A New Factor LapD Is Required for the Regulation of LpxC Amounts and Lipopolysaccharide Trafficking

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Abstract: Lipopolysaccharide (LPS) constitutes the major component of the outer membrane and is essential for bacteria, such as Escherichia coli. Recent work has revealed the essential roles of LapB and LapC proteins in regulating LPS amounts; although, if any additional partners are involved is unknown. Examination of proteins co-purifying with LapB identified LapD as a new partner. The purification of LapD reveals that it forms a complex with several proteins involved in LPS and phospholipid biosynthesis, including FtsH-LapA/B and Fab enzymes. Loss of LapD causes a reduction in LpxC amounts and vancomycin sensitivity, which can be restored by mutations that stabilize LpxC (mutations in lapB, ftsH and lpxC genes), revealing that LapD acts upstream of LapB-FtsH in regulating LpxC amounts. Interestingly, LapD absence results in the substantial retention of LPS in the inner membranes and synthetic lethality when either the lauroyl or the myristoyl acyl transferase is absent, which can be overcome by single-amino acid suppressor mutations in LPS flippase MsbA, suggesting LPS translocation defects in ΔlapD bacteria. Several genes whose products are involved in cell envelope homeostasis, including clsA, waaC, tig and micA, become essential in LapD’s absence. Furthermore, the overproduction of acyl carrier protein AcpP or transcriptional factors DksA, SrrA can overcome certain defects of the LapD-lacking strain.

Keywords: lipopolysaccharide; LpxC; cardiolipin synthase A; LPS assembly proteins LapB, LapC (YejM) and LapD; acyltransferases LpxL and LpxM; heptosyltransferase I WaaC; MsbA; trigger factor; MicA sRNA

1. Introduction

The most characteristic feature of Gram-negative bacteria, such as Escherichia coli, is the presence of an asymmetric outer membrane (OM), which is essential for their viability [1]. This asymmetric nature of OM is critical for endowing a permeability barrier to prevent the entry of bulky toxic molecules inside the cells and is based upon the unique distribution pattern that restricts the presence of lipopolysaccharide (LPS) in the outer leaflet of the cell envelope, with phospholipids facing its inner leaflet [1,2]. LPS comprises the major component of OM, covering nearly 75% of OM, and is the major virulence factor and the causative agent of sepsis due to Gram-negative bacteria [1,3,4]. Although the LPS composition is highly heterogenous, they overall share a common basic structure. Thus, LPS can be divided into three parts, with a highly conserved hydrophobic membrane-anchored lipid A, a core oligosaccharide, to which an oligosaccharide of variable length, called the O-antigen, is attached in bacteria with smooth LPS [2,3]. The most conserved part of LPS lipid A constitutes the endotoxin principal and in E. coli is composed of a phosphorylated β(1→6)-linked GlcN disaccharide, to which generally six asymmetric fatty acids are linked via ester and amide linkages. LPS biosynthesis begins with the acylation of UDP-GlcNAc by LpxA with (R)-3-hydroxyymristate derived from (R)-3-hydroxyymristoyl-ACP, followed by successive reactions catalyzed by additional enzymes with LpxC-mediated deacylation constituting the first committed step [3,5–8]. This generates a lipid IVα precursor to which two 3-deoxy-α-d-manno-oct-2-ulosonic acid (Kdo) residues are attached by the essential
enzyme WaaA, at the reducing GlcN residue [3,9]. This generates the key precursor intermediate, termed Kdo$_2$-lipid IV$_A$ [3,9]. This precursor species acts as an acceptor for the acylation by LpxL and LpxM generating hexa-acylated Kdo$_2$-lipid A, which is further extended by various glycosyltransferases for incorporating different sugar molecules for the completion of core biosynthesis [9,10].

The heterogeneity of LPS composition can arise due to changes in the lipid A acylation, modification of phosphate residues of lipid A by phosphoethanolamine (P-EtN), 4-amino-4-deoxy-L-arabinose (L-Ara4N), the non-stoichiometric incorporation of an additional third Kdo residue, uronic acid, rhamnose, modification of the second Kdo residue by phosphoethanolamine, truncation in the outer core and changes in the phosphorylation of the inner core [4,11,12]. This results in the presence of different glycoforms of LPS. This heterogeneity of LPS is regulated by regulon members of the cell envelope-responsive sigma factor RpoE, two-component systems such as BasS/R, PhoP/Q, PhoB/R and Rcs [11]. Thus, specific glycoforms are synthesized when the RpoE regulon is induced due to severe impairment in the cell envelope composition, either due to misfolding of outer membrane proteins (OMPs) or imbalance in their synthesis or when LPS biosynthesis is compromised [11]. These switches in the glycoform synthesis are regulated at the transcriptional level by a specific increase in the expression of certain genes as well as translational repression by sRNAs such as RybB and MgrR [11,13,14]. Although the structural analysis of LPS supports the role of such sRNAs, the physiological significance and molecular basis of specific mRNAs:sRNA interactions have not yet been elucidated. The incorporation of some of these non-stoichiometric modifications, such as P-EtN and L-Ara4N in the lipid A part, are known to confer resistance to cationic antimicrobial peptides such as polymyxin B and can be important in bacterial adaptation to various host and environmental niches [4].

The viability of all Gram-negative bacteria, including *E. coli*, requires a tight balance between phospholipids and LPS amounts, which is held at a constant ratio of (1:0.15) for the maintenance of outer membrane asymmetry [7]. This is achieved by the regulated turnover of LpxC via its proteolytic control and the activity of the FabZ dehydratase enzyme [15]. The regulation of LpxC amounts is critical as it mediates the first committed step in LPS biosynthesis, while FabZ initiates phospholipid biosynthesis [15–18]. Since these two essential enzymatic pathways use the same (R)-3-hydroxymyristate as the common metabolic precursor, its depletion due to diversion in either pathway is toxic for bacteria, and hence either excess or reduced amounts of LPS are lethal for bacteria [15,17]. The stability of LpxC and, in turn, LpxC amounts are regulated in a complex manner with several pathways involved in adjusting these amounts as per the cellular demand of LPS and also depends on the composition of fatty acids [2,17–19]. However, the molecular basis of alteration of the in vivo stability of LpxC and its amounts are not fully understood (Figure 1). One of the key enzymes that participate in the proteolysis of LpxC is the essential inner membrane (IM)-anchored ATP-dependent FtsH metalloprotease [15]. This degradation of LpxC by FtsH requires another essential factor called the LPS assembly protein LapB [17,20]. Thus, a deletion of either the *lapB* gene or the *ftsH* gene is lethal due to the stabilization of LpxC, which results in a toxic increase in the LPS synthesis [17]. However, this FtsH-LapB-mediated proteolysis can be counteracted by another essential protein designated LapC (previously YejM) [21–26]. The *lapC* gene was identified since a mutation in its coding sequence that causes truncation in the LapC’s periplasmic domain could allow the deletion of the essential *lapB* gene [21]. These genetic studies suggested that LapC antagonizes LapB-FtsH-mediated proteolysis [21,23]. Consistent with such a role for LapC, the truncation of its non-essential periplasmic domain or the depletion of *lapC* causes increased LpxC degradation, resulting in a concomitant reduction in LPS and LpxC amounts [21,23,25]. Furthermore, LapB and LapC co-purify, and both bind LPS [21,22]. However, how LapC and LapB adjust the rate of LpxC degradation is not understood (Figure 1). It is also not known if the lethality due to the excessive synthesis of LPS in *ftsH* and *lapB* mutants is due to the depletion of acyl-ACP pools or due to the retention of LPS in the IM and its poor translocation to the OM. Similarly, the physiological factors, other than increased...
LpxC degradation, which cause the lethality in the absence of LapC, are not identified. To add to this complexity, LpxC can also be degraded in vivo in an FtsH-LapB independent manner by the HslVU protease complex, and this degradation could be more relevant at high temperatures [21].

Figure 1. Key steps in the regulation of the first committed step in LPS biosynthesis catalyzed by LpxC and LPS transport mediated by MsbA across the inner membrane. Schematic illustration depicting utilization of the same metabolic precursor (R)-3-hydroxymyristate by LpxA and by FabZ in LPS and phospholipid biosynthesis, respectively. As the reaction catalyzed by LpxA is thermodynamically unfavorable, LpxC-mediated deacylation constitutes the first committed step in LPS biosynthesis. LpxC amounts are regulated by its turnover by the FtsH-LapB complex and at high temperature by HslVU protease. LapC acts as an antagonist of LapB to regulate LPS biosynthesis as per its demand. Once LPS is assembled, it is flipped across the inner membrane by MsbA for its further transport. Scissors depict proteolysis by FtsH and HslVU proteases. A newly identified LapD protein that co-purifies with LapB is depicted with a question mark.

LPS assembly further requires efficient LPS translocation with the first step of its flipping across the IM mediated by the essential ATP-dependent transporter MsbA [27]. In the subsequent steps, LPS is translocated to the OM by another essential trans-envelope machinery, comprising seven proteins that span all three compartments of the cell [28]. MsbA uses its hydrocarbon ruler properties to prevent or reduce the translocation of underacylated LPS species [29–32]. This preferential selectivity for hexa-acylated lipid A provides an essential checkpoint, ensuring only mature LPS is translocated to the OM [2]. Thus, not surprisingly, suppressor mutations that overcome the lethality of either Δ(lpxL lpxP lpxM) or ΔwaaA strains synthesizing lipid IV₅ derivatives map to the msbA gene, presumably by relaxing the selectivity of MsbA for the translocation of underacylated LPS [9,32]. In the translocation of underacylated LPS, MsbA is aided by cardiolipins [32,33]. Consistent with such a requirement for cardiolipins, mutational combinations of Δ(clsA msbA), Δ(clsA lpxL) and Δ(clsA waaA) are lethal, which can be overcome by suppressor mutations in the msbA gene [32]. However, the molecular basis of such lethality and how cardiolipins aid MsbA in LPS transport remains unknown.

To further understand the balanced regulation of LPS and phospholipid biosynthesis, we first carefully examined proteins that interact with LapB to identify if any factors had been previously missed. This analysis identified an additional protein YhcB, designated LapD, which co-purifies with LapA and LapB proteins (Figure 2A). This co-purification was also validated when the purification profile of LapD was analyzed, which showed that LapD co-purifies not only with LPS assembly proteins but also with several proteins involved in either LPS transport or its biosynthesis or the fatty acid synthesis (Figure 2A). We have previously shown that the lapD gene is required for the growth of E. coli at critical high temperatures [34]. LapD (YhcB) is an inner membrane protein and has recently been implicated in either the cell division process or the cell envelope homeostasis; although,
molecular mechanisms in either of these functions remain unknown [35–38]. In this work, we show that in the absence of LapD, LpxL and LpxM acyl transferases become essential and the synthetic lethality of either ∆(lpxL lapD) or ∆(lpxM lapD) can be overcome by extragenic suppressor mutations mapping to the essential msbA gene (Figure 2B). We further show that ∆lapD strains exhibit sensitivity to antibiotics such as vancomycin and reduced amounts of LpxC. Consistent with a role in the regulation of LPS amounts and the interaction with LapB protein, mutations that either render LpxC resistant to FtsH-mediated proteolysis or loss-of-function variants in the lapB gene can overcome the sensitivity of ∆lapD bacteria to vancomycin (Figure 2B). Consistent with a role in these essential processes, various growth defects of a ∆lapD derivative can be overcome when the acyl carrier protein AcpP is overproduced. Since the AcpP protein acts as a key component in the fatty acid synthesis pathway and interacts with various acyl transferases involved in the biosynthesis of lipid A and the phospholipid synthesis [39], its identification as a multicopy suppressor of ∆lapD defects is consistent with a critical role in balanced biosynthesis of LPS and phospholipids. We present genetic and biochemical data supporting a role for LapD acting upstream of LapB by acting in an antagonistic manner, thereby controlling LpxC levels and could also aid MsbA-mediated LPS translocation.

