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DOCTORAL DISSERTATION

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Regulacja homeostazy otoczki komórkowej przez białka Lap u *Escherichia coli*

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Prof. Dr. Satish Raina

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DESCRIPTION OF DOCTORAL DISSERTATION

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Keywords of doctoral dissertation in Polish: *Escherichia coli*, lipopolisacharyd (LPS), fosfolipid (PL), biogeneza błony zewnętrznej, LpxC, LapD, LapC, AcpP.

Keywords of doctoral dissertation in English: *Escherichia coli,* lipopolysaccharide (LPS), phospholipid (PL), outer membrane biogenesis, LpxC, LapD, LapC, AcpP.

Summary of doctoral dissertation in Polish: Niniejsze badania wyjaśniają molekularne sieci regulujące homeostazę błony zewnętrznej u *Escherichia coli* i ustalają kluczową rolę białka LapD. Bakterie Δ*lapD* wykazują letalność syntetyczną, gdy lipopolisacharyd jest skrócony, niedoacylowany lub gdy występują zaburzenia w biosyntezie kardiolipiny, co podkreśla kluczową rolę LapD w homeostazie otoczki komórkowej. Badania supresorów ujawniły mechanizmy leżące u podstaw tej niezbędności. Letalność z Δ*waaC* ulega supresji przez mutacje w genie kodujących LptD, koniecznym do transportu LPS, lub w genie *nlpI* kodującym regulator proteazy wymaganej do ekspansji peptydoglikanów, tworząc nową trójdzielną sieć integralności otoczki. Mutacje supresorowe, przezwyciężające letalność z Δ*clsA*, są mapowane w genach kodujących enzymy biosyntezy fosfolipidów: PgsA i CdsA. Wykazano również, że hamowanie biosyntezy kwasów tłuszczowych na etapie inicjacji, poprzez kompleks karboksylazy acetylo-CoA, lub nadprodukcja białka nośnikowego acylu lub tioesteraz, może kompensować brak LapD. Równoległe badania niezbędnego regulatora LPS, LapC, ujawniły hierarchiczną i nakładającą się sieć z LapD. Nadekspresja genu *lapD* prowadzi do supresji niektórych defektów bakterii z mutacją *lapC*, a LapD jest niezbędny, gdy funkcja LapC jest zaburzona. Badania supresorów pozwoliły na klasyfikację strategii w szlakach zależnych i niezależnych od LpxC. Podsumowując, niniejsza praca proponuje model, w którym kompleks LapB/C/D tworzy jednostkę składania i monitorowania, przy czym LapD występuje na styku i rekrutuje enzymy wymagane w biosyntezie PL i kwasów tłuszczowych.

Summary of doctoral dissertation in English: This study elucidates the molecular networks governing outer membrane homeostasis in *Escherichia coli*, establishing a pivotal role for LapD. Δ*lapD* bacteria exhibit synthetic lethality when lipopolysaccharide is either truncated or underacylated or when there are defects in cardiolipin biosynthesis, underscoring LapD's essential role in cell envelope homeostasis. Suppressor studies have revealed the mechanistic basis of this essentiality. Lethality with Δ*waaC* is rescued by mutations either in genes encoding LptD required for LPS transport or in protease regulator *nlpI* required for peptidoglycan expansion, forging a novel tripartite envelope integrity network. Suppressors mutation that overcome lethality with Δ*clsA* mapped to genes encoding phospholipid biosynthetic enzymes PgsA and CdsA. This analysis also revealed that the inhibition of fatty acid biosynthesis at the initiation step mediated by the acetyl-CoA carboxylase complex or overexpression of either acyl carrier protein or thioesterases can compensate for LapD's absence. A parallel investigation of the essential LPS regulator LapC revealed a hierarchical and overlapping network with LapD. *lapD* overexpression suppresses certain defects of *lapC* mutant bacteria, yet LapD is essential when LapC function is impaired. Suppressor screen in this background classified rescue strategies into LpxC and LpxC-independent pathways. Collectively, this thesis proposes a model in which the LapB/C/D complex forms an assembly and monitoring unit, with LapD at the interface to recruit enzymes involved in PL and fatty acid biosynthesis.

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STRESZCZENIE

Błona zewnętrzna (OM) bakterii Gram-ujemnych stanowi niezbędną asymetryczną barierę, której integralność wymaga zrównoważonej syntezy lipopolisacharydu (LPS) i fosfolipidów (PL). Równowaga ta jest regulowana przede wszystkim przez kontrolowaną proteolizę białka LpxC, które katalizuje pierwszy etap biosyntezy lipidu A, pośredniczoną przez proteazę FtsH, która do swojej funkcji wymaga białka błony wewnętrznej (IM) LapB. LapC (YejM/PbgA), niezbędne białko IM, wykrywa ilość LPS, aby stabilizować LpxC, wykorzystując swoją niezbędną N-końcową kotwicę transbłonową, która może oddziaływać zarówno z LapB, jak i LPS, regulując tempo biosyntezy LPS zgodnie z jego zapotrzebowaniem. LapD to kolejne białko tworzące kompleks wielobiałkowy, który koordynuje biogenezę otoczki. Badania typu pull-down wykazały, że LapD współoczyszcza się z kompleksem LapA/LapB, proteazą FtsH, białkami składania i transportu LPS oraz ważnymi enzymami biosyntezy kwasów tłuszczowych (FA) i PL. Mutanty Δ*lapD* wykazują wrażliwość na temperaturę, defekty przepuszczalności OM, aberracyjną morfologię komórek i obniżone poziomy białka LpxC. LapD staje się niezbędny, gdy LPS jest niedoacylowany, gdy rdzeń LPS jest skrócony lub gdy biosynteza PL jest zaburzona, o czym świadczy letalność syntetyczna z delecją genu *lpxM* lub *waaC* lub brakiem genu *clsA*. Mutacje supresorowe ratujące letalność Δ(*lapD waaC*) są zmapowane w genie *lptD*, co może wpływać na translokację LPS, oraz w genie *nlpI*, łącząc funkcję LapD z homeostazą peptydoglikanu (PGN). Mutacje supresorowe przezwyciężające letalność Δ(*lapD clsA*) zmapowano w genach *pgsA* i *cdsA*, które biorą udział w biosyntezie PL. Analiza wielokopijnych supresorów wykazała, że LapD odgrywa ważną rolę w metabolizmie kwasów tłuszczowych. Zgodnie z tą rolą, nadekspresja genu *acpP*, kodującego białko transportujące acyl, tłumiła defekty bakterii Δ*lapD*. Podobnie nadekspresja genów kodujących tioesterazy (*menI* i *tesA*') powodowała zmniejszenie ilości PL. Równoległa analiza wielokopijnych supresorów bakterii *lapC190* (pozbawionych domeny peryplazmatycznej LapC) zidentyfikowała geny, które po nadekspresji mogły przezwyciężyć defekty fenotypowe, takie jak fenotyp wrażliwości na temperaturę (Ts) szczepu mutanta *lapC*. Co więcej, nadekspresja genów *srrA*, *marA*, *yceJ* i *yfgM* może powodować supresję fenotypu Ts bakterii *lapC190* poprzez przywrócenie poziomu LpxC. Nadprodukcja nienaruszonego LapD hamuje fenotyp Ts bakterii *lapC190*, ale nie w przypadku mutacji w LapD: N-końcowym transbłonowym regionie kotwiczącym, C-końcowym (100–132 reszty aminokwasowe) lub specyficznych konserwowanych ewolucyjnie resztach aminokwasowych w domenie C-końcowej. Badania wykazały również, że geny *lapD*, *marA*, *srrA* i *pldA* są niezbędne dla bakterii z mutacją *lapC190*, definiując podstawową sieć genetyczną. Zatem białka Lap mogą fizycznie i funkcjonalnie integrować moduł regulacyjny ze szlakami metabolicznymi biosyntezy FA i PL.

Słowa kluczowe: *Escherichia coli*, lipopolisacharyd (LPS), fosfolipid (PL), biogeneza błony zewnętrznej, LpxC, LapD, LapC, AcpP.

Dziedzina nauki i techniki, zgodnie z wymogami OECD: nauki ścisłe i przyrodnicze, nauki chemiczne

ABSTRACT

The outer membrane (OM) of Gram-negative bacteria is an essential asymmetric barrier whose integrity requires the balanced synthesis of lipopolysaccharide (LPS) and phospholipids (PL). This balance is primarily governed by the regulated proteolysis of LpxC, which catalyzes the first committed step in lipid A biosynthesis, mediated by FtsH protease, which requires the inner membrane (IM) protein LapB for its function. LapC (YejM/PbgA), an essential IM protein, senses the amount of LPS to stabilize LpxC using its essential N-terminal transmembrane anchor that can interact either with LapB or LPS to adjust the rate of LPS biosynthesis as per its demand. LapD is another protein that forms a multiprotein complex that coordinates envelope biogenesis. Pull-down studies revealed that LapD co-purifies with the LapA/LapB complex, the FtsH protease, LPS assembly and transport proteins, and core enzymes of fatty acid (FA) and PL biosynthesis. Δ*lapD* bacteria exhibit temperature sensitivity, OM permeability defects, aberrant cell morphology, and reduced LpxC levels. LapD becomes essential when LPS is underacylated, or when the LPS core is truncated or when PL biosynthesis is impaired, as evidenced by synthetic/conditional lethality with deletion of either *lpxM*, *waaC* or the absence of *clsA*. Suppressor mutations rescuing Δ(*lapD waaC*) lethality mapped to the *lptD* gene, which could affect LPS translocation, and to the *nlpI* gene, linking LapD function to peptidoglycan (PGN) homeostasis. Suppressor mutations overcoming Δ(*lapD clsA*) lethality mapped to *pgsA* and *cdsA* genes, which are involved in PL biosynthesis. Multicopy suppressor analysis demonstrated that LapD plays an important role in fatty acid metabolism. Consistent with this role, overexpression of the *acpP* gene, which encodes an acyl carrier protein, suppressed the defects of Δ*lapD* bacteria. Similarly, overexpression of thioesterase-encoding genes (*menI*, *tesA'*) caused reduced the amount of PL. Parallel multicopy suppressor analysis of *lapC190* bacteria (lacking the LapC periplasmic domain) identified genes that, when overexpressed, could overcome phenotypic defects, such as the temperature-sensitive (Ts) phenotype of the *lapC* mutant strain. Moreover, overexpression of *srrA, marA, yceJ*, and *yfgM* genes can suppress the Ts phenotype of *lapC190* bacteria by restoring LpxC levels. The overproduction of the intact LapD suppresses the Ts phenotype of *lapC190*, but not when either its N-terminal transmembrane anchor, C-terminal (100-132 aa), or specific conserved amino acid residues in the C-terminal domain are mutated. This screen also revealed that *lapD*, *marA*, *srrA*, and *pldA* are essential in the *lapC190* mutant bacteria, defining a core genetic network. Thus, Lap proteins can physically and functionally integrate the regulatory module with the metabolic pathways of FA and PL biosynthesis.

Keywords: *Escherichia coli,* lipopolysaccharide (LPS), phospholipid (PL), outer membrane biogenesis, LpxC, LapD, LapC, AcpP.

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LIST OF IMPORTANT SYMBOLS AND ABBREVIATIONS

ACC	: Acetyl-CoA Carboxylase
ATP	: Adenosine Triphosphate
ABC	: ATP-Binding Cassette
BAM	: β-Barrel Assembly Machinery
CAMP	: Cationic Antimicrobial Peptide
CDP-DAG	: Cytidine Diphosphate Diacylglycerol
CFA	: Cyclopropane Fatty Acid
CFU	: Colony-Forming Units
CL	: Cardiolipin
CMP-Kdo	: Cytidine Monophosphate-Kdo
CoA	: Coenzyme A
EDTA	: Ethylenediaminetetraacetic Acid
ECF	: Extra Cytoplasmic Function
FA	: Fatty Acid
FAB	: Fatty Acid Biosynthesis
FAS	: Fatty Acid Synthesis
GC	: Gas Chromatography
Glc	: D-glucose
GlcN	: D-glucosamine
GlcNAc	: *N*-acetyl-D-glucosamine
GlcUA	: Glucuronic Acid
Galf	: D-galactofuranose
Gal	: D-galactose
Hep	: L-*glycero*-α-D-*manno*-heptose
His_6	: Hexahistidine Tag
IM	: Inner Membrane
IL-6	: Interleukin-6
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
Kdo	: 3-deoxy-α-D-*manno*-oct-2-ulosonic acid
LA	: Luria-Bertani Agar
LB	: Luria-Bertani Broth
L-Ara4N	: 4-amino-4-deoxy-L-arabinose
LBP	: LPS-Binding Protein

LPA	: Lysophosphatidic Acid
LPS	: Lipopolysaccharide
LOS	: Lipooligosaccharide
MALDI-TOF	: Matrix-Assisted Laser Desorption/Ionization-Time of Flight
MOI	: Multiplicity of Infection
MQ	: Millipore Quality
NBD	: Nucleotide-Binding Domain
OM	: Outer Membrane
OMP	: Outer Membrane Protein
ONPG	: o-Nitrophenyl-β-D-galactopyranoside
ORF	: Open Reading Frame
PA	: Phosphatidic Acid
PBPs	: Penicillin-Binding Proteins
PCR	: Polymerase Chain Reaction
P-EtN	: Phosphoethanolamine
PE	: Phosphatidylethanolamine
PGN	: Peptidoglycan
PG	: Phosphatidylglycerol
PVDF	: Polyvinylidene Fluoride
PL	: Phospholipid
SDS-PAGE	: SDS-Polyacrylamide Gel Electrophoresis
SEDS	: Shape, Elongation, Division, and Sporulation Proteins
SFA	: Saturated Fatty Acid
TAE	: Tris-Acetate-EDTA
TBST	: Tris-Buffered Saline with Tween 20
TCS	: Two-Component System
TLC	: Thin Layer Chromatography
TLR2/4	: Toll-like Receptor 2/4
TMD	: Transmembrane Domain
TPR	: Tetratricopeptide Repeat
Ts	: Temperature-sensitive
UFA	: Unsaturated Fatty Acid
UDP	: Uridine Diphosphate

1. INTRODUCTION

The bacterial cell envelope is a masterpiece of biological engineering, a composite structure that must be robust yet adaptable, impermeable yet communicative. In Gram-negative bacteria, such as *Escherichia coli*, this complexity is epitomized by the asymmetric outer membrane (OM), a formidable sieve of lipopolysaccharide (LPS) that confers innate resistance to many antimicrobials. Maintaining the precise composition of this barrier requires a dynamic equilibrium between the synthesis, transport, and remodelling/rebalancing of its constituent lipids and proteins, a fundamental cellular imperative. Disrupting this delicate homeostatic balance compromises viability, making the regulatory networks governing envelope biogenesis not only a fascinating subject of fundamental research but also a rich source of potential discoveries for future therapeutics[1,2].

1.1. Overview of the Gram-negative bacteria envelope

Unlike Gram-positive bacteria, which rely on a thick peptidoglycan (PGN) layer for structural integrity, Gram-negative species possess a tripartite envelope consisting of the inner membrane (IM), the periplasm with its PGN layer, and the OM. This multilayered structure provides both mechanical strength and a chemical barrier, making it essential for survival in hostile niches, soil, and aquatic environments[1,3]. While the Gram-negative bacterial envelope was initially regarded as a passive barrier, modern studies, especially those involving genetics, structural biology, and lipidomics, have revealed it as a dynamic, regulated structure that coordinates cellular physiology, stress adaptation, and pathogenesis[1,4]. Central to this complexity is the OM asymmetry, in which phospholipids (PLs) form the inner leaflet and LPS the outer leaflet. Thus the OM serves as a selective permeability barrier, excluding toxic hydrophobic compounds while allowing the controlled influx of nutrients via porins[5].

Importantly, studies from Prof. Satish Raina laboratory have shown that envelope stability is not merely a consequence of architecture but the outcome of tightly regulated genetic and proteolytic networks that balance outer membrane protein (OMP) folding, lipoprotein trafficking, and LPS synthesis[6]. This insight has reshaped the understanding of the Gram-negative envelope from a static barrier to a regulatory hub of bacterial life. The IM is a PL bilayer containing proteins involved in energy metabolism, transport, and signal transduction. Adjacent to the IM lies the periplasm, an aqueous compartment housing the PGN sacculus that provides mechanical rigidity. The OM is a unique asymmetric bilayer which encapsulates the cell. Key features include asymmetry, with PLs in the inner leaflet and LPS in the outer leaflet, anchoring via Braun's lipoprotein (Lpp). This covalently links the OM to PGN and ensures the structural integrity and barrier function. The presence of LPS excludes hydrophobic compounds, while porins mediate hydrophilic diffusion and envelope dynamics. This involves the transport systems such as β-barrel assembly machinery (BAM), Lol, Lpt, Mla that span the IM-periplasm-OM continuum, ensuring lipid and protein distribution[1,7]. This architecture is adaptable, enabling survival in diverse ecological niches. The Gram-negative envelope likely evolved as a defensive innovation, enabling colonization of harsh

environments despite the metabolic cost of its complex transport and surveillance systems[1,8,9]. Some authors argue that the envelope's evolution reflects a trade-off between impermeability and flexibility, while LPS confers resistance to many compounds, it also requires sophisticated export machinery (Lpt system) and surveillance by stress responses mediated by σ^E [10,11,12]. Nutrient acquisition is facilitated by porins, TonB-dependent receptors, and periplasmic binding proteins that allow the selective uptake of scarce resources. Osmotic balance is maintained as the PGN sacculus prevents cell lysis under hypoosmotic conditions[5]. Signal integration occurs through two-component systems, such as PhoP/PhoQ, BasS/BasR, PhoB/PhoR, CpxA/CpxR, and the Rcs system that use the envelope as a sensing module for environmental cues[12,13,14]. Proteostasis is managed by periplasmic chaperones, such as SurA and Skp, and proteases like DegP, which monitor OMP folding[15]. Thus, the envelope is not simply protective but also serves as a metabolic and signaling platform. It also contributes directly to host-pathogen interactions, for example, LPS acts as an endotoxin, activating host Toll-like receptor 4 (TLR4) and triggering inflammation[16]. Outer membrane vesicles (OMVs) derived from envelope blebbing deliver toxins and modulate host immunity[17]. Envelope remodelling during infection (e.g., lipid A modification by PhoP/PhoQ and BasS/BasR) confers resistance to antimicrobial peptides, directly linking envelope structure to virulence[18]. Although often portrayed as a nearly impenetrable fortress, the Gram-negative OM exhibits controlled permeability, mediated by porins[19]. The role of LPS in immune recognition remains nuanced, with lipid A variants modulating host responses. Studies on σ^E and fatty acid regulation stress pathways have reshaped understanding of envelope homeostasis[20].

1.1.1. Asymmetry of the outer membrane (OM): lipopolysaccharide (LPS) and phospholipid (PL)

One of the defining features of the Gram-negative envelope is the asymmetry of the OM which is caused by distribution of PL and LPS[1,21,22]. This arrangement is not a trivial peculiarity but a key determinant of bacterial physiology and resilience, shaping permeability, antibiotic resistance, and host-pathogen interactions[23]. Studies from Prof. Satish Raina's laboratory have highlighted that the balance between LPS and PLs is tightly regulated by proteins, such as LapB and its interacting partners, acting as key mediators of LPS and PL homeostasis[24]. LPS consists of lipid A, a core oligosaccharide, and the *O*-antigen polysaccharide. Its structural heterogeneity influences immune recognition and resistance[16]. The hydrophilic *O*-antigen and negatively charged core provide a chemical barrier, while divalent cations (Mg^{2+} and Ca^{2+}) cross-bridge adjacent LPS molecules to stabilize the membrane[25]. It is known that LPS acts as the canonical determinant of OM integrity with a prominent role for its non-stoichiometric modifications, such as incorporation of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (*P*-EtN) in the lipid A part, which can alter permeability and antibiotic susceptibility, particularly in polymyxin resistance[16]. The inner leaflet of the OM contains PLs, including phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL)[1]. These lipids provide flexibility and accommodate the insertion of OMPs and lipoproteins. The unique OM asymmetry confers multiple physiological advantages such as selective permeability (restricting hydrophobic compound entry via LPS while allowing hydrophilic

diffusion through porins), resistance to antimicrobial peptides via lipid A modifications, immune evasion through structural variation of *O*-antigen polysaccharides, and energetic efficiency conferred by PL lowering membrane rigidity[1,5,26]. However, asymmetry also creates fragility, as defects in LPS transport or PL retrograde trafficking (Mla pathway) collapse the OM, highlighting the necessity of homeostatic regulation[27,28].

1.1.2. The OM as a permeability barrier

Initial studies established that the OM acts as the primary permeability barrier in Gram-negative bacteria[5]. The permeability barrier arises from a synergy of structural features. Many antimicrobials, particularly large hydrophobic drugs, fail to cross the OM efficiently[29]. However, whether the OM is an absolute barrier or a conditional bottleneck remains debated. While the barrier model emphasizes OM impermeability, the porin leakage model demonstrates that porins create a controlled, non-specific permeability modulated by their structure and abundance[30]. Bacteria employ adaptive strategies, such as dynamically switching between porins like OmpF and OmpC and modifying LPS, to fine-tune membrane permeability in response to antibiotic challenge, balancing protection with essential influx[5,30,31]. Permeability is dynamically regulated and is condition-specific. In *E. coli*, high osmolarity downregulates OmpF and upregulates OmpC to reduce solute influx[32]. The σ^E and Cpx systems adjust porin expression and chaperone activity to maintain envelope integrity and under Mg^{2+} limitation, the PhoP/PhoQ system activates lipid A modifications[12,18,33]. These adaptive mechanisms reveal that the OM's barrier role is a regulated phenotype, not just a structural property.

1.2. Outer membrane proteins (OMPs)

OMPs are a defining feature of Gram-negative bacteria, representing a large and functionally diverse class of β-barrel proteins embedded in the OM. Unlike cytoplasmic or IM proteins, OMPs must fold and assemble in a lipid environment hostile to exposed hydrophobic domains, requiring specialized machinery for insertion and stability[34,35]. Their unique structural and functional properties make OMPs essential not only for cell survival but also for adaptation to environmental stresses and pathogenesis. Most OMPs adopt a β-barrel architecture, consisting of anti-parallel β-strands forming a cylindrical pore[34]. The number of β-strands typically ranges from 8 (small channels like OmpA) to 22 (large porins and autotransporters). The hydrophobic side chains face outward toward the lipid bilayer, while the hydrophilic residues line the pore interior. Porins, such as OmpF and OmpC, form trimeric complexes allowing the passive diffusion of small hydrophilic solutes. Specific transporters, such as FhuA or BtuB, mediate the uptake of scarce nutrients like siderophores and vitamin B12[36]. Autotransporters combine a C-terminal β-barrel with an N-terminal passenger domain, enabling the secretion of large virulence factors across the OM[35,37]. OMPs enable nutrient acquisition, defense and resistance to antibiotics, cellular communication, and mediation of virulence and host interaction[1,34]. Folding occurs without ATP or proton motive force, relying on chaperones and the BAM complex to achieve proper assembly. Misfolded OMPs induce envelope stress responses, underscoring the importance of conserved

folding catalysts[35,38,39]. The following subsections will examine the pathways ensuring their correct biogenesis, focusing on the Sec translocon, periplasmic chaperones, and the BAM complex.

1.2.1. Pathways of OMP biogenesis

The journey of OMPs from their synthesis in the cytoplasm to their final folded state in the OM is a highly orchestrated, multi-step process. This pathway is uniquely challenging because the OM lacks direct energy sources, such as ATP or a proton motive force, necessitating a reliance on specialized protein machineries and chaperones to ensure correct targeting, folding, and insertion[40]. OMP biogenesis can be broadly divided into four stages. The synthesis and translocation across the IM via the Sec translocon. Then the transit through the periplasm, guided by molecular chaperones that prevent misfolding and aggregation, and recognition and insertion into the OM by the BAM complex. Finally, quality control and degradation, ensuring envelope proteostasis (**Figure 1**). All OMPs are synthesized as precursors in the cytoplasm with N-terminal signal peptides that target them to the SecYEG translocon[41]. SecA ATPase drives post-translational translocation across the IM, and signal peptides are cleaved by signal peptidase I, releasing the unfolded OMP into the periplasm[42]. The proper timing of signal peptide cleavage is essential, as premature release can lead to periplasmic aggregation of the protein. Once in the periplasm, nascent OMPs are vulnerable to misfolding and proteolysis. The periplasm contains no ATP, therefore specialized chaperones guide proteins via hydrophobic shielding and transient binding. Key chaperones include SurA (primary chaperone), Skp (trimeric cavity for unfolded proteins), and DegP (dual protease/chaperone) [39,43].

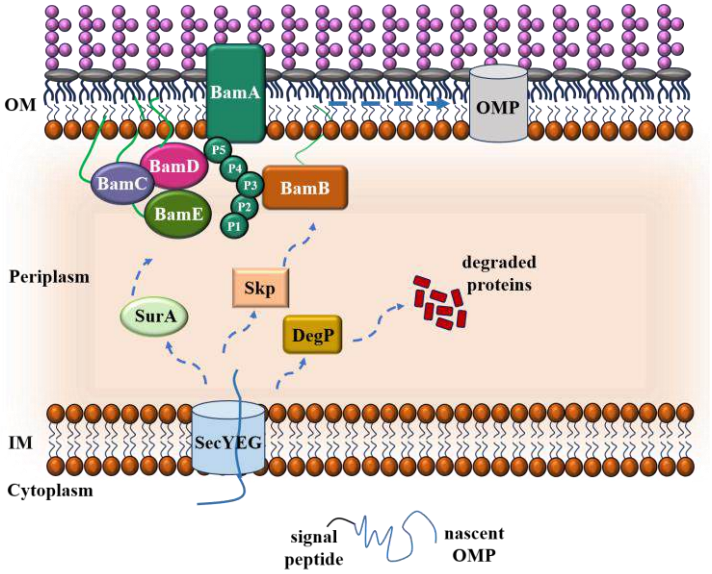

Figure 1: Biogenesis and folding of OMPs in *E. coli* (adapted from[44]).

Newly synthesized OMPs are translocated across the IM through the Sec pathway. Once in the periplasm, these unfolded OMPs are guided primarily by the SurA chaperone through the major folding pathway or alternatively by the Skp chaperone via a secondary route. DegP protease recognises and degrades the misfolded proteins. This prevents the accumulation of toxic intermediates. Properly folded OMPs are delivered to the BAM complex, which consists of BamA, BamB, BamC, BamD, and BamE, facilitating their insertion and assembly into the OM.

The final insertion into the OM is catalyzed by the BAM complex (BamA-BamE). BamA forms the core with a lateral gate, BamD acts as scaffold and regulator, BamB, BamC, and BamE enhance folding efficiency. OMPs are transferred from SurA/Skp to BAM for folding and membrane insertion[43,45]. To maintain OM integrity, bacteria deploy robust quality control systems. DegP protease degrades damaged or misfolded OMPs[46,15,47]. The σ^E stress response, first characterized by Profs. Raina and Gross laboratories, is upregulated when misfolded OMPs accumulate[11,48,49,50]. Envelope stress regulons, such as Cpx and Rcs, also indirectly influence OMP homeostasis[51]. This ensures a balance between OMP synthesis, insertion, and removal, preventing envelope instability.

1.2.2. The BAM complex - structure and function

First genetically identified in *E. coli* through BamA (originally Omp85), the BAM complex subsequently emerged as a paradigm of energy-independent membrane protein biogenesis[52,53]. BAM not only catalyzes the final step of OMP assembly but also integrates signals from periplasmic chaperones, stress responses, and envelope homeostasis pathways, positioning it at the crossroads of OM biology[1,54]. The *E. coli* BAM complex consists of five subunits: BamA, BamB, BamC, BamD, and BamE (**Figure 2**). Among these, BamA and BamD are essential, while the others enhance efficiency and stability[55]. BamA is a large OMP belonging to the Omp85 superfamily, with a 16-stranded β-barrel and five N-terminal periplasmic polypeptide transport-associated (POTRA) domains[56]. BamA forms the catalytic core and is directly involved in OMP insertion. BamB is a β-propeller protein that stabilizes interactions with BamA and enhances substrate recognition[57]. BamC, on the other hand, is a helical protein with peripheral association, thought to stabilize the complex but dispensable for viability[58]. BamD is an essential lipoprotein coordinating substrate binding and POTRA domain function. BamE is a small lipoprotein that interacts with BamD and contributes to the structural integrity of the complex[55].

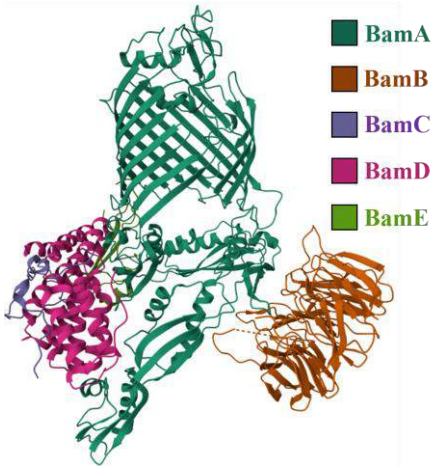

Figure 2: Crystal structure of the BAM complex from *E. coli* (PBD: 5AYW).
BamA (dark green), BamB (brown), BamC (purple), BamD (pink), BamE (light green) are shown.

Together, these subunits form a multi-protein machine embedded in the OM, spanning the periplasm to coordinate OMP folding. Despite significant advances, the exact mechanism by which BAM mediates OMP insertion remains debated. Advances in cryo-EM and X-ray crystallography

have transformed understanding of BAM. Cryo-EM studies of the intact BAM complex showed a dynamic, flexible architecture, with BamA undergoing conformational changes during substrate insertion[59]. These studies emphasize BAM as not just a passive channel, but a dynamic catalyst of membrane protein folding. BAM does not act in isolation. It functions downstream of SurA, Skp, and DegP. SurA appears to directly deliver substrates to the BamA POTRA domains. Genetic and biochemical evidence indicates that SurA deficiency leads to synthetic lethality with BamB or BamE absence, highlighting pathway redundancy and interdependence. This crosstalk underscores the integration of folding pathways, chaperones that buffer folding intermediates, while BAM provides the insertion catalyst[60].

