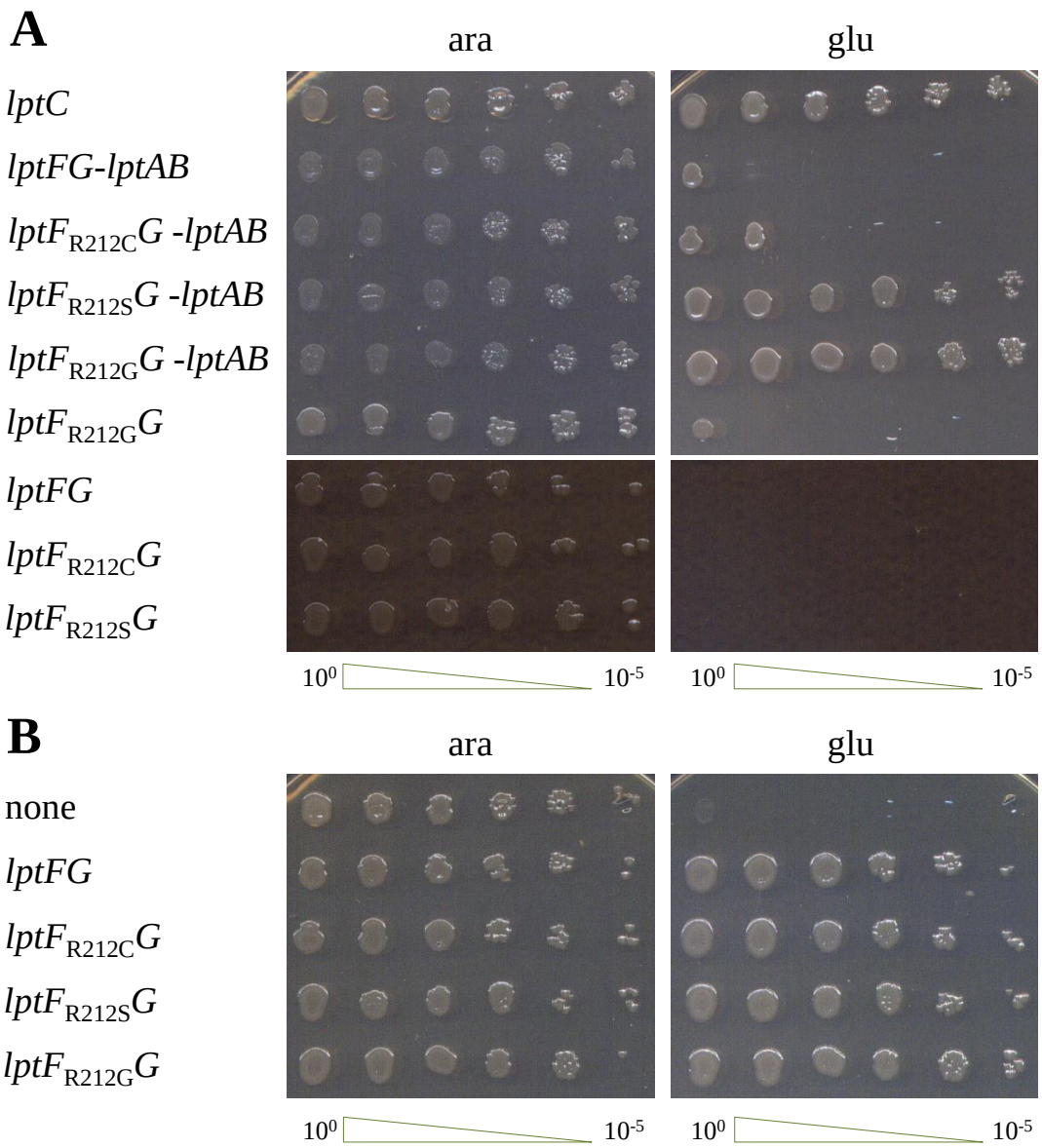


B



+ LptC + LptA **- LptC - LptA** **- LptC + LptA**

Fig. 4


A
ara
glu
lptC
lptFG-lptAB
lptFR212CG -lptAB
lptFR212SG -lptAB
lptFR212GG -lptAB
lptFR212GG
lptFG
lptFR212CG
lptFR212SG
10^0
10^-5
10^0
10^-5
B
ara
glu
none
lptFG
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lptFR212SG
lptFR212GG
10^0
10^-5
10^0
10^-5

Fig. 5

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Dissecting *Escherichia coli* Outer Membrane Biogenesis Using Differential Proteomics

Alessandra M. Martorana[1], Sara Motta[2], Dario Di Silvestre[2], Federica Falchi[3], Gianni Dehò[3], Pierluigi Mauri[2], Paola Sperandeo[1]*, Alessandra Polissi[1]*

1 Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, Milan, Italy, **2** Istituto di Tecnologie Biomediche (ITB-CNR), Segrate, Milan, Italy, **3** Dipartimento di Bioscienze, Università degli Studi di Milano, Milan, Italy

Abstract

The cell envelope of Gram-negative bacteria is a complex multi-layered structure comprising an inner cytoplasmic membrane and an additional asymmetric lipid bilayer, the outer membrane, which functions as a selective permeability barrier and is essential for viability. Lipopolysaccharide, an essential glycolipid located in the outer leaflet of the outer membrane, greatly contributes to the peculiar properties exhibited by the outer membrane. This complex molecule is transported to the cell surface by a molecular machine composed of seven essential proteins LptABCDEFG that form a transenvelope complex and function as a single device. While advances in understanding the mechanisms that govern the biogenesis of the cell envelope have been recently made, only few studies are available on how bacterial cells respond to severe envelope biogenesis defects on a global scale. Here we report the use of differential proteomics based on Multidimensional Protein Identification Technology (MudPIT) to investigate how *Escherichia coli* cells respond to a block of lipopolysaccharide transport to the outer membrane. We analysed the envelope proteome of a *lptC* conditional mutant grown under permissive and non permissive conditions and identified 123 proteins whose level is modulated upon LptC depletion. Most such proteins belong to pathways implicated in cell envelope biogenesis, peptidoglycan remodelling, cell division and protein folding. Overall these data contribute to our understanding on how *E. coli* cells respond to LPS transport defects to restore outer membrane functionality.

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* Email: alessandra.polissi@unimib.it (AP); paola.sperandeo@unimib.it (PS)

