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Characterization of the lipopolysaccharide transport machinery using an *Escherichia coli*/*Pseudomonas aeruginosa* hybrid system and *Escherichia coli lptC* mutants.

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Ph.D. Thesis

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CONTENTS

ABSTRACT

Characterization of the lipopolysaccharide transport machinery using an *Escherichia coli*/*Pseudomonas aeruginosa* hybrid system and *Escherichia coli lptC* mutants

The lipopolysaccharide (LPS) transport (Lpt) is an essential process for the biogenesis of the outer membrane (OM) in Gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. OM, the first bacterial defense against harsh environment and antimicrobial compounds, is composed by an inner leaflet of phospholipids, an outer leaflet of LPS, and outer membrane proteins.

Lpt system, originally identified in *E. coli*, is a protein machine responsible for LPS delivery from the inner membrane, where it is synthesized, to the outer membrane. It is composed of seven proteins, LptA through LptG, which form a complex spanning the IM and OM. LptBCFG are located at the IM, LptA in the periplasm, and LptDE at the OM. The *lpt* genes are evolutionarily conserved and appear to play an essential role in most Proteobacteria.

To understand how LPS is delivered to the OM crossing the hydrophilic periplasmic space, we focused on the role of the periplasmic components LptC and LptA by two main approaches:

i) comparison between *E. coli* and *P. aeruginosa* Lpt systems to search for conserved features shared by the Lpt machines of different Gram negative species. By plasmid shuffling technique we showed that *P. aeruginosa lptCAB* genes can complement *E. coli* Δ*lptCAB* mutants, thus indicating that the *Pseudomonas* proteins can interact with both the other proteins of the Lpt machine and the LPS of *E. coli*. Although *E. coli* and *P. aeruginosa* LptC and LptA proteins exhibit limited sequence identity and similarity, their 3D conformation is conserved. This suggests that LptA and LptC overall structure rather than their amino acid sequence may play a major role in Lpt assembly and LPS recognition and transport;

ii) analysis of *E. coli lptC* mutants in order to elucidate the role of LptC in the system. *E. coli* appears to tolerate several mutations in *lptC*, including deletion of the trans membrane domain; moreover the lethality of the *lptC* C-terminal region deletion can be suppressed by an appropriate expression of *lptB*. We thus tested whether *E. coli* could tolerate the lack of LptC, which was thought to be essential. By plasmid shuffling we obtained viable mutants missing *lptC*. Genome sequencing of such mutants revealed single amino acid substitutions at the R212 residue of the IM component LptF (*lptF*^{Sup}mutants); in complementation tests, *lptF*^{Sup} mutants suppress lethality of LptC conditional expression mutants. These data show that a specific mutation in LptF can compensate the lack of LptC and suggest that LptC may serve as a chaperon of the Lpt machine assembly and/or activity rather than an essential structural component.

PART I: INTRODUCTION AND OVERVIEW

STATE OF THE ART

In addition to the universally conserved cytoplasmic membrane, most bacteria have developed a wide variety of protective peripheral structures, collectively named the cell wall, which provide strength and protection to the cell and contribute to determine its shape (Beveridge, 1999; Ellen *et al.*, 2010; Silhavy *et al.*, 2010). Gram negative bacteria are surrounded by a peculiar additional bilayer, the outer membrane (OM), which is composed by an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS).

LPS is an essential glycolipid for the majority of Gram negative bacteria, with the notable exception of *Neisseria meningitidis* (Steeghs *et al.*, 1998). It forms a hydrophilic barrier against several toxic compounds such as antibiotics and detergents (e.g. bile salts) and makes Gram negative bacteria able to survive in hostile environments such as the gastrointestinal tracts of mammals (Nikaido, 2003; Gunn). OM proteins (OMPs) and lipoproteins complete the OM structure providing a variety of specific functions and making it a very complex system.

In Gram negative bacteria, the inner (cytoplasmic) membrane IM and the OM delimit the periplasmic space in which a thin layer of murein is located (Fig. 1).

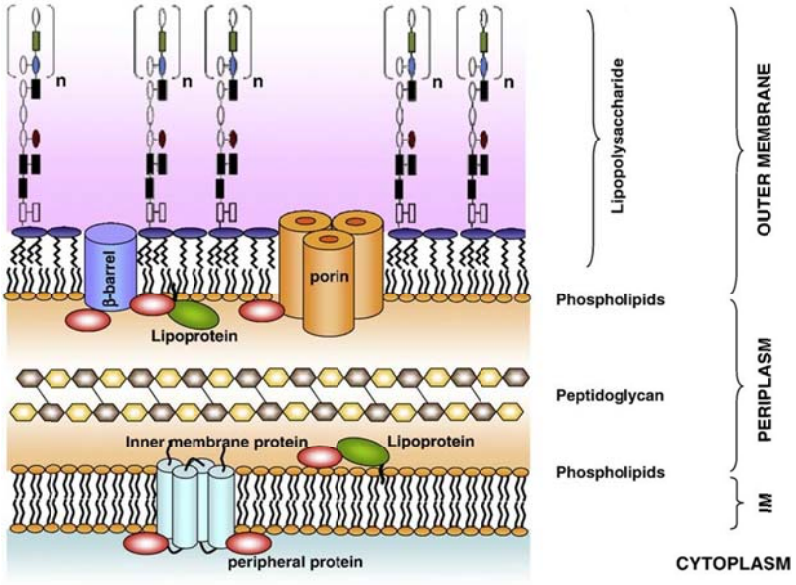

Fig. 1. Gram negative bacteria cell envelope (modified from (Sperandeo *et al.*, 2009)).

The OM biogenesis poses several problems since lipids and proteins, which are synthesized in the cytoplasm and/or at the IM need to cross the IM lipid bilayer, traverse the aqueous

periplasmic space, and be assembled at the amphipathic final destination. The cell compartments outside the IM lack ATP and other high energy carriers. Thus the energy required for the OM morphogenesis is either provided by exergonic reactions (thus involving substrates that have been energized before their translocation across the IM) or transduced by devices (usually protein machines) connected to the IM and capable to exploit the proton motive force or the energy released by ATP hydrolysis in the cytoplasm. LPS is an amphipathic molecule and its transport across the periplasm cannot occur by simple diffusion.

In *Escherichia coli,* the LPS transport (Lpt) from the IM, where it is synthesized, to the cell surface is performed by an essential protein machine composed by seven Lpt proteins (LptABCDEFG) located in every cellular compartment: cytoplasm, IM, periplasm and OM (Braun, and Silhavy, 2002) (Sperandeo *et al.*, 2006) (Sperandeo *et al.*, 2007) (Sperandeo *et al.*, 2008) (Wu *et al.*, 2006) (Ruiz *et al.*, 2008).

Through bioinformatic analysis, putative Lpt proteins and relative *lpt* genes were identified in a wide range of Gram negative bacteria, indicating that the system is evolutionarily conserved.

The inter-species analysis of the LPS transport machine could help to identify the basic requirements for its functioning. So in this work we focused on the Lpt system of two bacterial species: the model organism *Escherichia coli* and *Pseudomonas aeruginosa*. Both *E. coli* and *P. aeruginosa* are involved in infectious diseases: the first is implicated in severe gastrointestinal and extra-intestinal diseases whereas the latter is an opportunistic pathogen that causes a wide variety of infections in compromised patients such as those affected by cystic fibrosis.

Since *lpt* genes are thought to be essential for viability in most Proteobacteria, the Lpt pathway represents a potential target for the development of antibacterial drugs.

LIPOPOLYSACCHARIDE

Structure and functions

LPS is a glycolipid composed of three covalently linked structural domains: lipid A, the core oligosaccharide, and a highly variable polysaccharide, the O-antigen, made of repeating oligosaccharide units (Sperandeo *et al.*, 2014) (Fig. 2). The core can be further divided into inner (lipid A proximal) and outer core.

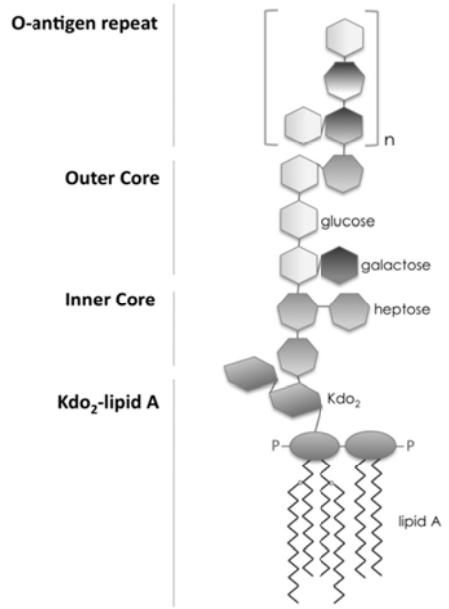

Fig. 2. Lipopolysaccharide of *Escherichia coli* (Sperandeo *et al.*, 2014).

The lipid A backbone is widely conserved and is composed by a bisphosphorilated diglucosamine substituted with six C12-C14 acyl chains, in *E. coli*, or five C10-C12 acyl chains, in *P. aeruginosa*. An additional C16 acyl chain is present in strains isolated from cycstic fibrosis patients (Erridge *et al.*, 2002) (Miller *et al.*, 2005). Both phosphates attached to diglucosamine can be further substituted with groups such as phosphate, ethanolamine, ethanolamine phosphate, ethanolamine diphosphate, GlcN, 4-amino-4-deoxy-L-arabinopyranose and D-arabino-furanose. Aminoarabinose are linked to phospates in *P. aeruginosa* isolated from cystic fibrosis patients (Pier, 2007).

The chemical structure of the outer core is variable, whereas the inner core tends to be quite conserved and in all species so far analyzed, it contains at least one residue of 3-deoxy-D-manno-oct-2 ulosonic acid (Kdo) linking the inner core to lipid A. Therefore, Kdo is a chemical

hallmark of LPS and a marker of LPS-producing Gram-negative bacteria (Holst, 2007). Both *E. coli* and *P. aeruginosa* have an inner core composed by two Kdo molecules, which is completed by three heptose in *E. coli* and by two heptose in *P. aeruginosa* (Sperandeo *et al.*, 2009) (Sperandeo *et al.*, 2014) (Pier, 2007).

The O-antigen is the distal, surface exposed, LPS moiety responsible of the immunogenic properties of this macromolecule (Raetz, and Whitfield). It is present in most wild type strains and clinical isolates where it contributes to virulence by protecting the bacteria from phagocytosis and complement-mediated lysis (Trent *et al.*, 2006), however it is not essential and is missing in common laboratory *E. coli* K12 strains.

The LPS structural requirements for bacterial viability may vary across genera/species. In *E. coli* the minimal LPS structure required for growth has been defined as Kdo2-lipid A (Raetz, and Whitfield), although the lethal phenotype of Kdo-deficient mutants may be rescued by several suppressor mutations (Meredith *et al.*, 2006). In contrast, *P. aeruginosa*, in addition to lipid A, requires the full inner core and at least part of the outer core to be viable (Rahim *et al.*, 2000; Walsh *et al.*, 2000).

The structural complexity of LPS correlates with its multiple functions. While the outer hydrophilic layer of LPS leaflet in the OM represents an effective barrier for passive diffusion of lipophilic compounds, the lipid A moiety, together with the phospholipids of the internal leaflet, forms a hydrophobic barrier. Moreover, LPS appears tightly packed at the outer OM leaflet thanks to strong lateral interaction between LPS molecules promoted by the bridging action of Mg^{2+} and Ca^{2+} divalent cations, which counteract the negative repulsive charges of phosphates and stabilize the structure (Nikaido, 2003) (Holst, 2007).

LPS is also a potent activator of the innate immune response and lipid A (known as endotoxin) is the conserved pathogen associated molecular pattern (PAMP) recognized by innate immune receptors to signal and activate complex signaling cascades that lead to the release of pro-inflammatory cytokines (Miller *et al.*, 2005). Recognition of lipid A requires the TLR4-MD2 complex (Medzhitov *et al.*, 1997) (Shimazu *et al.*, 1999) and the accessory protein CD14 and LPB (LPS binding protein) (Miyake, 2006).

Biosynthesis

LPS biosynthesis involves several independent pathways located in different cell compartments and converging in an ordered assembly line (reviewed by (Raetz *et al.*, 2007) (Raetz, and Whitfield, 2002)). The biosynthesis of core-lipid A takes place at the cytoplasmic face of the IM and needs the converging synthetic pathways of the lipid A moiety, the Kdo residue and the oligosaccharide core. Fatty acylation of two UDP-N-acetylglucosamine molecules leads to lipid IVA, the β-1–6 disaccharide backbone to which four fatty acid chains are linked. In *E. coli*, two activated CMP-Kdo residues (synthesized by a distinct pathway) are then added by a CMP-Kdo transferase to assemble the Kdo2-lipid IVA moiety. Two further acylation reactions lead to the synthesis of the hexaacylated Kdo2-lipid A. All the enzymes required in this part of the pathway are generally essential and constitutively expressed. The additional sugars composing the oligosaccharide core are added to Kdo2-lipid A by specific glycosyl-transferases to assemble the core-lipid A structure. The core-lipid A, which is anchored to the IM with its hydrophilic portion exposed to the cytoplasm, is then flipped across the IM by the ATP-binding cassette (ABC) transporter MsbA, thus becoming exposed to the periplasm.

O-antigen repeat units are synthesized in the cytoplasm and then flipped to the periplasmic face of the IM linked to the lipid carrier undecaprenyl diphosphate. The core-lipid A and O-antigen biosynthetic pathways converge with the ligation of O-antigen to core-lipid A at the periplasmic face of the IM mediated by the WaaL ligase, thus forming the mature LPS.

LPS TRANSPORT MACHINERY

Lpt proteins

The *E. coli* LptABCDEFG proteins (Tab. 1) were identified in the last decade through genetic, biochemical and bioinformatic approaches (Aono *et al.*, 1994) (Ruiz *et al.*, 2008) (Sampson *et al.*, 1989) (Serina *et al.*, 2004) (Sperandeo *et al.*, 2007) (Sperandeo *et al.*, 2008) (Sperandeo *et al.*, 2006) (Stenberg *et al.*, 2005) (Takase *et al.*, 1987) (Tran *et al.*, 2008) (Wu *et al.*, 2006).

Gene name	**Locus**	**Protein features**					
		n. aa	**kDa**	**Crystal structure**	**Lpt fold**	***Ec-Pa* identity (%)**	***Ec-Pa* similarity (%)**
lptB	*yrbG*	241	26.7	+	-	66	82
lptF	*lptF*	366	40.2	-	+	31	52
lptG	*lptF*	360	39.5	-	+	39	60
lptC	*yrbG*	191	21.6	+	+	19	42
lptA	*yrbG*	185	18.6	+	+	24	52
lptD	*lptD*	784	87	-	+	30	46
lptE	*leuS*	193	21.2	-	-	21	40

Table 1. Components of the LPS tansport machine in *E. coli*

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. In addition to the strong *yrbGp* promoter, two minor promoters (*lptAp1* and *lptAp2*) are located upstream of *lptA* within *lptC* (Martorana *et al.*, 2011). Although *lptAp1* promoter 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 (Martorana *et al.*, 2011), thus implying a specialized σ^E-dependent LPS stress signaling pathway. 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).

In *P. aeruginosa*, putative Lpt homologues were identified by bioinformatic predictions using the sequence of known Lpt proteins as template. They are LptH, PA4461, PA4459, OstA, PA3988, PA3828, PA3827, which for convenience are respectively named *Pa*-LptHBCDEFG (Bollati *et al.*, 2015). *Pa-lpt* genes are encoded at unlinked loci and clustered as in *E. coli*. Despite *E. coli* and *P. aeruginosa* proteins mostly exhibit low amino acidic identity and similarity, the crystal structure of *Pa*-LptH is superimposable to *Ec*-LptA (Bollati *et al.*, 2015) and bioinformatic predictions show that the fold of the other Pa-Lpt proteins seems conserved to that of the *E. coli* homologs.

The Lpt machinery may be divided in three subassemblies: LptBFGC, LptA, and LptDE, which are located at the IM, in the periplasm, and at the OM, respectively (Fig. 3).