1.3. Lipoproteins

Bacterial lipoproteins are lipid-modified membrane-anchored proteins crucial for envelope integrity, transport, signaling, and virulence in Gram-negative bacteria[61]. They contain a conserved lipobox motif (LXXC), where the cysteine is triacylated via the sequential actions of Lgt (diacylglycerol attachment), LspA (signal peptide cleavage), and Lnt (third fatty acid addition)[61,62]. In *E. coli*, over 90 lipoproteins exist and are distributed as: IM lipoproteins (involved in transport and signaling), OM lipoproteins (e.g., structural Lpp and Pal in the Tol-Pal complex), and periplasmic lipoproteins (e.g., chaperone LolA)[61,63]. Surface-exposed lipoproteins in pathogens are essential for adhesion, immune evasion, and colonization[64,65]. In pathogenic Gram-negative bacteria, lipoproteins contribute to survival within the host-immune evasion, such as modification of surface charge or masking epitopes[65]. Innate immune activation occurs when triacylated lipoproteins are recognized by mammalian TLR2/1 heterodimers, linking bacterial envelope biology to innate immunity[66]. These roles position lipoproteins at the intersection of bacterial physiology and host immunity. The biosynthesis of bacterial lipoproteins follows a highly conserved and tightly regulated pathway. This process involves co-translational translocation across the IM via the Sec system, followed by sequential enzymatic modifications that install lipid moieties at the conserved N-terminal cysteine[61,63]. These modifications are essential not only for stable membrane anchoring but also for the recognition and trafficking of lipoproteins by the Lol pathway. Pre-prolipoproteins are synthesized in the cytoplasm and translocated across the IM by the Sec translocon, guided by the SecA and SecYEG components. Unlike many periplasmic proteins, lipoproteins rely on signal peptidase II, rather than the canonical signal peptidase I, for processing[64]. Polypeptide folding during translocation is prevented by chaperones, ensuring that lipidation occurs only after translocation. The first enzymatic step is catalyzed by Lgt, which transfers a diacylglyceryl moiety from phosphatidylglycerol to the conserved cysteine thiol[67]. This lipidation step is essential for the further processing. *lgt* mutants accumulate unmodified prolipoproteins and display severe OM defects[67]. After lipidation, LspA cleaves the signal peptide between the lipobox cysteine and the preceding residue. This cleavage exposes the lipid-modified cysteine as the N-terminal residue. Inhibition of LspA by antibiotics, such as globomycin, prevents signal peptide removal, causing the lethal accumulation of prolipoproteins[68]. The final maturation step is catalyzed by Lnt, which transfers an acyl group from phosphatidylethanolamine to the free amino group of the cysteine.

This results in a triacylated lipoprotein, the prevalent form in most Gram-negative bacteria, essential for stable membrane anchoring. Interestingly, some Gram-positive bacteria lack Lnt and produce diacylated lipoproteins, which can still be functional but alter immune recognition[69]. Only fully mature triacylated lipoproteins are recognized and sorted by the Lol pathway. Non-lipidated or improperly modified lipoproteins fail to engage with LolCDE, leading to lethal accumulation in the IM. Thus, lipoprotein maturation and trafficking are coupled processes, integrating biogenesis with localization[70]. The Lol system in *E. coli* comprises five essential proteins. The IM ABC transporter LolCDE recognizes triacylated OM-destined lipoproteins and uses ATP hydrolysis to extract them. The soluble periplasmic chaperone LolA then accepts and shields the lipoproteins. Finally, the OM-anchored lipoprotein LolB acts as the receptor, mediating their insertion into the OM (**Figure 3**). Together, these proteins establish a relay-like mechanism for the safe, directed transport of lipoproteins across the periplasm[71]. The mechanism of lipoprotein transport is by the recognition by LolCDE of lipoproteins lacking IM-retention signals. ATP-driven extraction of lipoproteins from the IM by LolCDE and then transfer to LolA for encapsulation of lipid chains and protection during periplasmic transit. Reception by LolB, catalyses the integration into the OM. This stepwise process prevents the misfolding or aggregation of hydrophobic lipoproteins in the periplasm[72].

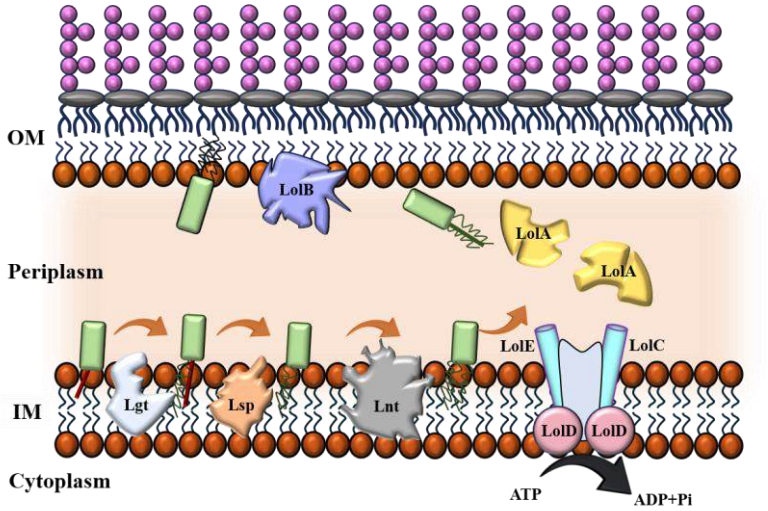

Figure 3: Maturation and transport pathway of bacterial lipoproteins in Gram-negative bacteria (adapted from[73]).

The mature lipoproteins are transported across the periplasm by the ABC transporter complex (LolCDE) with ATP hydrolysis. The chaperone LolA shuttles the lipoproteins through the periplasm, and they are finally inserted into the OM by LolB. This pathway ensures the proper localization and function of lipoproteins critical for cell envelope integrity.

Genetic studies confirm that all core Lol components (LolA, LolB, and LolCDE) are essential in *E. coli*, as their depletion causes OM destabilization and cell death. This highly conserved system is a selective antibacterial target, and LolCDE inhibitors show bactericidal activity[74,75]. Lipoproteins are integral to antibiotic resistance and host-pathogen interactions. The LspA inhibitor globomycin is lethal, and Lol pathway inhibitors can resensitize resistant strains[76,77].

1.4. Lipopolysaccharide (LPS)

Located exclusively in the outer leaflet of the OM, LPS functions as a protective barrier, shielding bacteria from hydrophobic compounds, detergents, and many antibiotics. Its structural and immunological properties make it a molecule of dual significance, indispensable for bacterial physiology and a potent trigger of host immune responses[1,5,16,18,78]. LPS consists of three covalently linked regions: lipid A, the core oligosaccharide, and the *O*-antigen. The *O*-antigen is a variable polysaccharide chain that extends outward from the core and is highly diverse across strains in terms of its sugar composition and structure serving as a key antigenic determinant. This tripartite organization ensures both structural stability and functional versatility. In *E. coli*, 75% of its surface is covered with LPS that is equal to approximately two million molecules of this glycolipid per cell[6].

1.4.1. Structure of LPS

LPS is the signature glycolipid of Gram-negative bacteria, forming the outer leaflet of the OM and providing both structural stability and a defensive barrier (**Figure 4**). Despite the conservation of its general tripartite design, the molecular structure of LPS exhibits significant species- and strain- specific variations, which critically influence bacterial physiology, virulence, and antibiotic resistance[79]. The asymmetric arrangement produces a tight, ordered barrier stabilized by divalent cations (Mg^{2+} and Ca^{2+}) bridging adjacent LPS phosphate groups[5]. The result is a membrane with very low permeability to hydrophobic compounds and many antibiotics, contributing to the intrinsic resistance of Gram-negative pathogens[80].

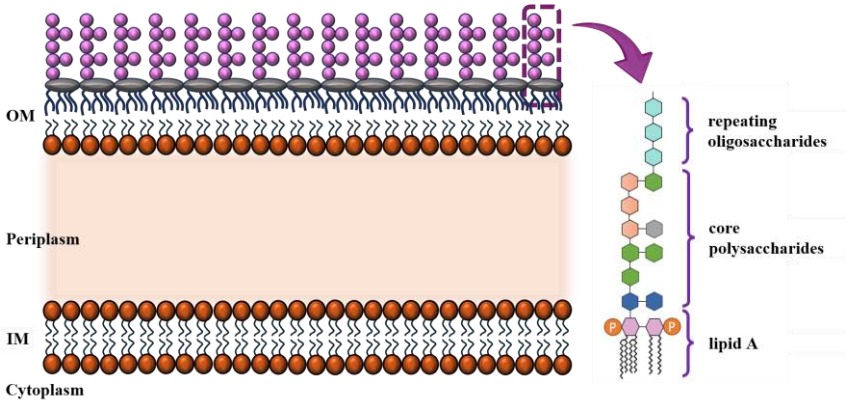

Figure 4: Architecture of the Gram-negative OM and LPS structure (adapted from[81]).
Schematic drawing of the structure and organization of the OM, with a detailed representation of the LPS architecture. The OM consists of an asymmetric lipid bilayer with LPS molecules embedded in the outer leaflet. The LPS comprises lipid A, a core polysaccharide, and repeating oligosaccharide (*O*-antigen) projecting outward.

Members of *Enterobacteriaceae* (e.g., *E. coli*, *Salmonella*) typically produce “smooth LPS” with full *O*-antigen chains, while laboratory *E. coli* K-12 strains make “rough LPS” lacking *O*-antigen[82]. *Neisseria* species express truncated LPS, termed lipooligosaccharide (LOS), lacking *O*-antigen but retaining immunological activity[83]. How bacteria balance envelope rigidity with structural variability to evade immunity remains an open evolutionary question.

1.4.2. Composition of LPS

While the tripartite structural organization of LPS is well-defined, and its chemical composition reveals a remarkable diversity in sugar residues, lipid modifications, and charge properties. These compositional variations are not random but reflect evolutionary strategies to balance the envelope stability, immune recognition, and environmental adaptation[16,84]. Understanding LPS composition is essential for dissecting its roles in antibiotic resistance, stress responses, and host-pathogen interactions[1]. Lipid A, which is the hydrophobic anchor having backbone of disaccharide of glucosamine, is typically phosphorylated at positions 1 and 4′ and acyl chains commonly hexa-acylated with C12-C14 fatty acids in *E. coli*, though chain length and number vary across species[16,85,86]. The core oligosaccharide acts as the structural bridge which links lipid A to the *O*-antigen and is divided into inner and outer regions. The inner core contains 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-α-D-*manno*-heptose (Hep), highly conserved and required for OM stability[16]. The outer core is more variable, containing hexoses, such as glucose, galactose, or *N*-acetylglucosamine, which contribute to the OM permeability barrier. Finally, the *O*-antigen, the variable extension which is composed of linear or branched polysaccharides composed of repeating oligosaccharide units, ranging from 1 to >100 repeats[84] (**Figure 5**).

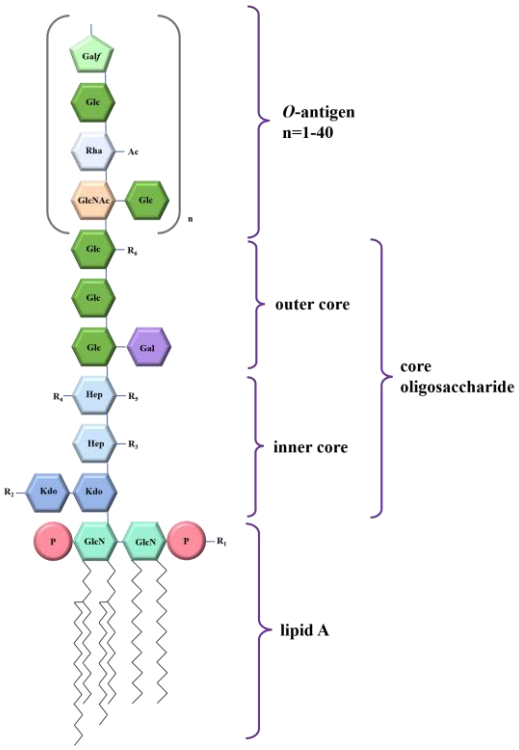

Figure 5: Schematic representation of *E. coli* K-12 LPS (adapted from[87]).

Abbreviations: GlcN: D-glucosamine; Kdo: 3-deoxy-α-D-*manno*-oct-2-ulosonic acid; Hep: L-*glycero*-α-D-*manno*-heptose; Glc: D-glucose; Gal: D-galactose; GlcNAc: *N*-acetyl-D-glucosamine; Rha: L-rhamnose; Galf: D-galactofuranose; P: phosphate; Ac: acetate. R groups mark common chemical modifications. R1: phosphate; R2: Kdo, rhamnose, or phosphoethanolamine; R3: phosphate or ethanolamine pyrophosphate; R4: phosphate; R5: heptose, GlcUA: glucuronic acid; R6: heptose or GlcNAc.

Unlike PLs, which are relatively homogeneous, LPS displays multiple glycoforms that coexist in a single bacterial population[88].

1.4.3. Structure of lipid A

The most conserved structural unit of LPS is the lipid A part, which serves as the hydrophobic anchor embedding LPS within the OM. Lipid A consists of a *β*(1→6)-linked disaccharide of glucosamine (GlcN) that is phosphorylated at the 1 and 4′ positions and acylated with (*R*)-3-hydroxymyristate chains at positions 2, 3, 2′, and 3′. Additionally, in *E. coli* K-12 the two (*R*)-3-hydroxyacyl groups attached to the nonreducing glucosamine are typically further esterified with laurate (C12) and myristate (C14)[1,89,90,91]. The O-6′ position of lipid A forms a glycosidic bond with Kdo I, which links lipid A to the core oligosaccharide region of LPS (**Figure 6**).

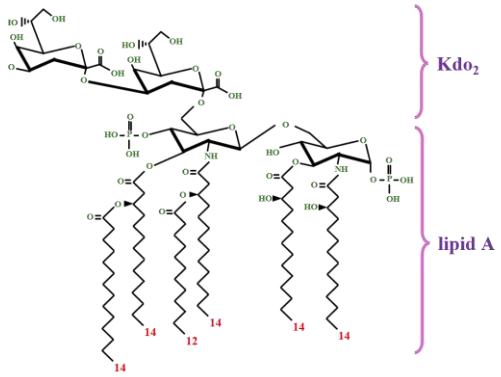

Figure 6: Covalent structure of *E. coli* K-12 Kdo_2-lipid A (adapted from[20]).
The number of carbon atoms in the fatty acyl moiety in lipid A is indicated using numbers 12 (lauroyl group) and 14 (myristoyl group).

E. coli and *Salmonella* generally produce hexaacylated lipid A, other bacteria generate tetraacylated or pentaacylated forms (e.g., *Helicobacter pylori*, *Francisella tularensis*), which are less immunostimulatory[92]. Hexaacylated lipid A with two first saccharides, which are two Kdo moieties (Kdo_2-lipid A), represents the first known minimal LPS structure that is sufficient for *E. coli* survival up to 42°C. The possibility to construct viable and suppressor-free strains was established by Professor Raina's group, that synthesize even smaller structures composed of only tetraacylated lipid A (lipid IV_A) or Kdo_2-lipid IV_A under slow-growing conditions (minimal medium at 23°C)[93]. Parallel work by Reynolds and Raetz similarly described the construction of strains with minimal LPS structures[94]. The strains that synthesize such minimal LPS are characterized with a very narrow growth range, defects in cell division, hyper-induced RpoE-regulated stress response, and Cpx signal transduction pathway, without the requirement for suppressors to be present in order to maintain *E. coli* viability under such growth conditions[93].

1.4.4. Structure of core oligosaccharide

The core oligosaccharide is the central linking region of LPS, connecting lipid A to the *O*-antigen. It plays a crucial structural role in stabilizing the OM and maintaining its permeability barrier function. Unlike the lipid A, the core oligosaccharide demonstrates moderate variability across species: the inner core is largely conserved, whereas the outer core is more heterogeneous. This organization reflects the dual requirement of structural conservation for survival and flexibility for adaptation[16,24]. The core oligosaccharide is typically 8-12 sugar residues long and divided into two

distinct domains. The first is the inner core, proximal to lipid A, which contains Kdo and Hep residues. The outer core, which is proximal to the *O*-antigen, is composed of hexoses, such as glucose, galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine[95]. The assembly of the inner core of *E. coli* LPS begins with the transfer of Kdo residues to lipid A, catalyzed by WaaA (KdtA-Kdo transferase), which transfers two Kdo molecules from CMP-Kdo, forming Kdo_2-lipid IV_A. To this precursor, late acyl transferases add lauroyl and myristoyl chains, generating the key intermediate Kdo_2-lipid A[96]. Subsequently, Hep residues are sequentially added from ADP-heptose donors by WaaC, WaaF, and WaaQ as heptosyltransferases I, II, and III, respectively[16,97] (**Figure 7**). The phosphorylation of Hep residues by WaaP and WaaY is critical for LPS structural integrity and interactions with divalent cations[18]. The outer core oligosaccharide varies by chemotype but extends from Hep II via WaaG, WaaO, WaaB, WaaR, and WaaU, acting sequentially using nucleotide sugar donors[98]. The mature lipid A-core serves as a substrate for the *O*-antigen ligase WaaL, which attaches the *O*-antigen from the undecaprenyl phosphate carrier to the core region[99].

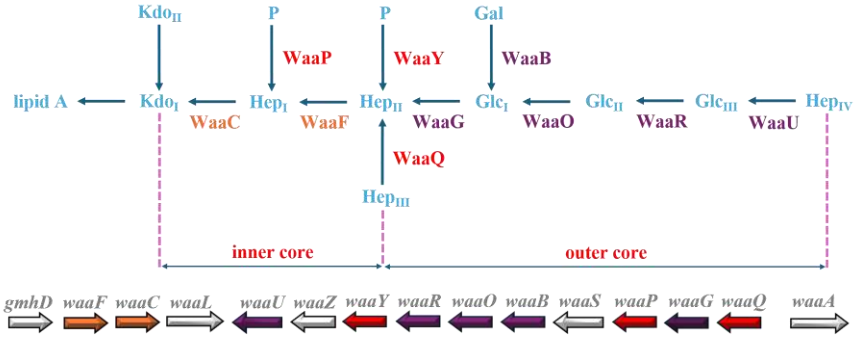

Figure 7: Genetic organization of the *waa locus* and core structure of *E. coli* K-12 W3110 LPS (adapted from[98]).

Glycosyltransferases construct the inner core backbone, and the genes encoding the enzymes are depicted as orange colour. Enzymes that modify the inner core structure and their corresponding genes are shown in red colour. Outer core enzymes and encoding genes are presented in purple colour.

Both the Kdo_2-lipid A and core regions exhibit considerable structural flexibility and undergo various modifications triggered by the activation of two-component systems, non-coding RNAs, and stress-responsive pathways. These alterations lead to the generation of seven distinct LPS glycoforms identified to date[20].

1.4.5. *O*-antigen

The *O*-antigen is the highly variable polysaccharide domain of LPS, extending outward from the bacterial surface. Unlike the conserved lipid A and inner core, the *O*-antigen exhibits extreme structural diversity, which is central to immune evasion, virulence, and host-pathogen interaction[100,101]. Chain length varies widely, from a few repeats (“short” *O*-antigen) to >100 units (“long” *O*-antigen), controlled by chain-length regulators such as Wzz proteins and is linked to the outer core via specific glycosyltransferases and ligase WaaL[16,102]. The *O*-antigen is synthesized independently of the lipid A-core and later ligated. One pathway is the Wzx/Wzy-dependent pathway, which is the most common and involves translocation of repeat unit across the IM via Wzx and polymerization by Wzy[103]. Another pathway is the ABC transporter-dependent pathway, in

which the entire *O*-antigen chains are transported across the IM. Ligation is performed by WaaL ligase, which attaches the completed *O*-antigen to the lipid A-core, yielding mature LPS. This modular biosynthesis enables flexible regulation and rapid evolutionary diversification[104].

1.4.6. LPS biosynthesis

In *E. coli* the Raetz pathway is the best-studied segment of LPS biosynthesis, which defines lipid A biosynthesis. This process can be divided into early cytoplasmic steps, late IM steps, and maturation events[105] (**Figure 9**). Early cytoplasmic steps include the catalyzation by LpxA. LpxA (UDP-*N*-acetylglucosamine *O*-acyltransferase) catalyzes the initial reaction in the lipid A biosynthetic pathway, transferring an (*R*)-3-hydroxymyristoyl group from its acyl carrier protein thioester donor to UDP-*N*-acetylglucosamine (UDP-GlcNAc)[106]. As the first enzyme of this essential pathway, LpxA represents a promising target for the development of novel antibiotics that inhibit LPS biosynthesis in Gram-negative bacteria[107]. LpxA's crystal structure revealed a unique left-handed parallel β-helix (LβH) motif, composed of successive parallel β-strands in a left-handed helical configuration[108]. This uncommon fold features non-canonical crossover connections between strands, deviating from conventional right-handed topologies. The LβH motif suggests LpxA shares structural and possible functional similarities with other enzymes containing a repeated hexapeptide motif, indicating a conserved framework among lipid A biosynthetic acyltransferases[108,109,110].

The enzyme UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC) catalyzes the first committed and rate-limiting step in the biosynthesis of lipid A, the essential anchor of LPS in Gram-negative bacteria[111] (**Figure 8**). As the essential enzyme controlling the flux into the LPS pathway, LpxC maintains OM integrity. Its unique fold, revealed by crystal structures, features a deep hydrophobic substrate acyl chain passage and a singular active site zinc ion[112]. The levels of LpxC are tightly regulated. Its cellular concentration is controlled by FtsH-mediated proteolysis, a process modulated by the IM proteins LapB and LapC in response to LPS levels[24,113,114].

Figure 8: Crystal structure of *E. coli* LpxC (PBD: 7PHJ).

Following LpxC-catalyzed deacetylation, the essential N-acylation step is performed by LpxD. This enzyme transfers a second (*R*)-3-hydroxymyristoyl chain from ACP to the glucosamine 2-amino group, forming UDP-2,3-diacylglucosamine and establishing lipid A backbone asymmetry[115]. Structurally, LpxD belongs to the LβH family, a fold whose constrained hydrophobic

channel acts as a "molecular ruler" for 14-carbon acyl chain specificity[110]. The pathway then transitions to the IM with the pyrophosphatases LpxH and LpxI, which catalyze the committed step of releasing the lipid A disaccharide precursor from its UDP carrier for membrane translocation[116]. Specifically, LpxH, found in proteobacteria like *E. coli*, hydrolyses the pyrophosphate bond of UDP-2,3-diacylglucosamine to yield UMP and the lipid carrier 2,3-diacylglucosamine-1-phosphate (lipid X), utilizing a magnesium-dependent mechanism that involves a unique "double-displacement" of the pyrophosphate moiety[117]. Strikingly, many other bacteria employ the structurally distinct, membrane-associated LpxI, which shares no homology with LpxH but performs the identical reaction[117]. This functional redundancy between unrelated enzymes for an essential step underscores the reaction's critical importance and the pathway's evolutionary flexibility[16].

The lipid A disaccharide scaffold is formed by the unique glycosyltransferase LpxB. It catalyzes the condensation of UDP-2,3-diacylglucosamine (donor) and lipid X (acceptor). The 6'-hydroxyl group of lipid X then attacks this complex, inverting the stereochemistry to form the characteristic *β*-1',6-glycosidic linkage and release disaccharide-1-phosphate. This Mg^{2+}-dependent reaction is essential for committing the pathway to mature lipid A assembly and distinguishes it from other glycan biosynthesis routes[116,118,119,120]. The lipid A disaccharide is subsequently phosphorylated by the cytosolic kinase LpxK, which catalyzes the ATP-dependent, Mg^{2+}-facilitated transfer of a phosphate group to the 4'-position, producing tetraacylated lipid IV_A (disaccharide-1,4'-bisphosphate)[121]. The phosphorylation by LpxK is indispensable, as the 4'-phosphate primes lipid A for further elaboration and is essential for OM integrity.

Subsequent maturation involves sequential glycosyl transfer and secondary acylation. The IM enzyme WaaA catalyzes the essential addition of two Kdo residues to lipid IV_A, forming Kdo_2-lipid IV_A[122]. This minimal structure is a critical bridge between lipid A and the core oligosaccharide and is a prerequisite for viability in *E. coli*. The presence of the Kdo disaccharide then licenses the subsequent secondary acylation of the molecule, a process mediated by dedicated acyltransferases that confer environmental adaptability[97]. The lauroyl transferase LpxL first adds a secondary laurate (C12) chain to the 2′-position in a Kdo-dependent manner, after which the myristoyl transferase LpxM incorporates a myristate (C14) chain at the 3′-position to yield the canonical symmetric hexaacylated lipid A species[123,124,125]. This constitutive pathway is complemented by inducible modifications that fine-tune the membrane properties. During cold shock, the palmitoleoyl transferase LpxP is upregulated to add a palmitoleate (C16:1) chain at the 2′ position, maintaining membrane fluidity[126]. Furthermore, in response to envelope stress, such as exposure to antimicrobial peptides, the PL-dependent palmitoyl transferase PagP remodels lipid A by transferring a palmitate (C16) chain to the hydroxyl group of a secondary acyl chain, enhancing bacterial resistance to host innate immunity[127].

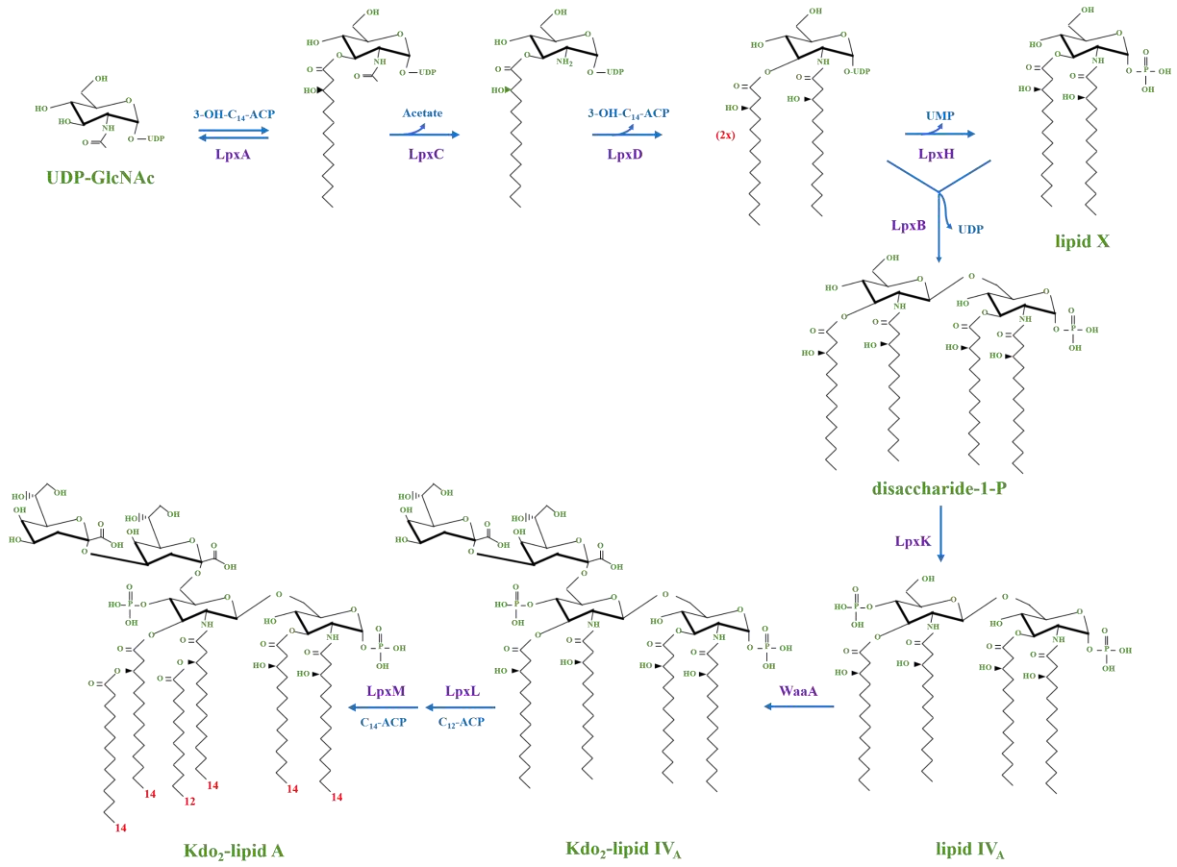

Figure 9: The Raetz pathway for constitutive biosynthesis of *E. coli* K-12 Kdo_2-lipid A (adapted from[128]).

Each enzyme of the constitutive Kdo_2-lipid A pathway is encoded by a single structural gene. The glucosamine disaccharide backbone of lipid A and the Kdo disaccharide are shown. LpxA, LpxC, and LpxD are soluble cytoplasmic proteins, whereas LpxH and LpxB are peripheral membrane proteins. The distal enzymes of the pathway, starting with LpxK, are integral IM proteins, the active sites of which face the cytoplasm. The single molecular species shown at the bottom left represents about 90% of the total lipid A in *E. coli*, with a C12 secondary acyl chain at third position.

Once formed, the lipid A-core molecule remains anchored in the inner leaflet of the IM until it is translocated across the membrane by the ABC transporter MsbA, exposing it to the periplasmic side[129]. The enzymes responsible for lipid A and Kdo biosynthesis are constitutively expressed[16]. However, in *E. coli*, the synthesis of Kdo_2-lipid A is post-transcriptionally regulated by the IM metalloprotease FtsH in cooperation with the heat shock protein LapB[24,114]. Among these enzymes, LpxC catalyses the first committed step in lipid A biosynthesis, whereas FabZ catalyses a key reaction in PL biosynthesis. Both pathways compete for the shared precursor (*R*)-3-hydroxymyristoyl-ACP (**Figure 10**). An imbalance favouring LpxC activity can be lethal due to excessive LPS accumulation relative to PLs, making FtsH-LapB regulation critical for maintaining membrane lipid homeostasis[114].