Introduction

The outer membrane (OM) of Gram-negative bacteria [1] is an asymmetric membrane containing phospholipids and a unique glycolipid lipopolysaccharide (LPS) in the inner and outer leaflet, respectively [2]. OM proteins (OMPs) and lipoproteins are also embedded and anchored, respectively, in the OM [3]. LPS is a complex molecule that can be structurally divided in three elements: lipid A, the hydrophobic moiety that anchors LPS in the outer membrane, the core oligosaccharide and the *O*-antigen (Figure 1). The OM mainly serves as a protective barrier enabling Gram-negative bacteria to survive in harsh environments and to exclude several toxic molecules effective against Gram-positive organisms [1]. LPS mainly contributes to the OM permeability barrier properties as LPS molecules are tightly packed in the OM and form a very effective barrier against hydrophobic compounds [2]. Biosynthesis of LPS components occurs in the cytoplasm and at the cytoplasmic side of the inner membrane (IM). The core-lipid A moiety is first flipped by the essential ABC transporter MsbA across the IM [4,5] ligated with the O-antigen and then transported across the periplasm by a transenvelope device, the Lpt protein machinery, composed in *E. coli* by seven essential proteins (LptABCDEFG) (reviewed by [6,7]) (Figure 1). At the IM, the LptBFG complex constitutes an ABC transporter that provides the energy for LPS transport. LptC is a small bitopic protein [8] that resides in the IM and interacts with the LptBFG complex [9] and with the periplasmic protein LptA [10] [11]. LptA is thought to transfer LPS to the LptDE protein complex of the OM. Thus, LptA is the periplasmic protein that connects the IM Lpt components to the OM LPS translocon (LptD and LptE), which ensures the assembly of LPS at the cell surface [12–14]. The Lpt machinery appears to operate as a single device as depletion of any Lpt component leads to common phenotypes that includes the appearance of an anomalous LPS form decorated by repeating units of colanic acid [8,15], and in such depleted strains the majority of *de novo* synthesised LPS accumulates in a novel membrane fraction (hIM) with higher density than the IM [8]. The process by which hydrophobic LPS is transported across the periplasm to the cell surface is not fully understood. The current model postulates that the Lpt proteins, through homologous domains interactions, create a transenvelope bridge that connects

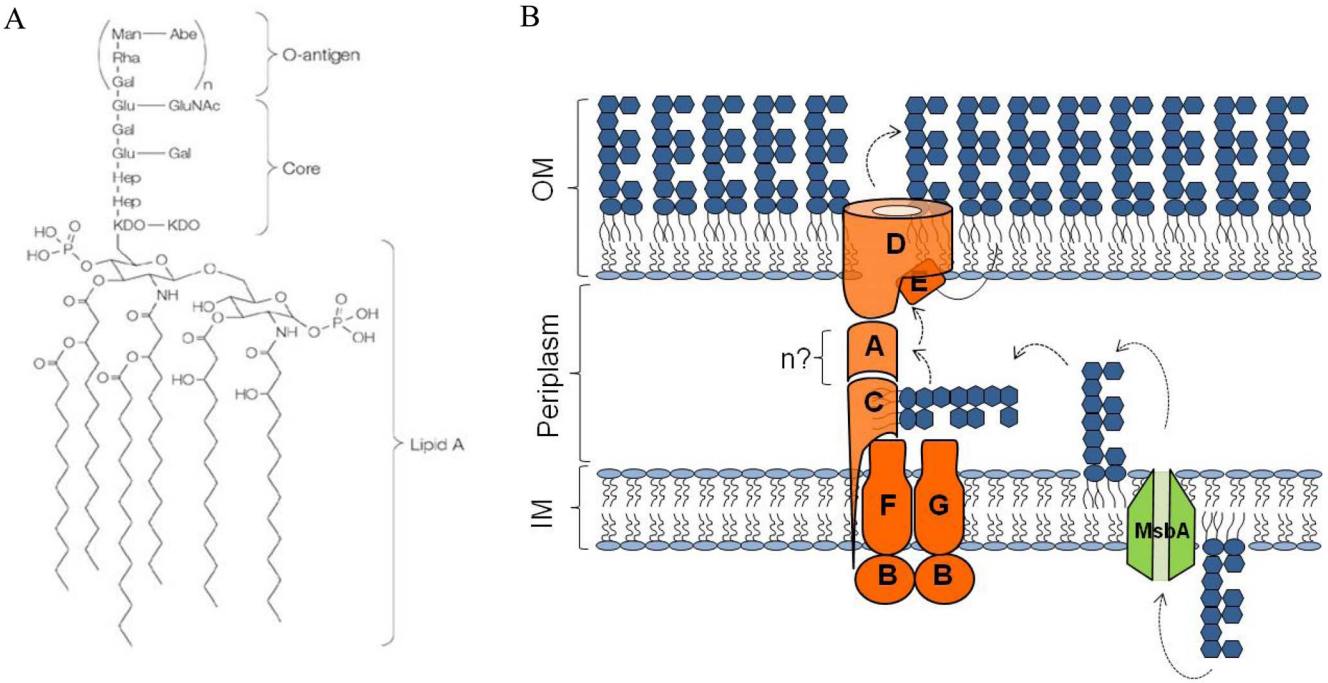

Figure 1. LPS structure and transport in *Escherichia coli*. A) Chemical structure of LPS. O-antigen is indicated in parenthesis as it is not synthesized in *E. coli* K12 derivatives. B) LPS transport from IM to OM. The MsbA protein catalyzes LPS flipping across the IM that is then exported to the cell surface by the Lpt machinery.
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IM and OM [14,16], thus forming a continuous channel through which LPS is moved to the cell surface [17].

The OM is an essential structure for bacterial survival and the first site of interaction with the mammalian host [18]; mutants defective in OM biogenesis typically display alterations of the OM permeability barrier properties [3]. The crucial role of this structure is highlighted by the fact that in *E. coli* at least five different pathways (Bae, Cpx, Psp, Rcs and σ^E) constitute signaling systems that detect and respond to alterations of the bacterial envelope [19–22]. These pathways regulate expression of complementary functions whose discrete contributions are integrated to mount a full adaptive response [23].

In this work we sought to analyze on a global level the response of *E. coli* cells to a severe OM biogenesis defect, namely the block of transport of LPS upon LptC depletion, to investigate on the cell response to an OM stress. To this purpose we used a proteomic approach based on two-dimensional chromatography coupled to tandem mass spectrometry (2DC-MS/MS), called MudPIT (Multidimensional Protein Identification Technology) [24] to monitor the cell envelope protein content of an arabinose dependent *lptC* conditional mutant [8] grown under permissive and non permissive conditions. Our results highlight pathways and strategies adopted by *E. coli* cells to respond to severe OM biogenesis defects.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are derivatives of AM604 (MC4100 ara$^+$; [25]. FL905 (AM604 Φ(*kan araC araBp-lptC)1*) is a conditional arabinose dependent mutant carrying *lptC* under the control of *araBp* promoter [8]. Bacteria were grown in LD medium [26] at 37°C. When required, 0.2% (w/v) L-arabinose (as an inducer of the *araBp* promoter), and 25 μg/ml kanamycin, were added. The strain PS200 (MC4100 *ara*$^+$ *asmA-SPA::kan*) was obtained by moving the *asmA-SPA::kan* allele from CAG64009 to AM604 by P1 transduction using standard procedures [8]. Subsequently, the *kan* cassette was removed from PS200 by pCP20-encoded Flp recombinase [27], generating the mutant PS201. The removal of the *kan* genes was verified by colony PCR. Finally, to construct strain PS202 [AM604 Φ(*kan araC araBp-lptC)1 asmA-SPA*], the *kan araC araBp-lptC* allele was moved from FL905 (AM604 Φ(*kan araC araBp-lptC)1*) into PS201 byP1 transduction and selecting on media containing kanamycin and 0.2% arabinose.

Transductions were verified by colony PCR and by immunoblotting on whole cell extract using anti-FLAG M2 antibodies (Sigma-Aldrich Inc., St.Louis, MO, USA).