Figure 2. Schematic depiction of various approaches that identify LapD as a regulator of LPS biosynthesis regulating LpxC amounts and assisting MsbA in the LPS transport. Identification of LapD as an IM protein associated with LapB and co-purification of LapD with proteins involved in LPS/phospholipid biosynthesis (A). Suppressors of vancomycin sensitivity of ∆lapD and of various synthetic lethal combinations identify suppressor mutations either in genes that stabilize LpxC or in the msbA gene, revealing that LapD acts upstream of LapB and aids MsbA-mediated LPS transport (B).

2. Results

2.1. LapD Is Part of LapA/LapB Complex and Co-Purifies with Several Proteins Involved in LPS and Phospholipid Biosynthesis

Examination of proteins that co-purify with LapB revealed the presence of a new component designated LapD in addition to previously known interacting partners such as LapA, FtsH, WaaC, FabZ and Lpt proteins (Figure 3). MALDI-TOF analysis identified peptides QQQALQYELEK, SAELLDTMAHDYR, SSSSLLPELSAEANPFR and LAESEASNDQAPVQMPRDISEGGLLR covering more than 52% of the entire LapD amino acid sequence. We had previously identified the lapD (yhcB) gene in a global screen of E. coli genomic knockouts, whose products are required for growth at high temperature [34]. Besides the temperature-sensitive (Ts) phenotype, ∆lapD bacteria are also sensitive to antibiotics such as vancomycin, suggesting defects in the OM barrier function (see below). We also had observed earlier that the deletion of the lapD gene cannot be tolerated in a strain devoid of six cytoplasmic peptidyl-prolyl cis/trans isomerases [40]. However, the molecular basis of such a lethality remained unknown. Although LapD has recently been
implicated in cell division or the maintenance of cell envelope homeostasis, its function has remained unknown [36,37].

Figure 3. LapD as a new interacting partner of LapB. Purification profile of His$_6$-tagged LapB protein from the IM fraction after elution with 250 mM imidazole. Lane 1 shows co-purifying proteins with LapB that include LapD. All major co-purifying proteins are indicated by arrows. In lane 2, purified LapD protein was applied. Proteins were resolved on a 12% SDS-PAGE, stained by Coomassie Brilliant Blue. Lane 3 shows pre-stained molecular weight standards.

To elucidate LapD function, a His$_6$-tagged derivative was purified from IM fractions and co-eluted proteins identified by MALDI-TOF to reveal its interacting partners (Figure 4). These experiments showed that the majority of proteins that co-purify with LapD are involved in either LPS biosynthesis/assembly or transport, which include (LpxM, FtsH, HldE, HldD, GmhA, WbbJ, LapA/LapB and LptB/C/D) and phospholipid/fatty acid biosynthesis (PssA, AccD and FabB/F/H/Y). A few proteins involved in cell shape and chromosomal segregation (MukB/F/E, MreC and ZapD) were also identified in such pull-down experiments (Figure 4). In addition, a cytoplasmic peptidyl-prolyl cis/trans isomerase FklB, belonging to the family of FK506-binding proteins, was identified among co-eluted proteins (Figure 4). Among co-eluting proteins, LpxM adds the last acyl chain to complete the synthesis of hexa-acylated lipid A after the addition of two Kdo residues [41], while FtsH is the essential IM protease, one of whose substrates is LpxC [15]. Other prominent co-purifying enzymes with LapD are involved in phospholipid biosynthesis. Thus, besides fatty acid biosynthetic Fab enzymes, PssA (phosphatidylserine synthase) mediates the first committed step for phosphatidylethanolamine biosynthesis [42]. These results demonstrate that LapD forms a complex in the IM with proteins involved in LPS assembly, its biogenesis and transport (LapA/B, LpxM and Lpt), and phospholipid biosynthesis.
2.2. LapD Is Required to Maintain Levels of LpxC

To further investigate the function of LapD and its requirement in the regulation of LPS, we analyzed the levels of the LpxC enzyme. Isogenic bacterial cultures of the wild type and a ΔlapD strain were grown at 30 °C (permissive growth conditions) and then shifted to 43 °C for 2 h. Such bacterial cultures were used to prepare whole cell lysates. As a control, we also included a previously well-characterized isogenic lapC190 bacterial strain, which lacks the periplasmic domain of LapC and exhibits diminished amounts of LpxC. The equivalent amounts of total proteins were resolved on a 12% SDS-PAGE, and LpxC amounts were analyzed by immunoblotting using LpxC-specific antibodies. Such experiments revealed that under such growth conditions, ΔlapD bacteria have reduced amounts of LpxC (Figure 5A, lane 2). This is consistent with previous results, where lapC190 mutant bacteria also exhibit reduced amounts of LpxC (Figure 5A, lane 3). Thus, lapC190 and ΔlapD bacteria both have reduced amounts of LpxC in contrast to the elevated levels of LpxC in lapB bacteria. As a control, we also estimated the amounts of LapB and FtsH in whole cell lysates obtained from the isogenic wild type and its ΔlapD derivative by immunoblotting with LapB- and FtsH-specific antibodies (Figure 5B,C). As can be seen, no major differences in LapB and FtsH amounts were observed, in contrast to a reduction in LpxC amounts in ΔlapD bacteria. Our results showing a reduction in LpxC amounts can explain phenotypic defects such as the loss of permeability, reflected in the sensitivity to antibiotics such as vancomycin when LapD is absent.
was somewhat lower than when stable lpxC::cm were first used as recipients to remove the lpxC::cm::Tn10 mutation by the introduction of tightly linked napA::Tn10 scoring for the loss of Cm resistant marker to have only an lpxC or ftsH chromosomal mutation. Thus, bacteria with a wild-type copy of the lpxC gene but with chromosomal lpxC single amino acid substitutions or a frameshift that render LpxC resistant to proteolysis (SR23812 lpxC R230C, SR23814 lpxC V37G, SR23816 lpxC V37L, SR23818 lpxC K270T, and SR23820 lpxC fs306 stop codon) and the strain SR23822 with ftsH A296V served as recipients (see Section 4.1). Into such lpxC and ftsH variants, the ΔlapD mutation was introduced by bacteriophage P1-mediated transductions and analyzed for restoration of resistance to vancomycin. All such strains with the deletion of the lapD gene were found to be resistant to vancomycin, unlike isogenic ΔlapD bacteria, which are sensitive (Figure 6). However, the restoration of growth of ΔlapD with ftsH A296V mutation on vancomycin was somewhat lower than when stable lpxC variants were introduced (Figure 6). In later sections (see Section 2.10), we have again verified that the above-mentioned mutations in the lpxC gene lead to increased accumulation of LpxC. Thus, we can conclude that a restoration of LpxC stability by introducing LpxC stable variants in ΔlapD bacteria can overcome membrane permeability defects.
Figure 6. Mutations that stabilize LpxC suppress vancomycin sensitivity of ΔlapD bacteria. Growth of isogenic cultures of strains with ΔlapD and with suppressor mutations in the lpxC gene was quantified by spot dilution on LA with and without supplementation of vancomycin. The relevant genotype and temperature of incubation are indicated.

2.4. Suppressor Mutations in the lapB Gene That Prevent LpxC Degradation and Restore the Growth of lapC190 Mutant Bacteria Can Also Restore the Wild-Type-like Growth of ΔlapD Bacteria on Vancomycin-Supplemented Growth Medium

We previously isolated several suppressor mutations that overcome Ts and permeability defects of lapC190 mutant bacteria mapping to the lapB gene [21]. Most of such suppressor mutations had severely reduced LapB amounts, which in turn prevented LpxC degradation [21]. As ΔlapD bacteria have reduced LpxC quite like lapC190 bacteria, we reasoned that the introduction of such lapB mutations should also restore the growth of a ΔlapD strain under conditions such as exposure to vancomycin. Thus, as described in the above section, firstly the lapC190 mutation was removed by introducing napA::Tn10, selecting for the loss of the CmR cassette that replaces the periplasmic domain of LapC to have only a single amino acid lapB suppressor mutation on the chromosome. Such isogenic strains with an intact copy of the lapC gene served as a recipient to bring in the ΔlapD deletion. This resulted in generating strains SR23857 (lapB H325P ΔlapD), SR23859 (lapB A88V ΔlapD), SR23861 (lapB H181R ΔlapD), SR23863 (lapB R115H ΔlapD), SR23865 (lapB D124Y ΔlapD) and SR23867 (lapB R125L ΔlapD) (see Methods section). Such isogenic strains along with parental ΔlapD were tested for the growth at permissive growth conditions and when growth medium was supplemented with vancomycin by spot dilution assay. Such experiments reveal that single amino acid substitutions in the lapB gene, which render LpxC stable, can confer vancomycin resistance to ΔlapD bacteria, although to a different extent (Figure 7). Among the tested lapB mutants, the introduction of lapB R113H, lapB D124Y, lapB R125L and lapB H181R in ΔlapD bacteria, conferred better suppression in terms of restoration of the growth on vancomycin-supplemented growth medium (Figure 7). Thus, we can conclude that the reduction in LpxC amounts in ΔlapD bacteria can be compensated when LpxC is stabilized by introducing loss-of-function mutations in the lapB gene in a manner similar to that previously observed with a lapC190 mutant strain. Hence, quite like LapC, LapD could function upstream of LapB and act as its antagonist to prevent excessive degradation of LpxC. However, it should be noted that the Ts phenotype of the ΔlapD derivative is not fully suppressed by mutations in the lapB gene, which is not the case with lapC190 mutant bacteria [21].
2.5. Reduction in the LPS Synthesis Is Lethal for ΔlapD Bacteria

If indeed, LapD regulates LpxC proteolysis in a manner antagonistic to LapB and acts in a pathway similar to LapC upstream of LapB to regulate LPS biosynthesis, any reduction in LPS biosynthesis should be toxic to ΔlapD bacteria. It should be noted that LapB becomes dispensable when LpxC/LPS amounts are reduced, as shown earlier, when the LPS synthesis is dampened in the presence of dysfunctional LapC or by introducing the lpxA2(ts) mutation [17]. Thus, we performed parallel transductions in SM101 lpxA2(ts), MN7 lpxB1(ts) and GK6075 (lapC190) bacteria by introducing a ΔlapD mutation, using appropriate controls (Table 1). It is known that SM101 lpxA2(ts), MN7 lpxB1(ts) and GK6075 (lapC190) have reduced amounts of LPS [15,17]. Most significantly, ΔlapD could not be introduced in the strains with mutations in either the lpxA gene or the lpxB gene or the lapC gene, while it could be introduced in the wild-type strain (Table 1). In contrast, a lapB deletion is readily accepted in lpxA2(ts), lpxB1(ts) and lapC190 mutant bacteria, consistent with our earlier results [17,21]. Thus, mutations in genes that cause a reduction in the LPS synthesis are lethal in a ΔlapD background and, in converse, the reduction in the LPS synthesis bypasses the lethality associated with ΔlapB. These results support the notion that LapD acts upstream of LapB, acting antagonistically, and has a function similar to LapC.

Table 1. A ΔlapD mutation is lethal when the LPS synthesis is impaired, which is the opposite in the case of ΔlapB strains.

| Genotype     | Number of Transductants | Viability in ΔlapD |
|--------------|-------------------------|-------------------|
| P1 ΔlapD LA 30 °C | P1 ΔlapB M9 30 °C     |                   |
| wt           | 953                     | 96 small colonies | viable          |
| lpxA2(ts)    | 12 sc 1                 | 650               | not viable      |
| lpxB1(ts)    | 9                       | 439               | not viable      |
| lapC190      | 17 sc                   | 475               | not viable      |

1 sc indicates small colony size.