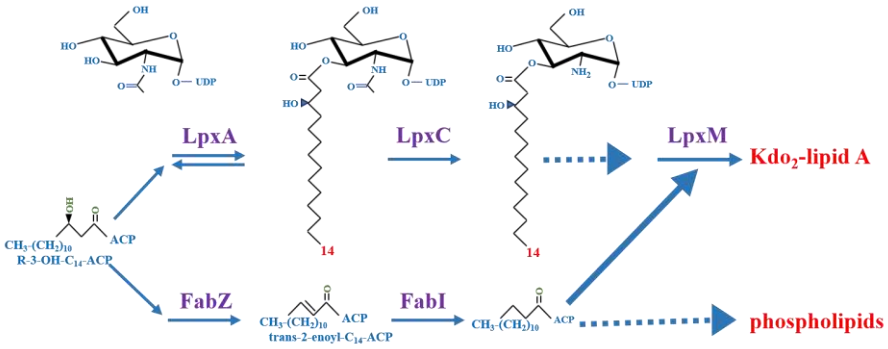

Figure 10: Schematic representation of the competing lipid A and PL biosynthesis pathways (adapted from[24]).

(*R*)-3-hydroxymyristoyl-ACP serves as a common substrate for both PL and Kdo_2-lipid A biosynthesis pathways. The branch point is defined by two key enzymes: FabZ dehydratase (for PLs) and LpxC deacetylase (for lipid A). LpxC's deacetylation of UDP-3-*O*-(acyl)-GlcNAc is a crucial early step in the lipid A pathway, necessary to overcome the unfavourable thermodynamics of the preceding UDP-GlcNAc acylation.

Absence of FtsH or LapB causes LpxC overaccumulation and LPS overproduction. This imbalance can be mitigated by suppressor mutations in the *fabZ* gene or its overexpression[114]. LapB interacts with WaaC and LPS transport (Lpt) components, suggesting that it scaffolds the IM assembly of LPS to ensure that only complete LPS is transferred[24]. Additionally, FtsH is known to regulate the degradation of WaaA, the enzyme responsible for Kdo incorporation into lipid IV_A[130]. While LpxC inhibitors are potent bactericidal agents, their toxicity has hindered clinical development[131].

As mentioned in the above section, at the heart of lipid homeostasis lies the first committed step of lipid A biosynthesis, catalyzed by the LpxC enzyme. Consequently, the cellular level of LpxC directly determines the flux of hydrocarbon chains into LPS versus PLs. The *lapB* gene was first identified in genetic screens for mutants exhibiting constitutive activation of the σ^E stress response, a hallmark of envelope defects[39]. Subsequent studies have shown that LapB plays a critical role in the regulation of LpxC[24]. LapB is an essential IM protein in *E. coli*, as its deletion is lethal in most of the strains and only conditionally viable in others due to toxic LPS overaccumulation when LpxC degradation is impaired[24]. Structurally, LapB contains nine cytosolic tetratricopeptide repeat (TPR) motifs, which mediate protein-protein interactions[24,132] (**Figure 11**). It delivers the substrate LpxC for proteolysis, co-purifying with the FtsH protease and LPS, and it interacts with components of the LPS transport (Lpt) system and lipid A biosynthetic enzymes. This positions LapB as a central regulatory hub that monitors LPS assembly flux and triggers LpxC degradation to maintain the LPS:PL balance[24].

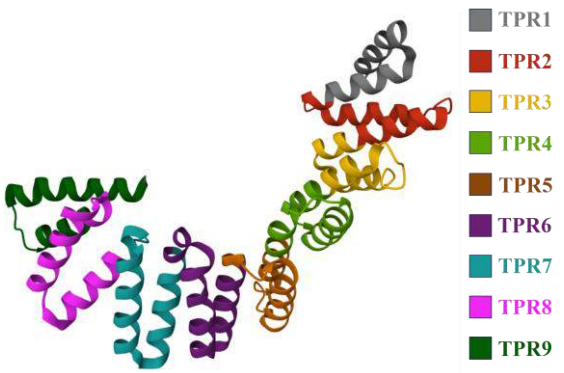

Figure 11: Crystal structure showing the monomer of LapB from *E. coli* (PDB: 4ZLH)
The position of the nine TPR motifs, TPR1 (grey), TPR2 (red), TPR3 (yellow), TPR4 (light green), TPR5 (brown), TPR6 (purple), TPR7 (cyan), TPR8 (pink), TPR9 (green) are shown.

The essential gene *lapC* was identified through two independent, complementary genetic strategies that converged on its critical role in regulating LpxC and maintaining LPS homeostasis. Also, work from other groups had established LapC as an essential IM metalloenzyme and a key modulator of the FtsH/LapB protease complex, which degrades LpxC to control LPS synthesis[133,134,135,136,137,138]. The first approach stemmed from the observation that a *lapB* deletion is lethal in the *E. coli* W3110 strain. A genetic suppressor approach identified the frameshift mutation *lapC377fs*, which truncates LapC's periplasmic domain. This loss-of-function mutation restored both the viability and normal LPS composition of Δ*lapB* bacteria, revealing that LapC antagonizes LapB-mediated LpxC degradation[113]. In a complementary approach, *lapC* was independently isolated from Ts mutants with constitutive *rpoE*P3 activation and hypersensitivity to LpxC inhibitor CHIR090, and MacConkey agar, yielding mutations including *lapC190*, *lapC377fs*, and *lapC* F349S[24,27,113]. The consistent identification of *lapC* mutations through these distinct strategies definitively established LapC as an essential component of the regulatory complex governing LpxC turnover and balanced LPS biosynthesis. LapC functions as an essential IM sensor that regulates LPS biosynthesis by controlling LpxC stability, acting in direct opposition to the LapB-FtsH proteolytic system to maintain a balanced envelope output[24,113]. Its crystal structure reveals a bipartite architecture with five transmembrane helices and a large periplasmic domain, supporting its role as an LPS-responsive switch[139] (**Figure 12**).

Figure 12: Crystal structure of LapC from *Salmonella enterica* serovar Typhimurium (PDB: 6V8Q).

The current model posits that LapC senses periplasmic LPS accumulation, triggering a conformational change transmitted across the membrane that modulates the LapB-FtsH complex's activity to adjust LpxC degradation and restore lipid homeostasis[78,113].

1.4.7. LPS transport across the inner membrane (IM)

Following its synthesis, the lipid A-core intermediate from the IM must be translocated across the IM. This process is mediated by the ABC transporter MsbA, which is essential for bacterial viability[140]. MsbA was initially discovered in *E. coli* as a multicopy suppressor capable of rescuing the Ts phenotype of *lpxL* (formerly *htrB*) insertion mutant[141]. *lpxL* knockout mutant bacteria exhibit lethality at temperatures above 33°C and display abnormal cell morphologies, including filamentation and blebbing. Interestingly, overexpression of the *msbA* gene from a plasmid can suppress these phenotypes. The discovery that tetra-acylated lipid A species, which is primarily seen in *lpxL* mutants, is poorly exported to the OM led to the emergence of a role for MsbA in LPS trafficking. Thus, strains either lacking LpxL or its derivatives lacking all three late acyl transferases Δ(*lpxL lpxM lpxP*) accumulate lipid IV_A precursors in the IM, and this defect can be overcome when MsbA is overexpressed[93,142,143]. This indicates that the elevated MsbA levels promote the translocation of the immature lipid A species to the OM, even though the missing acylation step is not restored. Conversely, MsbA-depleted bacteria accumulate fully acylated lipid A in the IM, confirming its indispensable role in LPS flipping[142].

MsbA's flippase function is validated by the requirement of a functional transporter for the periplasmic additions of L-Ara4N and *P*-EtN[144]. MsbA is a homodimeric ATP-binding cassette (ABC) transporter, with each monomer containing a nucleotide-binding domain (NBD) and a transmembrane domain (TMD)[145]. Its essential physiological substrate is lipid A part of LPS, and without MsbA, LPS accumulates in the IM causing lethality. While structurally homologous to multidrug exporters, MsbA is specific for lipid A, also transporting core-linked glycolipids and some PLs, acting as a key gatekeeper[146]. Landmark structural studies have captured its dynamic cycle (**Figure 13**). The initial structures revealed an inward-facing conformation with a cytoplasmic cavity suited for the lipid A's headgroup[147]. Subsequent structures with nucleotides show that ATP binding induces NBD dimerization, driving a conformational change to an outward-facing state[148]. This "alternating access" mechanism is described by the ATP-switch model, where ATP binding drives the outward-open state and hydrolysis resets the transporter (**Figure 13**). MsbA functions as a primary-active transporter, coupling ATP hydrolysis directly to substrate flipping. ATP hydrolysis is coordinated at the dimeric NBD interface by conserved Walker and signature motifs[149]. The cycle begins with the inward-facing apo state, which binds lipid A. ATP binding triggers cooperative NBD dimerization, providing a power stroke that forces the TMDs to twist and extrude the substrate to the periplasmic leaflet[150]. The translocation pathway is lined with residues that interact with both the acyl chains and the polar headgroup. Following ATP hydrolysis, the release of ADP and Pi resets the transporter to its inward-facing state[150,151,152]. Consistent with higher selectivity for hexaacylated lipid A derivatives, suppressor mutations that restore viability of Δ*waaA* and Δ(*clsA lpxM*) mutant bacteria mapped to the *msbA* gene, presumably by relaxing specificity for acyl chains[93,153].

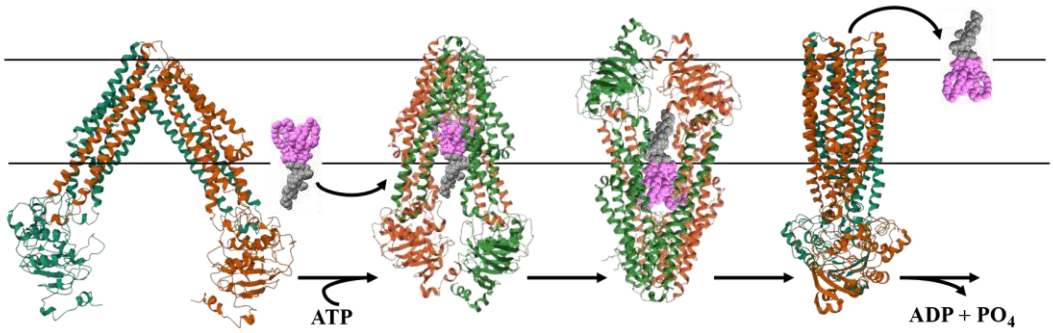

Figure 13: Conformational changes of MsbA during LPS transport (adapted from[152]).
LPS is depicted in pink and grey in sphere molecular representation. (PDB: 3B5W, 3B60, 5TTP).

The seven essential proteins, from LptA to LptG, are required to transport LPS to the outer leaflet of the OM, from the outer leaflet of the IM[154,155,156,157] . All seven Lpt proteins are essential, as the depletion of any particular Lpt component leads to the accumulation of LPS on the periplasmic surface of the IM[157,158]. LptB is a cytoplasmic nucleotide-binding ATPase containing characteristic Walker motifs and hydrolyses ATP to drive conformational changes in the transmembrane components[159]. LptF and LptG are the integral membrane subunits of the transporter, together with LptB they form a transporter complex that recognizes and binds LPS within a hydrophobic cavity[160]. The IM-anchored periplasmic protein that connects the ABC extractor to the periplasmic component, LptC helps transfer LPS from $LptB_2FG$ into the trans-envelope bridge and has been shown to modulate the ATPase activity of the transporter[22]. LptA is a soluble periplasmic bridging protein. It oligomerizes and interacts with LptC at the IM side and with LptD at the OM side, effectively forming a continuous hydrophobic groove that shields the lipid A portion of LPS as it traverses the periplasm[155]. Integral outer membrane β-barrel protein LptD together with LptE, forms the terminal translocon for LPS insertion. The N-terminal β-jellyroll domain of LptD interacts with LptA and receives LPS, while the β-barrel domain inserts lipid A into the outer leaflet of the OM[159]. LptE is a small lipoprotein that resides inside the barrel of LptD and is essential for the proper folding and function of the LptD translocon and also contributes to the final stages of LPS insertion[161]. Thus, essential Lpt system transports newly synthesized LPS from the IM to the OM to maintain the envelope barrier[162,163]. The trans-envelope bridge comprises three subcomplexes, the IM ABC transporter $LptB_2FGC$, the periplasmic bridge protein LptA, and the OM assembly complex LptDE has shown in **Figure 14**. In summary, the $LptB_2FGC$ complex extracts LPS from the IM using ATP hydrolysis by LptB, initiating transport via a “push-and-pull” mechanism[164,165]. Soluble LptA forms an elongated oligomeric bridge with a hydrophobic groove, guiding LPS across the periplasm and preventing backflow[163,166]. At the OM, the LptDE complex inserts LPS into the outer leaflet, with LptE acting as a chaperone for the LptD β-barrel[22].

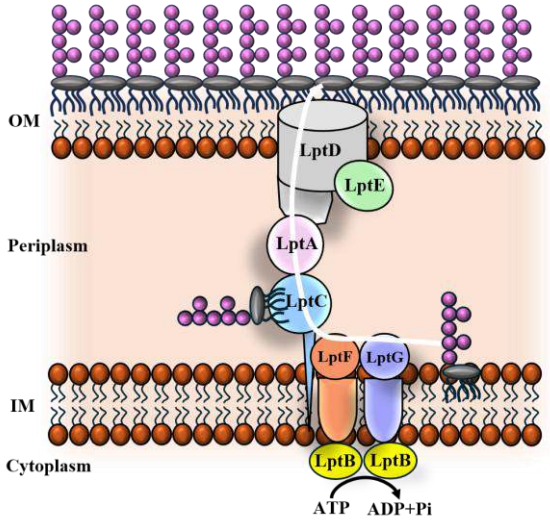

Figure 14: Schematic representation of the Lpt system for LPS transport (adapted from[167]).
The Lpt system transports LPS across the periplasm and inserts it into the OM at the expense of ATP hydrolysis. Lpt proteins are indicated from LptA to LptG.

The conserved lipoprotein LptM is a critical factor for the oxidative maturation of the LPS translocon. It assembles with the LptDE complex at the BAM machinery, stabilizing an LptD conformation that enables efficient formation of its native disulfide bonds[168,169]. When LptM is nonfunctional, the disulfide isomerase DsbC becomes essential, highlighting LptM's key role. Structural and biochemical evidence shows that LptM binds to sites on both LptD and LptE that are involved in LPS insertion. This suggests that LptM acts as an LPS structural mimic, facilitating LptD folding and disulfide bond formation to activate the translocon[169]. As the sole LPS translocation pathway, the Lpt system is indispensable and a promising antibiotic target[160].

1.4.8. Modification of lipid A

After translocation, the lipid A-core structure can undergo covalent modifications that are critical for environmental adaptation, such as during host colonization or immune evasion[128]. These modifications are regulated by two-component systems (TCSs). In *E. coli* and *Salmonella*, the PhoP/PhoQ and PmrA/PmrB (BasS/BasR) systems are key, while *Pseudomonas aeruginosa* uses systems like ParR/ParS[26]. In *E. coli* and *Salmonella*, induction of the BasS/BasR (PmrA/PmrB) TCS activates transcription of *arnT* and *eptA* genes, which catalyse the modification of the lipid A phosphate groups through the addition of L-Ara4N and *P*-EtN respectively[170]. These modifications occur at the periplasmic side. Unlike *eptA*, the *eptB* gene is not regulated by PmrA, but its expression is repressed by the small RNA MgrR, which itself is activated by the PhoP/PhoQ system[171,172]. The *phoP*/*phoQ* regulon is further modulated by MicA, another sRNA, while MicF negatively regulates the *lpxR* transcript encoding a lipid A deacylase in *Salmonella*. Post-translational control mechanisms also play a significant role in lipid A modification.

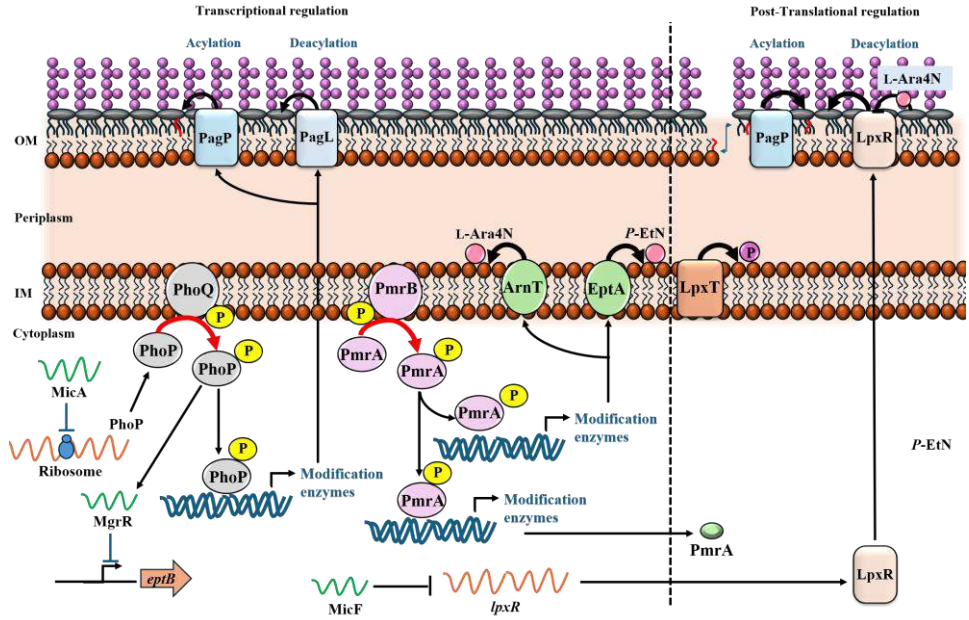

Figure 15: Multi-level regulation of lipid A modification systems in *E. coli* (adapted from[26]).
Representation of the transcriptional and post-transcriptional regulatory network mediating lipid A modifications. The two-component systems PhoP/PhoQ and PmrA/PmrB directly activate the genes encoding the modification enzymes. Fine-tuning is achieved through small RNAs (MgrR, MicA, and MicF) that repress specific targets, as indicated.

The enzyme LpxT, which transfers a phosphate group from undecaprenyl pyrophosphate to the 1-position phosphate of lipid A to form lipid A 1-diphosphate, is inhibited by the small peptide PmrR[173] (**Figure 16**). This modification increases the overall negative charge of the OM.

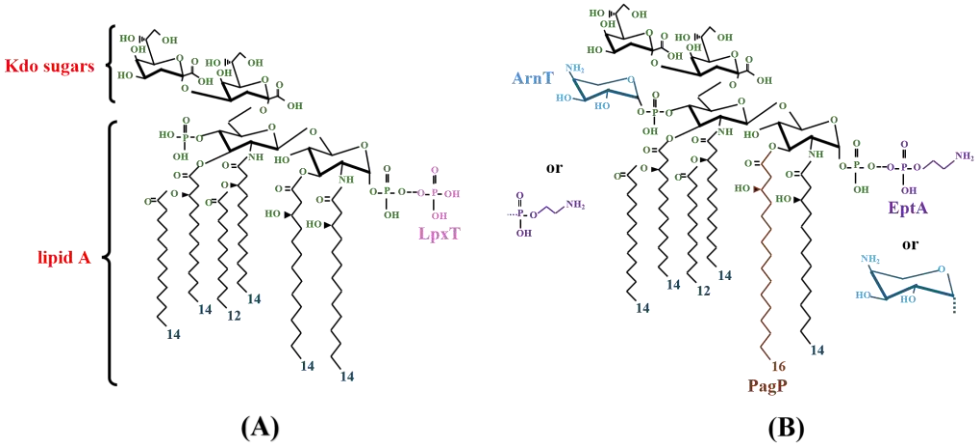

Figure 16: Structure and common modifications of *E. coli* Kdo_2-lipid A (adapted from[20,128]).
(**A**) Hexaacylated lipid A with two Kdo residues. The enzyme LpxT adds a phosphate to the 1-position, creating a bis-phosphorylated species. (**B**) Modified lipid A species carrying additional substitutions. ArnT**:** glycosyltransferase adding an amino sugar to the 1-phosphate. EptA**:** phosphoethanolamine transferase decorating the 1- or 4′-phosphate. PagP**:** OM acyltransferase adding a palmitate (C16) chain to produce a heptaacylated species.

In addition, other enzymes influence the acylation pattern of lipid A. The OMP PagP introduces a palmitate residue at the 2-position of the acyl chains, while LpxR and PagL, present in *Salmonella* but absent in *E. coli* K-12, remove the 3-linked acyl chains[174,175,176]. PagP-mediated palmitoylation occurs on the outer leaflet of the OM, utilizing PLs as palmitoyl donors[177]. Structural

studies indicate that the active site of PagP faces the cell exterior, suggesting that its activity is likely regulated by substrate accessibility at the OM surface[174]. Because these enzymes act at distinct locations and use LPS intermediates as substrates, they serve as useful biochemical reporters for studying LPS trafficking. This complex regulatory network is summarized in **Figure 15**.

1.4.9. Different LPS glycoforms

In *E. coli* K-12, at least seven distinct LPS glycoforms have been identified[18,20] (**Figure 17**). Under optimal growth conditions, glycoform I predominates (~70% of LPS), containing two Kdo residues, four heptoses, and four hexoses[178]. Minor species include glycoform II (~5%), which adds an *N*-acylglucosamine; glycoform III (~5%), lacking a terminal heptose; and glycoform IV (~20%), which has a branched tetrasaccharide with three Kdo residues and rhamnose but a truncated outer core[178]. Environmental and physiological cues drive transitions between these forms. Activation of the PhoB/PhoR and BasS/BasR systems by phosphate limitation, acidity, or high Fe^{3+} levels promote synthesis of glycoform IV[88,97].

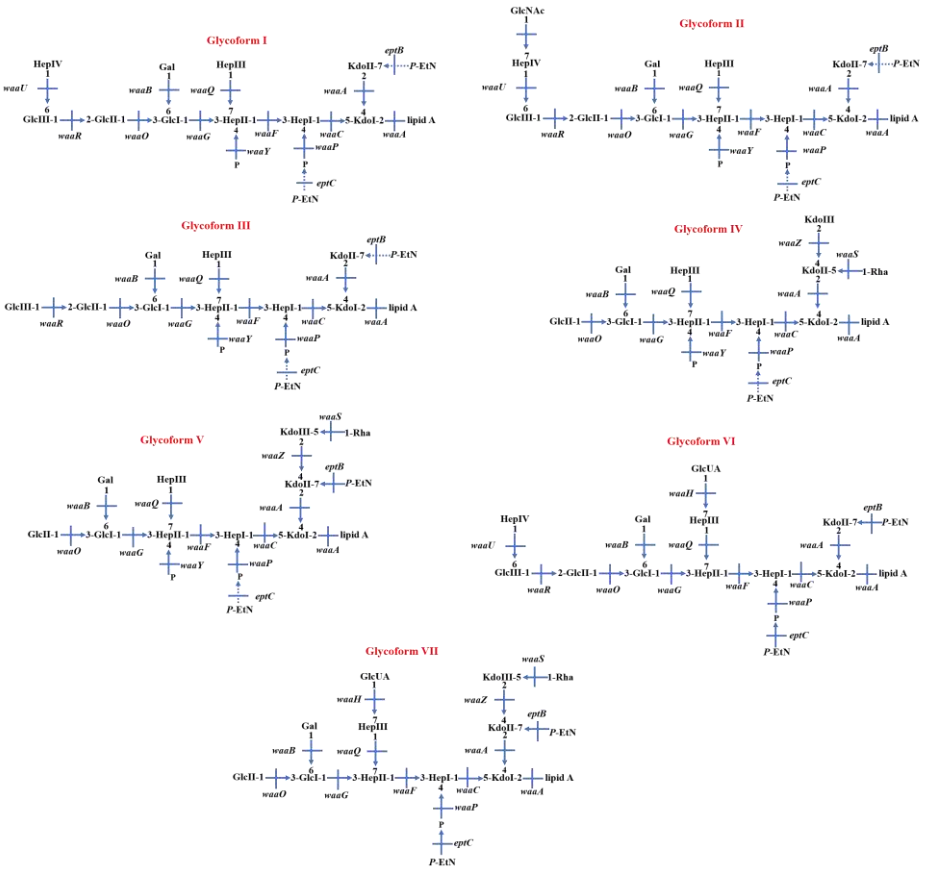

Figure 17: Schemas of *E. coli* K-12 LPS glycoforms I-VII (adapted from[97,178]).
Seven known glycoforms of *E. coli* K-12 are shown in the figure along with the indicated genes, whose products are responsible for the addition of particular moieties.

In this state, the PhoP/PhoQ-activated small RNA MgrR represses *eptB*, reducing phosphoethanolamine addition and allowing WaaS to attach rhamnose[20,88,171]. When the RpoE (σ^E) envelope stress response is activated, glycoform V becomes predominant LPS species. RpoE induction overrides MgrR repression, enabling EptB-mediated *P*-EtN addition and rhamnose

attachment[88]. Glycoform V arises under envelope stress conditions characterized by elevated RpoE activity. All forms of glycoforms containing three Kdo residues (IV, V, and VII) display truncations in the outer core due to inhibition of WaaR. WaaR is the glycosyltransferase responsible for adding the terminal glucose (Glc III) residue. Translation of *waaR* mRNA is suppressed by RpoE-induced small RNAs MicA and RybB. This explains the formation of truncated LPS cores under stress conditions[88]. Consistent with this model, Δ*waaR* bacteria were found to produce glycoform IV derivatives, even without the activation of σ^E. Overall, activation of the RpoE stress response leads to the synthesis of glycoforms IV, V, and VII, depending on the balance between MgrR repression, EptB activation, and other environmental factors. Glycoform IV accumulates when MgrR-mediated repression of *eptB* predominates. When RpoE activity is strongly induced, promoting *eptB* expression, glycoform V becomes dominant. Glycoform VII emerges under conditions combining RpoE activation with phosphate limitation and submillimolar concentrations of Zn^{2+} and Fe^{3+}, which induces the transcription of the gene encoding the WaaH glucuronic acid transferase. The main difference between glycoforms VI and VII lies in their regulatory requirements, while glycoform VII formation depends on envelope stress and nutrient limitation, glycoform VI synthesis occurs by the induction of PhoB/PhoR[18,97].

1.4.10. LPS in pathogenesis and immune recognition

LPS exemplifies a molecular duality, serving as an essential structural component for bacteria and the primary trigger of innate immunity. This role centers on the lipid A part of LPS, whose immunostimulatory activity is dictated by its acylation and phosphorylation pattern. The canonical hexaacylated lipid A of *E. coli* is a potent TLR4 agonist, while structural variants like the tetraacylated form from *Yersinia pestis* act as TLR4 antagonists, directly linking structure to pathogenesis and immune evasion[179]. Host detection is a multi-tiered process. LPS-binding protein (LBP) extracts and shuttles LPS to CD14, which loads it into the MD-2/TLR4 complex. This induces dimerization, activating two signaling pathways: the MyD88-dependent pathway for rapid pro-inflammatory cytokine production (e.g., TNF-α, and IL-6), and the TRIF-dependent pathway from endosomes to induce type I interferons[180,181]. When dysregulated, systemic LPS dissemination causes septic shock, while chronic low-grade exposure (metabolic endotoxemia) is linked to diseases like atherosclerosis and insulin resistance[182,183]. Pathogens evade this recognition using PhoP/PhoQ and PmrA/PmrB systems to modify lipid A via enzymes like ArnT, EptA, PagP, and LpxR, which mask the charge or alter acyl chains to enhance resistance[16,26].

1.5. Overview of bacterial phospholipid (PL)

In Gram-negative bacteria, PLs dominate the IM, while their distribution in the OM is tightly regulated to maintain asymmetry. LPS occupies the outer leaflet and PLs the inner leaflet[9]. The major PLs in *E. coli* are phosphatidylethanolamine (PE, 70-80%), phosphatidylglycerol (PG, 15-20%), and cardiolipin (CL, 5-10%)[184]. PE provides membrane curvature, PG contributes negative charge, and CL localizes to cell poles and division sites[185,186,187]. PL accumulation in the OM outer leaflet, leads to permeability defects and increased antibiotic sensitivity[188]. The Mla pathway actively

removes mislocalized PLs from the OM outer leaflet, preserving LPS dominance and thus OM barrier function[189]. PL and LPS biosynthesis are metabolically coordinated, sharing fatty acid precursors and coupled regulation through enzymes like LpxC[190]. The ratios of PE, PG, and CL shift under stress, enabling adaptive remodelling, though the systems-level coordination of PL and LPS synthesis remains poorly understood.

1.5.1. PL biosynthesis pathways

PL biosynthesis is a central, conserved process integrating fatty acid synthesis with membrane biogenesis[189]. The biosynthetic route responsible for the formation of major membrane PLs in *E. coli* was first elucidated through the pioneering work of Eugene P. Kennedy and his collaborators in the 1950s and 1960s. This metabolic framework, now known as the Kennedy pathway, describes the sequential enzymatic steps by which glycerophospholipids are synthesized from precursors, such as glycerol-3-phosphate and fatty acids[191,192].

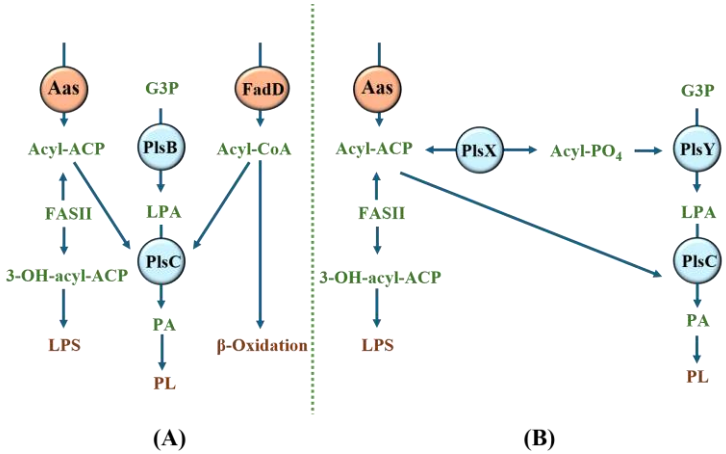

Figure 18: Pathways for PL synthesis in Gram-negative bacteria (adapted from[193]).

(**A**) In Gram-negative bacteria, PlsB/PlsC pathway for PL synthesis use either a FadD or an Aas. Acyl-CoAs are used for β-oxidation but are also substrates for PlsB/PlsC acyltransferases. (**B**) Other Gram-negative bacteria uses the PlsY/PlsC PL biosynthesis pathway. In these organisms, Aas is used to activate fatty acids that are incorporated at the PlsY step after conversion to acyl-PO_4 by PlsX. acyl-CoA synthetase (FadD), acyl-ACP synthetase (Aas).