Whole membrane proteins extraction

AM604 and FL905 cells were grown in LD supplemented with 0,2% arabinose up to OD600 = 0.2 at 37°C. Cells were then harvested, washed in LD, diluted five hundredfold (in fresh medium with or without 0,2% arabinose) and incubated with aeration at 37°C. After 330 minutes, 125 OD of cell cultures were harvested, re-suspended in 3 ml of 10 mM Tris (pH = 8.0), 1 mM EDTA, 1 mM PMSF, 0,2 mg/ml lisozyme and incubated on ice. After 30 minutes, 0,2 mg/ml DNAse was added and cells were disrupted by sonication (6 cycles of 10 seconds at 20% amplitude). The cleared lysates were then subjected to centrifugation at 100,000×g for 60 min at 4°C. Pellets, that contain whole cell membranes, were re-suspended in MilliQ water and lyophilized, or analysed by western blotting using anti-LptC, anti-LptE, anti-LptB, anti-AcrB or anti-FLAG M2 antibodies (Sigma-Aldrich Inc., St.Louis, MO, USA).

Cell fractionation

AM604 and FL905 cells were grown as described above. Cells were chilled in ice and harvested by centrifugation. IM and OM were separated by discontinuous sucrose density gradient centrifugation of a total membrane fraction obtained by spheroplast lysis as described previously [28]. Step gradients were prepared by layering 2 ml each of 50, 45, 40, 35, and 30% (wt/vol) sucrose solutions over a 55% sucrose cushion (0.5 ml). Fractions (300 µl) were collected from the top of the gradient, 50 µl of each fraction was assayed for NADH oxidase activity [28]. The total protein concentration of each fraction was determined by the Bradford assay (Thermo Fisher Scientific Inc. Waltham, MA) as recommended by the manufacturer. The protein profiles of OmpC, OmpF, and OmpA across the gradient were estimated by separating 20 to 40 µl of each fraction on 12.5% SDS-PAGE and by staining the gels with Coomassie blue. Fractions corresponding to IM, hIM, and OM were lyophilized and analyzed.

For the subcellular localization of AsmA-SPA, PS201 [AM604 *asmA-SPA*] and PS202 [AM604 Φ(*kan araC araBp-lptC)1 asmA-SPA*] cells were grown in LD or LD with 0.2% arabinose up to $OD_{600} = 0.2$. Cells were then harvested, washed in LD, diluted three hundredfold (in fresh medium with or without 0,2% arabinose) and incubated with aeration at 37°C. After 280 minutes, 125 OD of cell cultures were harvested. IM and OM were separated by discontinuous sucrose density gradient centrifugation of total membranes as described above. 20 µl of each fraction collected from the gradient were separated on 10% SDS-PAGE followed by immunoblot analysis using monoclonal anti-FLAG M2 antibodies (Sigma-Aldrich Inc., St.Louis, MO, USA) to detect AsmA-SPA.The 55 kDa IM protein that is detected by anti-LptD antibodies and the OM protein LamB were used as IM and OM markers, respectively [8].

Proteomic Analysis

Lyophilized samples were first resuspended in 0.1 M ammonium bicarbonate, pH 8.0, and then treated with RapiGest SF (Waters Corporation, Milford, MA, USA) at the final concentration of 0.2% (w/v). After incubation at 100°C for 5 min, the samples were cooled to room temperature and digested with trypsin (Sequencing Grade Modified Trypsin, Promega, Madison, WI, USA). Initially, trypsin was added to mixtures at an enzyme/substrate ratio of about 1:50 (w/w) and incubated at 37°C overnight, then another aliquot of enzyme was added at an enzyme/substrate ratio of 1:100 (w/w) and the samples were incubated at 37°C for 4 hours.

The enzymatic reactions were chemically stopped by acidification with TFA 0.5% (Sigma-Aldrich Inc., St.Louis, MO, USA), incubation at 37°C for 45 min and centrifugation at 13,000×g for 10 min in order to remove hydrolytic RapiGest SF by-products.

Before MudPIT analysis, samples were desalted by PepClean C-18 spin columns (Pierce Biothecnology Inc., Rockford, IL, USA), concentrated in a SpeedVac (Savant Instruments Farmingdale, NY, USA) at 60°C and finally resuspended in 0.1% formic acid (Sigma-Aldrich Inc., St.Louis, MO, USA).

Trypisn-digested peptides were analyzed by two dimensional micro-liquid chromatography coupled to ion trap mass spectrometry (Multidimensional Protein Identification Technology (MudPIT)) using ProteomeX-2 configuration (Thermo Electron Corporation, San Josè, CA, USA) [29]. 10 µl of the digested peptide mixtures were loaded by means of an autosampler (Suveyor AS Thermo) onto a strong cation exchange column (BioBasic-SCX, 0.32 i.d.×100 mm, 5 µm, Thermo Electron Corporation, Bellofonte, PA, USA) and then eluted using eight steps of increasing ammonium chloride concentration (0, 20, 40, 80, 120, 200, 400, 700 mM). Eluted peptides, obtained by each salt steps, were at first captured in turn onto two peptide traps (Zorbax 300 SB C-18, 5 µm, 0.3 id×5 mm, Agilent technologies, Santa Clara, CA, USA) mounted on a 10-port valve, for concentration and desalting, and subsequently loaded on a reversed phase C-18 column (BioBasic-18, 0.180 i.d.×100 mm, 5 µm, Thermo Electron Corporation, Bellofonte, PA, USA) for separation with an acetonitrile gradient. The gradient profile was: 5–10% eluent B in 5 min, 10–40% eluent B in 40 min, 40–80% eluent B in 8 min, 80–95% eluent B in 3 min, 95% eluent B for 10 min, 95–5% eluent B in 4 min and 5% eluent B for 15 min (eluent A, 0.1% formic acid in water; eluent B, 0.1% formic acid in acetonitrile). The flow rate was 100 µl/min slit in order to achieve a final flux of 1 µl/min.

The peptides eluted from the C-18 column were directly analysed with an ion trap mass spectrometer (LCQ Deca XP plus) equipped with a nano electrospray ionization source (nano-ESI) (Thermo Finnigan Corp., San Josè, CA, USA). The heated capillary was held at 185°C; full mass spectra were acquired in positive mode and over a 400–2000 m/z range, followed by three MS/MS events sequentially generated in a data-dependent manner on the first, second and third most-intense ions selected from the full MS spectrum, using dynamic exclusion for MS/MS analysis (collision energy 35%).

The experimental mass spectra produced by MudPIT analyses were correlated to tryptic peptide sequences by comparing with theoretical mass spectra, obtained by *in silico* digestion of *Escherichia coli* protein database downloaded from the NCBI website (www.ncbi.nlm.nih.gov/Ftp/index.htlm). Data processing was performed using the 3.3.1. Bioworks version, based on SEQUEST algorithm (University of Washington,licensed to Thermo Finnigan Corp., San Josè, CA, USA), and the following parameters: Xcorr scores greater than 1.5 for singly charged peptide ions and 2.0 and 2.5 for doubly and triply charged ions, respectively, the peptide probability ≤ 0.001 and the protein consensus score value ≥ 10. These filters guaranteed that the resulting proteins have a probability value $p \leq 0.001$.

Data were treated with an in-house algorithm called MAProMa [30] (Multidimensional Algorithm Protein Map), in particular a tool of MAProMa permits the comparison of the protein list obtain from the analysis of the samples.

Two biological replicates for the three samples of total membrane and for the samples obtained from the fractionation of the membranes were analysed and for each of them two technical replicates were made.

The reproducibility of the method was evaluated as described in our previous work [31].