2.6. LapD Is Essential When Either LpxL or LpxM Late Acyl Transferase Is Absent and the Conditional Lethality When Cardiolipin Synthase A or WaaC Heptosyl Transferase Is Absent

Data presented from several above-described experiments suggest physical (co-purification) or genetic interaction of LapD with several enzymes involved in LPS assembly or biosynthesis. To further investigate any specific requirement for LapD in these pathways, a series of transductions were performed using strains with a defined individual null mutation in otherwise non-essential genes whose products are known to be involved in either LPS or phospholipid biosynthesis. In the biosynthesis of hexa-acylated lipid A, only lpxL and lpxM are non-essential
genes; although, a deletion of the lpxL gene confers a Ts phenotype above 33 °C [43]. Thus, we attempted to construct Δ(lpxL lapD) and Δ(lpxM lapD) strains using bacteriophage P1-mediated transductions at 30 °C (Table 2). No viable transductants were observed and only when plated in large numbers a few suppressors were obtained (see below).

Table 2. Suppressor mutations mapping to the msbA gene can rescue the synthetic lethality of Δ(lpxM lapD) bacteria as judged by their viability.

| Genotype | P1 ΔlapD Number of Transductants LA 30 °C | Viability and Colony Size |
|----------|------------------------------------------|--------------------------|
| wt       | 1243                                     | viable                   |
| ΔlpxM    | 6                                        | not viable               |
| ΔlpxL    | 13                                       | not viable               |
| Δ(lpxM lapD) msbA S120L | 620 | viable small size |
| Δ(lpxM lapD) msbA M160I | 786 | viable medium size |
| Δ(lpxM lapD) msbA H777M | 730 | viable small size |
| Δ(lpxM lapD) msbA Y287A | 1490 | viable normal size |
| Δ(lpxM lapD) msbA D431Y | 735 | viable normal size |
| Δ(lpxM lapD) msbA S165C | 1130 | viable normal size |
| Δ(lpxM lapD) msbA D498Y | 1267 | viable normal size |
| ΔwaaC    | 433                                      | viable small size not viable at 42 °C |
| ΔclsA    | 378                                      | viable small size not viable at 42 °C |

After the minimal Kdo₂-lipid A LPS is synthesized, it becomes an acceptor for the incorporation of various sugars, with WaaC being the first enzyme mediating the transfer of the first heptose to the Kdo moiety. Thus, among various transductional combinations, Δ(waaC lapD) was constructed and analyzed for growth properties. Next, we examined the requirement of cardiolipins in the absence of LapD. In cardiolipin biosynthesis, ClsA is the main contributor [44,45]. Thus, Δ(clsA lapD) strains were also constructed and analyzed further (Table 2). Although Δ(waaC lapD) and Δ(clsA lapD) viable transductants were obtained at 30 °C, their colony size was smaller than that of the parental strains (Table 2). To quantify growth defects, panels of such strains were examined by spot-dilution assay at different temperatures. As shown, Δ(clsA lapD) bacteria form small-sized colonies at 30 and 37 °C, with a reduction of nearly 10⁵ in terms of colony forming units (cfu) (Figure 8A). At 42 °C, such bacteria exhibit a Ts phenotype, conditions under which ΔlapD and ΔclsA bacteria do not exhibit any major growth defects (Figure 8A). Regarding the growth properties of Δ(waaC lapD) bacteria, spot-dilution assays were performed at 30 °C and 42 °C. Even at 30 °C, Δ(waaC lapD) bacteria showed a 100-fold reduction in cfu (Figure 8B). At 42 °C, the Δ(waaC lapD) combination turns out to be lethal, which is permissive for the growth of either ΔwaaC or ΔlapD strains (Figure 8B). Thus, LapD is essential for the growth of E. coli when LPS is either underacylated or when bacteria synthesize the minimal LPS structure composed of Kdo₂-lipid A due to a lack of WaaC heptosyltransferase. LapD is also critically required for bacterial viability when cardiolipin biosynthesis is compromised, as shown by the conditional synthetic lethality of Δ(clsA lapD).
2.7. Single Amino Acid Suppressor Mutations in the msbA Gene Can Bypass the Lethality of Δ(lpxL lapD) and Δ(lpxM lapD) Bacteria

To further understand the molecular basis of the lethality of Δ(lpxL lapD) and Δ(lpxM lapD) combinations, we sought to isolate extragenic chromosomal suppressor mutations that can overcome this lethal phenotype. Thus, several rounds of bacteriophage P1-mediated transductions were performed by bringing in the null mutation of the lapD gene in defined ΔlpxL and ΔlpxM strains. As shown above, LpxL and LpxM are essential in the absence of LapD. Transductants were plated at 30 °C and few survivors could be obtained. Out of these, two such strains, SR23684 Δ(lpxL lapD) sup* and SR23685 Δ(lpxM lapD) sup*, were retained for further analysis. To identify the suppressor mutation, we PCR amplified coding regions of lpxC, lapA/B, lapC, ftsH, fabZ and msbA genes using the chromosomal DNA of SR23684 and SR23685 as templates. DNA sequencing analysis showed that SR23684 has a single nucleotide change in the codon CTG to CCG, resulting in a single amino acid exchange of L412P in the msbA gene. SR23685 was found to have also a single amino acid exchange of V287A due to the mutation of codon GTT to GCT. These two independent single amino substitutions in the MsbA structure show that the L412P substitution is in the nucleotide-biding domain and V287A is predicted to be located in the LPS-binding domain (Figure 9). Interestingly, we had recently isolated the msbA V287A mutation as a suppressor mutation that restored the growth of the Δ(lpxM clsA) derivative [32]. To ensure SR23684 and SR23685 do not carry an additional mutation, the replacement of the msbA suppressor by a wild-type copy did not allow restoration of growth using a linked marker in transductions. Isolation of suppressor mutations that overcome the synthetic lethality of Δ(lpxL lapD) and Δ(lpxM lapD) mapping to the msbA gene, whose product is required for flipping LPS from the inner leaflet of IM to its outer leaflet, suggests that the absence of LapD further retards LPS translocation across the IM, which is already reduced when lipid A is underacylated.
Mutations mapping to the msbA gene can bypass the synthetic lethality of Δ(lpxL lapD) and Δ(lpxM lapD) bacteria. Positions of various single amino acid substitutions in the structure of MsbA (PDB 6BPL) [30] are shown by arrows. The position of the LPS is also indicated and marked by the arrow.

To further reinforce these results, we next tested previously isolated single amino acid substitutions of msbA that restored the growth of strains synthesizing tetra-acylated lipid A Δ(lpxL lpxM lpxP) and Δ(lpxM clsA) to test if they could also restore the growth of Δ(lpxM lapD) bacteria. To achieve this goal, first, the deletion of the clsA gene from Δ(lpxM clsA) bacteria with the msbA sup* allele was eliminated by the introduction of a nearby oppA::spec marker, which is greater than 90% linked. The resulting ΔlpxM msbA sup* variants served as recipients to bring in the lapD deletion. In all cases, viable colonies were obtained; although, the transduction efficiency and colony size were variable, in contrast to the lethality of Δ(lpxM lapD) combination (Table 2). It should be noted that the presence of the oppA::spec marker does not influence the growth as Δ(lpxM oppA) still cannot accept a deletion of the lapD gene. Among various msbA suppressor-carrying strains in terms of viable colony size and number of transductants, the best suppression was observed when SR23711 (ΔlpxM msbA D498Y), SR23709 (ΔlpxM msbA S164C) and SR23707 (ΔlpxM msbA V287A) were used as recipients to bring in the lapD deletion (Table 2). A modest restoration of growth was also observed in strain backgrounds SR23705 (ΔlpxM msbA D431Y), SR23703 (ΔlpxM msbA M160I) and SR23701 (ΔlpxM msbA I177M) (Table 2). However, the colony size of transductants (although viable) with SR23699 (ΔlpxM msbA S120L) was smaller as compared to other msbA suppressor-carrying strains. It should be noted that Δ(lpxM lapD) is lethal in the absence of suppressor mutations mapping to the msbA gene. Thus, we can conclude that single amino acid substitutions that suppress the synthetic lethal phenotype of Δ(lpxM clsA) can also allow the growth of Δ(lpxM lapD) bacteria. Taken together, these results reveal an additional role of LapD in assisting MsbA-mediated LPS transport when the lipid A is either penta- or tetra-acylated. Thus, MsbA and LapD can collaborate in lipid A trafficking. This is more evident when mutant MsbA versions are examined, which are predicted to relax the carbon chain ruler or enhance ATP hydrolysis to accelerate LPS translocation (see Discussion section).

2.8. Absence of LapD Leads to Retention of LPS in the Inner Membrane

As presented in the above sections, LapD co-purifies with several proteins involved in either LPS biosynthesis or its translocation. Furthermore, LapD is absolutely required for
bacteria with either tetra- or penta-acylated lipid A as shown by the synthetic lethality of Δ(lpxl lapD) and Δ(lpxM lapD), respectively. Such underacylated LPS is poorly translocated by MsbA and, consistent with such results, suppressors that restore their growth were mapped to the msbA gene. Thus, we wondered if LPS in the absence of LapD is not efficiently translocated. To ascertain if indeed the absence of LapD results in defects in LPS translocation, isogenic cultures of wild type, ΔlapD, ΔwaaC, Δ(waaC lapD), ΔclsA and Δ(clsA lapD) were grown at permissive temperature and shifted to 42 °C for 2 h. After the harvesting of cultures by centrifugation, total cell extracts after the removal of soluble proteins were used to obtain IM and OM fractions using sucrose gradients. Pooled fractions from the IM were treated with Proteinase K. Such samples were analyzed on a 16% Tricine-SDS gel and LPS amounts were revealed by silver staining. Such experiments clearly show that very little LPS is retained in the IM in either the wild-type (Figure 10A) or ΔclsA (Figure 10B) or ΔwaaC strains (Figure 10C). However, a substantial amount of LPS was detected in the IM fraction of either ΔlapD or Δ(waaC lapD) or Δ(clsA lapD) bacteria (Figure 10). As an additional control, we also used LPS from IM fractions from Δ(tig) and Δ(tig lapD) derivatives (Figure 10C). Interestingly, a portion of LPS present in ΔlapD IM fractions also migrates much faster, resembling LPS of ΔwaaC bacteria, indicating the accumulation of premature species of LPS (Figure 10A, lane 2). These results show that LapD is required for efficient translocation of LPS and, in its absence, a significant portion of LPS is retained in the IM. Moreover, the absence of LapD results in the accumulation of LPS early intermediates. Thus, quite likely, LapD and MsbA cooperate in LPS translocation.

Figure 10. Lack of LapD causes the retention of significant amounts of LPS in the inner membrane. Total cell lysates obtained from isogenic derivatives of wild-type and ΔlapD bacteria were subjected to cellular fractionation to obtain the IM. Samples were treated with Proteinase K and resolved on a 16% Tricine-SDS gel. LPS was revealed by silver staining. The position of the LPS species is indicated by the arrow. Note the intense bands of LPS in the IM fraction of ΔlapD and its derivatives wt vs. ΔlapD (A), ΔclsA vs. Δ(clsA lapD) (B) and ΔwaaC, Δ(waaC lapD), Δtig, Δ(tig lapD) (C). The relevant genotype of strains used is indicated on the top of each panel.

2.9. Absence of LapD Causes the Constitutive Induction of LPS Defects Inducible RpoE-Dependent Stress Response

Previously, we have shown that severe defects in LPS biosynthesis, such as the synthesis of minimal LPS Kdo2-lipid IV_A or ΔwaaA or Δ(waaC lpxL lpxM lpxP), cause a constitutive induction of RpoE-dependent cell envelope stress response [9]. A similar induction of RpoE is also observed when LPS assembly is compromised by mutations in either the lapB gene or the lapC gene [17,21]. The RpoE regulon is known to control the expression of several genes whose products are involved in either OMP maturation, folding of envelope proteins or some steps in LPS translocation and assembly [46–48]. It is known that the signal of LPS defects stimulates transcription of the rpoEP3 promoter [49]. Thus, a lapD deletion was transduced in the wild-type strain carrying on the chromosome single-copy rpoEP3-lacZ fusion. To measure any impact on rpoE transcription, the isogenic wild-type strain carrying the rpoEP3-lacZ promoter fusion and its derivative were analyzed for the β-galactosidase...
activity when cultures were grown under permissive growth conditions. Measurement of the β-galactosidase activity reflecting the expression of the rpoE3 promoter activity showed a nearly 50% increase in strain with a deletion of the lapD gene under permissive growth conditions of 30 °C (Figure 11). As the rpoE3 promoter activity reflects the cellular response to LPS defects, it further establishes that LapD regulates LPS assembly and its absence causes LPS defects, which in turn induces the cell envelope stress response.