Fatty acid synthesis (FASII) serves as a critical point of connection, generating acyl-ACP precursors that supply hydrophobic building blocks for both PLs and LPS, coupling their biogenesis[194]. The commitment step is the acylation of *sn*-glycerol-3-phosphate, primarily catalyzed by PlsB to form lysophosphatidic acid (LPA), followed by PlsC to yield phosphatidic acid (PA)[195]. An alternative initiation route via PlsY underscores the functional redundancy in establishing the core lipid backbone[196,197] (**Figure 18**).

The enzyme CdsA is a critical metabolic node, converting phosphatidic acid (PA) into the universal precursor CDP-diacylglycerol (CDP-DAG) for all anionic PLs[198,199] (**Figure 19**). This magnesium-dependent cytidylyltransferase reactions is catalyzed by an integral membrane protein with a conserved CDP-alcohol phosphatidyltransferase superfamily architecture[200,201,202]. PE synthesis begins with phosphatidylserine synthase (PssA), which produces phosphatidylserine (PS) from CDP-DAG and serine[200,203]. PS is then decarboxylated by phosphatidylserine

decarboxylase (Psd) to yield PE, which is indispensable and its absence causes severe OM instability and cell inviability[204,205]. The major anionic PL, phosphatidylglycerol (PG), is synthesized via the committed step catalyzed by the essential enzyme phosphatidylglycerophosphate synthase (PgsA), which produces phosphatidylglycerol phosphate (PGP) from CDP-DAG[204]. PGP is subsequently dephosphorylated by Pgp enzymes to form PG[206,207]. PG is a structural component and the precursor for CL. CL is synthesized primarily by cardiolipin synthases ClsA, ClsB, and ClsC, which condense two PG molecules (with ClsB and ClsC using alternative substrates)[208,209]. However, this enzymatic redundancy is not reflected in their genetic regulation. The expression of *clsA* is upregulated upon entry into the stationary phase, while *clsB* and *clsC* are constitutively expressed at low levels. Despite the presence of three synthases, no single *cls* gene is essential for viability under standard laboratory conditions. However, triple Δ(*clsABC*) bacteria is completely devoid of CL, demonstrating that these enzymes constitute the complete synthetic repertoire for this lipid in *E. coli*[210].

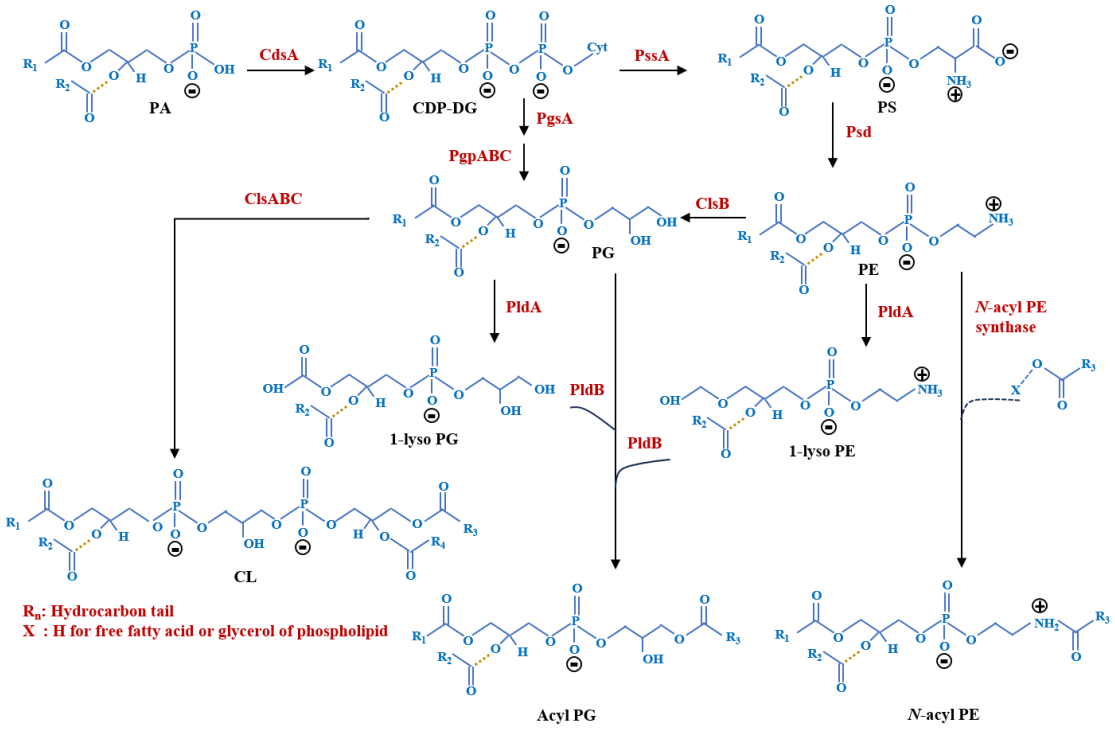

Figure 19: Schematic representation of PL biosynthetic pathway (adapted from[184]).
Illustration of the synthesis of PG, PE, and CL from PA. The enzymes involved in PL biosynthesis are also shown.

Previous studies demonstrated that CL is required for the survival of mutants deficient in either WaaA or LpxM[153]. Suppressor analysis of synthetic lethality of Δ(*clsA lpxM*) bacteria, identified compensatory mutations in the gene encoding the MsbA LPS flippase. This genetic evidence supports a model in which CL facilitates the transport of LPS across the IM[199,153,211]. The physiological importance of CL becomes evident under stress conditions. Cells lacking CL show pronounced defects in salt and pH tolerance, impaired biofilm formation, and aberrant cell division, often forming minicells due to a failure in properly localizing the division machinery to the midcell[212].

1.5.2. Maintenance of lipid asymmetry (Mla pathway)

Bacteria have evolved a dedicated quality control system to remove the mislocalized PLs and restore OM homeostasis. To restore asymmetry, bacteria employ the ATP-driven Mla retrograde transport pathway, which extracts mislocalized PLs from the OM and returns them to the IM[189]. This three-step process begins at the OM with the MlaA lipoprotein, which extracts PLs in complex with porins like OmpC/F[213]. The soluble periplasmic chaperone MlaC then shuttles the lipid via a dedicated hydrophobic pocket[214,215]. Finally, the IM ABC transporter complex MlaFEDB imports the PL. Here, MlaD receives the lipid from MlaC, MlaE forms the translocation conduit, and MlaF hydrolyzes ATP to power transport, a cycle regulated by MlaB[214,216,217]. The overall mechanism of the MlaFEDB complex follows the classic model of ABC transporters (**Figure 20**). The binding of the MlaC-PL complex to MlaD likely triggers ATP binding by MlaF. ATP hydrolysis then induces a conformational change in MlaE that translocates the lipid from the MlaD pocket into the IM bilayer. Subsequent phosphate release resets the complex for another cycle. Recent cryo-EM structural studies provide near-atomic resolution of the MlaFEDB complex, confirming an ATP-dependent retrograde lipid transport mechanism, though some data suggest possible bidirectional transport under specific conditions[218].

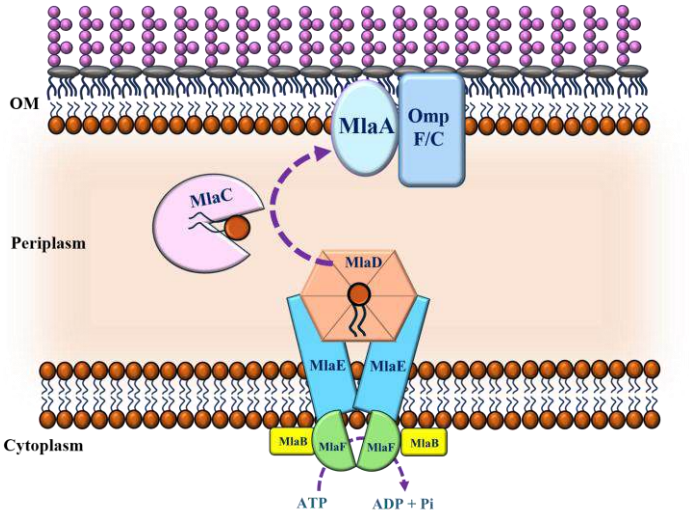

Figure 20: Schematic representation of the PL transport and Mla proteins (adapted from[219]).

The Mla system proteins are labelled as MlaA to MlaF. The IM is composed of PLs, while the OM is an asymmetric lipid bilayer with an inner leaflet composed of PLs and an outer leaflet of LPS.

The physiological importance of this pathway is profound. Mutations in any of the *mla* genes, particularly *mlaA* or *mlaF*, result in a characteristic hypersensitivity to detergents, bile salts, and a range of antibiotics, including novobiocin, which is normally excluded by an intact OM[189]. This phenotype directly links the Mla pathway to the barrier function of the OM.

1.5.3. PL in stress adaptation, virulence, and antibiotic resistance

PLs are dynamic regulators of bacterial physiology, with their composition and localization undergoing stress-responsive remodelling that impacts envelope integrity, virulence, and antibiotic resistance[220]. PE-deficient bacteria strongly induce the σ^E and Cpx responses, while CL deficiency

activates σ^S and Rcs pathways, with the severity depending on the specific PL missing and genetic background. At the protein level, PE-deficient bacteria overproduce DegP and alter sigma factor levels (increased RpoE, RpoH, RpoS, and decreased RpoD), whereas CL-deficient bacteria show a more moderate stress profile[186,212]. Also, comparative studies have shown that PE and CL deficient *E. coli* exhibit elevated expression of several envelope stress markers (*degP*, *cpxP*, and *spy*) even under non-stress conditions, indicating intrinsic membrane instability[186]. This suggests that different PL deficiencies elicit distinct adaptive responses, potentially involving compensatory mechanisms that maintain envelope function despite internal perturbations[186]. Altered PL distribution promotes biofilm development, particularly in *Pseudomonas aeruginosa*[221]. Additionally, PE-deficiency impair the ability of *E. coli* cells to adhere to surfaces. PL modification influences the physicochemical properties of cell[222]. PL play pivotal roles beyond structural support, enabling adaptation to stress, modulation of virulence, and resistance to antibiotics. Their remodelling, which is tightly integrated with LPS regulation, underpins Gram-negative bacterial resilience.

1.6. Fatty acid (FA) in bacterial physiology

Fatty acids (FAs) are essential structural components of bacterial membranes, forming the hydrophobic backbone of PLs and lipid A to determine membrane properties and act as regulatory molecules[223,224]. Changes in FA structure directly influence the envelope barrier function and stress adaptation[224,225]. Most bacteria synthesize straight-chain saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs), typically 14-18 carbons long. *E. coli*, for instance, produces palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and *cis*-vaccenic acid (C18:1) and two minor components, laurate (C12:0) and 3-hydroxymyristate[194]. These serve distinct roles, SFAs increase membrane rigidity and reduce permeability, whereas UFAs enhance fluidity, especially important during cold stress[194,226]. Cyclopropane fatty acids (CFAs), formed by the post-synthetic modification of UFAs, are associated with the stationary phase and acid stress adaptation[227]. Some pathogens also produce branched-chain or hydroxylated FAs, influencing virulence and immune recognition[224]. FAs contribute directly to multiple lipid classes of PLs such as PE, PG, and CL contain two FA chains. LPS (lipid A) molecules typically contain 4-6 FA chains, providing the hydrophobic anchor that stabilizes LPS in the OM. Thus, FA metabolism directly couples to PL and LPS biogenesis[6].

1.6.1. Central importance of AcpP

The acyl carrier protein AcpP is essential for all stages of fatty acid biosynthesis in *E. coli*, where intermediates remain bound as thioesters recognized by the corresponding enzymes of the pathway[228]. It is an acidic protein of ~8.9 kDa. Its structure is a rod-shaped, with three core α-helices and its activity depends on a 4′-phosphopantetheine prosthetic group attached to Ser-36[229]. The acyl chain binds within a hydrophobic cavity along the second helix, stabilizing a compact form, while charged intermediates induce a conformational expansion to facilitate catalysis[228]. In normally growing bacteria, the CoA concentration is approximately eightfold higher than that of AcpP, ensuring that the prosthetic group supply is not limiting for fatty acid biosynthesis. Nearly all AcpP

is present in its active holo form, and the turnover of its prosthetic group may contribute to CoA homeostasis. During exponential growth, a significant pool of unacylated holo-AcpP exists, but the inhibition of acyl-transfer reactions causes acyl-AcpP to accumulate and unacylated AcpP to decline sharply, indicating that disruptions in elongation or transfer transiently alter the acylated/unacylated balance[229]. AcpP also serves as a multifunctional hub, interacting with at least 35 partner proteins, and participates in processes like membrane-derived oligosaccharide synthesis and chromosome partitioning, indicating a role beyond lipid metabolism[230].

1.6.2. Fatty acid biosynthesis (FASII pathway)

De novo fatty acid biosynthesis in bacteria is governed by the dissociated FASII system. The pathway begins with the committed, rate-limiting step catalyzed by acetyl-CoA carboxylase (ACC), which produces malonyl-CoA[199,231]. Malonyl-CoA is transacylated to ACP by FabD, forming malonyl-ACP. Chain initiation is mediated by β-ketoacyl-ACP synthase III (FabH), which condenses an acyl-CoA primer (e.g., acetyl-CoA) with malonyl-ACP. FabH employs a Cys-His-Asn catalytic triad, and its acyl-CoA specificity determines the final FA profile in organisms like *E. coli*[228,232]. Elongation proceeds via an iterative four-step cycle. The condensation step, a key regulatory point, is performed by β-ketoacyl-ACP synthases FabB and FabF, which add two-carbon units from malonyl-ACP (**Figure 21**). At lower temperatures, FabF becomes more active in elongating *cis*-vaccenic acid (C18:1), allowing the bacterium to incorporate longer-chain UFAs into PLs to maintain the membrane[233]. The resulting β-ketoacyl-ACP is reduced by the NADPH-dependent, stereospecific reductase FabG[234].

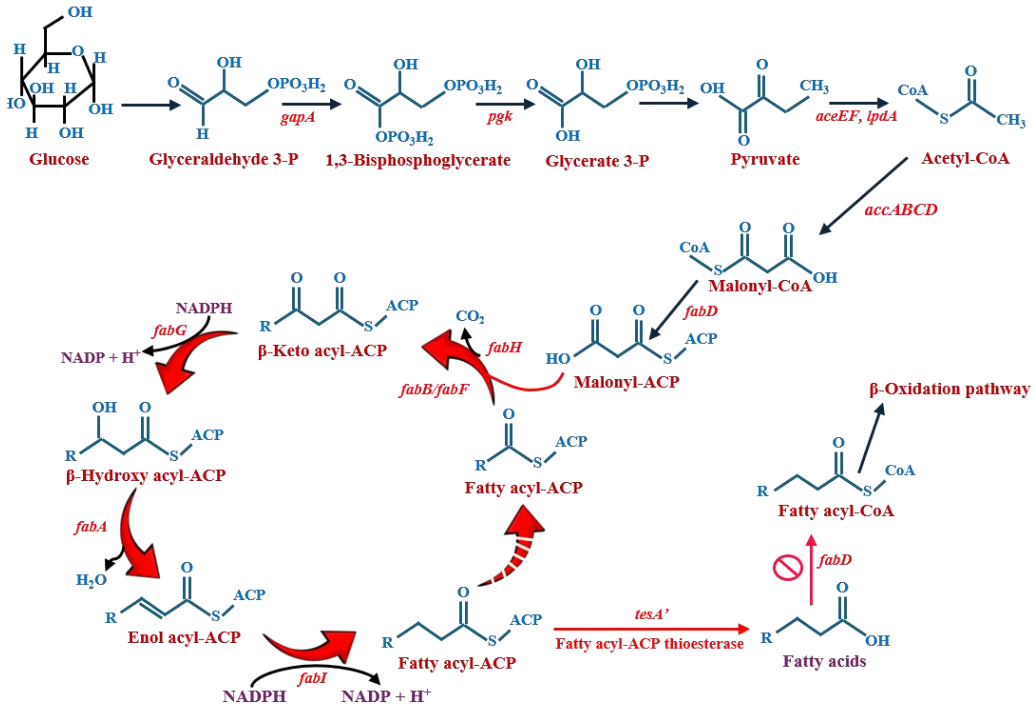

Figure 21: Schematic representation of the FA biosynthetic pathway (adapted from[235]).
accABCD, acetyl-CoA carboxylase; *fabD*, malonyl-CoA-ACP transacylase; *fabH*, β-ketoacyl-ACP synthase III; *fabB*, β-ketoacyl-ACP synthase I; *fabF*, β-ketoacyl-ACP synthase II; *fabG*, β-ketoacyl-ACP reductase; *fabA*, β-hydroxy acyl-ACP dehydrase; *fabI*, enol acyl-ACP reductase; *tesA'*, truncated multifunctional fatty acyl-CoA thioesterase; and *fadD*, fatty acyl-CoA synthetase.

Dehydration is catalyzed by FabZ for saturated FAs or the bifunctional FabA, which also isomerizes intermediates for UFA synthesis[236]. The final reduction of *trans*-2-enoyl-ACP is catalyzed by the NADH-dependent FabI, a target of inhibitors like triclosan[236,237]. This cycle repeats typically 5-7 times, until the acyl chain reaches its mature length (C14-C18). The chain termination occurs when the acyl-ACP is poorly recognized by condensing enzymes and is utilized by acyltransferases. These enzymes, including PlsB/PlsC for PL synthesis and LpxA/LpxD for lipid A biosynthesis, directly channel mature acyl-ACPs into envelope lipids, creating a critical metabolic node[238].

1.6.3. Regulation of fatty acid synthesis

FA metabolism is tightly regulated, as disruptions in FA pools compromise membrane integrity and viability[228]. The transcriptional regulator FadR regulates both FA degradation and biosynthesis. In the absence of FAs, FadR represses the *fad* regulon (e.g., *fadBA*, and *fadL*), preventing unnecessary β-oxidation. When long-chain FAs are present, they are converted to acyl-CoAs, which bind FadR's allosteric site, inducing a conformational change that reduces its DNA affinity. This derepresses the *fad* genes[239,240]. Consequently, *fadR* null mutants exhibit a constitutive β-oxidation phenotype, capable of metabolizing short-chain FAs without induction. FadR acts as a direct activator of FA biosynthesis, notably upregulating *fabA*, an essential gene for unsaturated FA synthesis[241]. This dual function is determined by the binding site position at activation targets like *fabA*. FadR binds near -40 to recruit RNA polymerase at repression targets, it binds downstream to block transcription[239]. Structurally, FadR contains a N-terminal DNA-binding domain and a C-terminal effector-binding domain. Crystal structures show that acyl-CoA binding induces a helical rotation, reorienting the dimer, and preventing DNA binding[242]. Thus, FadR senses acyl-CoA levels to switch cellular metabolism between net synthesis (low acyl-CoA: activates *fabA*, and represses *fad*) and net degradation (high acyl-CoA: dissociates from DNA, derepresses *fad*, and stops *fabA* activation), maintaining envelope homeostasis.

1.7. Peptidoglycan (PGN)

PGN, also known as murein, is one of the most fundamental components of bacterial cell envelopes. Its essentiality was first highlighted in the early days of antibiotic discovery when penicillin was found to target PGN biosynthesis, providing both a mechanistic understanding of bacterial cell death and a therapeutic breakthrough[243,244]. PGN is unique to bacteria, absent in eukaryotes, making it a prime antibiotic target. The widespread success of β-lactams, glycopeptides, and fosfomycin underscores the clinical relevance of this macromolecule[245]. In Gram-negative organisms, such as *E. coli*, PGN forms a thin but resilient sacculus located in the periplasm between the IM and the OM. It protects by shielding against osmotic stress, host defenses, and certain antibiotics[246]. The sacculus is a continuous net-like macromolecule that completely surrounds the cytoplasmic membrane, acting as a rigid exoskeleton without compromising cell growth and division[1]. While both Gram-positive and Gram-negative bacteria rely on PGN for structural stability, key differences exist, Gram-positive PGN is much thicker (20-80 nm)

and often cross-linked with teichoic acids, whereas Gram-negative PGN is much thinner but integrated into a more complex envelope containing LPS, PL, and lipoproteins[1,247].

1.7.1. PGN structure and composition

PGN is a mesh-like heteropolymer surrounding the cytoplasmic membrane, providing structural strength and adaptability[247]. Its glycan backbone consists of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β-1,4-glycosidic bonds[247]. The glycan chains typically range from 10 to 60 disaccharide units, forming long polymers that are covalently cross-linked by peptides[247]. Each MurNAc residue is substituted with a short stem peptide that provides the cross-linking capacity. In Gram-negative bacteria, such as *E. coli*, the canonical pentapeptide is: L-Ala-D-Glu-meso-diaminopimelic acid (m-DAP)-D-Ala-D-Ala[247]. Key features of L-Ala at position 1 is to provide the initial donor group. D-Glu (position 2), introduces chirality and prevents host protease degradation. m-DAP (position 3) is unique to Gram-negative PGN and provides the amino group for cross-linking. D-Ala-D-Ala (positions 4-5) serve as substrates for transpeptidation reactions. The terminal D-Ala is cleaved during cross-linking, leaving a tetrapeptide in the mature PGN[247]. Cross-linking forms a 3D sacculus by connecting the D-Ala at position 4 of one stem peptide to the m-DAP at position 3 of another, resulting in a lower cross-linking degree (~30-40%) compared to Gram-positive bacteria[247,248]. The degree of cross-linking in Gram-negative PGN is approximately 30-40%, lower than that in Gram-positives (~70-80%), reflecting their thinner PGN layers and reliance on the OM for additional mechanical stability. Although the basic framework of the PGN is conserved, variations exist across species. *E. coli* features the canonical L-Ala-D-Glu-m-DAP-D-Ala-D-Ala stem peptide[249].

1.7.2. Biosynthesis of PGN

PGN assembly begins in the cytoplasm, where the committed step is catalyzed by MurA, followed by MurB, and the ATP-dependent ligases MurC-MurF, which sequentially add the stem peptide amino acids to form UDP-MurNAc-pentapeptide[248,250,251] (**Figure 22 A**). This hydrophilic precursor is transferred to the lipid carrier undecaprenyl phosphate by MraY, and then MurG adds a GlcNAc residue to generate the complete lipid-linked precursor lipid II[252,253] (**Figure 22 B**). The flippase MurJ, assisted by SEDS-family proteins, translocates lipid II to the periplasm for polymerization, and the undecaprenyl phosphate carrier is recycled, linking PGN synthesis to other envelope polymers[254,255,256]. In the periplasm, lipid II is incorporated into the growing peptidoglycan meshwork by two key enzyme classes: glycosyltransferases (GTs) polymerize the disaccharide subunits into glycan strands, and transpeptidases (TPs) cross-link the stem peptides of adjacent strands[257].

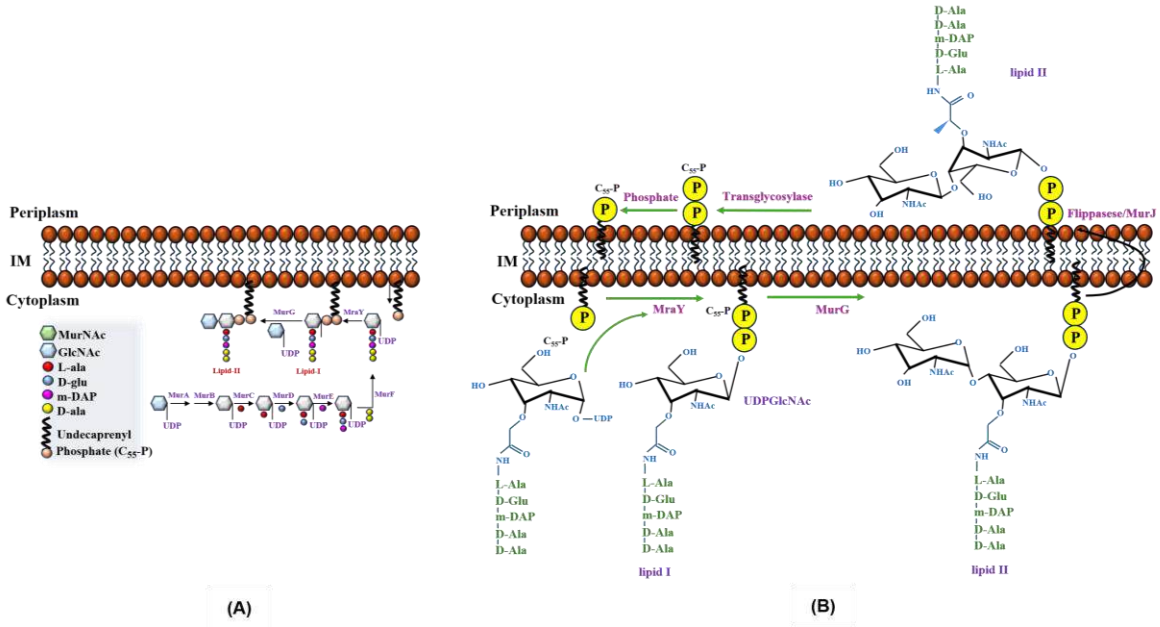

Figure 22: Synthesis of PGN precursors and lipid carrier steps at the IM (adapted from[250,258]).

(**A**) MurA-MurF enzymes consecutively assemble UDP-MurNAc-pentapeptide and then transfer it onto the undecaprenyl phosphate (C55-P) lipid carrier by MraY. This forms lipid I, followed by the addition of UDP-GlcNAc by MurG to generate lipid II prior to its translocation and polymerization into the cell wall. (**B**) Lipid I is formed on the cytoplasmic leaflet when MraY transfers the UDP-MurNAc-pentapeptide to undecaprenyl phosphate (C55-P), and MurG then converts lipid I to lipid II by adding UDP-GlcNAc before the flippase MurJ translocates lipid II to the periplasmic side, where transglycosylases polymerize the glycan strands and recycle the lipid carrier.

Insertion of new material requires remodelling of the existing sacculus by hydrolytic enzymes, the lytic transglycosylases cleave the glycan backbone, the DD-carboxypeptidases trim stem peptides and the endopeptidases/amidases cleave cross-links and separate daughter cells[259].

1.8. Envelope stress responses

Gram-negative bacteria maintain envelope integrity through two primary, complementary stress response systems: the RpoE (σ^E) pathway, which responds to OMP biogenesis defects and OM disturbances, and the Cpx two-component system, which senses IM and periplasmic protein-folding stress[12,260]. These dedicated systems are crucial due to unique envelope challenges. First, the ATP-free periplasm is vulnerable to misfolded OMP accumulation, a direct activator of the RpoE pathway[261]. Second, defects in LPS/PL asymmetry that increase permeability trigger responses to upregulate lipid A modification and retrograde transport[22]. *Pseudomonas aeruginosa* uses its RpoE homolog AlgU to control alginate biofilm production, *Neisseria meningitidis* requires its homologs for serum resistance[262,263]. The pioneering work by the laboratories of S. Raina, C. Gross, and T. Silhavy has been instrumental in mapping these networks[11,48,264]. This research established that envelope homeostasis is governed by a tightly regulated network dedicated to envelope quality control.

1.8.1. The σ^E regulon

The *rpoE* gene encodes an extracytoplasmic function (ECF) sigma factor known as σ^E (or σ^{24}), which serves as a specialized subunit of RNA polymerase[11]. It was first identified as the essential regulator of the P3 promoter of the *rpoH* gene, which encodes the heat shock sigma factor. This promoter is uniquely active at high temperatures[11,48,265]. σ^E also initiates transcription from the *rpoN* and *rpoD* genes, encoding σ^{54} and the primary sigma factor σ^{70}, respectively[266]. In *E. coli*, only σ^{70} and σ^E are essential for viability at all temperatures, primarily through their role in maintaining OM integrity[11,267]. The Eσ^E holoenzyme transcribes a wide array of genes responsible for the synthesis, modification, and transport of OM components. These include genes for PL biosynthesis (*plsB*), lipid A biosynthesis (*lpxA*, *lpxB*, *lpxD*, and *lpxP*), LPS core assembly (*rfaD*, *waaC*, *waaF*, *waaL*, and *waaU*), and the LPS transport system (*lptA*, *lptB*, and *lptD*)[266,268]. The RpoE regulon further includes genes, whose products are required for OM and periplasmic protein folding and assembly, such as the chaperones *skp* and *clpX*, proteases *degP*, *ecfE* (*rseP*), and *yfgC*, peptidyl-prolyl *cis*/*trans* isomerases *fkpA* and *surA*, the protein disulfide isomerase *dsbC*, and components of the BAM complex (*bamA*, *bamB*, *bamC*, and *bamD*). σ^E also regulates genes encoding OMPs (e.g., *ecfI*/*yidQ*), lipoproteins (*ecfH*/*yraP*, and *yeaY*), and various transport proteins (*sbmA*, *ecfG*/*ygiM*, *ecfL*/*yqjA*, *efcF*/*yggN*,and *ecfJ*/*ytfJ*)[266,269]. Additional regulon members are involved in cell envelope biosynthesis: *bacA* (for PGN, LPS, and teichoic acids) and *wzb*/*wzc* (for capsular polysaccharide) highlighting broad role of σ^E in envelope homeostasis[266,269,270,271]. Elevated expression levels of RpoE have been observed in bacterial strains that produce truncated forms of LPS, such as Kdo_2-lipid IV_A or free lipid IV_A. σ^E activity is strongly induced by an imbalance between LPS and PL composition in the OM[24,93]. Furthermore, mutants synthesizing heptoseless LPS exhibit constitutive *rpoE* activation, confirming that defects in LPS core biosynthesis potently trigger the σ^E stress response[93,272].