Proteins with significant differences in level, were identified by other two tools of MAProMA: DAve (Differential Average) and DCI (Differential Coefficient Index) [32]. These two algorithms are based on score values assigned by SEQUEST software to each identified protein in samples to be compared. Specifically, DAve is an index of the relative ratio between control and mutant and DCI is an index to evaluate the absolute variation of score value of each protein. Briefly, using MAProMA each identified protein in the two samples were aligned and then DAve and DCI indexes were calculated for all proteins. The threshold values imposed were very stringent: DAve >0.4 and DAve<-0.4, DCI>400 and DCI<-400. To increase the confidence, it is necessary that both indexes, DAve and DCI, satisfy these thresholds.

Results and Discussion

Proteomic profiles of cell envelope upon LptC depletion

To understand how cells respond to severe OM damage, we compared the cell envelope protein content of the conditional mutant FL905 grown under permissive and non-permissive conditions for LPS transport to the cell surface. In FL905 *lptC*, which encodes a component of the LPS transport (Lpt) machinery, is under control of the arabinose inducible *araBp* promoter and, upon LptC depletion in the absence of arabinose, LPS transport is blocked [8]. FL905 was grown under permissive (with 0.2% arabinose, FL905+A) and non-permissive (without arabinose, FL905–A) conditions and membrane proteins were extracted and analysed by MudPIT. As a control, the isogenic *lpt*$^{+}$ strain AM604 [25] was used. MudPIT has been extensively used for protein identification and characterization and provides a significant improvement over gel-based analysis, as it represents a fully automated and high-throughput technology. By this approach we identified about 300 proteins in each sample (Table S1). In particular, a total of 864 proteins were identified in the three samples of total membrane analysed in replicate and among these, 323 were detected in all the three samples. 115 were detected in AM604 and FL905+A and 35 in AM604 and FL905–A, whereas 47 were shared between FL905+A and FL905–A. 107, 78 and 159 unique proteins were exclusively detected in AM604 strain, in FL905+A and in FL905–A, respectively (Figure 2).

Using a in-house software called MAProMA [30], the protein lists identified in the three samples of total membranes were plotted on a 2-D map according to the theoretical MW and pI of identified proteins. Figure S1 reports as an example the 2D-map of the proteins obtained by the analysis of the FL905–A mutant membranome.

Using DAve and DCI algorithms of MAProMa software, the relative protein abundance, of AM604 and FL905 grown under permissive conditions, was evaluated by means of a label-free quantitative approach. DAve and DCI were calculated for each pairwise comparison. Only the proteins that exceed the set threshold values were considered. In FL905 the *araBp* promoter drives the expression of *lptCAB* [8]. Thus as expected we found

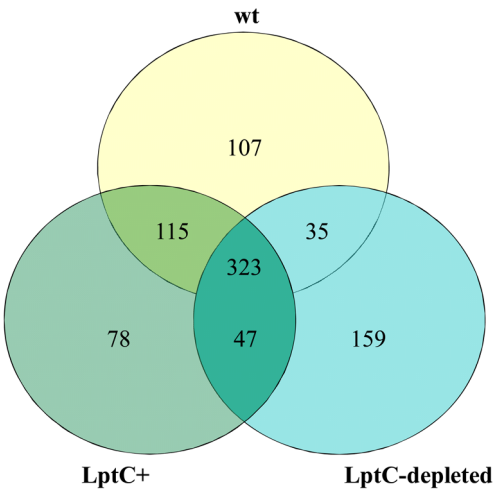

Figure 2. Venn diagram of proteins distribution across strains and growth conditions analysed. Proteins are identified from total membrane samples. wt, (PS201); LptC^{+} (PS202, *araBp-lptC*) grown under permissive condition (0,2% arabinose); LptC-depleted, PS202 grown under non permissive condition (without arabinose).
doi:10.1371/journal.pone.0100941.g002

increased abundance of LptC and the IM associated protein LptB in FL905 as compared with the wild type AM604 control. On the contrary, the periplasmic LptA protein was not detected in the membrane fraction of either strain. It is worth to mention that in the *lptC* depletion strain grown under non-permissive conditions *lptAB* are expressed from a σ^{E} dependent promoter located within the *lptC* coding sequence [33]. With the exception of proteins involved in arabinose metabolism (Table S1) no other significant differences in protein relative abundance were detected between AM604 and FL905+A. For this reason the latter was used as the reference condition in all comparisons with FL905 grown without arabinose (data not shown).

The comparative analysis of the cell envelope protein profile of FL905+A *versus* FL905–A cells showed 123 proteins differentially represented (Table S2). These proteins are related to different cellular pathways that collectively give a snapshot on cellular pathways modulated by *E. coli* cells to respond to severe OM biogenesis defects. As reported in the following paragraphs the functions assigned to the proteins differentially expressed fall in two main pathways: cell envelope biogenesis/remodeling, and protein transport/assembly at the OM. In addition we observed lower abundance for several proteins with a function in (i) central metabolism, (ii) translational apparatus (iii) protein folding/degradation. (Table S2). As lower abundance of these proteins may be correlated with growth arrest imposed by block of LPS transport [8,10,15,34], these functions will not be further discussed. Interestingly the expression of many functions identified by our analysis is under the control of signalling systems (Bae, Cpx, Rcs, and σ^{E}) that in *E. coli* detect and respond to alterations of the bacterial envelope [19–22].

Previous work [8,15] showed that mutant cells depleted of any Lpt component exhibit strikingly similar multilayer membranous bodies in the periplasm (hIM). In this contest, to understand the nature of the hIM and the changes that occur at the level of bacterial membrane in order to restore homeostasis, we used discontinuous sucrose density gradient centrifugation to fractionate IM, OM and hIM from FL905+A and FL905–A and we analysed the protein profile of each fraction by MudPIT (Table S1).

Below the main pathways showing different protein levels when comparing FL905+A and FL905–A are discussed.

Cell envelope biogenesis/remodeling

Peptidoglycan synthesis and cell division. Lpt proteins depletion leads to growth arrest and in the late phase of depletion cells show mostly short filaments [8,10]. In line with this phenotype we found that the level of many proteins implicated in peptidoglycan biosynthesis decreases (Table 1). The transglycosylase MurG, the transpeptidase MrdA (PBP2), peptidoglycan hydrolases AmiA, EmtA, and MltB all have a role during cell elongation [35,36] and show decreased abundance in FL905-A. The same applies to LpoA and LpoB OM lipoproteins that modulate the activity of transpeptidases involved in cell elongation [37]. TolB, a periplasmic protein that binds peptidoglycan via the Pal lipoprotein [36,38] and required for OM invagination also shows a decreased level whose significance is difficult to explain. On the contrary the level of proteins involved in peptidoglycan remodeling and cell division appears to increase in LptC depleted cells (Table 1). In fact we found increased abundance for DacA (PBP5) and DacC (PBP6) carboxypeptidases that remove terminal D-alanine residues from pentapeptide side chains thus preventing those side chains from serving as donors for transpeptidation [39]. Also the level of PBP1B a major peptidoglycan synthase involved in cell division [39], FtsZ the key player at the division machinery

Table 1. Envelope proteins exhibiting a significant variation upon LptC depletion: peptidoglycan synthesis/remodeling and cell division.