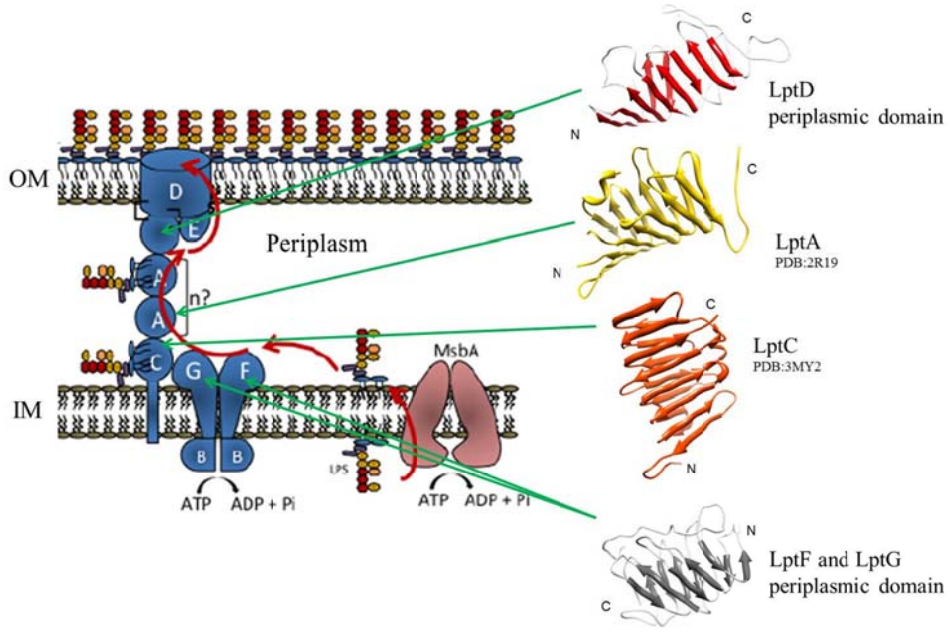

Fig. 3. Lpt proteins localization and Lpt fold (Sperandeo *et al.*, 2014), (Villa R., Ph.D. Thesis).

LptBFG (Ruiz *et al.*, 2008) (Ruiz *et al.*, 2009) (Narita, and Tokuda, 2009) is an ABC transporter composed by the ATP binding protein LptB (Saurin *et al.*, 1999), which is associated to the two transmembrane subunit LptF and LptG at the cytoplasmic face of the IM.

LptB is an ATPase which provides energy to the transport. Its crystal structure has been solved both before and after ATP hydrolysis showing that conformational changes occur between the two states and mutagenesis and biochemical experiments have been performed to identify the residue involved in ATP hydrolysis and LptB binding to LptFG (Sherman *et al.*, 2014) (Wang *et al.*, 2014). It is supposed that LptB conformational changes are involved in the transmission of the energetic impulse to LptFG (Sherman *et al.*, 2014).

Both LptF and LptG are composed by six trans membrane domains and a periplasmic domain with a predicted β-jellyroll fold (Villa *et al.*, 2013), however their sequence is highly divergent

by each other and to date the mechanism by which they participate to LPS extraction from the IM is unclear.

LptC is stably associated to the LptBFG complex and results an atypical subunit for an ABC transporter since, at least in vitro, does not affect its ATPase activity (Tokuda, 2009). It is a bitopic protein (Sperandeo *et al.*, 2008) composed by a transmembrane domain and a periplasmic domain with a slightly distorted β-jellyroll fold composed by 15 antiparallel β-strands (Tran *et al.*, 2010) (Villa *et al.*, 2013).

The LptC specific role and mechanism of action in the transport of LPS is unclear. Deletion of its transmembrane N-terminal domain is tolerated since does not impair neither LPS transport neither LptC assembly with the LptBFG IM complex, although the LptC periplasmic domain alone seems to interact with the IM complex less efficiently than the wild type protein or a chimera with a heterologous TM domain (Villa *et al.*, 2013).

Furthermore the *E. coli* ST190 strain and PA14 mutants are viable despite transposon insertions in the 3' region of *lptC* (Serina *et al.*, 2004) (ausubellab.mgh.harvard.edu). However, point mutations in the N-terminal periplasmic region (G56V) or in the C-terminal region (G153R) are not viable (Sperandeo *et al.*, 2011).

Biochemical assays assessed that the subunit ratio of the IM sub-complex is $LptB_2LptF_1LptG_1LptC_1$ (Narita, and Tokuda, 2009).

IM and OM subassemblies are connected by the periplasmic protein LptA (Sperandeo *et al.*, 2007) (Freinkman *et al.*, 2012) which has a slightly twisted β-jellyroll fold, composed of 16 antiparallel β-strands (Suits *et al.*, 2008) and show the propensity to form oligomeric fibrils in vitro and head-to-tail homodimers in vivo (Suits *et al.*, 2008) (Santambrogio *et al.*, 2013).

At the OM the LptDE subassembly is responsible of the final stage of LPS assembly at the cell surface (Wu *et al.*, 2006) (Chng *et al.*, 2010b). The solved crystal structures of the LptDE 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). Bioinformatic analysis predict a β-jellyroll fold for the periplasmic N-terminal domain of LptD (OstA superfamily domain).

It was demonstrated that LptE is required for a proper folding of LptD and that it interacts directly with some residues of a predicted LptD extracellular loop adopting a plug and barrel architecture (Freinkman *et al.*, 2012) (Freinkman *et al.*, 2011). Moreover LptE binds LPS in vitro (Chng *et al.*, 2010b), suggesting a dual role for this protein: a structural component of the LptDE complex and a recognition site for LPS at the OM (Chng *et al.*, 2010b) (Freinkman *et al.*, 2011).

The *E. coli* Lpt proteins or domains located in the periplasm appear to share the same slightly distorted β-jellyroll conformation, the so called Lpt fold. The Lpt β-jellyroll fold has an internal

hydrophobic cavity (Suits *et al.*, 2008) which is compatible with a role in hosting and shielding hydrophobic molecule portions such as the LPS lipid A, infact both LptC and LptA have been shown to bind LPS (Tran *et al.*, 2008) (Tran *et al.*, 2010) (Okuda *et al.*, 2012) (Sestito *et al.*, 2014).

Lpt machinery functioning model

Lpt proteins appear to act as a single device (Chng *et al.*, 2010a) formed by a proteinaceous bridge spanning from IM to OM.

All seven Lpt proteins co-sediment in a membrane fraction that contains both IM and OM (OM_L fraction) and co-purify as a single complex formed by physically interacting proteins (Chng *et al.*, 2010a).

LptC appears to interact with the LptBFG complex through its periplasmic domain (Villa *et al.*, 2013) and photo cross-linking experiments in vivo showed that it interacts with the N-terminal region of LptA, which in turn interacts with periplasmic domain of LptD through its C-terminal region (Freinkman *et al.*, 2012).

Based on structural data, it has been suggested that four superfamily domains are necessary to span the width of the periplasm (Suits *et al.*, 2008): if LptC and LptD periplasmic domains are taken into account, LptA would function as a dimer within the transenvelope bridge.

Alternatively, the periplasm could be constricted in the vicinity of Lpt bridges, and thus only one LptA molecule would be sufficient to build the bridge. It has been proposed that the bridge is based on the conserved structurally homologous jellyroll domain shared by five out of the seven Lpt components (Villa *et al.*, 2013).

The depletion of any of the Lpt proteins leads to the same phenotypes, which include inhibition of the LPS transport and accumulation of chemically modified LPS molecules (Sperandeo *et al.*, 2008) (Chng *et al.*, 2010a) (Sperandeo *et al.*, 2011). Moreover mutations impairing Lpt complex assembly result in degradation of the periplasmic component LptA (Sperandeo *et al.*, 2011), confirming the relevance of the transenvelope structure in vivo.

LPS transit along Lpt components seems to be an ordered and unidirectional process that requires LptBFG to transfer LPS to LptC and then LptBFGC to deliver LPS to LptA in line with their subcellular locations (Okuda *et al.*, 2012). This idea is supported by the ability of LptA to bind LPS with higher affinity than LptC and to displace it from LptC (Tran *et al.*, 2008) (Tran *et al.*, 2010) (Okuda *et al.*, 2012) (Sestito *et al.*, 2014).

The energy deriving from ATP hydrolysis is not required for assembly of the bridge, instead two energy dependent steps are necessary to translocate LPS from IM to OM: the first requiring energy step is the extraction of LPS from the IM and the second is the transfer of LPS from LptC to LptA (Okuda *et al.*, 2012). The current data indicate that the energy supplied by LptB is used to push a continuous stream of LPS through the transenvelope Lpt bridge in discrete steps against a concentration gradient (Sherman *et al.*, 2014) (Wang *et al.*, 2014) (Okuda *et al.*, 2012).

Then LPS pass from LptA to the LptDE subassembly which inserts it into the outer leaflet of the OM, as showed by mutagenesis, functional assays, and molecular dynamics simulations of the complex. 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 between strands β1C and β26C, delivers LPS hydrophilic portions across the OM lipidic bilayer (Gu *et al.*, 2015).

AIM OF THE PROJECT

Aim of the project is to get a deeper knowledge of the Lpt system, which is an essential protein machine involved in the outer membrane biogenesis of most Gram negative bacteria.

LPS transport is a complex mechanism which requires to overcome energetic, structural and physical barriers such as the distance interposed between the two membranes and the incompatibility between the hydrophobic lipid A and the aqueous periplasmic space.

There are unclear steps in the LPS transport. One of these regards the functioning of the LptBFGC complex and its functional interactions with LptA. It is unclear how the LPS molecule is extracted from the IM and how it is addressed to the Lpt periplasmic bridge. We do not know how LPS is managed by LptFG and it is unclear the role of LptC in in LPS extraction from the IM, the assembly if the IM complex and the the connection between IM and OM assemblies.

In the first part of this work we focused on an interspecies analysis of the Lpt system of *E. coli* and *P. aeruginosa*. The comparison of heterologous systems could help to identify essential conserved features shared by the Lpt machine of both species, in order to trace a more general model of LPS transport.

First of all we addressed the question if *Pa*-LptCHB proteins are functional in *E. coli*, in order to assess if they are the functional homologues of *Ec*-LptCAB. Thus we tested whether *Escherichia coli*/*Pseudomonas aeruginosa* hybrid strain in which one or more *E. coli* lpt genes are replaced by the *P. aeruginosa* heterologous genes.

Hybrid strains could be useful to highlight Lpt system interspecies common features and to analyze *P. aeruginosa* proteins in the more experimentally manageable host *E. coli*.

In the second part of the work we investigated more closely on the role of LptC.

Previous work showed that the absence of *lptC* gene expression is lethal. However the *E. coli* strain ST190 and *P. aeruginosa* mutants are viable despite the disruption *lptC* by transposon insertions in the 3 prime region of the gene (Serina *et al.*, 2004) (ausubellab.mgh.harvard.edu). Moreover a recent work shows that the trans-membrane domain of *Ec*-LptC is dispensable (Villa *et al.*, 2013).

Here we analyzed LptC mutants in order to characterize its functional domains and to understand how the *E. coli* Lpt machine tolerates defects in the C-terminal region of LptC. Then we tested whether the *E. coli* Lpt machine can function in absence of LptC, which was thought to be essential.

The isolation of *lptC* lacking mutants may be useful to clarify the role of LptC through a reverse genetics approach and to eventually select suppressor mutations able to restore viability in its absence.

The identification of suppressor mutations in genes involved in the LPS transport may help not only to investigate on the role of LptC but also to get information on other known or unknown components of the transport system.

The long term goal is to trace an inter-species model of the LPS transport, suitable for the rational design and synthesis of OM biogenesis inhibitors.

MAIN RESULTS

The main results obtained are presented in one published work and two draft manuscripts to be submitted for publication.

1. Published Paper.

Bollati, M., Villa, R., Gourlay, L. J., Benedet, M., Dehò, G., Polissi, A., Barbiroli, A., Martorana, A. M., Sperandeo, P., Bolognesi, M. and Nardini, M.

Crystal structure of LptH, the periplasmic component of the lipopolysaccharide transport machinery from *Pseudomonas aeruginosa*.

FEBS J. DOI: 10.1111/febs.13254

In this study, we focused on the *P. aeruginosa* PAO1 Lpt system and on its common features with the *E. coli* homologous machinery.

Using the BLAST bioinformatic tool, we identified a putative protein, renamed LptH, as the hypothetical *E. coli* LptA orthologue in *P. aeruginosa*.

We demonstrated by complementation assays that LptH is the genetic and functional homolog of *E. coli* LptA despite a low amino acidic identity. We were able to construct an *E. coli* strain in which the deletion of the essential *lptA* gene is complemented by lptH, although the OM functionality of the hybrid strain results to be at least partially impaired.

Then we solved the LptH crystal structure showing that it has a β-jellyroll fold and it is almost superimposable in its backbone to that of LptA .

The results support the general idea that the β-jellyroll is an inter-species fundamental structural element for proteins involved in LPS transport and that the Lpt fold seems to play a more important role than the amino acid sequence.

By site-directed mutagenesis we showed that mutations in a highly conserved glycine, LptAG138R and LptHG135R, result in severe *E. coli* growth defect. The mutated glycines are located in close proximity to the homodimerization interface that can link two adjacent subunits of LptA or LptH.

Since the complementation results are related to alteration of the oligomerization state of the mutant proteins and/or to a decrease of their structural stability we suggest that this residue may play a relevant role in protein–protein or ligand–protein interactions.

Furthermore, we modeled the N-terminal domain of *P. aeruginosa* LptD, based on the crystal structure of its homologue from *Shigella flexneri*.

We showed that the structure of LptH superimposes well with the modeled N-terminal β-jellyroll of *Pa*-LptD, supporting the relevance of a common Lpt fold for the LPS transport.

Our structural and genetic data on LptH provide a first step in the characterization of the Lpt system of the pathogenic *P. aeruginosa*.

2. Draft manuscript:

Martorana, A. M., Benedet, M., Maccagni, E. A., Sperandeo, P., Villa, R., Dehò, G. and Polissi, A.

Overexpression of ATP binding protein LptB suppresses defective forms of the lipopolysaccharide transport protein LptC in Escherichia coli. (To be submitted)

In this work we analyzed the functional domains of LptC through the study in *E. coli* of *Pa*-LptC, *Ec/Pa*-LptC chimeras and LptC C-terminal deletion mutants.

Complementation and biochemical assay using *Ec*/*Pa*-LptC chimeras, in which three LptC regions from the two organisms were swapped in all possible combinations, show that only the C-terminal region of *Ec*-LptC (aa 144-191) can be substituted by the *P. aeruginosa* homologous region. Indeed the resulting chimera both complements LptC depleted *E. coli* and assembles the Lpt complex.

The non-complementing *Ec*/*Pa*-LptC chimeras are proficient in interaction with *Ec*-LptA, although with different efficiency, but unable to bind the IM $LptB_2FG$ sub-complex as *Pa*-LptC. These data suggest that regions 1 and 2 (LptC aa 1-143) are more relevant for LptC function than region 3 and that their substitution with heterologous divergent sequences is not tolerated, as it impairs Lpt complex assembly, while the C-terminal region 3 may have an ancillary role.

On the other hand, despite the low amino acidic conservation of the region, it is possible that the fold of the C-terminal region may be more conserved between the two species than those of the other regions, thus resulting interchangeable.

Furthermore we show that LptB can suppress the transport defects caused by a defective LptC.

In an *E. coli* strain depleted of LptC and partially depleted of LptAB, the overexpression of LptB suppress the lack of complementation of *Ec*-LptC C-terminal deletion mutant ($LptC^{\Delta 139-191}$). LptB is able to stabilize the LptC C-terminal deletion mutant and LptA, suggesting that it could stabilize the defective complexes.

Overexpression of LptB also allows heterologous complementation by *Pa*-LptC. Thus we demonstrated that *Pa*-LptC is the genetic and functional homolog of *E. coli* LptA despite a low amino acidic identity.

Our data suggest a functional interaction between LptC and LptB. Studies are in progress to asses if LptB suppress LptC defects through a structural role or an ATPase dependent mechanism.

3. Draft manuscript:

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).

In this work we show for the first time 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.

The evidence that *E. coli* Lpt machinery can tolerate LptC defects led us to test more stringently whether LptC was absolutely essential for LPS transport and for cell viability. By plasmid shuffling, we isolated *E. coli* Δ*lptCA* mutants complemented by plasmids harboring *lptA* or *lptAB* genes and missing *lptC*. By genomic sequencing of three mutants we found they all shared a single amino acid substitutions at arginine 212 (either R212C or R212S). 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 harboring *lptC* under the control of the regulable *araBp* promoter and the chromosomal $lptF^{+}$ allele showed that$lptF^{R212G}$ and $lptF^{R212S}$ 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, all the three $lptF^{Sup}$ mutants were compatible with LptC both in the heterozygous and in the haploid state.

Our data show that mutations 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.

Studies are in progress to understand the structure and functioning of the six-protein Lpt system in order to understand how the $LptF^{Sup}$ mutants overcome the lack of LptC and to shed light on mechanism of action of the LptBFGC subassembly.

CONCLUSIONS AND FUTURE PROSPECTS

Comparison between *E. coli* and *P. aeruginosa* Lpt systems

We made a first step towards the comparison between the *E. coli* and *P. aeruginosa* Lpt systems and the identification of inter-species requirements for the LPS transport.

E. coli Lpt proteins and the predicted *P. aeruginosa* homologs share a low sequence identity and thus *P. aeruginosa* Lpt proteins can be considered as multiple mutants of the respective *E. coli* homologs.

Here we showed that *Pa*-LptCHB are able to replace *Ec*-LptCAB in an Lpt hybrid machine, demonstrating that they are *E. coli* the functional homologs and that at least these three components of the Lpt system share common features between the two species. Thus *Pa*-LptC and *Pa*-LptH can interact with both the other proteins of the Lpt machine and the LPS of *E. coli*; however, the hybrid strains increased sensitivity to toxic compounds indicates that the transport is somehow impaired.