Figure 11. The absence of LapD causes the constitutive induction of the LPS defects responsive rpoE3 promoter, even under permissive growth conditions. Exponentially grown wild type and its ΔlapD derivative carrying the single-copy chromosomal rpoE3-lacZ fusion were analyzed for the β-galactosidase activity. Bacterial cultures were adjusted to an OD595 of 0.05 and allowed to grow at 30 °C. Aliquots of samples were taken to measure the β-galactosidase activity. Error bars represent an S.E of three independent measurements.

2.10. The RpoE-Regulated MicA sRNA Is Required for the Viability of ΔlapD Bacteria

The evidence presented so far shows that any severe compromise in LPS assembly induces the RpoE-dependent stress response and a deletion combination of lapD with mutations in genes whose products are involved in LPS assembly/synthesis are severely compromised for the growth. Besides investigating various null combinations, as described in Section 2.6, we also investigated if the absence of any non-essential RpoE regulon members is critical for the growth of ΔlapD bacteria. We specifically focused on genes encoding sRNAs whose transcription either requires the RpoE sigma factor or other sRNAs that regulate LPS modifications. Thus, several multiple deletion strain combinations with ΔlapD were analyzed for their growth properties. We show a specific requirement for the MicA sRNA when LapD is absent. MicA, although initially identified for its posttranscriptional repression of major OMPs such as OmpA, has also been implicated in regulating glycoform switches and is known to regulate the translation of phoP mRNA and, hence, is linked to the regulation of LPS synthesis or its non-stoichiometric modifications [11,13,50]. MicA by itself is dispensable for bacterial growth (Figure 12A). However, significantly, for Δ(micA lapD) bacteria, although viable under normal growth conditions (30–33 °C), their colony size and their ability to grow were significantly impaired as determined by spot-dilution assay (Figure 12A). Moreover, Δ(micA lapD) bacteria are unable to propagate at temperatures above 42 °C (Figure 12A), exhibiting a synthetic lethal growth phenotype. Importantly, this synthetic lethality and severe growth defects of Δ(micA lapD) bacteria can be overcome when LpxC stable variants (lpxC V37G, lpxC V37L, lpxC K270T, and lpxC fs306 stop codon) are introduced. For such experiments, thus SR23838 (lpxC V37G ΔlapD), SR23840 (lpxC V37L ΔlapD), SR23842 (lpxC K270T ΔlapD) and SR23844 (lpxC fs306 stop codon ΔlapD) served as recipients to bring in a deletion of the micA gene. Viable transductants with the normal colony size were obtained at either 30 or 33 °C in all LpxC stable variant backgrounds. Comparative growth analysis of such Δ(lapD micA) with LpxC stable variants revealed the complete restoration of growth at either 30 or 37 °C as compared to very poor growth of a Δ(lapD micA) derivative (Figure 12B). Even at 42 °C, all four such derivatives with the lpxC mutation show the restoration of growth as compared to the
lethality of a Δ(lapD micA) strain (Figure 12B). However, it should be noted that a (lpxC K270T Δ(lapD micA)) derivative forms relatively smaller-sized colonies at 42 °C. Thus, MicA presence is essential for the viability of ΔlapD bacteria, and increasing the stability of LpxC can rescue the lethal phenotype of the Δ(lapD micA) combination.

Figure 12. The essentiality of MicA sRNA in the ΔlapD background can be bypassed by mutations in the lpxC gene. Growth of isogenic cultures of strains of wild type, ΔlapD, ΔmicA and Δ(micA lapD) was quantified by spot dilution on LA at 33 and 43 °C (A). Growth of isogenic cultures of Δ(micA lapD) and its derivatives carrying single amino acid mutations in the lpxC gene was quantified by spot dilution on LA at 30, 37 and 42 °C (B). An immunoblot of whole cell lysates obtained from isogenic strains with indicated genotypes using LpxC-specific antibodies. An equivalent amount of total proteins was resolved by a 12% SDS-PAGE prior to immunoblotting (C). Note the slower migration of LpxC-cross-reacting species in lane 7 due to a frame-shift mutation in the lpxC gene that adds 20 amino acids at the C-terminus. The relevant genotype and temperature of incubation are indicated.

Next, we analyzed LpxC levels of various Δ(lapD micA) combinations in the presence of different lpxC suppressor mutations by Western blotting. Isogenic cultures of wild type, ΔlapD, ΔmicA, Δ(lapD micA) and Δ(lapD micA) derivatives with lpxC suppressor mutations were grown at 30 °C and shifted for 2 h at 42 °C. Equivalent amounts of total proteins were resolved by SDS-PAGE and immunoblotted with LpxC-specific antibodies. The results from such an analysis clearly show that Δ(lapD micA) derivatives carrying the lpxC suppressor mutation that restore the growth at 42 °C reveal increased accumulation of LpxC (Figure 12C). This experiment again shows that ΔlapD bacteria have reduced amounts of LpxC (Figure 12C, lane 2). Thus, these experiments support a model wherein LapD regulates LpxC stability as ΔlapD bacteria have less LpxC and the main defects of such mutant bacteria stem from such a defect in regulating LpxC amounts.
2.11. Multicopy Suppressor Analysis to Identify Factors That Could Be Limiting When LapD Is Absent

To further understand the function of LapD and the reasons for the Ts phenotype and the sensitivity towards antibiotics, such as vancomycin, when the cognate gene is absent, we employed a multicopy suppressor approach. This approach can identify genes that, when mildly overexpressed, can overcome Ts and vancomycin sensitivity and help in identifying factors that are limiting for bacterial growth when LapD is absent. Thus, we used a whole genomic library of all ORFs of E. coli wherein the expression of each gene is inducible from a tightly regulated Pt5-lac promoter [51]. Plasmid DNA of pooled plasmids from this library was introduced into ΔlapD bacteria by transformation. Transformants were plated at either 44 °C or on LA medium supplemented with vancomycin (125 µg/mL) in the presence of 75 µM IPTG at 37 °C. This concentration of IPTG as an inducer of gene expression has been previously optimized with this library where the expression of most of the genes is moderate and not toxic [17,40]. ΔlapD transformants that grew at either 44 °C or on vancomycin-supplemented growth medium were grown to obtain plasmid DNA. Such plasmid DNA was used to retransform ΔlapD bacteria to ascertain the restoration of growth under non-permissive growth conditions. Validated suppressors were retained and their plasmid DNA was sequenced to identify genes whose overexpression can suppress growth defects of ΔlapD bacteria. This analysis identified certain genes, prominent among being acpP, dksA, srrA, accB, yfgM, ymgG, artJ and artI, which restored the growth at 44 °C (Table 3). Among these, the most robust restoration of growth at elevated temperatures was observed when the acpP gene is moderately overexpressed. This suppression was further verified by a spot-dilution assay in the presence of 75 mM IPTG using isogenic cultures of ΔlapD bacteria carrying different plasmids as compared to when only an empty vector was present. Data from such experiments show varying degrees of growth restoration at high temperature, with the nearly wild-type-like growth restoration when the acpP gene is present on the plasmid (Figure 13). The acpP gene encodes the acyl carrier protein. In E. coli, the acyl carrier protein (AcpP) plays a central role by sequestering and shuttling the growing acyl chain between fatty acid biosynthetic enzymes and also in providing acyl chains to LpxA, LpxD, LpxL and LpxM lipid A biosynthetic enzymes [39]. Concerning other multicopy suppressors, we previously showed that overexpression of dksA and srrA genes, which encode transcriptional factors, can restore growth at elevated temperatures when the protein folding machinery (absence of peptidyl-prolyl cis/trans isomerases) is impaired [40]. Using the same multicopy suppression approach showed that overexpression of rcsF and rcsA genes can restore resistance to vancomycin of a ΔlapD strain (Table 3). RcsF and RcsA belong to the two-component system that induces the expression of genes whose products are involved in colanic acid biosynthesis [52]. RcsF, located in the OM, can also sense perturbations in LPS biosynthesis and induce the signal of stress response [49,53]. Identification of AcpP as a multicopy suppressor of ΔlapD bacteria again reinforces the notion of the critical role played by LapD in LPS/fatty acid biosynthesis; although, which of the acceptors of AcpP are limiting requires further investigation.
Overexpression of specific genes, including the \textit{acpP} gene, restores the growth of \textit{\textDelta lapD} bacteria at 44 \degree C. Growth of isogenic cultures of \textit{\textDelta lapD} bacteria transformed with either plasmid DNA of the vector alone or when the specific multicopy suppressing gene is present on the plasmid was quantified by spot dilution on LA at 37 and 44 \degree C. The relevant genotype and temperature of incubation are indicated.

### Table 3. Identification of the most relevant multicopy suppressors of \textit{\textDelta lapD} strains.

| Name  | Number of Transformants | Function                                |
|-------|-------------------------|-----------------------------------------|
|       | LA 44 \degree C  |
|       | vancomycin  |
| vector alone | 9 small colonies | 6 small colonies |
| \textit{acpP}   | 750              | ND 1                                   |
| \textit{dksA}   | 486              | ND                                     |
| \textit{srrA}   | 520              | ND                                     |
| \textit{yfgM}   | 430              | ND                                     |
| \textit{accB}   | 511              | ND                                     |
| \textit{rcsF}   | ND               | 701 medium size colonies               |
| \textit{artJ}   | 473              | ND                                     |

\textsuperscript{1} ND denotes not determined since suppressors were isolated either at 44 \degree C or on vancomycin-supplemented growth medium at 37 \degree C.

#### 2.12. Impact on \textit{LpxC} Levels upon Overexpression of Genes That Overcome the Ts Phenotype of \textit{\textDelta lapD} Bacteria

As LapD absence results in the Ts phenotype with a concomitant reduction in \textit{LpxC} levels, we examined levels of \textit{LpxC} by immunoblotting. Thus, using total cell extracts from the wild type and its isogenic \textit{\textDelta lapD} derivatives with either an empty vector or when carrying an inducible gene whose overexpression restores the growth at high temperatures. Bacterial cultures were grown under permissive growth conditions at 30 \degree C and the gene expression was induced with the addition of 75 \textmu M IPTG at \textit{OD}_{595} 0.1. After 15 min of IPTG addition, an equivalent portion of each culture was shifted to 43 \degree C, and cultures were harvested after an additional incubation for 2 h. The equivalent amount of proteins was resolved on a 12\% SDS-PAGE, and \textit{LpxC} was detected by immunoblotting with \textit{LpxC}-specific antibodies. At both 30 and 43 \degree C, LapD with the vector alone had a reduced amount of \textit{LpxC} (Figure 14A, lane 2). Most significantly, only overexpression of the \textit{srrA} gene was found to restore \textit{LpxC} levels to nearly wild-type levels, particularly at 43 \degree C (Figure 14, lane 6). At 30 \degree C, also, overexpression of the \textit{srrA} gene shows a modest increase in \textit{LpxC} amounts. Surprisingly, overexpression of the \textit{acpP} gene, which confers the best suppression at elevated temperatures, did not cause any restoration of \textit{LpxC} amounts (Figure 14, lane 3). Thus, at least, we can explain that SrrA overproduction can suppress the Ts phenotype by restoring \textit{LpxC} amounts. The precise function of SrrA remains unknown, except that it was also identified as a multicopy suppressor that can restore the growth of strains lacking PPlases at high temperature [40].