1.8.2. Regulation of the *rpoE* gene

The activity and cellular concentration of σ^E are tightly regulated. The *rpoE* gene resides in a four-gene operon (*rpoE-rseA-rseB-rseC*) encoding σ^E, its anti-sigma factor RseA, and modulators RseB and RseC[260]. The discovery of the *rpoE* gene was accompanied by the identification of two distinct promoters responsible for its transcription[11,273]. Subsequent complementary studies demonstrated that *rpoE* expression is positively autoregulated through its proximal promoter (*rpoE*P2), which is specifically recognized by the Eσ^E holoenzyme. The activity of this promoter increases in response to disturbances in OMP homeostasis, highlighting its role in the envelope stress response. In contrast, the regulatory function of the distal promoter (*rpoE*P1) was not then fully elucidated at this stage[273] (**Figure 23**). More recent studies have identified six transcription start sites for *rpoE*. Among these, the promoters *rpoE*P2 and *rpoE*P3 are specifically activated by LPS perturbations, with *rpoE*P3 showing the strongest induction during LPS biosynthesis or assembly defects or upon polymyxin B exposure[273] (**Figure 23 B**). At elevated temperatures, transcription is primarily initiated from a σ^E-dependent promoter, whose activity can be repressed by phosphorylated CpxR, linking the σ^E and Cpx systems[11,274]. Two-component regulatory systems

are among the most widespread signal transduction mechanisms used by bacteria to adapt to environmental changes[275].

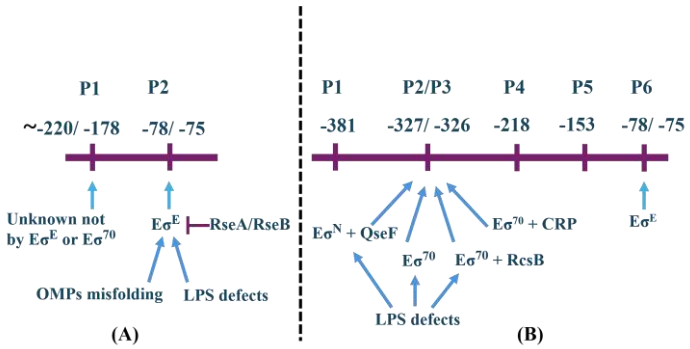

Figure 23: Transcriptional regulation of the *rpoE* gene (adapted from[273]).
(**A**) Schematic presentation of the transcriptional start sites of the *rpoE* gene known previously, since gene discovery. (**B**) Subsequent more precise identification by mapping of transcription start sites and verification by *in vitro* run-off assays[273]. Different sigma factors and regulatory proteins established in later study that affect the activity of certain promoters are presented.

The Cpx TCS consists of the sensor kinase CpxA and the response regulator CpxR. Under envelope stress, CpxA autophosphorylates and transfers the phosphate to CpxR, which then modulates target gene transcription[276]. The pathway is negatively regulated by the periplasmic inhibitor CpxP, which binds CpxA[277,278]. The Cpx and σ^E pathways functionally overlap in responding to extracytoplasmic protein misfolding[266,276]. Specific LPS defects, such as in Δ(*lapA lapB*), Δ*waaA*, or mutants synthesizing tetraacylated LPS precursors [e.g., Δ(*waaC lpxL*)], strongly induce both the σ^E and Cpx responses[18].

Under non-stress conditions, σ^E is sequestered at the IM by its anti-sigma factor RseA, which binds specifically to region 4.2 of σ^E, preventing its association with RNA polymerase core[279,280,281,282]. This inhibition is enhanced by the phosphorylation of RseA by Etk kinase and stabilized by the periplasmic protein RseB[279,283]. *rseC* is the final gene in this operon, which encodes an IM protein that plays a positive regulatory role in modulating the activity of σ^E. When bacteria encounter extracytoplasmic or envelope stress, such as the accumulation of misfolded OMPs or truncated LPS, RseB dissociates from RseA. This makes RseA accessible to regulated intramembrane proteolysis[260]. The process begins with cleavage of the periplasmic domain of RseA by the DegS protease, which is activated through binding of its PDZ domain to C-terminal peptides of unfolded OMPs[261,284,285]. Afterwards, the IM metalloprotease EcfE (also known as RseP) performs a second cleavage within the transmembrane region of RseA. This step facilitates the release of the cytoplasmic fragment of RseA, which is rapidly degraded by ATP-dependent proteases, such as ClpX/ClpP. As a result of this proteolytic cascade, active σ^E is released into the cytoplasm, where it binds to the RNA polymerase core to initiate transcription from its own operon as well as genes belonging to the RpoE regulon. These target genes encode proteins that assist in the proper folding, assembly, and biosynthesis of envelope components, thereby restoring membrane homeostasis[266].

1.8.3. σ^E and the Rcs two-component system, coordinated response to LPS and OM stress

The maintenance of the bacterial cell envelope requires a precisely coordinated interplay between multiple regulatory systems that sense and respond to environmental and physiological perturbations. In *E. coli*, the envelope stress response is governed by the RpoE regulon, which has been discussed in the above sections, and the Rcs (Regulator of Capsule Synthesis) TCS.

The Rcs system is composed of the OM lipoprotein RcsF, the IM sensor kinase RcsC, the phosphotransfer protein RcsD, and the response regulator RcsB[286]. These components collectively form a signal transduction cascade that translates envelope stress signals into transcriptional responses. Under non-stress conditions, RcsF forms stable complexes with abundant OMPs, such as OmpA, OmpC, and OmpF[287]. These interactions tether RcsF within the OM and suppress it signalling activity. However, when the OM is compromised, such as through LPS truncations, antibiotic exposure, or alterations in lipid organization, these complexes are destabilized. The RcsF lipoprotein then interacts with the periplasmic domain of the sensor kinase RcsC, triggering autophosphorylation and subsequent phosphotransfer through RcsD to the response regulator RcsB[287]. Phosphorylated RcsB (RcsB~P) then functions as a transcription factor, modulating the expression of genes that strengthen the envelope, promote exopolysaccharide synthesis, and regulate stress response operons[286].

The first clear evidence linking the Rcs system to σ^E regulation during LPS stress came from studies of *E. coli* mutants defective in LPS core biosynthesis. In these experiments, a sevenfold increase in transcription from the *rpoE*P3 promoter was observed in Δ*waaC* bacteria, which synthesize truncated LPS lacking the inner heptose residues of the core oligosaccharide[273]. Interestingly, this strong induction was almost completely abolished in Δ(*waaC rcsB*) bacteria, indicating that the Rcs two-component system is indispensable for RpoE activation under LPS-defective conditions[273]. This discovery provided the first mechanistic evidence that the Rcs phosphorelay acts upstream of σ^E in sensing and responding to LPS abnormalities. Further molecular analyses supported this conclusion. *In vitro* DNA-binding assays using purified RcsB demonstrated that this regulator specifically binds to a distinct motif within the *rpoE*P3 promoter region, confirming its role as a transcriptional activator of the *rpoE* gene[273]. When stress, including disturbance within the LPS structure, happens, the information is transduced to the RcsF C-terminal signalling domain located in the periplasm to activate the stress response[288]. These results collectively established that RcsB functions as a transcription factor that activates *rpoE* expression when LPS synthesis or OM architecture is perturbed[273].

The vital role of the Rcs system in detecting defects of LPS had been suggested even earlier during studies of deep-rough LPS mutant strains. In these mutants, particularly in the *rfa-1* strains that produce truncated LPS containing only the initial heptose residues, a strong induction of the *cps* (capsular polysaccharide synthesis) operon was observed. This induction was found to depend on Rcs signaling, as deletion of the *rcsF* gene abolished the activation of the *cps*

gene[288,289,290]. These findings imply that RcsF serves as a sensor for OM perturbations, including structural abnormalities of LPS. Further studies have revealed that RcsF forms complexes with OMPs. These RcsF-OMP assemblies are essential for detecting stress within the outer leaflet of the OM[287]. When structural defects of LPS or mechanical perturbations occur, these stresses alter the configuration of the RcsF-OMP complexes. This leads to transmission of a signal through the C-terminal domain of RcsF located in the periplasm. This signal activates the Rcs phosphorelay cascade, ultimately producing phosphorylated RcsB, which then induces transcription from the *rpoE*P3 promoter[273]. This mechanism links physical perturbations in the OM, particularly those caused by defective or truncated LPS, to transcriptional activation of the σ^E pathway through the Rcs signaling network. Collectively, these findings explained that RcsB-dependent activation of *rpoE* is a crucial regulatory step that connects LPS defect sensing with the broader envelope stress response. Thus, the Rcs system functions as both a sensor and an amplifier of σ^E-mediated signaling. By integrating structural cues from the OM and relaying them to transcriptional regulators, the Rcs phosphorelay ensures a rapid and proportional stress response[273,288].

2. AIM AND SCOPE OF WORK

The purpose of this study is to advance the mechanistic understanding of LPS regulation in *E. coli* by defining the specific roles of the LapB-interacting proteins, LapC and LapD. This investigation aims to characterize the molecular functions of LapC and LapD within the regulatory complex, which is essential for homeostatic control of LPS, PL, and other components of the envelope homeostasis.

2.1. Rationale of the work

While the foundational roles of LapB and LapC have been largely established, several key questions remain unanswered. The precise molecular mechanism by which LapB modulates FtsH activity and how it senses LPS flux is still not fully resolved. Furthermore, the network of protein interactions surrounding LapB is likely to be more extensive than currently known. At the beginning of this work, function of LapD (formerly YhcB) has remained elusive. However, it was shown that the absence of LapD renders *E. coli* unable to grow at critical high temperatures[291]. Furthermore, Δ*lapD* was shown to exhibit synthetic lethality in the absence of *rodZ*, suggesting a role in the maintenance of cell shape. Consistent with such findings around the time this work started, LapD was shown to exhibit genetic interactions with cell division-related proteins and was designated ZapG (Z-ring-associated protein G)[292]. The Δ*lapD* bacteria were also shown to exhibit cell division defects[292]. However, cell division defects are associated with several *E. coli* mutants, which are not strictly related to the direct role in the assembly of the Z-ring, such as when LPS is severely truncated and underacylated[93].

This study investigates the coordinated roles of LapB interacting proteins such as, LapC, and LapD in maintaining the overall envelope homeostasis. To define the architecture and functional logic of this regulatory module, this study employs a multi-faceted experimental approach. The discovery of LapD as a component of the LapB complex prompted a systematic biochemical and genetic dissection of its role. The experimental design mainly proceeds along three principal axes. First, the LapD interactome is defined through co-purification studies coupled with mass spectrometry to identify its physical network within the envelope biogenesis machinery. Second, the cellular essentiality of LapD is probed through synthetic genetic interaction screens, combining its deletion with mutations that perturb LPS (*waaC*) and PL metabolism (*clsA*). Also, extragenic suppressor analyses are employed to map the functional network to identify the role, and gain deeper insights into the importance of LapD in envelope homeostasis. Multicopy suppressor approach in which the identification of genes that are needed for overcoming the phenotypic defects of Δ*lapD* bacteria will further deepens our understanding. The absence of LapD leads to pleiotropic defects, including Ts growth, increased OM permeability (e.g., vancomycin sensitivity), and filamentation.

In parallel, factors that are limiting in backgrounds with partial loss of function LapC protein is undertaken**.** Given that a truncated *lapC190* bacteria exhibits pleiotropic envelope defects stemming from LpxC hyperdegradation, a multicopy suppressor analysis for overcoming Ts and

defects in permeability is conducted to identify genes that can bypass the requirement when LapC is impaired (**Figure 24**).

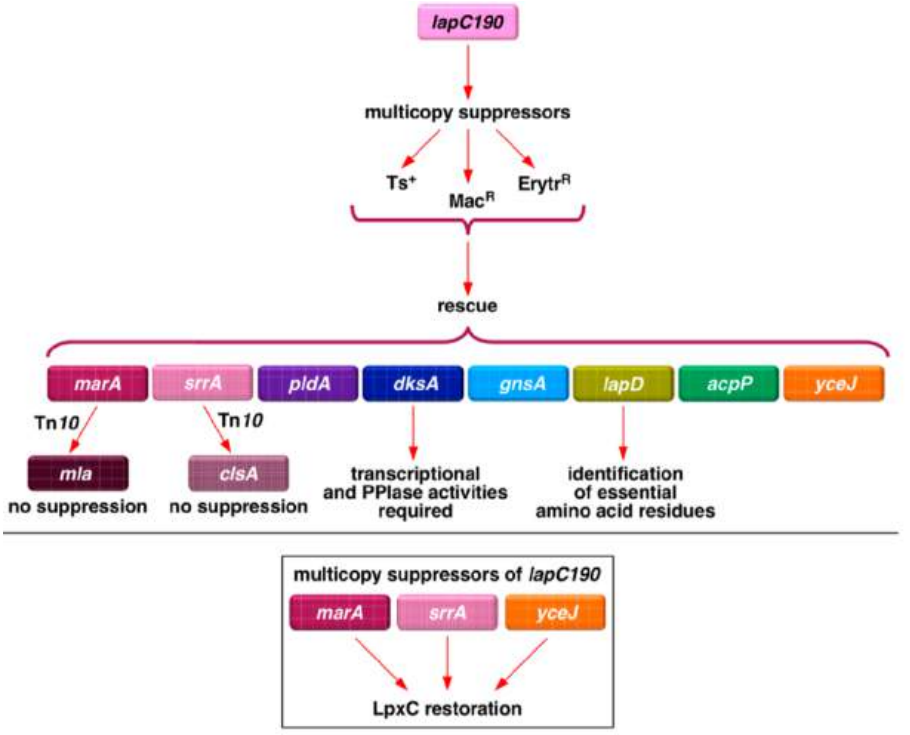

Figure 24: Schematic depiction of various approaches that identify new regulators of LpxC[293].

Multicopy suppressors of sensitivity to high temperature, growth on either MacConkey agar or erythromycin of *lapC190* identified several genes, some of which act by increasing LpxC and LPS amounts (bottom panel). Transposon mutagenesis showed that while as MarA-mediated suppression requires the functional presence of Mla system.

The subsequent genetic and biochemical characterization of these suppressors aims to delineate the pathways that interface with LapC-dependent regulation. Furthermore, systematic genetic interaction studies are performed to test for synthetic lethality between *lapC190* and deletions of the identified suppressor genes, revealing the connections within the network that are essential for viability when LapC is compromised. A complementary suite of biochemical and cell biological assays, including analysis of LpxC stability, LPS amounts, PL composition, and activation of stress responses, provides mechanistic insight into the phenotypes observed. By integrating these genetic, biochemical, and molecular biology approaches, this work aims to fill critical gaps in our understanding of how these proteins senses and coordinates the biogenesis of the Gram-negative envelope. In this study, extragenic suppressors of Δ(*lapD waaC*), Δ(*lapD clsA*) bacteria were isolated and characterized (**Figure 25**).

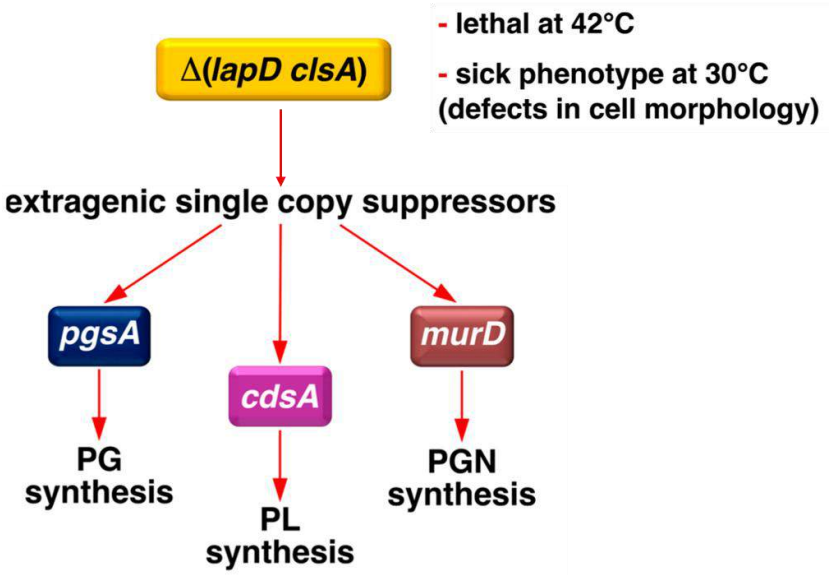

Figure 25: Schematic drawing of the extragenic suppressor approach used to address the molecular basis of the lethality of Δ(*lapD clsA*) bacteria[199].

Extragenic single-copy suppressors that restore growth at elevated temperatures were mapped to either PL biosynthetic (*pgsA* and *cdsA*) genes or those involved in peptidoglycan (PGN) formation (*murD*).

Suppressors of Δ(*lapD clsA*) bacteria mapped to genes, whose products are dedicated to PL biosynthesis, such as *pgsA* and *cdsA*. Similarly, overexpression of *acpP* and specific thioesterases encoding genes was found to mitigate cell division defects and reduce elevated accumulation of PLs. Overall, it is shown that inhibition of fatty acid biosynthesis can overcome growth defects of strains lacking either LapD alone or in combinations when LapD is conditionally essential particularly with compromised LPS or PL biosynthesis. The findings will provide a more complete picture of the complex network that safeguards envelope integrity, a system fundamental to bacterial physiology and a potential target for novel antimicrobial strategies.

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3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Bacterial strains and plasmids

Table 1 contains a list of strains used in this study.

Table 1: List of strains and genotypes.

Strains	Genotype	Reference
BW25113	*lacI*^{q}, *rrnB*$_{T14}$, Δ*lacZ*$_{WJ16}$, *hsdR514*, Δ*araBAD*$_{AH33}$, Δ*rhaBAD*$_{LD78}$	294
GK1942	BW25113 + pKD46	24
W3110	λ^{-}, *IN* (*rrnD-rrnE*)*1, rph-1*	CGSC, Yale
GK6075	BW25113 *lapC190*<>*cat*	113
SR23529	GK6075 *lapC190*<>*frt*	293
SR23583	BW25113 *lapC190*<>*aph*	293
SR23679	BW25113 *lapD*<>*aph*	188
SR23743	SR23679 *lapD*<>*frt*	188
SR23605	BW25113 *marA*<>*aph*	293
SR9073	BW25113 *mlaC*<>*aph*	88
SR9078	BW25113 *mlaD*<>*aph*	88
SR23627	BW25113 *mlaCD*<>*aph*	293
SR23630	GK6075 *mlaCD*<>*aph*	293
SR7092	BW25113 *clsA*<> *aph*	Prof. Rains's laboratory strain
SR23320	BW25113 *clsA*<> *frt*	188
SR24224	BW25113 *pldA*<>*aph*	293
GK1275	W3110 *lpxM*<>*aph*	93
GK1077	W3110 *lpxL*<>*aph*	93
SR19761	BW25113 + pCA24N	293
SR23992	SR23529 + p*yfgM*$^{+}$	293
SR23994	SR23529 + p*dksA*$^{+}$	293
SR23996	SR23529 + p*artJ*$^{+}$	293
SR23998	SR23529 + p*acpP*$^{+}$	293
SR24000	SR23529 + p*accB*$^{+}$	293
SR24027	SR23529 + p*lapD*$^{+}$	293
SR24097	SR23529 + p*pldA*$^{+}$	293

SR24101	SR23529 + p*gnsA*$^{+}$	293
SR24103	SR23529 + p*yceJ*$^{+}$	293
SR24105	SR23529 + p*acpT*$^{+}$	293
SR24107	SR23529 + p*ymgG*$^{+}$	293
GK3592	BW25113 *sfhC21 zad220*::Tn*10* Δ*ftsH3*::Kan	93
SR24056	SR23529 + p*lapD* ΔN20	293
SR24058	SR23529 + p*lapD* Δ(100-132)	293
SR24167	SR23529 + p*lapD* D69A Y70A R71A	293
SR24170	SR23529 + p*lapD* L84A L85A P86A	293
SR24173	SR23529 + p*lapD* N93A P94A F95A	293
SR23678	BW25113 *lapD<>aph*	188
SR23743	BW25113 *lapD<>frt*	188
SR17532	BW25113 φ(*rpoE*P3-*lacZ*)	93
SR23686	SR17532 *lapD<>aph*	188
SR8233	W3110 *waaC<>cat*	93
SR23691	SR23678 *waaC<>cat*	188
SR23798	SR23678 + pCA24N	188
SR23790	SR23678 + p*acpP*$^{+}$	188
SR23784	SR23678 + p*yfgM*$^{+}$	188
SR23786	SR23678 + p*dksA*$^{+}$	188
SR23792	SR23678 + p*srrA*$^{+}$	188
SR23796	SR23678 + p*accD*$^{+}$	188
SR23794	SR23678 + p*ymgG*$^{+}$	188
SR23788	SR23678 + p*artJ*$^{+}$	188
SR23738	SR23678 + pBAD24	188
SR8035	W3110 *waaC<>aph*	93
SR25196	W3110 *lapD<>aph*	96
SR25204	SR25196 *lapD<>frt*	96
SR25651	SR25204 *lapD<>frt waaC<>aph*	96
SR25660	SR25204 *lapD<>frt waaC<>aph lptD* L651P	96
SR25661	SR25204 *lapD<>frt waaC<>aph nlpI* Y243H	96
SR25662	SR25204 *lapD<>frt waaC<>aph lptD* L651P *bamC* F248L	96
RU857	Δ*pgsA* Δ(*clsABC*) *lpp*2	295

SR25199	SR25204 *lapD*<>*frt* + p*acpP*$^{+}$	96
SR25203	SR25204 *lapD*<>*frt* + p*accB*$^{+}$	96
SR25870	SR25204 *lapD*<>*frt* + p*tesA'*	96
SR25872	SR25204 *lapD*<>*frt* + p*menI*$^{+}$	96
SR25874	W3110 + pCA24N	96
SR23799	SR25204 *lapD*<>*frt* + pCA24N	96
SR25878	W3110 + pTTQ18	96
SR25880	SR25204 *lapD*<>*frt* + pTTQ18	96
SR25533	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* I99N	199
SR25534	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* T7A	199
SR25537	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* T60A	199

Table 2 contains a list of plasmids used in this study.

Table 2: List of plasmids and genotypes.

Strains	**Genotype**	**Reference**
pCA24N	IPTG-inducible expression vector cm^{R}	296
pDUET	expression vector	Prof Raina's laboratory plasmids
pKD3	*oriR6K$_g$*, *bla*(Amp^{R}), *kan*, *rgnB*(Ter), *cat*	294
pKD13	*oriR6K$_g$*, *bla*(Amp^{R}), *kan*, *rgnB*(Ter)	294
pKD46	*araBp-gam-bet-exo*, *bla*(Amp^{R}), *repA101*(ts)	294
pCP20	ts replicon with inducible FLP recombinase	294
JW0141	*dksA*$^{+}$ in pCA24N cm^{R}	296
JW0844	*artJ*$^{+}$ in pCA24N cm^{R}	296
JW0976	*gnsA*$^{+}$ in pCA24N cm^{R}	296
JW1044	*yceJ*$^{+}$ in pCA24N cm^{R}	296
JW1080	*acpP*$^{+}$ in pCA24N cm^{R}	296
JW2497	*yfgM*$^{+}$ in pCA24N cm^{R}	296
JW3223	*accB*$^{+}$ in pCA24N cm^{R}	296
JW3440	*acpT*$^{+}$ in pCA24N cm^{R}	296
JW3794	*pldA*$^{+}$ in pCA24N cm^{R}	296
JW5178	*ymgG*$^{+}$ in pCA24N cm^{R}	296
JW5249	*marA*$^{+}$ in pCA24N cm^{R}	296
JW5539	*lapD*$^{+}$ in pCA24N cm^{R}	296

pSR14857	*srrA*$^{+}$ in pCA24N cm^{R}	293
pTTQ18	expression vector with LacI repressor	297
pSR25845	*tesA'* in pTTQ18	96
JW3223	*accB*$^{+}$ in pCA24N cm^{R}	296
JW1676	*menI*$^{+}$ in pCA24N cm^{R}	296
JW5539	*lapD*$^{+}$ in pCA24N cm^{R}	296
pREG	cosmid cloning vector	298

3.1.2. Oligonucleotides

Table 3 lists the oligosaccharides used in this study.

Table 3: List of oligonucleotides.

Name		**Sequence**
lapC	Forward	5'-CTC TAT CAA CGA AGA CAA AGC GCA CTA AGG GAA ACA GAT AAC AGG TTA TGA TTC CGG GGA TCC GTC GAC CC-3'
	Reverse	5'-AGA TAT TTC GCT AAC TGA TTT ATA ATT AAT CAG TTA G CG ATA AAA CGC TTT GTA GGC TGG AGC TGC TTC G-3'
lapD Kan	Forward	5'-GCC TGA AGC CAA CAC CCT ACG GAA ACA AAA GAC AAC GGG AGA TGT TCA TGA TTC CGG GGA TCC GTC GAC CC-3'
	Reverse	5'-GAG GGC GCA ATG GCT GCG CCC GAA AAA TAA ATT AGT CGC GCT TCG CGC CAT GTA GGC TGG AGC TGC TTC G-3'
lapD ΔN20	Forward	5'-ATG CGT TTT GGT AAT CGT AA-3'
	Reverse	5'-TCG CGC TTC GCG CCA GTA-3'
lapD Δ(100-132)	Forward	5'-GAC CTG GGA ATA TGC GCT AAT-3'
	Reverse	5'-TGC CAG ACG ATT ACG GAA CGG-3'
tesA	Forward	5'-CAT TAG GAA TTC TAA GGA GGA CGT ACA TGG CGG ACA CGT TAT TGA TTC TGG GTG-3'
	Reverse	5'-CAT TAG GGA TCC TTA TGA GTC ATG ATT TAC TAA AGG C-3'

3.1.3. Media

Table 4 contains the list of media, its composition, and manufacturers used in this study.

Table 4: List of media, composition, and manufacturers.

Media	Composition
LB medium	per litre: (Difco) 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7 25 g of LB powder dissolved in Millipore quality (MQ) water and adjusted to 1000 mL, sterilized by autoclaving for 30 min at 121°C under 1.5 atm
LB agar	per litre: (Difco) 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar-agar, pH 7 40 g of LB agar powder dissolved in MQ water and adjusted to 1000 mL, sterilized by autoclaving for 30 min at 121°C under 1.5 atm
LB soft agar	per litre: (Difco) 25 g of LB powder (Difco), 7 g agar-agar (Difco) dissolved in MQ water and adjusted to 1000 mL, sterilized by autoclaving for 30 min at 121°C under 1.5 atm
M9 minimal medium	per litre: 200 mL 5× M9 salts (Difco), 1 mL 1 M $MgSO_4$ (Sigma-Aldrich) ,1 mL 0.01 M $FeCl_2$ (Sigma-Aldrich), 0.1 mL 1 M $CaCl_2$, (Sigma-Aldrich), 1 mL B1 (10 mg/mL) (Sigma-Aldrich), 5 mL 10% casamino acids (Difco), 15 mL 20% glucose (Sigma-Aldrich), 876.9 mL H_2O
M9 minimal agar	per litre: 500 mL 2× agar-agar (Difco), 200 mL 5× M9 salts (Difco), 1 mL 1 M $MgSO_4$ (Sigma-Aldrich), 1 mL 0.01 M $FeCl_2$ (Sigma-Aldrich), 100 µl 1 M $CaCl_2$ (Sigma-Aldrich), 1 mL B1 (10 mg/mL) (Sigma-Aldrich), 5 mL 10% casamino acids (Difco), 15 mL 20% glucose (Sigma-Aldrich), 376.9 mL H_2O

MacConkey agar	per litre: (BD) (MacConkey agar: 17 g pancreatic digest of gelatin, 3 g peptones (meat and casein), 10 g lactose, 1.5 g bile salts, 5 g NaCl, 13.5 g agar, 0.03 g neutral red, 0.001 g crystal violet, pH 7.1). 50 g of MacConkey agar powder briefly boiled in 1000 mL of MQ water until dissolved and aliquoted in sterile Petri dishes
MOPS medium	per litre: 10 g MOPS powder (Sigma-Aldrich), 30 g tryptone (Difco) 20 g yeast extract (Difco), dissolved in 1000 mL of MQ water, pH adjusted with NaOH to 7.0, sterilized by autoclaving for 30 min at 121°C under 1.5 atm
SOC medium	per litre: 20 g bacto-tryptone (Difco), 5 g yeast extract (Difco), 0.5 g NaCl (Sigma-Aldrich), 1.86 g KCl (Sigma-Aldrich), 19 g $MgCl_2$ (Sigma-Aldrich), 1.2 g $MgSO_4$ (Sigma-Aldrich), 18 g glucose (Sigma-Aldrich), pH adjusted with NaOH (Sigma-Aldrich) to 7.0, MQ water added to 1000 mL, sterilized by autoclaving for 30 min at 121°C under 1.5 atm

3.1.4. Buffers, gels, and solutions

Table 5 lists the buffers and its composition used in this study.

Table 5: List of buffers and compositions.

Buffer	Composition
50× TAE	per litre: 242 g Tris Base (Sigma-Aldrich), 18.61 g ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), 57.1 mL acetic acid glacial (Sigma-Aldrich), in MQ water adjusted up to 1000 mL
Z-buffer	per litre: 16.1 g $Na_2HPO_4 .7H_2O$ (Sigma-Aldrich), 5.5 g $NaH_2PO_4 .H_2O$ (Sigma-Aldrich), 0.75 g KCl (Sigma-Aldrich), 0.246 g $MgSO_4.7H_2O$ (Sigma-Aldrich), 2.7 mL *β*-mercaptoethanol (Sigma-Aldrich), pH 7.0

1× SDS-PAGE running buffer	14.4 g glycine (Sigma-Aldrich), 3.03 g Tris Base (Sigma-Aldrich), 1 g sodium dodecyl sulfate (SDS) (Sigma-Aldrich)
1 M Tris pH 6.8	per litre: 121 g Tris Base (Sigma-Aldrich) dissolved in 808 mL of MQ water, pH adjusted with HCl (Sigma-Aldrich) to 6.8, water added to total volume of 1000 mL
1 M Tris pH 8.8	per litre: 121 g Tris Base (Sigma-Aldrich) dissolved in 808 mL of MQ water, pH adjusted with HCl (Sigma-Aldrich) to 8.8, water added to total volume of 1000 mL
8× phosphate buffer	0.16 M sodium phosphate, 4 M NaCl, pH 7.4 (GE HealthCare)
2× binding buffer (20 mM imidazole)	per 10 mL: 2.5 mL 8× phosphate buffer (GE HealthCare), 0.1 mL 2 M imidazole (GE HealthCare), 7.4 mL MQ water
Elution buffers for protein purification	Elution buffers (10 mL) for protein purification were prepared by mixing appropriate volume of 2 M imidazole solution (GE HealthCare) with 1.25 mL of 8× phosphate buffer (GE HealthCare) and equivalent volume of sterile MQ water up to 10 mL. If necessary, binding and elution buffers were supplemented with appropriate volume of 0.1% octyl *β*-D-glucopyranoside
Gel buffer for LPS	3 M Tris Base (Sigma-Aldrich), 0.3% SDS (Sigma-Aldrich), pH 8.45
6× lysis buffer	0.001% bromophenol blue (Sigma-Aldrich), 10% glycerol (Sigma-Aldrich), 2% SDS (Sigma-Aldrich), 0.0625 M Tris-HCl pH 6.8, 5% *β*-mercaptoethanol (Sigma-Aldrich)
10× top buffer for LPS	2 M Tris Base (Sigma-Aldrich) pH 8.35, 1% SDS (Sigma-Aldrich), 1 M Tricine (Sigma-Aldrich)
10× bottom buffer for LPS	2 M Tris Base (Sigma-Aldrich), pH 8.9

Transfer buffer for Western blotting	per litre: 14.4 g glycine (Sigma-Aldrich), 3.026 g Tris Base (Sigma-Aldrich), 200 mL methanol (Sigma-Aldrich), MQ water added to total volume of 1000 mL
Blocking buffer for Western blotting	StartingBlock™ T20 (TBS) Blocking Buffer (Thermo Scientific™)
Washing buffer for Western blotting	20× TBS Buffer (Thermo Scientific™ Pierce™)
6× DNA loading buffer	30% glycerol (Sigma-Aldrich), 0.25% bromophenol blue (Sigma-Aldrich), 0.25% xylene cyanol FF (Sigma-Aldrich), MQ quality sterile water

Table 6 presents composition of gels used in this study.