Interestingly, it appears that the outer membrane of *E. coli* with *Pa*-LptC in the hybrid Lpt transporter is less defective than that with *Pa*-LptH, despite the *Pa*- end *Ec*-LptC proteins share with each other the lowest sequence identity among the different Lpt proteins.

Bioinformatic analysis predicts a conserved Lpt fold between *E. coli* and *P. aeruginosa* proteins. Our data suggests that LptA and LptC overall structure, rather than their amino acid sequence, may play a major role in Lpt assembly and LPS recognition and transport. This is supported by the crystal structure of *Pa*-LptH, which is superimposable to that of *Ec*-LptA. However, the defective LptAG138R and LptHG153R indicate that at least this amino acidic substitution in an *E. coli-P. aeruginosa* conserved residue is not tolerated. Thus it could be useful to understand whether the conserved residues in *E. coli-P. aeruginosa* LptCA(H) are relevant for the functioning of the Lpt machine.

Analysis of *E. coli lptC* mutants

In this work we focused on the LPS transport protein LptC, showing that the *E. coli* Lpt machine can function despite defects or even the absence of this subunit.

We showed that in *E. coli* the overexpression of LptB can suppress defects in the functioning of the machine caused by the lack of the LptC C-terminal region ($LptC^{\Delta 139\text{-}191}$ mutant) or by the substitution of *Ec*-LptC with the *P. aeruginosa* homologous protein *Pa*-LptC. Furthermore we tested for the possibility to isolate Δ*lptC* or Δ*lptA* strains on the basis of two considerations: 1) LptC and LptA share a common structure and thus it is possible that in selective conditions the first one can substitute for the second one and *vice versa*; 2) the Lpt system tolerates highly divergent LptC and LptA xenogeneic proteins as well as large deletions of LptC.

By plasmid shuffling we were able to isolate Δ*lptC* viable mutants and through genome sequencing we identify single amino acid substitutions at a unique position (R212) in the periplasmic domain of the IM component LptF ($LptF^{Sup}$). Complementation assays showed that $LptF^{Sup}$ are able to suppress the lethal effect associated to the lack of LptC. On the other hand we did not obtain Δ*lptA* mutants. Thus either LptC cannot replace LptA or the selection efficiency of suppressor mutations able to overcome the lack of LptA was too low to be detected in the plasmid shuffling experiment performed. Our data suggest that LptC may serve as a chaperon of the Lpt machine assembly and/or activity rather than be an essential functional component and implicate the LptF periplasmic region in the LPS delivering from the IM and in the formation of the Lpt bridge between IM and OM complexes.

The isolation of several independent Δ*lptC* suppressor mutations in LptF R212 residue highlights the relevance of the putative LptF periplasmic domain in the transport mechanisms. $LptF^{Sup}$ could replace LptC in the mediation of LptFG-LptA interactions for the assembly of the Lpt complex. Photo crosslinking experiments in vivo could allow to elucidate differences in LptF and $LptF^{Sup}$ interactions with LptA and affinity purification experiments will be performed to test if $LptF^{Sup}$ can promote the assembly of a stable Lpt bridge interacting with LptA in absence of LptC. On the other hand, it could be possible that in Δ*lptC* mutants the LPS transport could occur in absence of a periplasmic bridge and through transient interactions between $LptBF^{Sup}G$ and LptA.

The crystal structure of both LptF and $LptF^{Sup}$ will be useful to asses if the suppressor mutations have an impact on the LptF fold, modifying the LptF periplasmic domain interactions with the other components of the system and/or with LPS.

A recent work (Gu *et al.*, 2015) showed that LptD arginine residues (R145 and R191) are involved in LPS transport and hypothesized that these positively charged residues are involved in

the interaction with the negatively charged LPS. Photo crosslinking experiments in vivo could help to asses which LptF residues, R212 *in primis*, and regions are involved in interaction with LPS. If the LptF R212 residue interacts with LPS it could be possible that in $LptF^{Sup}$ LPS is more able to flow from LptFG to LptA. Thus in the wt machine LptC could act as a switch to promote and regulate LPS unblocking form LptFG.

In order to investigate on the role of the LptF R212 residue and on its surrounding region, mutagenesis experiments will be performed to asses which kinds of R212 mutations are functional or lethal in presence or absence of LptC. The identification of amino acids groups relating with *E. coli* viability or lethality in these conditions could help to clarify which physical properties are required for the R212 region interaction with LPS. Alternatively if LptF R212 region does not interact with LPS, the effect on viability, in presence or absence of LptC, of the mutagenesis of residue predicted to face on R212 region could allow to identify R212 partner residue and to give us structural information about LptF periplasmic region.

Another key point is to understand how LptF and LptG interact with each other and with LPS since we have to keep in consideration the possibility that LptF R212 mutations can determine a change in the not characterized interactions between LptF and LptG.

It might be of interest to evaluate if *E. coli* adaptation to LptC defects occurs also in presence of an LPS with O-antigen. In any case the efforts for the development of new antibacterial drugs should take in account the possibility to select for LptF mutants in nature. For these reason drugs which target steps requiring LptC could be at risk for the selection of LptF mutants, leading to the widespread of resistant bacterial species. Conversely the development of antibiotics targeting LptC independent steps appears of higher interest.

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PART II: PUBLISHED WORK AND MANUSCRIPTS

1. Published Paper.

Bollati, M., Villa, R., Gourlay, L. J., Benedet, M., Dehò, G., Polissi, A., Barbiroli, A., Martorana, A. M., Sperandeo, P., Bolognesi, M. and Nardini, M.

Crystal structure of LptH, the periplasmic component of the lipopolysaccharide transport machinery from *Pseudomonas aeruginosa*.

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2. Draft manuscript:

Martorana, A. M., Benedet, M., Maccagni, E. A., Sperandeo, P., Villa, R., Dehò, G. and Polissi, A.

Overexpression of ATP binding protein LptB suppresses defective forms of the lipopolysaccharide transport protein LptC in *Escherichia coli*.

3. Draft manuscript:

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.

1. Published Paper.

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2. Draft manuscript:

Overexpression of ATP binding protein LptB suppresses defective forms of the lipopolysaccharide transport protein LptC in *Escherichia coli*

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ABSTRACT

The outer membrane (OM) of Gram-negative bacteria contains a layer of lipopolysaccharide (LPS) that provides a barrier against the entry of many toxic compounds. LPS assembly at the cell surface requires a trans-envelope LPS transport (Lpt) complex made of seven essential proteins located in the inner membrane (IM) (LptBCFG), periplasm (LptA) and OM (LptDE). LptBFG constitute an unusual ABC transporter that is thought to extract LPS from the IM and to provide the energy for its export across the periplasm to the cell surface. LptC is a small IM bitopic protein that associates to LptBFG although it does not modulates its ATPase activity and whose molecular role in LPS transport is not completely understood. Here we show that LptB overexpression suppresses the lethality of deletions in the C-terminal region of LptC and allows the functioning of a hybrid Lpt machinery that carries the highly divergent LptC orthologue from *Pseudomonas aeruginosa*. Overall our data point to a functional interaction between LptC and LptB and suggest that they play a key role in the assembly of the Lpt machinery.

INTRODUCTION

Gram-negative bacteria are typically surrounded by two lipid bilayers, the inner and outer membrane (IM and OM), showing distinct composition, structural and functional properties (Silhavy *et al.*, 2010). The two membranes delimit an aqueous compartment, the periplasm, containing a thin peptidoglycan layer. The IM is a symmetric bilayer made of phospholipids, whereas the OM is an asymmetric membrane composed of glycerophospholipids in the inner leaflet, and lipopolysaccharide (LPS) in the outer leaflet (Silhavy *et al.*, 2010). LPS is a complex glycolipid composed of three elements: lipid A, the hydrophobic moiety, which anchors LPS to the OM, the oligosaccharide core, and the O-antigen. LPS molecules are assembled at the outer leaflet of the OM to form a permeability barrier that prevents entry of many hydrophobic toxic compounds, including antibiotics (Nikaido, 2003). In *Escherichia coli,* LPS export to the cell surface is performed by seven essential Lpt proteins (LptABCDEFG) located in every cellular compartment: cytoplasm, IM, periplasm and OM (Wu *et al.*, 2006, Sperandeo *et al.*, 2007, Sperandeo *et al.*, 2008, Ruiz *et al.*, 2008, Chng *et al.*, 2010) that assemble to form a transenvelope complex (Chng *et al.*, 2010). At the IM, $LptB_2FG$ constitute an ABC transporter that provides energy for LPS transport across the periplasm and its insertion at the outer leaflet of the OM (Narita & Tokuda, 2009, Okuda *et al.*, 2012, Sherman *et al.*, 2014). LptC is a bitopic IM protein that associates to the IM $LptB_2FG$ transporter (Narita & Tokuda, 2009). The β-barrel LptD protein and the LptE lipoprotein constitute the OM translocon, characterized by a unique plug and barrel architecture, responsible for the final stages of LPS assembly at the cell surface (Freinkman *et al.*, 2011, Qiao *et al.*, 2014, Wang *et al.*, 2014). LptA is the key periplasmic component of the machinery that connects IM and OM complexes (Sperandeo *et al.*, 2008, Sperandeo *et al.*, 2011, Freinkman *et al.*, 2012).

The crystal structures of five components of the Lpt machinery, LptC from *E. coli* (Tran *et al.*, 2010), LptA from *E. coli* and *Pseudomonas aeruginosa* (Suits *et al.*, 2008, Bollati *et al.*, 2015), the LptD-LptE complex from *Shigella flexneri* and *Salmonella enterica* sv. Typhimurium (Qiao *et al.*, 2014, Wang *et al.*, 2014), and the cytoplasmic ABC protein LptB (Sherman *et al.*, 2014, Wang *et al.*, 2014) have been solved. Interestingly, LptA, the periplasmic region of LptC, and the periplasmic N-terminal region of LptD share a very similar β-jellyroll fold, made of juxtaposition of a variable number of antiparallel β-strands, despite a lack of sequence similarity. The β-jellyroll fold is a key element in driving the assembly of the Lpt machinery. In fact using these homologous domains the C-terminus of LptC interacts with the N-terminus of LptA and the C-terminus of LptA interacts with the N-terminal periplasmic domain of LptD to form the protein bridge that connects IM and OM (Freinkman *et al.*, 2012, Villa *et al.*, 2013, Qiao *et al.*, 2014, Dong *et al.*, 2014).

The assembly of the transenvelope bridge appears to be finely regulated to prevent LPS mistargeting. Proper interaction of LptC with $LptB_2FG$ is necessary for LptA recruitment (Villa *et al.*, 2013). Interaction of the N-terminal domain of LptD with LptA requires the correct maturation of the LptDE complex that in turns depends on non-consecutive disulphide bonds formation in LptD (Freinkman *et al.*, 2012); LptE and the chaperone/protease BepA have been implicated in this process (Narita *et al.*, 2013). Based on in vivo photo-cross-linking experiments LPS molecules appear to cross the periplasm inside the β-jellyroll of LptC and LptA (Okuda *et al.*, 2012). ATP hydrolysis by LptB, the cytoplasmic ATPase of the $LptB_2FG$ transporter, is required to extract LPS from the IM and transfer it to LptA via LptC, on the contrary energy is not required for the assembly of the transenvelope bridge (Okuda *et al.*, 2012). The molecular role of LptC in LPS transport is still elusive: it does not seem to be a functional component of the IM ABC transporter as its association to the $LptB_2FG$ complex does not affect its ATPase activity (Narita & Tokuda, 2009), on the other end it seems relevant for proper IM complex assembly (Villa *et al.*, 2013). Mutational analyses suggest that the interaction of LptC to the IM $LptB_2FG$ complex is mediated by the N-terminal region of the jellyroll while its transmembrane domain appears to be dispensable, as a periplasmic soluble version of LptC or a LptC chimera carrying a heterologous transmembrane segment are both functional and proficient in Lpt complex assembly (Villa *et al.*, 2013).

To gain further insights in the LptC role in the LPS export pathway we dissected *E. coli* LptC (*Ec*-LptC) functional domains by analyzing the phenotype of *E. coli lptC* mutants and the properties of LptC from *P. aeruginosa* (*Pa*-LptC) and *Ec/Pa*-LptC chimeras in *E. coli*. We show that *lptB* ectopic expression suppresses lethality of C-terminal deletion of LptC and allows the functioning of a hybrid Lpt machinery that carries the highly divergent LptC orthologue from *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in LD medium (Ghisotti *et al.*, 1992). When required, 0.2% (w/v) L-arabinose (as an inducer of the *araBp* promoter), 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), 25 μg/ml chloramphenicol, 100 μg/ml ampicillin were added. Solid media were prepared as described above with 1% (w/v) agar.

Plasmids construction.

Plasmids used in this study are listed in Table 1. The oligonucleotides used are listed in Table 2. Plasmid pBAD24-LptA-HA expresses LptA fused to hemagglutinin (HA) epitope (YPYDVPDYA) at the C-terminal end. The DNA encoding LptA-HA was amplified by MG1655 genomic DNA using AP329 and AP330 hybrid primers. The PCR fragment was digested with *Eco*RI and *Hind*III and inserted into pBAD24 cut with the same enzymes. The *Eco*RI-*Hind*III insert was verified by sequencing. Plasmid pGS111 and pGS200 express Pa-LptC and Pa-LptC-H with C-terminal 6xHis tag, respectively. Pa-LptC and Pa-LptC-H encoding fragments were obtained by PCR using AP175/AP176 and AP175/AP237 pair of primers with *P. aeruginosa* (PAO1) genomic DNA as template. The PCR fragments were digested with *Eco*RI and *Hind*III, inserted in pGS100 cut with the same enzymes and verified by DNA sequencing. The swapping of motifs between Ec-LptC and Pa-LptC was achieved using *E. coli/P. aeruginosa* hybrid primers by three-step PCR (Li & Shapiro, 1993). For CPP chimera the first round PCR products was generated using AP91/AP192 with pGS103 as template (to amplify C1) and AP92/AP191 with pGS111 as template (to amplify P2-P3). Similarly, PCC chimera was constructed using AP91/AP195 primers with pGS111 as template (to amplify P1) and AP92/AP196 primers with pGS103 as template (to amplify C2-C3). The amplified (C1+P2-P3 and P1+C2-C3) products were used in a second round of PCR using AP91/AP92 primers to generate the CPP and PCC inserts, respectively. CPC and PCP were constructed using AP91/AP194 and AP91/AP198 primers using CPP and PCC as template, respectively, to obtain C1-P2 and P1-C2 products. Amplified products at C-terminal regions were generated using AP92/AP193 with pGS103 as template (to obtain C3) and AP92/AP197 with pGS111 as template (to obtain P3). The amplified (C1-P2+C3 and P1-C2+P3) products were used in a second round of PCR with AP91 and AP92 to generate the CPC and PCP inserts, respectively. For CCP chimera the C1-C2 product was obtained using AP91/AP198 primers with pGS103 as template and the P3 product using AP92/AP197 primers with pGS111 as template. For PPC chimera P1-P2 product was obtained using AP91/AP194 primers with pGS111 as template and the C3 fragment was obtained using AP92/AP193 primers with pGS103 as template. The amplified (C1-C2 + P3 and P1-P2 + P3) products were used as template in a second round of PCR with primers AP91 and AP92 to obtain the CCP and PPC inserts, respectively. All PCR products were cloned into pGS100 using *Eco*RI and *Hind*III and verified by DNA

sequence. C-terminal His tags were introduced into LptC chimera constructs using PCR. DNA was amplified using AP91 and the required His primers (AP63 for Ec-LptC C-terminal or AP237 for Pa-LptC C-terminal) with the suitable plasmid as template. Products were digested with *Eco*RI and *Xba*I and cloned in pGS100 cut with the same enzymes. All inserts were verified by DNA sequence. The CC- and CC-H truncated Ec-*lptC* alleles were generated using primers AP91 and either AP363 or AP361 with pGS103 as template. AP91/AP363 and AP91/AP361 PCR products were cloned into pGS100 using *Eco*RI and *Hind*III and verified by DNA sequence. pGS401 (Bollati *et al.*, 2015) derivatives were obtained by cloning in its two cloning sites the genes indicated in Table 1 obtained either by PCR with primers listed in Table 2, or as DNA restriction fragments from suitable plasmid constructs.

Identification of motifs in LptC.

The MM algorithm (Bailey & Elkan, 1994) from the MEME suite (Bailey *et al.*, 2009) (http://meme.nbcr.net/meme/tools/meme) was applied to analyze protein sequences for the occurrence of amino acid motifs. A motif is a sequence pattern that occurs repeatedly in a group of evolutionary related protein. MM is capable of discovering different motifs with different number occurrences in a single data set. The algorithm estimates how many times each motif occurs in each sequence in the data set, ordering the motifs found with a statistical significance. Default settings have been applied to perform an *ab initio* motif discovery procedure to search for no more than three motifs on amino acid sequences of LptC homologues from a set of 15 γ-Proteobacteria (*Escherichia coli*, *Salmonella enterica, Pectobacterium atrosepticum, Yersinia enterocolitica, Photorhabdus luminescens, Haemophilus influenzae, Actinobacillus pleuropneumoniae, Vibrio cholerae* O1*, Shewanella* sp.*, Pasteurella multocida, P. aeruginosa, Aeromonas salmonicida*, *Legionella pneumophila*). In an order based on statistical significance Motif 1 spans from amino acid 94 to 144 (*E. coli* coordinates), motif 2 from amino acid 37 to 82, and motif 3 from amino acid 152 to 182 (Fig. 1A).