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**Figure 13.** Overexpression of specific genes, including the \textit{acpP} gene, restores the growth of \textit{\textDelta lapD} bacteria at 44 \degree C. Growth of isogenic cultures of \textit{\textDelta lapD} bacteria transformed with either plasmid DNA of the vector alone or when the specific multicopy suppressing gene is present on the plasmid was quantified by spot dilution on LA at 37 and 44 \degree C. The relevant genotype and temperature of incubation are indicated.
The quantification of the q-RT-PCR analysis was undertaken using gene-specific oligonucleotides for the synthesis of cDNA. For such experiments, total RNA was extracted from isogenic strains of bacteria grown either at 30 °C, following a temperature shift for 2 h at 43 °C (A) or when grown at 30 °C (B). For immunoblotting, LpxC-specific antibodies were used and the relevant genotype is indicated. An equivalent amount of total proteins was resolved by an SDS-PAGE prior to immunoblotting. Note the restoration of LpxC levels when the srrA gene is overexpressed, particularly at 43 °C.

2.13. SrrA Does Not Regulate Transcription of the lpxC Gene

SrrA bears features of a transcriptional regulator with a conserved helix-turn-helix motif [40]. Thus, to determine if SrrA directly controls the expression of the lpxC gene, q-RT-PCR analysis was undertaken using gene-specific oligonucleotides for the synthesis and quantification of cDNA. For such experiments, total RNA was extracted from isogenic cultures of wild-type and ΔsrrA bacteria grown at 37 °C. In parallel, RNA was also extracted from wild-type bacteria transformed with either the empty vector DNA alone or with plasmid DNA carrying the inducible srrA gene after a transient shift to 43 °C. The quantification of the lpxC transcription pattern showed nearly similar abundance of lpxC transcripts between the wild-type and ΔsrrA bacteria (Figure 15). A shift to 43 °C showed a minor increase in lpxC transcripts in the wild type with the empty vector as well as when the expression of the srrA gene was induced (Figure 15). Thus, we can conclude that SrrA does not directly regulate lpxC transcription and that the increased accumulation of LpxC when SrrA is overproduced occurs at a post-transcriptional level.

Figure 15. Transcription of the lpxC gene is not positively regulated by SrrA. q-RT-PCR analysis of mRNA extracted from wild-type bacteria, its ΔsrrA derivative and transformed with either the vector DNA alone or when the srrA gene expression is induced from the IPTG-inducible promoter present in the plasmid. Isogenic bacterial cultures were grown with or without temperature shifts at the indicated temperatures. Data presented are from RNA isolated from three biological replicates and error bars are shown.
2.14. Catalytic Activity of AcpP Is Required for Its Multicopy Suppression of Growth Defects of ΔlapD Bacteria

As mentioned above, the acyl carrier protein (AcpP) plays key roles in the fatty acid and lipid A synthesis systems by mediating acyl group delivery and shuttling. ACP function requires the modification of the protein by the attachment of 4′-phosphopantetheine to a conserved Ser 36 [39,54,55]. The phosphopantetheine thiol acts to tether the starting materials and intermediates as their thioesters. Thus, in E. coli, AcpP is functional only in LPS and fatty acid biosynthesis after it has been posttranslationally modified by the covalent attachment of a 4′-phosphopantetheinyl (4′-PP) moiety [56]. As the acpP gene was identified as a multicopy suppressor of the Ts phenotype of ΔlapD bacteria, we tested if this suppression by AcpP requires it to be catalytically active. Thus, plasmid DNA of the pBAD24 vector containing either the acpP S36C gene or the acpP S36T gene was introduced into ΔlapD bacteria by transformation. In parallel, ΔlapD bacteria transformed with a cloned wild-type acpP gene or with the vector alone were used as controls. Such isogenic cultures were cultivated in the presence of glucose (0.3%) under permissive growth conditions and tested for the restoration of growth at 44 °C in the presence of 0.05% arabinose using a spot-dilution assay. The concentration of inducer arabinose was kept deliberately low since it is known that excess of ACP is toxic to bacteria. Results from such experiments reveal that the induction of expression of acpP S36C and acpP S36T cannot suppress the Ts phenotype of ΔlapD bacteria, while the induction of expression of the wild-type acpP gene can restore the growth under identical conditions (Figure 16). Results from such an experiment allow us to conclude that quite like the requirement of Ser 36 residue of AcpP in mediating acyl chain transfer in fatty acid biosynthesis, this catalytic activity is also required for AcpP to act as a dosage-dependent suppressor of ΔlapD bacteria. Thus, although AcpP is a very abundant protein, its increased amounts are required when LapD is absent to carry out its normal function to shuttle a growing acyl chain between biosynthetic enzymes. However, since AcpP also interacts with many other proteins that are not directly involved in the fatty acid synthesis, further experiments are required to identify the partner(s) of AcpP that are limiting in the absence of LapD.

![Figure 16. Multicopy suppression of ΔlapD bacteria by the acpP gene requires its product to retain its active site Ser 36 amino acid residue. Growth of isogenic cultures of ΔlapD bacteria transformed with either plasmid DNA of the vector alone or with plasmids carrying the wild-type acpP gene or its active site variants. The expression of the acpP gene is induced by the addition of 0.05% arabinose. Bacterial growth was quantified by a spot-dilution assay on LA at 30 and 44 °C. The relevant genotype and temperature of incubation are indicated.](image)

2.15. LapD Is Required for Bacteria That Lack Six Major Cytoplasmic Peptidyl-Prolyl Cis/Trans Isomerases, Which Is Due to a Specific Requirement for Trigger Factor

The cytoplasm of E. coli contains six well-characterized peptidyl-prolyl cis/trans isomerases (PPIs), which include PpiB, Tig, SlyD, FkpB, FklB and PpiC [57]. We also recently described that DksA, Cmk and MetL exhibit the PPIase activity that can be inhibited by FK506 [40]. As DksA is a multicopy suppressor of Δ6ppi and also of ΔlapD bacteria, we
examined if LapD is required when PPIs are individually or collectively absent. This was further necessitated since FklB was found to co-purify with LapD (Figure 3). Furthermore, some of the lipid A biosynthetic enzymes are known to aggregate in Δ6ppi strains [57]. Thus, a systemic series of bacteriophage P1-mediated transductions were executed using Δ6ppi bacteria as recipients. No viable transductants Δ(6ppi lapD) were obtained under conditions when Δ6ppi strains can grow (Table 4). Regarding individual PPI encoding genes, normal transductants were obtained when deletion derivatives of ppiC, fkpB or slyD served as recipients (Table 4). Δ(fklB lapD), although viable, formed smaller-sized colonies. However, severe growth defects were observed when the growth properties of Δ(tig lapD) were analyzed (Table 4, Figure 8). Δ(tig lapD) bacteria exhibited a nearly 100-fold reduction in cfu at 30 and 37 °C and with more than 10³-fold reduction at 42 °C. In all conditions, the colony size was severely reduced, revealing a synthetic sick phenotype of Δ(tig lapD) bacteria. Regarding Δ(slyD lapD) derivative, although viable up to 42 °C, a reduction in colony size was observed at elevated temperatures (Table 4). In contrast, no viable transductants were obtained when a lapD deletion was introduced in a strain lacking the ppiB gene. As the lethality of Δ(ppiB lapD) was unexpected, we reasoned that the ppiB deletion could be polar on the downstream essential lpxH gene. Consistent with such a presumption, ΔlapD could be readily introduced when ΔppiB carrying the lpxH gene on a plasmid was used as a recipient (Table 4).

Table 4. Requirement of LapD in strains lacking cytoplasmic PPIases.

| Name                  | P1 ΔlapD | Number of Transductants LA 33 °C | Features                                      |
|-----------------------|----------|----------------------------------|-----------------------------------------------|
| wt                    |          | 1300                             | viable                                        |
| Δ6ppi                 |          | 5                                | not viable                                    |
| ΔppiB                 |          | 9                                | not viable                                    |
| ΔppiB + ppxH⁺         |          | 920                              | viable                                        |
| ΔppiC                 |          | 1430                             | viable                                        |
| Δtig                  |          | 230 sc ¹                         | conditional lethality at high temperatures     |
| ΔfklB                 |          | 490 sc                           | viable but smaller colony size                 |
| ΔslyD                 |          | 735                              | viable                                        |
| ΔslyD                 |          | 506                              | viable but colony size reduced above 42 °C     |

¹ sc indicates small colony size.

As DksA and Cmk exhibit a weak PPIase activity and their overproduction can restore Δ6ppi bacterial growth on rich medium at elevated temperature, we also examined their requirement. Viable transductants at a normal frequency could be obtained when a lapD deletion was introduced in a ΔdksA background at either 33 or 37 °C; however, the colony size of Δ(dksA lapD) bacteria is highly heterogeneous (Table 4). Significantly, Δ(cmk lapD) turned out to be lethal.

Taken together, we can conclude that the lethality of ΔlapD in Δ6ppi can mainly be attributed to a requirement of Tig and a deletion of the ppiB gene is not tolerated due to the polar effect on the expression of the downstream lpxH gene. Reduction in the amounts of LpxH can reduce lipid A synthesis and such results are consistent with the essentiality of LapD in strains with point mutations in genes required for the early steps of lipid A biosynthesis.

3. Discussion

The pivotal enzyme LpxC catalyzes the first committed step in LPS biosynthesis and the regulation of LpxC turnover is key to maintaining a balance between phospholipid and LPS biosynthesis [15,17,58]. LpxC is an unstable protein and its proteolysis is regulated by the FtsH-LapB complex [17]. This FtsH-LapB proteolysis is adjusted to match the demand for the LPS synthesis by a negative control exerted by LapC [21,22]. To fine-tune LpxC amounts, the HslVU protease complex can also degrade LpxC, which could be particularly
utilized under heat shock conditions since genes encoding these proteases are regulated at the transcriptional level by the RpoH sigma factor [21]. However, we still lack complete knowledge of LpxC regulation by FtsH-LapB and LapC in terms of how they sense the LPS concentration and if they recruit any additional partners. It is also not known what are the contributions of different signals that either enhance LpxC degradation or rather render it resistant to proteolysis. Regulation of the LpxC amounts also depends on: the accumulation of precursor components of lipid A biosynthesis, levels of acyl-ACP pools, acyl-CoA, the fatty acid synthesis, growth-rate-dependent proteolysis and their individual contributions remain poorly understood [2,15,16,18,19]. In this work, we started by performing a more elaborated analysis of the LapB interactome, which revealed LapD inner membrane protein as a new additional partner that physically interacts with LapA and LapB proteins. This physical interaction was further substantiated when LapD was purified. The gene lapD, previously yhcB, was earlier identified in a screen for genes whose products are required for growth at high temperatures [34], which was again confirmed in recent studies [38]. Purification of LapD provided strong clues that LapD could be involved in LPS assembly and biosynthesis of membrane lipids since most of the LapD interactome members either participate in the LPS synthesis/transport or are involved in fatty acid biosynthesis. It needs to be emphasized that, in this study, we again observe that LapB serves as a key hub of interaction coupling LPS biosynthesis with transport. Additionally, LapB also links LpxC degradation rate with phospholipid biosynthesis since FabZ dehydratase mediating the first committed step in this pathway was also found to co-purify with FabZ. This is consistent with the previous immunoprecipitation of FabZ with LapB [17].