Table 6: List of gel composition.

Type of gel	Composition
SDS-PAGE 4% stacking gel	1.47 mL H_2O, 260 µl 30% acrylamide/bis solution (37.5:1) (Bio-Rad), 250 µl buffer (1 M Tris Base pH 6.8) (Sigma-Aldrich), 20 µl 10% sodium dodecyl sulfate (SDS) (Sigma-Aldrich), 20 µl 10% ammonium persulfate (APS) (Sigma-Aldrich), 4 µl N,N,N',N'-tetramethylenediamine (TEMED) (Bio-Rad)
SDS-PAGE 12% resolving gel	1.905 mL H_2O, 1.8 mL 30% acrylamide/bis solution (37.5:1) (Bio-Rad), 750 µl buffer (1 M Tris Base pH 8.8) (Sigma Aldrich), 45 µl 10% SDS (Sigma-Aldrich), 22.5 µl 10% APS, 2.25 µl TEMED (Bio-Rad)
SDS-PAGE 12.5% resolving gel	1.83 mL H_2O, 1.875 mL 30% acrylamide/bis solution (37.5:1) (Bio-Rad), 750 µl buffer (1 M Tris Base pH 8.8) (Sigma Aldrich), 45 µl 10% SDS (Sigma-Aldrich), 22.5 µl 10% APS (Sigma-Aldrich), 2.25 µl TEMED (Bio-Rad)
14% Tricine-SDS resolving gel for LPS	1.142 mL H_2O, 0.410 mL 49.5% acrylamide/bis solution (Bio-Rad), 1.494 mL gel buffer (3 M Tris, 0.3% SDS, pH 8.45) (Sigma-Aldrich), 0.468 mL 100% glycerol (Sigma-Aldrich), 12 µl 10% APS (Sigma-Aldrich), 7.2 µl TEMED (Bio-Rad)

16% Tricine-SDS resolving gel for LPS	1.084 mL H_2O, 0.468 mL 49.5% acrylamide/bis solution (Bio-Rad), 1.494 mL gel buffer (3 M Tris, 0.3% SDS, pH 8.45) (Sigma-Aldrich), 0.468 mL 100% glycerol (Sigma-Aldrich), 12 µl 10% APS (Sigma-Aldrich), 7.2 µl TEMED (Bio-Rad)
Stacking gel Tricine-SDS for LPS	1.260 mL H_2O, 0.150 mL 49.5% acrylamide/bis solution (Bio-Rad), 0.465 mL gel buffer (3 M Tris, 0.3% SDS, pH 8.45) (Sigma-Aldrich), 30 µl 10% APS (Sigma-Aldrich), 13 µl TEMED (Bio-Rad)

Table 7 contains the list of solutions used in this study.

Table 7: Lists the solutions.

Solution	Description
IPTG Isopropyl *β*-D-1-thiogalactopyranoside (Sigma-Aldrich)	IPTG reagent was prepared as a 1 M water solution and added accordingly, to the indicated concentration when induction of expression from the lactose-induced promoter was required
1 M sodium citrate (Sigma-Aldrich)	Sodium citrate was prepared as a 1 M water solution, sterilized by autoclaving for 30 min at 121°C under 1.5 atm, and added accordingly to indicated concentration when needed during bacteriophages involved experiments
20% glucose (Sigma-Aldrich)	Glucose was prepared as a 20% water solution, sterilized by filtration, and added accordingly to the indicated concentration when needed as a carbon source or catabolic repressor
5× M9 salts (Difco)	Composition per litre: 60 g Na_2HPO_4, 30 g KH_2PO_4, 10 g NH_4Cl, and 5 g NaCl. Salts were dissolved in Millipore Quality water and sterilized by autoclaving for 30 min at 121°C under 1.5 atm
2× agar-agar (Difco)	Per litre: 20 g of agar-agar powder (Difco) was dissolved in 1000 mL of MQ H_2O and sterilized at 121°C under 1.5 atm pressure for 30 min

1 M $MgSO_4$ (Sigma-Aldrich)	Magnesium sulfate was prepared as a 1 M water solution, sterilized by autoclaving for 30 min at 121°C under 1.5 atm, and added accordingly to the indicated concentration or diluted with sterile MQ water to obtain the concentrations indicated in the methods
1 M $MgCl_2$ (Sigma-Aldrich)	Magnesium chloride was prepared as a 1 M water solution, sterilized by autoclaving for 30 min at 121°C under 1.5 atm, and added accordingly to the indicated concentration or diluted with sterile MQ water to obtain the concentrations indicated in the methods
1 M $CaCl_2$ (Sigma-Aldrich)	Calcium chloride was prepared as a 1 M water solution, sterilized by autoclaving for 30 min at 121°C under 1.5 atm, and added accordingly to the indicated concentration or diluted with sterile MQ water to obtain the concentrations indicated in the methods
80% glycerol (Sigma-Aldrich)	Glycerol was prepared as a 80% water solution, sterilized by autoclaving for 30 min at 121°C under 1.5 atm, and added accordingly to the indicated concentration or diluted with sterile MQ water to obtain the concentrations indicated in the methods
10% SDS (Sigma-Aldrich)	SDS was prepared as a 10% water solution
ONPG o-nitrophenyl-*β*-D-galactopyranoside (Sigma-Aldrich)	ONPG was prepared as a 4 mg/mL solution in the Z-buffer
1 M Na_2CO_3 (Sigma-Aldrich)	Disodium carbonate was prepared as a 1 M solution in MQ water
2 M imidazole (GE HealthCare)	Imidazole was prepared as a 2 M solution in MQ water
Lysozyme (Sigma-Aldrich)	Lysozyme was prepared as a 20 mg/mL solution in sterile MQ water
Fixing solution for silver staining	40% ethanol (Sigma-Aldrich), 5% acetic acid (Sigma-Aldrich)

3.1.5. Other chemicals

Table 8 contains enzymes, chemicals, kits, other materials, and solutions used in this study.

Table 8: List of enzyme, chemicals, kits, and other materials and solutions.

Name	**Manufacturer**
Lysozyme	Sigma-Aldrich
Proteinase K	QIAGEN
B-PER (Bacterial Protein Extraction Reagent)	Thermo Scientific Pierce
PMSF (phenylmethanesulfonyl fluoride)	Sigma-Aldrich
Benzonase	Merck
T4 DNA ligase	Thermo Scientific
Platinum SuperFi II DNA Polymerase	Invitrogen
Triethylamine	Merck
Acetic acid	Merck
Ethanol	Merck
Isopropanol	Merck
Methanol	Merck
Hexane	Merck
Phenol	Thermo Scientific
Chloroform	Merck
Petroleum ether	Thermo Scientific
DAPI (4′,6-diamidino-2-phenylindole)	Thermo Scientific
FAME standard mixture	Merck
Heptadecanoic acid	Merck
GeneRuler 1kb DNA Ladder	Thermo Scientific

SeaKem™ LE Agarose	Lonza
Ethidium Bromide	Sigma-Aldrich
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific
Plasmid DNA Extraction GPB Kit	GenoPlast Biochemicals
GenElute™ Bacterial Genomic DNA Kit	Sigma-Aldrich
GeneJET Gel Extraction Kit	Thermo Scientific
GeneJET PCR Purification Kit	Thermo Scientific
Silica gel plates 20 × 20 cm	Merck

3.1.6. Antibiotics

Media were supplemented with kanamycin (30 or 50 µg/mL), ampicillin (100 µg/mL), chloramphenicol (10 or 30 µg/mL), tetracycline (10 µg/mL), and vancomycin (125 µg/mL), all obtained from Sigma-Aldrich.

3.2. Methods

3.2.1. Preparation of chemically competent *E. coli* cells

An overnight culture of *E. coli* was diluted 1:100 in LB broth or M9 minimal medium, supplemented with the appropriate antibiotic. Cultures were grown with shaking at 30°C or 37°C to the mid-exponential phase ($OD_{595} \approx 0.3$). Bacteria were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. The pellet was resuspended in half the original culture volume of ice-cold 10 mM $MgCl_2$ and centrifuged again at 5,000 × *g* for 10 min at 4°C. The final pellet was resuspended in half the original volume of ice-cold 100 mM $CaCl_2$ and incubated on ice for 20 min. Bacteria were then centrifuged (5,000 × *g*, 10 min, 4°C) and resuspended in 1/20th of the initial volume of ice-cold 100 mM $CaCl_2$. For storage, 100 µL aliquots in 10% glycerol were frozen at -80°C.

3.2.2. Transformation of chemically competent *E. coli* cells

Plasmid DNA was mixed with 100 µL of thawed competent cells by gently flicking the tube. The mixture was then incubated on ice for 45 min. A heat shock was applied by transferring the tube to a 43°C heating block for 45 s, followed by a 2-minute incubation on ice. To allow for expression, 700 µL of liquid medium (LB or supplemented M9) was added, and the culture was shaken for 1 h at the permissive temperature (30°C or 37°C). Bacteria were then harvested by

centrifugation (5,000 × *g*, 3 min, 23°C), resuspended in 100 µL of LB, and plated onto selective agar (LA or M9 containing the relevant antibiotics).

3.2.3. Plasmid DNA isolation

Bacterial cells from 1.5 ml overnight cultures were collected by centrifugation (5000 × *g*, 10 min, 4°C). Pellets were thoroughly resuspended in 250 µL of resuspension buffer (50 mM glucose, 25 mM Tris-HCl pH 8,10 mM EDTA, 100 µg/mL RNase A) and incubated for 5-10 min at ambient temperature. Cell lysis was achieved by adding an equal volume of alkaline lysis solution (1% SDS, and 0.2 N NaOH), mixing by gentle inversion until the solution cleared. This was followed by the addition of 350 µL of neutralizing buffer (3 M potassium acetate, pH 5.5). The mixture was kept on ice for 5 min to allow complete precipitation of the proteins and genomic DNA. Following centrifugation (16000 × *g*, 5 min, 23°C), the supernatant was loaded onto a silica-membrane purification column (Thermo Scientific). The column was washed twice with 500 µL wash buffer (70% ethanol) under centrifugation (13000-16000 × *g*, 1 min per wash, 23°C) and dried by a final spin (16000 × *g*, 3 min, 23°C). Purified plasmid DNA was eluted with 50 µL of pre-heated (65°C) elution buffer or nuclease-free water and stored at -20°C.

3.2.4. Chromosomal DNA isolation

Bacterial cells from 1.5 to 3 mL were collected by centrifugation at 10,000 × g for 3 min at 23°C. Isolation of chromosomal DNA was performed using the bacterial genomic DNA isolation kit from QIAGEN according to the protocol provided by the manufacturer.

3.2.5. PCR amplification

DNA fragments were amplified using standard or high-fidelity polymerases (Platinum™ SuperFi II PCR Master Mix, Invitrogen). Reaction mixtures were assembled on ice and typically included a commercial PCR master mix, 0.2-0.5 µM of each oligonucleotide primer, and 1-100 ng of template DNA. The final reaction volume was adjusted with nuclease-free water. Protocol for PCR amplification is as per manual MAN0018860 from Invitrogen. The thermal cycling conditions consisted of an initial denaturation step at 98°C for 30 s, followed by 25-35 cycles of denaturation at 98°C for 5-10 s, primer annealing at 60°C for 10 s, and extension at 72°C for 15-30 s per kb. A final extension step at 72°C for 5 min completed the program. PCR products were analyzed by agarose gel electrophoresis (0.8-1.2% agarose in 1× TAE buffer) alongside a DNA ladder (GeneRuler™ 1 kb DNA Ladder, Thermo Fisher Scientific). Bands of the expected size were excised under UV illumination for downstream applications.

3.2.6. Purification of DNA fragments by gel extraction

Target DNA bands were excised from the agarose gels following electrophoresis and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Gel slices were weighed and dissolved in binding buffer using an equal mass-to-volume ratio (1 mg gel: 1 µL buffer) at 55°C for 10 min with intermittent mixing. Equal

volume of isopropanol was then added, and the mixture was applied to a purification column and centrifuged at 13,000 × *g* for 1 min at 23°C. The column was washed twice with 700 µL wash buffer, followed by centrifugation and a final drying spin. DNA was eluted in 20-30 µL pre-warmed elution buffer (10 mM Tris-HCl, pH 8.5) and stored at -20°C.

3.2.7. Restriction enzyme digestion of DNA

DNA fragments or plasmids were digested using FastDigest enzymes (Thermo Fisher Scientific). Standard 20 µL reactions contained 15 µL of nuclease-free water, 2 µL 10× FastDigest buffer, 2 µL of DNA, and 1 µL of FastDigest enzymes (1 µL of FastDigest enzyme cleaves 1 µg of substrate DNA). The reactions were incubated at 37°C for 5-15 min. The digestion completeness was verified by agarose gel electrophoresis. The desired DNA fragments were excised and purified using the GeneJET Gel Extraction Kit, as described above.

3.2.8. Ligation of DNA fragments

Purified insert and linearized vector DNA were ligated using T4 DNA Ligase. Reactions (20 µL total volume) contained 1× T4 DNA ligase buffer, 20-100 ng of vector, a 3:1 molar ratio of insert-to-vector, and 1 µL of T4 DNA ligase. The ligation reactions were incubated at 23°C for 45 min. For blunt ends, a 1:1 molar ratio of insert-to-vector was used for ligation. Ligase was inactivated at 65°C for 10 min prior to transformation.

3.2.9. Bacteriophage P1 lysates

Overnight culture was started in 1 mL of LB broth and grown overnight at 30°C or 37°C with shaking. This culture was diluted 1:100 into fresh LB supplemented with 5 mM $CaCl_2$ in a flask and incubated at 30°C or 37°C with shaking until it reached an OD_{595} of 0.3. A P1 lysate stock, stored at 4°C, was added at a multiplicity of infection (MOI) of ~0.1. The culture was incubated statically for 20 min at 30°C or 37°C to allow for phage adsorption, and then returned to shaking until complete lysis was observed (typically 2-3 h, indicated by significant bacterial culture clarification). To ensure complete lysis and killing of resistant bacteria, chloroform was added and the mixture was shaken for an additional 20 min at 30°C or 37°C. Cellular debris was removed by centrifugation at 5000 × *g* for 10 min at 4°C. The supernatant, which constituted the clarified P1 lysate, was carefully transferred to a fresh tube. The lysates were stored at 4°C.

3.2.10. Bacteriophage P1-mediated transduction

An overnight culture of the recipient *E. coli* strain was diluted 1:100 in LB broth supplemented with 5 mM $CaCl_2$ and grown at 30°C or 37°C with shaking to an $OD_{595} \approx 1.0$. A 1.2 mL aliquot of this culture was transferred to a sterile 1.5 mL microcentrifuge tube, and 70-90 µL of the P1 lysate was added. The mixture was incubated statically at 30°C for 19 min to allow for the adsorption of transducing particles. The reaction was terminated by adding 130 µL of 1 M sodium citrate solution. Bacteria were harvested by centrifugation (5000 × *g*, 3 min, 23°C), and the supernatant was discarded. The final cell pellet was resuspended in 100 µL of LB and spread onto

LA plates containing 10 mM sodium citrate and the appropriate antibiotic for the selection of the transduced marker. The plates were incubated at 30°C or 37°C for 24-36 h. The obtained transductant colonies were purified by streaking onto fresh selective plates containing 10 mM sodium citrate and the appropriate antibiotic.

To generate the Δ(*lapD waaC*) and Δ(*lapD clsA*) combinations, non-polar deletion of the *lapD* gene was transduced into the recipient Δ*waaC* and Δ*clsA* single mutants by bacteriophage T4- and P1-mediated transduction respectively. Transductants were plated on LA plates supplemented with 10 mM sodium citrate and kanamycin at 30°C.

Synthetic lethal interactions with *lapC190* derivative were analyzed by P1-mediated transductions using the standard protocol described above. Deletions of genes of interest were introduced into the *lapC190* recipient GK6075. Similarly, the *lapC190* mutation was introduced into the corresponding single-deletion recipients with either a vector alone or a covering plasmid. In-frame deletions Δ*mlaC*, Δ*mlaD*, and Δ(*mlaC mlaD*) in *lapC190* bacteria were also introduced by P1-mediated transductions Transductants were plated on LA plates supplemented with 10 mM sodium citrate and the appropriate antibiotic at 30°C. Resulting strains were analysed for MarA-dependent suppression by comparing growth on plates with 75 μM IPTG at 43°C and 30°C.

3.2.11. Protein analysis by SDS-PAGE

Protein samples were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Discontinuous gels were manually cast using a vertical gel-casting system. A resolving gel (12.5%) was prepared, and the solution was poured between glass plates. After 60 min of polymerization, a stacking gel (4%) was prepared. The stacking gel was poured, a 10-well comb inserted, and polymerization proceeded for 45 min. Protein samples were mixed at a ratio of 5:1 with 6× Laemmli buffer (375 mM Tris-HCl pH 6.8, 12% SDS, 60% glycerol, 600 mM DTT, 0.06% bromophenol blue), heated at 95°C for 10 min, and briefly centrifuged. Samples and a prestained protein ladder (PageRuler, Thermo Fisher Scientific) were applied to the gel. Gels were run at 200 V for ~45 min in SDS-PAGE running buffer using a Mini-PROTEAN Tetra Cell (Bio-Rad). Gels were fixed in 40% methanol and, 10% acetic acid for 20 min with agitation, and then stained in 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and, 10% acetic acid for 1 h. Gels were destained in 40% methanol and, 10% acetic acid until clear and imaged using a Gel Doc XR+ Imaging System (Bio-Rad). For Western blot analysis, the gel was processed for protein transfer immediately after the water rinse, as described in the subsequent section.

3.2.12. Western blotting

Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) transfer membrane 0.2 μm (Thermo Fisher Scientific) using a wet transfer system (Bio-Rad). The membrane was activated in methanol, equilibrated in transfer buffer (25 mM Tris Base, 192 mM glycine, 20% methanol), and assembled in a transfer stack with the gel and gel blotting paper Whatmann 3MM thickness 0.34 mm. The transfer was performed at 100 V for 90 min at 4°C. The

membrane was blocked with Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at 23°C. It was then incubated with primary antibody diluted 1:20 000 in blocking buffer for 1 h, washed three times for 15 min in TBST, incubated with secondary antibody [goat anti-rabbit IgG (HRP) or goat anti-mouse IgG (HRP) diluted 1:20 000 from Invitrogen in TBST] for 1 h, and washed five times for 10 min in TBST. The membrane was incubated with a commercial enhanced chemiluminescence substrate kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

To estimate LpxC levels, isogenic cultures of the wild type, Δ*lapD*, and *lapC190* strains were grown in LB at 30°C, shifted to 43°C for 2 h, and harvested by centrifugation. For the suppressor analysis, the wild type, *lapC190* with the empty vector, and *lapC190* strains carrying IPTG-inducible genes cloned in plasmids were grown in LB at 30°C to an OD_{595} of 0.2, induced with 75 μM IPTG, shifted to 43°C for 2 h, and harvested. Protein samples from whole-cell lysates were prepared and resolved using SDS-PAGE. Proteins were transferred by Western blotting, as described. For the detection of LpxC, membranes were probed with polyclonal anti-LpxC primary antibodies[113]. Loading and specificity controls included anti-LapB, anti-FtsH, and anti-TrxA antibodies.

For LPS analysis, whole-cell lysates were prepared from isogenic strains grown in LB at 30°C to an OD_{595} of 0.5 as per procedure[299]. Cell pellets were resuspended in 1× Laemmli sample buffer, boiled for 10 min, and treated with Proteinase K at 37°C for 2 h. Samples were then separated on 15.5% Tris-Tricine gels. Resolved LPS was transferred to a PVDF membrane and processed for immunoblotting, as previously described. Membranes were probed with monoclonal antibody WN1 222-5 from mouse and visualized with a chemiluminescent substrate kit (Thermo Scientific)[300]. LPS band intensities were compared across the strains to evaluate the relative changes in LPS abundance.

3.2.13. Preparation of electrocompetent cells

An overnight culture was diluted in MOPS medium to an initial OD_{595} of 0.05 and grown at 30°C with 0.5% L-arabinose induction until mid-exponential phase (OD_{595} ≈ 0.6). The culture was then immediately transferred to pre-chilled centrifuge tubes and incubated on ice for 30 min. The cells were then harvested by centrifugation at 5,000 × *g* for 15 min at 4°C. The supernatant was carefully discarded, and the cell pellet was gently resuspended in an equivalent volume of ice-cold, sterile water. This washing step was repeated once, the suspension was centrifuged again under the same conditions, and the pellet was resuspended in ice-cold water (half of the original culture volume). Following the final centrifugation, the supernatant was completely removed. The pellet was resuspended in ice-cold 10% glycerol at $1/300^{th}$ of the initial culture volume. The resulting cell suspension was used for electroporation immediately. For transformation with plasmid DNA libraries, electrocompetent cells of Δ*lapD*, *lapC190* bacteria, and various mutational combinations were prepared without the addition of L-arabinose and in the absence of the pKD46 plasmid.

3.2.14. Electroporation

A 100 µL aliquot of electrocompetent cells was incubated on ice and gently mixed with 1-5 µL of plasmid DNA (<100 ng). The mixture was transferred to a pre-chilled 1 mm Gene Pulser Electroporation Cuvette (Bio-Rad). A single pulse was delivered using a Bio-Rad MicroPulser electroporator with the *E. coli* preset protocol (1.8 kV, 18 kV/cm, yielding a 4-5 ms time constant). Immediately following the pulse, 1 mL of pre-chilled SOC medium was added to the cuvette. The cells were resuspended, transferred to a culture tube, and recovered by shaking at the appropriate temperature (30°C or 37°C) for 75 min. Aliquots (10-100 µL) of the culture were spread onto selective LA or M9 plates and incubated for 18 h. Transformation efficiency was calculated as colony-forming units per microgram of DNA.

3.2.15. Gene disruption strategy

Chromosomal gene deletions were engineered using the λ-Red recombinase system, which is a highly efficient method for homologous recombination in *E. coli*[294]. Gene-specific primers containing 50-nucleotide homology extensions to the target *locus*, followed by 20-nucleotide sequences complementary to plasmids pKD13 or pKD3 were used to amplify an FRT-flanked kanamycin or chloramphenicol resistance cassette. The PCR product was electroporated into the recipient strain GK1942 harbouring the arabinose-inducible plasmid pKD46. Following electroporation, cells were recovered in SOC medium at 30°C and plated on LA containing the appropriate antibiotic. The temperature-sensitive FLP recombinase plasmid pCP20 was then introduced to excise the *aph* cassette via FRT site recombination. Transformants were streaked at 30°C on parallel LA plates with and without antibiotics and screened for the loss of pCP20 and the marker was confirmed by sensitivity to kanamycin and ampicillin. Excision of the antibiotic marker and the presence of gene disruption were verified by PCR. For further strain constructions, deletions were introduced into clean genetic backgrounds, selecting for Kan^R as required for specific experiments. This was achieved using either P1 or T4 bacteriophage-mediated transductions, as detailed in the previous section.

3.2.16. Bacterial growth measurement by spot-dilution assay

To compare growth phenotypes, cells from exponentially growing cultures were harvested, washed, and resuspended in sterile 10 mM $MgSO_4$ to an OD_{595} of 0.1. Ten-fold serial dilutions were prepared in 10 mM $MgSO_4$. Aliquots (5 µL) from each dilution were spotted onto the surface of LA plates containing the relevant supplements or antibiotics. After allowing the spots to absorb into the agar at room temperature for 5-8 min, the plates were incubated at defined temperatures (30°C, 37°C, 42°C, 43°C or 44°C) for 18 h or as required. Strains were spotted in parallel on identical media to directly compare the growth at permissive and restrictive temperatures.

This assay was used to examine whether *lapD* overexpression suppresses the Ts growth defect of the *lapC190* mutant bacteria. The *lapC190* derivative GK6075 was transformed with the empty vector pCA24N and the *lapD*-expression plasmid pCA24N-*lapD*. Also, the *lapC190* bacteria

were transformed with various plasmid-born *lapD* mutations. Mutations in the *lapD* gene were generated using PCR. DNA fragments encoding either the soluble domain alone or a version lacking the coding sequence for the final 32 C-terminal amino acid residues were PCR amplified. For targeted mutagenesis, alanine substitutions were designed within the conserved cytoplasmic motifs. Triple-alanine mutations were constructed at residues D69/Y70/R71, L84/L85/P86, and N93/P94/F95 using gene synthesis from the service provided by Invitrogen. To enable functional complementation assays in *lapC190* bacteria, the coding sequences of these *lapD* variants were PCR-amplified with primers introducing appropriate restriction sites and subsequently subcloned into the expression vector pCA24N. The resulting constructs (pCA24N-*lapD* variants) were verified and used to transform *lapC190* bacteria. Overnight cultures grown in LB at 30°C were diluted and incubated with shaking at 30°C until an $OD_{595} \approx 0.5$. The cells were then processed for the spot-dilution assay as described above. Spotted plates were incubated at the permissive temperature (30°C) and the restrictive temperature (43°C, 43.7°C with IPTG to induce gene expression).

3.2.17. Purification of LapD protein

The *lapD* coding sequence was cloned into the T7 expression vector pDUET to generate an N-terminal hexahistidine (His_6) fusion. The resulting plasmid was transformed into *E. coli* BL21(DE3). A 1 L culture was grown at 28°C in LB to OD_{600} 0.1, induced with 300 µM IPTG, and grown to OD_{600} 0.2. When required, rifampicin (200 µg/mL) was added to inhibit host transcription, and incubation continued for 2 h. Cells were harvested by centrifugation (13000 × *g*, 30 min, 4°C), and pellets were stored at -80°C. Frozen cell pellets were thawed and resuspended in 2× B-PER reagent (Thermo Scientific) supplemented with 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, lysozyme (200 µg/mL), PMSF, protease inhibitor cocktail (Sigma-Aldrich), and 30 units of benzonase (Merck). After 45 min on ice, the lysate was centrifuged (45,000 × *g*, 90 min, 4°C). The membrane pellet was solubilized in the same buffer containing 2% (w/v) octyl-*β*-D-glucoside. The solubilized membrane fraction was applied to a column packed with Ni-NTA agarose resin (Qiagen). Eluted proteins were collected using a linear imidazole gradient (50-500 mM) in buffer containing octyl-*β*-D-glucoside. Protein-containing fractions were resolved by 12% SDS-PAGE, and co-eluting species were identified by MALDI-TOF mass spectrometry, as described[188].

3.2.18. Isolation and mapping of extragenic suppressors of the Δ(*lapD waaC*) and Δ(*lapD clsA*) bacteria

Spontaneous suppressor mutations that overcome conditional lethal phenotype of Δ(*lapD waaC*) bacteria were isolated by plating cultures on LA medium at the restrictive temperature of 42°C. Temperature-resistant (Ts^+) colonies were purified. Each suppressor mutation was marked with a mini-Tn*10* transposon carrying a chloramphenicol-resistance gene (*Cm*) and verified that the Tn*10*-linked suppressor mutation breeds true[301]. Cosmids that recombined the Tn*10*-marked mutation were isolated using a single-copy cosmid library[113,298]. Tn*10* containing fragments, along with their flanking genomic DNA, were PCR amplified from recombinant cosmid clones and sequenced. DNA from such cosmids were used for complementation analysis. To identify the

specific suppressor mutation, candidate open reading frames (ORFs) were PCR-amplified from the chromosomal DNA of suppressor strains and sequenced. This suppressor mapping and identification strategy was applied as described[96]. Similar methodology was executed in the isolation of suppressors of Δ(*lapD clsA*) as well. Here the suppressor mutations were marked with mini-Tn*10* Tet, and suppressors were identified as described[199,301].

To generate the Δ(*lapD clsA*) combination, reciprocal bacteriophage P1-mediated transduction was performed. Transductants were selected on LA agar and incubated at 30°C. As controls, each recipient strain used in the transduction was complemented with a plasmid expressing either *lapD* or *clsA*. To isolate extragenic chromosomal suppressors, several independently derived Δ(*lapD clsA*) transductants, initially propagated at 30°C, were subsequently plated and incubated at 42°C. Colonies exhibiting a temperature-sensitive (Ts^+) phenotype were confirmed by re-streaking. From this selection, 65 candidate suppressor-containing strains derived from Δ(*lapD clsA*) bacteria were retained. Suppressor mutations were marked using a mini-Tn*10* Tet element, and stable inheritance of the Tn*10*-linked suppressors was confirmed[301]. To identify the suppressor mutations, Tn*10*-linked mutations were recombined onto single-copy cosmid clones obtained from a previously established genomic DNA library[96,298]. Candidate genes were further refined either by subcloning DNA fragments from recombinant cosmids and selecting for both tetracycline (Tn*10* Tet) and ampicillin resistance markers, or by using inverse PCR on chromosomal DNA to map Tn*10* insertion sites[302]. Recombinant plasmids, including the Tn*10* junction regions and flanking DNA were sequenced using inverse PCR as described earlier[113,302]. Using this strategy, 14 suppressor mutations were assigned to three distinct complementation groups. The corresponding cosmid DNAs were subsequently employed to sequence the implicated candidate genes using gene-specific oligonucleotides as described[199].