Complementation assay.

FL905 (*araBp-lptC*) carrying plasmids expressing different *Ec-lptC* or *Pa-lptC* alleles or chimeras, alone or in combination with *Ec-lptA*, *lptB* or *lptAB,* (see Table 1) were grown at 30 °C in LD containing chloramphenicol (25 µg/ml) and arabinose (0.2%) for 18 hours. Serial tenfold dilutions in microtiter wells of the cultures were then replica plated on agar plates containing 25 µg/ml chloramphenicol, with or without 0.2% arabinose and incubated overnight at 37 °C.

Determination of LptA, LptC LptB and LptE levels.

LptA, LptC, LptB and LptE levels were assessed in FL905 co-expressing wild type or truncated LptC proteins with LptA or LptB or LptAB by Western blot analysis using polyclonal antibody raised in mouse or rabbit against peptides (LptA and LptB), or whole proteins (LptE, LptC). Bacterial cultures grown up to OD_{600} = 0.2 at 37 °C in LD supplemented with 0.2% arabinose and 25 µg/ml of chloramphenicol were harvested by centrifugation, washed in LD, and diluted 500 fold in fresh media with or without 0.2% arabinose and with 25 µg/ml of chloramphenicol. Growth was monitored by measuring OD at 600 mn. Samples for protein analysis were collected 240 min after the shift to nonpermissive conditions, centrifuged (16,000*g*, 5 min), and pellets were resuspended in a volume (in ml) of SDS sample buffer equal to 1/24 of the total OD of the sample. Samples were boiled for 10 min, and equal volumes (20 µl) were analyzed 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 LptC and LptA (GenScript Corporation) were used as primary antibody at a dilution of 1:500, whereas polyclonal sera against LptE (kindly provided by D. Khane) LptB (kindly provided by N. Ruiz) were used at a dilution of 1:5000 and 1:1000, respectively. As secondary antibodies, goat antirabbit immunoglobulin (LI-COR) were used at a dilution of 1:15000.

Co-purification of LptA-LptC complex.

AM604 carrying pBAD24-LptA-HA and plasmids expressing the different his-tagged LptC constructs were grown at 30°C in LD containing ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml) up to OD_{600} 0,4. Expression of LptA-HA harboured by pBAD24 and the His-tagged version of *Ec*-LptC, *Pa*-LptC or LptC chimeras harboured by pGS100 derivatives were induced for 60 minutes by adding 0.2% arabinose and 0.1 mM IPTG. Cells were harvested by centrifugation (5,000xg, 10 minutes), washed in 20 mM potassium phosphate buffer and stored at -20 °C. Cell pellets were resuspended in 4 ml of 50 mM potassium phosphate buffer (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 1% ZW3-14 (*n*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate) containing lysozyme (50 µg/ml), DNase I (50 µg/ml), RNase I (50 µg/ml), and lysed by shaking for 20 min at room temperature. To remove cell debris after lysis, the mixture was then centrifuged at 10,000*g* for 10 min. To the cleared lysate (whole-cell extract) 20 mM imidazole (pH 8.0) was added and the final mixture was loaded onto a 0.4-ml Ni-NTA column. The column was first washed with 8 ml of 50 mM potassium phosphate buffer (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.1% Triton X-100, and 0.1% SDS and then eluted with 4 ml of 50 mM potassium phosphate buffer (pH 8.0), 300 mM NaCl, and 200 mM imidazole. The eluate was concentrated in an ultrafiltration device (Amicon Ultra; Millipore; MWCO 10K) by centrifugation at 5,000 g. The concentrated sample was used for SDS-PAGE and Western blot analysis using anti HA (1:10,000) (Pierce™, Thermo scientific) and anti-His monoclonal antibodies (1:3,000) (Sigma Aldrich).

Affinity purifications.

AMM04 strain harbouring plasmids expressing His tagged *Ec*-LptC, *Pa*-LptC and LptC chimeras were affinity purified as previously described (Chng *et al.*, 2010) with few modifications. The 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 x g for 1 h. The 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 x g for 1 h and insoluble material was discarded. The supernatant was incubated with 0.5 ml TALON resin suspension for 30 min at 4 °C and the mixture was then loaded onto a column. The column was washed with 10 ml of 50 mM Tris-HCl, pH 7.4, 10% glycerol, 0.05% dodecylmaltoside, 5 mM imidazole and eluted with 5 ml of 50 mM Tris-HCl, pH 7.4, 10% glycerol, 0.05% dodecylmaltoside, 200 mM imidazole. The eluate was concentrated using an ultrafiltration device (Amicon Ultra, Millipore) by centrifugation at 5,000 x g to a final volume of 50 µl. Samples were mixed with 1.1 2x loading buffer, boiled and separated on SDS-PAGE gel, electroblotted and immunodetected using the anti-Flag monoclonal antibody (1:2,000) (Sigma Aldrich) to detect LptD-SPA, anti-His monoclonal antibodies (1:3,000) (Sigma Aldrich) to detect wild type *Ec*- and *Pa*- LptC-H and LptC-H chimeras, and anti-LptA (1:2,000), LptE (1:5,000), LptF (1:2,000) as previously described (Villa *et al.*, 2013).

RESULTS

Complementation of E. coli lptC conditional mutant by lptC from P. aeruginosa.

Blast (Basic Local Alignment Search Tool: http://blast.ncbi.nlm.nih.gov/Blast.cgi) search using the amino acid sequence of LptC from *Escherichia coli* K12 (Uniprot Accession number P0ADV9) as a query against the translated *P. aeruginosa* strain PAO1 genomic sequence (Accession number NC_002516.2; http://www.pseudomonas.com/), identified a conserved hypothetical protein of 190 amino acids with ~20% sequence identity (Fig. 1A), encoded by PA4459. This hypothetical gene is located upstream of *lptH* (PA4460), recently identified as the *Pseudomonas* homologue of *lptA* (Bollati *et al.*, 2015). Despite the low sequence similarity, the Pfam algorithm (Punta *et al.*, 2015) predicts for the PA4459-encoded protein the same β-jellyroll domain found in *Ec*-LptC (Tran *et al.*, 2010) (Fig 1B). Overall these data strongly suggest that PA4459 is homologous to *Ec-lptC* and is herewith designated *Pa-lptC*.

We then tested whether *Pa-lptC* could complement *E. coli lptC* conditional expression mutant under non permissive conditions. FL905, an *E. coli araBp-lptC* arabinose dependent mutant (Sperandeo *et al.*, 2008), was thus transformed with plasmid pGS200, which expresses *Pa-lptC* from *ptac* promoter. As shown in Fig. 2, expression of *Pa-lptC*, unlike the *Ec-lptC* control, did not complement FL905 for growth in the absence of arabinose.

Construction and expression of E. coli-P. aeruginosa LptC chimeras

Although *Ec*-LptC and *Pa*-LptC share low sequence identity (20%), the predicted three-dimensional structure of Pa-LptC appears to be very similar to that of Ec-LptC (Fig. 1B). Sequence alignments among LptC homologues in a subset of representative γ-Proteobacteria using the MEME suite software (Bailey *et al.*, 2009) were performed to identify conserved sequence motifs in the LptC family of proteins. LptC multiple alignments recognized three sequence motifs in all representative LptC homologues taken into consideration with the exception of *P. aeruginosa* and *Legionella pneumophila* (Fig. 1C). According to MEME analysis, motif 3 (which spans from amino acid 152 to 182) is the least conserved across species and is not recognized in *Legionella*-LptC, whereas motif 1 (from amino acid 94 to 144), which is the most conserved in other bacteria was not recognized both in *Legionella* and *Pseudomonas* LptC. As shown in fig 1A, motif 2 is located in the N-terminal region of LptC (region 1), the most conserved motif 1 lies in the central portion of the protein (region 2) whereas motif 3 is located in the C-terminal end of LptC (region 3). To assess whether the lack of complementation by Pa-LptC could be imputed to any of the three regions, we swapped these three different regions of *Ec*- and *Pa*-LptC in all possible combinations and cloned the entire set of chimeric constructs in the pGS100 plasmid vector under control of the *ptac* promoter.

The *lptC* chimeras were then tested for the ability to complement the *araBp-lptC* conditional expression mutant under non-permissive conditions (no arabinose). Only the chimeric protein that carries *E.*

coli regions 1 and 2 and *P. aeruginosa* region 3 (CCP chimera) was able to support the growth of *araBp-lptC* conditional mutant (Fig. 2).

Association of LptC chimeras with LptA and Lpt complex assembly.

LptC is a component of the Lpt machinery and interacts with both the IM protein complex LptBFG and the periplasmic component LptA (Narita & Tokuda, 2009, Sperandeo *et al.*, 2011, Villa *et al.*, 2013). We thus assessed the ability of *Pa*- and chimeric LptC proteins to interact with *Ec*-LptA by affinity purification followed by immunoblotting. C-terminal His tagged *Ec*-LptC, *Pa*-LptC, and LptC chimeras expressed from the pGS100 vector in AM604 strain were used as baits in co-purification experiments. AM604 cells harboring the different LptC constructs were transformed with plasmid pBAD24-LptA-HA carrying *Ec*-LptA fused to hemagglutinin (HA) tag at the C-terminal end. As shown in Fig. 3, His tagged Pa-LptC and all His tagged LptC chimeras were able to co-purify *Ec*-LptA although with different efficiencies. It is worth mentioning that this experimental set up can reveal protein-protein interactions occurring post cell disruption, as suggested by the fact that the His-tagged LptC bait also co-purifies non-processed LptA (Fig. 3, top and middle panels). On the other end, a truncated LptC protein missing the entire region 3 (CCΔ carrying amino acids 1-143, see Fig. 1) was not able to co-purify *Ec*-LptA (Fig. 3). The lower LptA level in AM604 expressing a LptC truncated protein (Fig. 3, lower panel) is in line with previous data showing that the inability of the LptA protein to properly interact with IM through LptC results in its destabilization and therefore in a lower level in the cell (Sperandeo *et al.*, 2011). Overall it appears that interaction with *Ec*-LptA can be established by LptC region 3 from either *E. coli* or *P. aeruginosa,* thus suggesting that the structure and not a specific LptC amino acid sequence is the driving element in the LptC-LptA interaction.

The assembly of the Lpt machinery is a highly coordinated multi step process, as interaction of LptC with the IM complex LptBFG is required to recruit LptA and the OM complex LptDE (Villa *et al.*, 2013). Thus, the ability of LptC chimeras to bind LptA *in vitro* does not necessarily imply that these proteins can assemble the Lpt complex *in vivo*. We therefore investigated whether the LptC chimeras are able to properly interact with the IM Lpt components and to assemble the Lpt export machinery by affinity purification experiments on the purified membrane fraction as previously described (Chng *et al.*, 2010, Villa *et al.*, 2013). Total membranes collected from AMM04 cells ectopically expressing C-terminally His-tagged *Ec*-*Pa*-, or chimeric LptC proteins from pGS100 vector were solubilized and the Lpt complexes with the different LptC baits were affinity purified. Samples were then processed by immunoblotting with a panel of specific antibodies. First we assessed the ability of *Pa*-LptC and the chimeric proteins to recruit the IM complex LptBFG by revealing the presence of LptF with anti LptF antibodies. As judged by the co-purification profile shown in Fig. 4A it appears that only the complementing CCP chimera binds to the inner membrane component LptF at a level comparable to that of *Ec*-LptC. On the contrary *Pa*-LptC as well as the non-complementing chimeras fail to assemble the IM complex, as they are all unable to interact with LptF (Fig. 4A). Indeed, based on the co-purification profile of LptA, LptD, and LptE shown in Fig 4B, CCP

chimera but not *Pa*-LptC is able to recruit LptA and the LptDE complex and thus to assemble the whole Lpt machine.

Overall these data suggest that *E. coli* regions 1 and 2 are required for the interaction with the LptBFG complex and further support the hypothesis that binding with *Ec*-LptA does not require LptC region 3 from the same species, thus suggesting that either LptC region 3 is not relevant for LptC functionality or that its three dimensional structure, rather than a specific amino acid sequence, is required to fulfil LptC function(s).

LptC region 3 may be dispensable.

Previous data (Sperandeo *et al.*, 2011, Freinkman *et al.*, 2012, Villa *et al.*, 2013) implicated the C-terminal region of *Ec*-LptC (which encompasses region 3; Fig 1A) in binding with LptA. In particular, the *Ec*-LptC residue A172 is thought to interact with LptA (Freinkman *et al.*, 2012), whereas the *Ec-lptC*^{G153R} mutation not only is lethal but also impairs LptC-LptA co-purification (Sperandeo *et al.*, 2011, Villa *et al.*, 2013). However, the results presented here suggest that structural features rather than specific amino acids residues are relevant to establish LptA-LptC interaction.

To gain further insights into this problem, we revisited a previously described conditional expression mutant ST-190 (Serina *et al.*, 2004) in which a mini transposon with the arabinose inducible *araBp* promoter oriented towards *lptAB* is inserted after nucleotide 403 of *lptC*. Thus this mutant expresses, from the major promoter of the *yrbG* operon, an *lptC* substitution allele (henceforth named *lptC190N*) which encodes the 1-138 N-terminal peptide of LptC fused to a 19 amino acids long C-terminus encoded by the left end of the inserted mini transposon (Fig. 1). Therefore the LptC truncation in ST-190 largely overlaps the region 3 of the protein. The mini transposon insertion is not thought to disrupt the downstream minor promoters *lptAp1* and *lptAp2* (σ^E and σ^D dependent, respectively) located within the *lptC* coding region (Martorana *et al.*, 2011). The expression of *lptAB* could thus be driven by these minor promoters and/or, in permissive condition, by *araBp*. The viability of ST-190 in the presence of arabinose (Serina *et al.*, 2004) indicates that under such conditions LptC190N is functional, whereas the non-viability of the mutant in non-permissive condition suggests that *lptAB* expression is either lacking or insufficient. Here we show (Fig. 5) that ST-190 viability in non-permissive condition can be rescued not only by ectopic expression of *lptC*, but also by a plasmid harboring *lptAB*. This indicates that in this mutant strain i) *lptAB* are indeed expressed from the minor promoters and their expression level is sufficient for viability in the presence of a wild type LptC; ii) the truncated *lptC190N* allele is defective when *lptAB* is expressed only from the ancillary promoters *lptAp1p2*; iii) a higher expression level of *lptAB* (from *araBp* in permissive condition and/or from the complementing plasmid in the absence of arabinose) suppresses the *lptC190N* defect. It thus appears that the C-terminal 53 residues of LptC are dispensable at least in conditions of *lptAB* expression level higher than that granted by *lptAp1p2*. Henceforth, the term "overexpression" will be used solely to indicate this fact.

To rule out that the observed phenotype depends on the ST-190 genetic background or the 19 amino acid C-terminal substitution of the *lptC190* allele, we tested whether plasmids expressing the *lptC190N* or the truncated *lptC*$^{\Delta 139\text{-}191}$ allele (either alone or with *lptA*, *lptB*, or both), could complement FL905 under non-permissive conditions. As shown in Table 3, both *lptC190N* and *lptC*$^{\Delta 139\text{-}191}$ mutants supported the growth of the conditional FL905 mutant under non-permissive conditions (- arabinose) when co-expressed with *lptAB*, whereas neither *lptC* mutant could complement when expressed alone or with *lptA* only. Interestingly, co-expression of either *lptC190N* or *lptC*$^{\Delta 139\text{-}191}$ with *lptB* only was also able to rescue FL905 growth under non-permissive conditions (Table 3). It thus appears that expression over the basal *lptAp1p2* level of *lptB*, but not of *lptA*, is required to suppress the *lptC*$^{\Delta 139\text{-}191}$ defect. On the contrary the defective *lptC*^{G153R} mutation was not suppressed by *lptB* overexpression (Table 3; see discussion).