Besides the co-purification of LapD with LPS assembly proteins (LapA/LapB) and several proteins involved in LPS biosynthesis/transport, we carried out a systematic genetic and biochemical analysis to elaborate on the LapD function. Our data provide strong evidence that LapD plays an important role in the LPS assembly/transport and regulating LpxC amounts. This is based on: (i) An absence of LapD results in a reduction in LpxC amounts and the sensitivity towards vancomycin (membrane permeability defect). (ii) Mutations that reduce the LPS synthesis, such as lpxA2(ts), are synthetically lethal in ΔlapD bacteria. (iii) In converse, mutations that either stabilize LpxC due to mutations in the lpxC gene or prevent LpxC degradation (loss-of-function mutations in either the fisH gene or the lapB gene) restore vancomycin resistance in ΔlapD bacteria. These very mutations in lpxC or fisH or lapB genes were earlier shown to suppress the Ts phenotype of lapC mutants lacking its periplasmic domain and restore LpxC amounts. Thus, ΔlapD phenocopies a lapC190 mutation that has a truncation of the periplasmic domain and also results in reduced amounts of LpxC. These results suggest that, quite like LapC, LapD acts upstream of LapB-FtsH in regulating LpxC levels. (iv) A deletion of the lapD gene is synthetically lethal with the absence of either LpxL (lauroyl acyl transferase) or LpxM (myristoyl acyl transferase). LpxL and LpxM are known to sequentially use Kdo2-lipid IV_A as a substrate to generate hexa-acylated Kdo2-lipid A. Critically, it is known that tetra-acylated lipid A species are selected at 1000-fold reduced efficiency by MsbA for their transport and penta-acylated lipid A derivatives could as well be transported poorly by MsbA. Thus, the synthetic lethality of Δ(lpxL lapD) and Δ(lpxM lapD) posits LapD’s involvement in LPS transport. (v) Consistent with the proposed role of LapD in LPS transport, suppressors that relieve the lethality of Δ(lpxL lapD) and Δ(lpxM lapD) bacteria map to the msbA gene. To strengthen the notion of LapD assisting MsbA-mediated transport of LPS, previously well-established suppressor mutations that restore the growth of strains synthesizing tetra-acylated LPS Δ(waaC lpxL lpxM lpxP) such as MsbA D498Y also suppress the lethality of Δ(lpxM lapD) bacteria. Similarly, all suppressors mapping to the msbA gene that relieve synthetic lethality of Δ(lpxM clsA) also confer the viability to Δ(lpxM lapD) bacteria. All such suppressor mutations are predicted to map either in the ATP-binding site of MsbA or are located in lipid A-binding/exit portals [32]. Such mutations could enhance lipid A trafficking by increasing the ATPase activity and altering the carbon chain ruler properties of MsbA, conferring a relaxed specificity to transport underacylated LPS [29–32].
(vi) Consistent with a predicted role in LPS assembly/transport, ∆lapD bacteria retain a substantial fraction of LPS in the IM, which is not the case when either WaaC or ClsA is absent, particularly when shifted to elevated temperatures. (vii) Deletion derivatives of lapD that synthesize only Kdo3-lipid A LPS due to lack of WaaC heptosyltransferase I are synthetically lethal at 42 °C and are very poorly tolerated even at 30 °C. The same synthetic lethal phenotype is observed when the cardiolipin synthase A encoding gene is removed in a ∆lapD background. Thus, defects in either early steps of LPS core biosynthesis or underacylation of lipid A and disturbance of glycerophospholipid are not tolerated when LPS assembly is impaired in the absence of LapD. However, how LapD regulates LpxC amounts via interaction with LapB needs further detailed studies, and possible mechanisms are discussed below.

Additional support for the requirement of LapD in LPS biogenesis and maintaining the cell envelope homeostasis comes from experimental evidence that ∆lapD bacteria exhibit a constitutive induction of the rpoEP3 promoter even under permissive growth conditions. Transcription of the rpoE gene is directed from six promoters, out of which the rpoEP3 promoter responds specifically to LPS defects [49]. The RpoE sigma factor regulates transcription of several genes, whose products are required for either OMP maturation (surA, RpaA and skp), some steps in LPS modifications (eptB), LPS translocation (some of the lpt genes), the quality control in the periplasm (degP) and a long operon that includes fabZ and lpxD genes [46, 48, 59]. The constitutive induction of the RpoE regulon could stem from LPS defects, which can also cause changes in OMP maturation. RpoE is also required for the transcription of micA, rypB and strA sRNAs, constituting the non-coding repressing arm of this regulatory system [17, 50]. Quite interestingly, we show that MicA sRNA becomes essential in the absence of LapD. Although a Δ(micA lapD) strain can be constructed, such bacteria grow extremely poorly with a small colony size in the temperature range of 30–37 °C and such bacteria are not viable at 42 °C. Since the major defect of ΔlapD bacteria is a reduction in LpxC amounts, the introduction of mutations in the lpxC gene that render encoding mutant proteins resistant to proteolysis can tolerate Δ(micA lapD) even at 42 °C. MicA is known to repress the synthesis of major OMPs such as OmpA and non-OMP targets such as PhoP/Q at the posttranscriptional control of gene expression [13, 14, 60]. A deletion of the micA gene in the ∆lapD background could thus lead to the alteration in the amounts of OMPs and relieve the repression of phoPQ mRNA translation. The PhoP/Q two-component system regulates lipid A modifications and also positively regulates transcription of the mgrR sRNA encoding gene, which represses the expression of the eptB gene whose product is required for the modification of the second Kdo [11, 13, 61]. However, how MicA absence limits LpxC amounts remains to be addressed. Thus, any major perturbation in OMP composition and in either lipid A biosynthesis or the truncation of the core region of LPS, and even potential non-stoichiometric alterations in the lipid A region of LPS are not tolerated in the absence of LapD.

While addressing the cellular requirement of LapD, we found that ∆lapD could not be introduced into strains lacking six known major cytoplasmic PPIases. This essentiality of LapD in the Δ6ppi derivative was not surprising since we have earlier shown that several enzymes involved in lipid A and phospholipid biosynthesis aggregate when all six PPIs are absent [57]. This essentiality could be attributed specifically to Tig and, to some extent, to FklB. Tig PPIase acts as a nascent chain ribosome-associated chaperone with the PPIase activity, and it has several substrates and β-barrel outer-membrane proteins constitute its most prominent substrates [62]. Thus, the absence of Tig could accentuate defects in OMP maturation and hence its essentiality in ∆lapD bacteria. As a consequence, Tig not only shows the synthetic lethality in a ΔdnaK or ΔdnaKJ background [63, 64] but also in the absence of LapD. Δ(fklB lapD) bacteria, although viable, form small colonies, which is consistent with the co-purification of FklB with LapD. In line with such findings, overexpression of the dksA gene that overcomes growth defects of either Δ6ppi strains or a ΔdnaKJ derivative [40, 65] was also found to suppress the Ts phenotype of ΔlapD bacteria. Quite interestingly, another multicopy suppressor of Δ6ppi bacteria, srrA [40], was also found...
to suppress the Ts phenotype of ΔlapD bacteria. SrrA is predicted to be a transcriptional regulator; however, genes whose expression it regulates have not been identified thus far and are currently being investigated. More related to this work, SrrA overproduction restored LpxC levels to nearly wild-type levels in ΔlapD bacteria, without increasing lpxC transcription. Thus, SrrA could regulate the expression of some genes whose products enhance either LpxC stability or prevent its degradation. Of further interest, another multicopy suppressor of Δ6ppi, encoded by the cmk gene, becomes indispensable in the ΔlapD background. Cytidylate kinase Cmk phosphorylates CMP and dCMP, which are produced by the turnover of CDP diglycerides and nucleic acids [66,67]. It is well established that CTP and dCTP, besides being precursors for nucleic acid synthesis, are also involved in phospholipid biosynthesis. This provides a rationale explanation for the synthetic lethal phenotype of a Δ(lapD cmk) combination.

Besides looking for extragenic single-copy chromosomal suppressors, we also undertook a multicopy suppressor approach that can rescue the Ts or vancomycin-sensitive phenotype of ΔlapD bacteria. Most interestingly, we found that a mild overexpression of acyl carrier protein encoded by the acpP gene can effectively restore the growth of a ΔlapD strain at elevated temperatures. An acyl carrier protein is a universally conserved carrier of acyl intermediates during fatty acid synthesis [39]. The major destinations of fatty acids in bacteria are glycerophospholipids, present in the IM and the inner leaflet of OM, and the lipid A part of LPS. Identification of the acpP gene as a multicopy suppressor of ΔlapD bacteria is intriguing since ACP is one of the most abundant proteins in E. coli, comprising nearly 0.25% of the total soluble protein [68]. Since long-chain acyl-ACPs represent only a small proportion of the total ACP pool, it is likely that in ΔlapD bacteria there is an alteration in the destination of acyl products, which could alter the ratio between saturated and unsaturated fatty acids. However, more studies are required to address such issues. Since the synthesis of hexa-acylated lipid A requires four ACP-dependent acyltransferases, namely, LpxA, LpxD, LpxL and LpxM, we also examined if LpxC amounts that are reduced in ΔlapD bacteria are altered by the induction of acpP gene expression. Estimation of LpxC levels did not show any restoration when the acpP gene was overexpressed. Despite such results, we find that for the multicopy suppression of ΔlapD Ts phenotype by AcpP, it is required for it to be catalytically active. This was demonstrated by mutational alteration of the active site residue Ser36 of AcpP, which is the site of prosthetic group attachment. A substitution of Ser36 by either Thr or Cys residue abrogates the suppressing ability of AcpP. Since the 4′-PP prosthetic group is attached to the hydroxyl group of a centrally located Ser36 residue by the AcpS 4′-PP transferase, any replacement of this residue results in the loss of function of AcpP in shuttling acyl chains in fatty acid biosynthesis [69]. However, it is also pertinent to point out that AcpP is known to interact with more than three dozen proteins, and all of them are not involved in fatty acid metabolism, and hence a more detailed study is required to further understand the mechanism of suppression by acpP overexpression. The co-purification of LapD with proteins involved in LPS and fatty acid biosynthesis pathways also identified many proteins that are also known to be part of the AcpP interactome, including LpxM, PssA and Fab enzymes. Besides these proteins, co-purifying proteins such as MukB are also known to interact with ACP [70,71]. It is likely that AcpP may also show some physical interaction with LapD and may account for some phenotypes related to cell division/chromosome segregation.

During the progression of this study, it has been suggested that LapD (YhcB) plays a role in cell division based on morphological defects and also the co-purification with some components of cell shape determination and cell division [37]. We also observed that ΔlapD bacteria exhibit filamentous morphology, and our own co-purification results show the interaction with ZapD, Muk proteins and MreC. Earlier studies based on the bacterial two-hybrid system have also found that LapD interacts with MreC, RodZ and LapA [35]. However, subsequent studies did not find RodZ–LapD interaction [38]. At the same time, it is important to point out that several defects in LPS biosynthesis and assembly also lead to filamentous morphology, as shown for gmhD, waaC, lpxL and lapB, (waaC lpxL lpxM lpxP)
mutant bacteria [9,43]. Our studies do not rule out a direct link between LapD and cell division machinery; however, our suppressor approach clearly shows suppressors that either increase LpxC amounts (mutations in lpxC, ftsH and lapB) or enhance LPS translocation (msbA suppressor mutations) support direct participation of LapD in LPS assembly. Another study using whole genome transposon mutagenesis approaches also proposes that LapD (YhcB) functions at the junction of several envelope biosynthetic pathways including peptidoglycan biogenesis [36]. Some phenotypes, such as defects in biofilm formation of ΔlapD mutant bacteria [72], can be explained as an indirect consequence of the alteration in LPS amounts.