3.2.19. Separation of inner and outer membranes to quantify LPS

Isogenic wild-type and mutant bacterial strains [Δ*lapD*, Δ(*waaC lapD*), Δ*clsA*, and Δ(*clsA lapD*)] were cultured under permissive conditions to an OD_{595} of 0.8. Cells were harvested and disrupted using French press, after which cellular debris was removed by sequential centrifugation to pellet the membrane fraction. Membrane pellets were resuspended in a buffer containing 20% sucrose and 1 mM Tris-HCl (pH 7.5). Ultracentrifugation on a two-step sucrose gradient at 23,000 × *g* for 8 h at 4°C was used to accomplish membrane separation. The IM fractions, which were found between the 20% and 53% sucrose layers, were collected, combined, and then treated with Proteinase K for 2 h. Samples from this fraction were applied to a 16% Tricine-SDS PAGE. LPS was revealed by silver staining as previously described[188].

3.2.20. Identification of multicopy suppressors whose overexpression suppresses the phenotypic defects of the mutant bacteria

A multicopy suppressor selection was employed to identify genes whose overexpression could compensate for the phenotypic characteristics, thereby revealing potential functional

interactors or limiting cellular factors in the mutant background. The multicopy suppressor selection was performed using the complete ASKA library (a collection of all predicted *E. coli* open reading frames individually cloned into the pCA24N expression vector under the control of an IPTG-inducible P_{T5}-*lac* promoter[296]. To isolate multicopy suppressors, both Δ*lapD* (strains SR23678 and SR25204) and *lapC190* (strains SR23529 and SR23583) mutant backgrounds were transformed by DNA obtained from the ASKA library. Selection was performed under conditions specific to each mutant's phenotype. For the Δ*lapD* strains, suppressors of the Ts phenotype were selected on LA plates containing 75 μM IPTG at 44°C, while suppressors of vancomycin hypersensitivity were selected on plates supplemented with 75 μM IPTG and 125 μg/mL vancomycin at 37°C. For the *lapC190* strains, suppressors were selected on LA plates with 75 μM IPTG at the restrictive temperature of 43°C and also on MacConkey agar. Candidate colonies from all selections were purified, and plasmid DNA was isolated for subsequent confirmation.

Due to the occurrence of background clones resulting from complementation by previously deleted genes, an alternative *in vivo* cloning strategy using a mini-Mu system was employed[303]. For this purpose, the mutant strain was first lysogenized with a temperature-sensitive mini-Mu prophage. Mini-Mu lysates were generated by thermal induction and used to transduce into the mutant strain. Transductants were selected for restoration of growth at the non-permissive temperature. Plasmid DNA from temperature-resistant derivatives was isolated and subcloned into a medium-copy plasmid vector to identify the minimal DNA fragment required for suppression. Plasmids that reproducibly restored growth were subjected to DNA sequencing to determine the identity of the suppressor gene as described[199].

3.2.21. Construction of a cytosolic TesA' expression system and analysis of cellular morphology

In order to enable controlled cytosolic expression, the gene encoding TesA' (the mature thioesterase without its signal sequence) was cloned into expression vector pTTQ18, with a tightly inducible p*tac* promoter[297]. Along with the wild-type controls, the Δ*lapD* strain carrying pTTQ18-*tesA*' or the empty vector was grown in LB with 0.3% glucose at 37°C. Fifty μM IPTG was used to induce the expression of *tesA*' at an OD_{595} of 0.1. The cultures were grown to an OD_{595} of 0.6. After harvesting, the cells were immobilized on 1% agarose pads, washed, and stained with 10 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in Tris-buffered saline for 10 min in the dark. A Zeiss Apotome microscope equipped with differential interference contrast (DIC) and DAPI fluorescence filters (excitation 370-410 nm, emission 430-470 nm) was used to examine the morphology. A Zeiss Apotome microscope (Carl Zeiss, Jena, Germany) was used to examine the cells as described[96].

3.2.22. Isolation and analysis of ^{32}P-labeled PL species

To analyze changes in PL composition resulting from overexpression of suppressor genes (*acpP*, *accB*, *menI*, and *tesA'*), a protocol involving metabolic radiolabelling with $^{32}P_i$, lipid extraction, and chromatographic separation was employed[304,305,306]. Overnight cultures of the relevant strains

were diluted to an OD_{595} of 0.05 in a fresh medium. To minimize phosphate carryover, the cultures were washed with LB prior to the radiolabelling.

Radiolabelling was initiated by the addition of 2.5 µCi/mL $^{32}P_i$. Gene expression was induced during labelling with different concentrations of IPTG (75 µM IPTG for *acpP*, *accB*, and *menI* under P_{T5}-*lac* or 50 µM IPTG for *tesA'* under p*tac*). Cultures were grown under shaking conditions to an OD_{595} of 1.0, prior to extraction. PLs were extracted from cell pellets using the method described by Bligh and Dyer as described[96,304,305]. The organic phase was resuspended in 50 µL of chloroform/methanol (2:1, v/v) after drying under a stream of nitrogen. Total protein was quantified from an aliquot of each culture using the Pierce BCA Protein Assay Kit for protein normalization. The PL samples were standardized by integrated radioactivity (10,000 cpm per lane) and protein content before being spotted onto silica gel plates for thin-layer chromatography (TLC). A solvent system of chloroform/ethanol/water/triethylamine (30:35:7:35, v/v/v/v) was used for the separation of PLs as described[306]. Radiolabelled PLs were visualized by exposure to a PhosphorImager screen and quantified using an Amersham Typhoon Biomolecular Imager. Densitometric analysis was performed using the Image Lab software, as described[96].

3.2.23. Isolation of fatty acids and their analysis by gas chromatography

An established method was used to extract the free fatty acids[307]. Bacterial cultures were grown under phospholipid extraction conditions, excluding radioactive phosphate supplementation. Following centrifugation, cell pellets were resuspended in water and homogenized. Heptadecanoic acid (C17:0) (Merck) was added as an internal reference compound. Fatty acid methyl esters were obtained by treating the dried extract with 0.5 mL of 1.25 M HCl in methanol and incubated at 50 °C for 15 h. Fatty acids were extracted in 1 mL of hexane and separated by gas chromatography using a Shimadzu GC-2010 Pro system. Samples were introduced via an autosampler under split injection mode, and chromatographic separation was performed under controlled injector and column temperature conditions. Retention times were assigned using a commercial fatty acid methyl ester standard mixture (Merck), and chromatograms were processed using the manufacturer-provided software, as described[96].

3.2.24. Transposon mutagenesis

To investigate the genetic basis underlying suppressor-mediated restoration of growth in Ts mutants, random transposon mutagenesis was employed using a λTn*10* transposon delivery system. Recipient strains harbouring plasmid-borne suppressor genes were subjected to saturation transposon mutagenesis. Mutant strains exhibiting a Ts phenotype were transformed with pCA24N plasmids carrying candidate suppressor genes. These strains were then used as recipient to generate random chromosomal insertions. 50,000 independent transposon mutants were screened. To identify insertions that disrupted suppressor-mediated rescue, the mutant libraries were streaked in parallel on LA plates containing kanamycin, 75 µM IPTG, and incubated at the restrictive temperature (43.5°C for the *lapC190* background) and at 30°C as well. Colonies that failed to grow under these restrictive conditions but grew at 30°C were selected as candidate

mutants. Candidate Ts mutants were purified. To confirm that the Ts phenotype was linked to the transposon insertion, a P1 lysate was prepared on each candidate and used to transduce the original suppressor-expressing strain. Transductants were again tested for growth at 43.5°C on IPTG-containing plates. Insertions that consistently co-transduced with the loss of suppression were considered validated. To exclude insertions causing nonspecific sickness, validated Tn*10* insertions were moved using P1 transduction into a wild-type strain and assessed for growth at 30°C and 43.5°C. Insertions that did not confer a Ts phenotype in the wild-type background were considered specific disruptors of the suppressor pathway. Genomic DNA from validated mutants was isolated, and the position of Tn*10* insertion junction was obtained by sequencing of inverse PCR products[24].

3.2.25. LPS extraction and structural analysis by mass spectrometry

Isogenic cultures of wild-type and its, Δ*mlaC*, Δ*mlaD*, and Δ(*mlaC mlaD*) derivatives were grown in 400 mL of phosphate-limiting medium at 37°C with shaking. Cells were harvested by centrifugation (10,000 × g, 30 min, 4°C). The resulting pellets were lyophilized overnight. LPS was extracted using the phenol/chloroform/petroleum ether method as described previously[308]. The extracted LPS was solubilized in water at a concentration of 2 mg/mL with brief sonication to ensure complete dispersion. Intact LPS was analyzed by negative-ion mode electrospray ionization fourier-transform ion cyclotron resonance mass spectrometry (ESI-FT-ICR-MS) using an APEX QE instrument (Bruker Daltonics) equipped with a 7 Tesla magnet. Data were charge-deconvoluted, and reported masses correspond to monoisotopic peaks. Instrument calibration and data acquisition were performed as described[93,293].

3.2.26. Measurement of *β*-galactosidase activity

Single-copy chromosomal *lacZ* transcriptional fusions were used to monitor the promoter activity. For analysis of the RpoE-dependent *rpoE*P6 promoter, the relevant fusion was introduced into isogenic strain Δ*lapD* and its suppressor derivatives. For the assay, the bacterial cultures were grown overnight at 30°C and diluted to an OD_{595} of 0.05 in fresh LB. At the early exponential phase, the expression from inducible plasmids was induced with the supplementation of IPTG (75 μM), where applicable. At defined intervals of time, culture aliquots were taken for OD_{595} measurement and the enzymatic assay. The aliquots were added to the Z-buffer (final volume 1 mL). Then to this, 20 μL each of 10 % SDS and chloroform was added, followed by vigorous vortexing for 5 s. The reaction was initiated by the addition of o-nitrophenyl-*β*-D-galactopyranoside (ONPG) (200 μL) and incubated for 10 min at 30°C. The reaction was stopped by adding 1 M Na_2CO_3 (500 μL). The sample was vortexed, and the clarified aqueous phase was transferred to a cuvette for absorbance measurement. The absorbance of the reaction mixture was measured at two wavelengths: 420 nm (A_{420}), the absorbance maximum for the reaction product *o*-nitrophenol, and 550 nm (A_{550}), to correct for light scattering caused by cell debris. The *β*-galactosidase activity was calculated in Miller Units using the following standard formula:

$$\text{Miller Units} = 1000\,\frac{A_{420}-(1.75\times A_{550})}{T\times V\times OD_{595}}$$

Where:

A_{420} = Absorbance at 420 nm

A_{550} = Absorbance at 550 nm

T = Reaction time (in minutes)

V = Volume of culture used in the assay (in mL)

OD_{595} = Optical density of the culture at the time of sampling

For each strain and condition, assays were performed in triplicate using three independent biological cultures, and the results are presented as the mean with the standard error.

4. RESULTS

4.1. LapD

4.1.1. LapD is part of LapA/LapB complex and co-purifies with several proteins involved in LPS and PL biosynthesis (results from[188])

As the function of LapD has remained unknown, in this work, first biochemical studies were undertaken by the purification of LapD and its characterization using pull-down experiments. Consistent with its predicted the IM localization, LapD-His_6 was purified from the IM fractions. Affinity purification revealed that its co-eluted proteins are involved in critical cell envelope functions (**Figure 26**). Co-eluting proteins were identified by MALDI-TOF mass spectrometry analysis, revealing that LapD could form a complex with proteins involved in LPS assembly, transport and fatty acid biosynthesis. One set of co-eluting proteins includes key enzymes and transporters such as LpxM, LapA/LapB, FtsH, LptB/C/D, HldE, GmhA, HldD and WbbJ. The next set of proteins that copurified are involved in the PL and FA biosynthetic pathways (PssA, AccD, and FabB/F/H/Y).

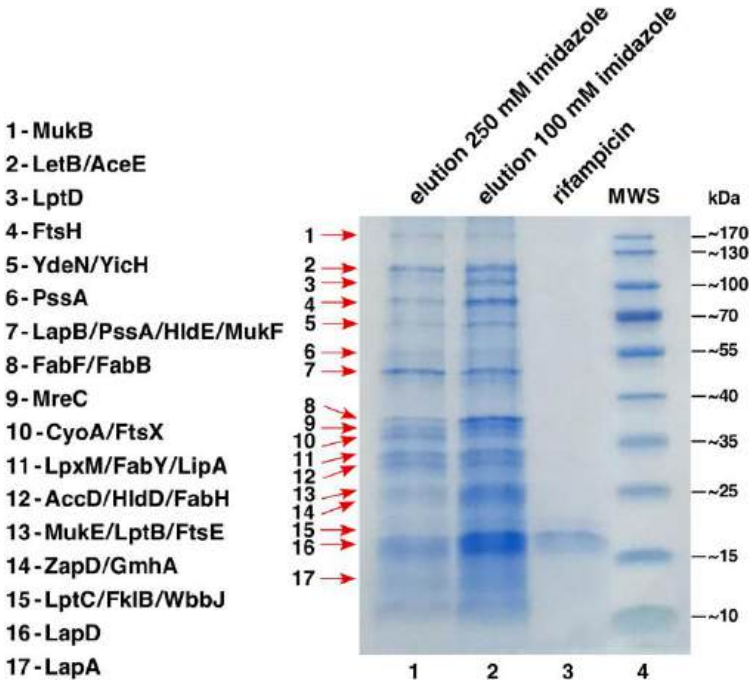

Figure 26: LapD forms a complex in the IM with proteins involved in LPS and PL biosynthesis[188].

Purification profile of His_6-tagged LapD protein from the IM fraction after elution with 250 mM and 100 mM imidazole (lanes 1 and 2, respectively). Lane 3 shows the migration of His_6-tagged LapD protein obtained after rifampicin treatment during the induction of its synthesis. All major co-purifying proteins are indicated by arrows. Proteins were resolved on a 12% SDS-PAGE, stained by Coomassie Brilliant Blue. Lane 4 shows pre-stained molecular weight standards.

Furthermore, the pull-down assays identified a smaller cohort of proteins that can be part of cell envelope biogenesis, including factors responsible for maintaining cell shape and ensuring proper chromosomal segregation (MukB/F/E, MreC, and ZapD). Intriguingly, a cytoplasmic peptidyl-prolyl cis/trans isomerase, FklB, which belongs to the FK506-binding protein family, was also detected among the co-eluting proteins, implying a potential regulatory interaction (**Figure 26**).

4.1.2. LapD is required to maintain levels of LpxC at elevated temperatures (results from[188])

To examine the role of LapD in regulating LPS biosynthesis, we measured the levels of LpxC in Δ*lapD* bacteria. As a reference, an isogenic *lapC190* strain, which expresses a truncated LapC lacking its periplasmic domain, was included due to its known reduced LpxC levels. Bacterial cultures were initially grown under permissive conditions and then shifted to elevated temperature for 2 h before harvesting the whole-cell lysates. Equal amounts of total protein were separated using 12% SDS-PAGE and analyzed by immunoblotting with antibodies specific to LpxC. The Δ*lapD* strain showed a noticeable decrease in LpxC levels compared to the wild type, while *lapC190* contained significantly much lower amounts of LpxC (**Figure 27 A**).

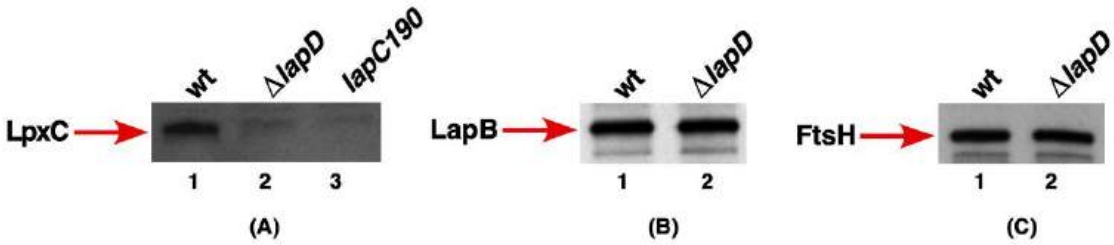

Figure 27: Reduction in LpxC level in Δ*lapD* bacteria at elevated temperature[188].
An immunoblot of whole cell lysates obtained from isogenic strains with indicated genotypes using LpxC-specific antibodies (**A**). In parallel, samples from the wild type and Δ*lapD* were immunoblotted with LapB-specific antibodies (**B**) and with FtsH-specific antibodies (**C**). An equivalent amount of total proteins was resolved by SDS-PAGE prior to immunoblotting.

This stands in direct contrast to the previously documented elevation of LpxC observed in the *lapB* mutant bacteria, highlighting the distinct regulatory nodes within this pathway. Control blots for LapB and FtsH demonstrated that the levels of these proteins remained largely unchanged in the absence of LapD, suggesting that the reduction of LpxC is a specific effect rather than a general disruption of the regulatory network (**Figure 27 B, C**). These results indicated that the reduction in LpxC amounts at elevated temperatures is a specific consequence of LapD absence and is not attributable to the downregulation of other core components of the LPS regulatory system.

4.1.3. Conditional synthetic lethality of Δ*lapD*, when either cardiolipin synthase A or WaaC heptosyl transferase is absent (results from[188])

Following biochemical evidence suggesting that LapD co-purifies with proteins involved in envelope biogenesis, we investigated whether its cellular role becomes essential under specific perturbations of LPS or PL metabolism. To test this, we generated strains by combining *lapD* deletion with mutations in genes encoding nonessential enzymes from these pathways.

Since WaaC catalyzes the addition of the first heptose to the Kdo_2-lipid A precursor during LPS core assembly, a Δ(*waaC lapD*) derivative was constructed using bacteriophage T4-mediated transduction and evaluated for growth properties. To examine the potential interactions with PL biosynthesis, we also deleted the *clsA* gene, which encodes the dominant cardiolipin synthase, in a *lapD*-deficient background.

Thus, a Δ(*clsA lapD*) deletion strain was generated by bacteriophage P1-mediated transductions for phenotypic characterization. For such strain constructions BW25113 was initially used[188]. However, because of the presence of missense mutation in the *fabR* gene in BW25113, for subsequent work, W3110 strain served as the parental strain[96]. The *fabR* gene product represses the expression of *fabA* and *fabB* genes, whose products are required for the synthesis of unsaturated fatty acids. The misense mutation is the *fabR* gene in BW25113 renders the gene nonfunctional[309]. These experiments revealed that both the Δ(*waaC lapD*) and Δ(*clsA lapD*) bacteria were viable at the permissive temperature of 30°C[188]. At higher incubation temperatures, both strains exhibited a pronounced reduction in the size compared to their isogenic parental single mutants, indicating a significant growth defect. The spot-dilution assays were conducted across a temperature gradient (30°C, 37°C, and 42°C) inorder to quantitatively assess these to determine their temperature sensitivity. The analysis of the Δ(*clsA lapD*) strain uncovered a synthetic sickness phenotype. Δ(*clsA lapD*) bacteria exhibited a reduced colony size at both 30°C and 37°C, with nearly 10^3-fold decrease in viable colony-forming units (CFU). This Δ(*clsA lapD*) combination became synthetically lethal at 42°C, where no growth was observed. Under identical conditions, it is critical to note that the single Δ*lapD* and Δ*clsA* mutant bacteria displayed no major growth defects. This confirms that the severe phenotype is a specific consequence of the combined deletions (**Figure 28 A**).

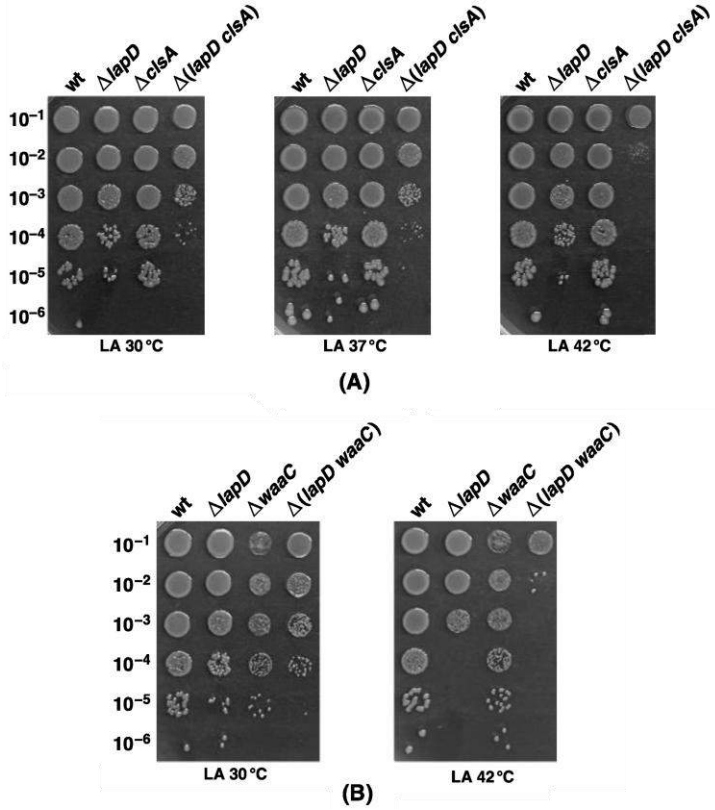

Figure 28: Conditional synthetic lethality of Δ*lapD* with mutation causing LPS truncation or cardiolipin biosynthesis disruption[188].

Growth of isogenic cultures of strains of wild type, Δ*lapD*, Δ*clsA*, Δ*waaC*, and various null combinations was quantified by spot dilution on LA at different temperatures [Panels (**A**, **B**)]. The relevant genotype and temperature of incubation are indicated. Pictures from a representative experiment from three biological repeats is presented.

In parallel, equally severe growth defect was observed for the Δ(*waaC lapD*) combination. Δ(*waaC lapD*) bacteria exhibited a substantial 100-fold reduction in CFU, even at the base temperature of 30°C. Furthermore, the combination proved to be synthetically lethal at 42°C, whereas the individual Δ*waaC* and Δ*lapD* bacteria are viable at such a temperature (**Figure 28 B**). The conditional synthetic lethality observed with *waaC* deletion underscores that LapD function is critical when LPS is composed of only Kdo_2-lipid A. Similarly, the critical requirement for LapD in the absence of cardiolipin synthase A highlights its vital role in preserving membrane integrity when PL composition is altered. It should be noted that synthetic lethality or conditional synthetic lethal phenotypes were observed in BW25113 as well as in W3110 backgrounds. Collectively, these findings position LapD as one of the factors that coordinate LPS assembly with PL homeostasis, a requirement that is fundamental for the stability and function of the OM in *E. coli*.

4.1.4. Suppressor mutations mapping to *lptD* and *nlpI* genes can bypass the synthetic lethal phenotype of Δ(*lapD waaC*) bacteria at 42°C (results from[96])

At 42°C, Δ(*lapD waaC*) bacteria exhibit synthetic lethal phenotype, whereas the single deletion of either gene is fully permissive at this temperature. To unravel the molecular basis underlying this lethality and to identify pathways that functionally intersect with LapD, an extragenic suppressor analysis was performed that could restore the viability of the Δ(*lapD waaC*) bacteria at the non-permissive temperature. The frequency of Ts^+ suppressor mutations ranges from 10^{-6} to 10^{-7}. Following their isolation, suppressor mutations conferring Ts^+ phenotype were marked with Tn*10*. Linked Tn*10* suppressor mutations were backcrossed into the parental Δ(*lapD waaC*) background to validate suppression. These Tn*10*-linked mutations were recombined on cosmids and used for mapping and DNA sequence analysis. For further analysis, three independent Ts^+ suppressors were selected. Based on DNA sequencing, one suppressor strain, SR25560, carried a single nucleotide change, resulting in a Leu-to-Pro substitution at the 651st position (L651P) in the essential OMP LptD. The second suppressor-carrying strain, SR25561, carried a Tyr-to-His substitution at position 243 (Y243H) in the gene encoding the OM lipoprotein NlpI. A third suppressor, SR25562, was not further followed for additional research after it was discovered to have two related Tn*10*-marked mutations (LptD L651P and BamC F248L). Spot-dilution assays were performed to measure the restorative capacity of these suppressor mutations. Isogenic cultures of the wild type, Δ*lapD*, Δ(*lapD waaC*), and the two suppressor strains SR25560 and SR25561 were serially diluted and spotted onto agar plates to estimate colony-forming ability at 30°C and 42°C. The results demonstrated restoration of viability, with both the Δ(*lapD waaC*) *lptD* L651P and Δ(*lapD waaC*) *nlpI* Y243H strains exhibiting an increase of up to 10^4-fold in survival at 42°C compared to the non-suppressed parental Δ(*lapD waaC*) strain (**Figure 29**). The Δ(*lapD waaC*) strain itself was inviable at 42°C and formed mucoid colonies at 30°C, a characteristic phenotype of deep rough LPS mutants, such as Δ*waaC*, that results from the induction of capsular polysaccharide synthesis[272]. Notably, at 42°C, the suppression conferred by the LptD L651P mutation was approximately 10-fold more efficient than that conferred by the NlpI Y243H mutation.

Furthermore, at 30°C, the LptD L651P mutation also fully suppressed the mucoid colony morphology (**Figure 29**).

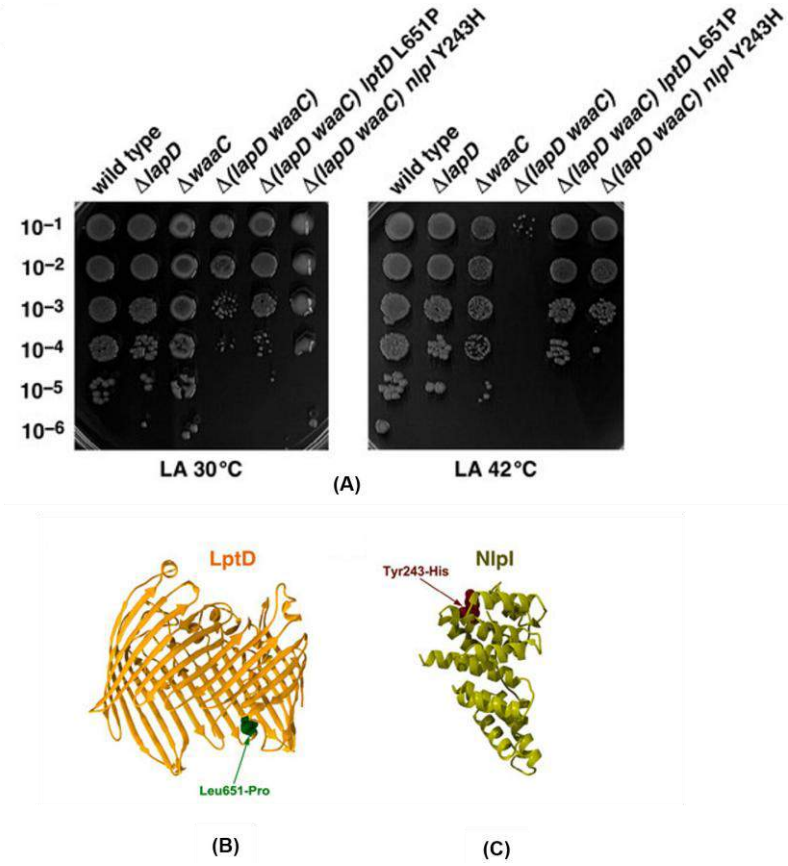

Figure 29: Single amino acid substitutions in either *lptD* or *nlpI* can overcome the synthetic lethality of Δ(*lapD waaC*) bacteria[96].

(**A**) The growth of isogenic cultures of the wild type, its Δ*lapD*, Δ(*lapD waaC*), Δ(*lapD waaC*) *lptD* L615P, and Δ(*lapD waaC*) *nlpI* Y243H derivatives was quantified by spot dilution on LA medium at 30 and 42 °C. The relevant genotypes and incubation temperatures are indicated. A representative image of the spot dilution assay after 24 h of incubation is shown. Pictures from a representative experiment from three biological repeats is presented. (**B**) Positions of single amino acid substitutions in the structures of LptD PDB 4RHB (**C**) NlpI PDB 1XNF are shown.

The suppressor mutation in the *lptD* gene may likely compensates for the LPS translocation defect potentially by enhancing the efficiency of LPS assembly in the OM, even when the LPS core is truncated. On the other hand, the discovery of a suppressor in *nlpI* suggests a connection between LapD and PGN. Therefore, the function of LapD extends beyond its role in FA biosynthesis regulation[96]. It appears to be one of the key players in ensuring the coordinated biosynthesis and assembly of the three major macromolecular structures of the cell envelope: the PL bilayer, LPS, and PGN.

4.1.5. Suppressors that allow Δ(*lapD clsA*) bacterial growth at elevated temperatures map to PL biosynthetic genes *pgsA*, *cdsA*, and those involved in PGN biosynthesis (results from[199])

To further elucidate the functional network of LapD and define the basis of its conditional synthetic lethality in the absence of cardiolipin synthase (ClsA), Ts^+ extragenic single-copy

suppressors were isolated as Δ(*lapD clsA*) bacteria are inviable at 42°C. Therefore, we isolated spontaneous Ts^+ suppressor mutations from cultures of Δ(*lapD clsA*) bacteria grown at the permissive temperature of 30°C. Ts^+ colonies arose at a frequency of approximately 10^{-8}. Ts^+ suppressors were marked with Tn*10* Tet. Linkage analysis of DNA placed 14 Ts^+ suppressors in three complementation groups, revealing that the independent Ts^+ suppressors harboured single nucleotide substitutions within the *pgsA* gene, which encodes phosphatidylglycerophosphate synthase, the enzyme committed to PG and CL biosynthesis. The identified mutations resulted in the amino acid substitutions T7A, T60A, T60P, and I99N (**Table 9**). Notably, Thr60 is a known catalytically essential residue for PgsA activity. The next suppressor mutation was mapped to the *cdsA* gene (D249G), which encodes CDP-diacylglycerol synthase, a key upstream enzyme in the PL biosynthetic pathway responsible for generating the activated lipid precursor CDP-diacylglycerol. Another suppressor mapped to the *murD* gene (M407V).

Table 9: Mapping of extragenic chromosomal suppressors of Δ(*lapD clsA*) bacteria.