Category[a]	GI Accession[b]	Protein	Activity	DAve[c]
Peptidoglycan synthesis	16128083	MurG	Precursors synthesis - GTases	0,50
	16128142	PBP1B	Synthesis - GTases and DD-TPases	−1,23
	16128615	PBP5	Regulation of structure-DD-CPases	−0,58
	16128618	PBP2	Synthesis - DD-TPases	2,00
	16128807	PBP6	Regulation of structure-DD-CPases	−2,00
	16129068	LpoB	Regulation of synthesis-Synthase activators	0,50
	16129156	EmtA	Hydrolysis - Lytic transglycosylases	1,00
	16129736	MipA	Synthesis -Scaffolding protein	0,66
	16130360	AmiA	Hydrolysis - Amidases	1,19
	16130608	MltB	Hydrolysis - Lytic transglycosylases	0,85
	16131039	LpoA	Regulation of synthesis - Synthase activators	0,65
Cell division	16128088	FtsZ	Cytosckeletal structure	−1,26
	16128715	TolB	Outer membrane invagination	0,46
	16130338	ZipA	Early association with Z ring	−0,76

[a]inferred from ecocyc.org.
[b]NCBI accession number.
[c]DAve value ranges from −2 and +2; positive value for DAve indicates that the protein is more abundant in LptC+ (grown with 0.2% arabinose); negative value for DAve indicates that the protein is more abundant in LptC-depleted (grown without arabinose).
doi:10.1371/journal.pone.0100941.t001

apparatus [40] and ZipA a protein interacting with FtsZ and required for preseptal peptidoglycan synthesis [41], increases upon LptC depletion.

Thus it appears that in LptC depleted cells the synthesis of peptidoglycan is inhibited and that the arrest of cell wall growth shifts LptC depleted bacteria to the cell division program as suggested by the increased level of functions implicated in septation; this is in line with the notion that bacteria must somehow coordinate peptidoglycan synthesis with OM assembly. The recent discovery of OM lipoproteins LpoA and LpoB as modulators of PBPs activity suggests that they may play a crucial role in coupling OM biogenesis with PG synthesis [37]. Interestingly we found that the level of both lipoproteins decreases in LptC depleted cells further strengthening the idea that peptidoglycan synthesis is also controlled from outside of the sacculus.

Known/putative proteins involved in LPS/OMP biogenesis. Upon LptC depletion the level of proteins involved in LPS biogenesis (LptD, MsbA, WbbK and WbbI) decreases (Table 2). The decreased level of the OM LptD protein (Table 2) is in line with our previous observation that the steady state level of LptA is affected by depletion of LptC or LptD/E [11]. In fact depletion of LptC or LptD removes the IM and OM docking site, respectively, of LptA resulting in LptA degradation [11]. Assembly of LptD requires lipoprotein LptE [42] and the Bam complex [43]. LptE was not detectable even in samples grown under permissive conditions, possibly because it resides within the LptD β-barrel [13] and therefore it may be protected from trypsin degradation. However, LptE level does not change upon LptC depletion (Figure 3A) in line with previous data [11]. LptF, and LptG were not detectable even in samples grown under permissive conditions due to their very low abundance in the cell [15]. Interestingly our data show a decreased level of BamA and BamD, two members of the Bam machinery (see Protein transport/assembly paragraph below). The lower LptD level may thus be the result of a less efficient assembly at the OM. Recently it has been shown that the periplasmic protease BepA, whose expression is regulated by σ^E, specifically degrades LptD when it fails to form the OM LPS translocon [44]. As an alternative hypothesis we suggest that LptC depletion might affect the formation of a functional OM translocon that undergoes degradation by BepA [15]. Overall, from our data it is not possible to discriminate whether decreased LptD level is the result of lower synthesis, less efficient assembly or degradation of non-functional translocon.

MsbA is the essential ABC transporter implicated in flipping LPS across the IM [4,5] and operates in the LPS export pathway just upstream the Lpt protein machinery (Table 2). No physical interactions have been detected so far between MsbA and the Lpt machinery. Our data for the first time point to a functional interaction between the two systems and suggest that arrest of LPS transport and its accumulation at the periplamic face of the IM somehow affects MsbA level.

We found that the level of WbbI (galactofuranosyl transferase) and WbbK (glucosyl transferase) decreased upon LptC depletion (Table 2). The *wbbI* and *wbbK* genes map at a locus encoding genes involved in O-antigen subunits and other exopolysaccharides (including colanic acid) biosynthesis [2]; in particular, *wbbIJKL* genes have been implicated in biosynthesis of O16 antigen subunit [45]. *E. coli* K12 LPS, however, is lacking the O-antigen as *wbbL*, which codes for a rhamnosyltransferase, is interrupted by an IS*5* insertion [46]. It thus appears that in strain FL905 (an *E. coli* K12 derivative) the loss of *wbbL* does not prevents the expression of other *wbb* genes, whereas the block of LPS transport affects *wbb* genes expression resulting in lower levels of WbbI and WbbK.

Finally, in LptC depleted cells the level of AsmA, a non-essential IM protein of unknown function (Table 2, Figure 3A) for which a possible role in OM biogenesis has been previously proposed [47], decreases. The role of AsmA in OM biogenesis stems from the observation that a mutant *asmA* allele is able to correct the assembly defect of mutated OmpC and OmpF proteins [48,49]. In agreement with a putative role in OM biogenesis, *asmA* null mutants show increased sensitivity to hydrophobic antibiotics and

Table 2. Envelope proteins exhibiting a significant variation upon LptC depletion: cell envelope biogenesis.

Category[a]	GI Accession[b]	Protein	Activity	DAve[c]
Known proteins involved in LPS biogenesis	16128048	LptD	Transporter	0,43
	16128881	MsbA	Transporter	0,80
	16129972	WbbK	Biosynthesis-O antigen	1,40
	16129974	WbbI	Biosynthesis-O antigen	0,85
Putative proteins involved in OM biogenesis	16130004	AsmA	Assembly of OM proteins?	2,00
Functions involved in OM homeostasis	16128173	FabZ	Biosynthesis - β-hydroxyacyl-ACP dehydratases	−1,19
	16128178	AccA	Biosynthesis - Acetyl-CoA carboxylase A	−0,54
	16128757	YbhO	Biosynthesis - Cardiolipin synthase	−2,00
	16129985	WcaK	Synthesis - glycosyl transferase	−2,00
	16129990	WcaI	Synthesis - glycosyl transferase	−2,00
	16129993	Gmd	Synthesis - GDP-mannose 4,6-dehydratase	−2,00
	16129997	WcaC	Synthesis - glycosyl transferase	−2,00
	16130002	Wza	Export - capsular polysaccharide	−2,00
	16130251	AccD	Biosynthesis - Acetyl-CoA carboxylase D	−2,00
	16130740	Aas	Biosynthesis - Hydroxycinnamate-CoA ligase	1,19
	16131083	MlaD	Transporter - Phospholipids	−1,19
	16131084	MlaE	Transporter - Phospholipids	−2,00
	16131641	WzzE	Regulator of O length	−0,50
	16131985	Psd	Biosynthesis - Phosphatidylserine decarboxylase	−1,13
	33347613	WzzB	Regulator of O length	−0,99
	33347615	Wzc	Export - capsular polysaccharide	−2,00
	33347817	PlsB	Biosynthesis - Glycerol-3-phosphate acyltransferase	−0,77
Protein membrane turnover	16128154	DegP	Protease - Periplasmic serine protease	−1,52
	16131068	FtsH	Protease - ATP-dependent metalloprotease	−1,35
	16131996	HflK	Regulator of FtsH	−1,55
	16131997	HflC	Regulator of FtsH	−1,41