The model for LapD function: Based on data presented in this work, we propose that LapD functions upstream of LapB in the regulation of LpxC turnover since suppressors mapping to either the lapB gene (loss of function) or stable variants of LpxC that are resilient to proteolysis by FtsH overcome vancomycin sensitivity and Ts phenotype in certain combinations when LapD is absent (Figure 17). This places LapD at a junction that has so far only been assigned to LapC as an antagonist of LapB (Figure 17). All the genetic data support such a model. This model is further supported by the observed physical and genetic interactions between LapB and LapD. However, LapD function may be specifically required under conditions such as when bacteria enter a stationary phase or when OM asymmetry is compromised. In support of such a model, the stationary phase-regulated sRNA (toxin) SdsR has been shown to repress the synthesis of LapD leading to cell lysis upon its overproduction [73]. For LapD- and LapC-mediated regulation of LpxC, we need to understand some major differences. While LapC is essential, LapD is required for bacterial growth at elevated temperatures and when challenged with antibiotics such as vancomycin. The IM anchor of LapC is essential and required for the interaction with LapB, but the function of the LapD IM region could be dispensable. Although we have not addressed the requirement of the N-terminal single IM anchor of LapD, some reports find it to be dispensable [36,38], while another report suggests its requirement for LapD functionality [37]. We suggest that the LapD soluble domain could interact with the LapB cytoplasmic domain containing TPR (Tetratricopeptide Repeat) elements. Several mutations in TPR elements of LapB exhibit loss-of-function properties and structural alterations [17,21,74]. In such an interaction, LapD could act as an anti-adaptor protein, preventing excessive degradation of LpxC for proteolysis by the FtsH-LapB complex (Figure 17). In support of such a model, several loss-of-function single amino acid mutations in TPR repeats of LapB were earlier shown to suppress growth defects of lapC mutant bacteria [21] and in this work were shown to restore vancomycin sensitivity of ΔlapD bacteria as well. Alternatively, it is also possible that LapA and LapB TM regions could interact with LapD, preventing FtsH-mediated proteolysis of LpxC. Since suppressors of Δ(lpxL lapD) and Δ(lpxM lapD) map to the msbA gene, LapD could assist MsbA in selecting underacylated lipid A derivatives (Figure 17). However, a biochemical proof for such a LapD-MsbA interaction needs to be established. In this process of interaction with MsbA, LapC involvement is not known. LapD and cardiolipins may act similarly in assisting MsbA-mediated transport as suppressors of Δ(lpxM clsA) lethality mapping to the msbA gene also suppress the Δ(lpxM lapD) lethality. Consistent with such a role, ΔlapD bacteria retain significant amounts of LPS. This retention of LPS in the IM in ΔlapD bacteria makes it different from ClsA-MsbA assisting LPS transport since ΔclsA bacteria do not exhibit any enhanced retention of LPS, as observed in the case with the absence of LapD. Thus, in summary, we propose that LapD plays an important role in LPS assembly by regulating LpxC degradation, acting as an antagonist of LapB, and in assisting MsbA-mediated LPS translocation across the IM. As LapD is conserved in gamma-proteobacteria, this model of LpxC regulation and LPS transport could be applicable to all such bacteria in general.
Figure 17. Model of LapD action in regulating LpxC levels and in assisting MsbA-mediated LPS translocation. Based on the co-purification of LapD with the LapA/LapB-FtsH complex and a reduction in LpxC amounts in the absence of LapD, it is proposed that LapD forms a complex in the IM to regulate LpxC amounts. As suppressors mapping to lapB, ftsH and lpxC genes restore growth defects of ΔlapD bacteria, LapD can act upstream of LapB as an antagonist of FtsH-mediated degradation of LpxC. This role could be similar to that earlier proposed for LapC [2]. As Δ(lpxL lapD) and Δ(lpxM lapD) are synthetically lethal and this lethality is overcome by mutations in the msbA gene, LapD can also assist MsbA-mediated transport of underacylated LPS species.

4. Materials and Methods

4.1. Bacterial Strains, Plasmids and Media

The various bacterial strains and plasmids used in this study are described in Table 5. Luria-Bertani (LB) broth (Difco, Franklin Lakes, NJ, USA), M9 (Difco) and M9 minimal media were prepared as described previously [9,17]. Whenever required, growth media were supplemented with kanamycin (50 µg/mL), ampicillin (100 µg/mL), chloramphenicol (10 or 30 µg/mL), tetracycline (10 µg/mL) and vancomycin (125 µg/mL). All strains used in this study were derived from E. coli K-12 BW25113 strain [75], unless indicated. The construction of some of the deletion derivatives used in this study, ΔlapB, ΔwaaC, Δtig, ΔdksA, ΔsrrA, ΔmicA, Δcmk, ΔclsA, ΔlpxL, ΔlpxM and lapC190, has been previously described [9,17,21,32,40]. A non-polar ΔlapD antibiotic-free deletion mutation was constructed by using the λ Red recombinase/FLP-mediated recombination system [75]. The kanamycin resistance cassette was amplified using pKD13 as a template [75]. A PCR product from
such an amplification reaction was electroporated into BW25113 containing the λ Red recombinase-encoding plasmid pKD46. The aph cassette was removed using the plasmid pCP20 expressing FLP recombinase at either 30 °C or 33 °C. Isogenic multiple deletion combinations were constructed using bacteriophage P1-mediated transductions at either
30 or 33 °C.

Table 5. Bacterial strains and plasmids used in this study.

| Strains          | Genotype                          | Reference |
|------------------|-----------------------------------|-----------|
| BW25113          | lacI trpB T14 ΔlacZ W14 hsdR514 ΔaraBAD AH33 ΔrhaBAD LCD8           | [75]      |
| SR23678          | BW25113 lapD<>aph                  | This study|
| SR23743          | BW25113 lapD<>frt                 | This study|
| SR17532          | BW25113 φ(pcoEP3-lacZ)            | [9]       |
| SR23686          | SR17532 lapD<>saph                | This study|
| SR8233           | W3110 waaC<>cat                   | [9]       |
| SR23691          | SR23678 waaC<>cat                 | This study|
| SR23320          | BW25113 claA<>cat                 | This study|
| SR23769          | SR32320 lapD<>saph                | This study|
| SR8522           | W3110 micA<>cat                   | [11]      |
| SR23852          | BW25113 micA<>cat                 | This study|
| SR23900          | SR23678 micA<>cat                 | This study|
| SR21068          | BW25113 tig<>cat                  | [57]      |
| SR23773          | SR21068 lapD<>saph                | This study|
| SR23684          | Δ(lpxL lapD) msbA L412P            | This study|
| SR23685          | Δ(lpxM lapD) msbA V287A            | This study|
| SR23699          | BW25113 lpxM msbA S120L           | This study|
| SR23701          | BW25113 lpxM msbA I177M           | This study|
| SR23703          | BW25113 lpxM msbA M160I           | This study|
| SR23705          | BW25113 lpxM msbA D431Y           | This study|
| SR23707          | BW25113 lpxM msbA V287A           | This study|
| SR23709          | BW25113 lpxM msbA S164C           | This study|
| SR23711          | BW25113 lpxM msbA D498Y           | This study|
| GK6075           | BW25113 lapC190                   | [21]      |
| GK6078           | lapC190 lpxC K270T                | [21]      |
| GK6094           | lapC190 lpxC fs306 stop codon     | [21]      |
| SR22727          | lapC190 lpxC R230C                | [21]      |
| SR22731          | lapC190 lpxC V37G                 | [21]      |
| SR22738          | lapC190 lpxC V37L                 | [21]      |
| GK6095           | lapC190 fshA A296V                | [21]      |
| SR22724          | lapC190 lapB H325P                | [21]      |
| SR22726          | lapC190 lapB A88V                 | [21]      |
| SR22730          | lapC190 lapB H181R                | [21]      |
| SR22733          | lapC190 lapB R115H                | [21]      |
| GK6084           | lapC190 lapB D124Y                | [21]      |
| GK6087           | lapC190 lapB R125L                | [21]      |
| SR23812          | lpxC R230C                        | This study|
| SR23814          | lpxC V37G                         | This study|
| SR23816          | lpxC V37L                         | This study|
| SR23818          | lpxC K270T                        | This study|
| SR23820          | lpxC fs306 stop codon             | This study|
| SR23822          | fshA A296V                        | This study|
| SR23836          | lpxC R230C lapD<>aph              | This study|
| SR23838          | lpxC V37G lapD<>aph               | This study|
| SR23840          | lpxC V37L lapD<>aph               | This study|
| SR23842          | lpxC K270T lapD<>aph              | This study|
Table 5. Cont.

| Strains   | Genotype            | Reference       |
|-----------|---------------------|-----------------|
| SR23844   | lpxC fs306 stop codon lapD<>aph | This study     |
| SR23905   | SR23840 micA<>cat | This study     |
| SR23907   | SR23842 micA<>cat | This study     |
| SR23909   | SR23844 micA<>cat | This study     |
| SR23911   | SR23838 micA<>cat | This study     |
| SR23846   | fshA A296V lapD<>aph | This study     |
| SR23857   | lapB H325P lapD<>aph | This study     |
| SR23859   | lapB A88V lapD<>aph | This study     |
| SR23861   | lapB H181R lapD<>aph | This study     |
| SR23863   | lapB R115H lapD<>aph | This study     |
| SR23865   | lapB D124Y lapD<>aph | This study     |
| SR23867   | lapB R125L lapD<>aph | This study     |
| SR22995   | SR23138 BW25113 oppA<>ada | This study     |
| SM101     | lpxA2(ts) | CGSC, Yale |
| MN7       | lpxB1(ts) | CGSC, Yale |
| SR23798   | SR23678 + pCA24N | This study     |
| SR23790   | SR23678 + pacpP+ | This study     |
| SR23784   | SR23678 + pufgM+ | This study     |
| SR23786   | SR23678 + pkdsA+ | This study     |
| SR23792   | SR23678 + pssrA+ | This study     |
| SR23796   | SR23678 + pacCD+ | This study     |
| SR23794   | SR23678 + pygG+ | This study     |
| SR23788   | SR23678 + parf+ | This study     |
| SR23792   | SR23678 + pEB540 pBAD-CBP-ACP | This study     |
| SR23732   | SR23678 + pEB547 pBAD-CBP-ACP (S36C) | This study     |
| SR23735   | SR23678 + pEB797 pBAD-CBP-ACP (S36T) | This study     |
| SR23738   | SR23678 + pBAD24 | This study     |
| SR23729   | SR23678 + pEB540 pBAD-CBP-ACP | This study     |
| SR23732   | SR23678 + pEB547 pBAD-CBP-ACP (S36C) | This study     |
| SR23735   | SR23678 + pEB797 pBAD-CBP-ACP (S36T) | This study     |
| SR23738   | SR23678 + pBAD24 | This study     |

Plasmids Genotype Reference

| Plasmids | Genotype | Reference |
|----------|----------|-----------|
| pCA24N   | IPTG-inducible expression vector cm\(^R\) | [51] |
| pDUET    | expression vector | Our collection |
| pKD3     | oriR6K\(_p\), bla(Amp\(^R\)), kan, rgnB(Ter), cat | [75] |
| pKD13    | oriR6K\(_p\), bla(Amp\(^R\)), kan, rgnB(Ter) | [75] |
| pKD46    | araB-gam-bet-exo, bla(Amp\(^R\)), repA101(ts) | [75] |
| pCP20    | ts replicon with inducible FLP recombinase | [75] |
| JW5539   | lapD\(^+\) in pCA24N | [51] |
| pSR23599 | lapD\(^+\) in pDUET | This study |
| pSR23790 | acpP\(^+\) in pCA24N | This study |
| pEB540   | pBAD-CBP-ACP | [55] |
| pEB547   | pBAD-CBP-ACP (S36C) | [55] |
| pEB797   | pBAD-CBP-ACP (S36T) | [55] |