Suppression of LptC defects by increased expression of LptB is intriguing as it reveals functional interactions between the cytoplasmic LptB and the bitopic LptC protein. LptB has been implicated not only in providing energy through ATP hydrolysis, but also in the assembly of the IM complex (Sherman *et al.*, 2014). It could be hypothesized that the assembly of LptC lacking the C-terminal domain is partially defective, thus leading to LptC instability, and that a level of *lptB* higher than that provided by the ancillary promoters *lptAp1* and *lptAp2* is necessary for LptC assembly and stability. We therefore examined the levels of C-terminally truncated LptC with LptB expressed from a plasmid. FL905 cells co-expressing wild type *lptC* or *lptC*$^{\Delta 139\text{-}191}$ with *lptA*, *lptB* or *lptAB* were grown up to the exponential phase and shifted into a medium lacking arabinose to deplete the chromosomally encoded wild type LptC while allowing expression of the alleles from the complementing plasmids. Samples were then taken from cultures grown in the presence or in the absence of arabinose 240 min after the shift to non-permissive conditions and analysed by Western blotting using anti-LptA, anti-LptC and anti-LptB antibodies. The level of the LptE protein was used as a sample loading control. As shown in Fig. 6 the expression level of LptC$^{\Delta 139\text{-}191}$ in depleted FL905 cells is detectable when *lptAB* or *lptB* alone are also ectopically expressed, whereas it is not when the *lptAB* operon is only expressed from the minor chromosomal promoters. Interestingly, in the *lptC*$^{\Delta 139\text{-}191}$ mutant background, the LptA level also increases when *lptA* is co-expressed with *lptB* (compare lanes LptC$^{\Delta 139\text{-}191}$ LptAB and LptC$^{\Delta 139\text{-}191}$ LptA in Fig. 6B), in keeping with our previous observations (Sperandeo *et al.*, 2011) that the increased amount of wild type or truncated LptC helps in stabilizing LptA.

Overexpression of *lptB* rescues LptC depleted cells complemented by *Pa-lptC*.

We showed (Fig. 2) that ectopic expression of the highly divergent *Pa-lptC* plasmid did not complement FL905 for growth in the absence of arabinose. We thus tested whether co-expression of *lptB* could rescue growth of FL905 upon overexpression of *lptB*. FL905 cells were thus transformed with plasmids co-expressing *Pa-lptC* with *lptA*, *lptB*, or *lptAB* from *E. coli*. As shown in Table 3, ectopic

expression of *lptB* is necessary and sufficient to rescues growth of FL905 complemented by *Pa-lptC.* This confirms that *P. aeruginosa* ORF PA4459 encodes the functional homologue of *E. coli* LptC.

DISCUSSION

The *E. coli* lipopolysaccharide transporter Lpt exhibits the overall organization of a trans-envelope ABC transporter (Davidson *et al.*, 2008). The IM complex ($LptB_2FG$), which provides energy to the system through the LptB ATPase, is connected to the OM LptDE complex *via* the periplasmic LptA and the bitopic LptC proteins (Freinkman *et al.*, 2012, Villa *et al.*, 2013). LptC role in LPS transport remains unclear. Its transmembrane N-terminal domain is dispensable whereas the soluble periplasmic domain interacts with both LptA and the IM $LptB_2FG$ complex via the C- and N-terminal regions, respectively (Villa *et al.*, 2013). LptC also binds LPS and transfers it to LptA in an energy dependent way (Tran *et al.*, 2010, Okuda *et al.*, 2012). Here we have further dissected the LptC functional domains by analyzing the properties of *Pa*-LptC and *Ec/Pa*-LptC chimeras and of LptC C-terminal deletions in *E. coli*.

Ec-LptC C-terminal region can be replaced by the equivalent *P. aeruginosa* region.

Among the seven components of the Lpt machinery, *Pa*-LptC is the most divergent from the *E. coli* homologue (~20% amino acid sequence identity). Ectopic expression of *Pa*-LptC, unlike *Ec*-LptC expression, does not complement LptC-depleted *E. coli* mutants. We exploited these non-complementing conditions to dissect *Pa*-LptC and implicate specific regions in this phenotype. Three conserved motifs were identified in LptC based on homologues sequences alignments and the most conserved motif 1 (encompassing amino acids 94-143, Fig. 1) was not recognized in Pa-LptC. The Pa-LptC protein appears to be proficient in interaction with Ec-LptA however it is unable to assemble the Lpt multi-protein machinery, as it does not interact with the $LptB_2FG$ IM sub-complex (Fig. 4). It should be reminded here that the assembly of the Lpt machinery is regulated and LptC interaction with $LptB_2FG$ is necessary for LptA recruitment (Villa *et al.*, 2013).

Analysis of *Ec/Pa*-LptC chimeras, in which the three LptC regions from the two organisms were swapped in all possible combinations, indicate that the C-terminal region of the *E. coli* protein tolerates the substitution with the *P. aeruginosa* homologous region. Indeed the CCP-LptC chimera complements LptC-depleted *E. coli* (Fig. 2) and assembles the LptC complex (Fig. 4B). The non-complementing *Ec/Pa*-LptC chimeras are proficient in interaction with Ec-LptA, although with different efficiency but, like *Pa*-LptC, are unable to bind the IM $LptB_2FG$ sub-complex.

These data suggest that regions 1 and 2 are more relevant for LptC function and that their substitution with heterologous divergent sequences is not tolerated as it impairs Lpt complex assembly, while the C-terminal region 3 may have an ancillary role (see below).

LptB overexpression allows functioning of mutant or hybrid Lpt machines.

The C-terminal region of LptC has been previously implicated in binding to LptA. Indeed, the unviable LptC^{G153R} mutant fails to interact with LptA and to assemble the transenvelope complex, although it associates with the IM LptBFG sub-complex (Sperandeo *et al.*, 2011, Villa *et al.*, 2013). Moreover, by in-vivo photo-cross-linking, the C-terminal LptC residues A172 and Y182 have been implicated in binding with LptA (Freinkman *et al.*, 2012). These three residues, therefore, define the LptA-LptC interaction interface. In contrast, here we show i) that the substitution of the C-terminal region with the highly divergent *P. aeruginosa* homologous region is viable (see above) and ii) several *lptC*-truncated alleles are viable under conditions of non-limiting expression of *lptB*. In fact, FL905 cells grown under non-permissive conditions and complemented by a truncated *lptC* allele missing the C-terminal 53 amino acids (Δ139-191) are viable when *lptB* is ectopically expressed from a plasmid. It should be noted that in the LptC depletion strain FL905, in the absence of arabinose, expression of *lptAB* is driven by the ancillary promoters *lptAp1* and *lptAp2* (σ^E- and σ^D-dependent, respectively) located within the 3'-end of *lptC* (Martorana *et al.*, 2011). These promoters ensure sufficient *lptAB* expression when FL905 cells grown in the absence of arabinose are complemented by wild type *lptC*. However in ST-190 (carrying the *lptC190N* allele) growth under non-permissive conditions requires both *lptA* and *lptB* overexpression. Likely in this mutant strain the mini-transposon insertion affects expression from the chromosomal downstream *lptAp1* and *lptAp2* promoters. In fact, FL905 complemented by both *lptC190N* and *lptC*$^{\Delta 139-191}$ is viable when *lptB* alone is ectopically expressed suggesting that in this strain *lptA* expression from *lptAp1p2* promoters is sufficient for growth/viability. LptC$^{\Delta 139-191}$ stabilization appears crucial for FL905 viability under non-permissive conditions: indeed LptC$^{\Delta 139-191}$ steady state level increases when *lptB* but not *lptA* is overexpressed (Fig. 6). Interestingly, wild type LptC and the truncated LptC$^{\Delta 139-191}$ proteins seem also to positively affect LptA stability (Fig. 6), suggesting a co-dependence between the three proteins. The tight genetic association of *lptCAB*, which belong to the same *yrbG* operon, and the presence of two ancillary promoters for the nested *lptAC* operon within *lptC* may be instrumental in maintaining balanced expression levels of the three encoded proteins.

Deletions of the C-terminal regions of LptC appear to be tolerated also by species other than *E. coli* as witnessed by the isolation of a *Salmonella enterica* mutant carrying a *lptC* allele with a frame shift mutation resulting in a premature stop codon after amino acid 134 that is viable and also bile resistant (Hernandez *et al.*, 2012). Moreover, *P. aeruginosa* transposon insertion mutants in the C-terminal region of LptC have also been isolated (http://ausubellab.mgh.harvard.edu; see Fig. 1). Overall these data further support the notion that the C-terminal region of LptC might be dispensable.

We recently showed that *Pa*-LptH (the LptA homologue of *P. aeruginosa*) can replace LptA in that a hybrid Lpt machinery can ensure *E. coli* growth/viability (Bollati *et al.*, 2015). Interestingly, *Pa*-LptC was able to complement LptC-depleted *E. coli* cells when co-expressed with the *Ec-lptB* (Table 3), indicating that

Pa-LptC is the functional homologue of *Ec*-LptC. It thus appears that overexpression of *lptB* also allows functioning of a hybrid Lpt machinery carrying a heterologous highly divergent LptC protein.

The finding that ectopic expression of *lptB* allows functioning of mutated or hybrid Lpt machineries raises questions on the functional interactions between LptC and LptB. LptB is the IM-associated ATPase that interacts with the IM complex and provides the energy required for LPS transport (Narita & Tokuda, 2009, Okuda *et al.*, 2012). It is possible that the requirement for a higher LptB expression level reflects an increased energetic demand by a mutated Lpt machine that may be less efficient due to the presence of heterologous or truncated and unstable LptC component(s). Alternatively, assembly of LptB to the IM Lpt complex could be somewhat impaired by the truncated/hybrid component, thus implying a structural role of LptB and LptC in the biogenesis of the Lpt complex. This finding is in line with recent data showing that the ATPase activity and the assembly function of LptB can be separated genetically and that ATP hydrolysis is not required for the formation of the Lpt IM complex (Sherman et al., 2014).

The molecular function of LptC in the LPS export pathway still remains elusive. We previously showed that LptC transmembrane region is dispensable for function in that it can be either deleted or substituted by a heterologous sequence and that the connection to $LptB_2FG$ and LptA is established by LptC periplasmic domain (Villa *et al.*, 2013). LptC binds LPS *in vivo* at the N-terminal region (via residue T47) and transfers it to LptA, both steps require ATP hydrolysis by the $LptB_2FG$ complex (Okuda *et al.*, 2012). However, association of LptC to the $LptB_2FG$ complex does not affect the ATPase activity of the ABC transporter (Narita & Tokuda, 2009) and no energy seems to be required to assemble the IM Lpt sub-complex (Okuda *et al.*, 2012). Here we show that LPS binding and transfer to LptA may be accomplished either by a C-terminally truncated LptC protein or by highly divergent heterologous sequences under specific conditions (*lptB* over expression). What are the structural bases for the functioning of such Lpt machineries is not clear. Despite extensive studies on the Lpt machinery we still lack structural information on LptC-LptA interaction as well as on the determinants for LptC-LPS interaction both in the protein and in the ligand. Such structural information will be crucial to understand at the molecular level the LPS export process.

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TABLES

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
***E. coli* strains**		
AM604	MC4100 Ara^+	(Wu *et al.*, 2006)
AMM04	AM604 *lptD-SPA::Kan*	(Villa *et al.*, 2013)
DH5α	Δ*(argF-lac169) 80dlacZ58(M15) glnV44(AS)λ⁻ rfbD1 gyrA96 recA1 endA1 spoT1 thi-1 hsdR17*	(Hanahan, 1983)
DH10B		(Grant *et al.*, 1990)
FL905	AM604 Φ(*kan araC araBplptC*)	(Sperandeo *et al.*, 2008)
MC4100	F^- *araD139 Δ(argF-lac)U169 rpsL150* (Str^R) *relA1 flbB5301 deoC1 ptsF25 rbsR*	(Casadaban, 1976)
MG1655		(Bachmann, 1987)
ST-190	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB*(Rif^R) *argE*(am) *recA1* λ^R *lptC*::TnSS2	(Serina *et al.*, 2004)
***P. aeruginosa* strain**		
PAO1	reference strain	(Stover *et al.*, 2000)
Plasmids		
pBAD24	pBRpBR322/ColE1	(Guzman *et al.*, 1995)
pBAD24-LptA-HA	pBAD24 *araB*p*lptAHA, bla*	This study
pGS100	pGZ119EH derivative	(Sperandeo *et al.*, 2006)
pGS103	pGS100 *ptac-lptC*	(Sperandeo *et al.*, 2006)
pGS108	pGS100 *ptac-lptC*-H, *cat*	(Sperandeo *et al.*, 2006)
pGS111	pGS100 *ptac-Pa-lptC, cat*	This study
pGS200	pGS100 *ptac- Pa-lptC*-H*, cat*	This study
pGS201	pGS100 *ptac-lptC-CPP*, *cat*	This study
pGS201H	pGS100 *ptac-lptC-CPP*-H, *cat*	This study
pGS202	pGS100 *ptac-lptC-PCC*, *cat*	This study
pGS202H	pGS100 *ptac-lptC-PCC*-H, *cat*	This study
pGS203	pGS100 *ptac-lptC-CPC*, *cat*	This study
pGS203H	pGS100 *ptac-lptC-CPC*-H, *cat*	This study
pGS204	pGS100 *ptac-lptC-PCP*, *cat*	This study
pGS204H	pGS100 *ptac-lptC-PCP*-H, *cat*	This study
pGS206	pGS100 *ptac-lptC-CCP*, *cat*	This study
pGS206H	pGS100 *ptac-lptC-CCP*-H, *cat*	This study
pGS207	pGS100 *ptac-lptC-PPC*, *cat*	This study
pGS208	pGS100 *ptac-lptC-CC*Δ, *cat*	This study
pGS208H	pGS100 *ptac-lptC-CC*Δ*-H*, *cat*	This study
pGS207H	pGS100 *ptac-lptC-PPC*-H, *cat*	This study
pGS103G153R	pGS100 *ptac-lptC*^{G153R}, *cat*	(Airoldi *et al.*, 2011)
pGS401	pGS100 *ptac*-SD1-*Eco*RI-*Xba*I-SD2-*Sal*I-*Hin*dIII, *cat*	(Bollati *et al.*, 2015)
pGS402	pGS401 *ptac-lptC*, *cat*	(Bollati *et al.*, 2015)
pGS403	pGS401 *ptac-Pa-lptC*, *cat*	This study
pGS404	pGS401 *ptac-lptC-lptA*, *cat*	Bollati *et al*., 2015
pGS407	pGS401 *ptac-Pa-lptC-lptA*, *cat*	This study
pGS408	pGS401 *ptac-lptC190N*, *cat*	This study

pGS411	pGS401 *ptac-lptC190N-lptA*, *cat*	This study
pGS412	pGS401 *ptac-lptC*^{G153R}*-lptA*, *cat*	This study
pGS413	pGS401 *ptac-lptC*^{G153R}*-lptAB*, *cat*	This study
pGS414	pGS401 *ptac-lptC190N-lptAB*, *cat*	This study
pGS415	pGS401 *ptac-lptC-lptAB*, *cat*	This study
pGS416	pGS401 *ptac*-void-*lptAB*, *cat*	Bollati *et al*., 2015
pGS417	pGS401 *ptac-lptC*$^{\Delta 139\text{-}191}$, *cat*	This study
pGS418	pGS401 *ptac-lptC*$^{\Delta 139\text{-}191}$*-lptA*, *cat*	This study
pGS419	pGS401 *ptac-lptC*$^{\Delta 139\text{-}191}$*-lptAB*, *cat*	This study
pGS428	pGS401 *ptac*-void-*lptB*, *cat*	Bollati *et al*., 2015
pGS429	pGS401 *ptac-lptC-lptB*, *cat*	This study
pGS430	pGS401 *ptac-lptC190N-lptB*, *cat*	This study
pGS431	pGS401 *ptac-lptC*$^{\Delta 139\text{-}191}$*-lptB*, *cat*	This study
pGS434	pGS401 *ptac-lptC*^{G153R}*-lptB*, *cat*	This study
pGS439	pGS401 *ptac-lptC*^{G153R}, *cat*	This study
pGS440	pGS401 *ptac*-void-*lptA*, *cat*	This study
pGS448	pGS401 *ptac-Pa-lptC-lptAB*, *cat*	This study
pGS456	pGS401 *ptac-Pa-lptC-lptB*, *cat*	This study

Table 2. Oligonucleotides

Name	Sequence (5'-3')[a]	Use and/or description
AP63	**gtgatcacatctagat***CAGTGGTGGTGGTGGTGGTG* AGGCTGAGTTTGTTTGTTTTG	Construction of His tagged chimeras version; XbaI
AP91	CATCGGCTCGTATAATGTG	Construction of pGS200 derivatives
AP92	CTGCGTTCTGATTTAATCTG	Construction of pGS200 derivatives
AP175	**cgagagaggaattcacc**atgccgaagacactacgc	Pa-LptC and Pa-LptC-H construction for pGS111, pGS200, pGS403 and pGS456; EcoRI
AP176	**aagcttctaga**ttaacgaacctcatgctgacc	Pa-LptC construction for pGS111, pGS403 and pGS456; XbaI
AP191	AAGGATAAAATCCCGACAtggcacatccagagcgcc	CPP construction
AP192	ggcgctctggatgtgccaTGTCGGGATTTTATCCTT	CPP construction
AP193	CCCGACAAGAATTATGCAacctctgaagacctcgtc	CPC and PPC construction
AP194	gacgaggtcttcagaggtTGCATAATTCTTGTCGGG	CPC, and PPC construction
AP195	CGTGGCAACGTGCAACCCtggtccgtaaaagcagat	PCC construction
AP196	atctgcttttacggaccaGGGTTGCACGTTGCCACG	PCC construction
AP197	CTGGTGACGCAGGATGTT cagaccgagcaagccg tt	PCP and CCP construction
AP198	aacggcttgctcggtctg AACATCCTGCGTCACCAG	PCP and CCP construction
AP237	**aagcttctaga**TCA*GTGGTGGTGGTGGTGGTG* acgaacctcatgctgacct	Pa-LptC-H construction for pGS200; XbaI
AP329	**gaattcacc**ATGAAATTCAAAACAAACAAACT C	LptA-HA construction for pBAD24LptHA; EcoRI
AP330	**aagctt**TTA*AGCGTAATCTGGAACATCGTATGG GTA*ATTACCCTTCTTCTGTGCCGGGG	LptA-HA construction for pBAD24LptHA; HindIII
AP361	**cccaagcttctaga**TCA*GTGGTGGTGGTGGTGGT GCTG*AACATCCTGCGTCACC	CC-H construction for pGS208H; HindIII
AP363	**cccaagcttctaga**TCAAACATCCTGCGTCACC	CC- construction for pGS208; HindIII
FG2935	**catattcgtctcgtcgacacc**ATGAAATTCAAAACA AACAAACTCAGCC	*lptA* and *lptAB* construction for pGS407, pGS413, pGS414 and pGS415; Esp3I-SalI
FG2936	**ggccttcgtctcaagctt**TTAATTACCCTTCTTCTGT GCCG	*lptA* construction for pGS407; Esp3I-HindIII
FG2978	**catattcgtctcgaattcacc**ATGAGTAAAGCCAGAC GTTGGG	*lptC190N* and *lptC*$^{\Delta 139\text{-}191}$ construction for pGS408, pGS411, pGS417, pGS418 and pGS419; Esp3I-EcoRI
FG2979	**caggttcgtctctctaga**TCAATCAATCACCGGATC CCC	*lptC190N* construction for pGS408 and pGS411; Esp3I-XbaI
FG3058	**ccttcgtctcaagctt**TCAGAGTCTGAAGTCTTCCC C	*lptAB* construction for pGS413, pGS414 and pGS415; Esp3I-HindIII

FG3088	**caggttcgtctctctaga**TTAGAGATTGATCTGCGC GTTATC	*lptC*$^{\Delta 139\text{-}191}$ construction for pGS417, pGS418, pGS419, pGS431; Esp3I-XbaI

[a] Upper case*, E. coli* genomic sequence; lower case, *P. aeruginosa* genomic sequence; bold lower case, oligonucleotide tail; lower bold underlined case, restriction site; italic upper case, HA or 6xHis coding sequence.