[a]inferred from ecocyc.org.
[b]NCBI accession number.
[c]DAve value ranges from −2 and +2; positive value for DAve indicat that the protein is more abundant in LptC+ (grown with 0.2% arabinose); negative value for DAve indicates that the protein is more abundant in LptC-depleted (grown without arabinose).
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a decreased LPS level [47]. In contrast to previous reports [47] we found that in wild type and in non LptC-depleted cells AsmA localizes in both the IM and OM (Table 3), a typical behaviour of membrane fusion proteins (MFP) that function in bridging IM and OM in Gram-negative bacteria [50]. Interestingly, in LptC depleted cells not only the level of AsmA decreased but also its subcellular localization changed as the protein disappeared from the IM and localized at the hIM and OM in LptC depleted cells (Table 3 and Figure 3B). Our data support the role of AsmA in OM biogenesis highlighting a functional link between the assembly of OM proteins and that of LPS as the correct balance of both OM components is required to build a functional OM.

Functions involved in OM biogenesis/ remodelling. Several proteins (WzzE, WzzB, WcaC, WcaK, Gmd, WcaI, Wza, Wzc) belonging to the 19 genes *wca* (*cps*) cluster and implicated in colanic acid biosynthesis [51,52] showed increased abundance in LptC depleted cells (Table 2). This finding is in agreement with our previous observation that depletion of any Lpt protein leads to the production of LPS decorated by colanic acid, indeed this modification is diagnostic of defects in LPS transport occurring downstream of MsbA mediated lipid A-core flipping across IM [8]. Interestingly, *Salmonella enterica* mutants defective in LPS transport due to mutations in *lptC* also show an altered LPS profile [53]. Although the chemical nature of such modification is not known it is reminiscent of the high molecular weight ladder observed upon Lpt proteins depletion. Colanic acid is a cell surface polysaccharide synthesised by enteric bacteria in response to envelope damaging conditions such as osmotic, acid and cold stresses [21,54]. Expression of *cps* cluster is controlled by the Rcs proteins that constitute a complex phosphorelay system known to extend well beyond regulation of colanic acid synthesis [21]. We recently showed that *E. coli* cells treated with ammonium metavanadate, a phosphatase inhibitor known to induce covalent modification of lipid A [55], also produce LPS decorated by colanic acid [33]. The signals that activate such pathways are not well known, however our data support the idea that a specific "LPS" stress may induce the Rcs system therefore activating functions needed for surface remodelling.

In agreement with the notion that block of LPS export pathway results in migration of phospholipids in the outer leaflet of the OM, we found that the level of two components of Mla pathway,

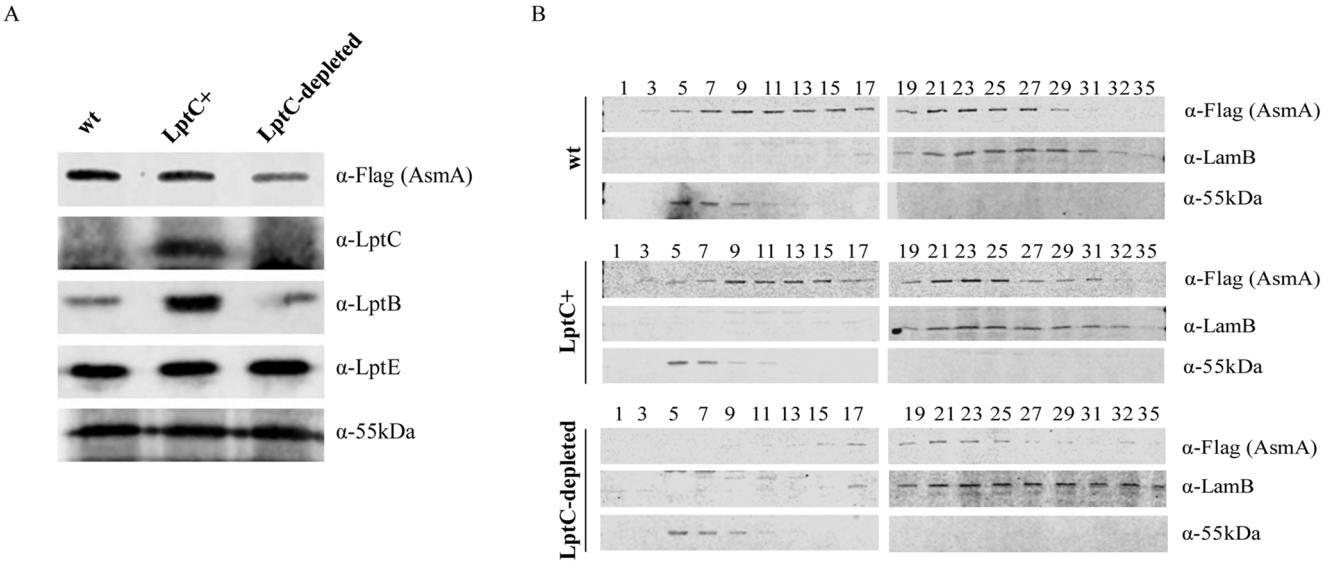

Figure 3. AsmA protein abundance and subcellular localization. PS201 (*asmA-SPA lptC*$^{+}$) and PS202 (*asmA-SPA araBp-lptC*) cells were grown with or without arabinose as indicated. Total membrane protein extracts prepared as described in Materials and Methods were analysed by immunoblotting (panel A) or fractionated by sucrose density gradient (panel B). A) 10 μg of total membrane proteins were loaded in each lane. 55-kDa protein was used as loading control. B) Fractions were collected from the top of the gradient and immunoblotted using antibodies recognizing the 55-kDa protein as IM marker, LamB as OM marker. α-Flag antibodies were used to detect AsmA-SPA protein. wt, PS201; LptC^{+}, PS202 (*araBp-lptC*) grown under permissive condition (with 0,2% arabinose); LptC-depleted, PS202 grown under non permissive condition (without arabinose).
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MlaD and MlaE, increases upon LptC depletion (Table 2). The Mla (Maintenance of OM lipid asymmetry) proteins function as an inter-membrane transport system to prevent surface exposure of phospholipids upon stressful conditions thus maintaining OM asymmetry [56]. Several mutants in LPS biogenesis display increased OM permeability as phospholipids may migrate from the inner to the external leaflet of the OM thus generating locally symmetric bilayer rafts freely permeable to hydrophobic compounds [57]. As the Mla system appears to function by retrograde trafficking of phospholipids from the OM to the IM, the increased level of MlaD and MlaE proteins upon LptC depletion might be needed to maintain lipid asymmetry and therefore OM homeostasis. Our data provide the first functional connection between the LPS export and the OM phospholipid removal pathways.