4.2. Purification of LapD and LapB

The LapB protein was purified from solubilized IM fractions essentially as described earlier [17]. To induce the expression of the lapD gene, we used the minimal ORF cloned in the pCA24N expression plasmid (JW5539) [51]. In this plasmid, the expression is inducible from the P\(_T5\)-lac promoter. The plasmid DNA was used to transform the wild-type strain BW25113 and the expression was induced with the addition of 300 \(\mu\)M IPTG at an OD\(_{600}\) 0.1 in a 1 L culture medium at 28 °C. Cultures were grown for another 5 h prior to harvesting by centrifugation at 12,000 rpm for 30 min. To obtain a relatively pure LapD protein without contamination from host proteins, the minimal coding region was cloned into the low-copy T7 promoter-based pDUET expression vector (Novagen, Warsaw, Poland) with an in-frame His\(_6\) tag at the N-terminus of LapD. For such experiments, the expression of the lapD gene was induced in BL21(DE3) derivative by the addition of 300 \(\mu\)M IPTG at an OD\(_{600}\) 0.1 in a 1 L culture medium at 28 °C. Cultures were further shaken till they reached...
an OD$_{600}$ 0.2, followed by an addition of 200 µg/mL of rifampicin to prevent the host protein synthesis and incubated for another 2 h. Cultures were harvested by centrifugation at 12,000 rpm for 30 min at 4 °C. Pellets were frozen at −80 °C and used for further protein extraction when required. To the frozen pellet, 2X B-PER reagent (Thermo Scientific, Warsaw, Poland) was added and allowed to thaw. This mixture was adjusted to contain 50 mM Na$_2$HPO$_4$, 300 mM NaCl, 10 mM imidazole (buffer A), supplemented with lysozyme to a final concentration of 200 µg/mL, PMSF and a cocktail of protease inhibitors (Sigma Aldrich, Poznan, Poland) and 30 units of benzonase (Merck, Poznan, Poland). This mixture was incubated on ice for 45 min with gentle mixing. The lysate was centrifuged at 45,000 × g for 90 min at 4 °C and pellets containing IM and OM proteins were retained. LapA/B and LapD proteins were extracted using 2% octyl-β-D-glucoside for solubilization of IM proteins in buffer A supplemented by PMSF and a cocktail of protease inhibitors. Solubilized IM proteins were applied over nickel-nitrilotriacetic acid beads (Qiagen, Geneva, Switzerland) and Lap proteins eluted with a linear gradient (50–500 mM) of imidazole in the presence of octyl-β-D-glucoside. Eluting protein fractions were analyzed by resolving on a 12% SDS-PAGE. The identity of co-eluting proteins was obtained by MALDI-TOF.

4.3. Immunoblotting to Estimate Amounts of LpxC

The isogenic bacterial culture of wild type, ΔlapD with the vector alone, and its isogenic derivatives carrying multicopy suppressor encoding genes were grown in LB medium at 30 °C, adjusted to an OD$_{595}$ of 0.05 and allowed further growth up to an OD$_{595}$ of 0.2. To induce the expression of the suppressing gene, IPTG at the final concentration of 75 µM was added and shifted in prewarmed flasks held at 42 °C. Cultures were harvested by centrifugation and pellets were resuspended in sample buffer. For estimating LpxC levels in the Δ(micA lapD) derivative with and without the presence of extragenic suppressors mapping to the lpxC gene, isogenic cultures were grown in LB medium at 30 °C, adjusted to an OD$_{595}$ of 0.05 and allowed to grow for another 90 min, followed by shifting to 42 °C for another 2 h. Cultures were harvested by centrifugation. Equivalent amounts of proteins were applied to a 12% SDS-PAGE and transferred by Western blotting. Blots were probed with polyclonal antibodies against LpxC, as described previously [21]. Blots were revealed by a chemiluminescence kit from Thermo Scientific as per manufacturer’s instructions.

4.4. Identification of Multicopy Suppressors Whose Overexpression Suppresses Temperature and Vancomycin Sensitivity of ΔlapD Bacteria

A multicopy suppressor approach to identify either limiting factors in ΔlapD bacteria or find additional proteins with a function in the same pathway was essentially as previously described [76] with the following modification. The complete genomic library of all predicted ORFs of E. coli cloned in pCA24N [51] was used to transform ΔlapD strain SR23678. Transformants were plated at 44 °C on LA medium in the presence of 75 µM IPTG. In parallel, transformants were also plated on LA medium supplemented by 125 µg/mL of vancomycin at 37 °C in the presence of 75 µM IPTG. Obtained temperature-resistant or vancomycin-resistant colonies were retained. Bacterial cultures were grown from such suppressing clones and used to retransform ΔlapD strain SR23678 to verify the suppression. DNA insert of all relevant plasmids that yielded reproducible results was sequenced to obtain the identity of the multicopy suppressing gene.

4.5. Introduction of Various Suppressor Mutations Mapping to lpxC, lapB, ftsH and msbA in ΔlapD and Its Derivatives

We previously described the isolation of extragenic suppressors of GK6075 with a Cm cassette replacing the entire periplasmic domain of the LapC (lapC190) strain [21]. Such single amino acid substitutions mapped to either lpxC or lapB or ftsH genes restored the growth at elevated temperatures and suppressed permeability defects. To test if such suppressor mutations can also overcome permeability defects (vancomycin sensitivity), the lapC190 mutation was replaced by a wild-type copy of the lapC gene by bringing in a closely linked marker using bacteriophage PI-mediated transduction. Thus, a bacterio-
phage P1 was grown on a strain (SR9710) carrying a napA::Tn10 insertion (70% linked to the lapC gene) with an intact wild-type lapC gene and used as a donor selecting for Tet resistance with strains SR22731, SR22738, SR22727, GK6098 and GK6094 serving as recipients (Table 5). All such recipient strains contain the lapC190::cm mutation and a single amino acid substitution in the lpxC gene (Table 5). TetR colonies that lost the Cm cassette were retained. A representative strain from each transduction TetR CmR was first verified to have retained the lpxC suppressor mutation with the wild-type copy of the lapC gene by DNA sequence analysis of PCR products using specific oligonucleotides to amplify coding regions of lpxC and lapC genes. After such verification, one strain each with a different lpxC suppressor mutation (SR23812, SR23814, SR23816, SR23818 and SR23820, Table 5) served as a recipient to bring in ΔlapD KanR replacement. Transductants were plated at 33 °C and analyzed further. The same strategy of replacing the chromosomal lapC190 mutation with the wild-type copy of the lapC gene was used for testing the suppression of lethality of Δ(lpxM lapD), msbA suppression mutations that overcome the lethality of Δ(lpxM clsA) combination were used for such a reconstruction. To achieve this, previously constructed strains SR23302, SR23303, SR23305, SR23309, SR23313, SR23315 and SR23316 [32], all carrying different single amino acid substitutions in the msbA gene with a chromosomal deletion combination of lpxM and clsA genes, served as recipients to first replace the deletion of the clsA gene by the wild-type copy of this gene. To achieve this, a bacteriophage P1 lysate was grown on strain SR23138 with an oppA::ada mutation, which served as a donor for the above-mentioned strains with Δ(lpxM clsA) msbA* combinations. The oppA gene is more than 90% linked to the clsA gene. Thus, SpecR transductants were selected and those that were KanR (replacement of AclsA by the wild-type copy) were retained. The presence of a specific msbA suppressor was verified by PCR amplification. The resulting strains then served as recipients to introduce a deletion of the lapD gene in the ΔlpxM background.

4.6. Isolation of Suppressor Mutations That Confer Viability to Δ(lpxM lapD) and Δ(lpxM lapD) Derivatives and Their Mapping

As Δ(lpxL lapD) and Δ(lpxM lapD) combinations turned out to be lethal, we sought suppressor mutations that allow their growth. Towards this goal, multiple rounds of transductions were executed in ΔlpxL and ΔlpxM backgrounds to bring in a deletion of the lapD gene. Transductants were plated on LA medium at 30 °C and incubated for 72 h. Surviving transductants were streak purified and one strain from each combination was retained. Chromosomal DNA from such strains SR23684 Δ(lpxL lapD) and SR23685 Δ(lpxM lapD) was used as a template to amplify several candidate genes that included lpxC, lapA, lapB, fabZ, fabH and msbA. As both of them had a different single-amino acid substitution in the msbA gene, we ruled out the presence of any additional mutation by replacement of msbA* with the wild-type copy using a linked marker.

4.7. RNA Purification and q-RT-PCR Analysis

Exponentially grown isogenic cultures of wild type and its ΔsrrA derivative, and strains carrying the inducible srrA gene present on a plasmid were grown at 37 °C in LB medium, adjusted to an OD695 of 0.05 and allowed to further grow up to an OD695 of 0.2. In the case of strains with either a vector alone or when the srrA gene was present on the plasmid, 75 µM IPTG was added prior to the shift up of temperature. For heat shock, aliquots were shifted to prewarmed medium held at 43 °C and incubated for 15 min. Total RNA was purified by hot phenol extraction as described [77]. Purified RNA was treated with RQI RNase-free DNase (Promega, Madison, WI, USA) to remove any chromosomal DNA, and RNA was ethanol precipitated and resuspended in DEPC-treated water. RNA amounts were quantified and their integrity verified by agarose gel
electrophoresis. q-RT-PCR was used to quantify changes in the lpxC gene expression in ∆srrA and the wild type and when the expression of the srrA gene was induced, using gene-specific primers. Purified mRNA (2 µg) was converted to cDNA using Maxima H-Minus Reverse Transcriptase (Thermo Scientific). Reactions were carried out for 40 cycles using PowerUp SYBR® Green PCR Master Mix (Thermo Scientific), as described previously [57]. q-RT-PCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad, Warsaw, Poland). Data were analyzed by software Bio-Rad CFX Maestro.

4.8. Separation of Inner and Outer Membranes to Quantify LPS

Isogenic cultures of the wild type, its ΔlapD, Δ(waaC lapD), ΔclsA and Δ(clsA lapD) derivatives were grown under permissive growth conditions (LB 30 °C) up to an OD$_{595}$ 0.8. Cultures were harvested by centrifugation and cells were broken by French Press. Unbroken cells were removed by centrifugation at 3500 rpm for 15 min. The total cell lysate was subjected to centrifugation at 20,000 rpm for 90 min to remove soluble proteins and the membrane fraction resuspended in 1 mM Tris-HCl, pH 7.5, 20% sucrose. Samples were applied to a two-step sucrose gradient. The IM and the OM were separated by ultracentrifugation at 23,000 rpm for 18 h at 4 °C using an SW28 rotor (Beckman, Warsaw, Poland). The IM fractions located between 20% and 53% sucrose were pooled, treated with Proteinase K for 2 h and resolved on a 16% Tricine-SDS. LPS was visualized by silver staining.

4.9. Growth Analysis and Measurement of β-galactosidase Activity

For the quantification of bacterial growth and measurement of sensitivity to vancomycin, exponentially grown cultures were adjusted to an optical density OD$_{595}$ of 0.1. Samples were prepared using ten-fold dilutions and analyzed by spot-dilution assay on agar plates at different temperatures or when supplemented by 125 µg/mL of vancomycin. An amount of 5 µl of each dilution was spotted on agar plates and bacterial growth analyzed after incubation for 18–24 h at indicated temperatures. To measure the impact on the envelope stress response, isogenic cultures of the wild type and its lapD deletion derivative carrying the rpoEP3-lacZ promoter fusion were grown at 30 °C. Cultures were adjusted to an optical density OD$_{595}$ of 0.05 and allowed to grow at 30 °C for another 45 min. Aliquots of cultures were taken after different time intervals of growth and analyzed for β-galactosidase activity as described previously [49]. For each assay, three independent cultures were used and the average of each was plotted.

**Author Contributions:** Conceptualization, methodology, validation, writing, review and editing, S.R. and G.K.; investigation, A.W., A.S., A.M., M.S., G.K. and S.R.; supervision, S.R.; funding acquisition, S.R. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by National Science Center (NCN) Grant 2017/25/B/NZ6/02021 to S.R.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article.

**Acknowledgments:** We gratefully acknowledge E. Bouveret for the kind gift of plasmids, F. Narberhaus for LpxC-specific antibodies, K. Ito for FtsH-specific antibodies and M. Szuster for help at the early stages of this work.

**Conflicts of Interest:** The authors declare no conflict of interest.

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