Table 3. Complementation assays in LptC-depleted cells with additional ectopic expression of *lptAB*

Suppressing genes	Complementing *lptC* allele					
	none	*Ec-lptC*	*Ec-lptC190N*	*Ec-lptC*$^{\Delta 139\text{-}191}$	*Ec-lptC*^{G153R}	*Pa-lptC*
none	-	+	-	-	-	-
lptA	-	+	-	-	-	-
lptB	-	+	+	+	-	+
lptAB	-	+	+	+	-	±

[a] FL905 strains harboring pGS401 derivatives expressing different combinations of *lptC* complementing alleles and *lptA lptB* suppressor genes were grown in LD with 0.2% arabinose and 25 μg/ml chloramphenicol. Serial dilutions in microtiter plates were replica plated on the same medium or without arabinose. Efficiency of plating (e.o.p.) in the nonpermissive condition (no arabinose) is expressed as follows: +, about 1;±, between 10^{-2} and 10^{-3}; -, $<10^{-3}$.

FIGURE LEGENDS

Figure 1. Comparison of *E. coli* and *P. aeruginosa* LptC amino acid sequences and structures. Panel A. Amino acid sequence alignment of LptC from *E. coli* (*Ec*) and *P. aeruginosa* (*Pa*). Aminoacid identity (*) and similarity (: and .) are labelled. Leader peptide (LP) and transmembrane regions are indicated. Regions 1, 2, and 3 swapped in chimeras construction are delimited by arrowheads at the end of double underlining. Sequences corresponding to the MEME motifs are overlined and colour coded: blu: motif 1; red, motif 2; green, motif 3. Relevant LptC mutations are indicated above and below the *E. coli* and *P. aeruginosa* sequences, respectively. Vertical arrows indicate the mutated aminoacid (for point mutations), the first deleted aminoacid (for deletions) and the first aminoacid at the right transposon insertions. ST-190: transposon insertion in ST-190 (Serina et al., 2004); 23553 and 39714 indicate two transposon insertions in *Pa-lptC* (http://ausubellab.mgh.harvard.edu). o and <u>o</u> indicate amino-acids photo-cross-linked to LPS and LptA, respectively (Freinkman *et al.*, 2012, Okuda *et al.*, 2012). Tilde marks (~) indicate aminoacids in β strands, progressively numbered (Tran *et al.*, 2010).

Panel B. Structure of *Pa*-LptC predicted by I-TASSER is shown next to the *Ec*-LptC structure 3MY2 (Tran *et al.*, 2010). Panel C. Motifs predicted in *E. coli* LptC by MM algorithm. TM (Transmembrane Region, from residue 7 to 25), Motif 2 (from residue 37 to 82), Motif 1 (from residue 94 to 144), Motif 3 (from 152 to 182) are indicated. Consensus polypeptides from each motif are represented as a sequence logo graphic, the relative size of the letters indicates their frequency in the sequences.

Figure 2. Complementation test of LptC depletion mutants with different *E. coli* and *P. aeruginosa* wild type or chimeric LptC constructs. Cultures of FL905 (*araBp-lptC*) strains freshly transformed with pGS100 derivatives expressing *Ec*-LptC (CCC), *Pa*-LptC (PPP), LptC chimeras, or a truncated *Ec*-LptC protein missing region 3 (CCΔ) and grown in LD-chloramphenicol-arabinose were serially diluted 1:10 in microtiter wells and replica plated in agar plates with (+ ara) or without (- ara) arabinose or with glucose (+ glu) to fully repress the *araBp* promoter. The log of the serial dilutions is indicated on the right of the panel.

Figure 3. Interaction of Pa-LptC and LptC chimeras to Ec-LptA in vitro. Affinity chromatography was performed as described in Materials and Methods with AM604 cells expressing Ec-LptA-HA and the different pGS100 derivatives harboring Ec-LptC-H (CCC), Pa-LptC-H (PPP), His tagged LptC chimeras, or a His-tagged truncated LptC protein missing region 3 (CCΔ). LptA, LptC or LptC chimeras were detected in Ni-NTA column enriched fractions by Western blot analysis with anti-His (upper panel) and anti-HA (middle panel) antibodies. Lower panel: the level of LptA-HA was detected in crude extracts by anti-HA antibodies. The asterisks indicate the non-processed form of LptA.

Figure 4. Assembly of the Lpt complex by Pa-LptC and LptC chimeras. Dodecyl β-D-maltoside (DDM)-solubilized total membranes from AMM04 strains harboring pGS100 derivatives expressing the His-tagged proteins of interest were affinity purified using a Talon metal affinity resin, as described in Materials and Methods. Proteins were then fractionated by SDS-PAGE and immunoblotted with suitable antibodies to detect the corresponding proteins in the fraction. Panel A. Assembly of the Lpt IM complex. Samples were prepared from AMM04 harboring pGS100 derivatives expressing Ec-LptC-H (CCC), Pa-LptC-H (PPP), His tagged LptC chimeras Immunoblotting was performed with anti-LptF and anti-His antibodies to detect LptF and the different His-tagged LptC forms, respectively, which display different electrophoretic mobility on SDS-PAGE. Panel B. Assembly of the trans-envelope Lpt complex. Samples were prepared from AMM04 harboring Ec-LptC-H (CCC), Pa-LptC-H (PPP), the His tagged LptC CCP chimera (CCP), or pGS100 expressing the His tag (-) as a negative control. Immunoblotting was performed with antibodies anti-LptA, anti-LptE, anti-His (to detect the different LptC forms) and anti-Flag (to detect LptD-SPA).

Figure 5. Growth of ST-190 conditional expression mutant with different levels of LptA and/or LptB expression. Cultures of ST-190 transformed with pGS100 derivatives expressing the genes indicated on top of each lane and grown in LD-chloramphenicol-arabinose were serially diluted 1:10 in microtiter wells and replica plated in agar plates supplemented (+ ara) or not (- ara) with arabinose. The log of the serial dilutions is indicated on the right of the panel.

Figure 6. Expression levels of LptA, LptB, and LptC or $LptC^{\Delta 139\text{-}191}$ proteins in FL905 upon depletion of the chromosomally encoded LptC. FL905 cells transformed with pGS100 derivatives harboring wild type LptC (C) or $LptC^{\Delta 139\text{-}191}$ (C^{Δ}) as indicated on the left of Panel A and Panel B, respectively, co-expressed with LptA (A), LptB (B), or LptAB (AB) as indicated on top of the lanes, were grown in the presence (+ ara) or in the absence (- ara) in LD with arabinose and chloramphenicol as described in Materials and Methods. Samples collected 4 h after shift to the non-permissive condition were analyzed by Western blotting using anti-LptA, anti-LptB, anti-LptC, and anti-LptE antibodies. Equal amount of cells (0.6 OD_{600} units) were loaded onto each lane.

The last lane of both images in Panel B (C^{Δ}+IPTG) were loaded with 10 µl of cell extract of FL905 harboring $LptC^{\Delta 139\text{-}191}$ arabinose-depleted for 3 h and further incubated 1 h with 0.1 mM IPTG to induce expression of the truncated LptC protein. LptE level was used as a sample loading control.

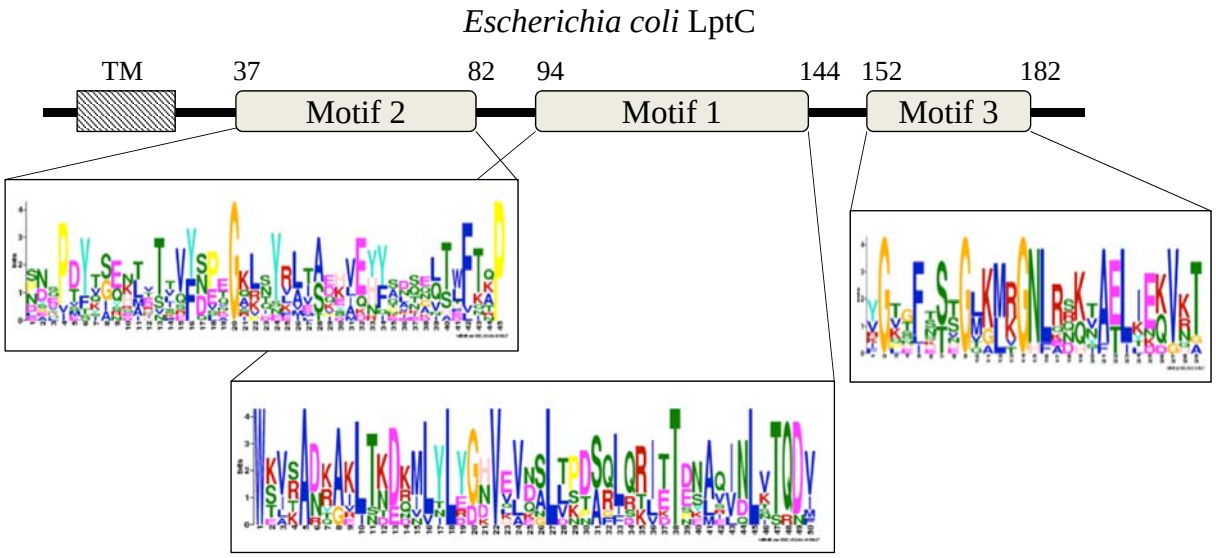

Escherichia coli LptC
TM
37
82
94
144
152
182
Motif 2
Motif 1
Motif 3

Fig. 1C

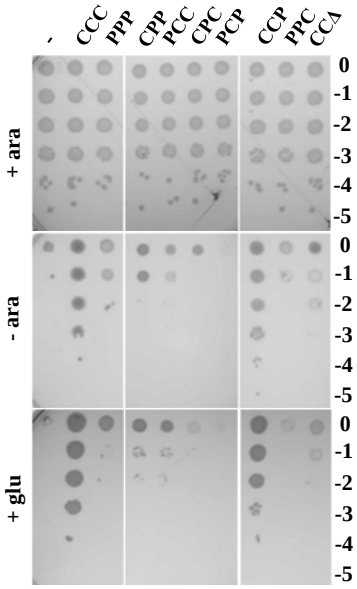

-
CCC
PPP
CPP
PCC
CPC
PCP
CCP
PPC
CCA
+ ara
- ara
+ glu
0
-1
-2
-3
-4
-5

Fig. 2

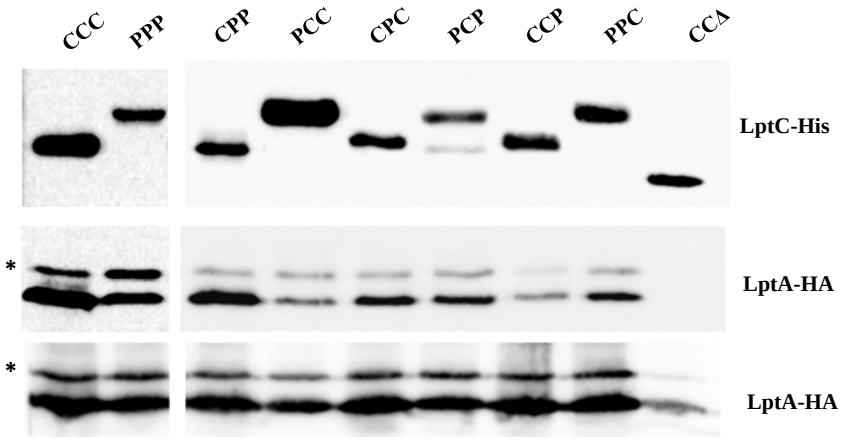
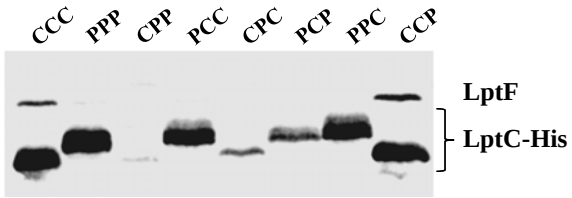

CCC
PPP
CPP
PCC
CPC
PCP
CCP
PPC
CCA
LptC-His
*
LptA-HA
*
LptA-HA

Fig. 3

A


CCC
PPP
CPP
PCC
CPC
PCP
PPC
CCP
LptF
LptC-His

B

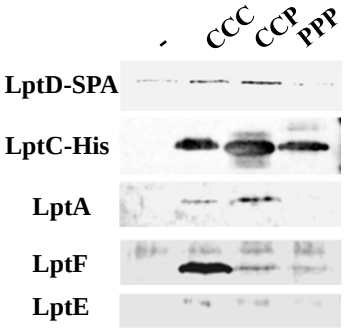

CCC
CCP
PPP
LptD-SPA
LptC-His
LptA
LptF
LptE

Fig. 4

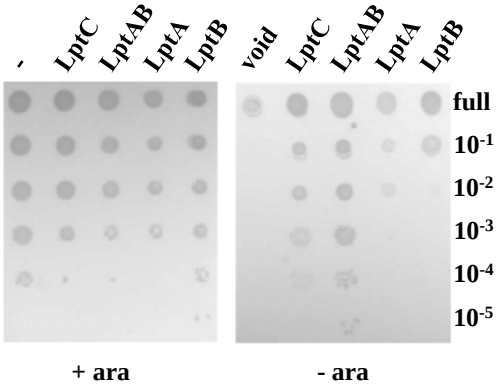

-
LptC
LptAB
LptA
LptB
void
LptC
LptAB
LptA
LptB
full
10-1
10-2
10-3
10-4
10-5
+ ara
- ara

Fig. 5

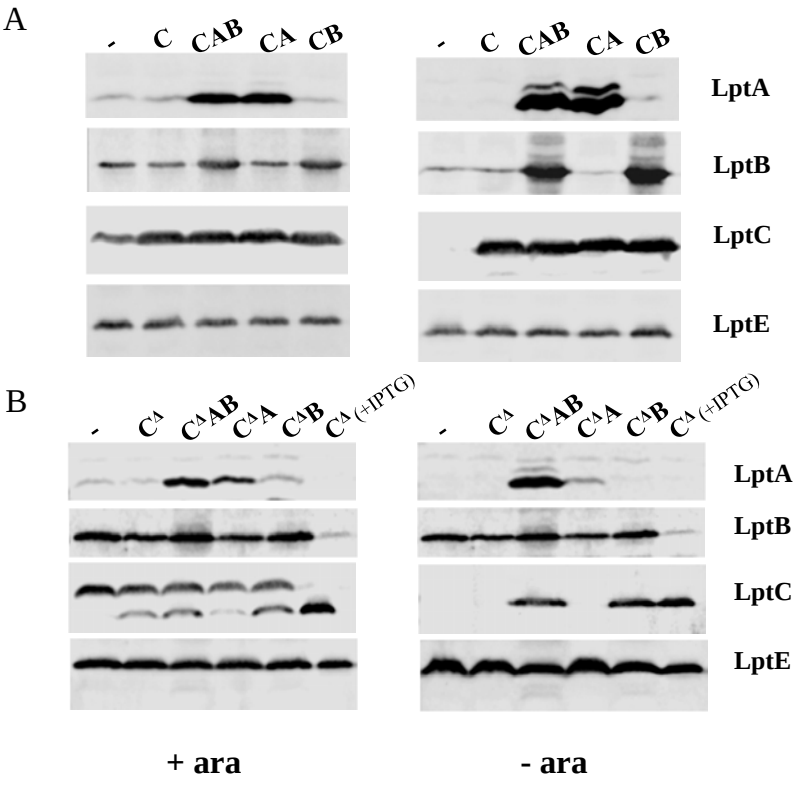

A
-
C
CAB
CA
CB
LptA
LptB
LptC
LptE
B
CΔ
CΔAB
CΔA
CΔB
CΔ (+IPTG)
+ ara
- ara

Fig. 6

3. 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

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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 (R212S in strains KG-292, and R212C 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-lptA*)*1*			Sperandeo et al., 2008
FL905/pGS442	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptFG cat*	by transformation	this work
FL905/pGS443	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptF*^{R212C}*G cat*	by transformation	this work
FL905/pGS444	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptF*^{R212S}*G cat*	by transformation	this work
FL905/pGS445	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptFG_lptAB cat*	by transformation	this work
FL905/pGS446	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptF*^{R212C}*G_lptAB cat*	by transformation	this work
FL905/pGS447	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptF*^{R212S}*G_lptAB cat*	by transformation	this work

FL905/pGS450	FL905	Φ(*kan araC araBp-lptA*)*1*	*ptac-lptF*^{R212G}*G cat*	by transformation	this work
FL905/pGS451	FL905	Φ(*kan araC araBp-lptA*)*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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 *EcoR*I-*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.