Proteins implicated in *de novo* fatty acids (AccD, AccA, FabZ) and in phospholipids biosynthesis (PlsB, Psd) (Table 2) also show an increased level in LptC depleted cells. AccA and AccD constitute the heterodimeric carboxyltransferase involved in the first reaction of fatty acid synthesis. FabZ is an R-3 hydroxyacyl-ACP dehydrase which provides precursors for phospholipids synthesis [58]. Interestingly, FabZ shares the substrate R-3 hydroxyacyl-ACP with LpxA and LpxD two enzymes involved in lipid A biosynthesis [59] and is therefore a key enzyme in controlling phospholipids and LPS synthesis. Our data suggest that the modulation of levels of FabZ which competes with LpxA and LpxD for the shared precursor, can be a strategy to shift the synthesis towards the production of phospholipids thus limiting lipid A synthesis. Indeed in LptC depleted cells LPS can not be inserted in the outer leaflet of the OM and consequently cells need to fill the "void" with phospholipids. On the other hand, the increased level of PlsB and Psd (Table 2), which are involved in phosphatidlyethanolamine (PE) synthesis [60] suggests that increased synthesis of this specific phospholipid may help cells upon severe cell envelope damage. In fact surface exposed phosphatidlyethanolamine (PE) is the substrate of the inducible EptA enzyme [61] that removes phosphoethanolamine from its donor substrate (PE) and transfers it to the 1-phophate group of lipid A. Such modification has been detected in lipid A of several mutants defective in LPS transport [13,15] and contributes to polymixin resistance in several organisms such as *Helicobacter pylori* [62], *Neisseria meningitidis* [63] and *Campylobacter jejuni* [64]. Lipid A modifications by EptA are thought to stabilize and/or balance the

Table 3. AsmA level in membrane fractions.

Strain/condition	IM		hIM		OM	
	Score[a]	Hits[b]	Score[a]	Hits[b]	Score[a]	Hits[b]
wt	40,25	7	nd	nd	50,26	9
LptC+	30,23	4	nd	nd	60,21	8
LptC-depleted	0	0	50,30	7	30,33	3

[a]SEQUEST score value, related to the confidence of identification.
[b]number of identified peptides.
doi:10.1371/journal.pone.0100941.t003

surface electrostatics of the OM and can be thus considered an additional mechanism to restore the OM barrier function when LPS transport is defective. It is thus possible that in LptC depleted cells PE synthesis increases the donor substrate for lipid A modification as a strategy to restore the OM functionality.

Finally, we found increased level for YhbO, a conserved protein with unknown function that has been implicated in protection against diverse environmental stresses such as oxidative, thermal, osmotic and pH stresses [65]. YhbO increased level upon LptC depletion may also help cells to recover from envelope damaging stresses.

A decreased expression level upon LptC depletion has been observed for Aas (Table 2), an enzyme implicated in membrane phospholipid turnover [66]. The regulation of Aas expression has been poorly investigated and the meaning of our observation is presently not obvious.

Membrane protein turnover. The level of FtsH, HflK and HflC proteins increases upon LptC depletion (Table 2). FtsH (also named HflB) is an essential ATP dependent IM protease that interacts with HflK and HflC membrane proteins to form a large holoenzyme complex with a role in quality control and degradation of membrane proteins [67]. FtsH plays also a key role in modulating LPS biosynthesis as it controls by proteolysis the amount of LpxC, which catalyses the first committed reaction in lipid A biosynthesis, and of KdtA, a glycosyltransferase involved in Kdo synthesis [68]. The increased amount of FtsH in LptC depleted cells might lower LPS synthesis as a strategy to cope with the block of its transport and to prevent its accumulation at the IM. Interestingly, in addition to the σ^{70} dependent promoter, a σ^{E} promoter has been predicted upstream the *ftsH* gene [69]. We previously showed that LptC depletion activates the σ^{E} dependent regulon [33]. Our data suggest that the predicted σ^{E} sequence upstream of *ftsH* may represent a functional promoter.

DegP is a periplasmic protein functioning both as a protease and as a chaperone whose expression is induced upon cell envelope stresses [70]. DegP increased level in LptC depleted cells (Table 2) fits with its dual role of protease and chaperone in being able to both degrade irreversibly damaged proteins and to assist/promote folding of partially unfolded periplasmic or OM proteins.

Protein transport/assembly

Upon LptC depletion we observe a decreased level for BamA and BamD (Table 4) the essential components of the multiprotein machinery responsible for OMP assembly at the OM [43]. LptD, the only known essential OMP, is also a Bam complex substrate. BamA, whose expression is regulated by σ^{E} [71], has recently been shown to be a substrate of the BepA protease when BamA assembly at the OM is compromised [44]. BamD interaction with BamA appears to stabilize the Bam complex [72,73]; therefore BamD may be an additional BepA substrate or may be degraded by not yet known proteases. The non-essential OM lipoproteins BamB [74] and YiaD instead showed increased abundance (Table 4). YiaD has been identified as multicopy suppressor of a temperature sensitive *bamD* allele [75] and BamB expression is under σ^{E} control [76] possibly explaining their increased level. It thus appears that in Lpt depleted cells growth arrest and damaged envelope both contribute to a general reduction of protein synthesis and therefore to lower level of components of the machinery that insert β-barrel proteins in the OM.

An important function of the OM is the control of influx and efflux of nutrients and toxic compounds playing an important role in the adaptation to different environmental conditions. Accordingly, in LptC depleted cells where the OM is damaged we observed modulation of the level of proteins that regulate the intracellular influx of toxic compounds. OmpF is a porin with a role in the influx of small molecules [1] whose level decreases. Instead, the level of components of efflux pumps, such as AcrA, AcrB, MdtA increases (Table 4). AcrA, AcrB and MdtA are proteins belonging to multidrug efflux pumps, whose expression is up-regulated in response to envelope-damaging agents [77,78]. We confirmed by western blotting that AcrB level indeed increases upon LptC depletion (Figure S2).

Extra-cytoplasmatic stress response

The OM functionality is essential for survival in Gram-negative bacteria and therefore its integrity in *E. coli* is monitored by at least five different but overlapping stress response systems (RpoE, Rcs, BaeR, Cpx, Psp) [79]. In LptC depleted cells the relative abundance of 26 proteins belonging to four such pathways (RpoE, Rcs, BaeR, Cpx) increased and three (out of 10) belonging to RpoE pathway (BamE, BamD, OmpF) decreased (Figure 4A). Interestingly, this list includes most of the functions that have been discussed in the previous paragraphs thus highlighting the importance of such pathways in triggering adaptive responses to OM dysfunction (Figure 4B).

Proteins of hIM

Depletion of any Lpt proteins results in appearance of a novel membrane fraction with higher density of the IM (hIM) where most of *the novo* synthesised LPS accumulates [8,10,15]. To better clarify the nature of such fraction we analysed its proteome. We considered hIM proteins those showing a score higher that 30 (Table S3). Out of the 53 hIM proteins matching the selected parameter 44% are proteins normally localized at the IM, 21% are OM proteins and the remaining fraction is represented by cytoplasmic (26%), periplasmic (2%) and unknown (7%) proteins (Figure S3). Most of the proteins enriched in the hIM fraction are related to transport systems whereas the rest form a miscellaneous group; notably none of the proteins displaying an increased level in LptC depleted cells was found in hIM (Table S3). These data suggest that that hIM is more similar to the IM than the OM and are in line with the hypothesis that hIM corresponds to the abnormal membrane structures visible by electron microscopy in Lpt depleted cells [8,10,15] where proteins belonging to different biological processes, unrelated to each other, remain trapped.