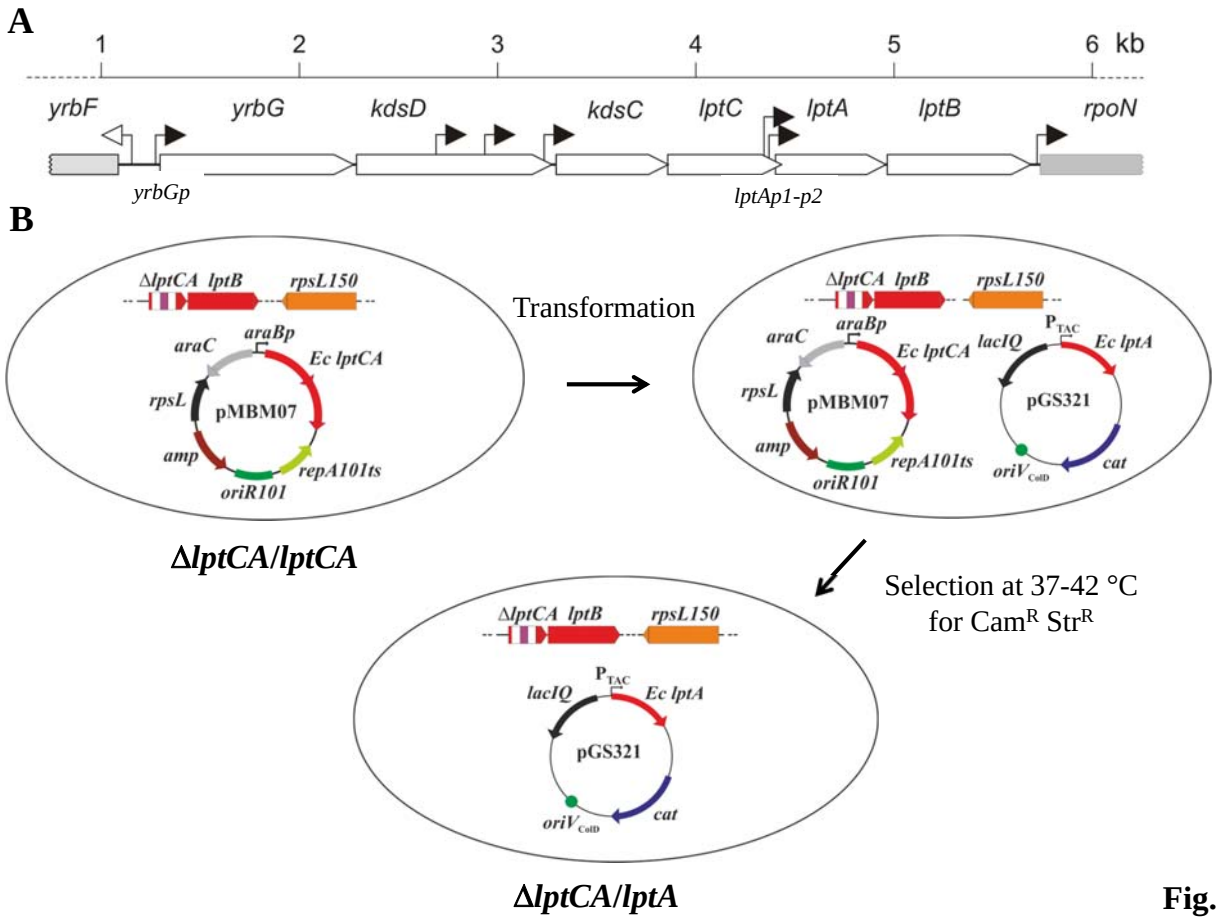
Fig. 2. Screening for the presence of *lptA* and *lptC* in plasmid shuffled clones by PCR and Southern blotting analysis. A. Electrophoretic analysis of amplicons obtained with *lptC*- and *lptA*-specific primers, as indicated on the bottom of the panel, from plasmid shuffled and control strains. On 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.


A
1
2
3
4
5
6 kb
yrbF
yrbG
kdsD
kdsC
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lptA
lptB
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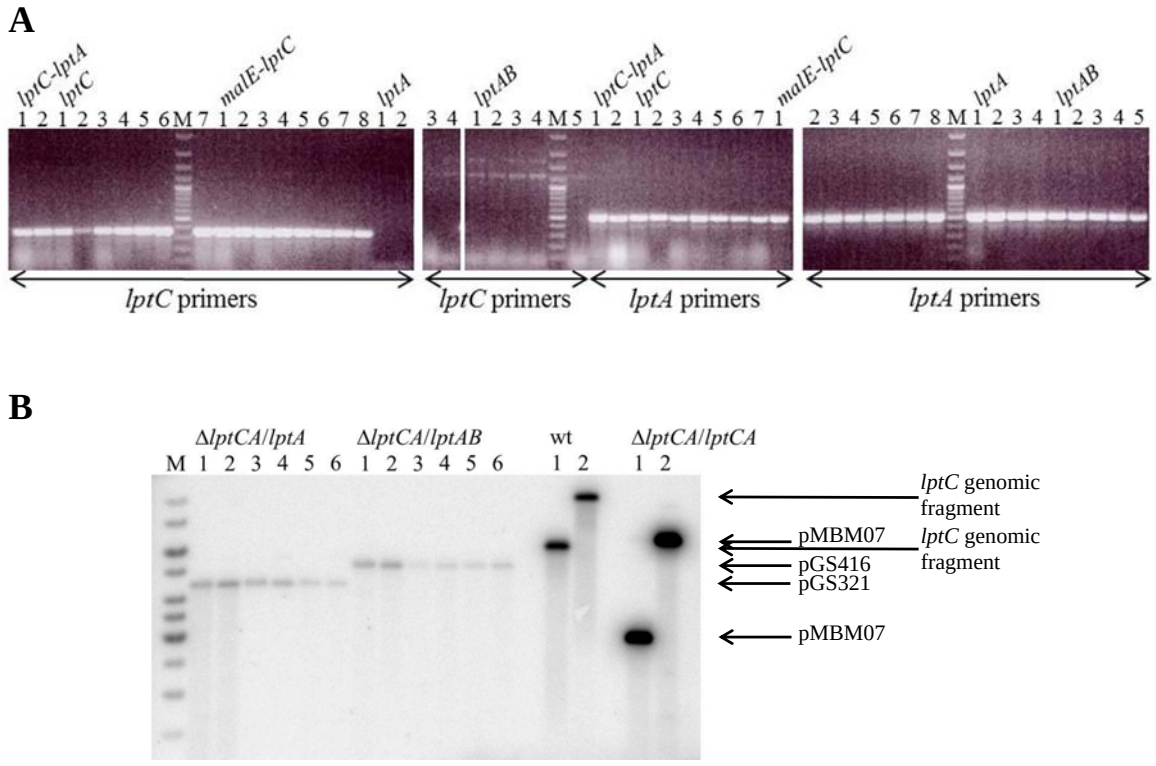
yrbGp
lptAp1-p2
B
ΔlptCA lptB
rpsL150
araC
araBp
Ec lptCA
rpsL
pMBM07
amp
repA101ts
oriR101
Transformation
lacIQ
P TAC
Ec lptA
pGS321
oriV ColD
cat
ΔlptCA/lptCA
Selection at 37-42 °C
for Cam R Str R
ΔlptCA/lptA

Fig. 1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lptC
malE-lptC
lptA
lptAB
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3 4 1 2 3 4 M 5 1 2 1 2 3 4 5 6 7 1
2 3 4 5 6 7 8 M 1 2 3 4 1 2 3 4 5
lptC primers
lptC primers
lptA primers
lptA primers
B
ΔlptCA/lptA
ΔlptCA/lptAB
wt
ΔlptCA/lptCA
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lptC genomic fragment
pMBM07
lptC genomic fragment
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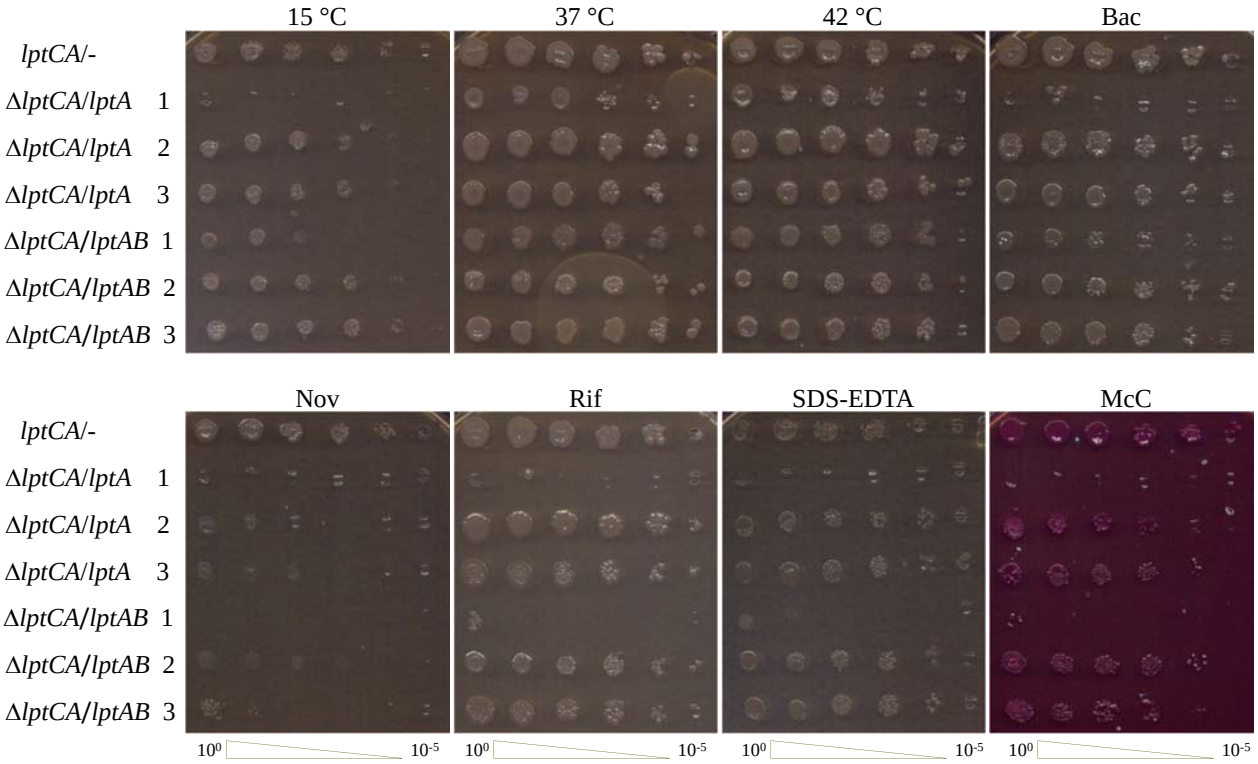
pGS321
pMBM07

Fig. 2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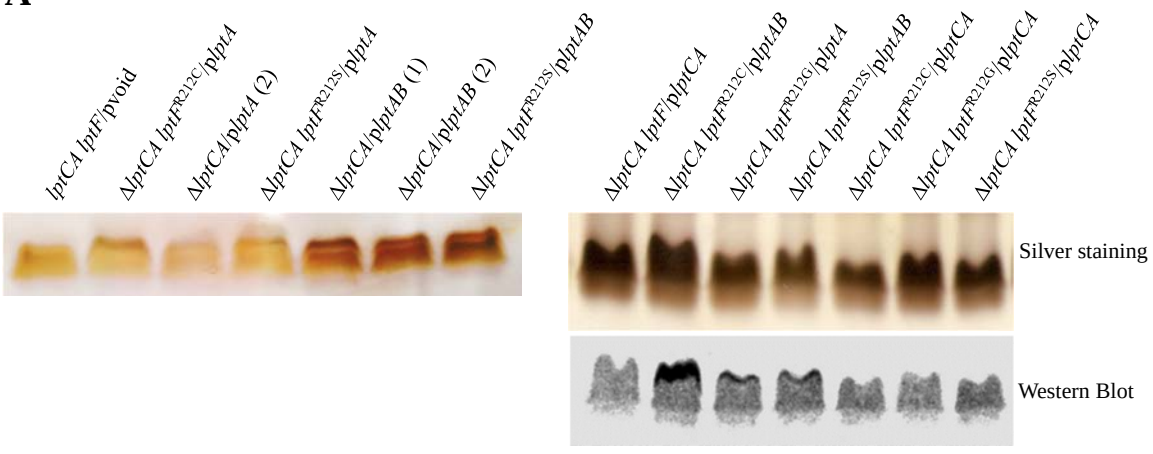
37 °C
42 °C
Bac
lptCA/-
ΔlptCA/lptA 1
ΔlptCA/lptA 2
ΔlptCA/lptA 3
ΔlptCA/lptAB 1
ΔlptCA/lptAB 2
ΔlptCA/lptAB 3
Nov
Rif
SDS-EDTA
McC
lptCA/-
ΔlptCA/lptA 1
ΔlptCA/lptA 2
ΔlptCA/lptA 3
ΔlptCA/lptAB 1
ΔlptCA/lptAB 2
ΔlptCA/lptAB 3
10⁰ 10⁻⁵
10⁰ 10⁻⁵
10⁰ 10⁻⁵
10⁰ 10⁻⁵

Fig. 3

A


lptCA lptF/pvoid
ΔlptCA lptF^{R212C}/plptA
ΔlptCA/plptA (2)
ΔlptCA lptF^{R212S}/plptA
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ΔlptCA/plptAB (2)
ΔlptCA lptF^{R212S}/plptAB
ΔlptCA lptF/plptCA
ΔlptCA lptF^{R212C}/plptAB
ΔlptCA lptF^{R212G}/plptA
ΔlptCA lptF^{R212S}/plptAB
ΔlptCA lptF^{R212C}/plptCA
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Silver staining
Western Blot

B

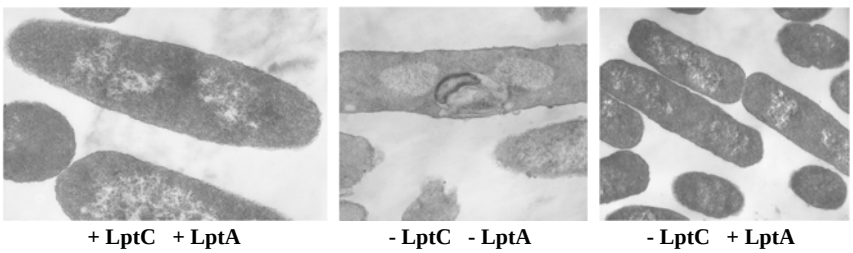
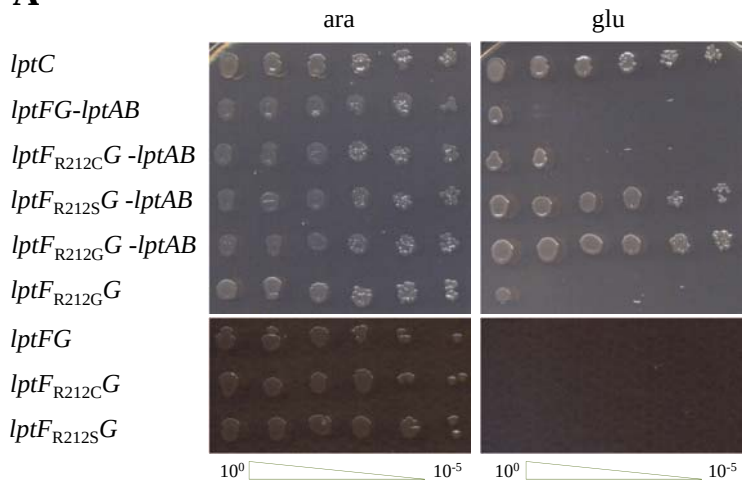

+ LptC + LptA
- LptC - LptA
- LptC + LptA

Fig. 4

A


ara
glu
lptC
lptFG-lptAB
lptF$_{R212C}$G -lptAB
lptF$_{R212S}$G -lptAB
lptF$_{R212G}$G -lptAB
lptF$_{R212G}$G
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lptF$_{R212C}$G
lptF$_{R212S}$G
10^0
10^{-5}
10^0
10^{-5}

B

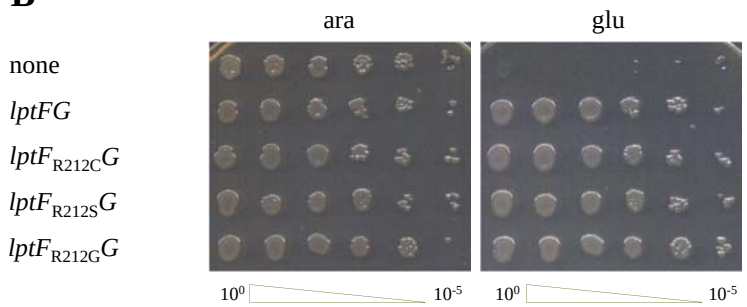
ara
glu
none
lptFG
lptF$_{R212C}$G
lptF$_{R212S}$G
lptF$_{R212G}$G
10^0
10^{-5}
10^0
10^{-5}

Fig. 5

PART III: SUPPLEMENT AND ADDITIONAL RESULTS

PLASMID SHUFFLING TECHNIQUE

Plasmid shuffling technique consists in the substitution of a resident plasmid harbored by an host organism with a chasing plasmid. The two plasmids usually harbor genes which differ between them, in order to test the effects of genes substitution on the host.

A strain which harbors the chasing plasmid and has lost the resident plasmid must be selectable. A simple way is to use plasmids harboring non-compatible replication origins and different marker resistances, in order to select for the marker of the chasing plasmid and to screen for the loss of the resident plasmid resistance.

In this work the first plasmid shuffling experiments were performed using a resident plasmid and a chasing plasmid harboring the $oriV_{ColD}$ replication origin and *cat* (Cam^R) and *kan* (Kan^R) resistance marker respectively. Both plasmids harbor a *ptac* promoter. However if the chasing plasmid harbors genes less functional or more toxic for the host than those harbored by the resident plasmid, the frequency of loss of the resident plasmid may be very low or nil.

In order to improve the selection efficiency of shuffled clones I prepared a system in which the resident plasmid harbors *cat* and the $rpsL^+$ (Str^S) allele, which is dominant on the chromosomal *rpsL150* (Str^R) allele of the host strain and confers streptomycin sensitivity. The chasing plasmid harbors *kan*. Therefore, I directly selected clones on streptomycin for the loss of the resident plasmid and on kanamycin for the presence of the chasing plasmid. Using this system I performed plasmid shuffling in *E. coli* AM604 derivative strains harboring *rpsL150* (Str^R) and the chromosomal *lptCAB* or *lptCA* deletions complemented by a resident plasmid harboring *Ec-lptCAB* (pGS315).

I was able to obtain the following strains:

KG282/pGS312 Δ*lptCAB*/*Pa-lptCAB*

KG286/pGS311 Δ*lptCA*/*Pa-lptCA*

Then to further improve the technique I prepared a new resident plasmids (pMBM06). It has a *bla* marker (Amp^R), an inducible *araBp* promoter, a thermosensitive replication origin (*oriR101, repA101ts*) and $rpsL^+$. In this way I could select for shuffled at 42° or 37°C (temperature non-permissive for the replication of the resident plasmid) and streptomycin; glucose was also added for repression of the genes harbored by the resident plasmid (making their presence superfluous),. A new chasing plasmid (pGS401) was constructed to have two polylinkers with independent Shine-

Dalgarno, under the control of the same *ptac* promoter in order to clone two genes independently by each other.

Using this system I performed plasmid shuffling in an *E. coli* AM604 derivative strain harboring *rpsL150* (Str^R) and the chromosomal *lptCA* deletion complemented by a pMBM06 derivative harboring *Ec-lptCA* (pMBM07).

I was able to obtain the following strains:

KG286/pGS405 Δ*lptCA*/*Pa-lptC_Pa-lptA*

KG286/pGS407 Δ*lptCA*/*Pa-lptC_Ec-lptA*

KG286/pGS406 Δ*lptCA*/*Ec-lptC_Pa-lptA*

KG286/pGS412 Δ*lptCA*/*Ec-lptC*^{G153R}*_Ec-lptA*

KG286/pGS321 Δ*lptCA*/*Ec-lptA*

KG286/pGS416 Δ*lptCA*/*Ec-lptAB*

Escherichia coli/*Pseudomonas aerugionosa* HYBRID STRAINS

An *E. coli*/*P. aeruginosa* Lpt hybrid system consists in the substitution of one or more *E. coli* lpt genes with the *P. aeruginosa* xenogeneic genes, thus resulting in an Lpt hybrid machine (Fig. 1). This system can be useful to study *P. aeruginosa* Lpt proteins and to search for conserved features shared by the Lpt system of different Gram negative species.

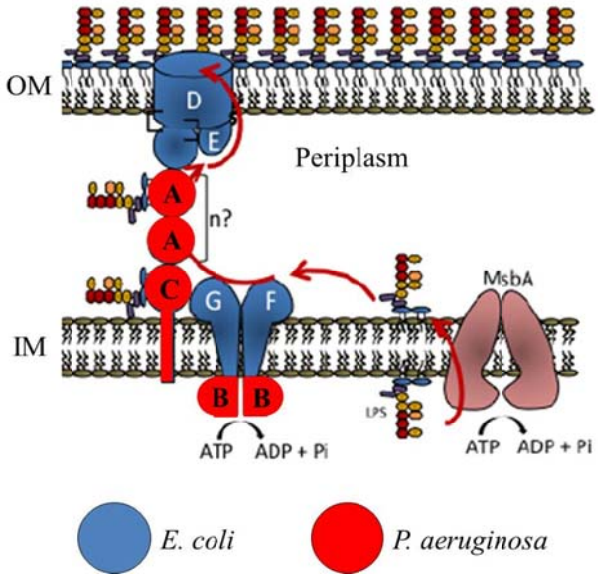

Fig. 1. *E. coli*/*P. aeruginosa* hybrid machine.

In order to obtain hybrid strains I devised an approach based on plasmid shuffling technique in an *E. coli* mutant deleted for one or more lpt genes. I used a resident plasmid harboring *E. coli* genes and a chasing plasmid harboring *P. aeruginosa* genes. I was able to obtain *E. coli lptCAB* and *lptCA* deletion mutants complemented by *Pa-lptCAB* and *Pa-lptCA*, respectively.

The result shows that *Pa*-LptC and *Pa*-LptA can form a complex compatible with the remaining *E. coli* Lpt proteins and can transport the *Ec*-LPS.

Sequenced *Ec-lptFGDE* and *Pa-lptCAB* genes were without mutations, therefore "adaptive" mutations in lpt genes of the Δ*lptCAB*/*Pa-lptCAB* strain are not required to accommodate Pa-LptB and the Pa-LptC-LptA complex in the *E. coli* machine and thus that LptB-LptFG, LptC-LptFG and LptA-LptD interactions are not species-specific.

Hybrid strains show modified LPS (Fig. 2) and higher sensitivity than the wt to low and high temperature and to toxic compounds (Tab. 1), indicating a compromised OM functionality.

To assess whether the interaction between LptC and LptA is species-specific I improved plasmid shuffling technique preparing new resident and chasing plasmids. I prepared chasing plasmids with:

Ec-lptC_Ec-lptA, *Pa-lptC_Pa-lptA*, *Ec-lptC_Pa-lptA* and *Pa-lptC_Ec-lptA*. I obtained *E. coli* lptCA deletion mutants complemented by all the described chasing plasmids and I assessed their correct structure by PCR. Sequencing of *lptC* and *lptA* indicates that no mutations are necessary to tune the single *Pa*-LptC or *Pa*-LptA with its *E. coli* counterparts.

The hybrid strains show an increased permeability to hydrophobic toxic compounds; however their viability indicates that *Pa*-LptC and *Pa*-LptH can interact with both the other proteins of the Lpt machine and the LPS of *E. coli*.

Thus the interaction between LptC and LptA is not species-specific.

LPS analysis (Fig. 2) and growth on stress conditions (Tab. 1) indicate a less compromised OM in the strain complemented with the couple *Pa-lptC_Ec-lptA* than in the strains complemented with *Ec-lptC-Pa-lptA* or *Pa-lptC-Pa-lptA*. Therefore *Pa*-LptC is more compatible with the remaining *E. coli* Lpt proteins than *Pa*-LptA.

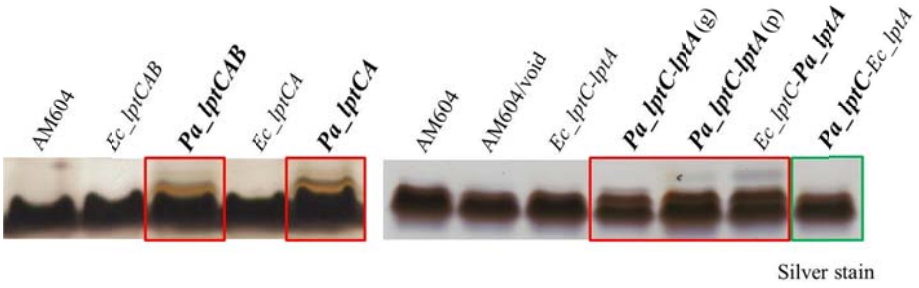

Fig. 2. Analysis of LPS extracted from hybrid strains.

Δ*lptCAB* or Δ*lptCA* complemented by:	Temperature °C					Toxic compounds, 37°C			
	12	20	28	37	42	Bac	Nov	Rif	McC
Ec_lptCAB	+	+	+	+	+	+	+	+	+
Pa_lptCAB	-	+	+	+	+/-	-	-	-	-
Ec_lptCA	+	+	+	+	+	+	+	+	+
Pa_lptCA	-	-	-	+	+/-	-	-	-	-
Pa_lptC-Pa_lptA	-	-/+	+/-	+	+	-	-	-	-
*Ec_lptC-**Pa_lptA***	-	-/+	+/-	+	+	-	-	-	-
***Pa_lptC**-Ec_lptA*	+	+	+	+	+	+	+/-	+/-	-/+

Table 1. Hybrid strains growth in stress conditions.

In this work we assessed that the *Pa*-LptCHB proteins are able to replace *Ec*- LptCAB in *E. coli* and that *Pa*-LptCHB are the functional homologue of *Ec*-LptCAB. Thus despite low amino acidic conservation *Pa*-LptCAB are functionally conserved to *E. coli* homologous proteins, in particular LptC. Our data suggest that LptA and LptC overall structure may play a major role in LPS recognition and transport.

Δ*lptCA*/*lptC*^{G153R}-*lptA* STRAIN

lptC^{G153R} is an lptC mutant allele unable to complement the *araBp-lptC* FL905 strain in non permissive conditions (-arabinose; lack of *lptC* expression and *lptAB* expression only by the *lptAp1p2* ancillary promoters), neither alone or in co-expression with LptB (Sperandeo *et al.*, 2011) (DRAFT1).

Federica Falchi and I performed a plasmid shuffling experiment (Bollati *et al.*, 2015) (Draft 2) using the *E. coli* strain KG286/pMBM07 and a chasing plasmid harboring *lptC*^{G153R} and *lptA* (pGS412 plasmid, pGS401 derivative, *ptac-lptC*^{G153R}*-lptA, cat, oriV*$_{ColD}$), with the purpose to search for suppressor mutations of the lethal phenotype.

Cam^{R} Str^{R} Ts^{+} transformants were obtained, as described in DRAFT 2, with frequencies of 4 and 14.7 transformants per ng of plasmid DNA at 42 and 37 °C respectively on LD supplemented as described in DRAFT 2 (Table 2).

After selection transformants were purified and grown at 37 °C in LD supplemented with 0.2% glucose and 34 μg/ml chloramphenicol.

Plasmid	**Genes**	**Selection**	
		42 °C Str Cam	**37 °C Str Cam**
pGS401[a]	none	<0.25	<0.25
pGS404[a]	*lptC-lptA*	>2000	>2000
pGS412	*lptC*^{G153R}	4	14.7

Table 2. Frequency of transformants upon selection for plasmid shuffling in an ectopically complemented Δ*lptCA* mutant.

[a] control data from an analogue plasmid shuffling experiment (see DRAFT 2).

Fourteen out of fourteen clones of the KG286/pGS412 strain were found to be Amp^S and sequencing of plasmid DNA extracted by one clone selected at 42 °C (KG286/pGS412 A) and one clone selected at 37 °C (KG286/pGS412 B) revealed no mutations in the $lptC^{G153R}$ and *lptA* genes. Through Southern blotting analysis of the same clones we assessed the absence of *lptC* alleles integrated in the genome (Fig. 3). Only signal corresponding to the plasmid $lptC^{G153R}$ is visible. The DNA probe covers the *lptC* 148-293 region comprising the gga → aga mutation (G153R) and was obtained by PCR amplification with primers FG2760-FG3045.

Thus we obtained *bona fide E. coli* $lptC^{G153R}$ viable mutants. Impaired LPS transport may lead to growth defects such as increased sensitivity to toxic chemicals. As shown in Table 3 the outer membrane of $lptC^{G153R}$ strains appears at least in part impaired. Genetic and biochemical experiments will be useful to asses whether in this mutant strain $LptC^{G153R}$ is an active component of the Lpt machine or the Lpt machine functions without LptC, like in the Δ*lptC* strains.

We shall assess by *lptF* sequencing whether a putative suppressor of the $lptC^{G153R}$ lethal phenotype could be found in *lptF*; alternatively, sequencing of the entire genome could allow the identification of new suppressor mutations for LptC defects.

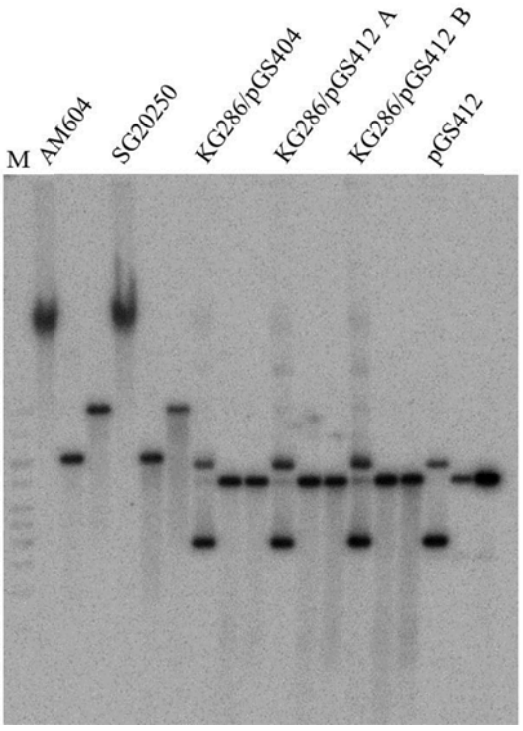

Fig. 3. Southern blotting analysis of KG286/pGS412 A and KG286/pGS412 B total DNA. AM604, SG20250 and KG286/pGS404 total DNA and pGS412 plasmid were used as control. For each seample, from the left: not digested, *Sal*I digested, *Hind*III digested. M: marker.

Strain	Condition						
	12°C	42°C	Bac 50µg/ml	Nov 10µg/ml	Rif 2,5µg/ml	McC	SDS 0,5% EDTA 0,25mM
AM604	1,00E+00	4,00E+00	4,00E-02	3,00E+00	3,00E-02	3,00E+00	7,00E+00
NR698	2,50E-02	1,25E+00	< 1,25E-05	< 1,25E-05	< 1,25E-05	< 1,25E-05	< 1,25E-05
KG286/pGS412 A	2,00E-01	1,10E+00	1,00E-01	< 1,00E-04	1,00E+00	7,00E-01	3,00E-02
KG286/pGS412 B	1,00E+00	1,00E+00	1,00E-01	< 1,00E-04	7,00E-01	1,40E-02	2,00E-02

Table 3. KG286/pGS412 A and KG286/pGS412 B growth at different temperatures and in presence of toxic compounds. AM604 was used as not sensitive control strain, while NR698 (Ruiz *et al.*, 2005) was used as sensitive control strain. Growth efficiency are referred to 37 °C.