Conclusions

We used the MudPIT technology to analyze the envelope proteome in LptC depleted cells, which experience a severe OM biogenesis defect due to block of LPS transport to the cell surface. The comparative proteomic analysis between LptC depleted and not-depleted cells highlighted strategies adopted by bacteria to maintain OM homeostasis. The envelope proteome of LptC depleted cells displayed higher abundance of functions that collectively may contribute to repair the OM and restore its permeability barrier properties. Such functions include proteins implicated in maintaining OM asymmetry or involved in the synthesis of phospholipids and exopolysaccharides as substrates for lipid A-core modification enzymes. Lipid A modifications are generally not required for growth under laboratory conditions but confer selective advantages, such as resistance to antimicrobial peptides or the ability to evade the innate immune system [80]. Interestingly, we found that the level of several enzymes implicated in peptidoglycan synthesis/remodeling changes in LptC depleted cells. Indeed, our results suggest that when growth of the OM is compromised by block of LPS transport cells switch from the "elongation mode" of peptidoglycan synthesis to the "constrictive

Table 4. Envelope proteins exhibiting a significant variation upon LptC depletion: transport/assembly.

Category[a]	GI Accession[b]	Protein	Activity	DAve[c]
Protein transport	16128091	SecA	Transporter -Sec Translocation Complex	1,55
	16128170	BamA	Transporter - OMPs	0,54
	16128392	YajC	Transporter -Sec Translocation Complex	0,66
	16128393	SecD	Transporter -Sec Translocation Complex	0,93
	16128394	SecF	Transporter -Sec Translocation Complex	0,66
	16130437	BamB	Transporter - OMPs	−1,19
	16130516	BamD	Transporter - OMPs	0,57
	16131423	YiaD	Transporter - OMPs	−1,42
Transport	16128446	AcrA	Membrane fusion protein	−1,63
	16128447	AcrD	Permease	−1,17
	16128896	OmpF	General Bacterial Porin	0,83
	16130014	MdtA	Membrane fusion protein	−1,32

[a]inferred from ecocyc.org.
[b]NCBI accession number.
[c]DAve value ranges from −2 and +2; positive value for DAve indicates that the protein is more abundant in LptC+ (FL905 grown with 0.2% arabinose); negative value for DAve indicates that the protein is more abundant in LptC-depleted (FL905 grown without arabinose).
doi:10.1371/journal.pone.0100941.t004

mode'' to direct cells towards the cell division program. It has been recently shown that in *E. coli* the enlargement of the peptidoglycan layer requires control or activation not only from the inside of the cells but also from outside by proteins associated to the OM [37,81]. Overall, our data further support the notion that OM biogenesis and PG synthesis are tightly coordinated pathways.

The block of LPS transport result in growth arrest and as a consequence the level of ribosomal and transport proteins as well as many folding factors decreases in LptC depleted cells. Conversely, the level of several IM, periplasmic and OM proteases increases to cope with increased abundance of proteins that are not assembled at the OM. Our data are consistent with the notion that the extracytoplasmic stress response is activated upon block of LPS transport as the expression of many functions implicated in OM biogenesis, OM remodeling, protein folding/degradation showing increased abundance in LptC depleted cells are under the control of the Bae, Cpx, Rcs and σ^E signaling systems [23]. Of note is the finding that the level FtsH, an essential IM anchored protease, increases in LptC depleted cells. FtsH is known to control LPS biosynthesis by degrading LpxC the enzyme that catalyzes the first committed step of lipid A biosynthesis [82]. Our data point for the first time to a feed-back control on lipid A synthesis signaled from the external surface of the cell when LPS transport to the OM is compromised. We do not know how such signal may be transduced inside the cell; a possible candidate in such signal transduction pathway is the recently identified YciM IM protein that has been suggested to act in concert with FtsH to regulate synthesis of lipid A [83,84,85].

Overall our results show a snapshot of pathways modulated by *E. coli* cells to respond to a severe OM biogenesis defect namely block of LPS transport, that act integrating complementary functions to restore OM functionality.

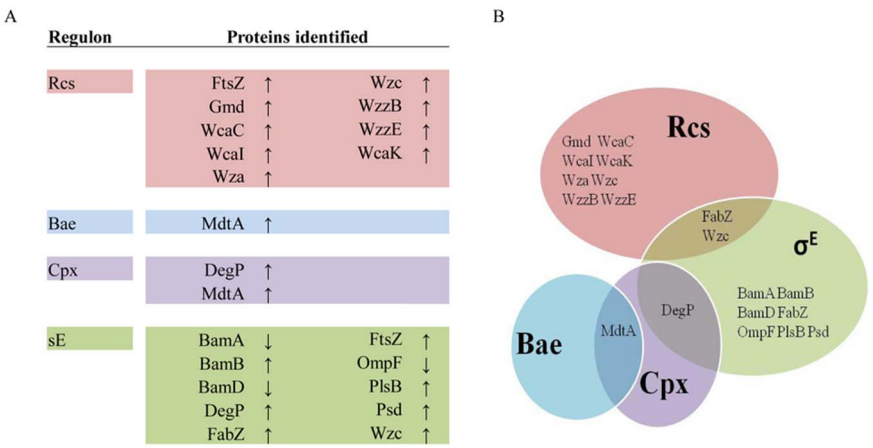

Figure 4. List of proteins belonging to cell envelope stress response pathways whose level changes upon LptC depletion. A. Relative abundance is calculated as the ratio beetween FL905 grown under permissive and non permissive conditions: ↑, increased; ↓, decreased (see Tables 1, 2. 4). B. Venn diagram showing functions whose regulations is shared by multiple envelope signaling systems.
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Supporting Information

Figure S1 2D map of all the proteins identified in the analysis of total membrane. MAProMa software plots all the proteins according to the theoretical pI and MW. A color/shape code is assigned to each protein according to relative SC value. Proteins with SC≥35 are reported as red/circle, proteins with SC<35 and >15 are reported as blue/square, and proteins with SC≤15 are reported as yellow/triangle code. The dashed box indicates the typical pI and MW ranges for 2-DE.
(TIF)

Figure S2 AcrB protein abundance upon LptC depletion. PS201 (*asmA-SPA lptC*$^{+}$) and PS202 (*asmA-SPA araBp-lptC*) cells were grown with or without arabinose. Extracts of total membrane proteins prepared as described in Material and Methods were analysed by immunoblotting using anti-AcrB anti-LptC antibodies. An IM 55-kDa protein was used as loading control. 10µg of proteins were loaded in each lane. wt, PS201; LptC^{+}, PS202 grown under permissive condition (0,2% arabinose); LptC-depleted, PS202 grown under non permissive condition (without arabinose). The asterisk (*) indicates a band cross reacting with anti-AcrB antibodies.
(TIF)

Figure S3 Localization of proteins identified in hIM.
(TIF)

Table S1 List of proteins identified in AM604, FL905+A and FL905-A (total membrane, inner membrane, outer membrane, heavy inner membrane (hIM)).
(XLS)

Table S2 Proteins identified by comparative analysis of the cell envelope proteome of FL905+A versus FL905–A cells.
(XLSX)

Table S3 List of proteins identified in the heavy inner membrane (hIM) of FL905–A.
(XLSX)

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Author Contributions

Conceived and designed the experiments: PM AP PS. Performed the experiments: AMM SM FF DDS. Analyzed the data: AMM DDS GD. Contributed to the writing of the manuscript: AMM GD AP PS.

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