Gene	Function	Mutation position	Number of isolates
pgsA	phosphatidylglycerophosphate synthase	T60A (ACC to GCC)	4
		T60P (ACC to CCC)	1
		T7A (ACG to GCG)	3
		I99N (ATC to AAC)	2
cdsA	CDP-diglyceride synthetase	D249G (GAC to GGC)	3
murD	UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase	M407V (ATG to GCG)	1

A spot dilution assay was performed at 30°C and 42°C to assess growth properties. The Δ(*lapD clsA*) strain was not able to grow at 42°C (**Figure 30 A**). Suppressor mutations in the *pgsA* gene allowed the growth of Δ(*lapD clsA*) bacteria at 42°C.

The identification of these suppressors provides a link between LapD function and the homeostasis of anionic PLs. PgsA catalyzes the committed step in PG and CL synthesis. Therefore, mutations that reduce or abolish its activity are expected to reduce the cellular pool of anionic PLs. Similarly, a suppressor mutation in the *cdsA* gene would likely reduce the overall flux into all major PL pathways by limiting the supply of the central precursor CDP-diacylglycerol.

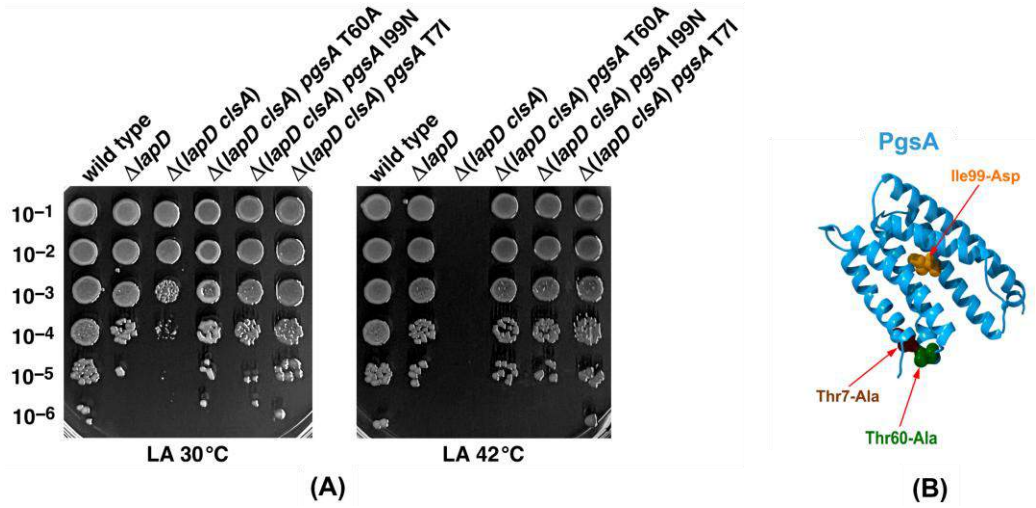

Figure 30: Mutations mapping to the *pgsA* gene encoding phosphatidylglycerophosphate synthase overcome the synthetic lethality of Δ(*lapD clsA*) bacteria[199].

(**A**) Exponentially grown cultures of the wild type, Δ*lapD*, and Δ(*lapD clsA*) derivatives with and without suppressor mutations mapping to the *pgsA* gene grown at 30°C. The cultures were adjusted to an OD_{595} of 0.1 and serially diluted. Bacterial growth was estimated by spot dilution on LA media at 30 and 42°C. The relevant genotypes and incubation temperatures are indicated. Pictures from a representative experiment from three biological repeats is presented. (**B**) Positions of various single amino acid substitutions in the structure of PgsA AlphaFold AF-P0ABF8-F1 are shown.

This genetic evidence strongly suggests that the synthetic lethality of Δ(*lapD clsA*) bacteria stems from a toxic imbalance in anionic PL levels or ratios and that suppressing this lethality requires a fundamental rewiring of PL metabolism. The isolation of suppressors in core PL biosynthetic enzymes demonstrates that LapD is integral to a broader metabolic network that coordinates fatty acid flux with the synthesis of specific PL classes. The conditional synthetic lethality with deletion of the *clsA* gene and its suppression by *pgsA* mutations indicate that in the absence of CL, LapD becomes essential to manage the imbalance by regulating the PG/CL ratio at the membrane.

4.1.6. Absence of LapD leads to retention of LPS in the IM (results from[188])

The data presented in the preceding sections establish two key points. First, that LapD physically associates with a network of proteins dedicated to LPS biosynthesis and transport and second, that it is conditionally essential for the viability of bacteria lacking either *waaC* or *clsA* genes. It has been shown by other members of our laboratory about the essentiality of LapD for the viability of bacteria producing underacylated lipid A, as demonstrated by the synthetic lethality of Δ(*lpxL lapD*) and Δ(*lpxM lapD*) combinations[188].

We postulated that LapD might be involved in the LPS translocation process itself because it is known that the MsbA flippase translocates such underacylated LPS species across the IM inefficiently. Therefore, we sought to determine whether the absence of LapD leads to a general defect in the transport of LPS from the IM to the OM. To evaluate this, we examined the membrane distribution of LPS in wild-type bacteria and its isogenic multiple mutant derivatives, including Δ*lapD*, Δ*waaC*, Δ*clsA*, and their corresponding double mutational combinations. Cultures were

grown at permissive temperature and shifted to 42°C before membranes were isolated and separated into inner and outer membrane fractions by sucrose density gradient centrifugation. LPS present in these fractions was detected using Tricine-SDS-PAGE, followed by silver staining. In wild-type bacteria and in single mutants lacking either *waaC* or *clsA* genes, LPS was largely absent from the IM fractions, consistent with efficient export. In contrast, cells lacking *lapD*, either alone or in combination with *waaC* or *clsA* deletions, exhibited pronounced retention of LPS in the IM. Additionally, a subset of the retained material migrated more rapidly during electrophoresis, suggesting the accumulation of immature LPS species similar to those observed in core-defective mutants (**Figure 31**). As an additional control, we also used LPS from IM fractions from the Δ*tig* and Δ(*tig lapD*) derivatives (**Figure 31 C**). This suggests that in the absence of LapD, there is not only a general defect in translocation but also a specific accumulation of immature LPS intermediates, implying a potential role for LapD in ensuring the transport of mature LPS species prior to their transport.

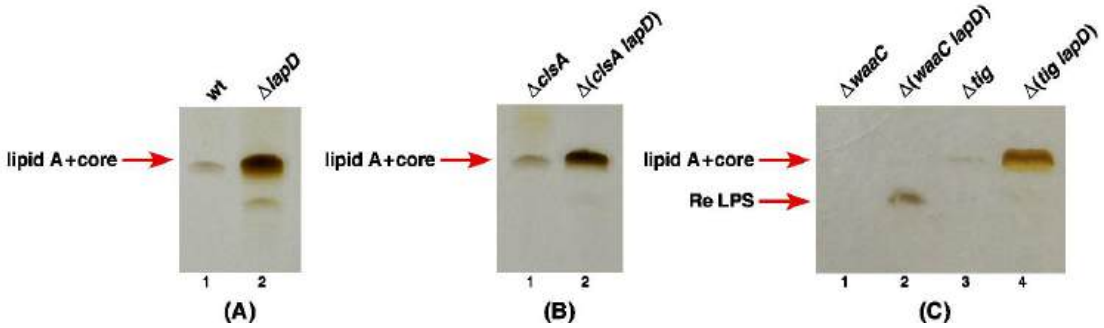

Figure 31: Lack of LapD causes the retention of significant amounts of LPS in the IM[188].
Total cell lysates obtained from isogenic derivatives of wild-type and Δ*lapD* bacteria were subjected to cellular fractionation to obtain the IM. Samples were treated with Proteinase K and resolved on a 16% Tricine-SDS 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 Δ*lapD* and its derivatives wt vs. Δ*lapD* (**A**), Δ*clsA* vs. Δ(*clsA lapD*) (**B**) and Δ*waaC*, Δ(*waaC lapD*), Δ*tig*, Δ(*tig lapD*) (**C**). The relevant genotype of strains used is indicated on the top of each panel.

All these findings point that LapD is required for the effective translocation of LPS from the IM. Therefore, a significant pool of LPS, including premature forms, is retained in the IM in its absence.

4.1.7. Multicopy suppressor analysis to identify factors that could be limiting when LapD is absent (results from[188])

To investigate why *lapD*-deficient strains exhibit temperature sensitivity and increased vancomycin susceptibility, a multicopy suppressor analysis was carried out. This method identifies genes whose elevated expression overcomes the phenotypic defects. This selection approach identified several genes (*acpP, dksA, srrA, accB, yfgM, ymgG, artJ,* and *artI*) capable of restoring growth at 44°C (**Table 10**). Among these candidates, *acpP* exhibited the strongest phenotypic rescue.

Table 10: Identification of the most relevant multicopy suppressors of the Δ*lapD* strains.

Gene	Number of transformants		Function
	LA 44°C	**Vancomycin**	
vector alone	9 small colonies	6 small colonies	
acpP	750	ND [1]	acyl carrier protein
dksA	486	ND	transcriptional regulator
srrA	520	ND	transcriptional regulator
yfgM	430	ND	IM protein, substrate of FtsH
accB	511	ND	acetyl-CoA carboxylase
rcsF	ND	701 medium size colonies	colanic acid regulator
artJ	473	ND	L-arginine ABC transporter

[1]ND denotes not determined since suppressors were isolated either at 44°C or on vancomycin supplemented growth medium at 37°C.

This effect was confirmed through spot dilution assays, where strains expressing the *acpP* gene showed improved viability relative to vector controls under restrictive conditions (44°C in the presence of 75 µM IPTG) (**Figure 32**).

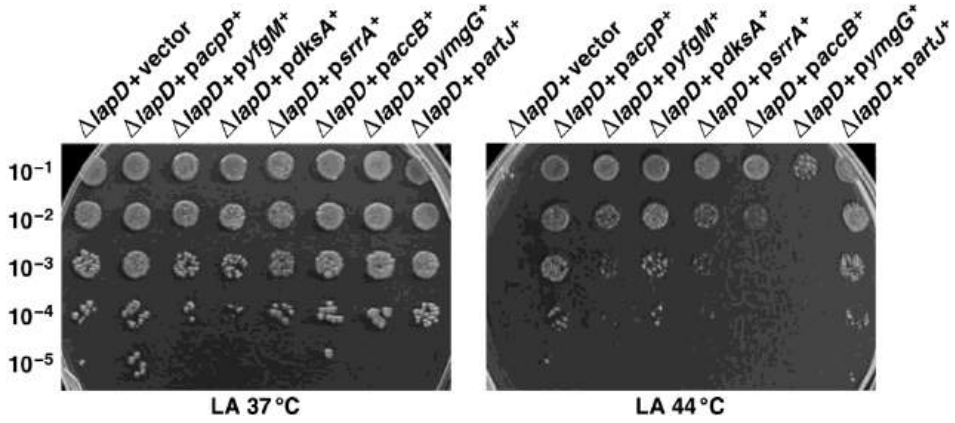

Figure 32: Overexpression of specific genes, including the *acpP* gene, restores the growth of Δ*lapD* bacteria at 44°C[188].

The growth of isogenic cultures of Δ*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°C. The relevant genotypes and temperature of incubation are indicated. Pictures from a representative experiment from three biological repeats is presented.

The ability of *acpP* overexpression to suppress Δ*lapD* phenotypes underscores the functional connection between LapD and lipid metabolism, particularly in the synthesis of LPS and FAs[188]. Additionally, other identified suppressors provided valuable insights into compensatory

mechanisms. The transcriptional regulators *dksA* and *srrA*, when overexpressed, also enhanced bacterial growth at elevated temperatures.

4.1.8. Overexpression of either *acpP* or *menI* genes overcomes vancomycin sensitivity of Δ*lapD* bacteria (results from[96])

The Δ*lapD* bacteria displays both Ts growth and susceptibility to vancomycin, indicating a defect in envelope integrity. Although the *acpP* gene had previously been identified as a suppressor of the Ts phenotype, its ability to restore antibiotic resistance had not been examined in the W3110 background. To address this, an additional round of multicopy suppressor approach was used to identify genes, whose overexpression could enhance the survival of Δ*lapD* bacteria in the presence of vancomycin. This approach identified two suppressors encoded by *acpP* and *menI* genes. The next approach is a quantitative assessment of the strength of this suppression. For this, spot-dilution growth assays on LA media supplemented with vancomycin (125 µg/mL) and an inducer (75 µM IPTG) to drive the gene expression from the plasmid was performed. The results revealed that induced overexpression of the *menI* gene is capable of restoring vancomycin resistance to the Δ*lapD* bacteria. This result is comparable to that of overexpression of the *acpP* gene (**Figure 33**). The identification of *menI* as a suppressor is particularly interesting. It has been biochemically characterized as possessing a broad substrate range, acting on various acyl-CoA thioesters, and its role in the menaquinone biosynthesis pathway is well-documented[310].

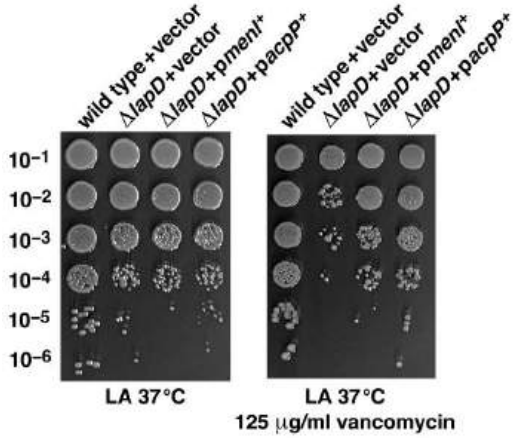

Figure 33: Overexpression of either *menI* or *acpP* genes can suppress vancomycin sensitivity of Δ*lapD* bacteria[96].

The growth of isogenic cultures of wild type with vector alone, its Δ*lapD* derivative with empty vector, and Δ*lapD* bacteria carrying a plasmid expressing either *menI* or *acpP* genes was quantified by spot dilution on LA with and without vancomycin supplementation. The relevant genotypes and vancomycin concentration are indicated. All experiments were performed with three biological repeats and a representative picture from each set is shown.

The mechanism by which MenI overproduction suppresses the vancomycin sensitivity of Δ*lapD* bacteria likely stems from its degenerate thioesterase activity. The multicopy suppressing ability by the *acpP* gene could arise from a specific requirement of providing acyl-ACP intermediates that can regulate fatty acid biosynthesis and alter their relative distribution. The overexpression of *acpP* or *menI* thus appears to alleviate a critical bottleneck in envelope biogenesis that is created by the absence of the *lapD* gene.

4.1.9. Overexpression signal sequence-less TesA (TesA') overcome cell morphology defects of Δ*lapD* bacteria (results from[96], kindly provided by Aravind Ayyolath)

The successful suppression of the Δ*lapD* phenotypic defects by MenI, a thioesterase with broad substrate specificity, led us to hypothesize that this suppression mechanism might be a general function of thioesterase activity rather than a unique property of MenI. We therefore reasoned that overexpression of a distinct, well-characterized thioesterase could similarly ameliorate certain abnormalities in the Δ*lapD* bacteria. To test this, we selected TesA, a periplasmic protein known to significantly alter cellular FA composition when artificially retained in the cytoplasm[311].

Since the native TesA protein is exported to the periplasm, we engineered a variant for cytoplasmic expression. The *tesA* gene was cloned lacking its signal sequence (hereafter referred to as *tesA'*), into the pTTQ18 expression vector, placing it under the control of the tightly regulated p*tac* promoter. The resulting *tesA'*-expressing plasmid DNA and an empty vector control were then introduced into the Δ*lapD* bacteria. Transformants were examined for morphological changes following induction of the gene expression with 50 µM IPTG. Strikingly, microscopic analysis revealed that overexpression of *tesA'* in the cytoplasm effectively suppressed the pronounced filamentous morphology that is characteristic of Δ*lapD* (**Figure 34**).

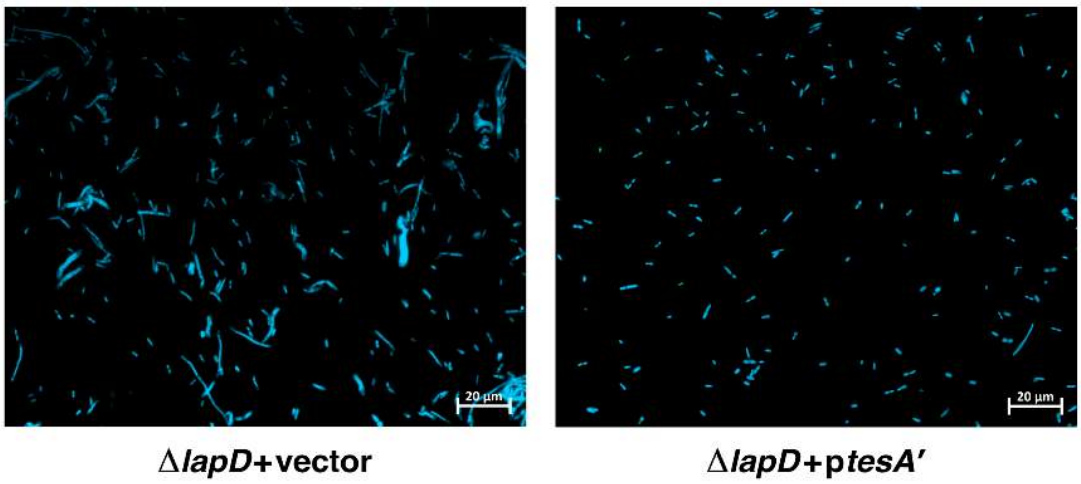

Figure 34: Induction of signal sequence-less TesA' restores normal cellular morphology of Δ*lapD* bacteria[96].

Figure shows fluorescence microscopy analysis at ×1000 magnification with a 20 µm scale of DAPI-stained bacterial cells. Restoration of the normal cellular morphology of the Δ*lapD* bacteria by the induction of the signal sequence-less TesA'. Microscopic images of the wild type, its isogenic Δ*lapD* with the empty vector, and Δ*lapD* expressing *tesA'* were investigated.

This finding indicates that the elevation of cytoplasmic thioesterase activity is sufficient to rescue the cell division defect associated with the absence of LapD. This result reinforces the model that fine-tuning the acyl-CoA pool or FA metabolism in the cytoplasm is a critical function that becomes dysregulated in the absence of LapD, and that this imbalance can be corrected by the action of overproduced thioesterases.

4.1.10. Induction of either AcpP or AccB subunit of the ACC enzyme or TesA' represses PL biosynthesis in Δ*lapD* bacteria (results from[96] kindly provided by Professor Gracjana Klein)

The Δ*lapD* strain is characterized by a pronounced filamentous morphology, a defect indicative of a failure in cell division whose underlying metabolic cause is not fully understood[292]. Given the established critical link between cellular dimensions and FA synthesis, we hypothesized that this defect arises from defects in the PL content[312]. To test this, complementary studies conducted in our laboratory revealed that the cellular filamentation phenotype of the Δ*lapD* bacteria was effectively suppressed by overexpression of *acpP* or *accB* genes. To define the biochemical consequences underlying these morphological corrections and to understand the mechanism of other suppressors, a comprehensive analysis of cellular PL composition was performed. PLs were extracted from the Δ*lapD* strains overexpressing *acpP*, *accB*, the cytoplasmic thioesterase-encoding gene *tesA'*, and *menI*. The PLs were separated by thin-layer chromatography (TLC) and quantified by densitometry.

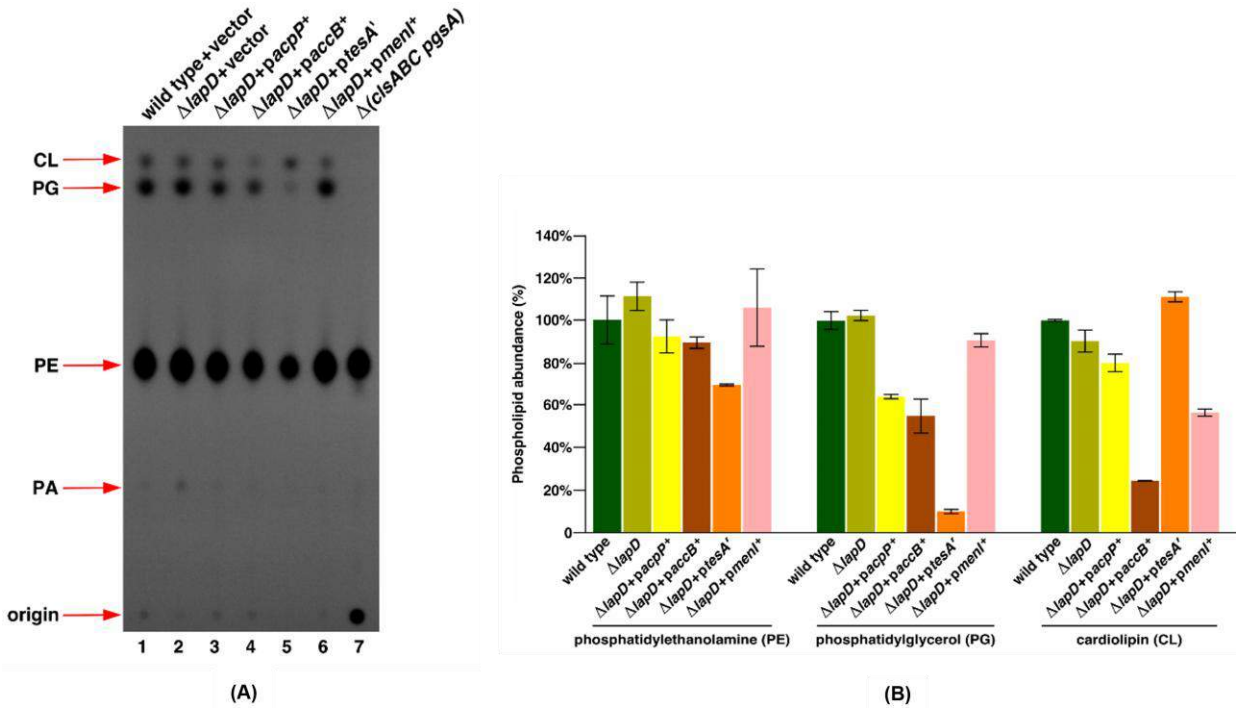

Figure 35: Induction of expression of either *acpP*, *accB* or *tesA'* genes represses PL biosynthesis in Δ*lapD* bacteria[96].

(**A**) Thin-layer chromatography of ^{32}P-labeled PLs extracted from isogenic strains carrying either vector alone or individually expressing *acpP*, *accB*, *tesA'*, and *menI* genes. As a control, ^{32}P-labeled PLs were extracted from the RU857 strain, which cannot produce CL and PG and synthesizes only PE, serving as a marker for three major PL species. A representative autoradiogram is presented. The arrows indicate the positions of major PL species. (**B**) Bar graph representing the relative amounts of CL, PG, and PE species quantified using densitometry from three biological replicates. Error bars represent a S.E. of three independent measurements.

The wild-type strain displayed a canonical *E. coli* K-12 PL profile[186], with PE at 77.69% ± 3.6, PG at 17.93% ± 0.8, and CL at 4.2% ± 0.4 (**Figure 35**, **lane 1**). Strikingly, the suppressors that corrected cell morphology (*acpP*, *accB*, and *tesA'*), all resulted in a significant reduction in the overall abundance of acidic PLs (PG and CL). The most significant effect was overexpression of

accB, which reduced PE and drastically reduced CL and PG to approximately 24% and 54% of the wild-type values, respectively (**Figure 35**). On the other hand, the levels of the main PE and PG species were not significantly changed by the stimulation of *menI* gene expression. Rather, its main impact was a modest 56% decrease in the CL content when compared to the wild type (**Figure 35, lane 6**). When compared to the wild type, the Δ*lapD* bacteria did not show major alterations in the abundance of the primary PL classes (PE, PG, and CL) (**Figure 35, compare lane 2 with lane 1**), but the accumulation of a phosphatidic acid (PA) species (less than 1%) was observed, which was not seen in the wild-type extracts (**Figure 35, lane 2 vs. lane 1**). This points to a perturbation in the metabolic flux through the PL biosynthetic pathway in the absence of LapD. In conclusion, our integrated analysis demonstrates that diverse genetic suppressors of the Δ*lapD* defects, whether correcting division or permeability, converge on a common endpoint, the rebalancing of the membrane PL composition. The data support a model where LapD functions as a crucial regulator of lipid homeostasis, and its loss creates a metabolic imbalance that can be compensated by redirecting flux away from specific PL branches with different suppressors fine-tuning this process in distinct yet functionally complementary ways. Thus, overproduction of either AcpP or AccB can adjust the balance between LPS and PL amounts by reducing the amount of PL.

4.1.11. Overexpression of the *acpP* gene suppresses elevated RpoE-regulated envelope stress response of Δ*lapD* bacteria (results from[96])

The alternative sigma factor RpoE is a master regulator of the bacterial envelope stress response, which is transcriptionally activated by a variety of perturbations in the periplasm and OM. These include defects in OMP biogenesis and folding, as well as disruptions in the homeostasis of LPS and PLs[24,113,313,314]. Consistent with this, the deletion of the *lapD* gene was shown to trigger an induction of the RpoE regulon, a phenotype shared with other mutants, such as *lapB* and *lapC*, that are impaired in LPS biosynthesis and regulation[188]. After establishing that certain phenotypic defects of the Δ*lapD* bacteria, such as aberrant cell morphology and hypersensitivity to vancomycin, can be overcome by moderate overexpression of the *acpP* gene, we next investigated whether this suppression also functions at the level of cellular stress signaling. We hypothesized that if AcpP overproduction rectifies the underlying envelope defect, it should correspondingly attenuate the induced stress response. To verify this, we monitored RpoE activity using a transcriptional fusion of the *rpoE*P6 promoter to a *lacZ* reporter gene. As anticipated, the Δ*lapD* bacteria exhibited a significant, approximately 4-fold induction in *rpoE*P6-*lacZ* activity compared to the wild-type strain, confirming the presence of a chronic envelope stress condition (**Figure 36**). Strikingly, the mild overexpression of the *acpP* gene in the Δ*lapD* background resulted in a substantial reduction of this stress signature, decreasing elevated RpoE activity by nearly 50% as compared to the Δ*lapD* bacteria (**Figure 36**).

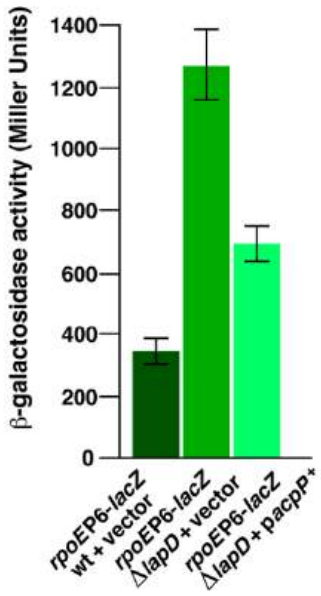

Figure 36: Overexpression of the *acpP* gene represses elevated envelope stress-responsive *rpoE* promoter activity[96].

Exponentially grown wild type, its isogenic Δ*lapD* derivative with the vector alone, and its derivative expressing the *acpP* gene, carrying the single-copy chromosomal *rpoE*P6-*lacZ* fusion, were analyzed for the *β*-galactosidase activity. The cultures were adjusted to an OD_{595} of 0.05. IPTG (75 μM) was added to induce *acpP* expression, and the cultures were allowed to grow in LB medium at 37°C. Aliquots of samples were drawn after different time intervals and used to measure the *β*-galactosidase activity. Error bars represent a S.E. of three independent measurements.

This finding provides a direct transcriptional correlation to the observed phenotypic suppressions. The ability of AcpP to dampen the RpoE response is entirely consistent with its role as a multicopy suppressor that acts by overcoming defects in the accumulation of PLs. The *acpP* overexpression appears to correct the core envelope defect that triggers the RpoE-mediated stress response in the Δ*lapD* bacteria.

4.2. LapC

4.2.1. Genes whose overexpression overcomes the temperature sensitivity of *lapC* mutant bacteria with a truncation of LapC periplasmic domain identify new players that can regulate LpxC amounts (in collaboration with Aravind Ayyolath, another PhD student in the laboratory of bacterial genetics - results from[293])

Previous work from our laboratory demonstrated that certain aspects of this phenotype could be suppressed by specific chromosomal mutations in genes such as *lpxC*, *ftsH*, and the *lapA/B* operon, primarily through mechanisms that stabilize the LpxC protein[113]. However, given the complex, multi-factorial nature of LpxC regulation, we sought to conduct additional studies to identify the full spectrum of cellular pathways capable of influencing this critical regulatory node. To this end, we employed a multicopy suppressor approach to identify genes whose overexpression could overcome defects in LapC function. We utilized the *lapC190* strain and selected for multicopy suppressors capable of restoring growth at the non-permissive temperature of 43.5°C and overcoming sensitivity to growth on MacConkey agar medium. Plasmid DNA pools from this library

were used to transform competent cells of *lapC190* bacteria, and transformants were selected on LA plate (supplemented with 75 μM IPTG). This resulted in the identification of 13 unique genes, whose moderate overexpression is sufficient to rescue the phenotypic defects of the *lapC190* bacteria (**Table 11**).

Table 11: Identification of multicopy suppressors of the *lapC190* bacteria.

Gene	LA 43°C	MacConkey agar 37°C	Δ*lapD*	Function
yfgM	+	+	+	FtsH substrate, ancillary SecYEG translocon subunit
dksA	+	+	+	RNA polymerase-binding transcription factor
artJ	+	+	+	L-arginine ABC transporter periplasmic binding protein
acpP	+	+	+	acyl carrier protein
accB	+	-	+	biotin carboxyl carrier protein
lapD	+	ND	+	LPS assembly protein
pldA	+	-	-	outer membrane phospholipase A
marA	+	±	-	DNA-binding transcriptional dual regulator
yceJ	+	-	-	putative cytochrome b561
gnsA	+	-	-	putative phosphatidylethanolamine synthesis regulator
ymgG	+	-	±	PF13436 family protein toxin-antitoxin system
srrA	+	+	+	transcription (stress response regulator A)
acpT	+	+	-	*holo*-(acyl carrier protein) synthase 2

ND denotes not determined. Sign + indicates restoration of growth, - indicates lack of growth and ± refers to partial restoration of growth.

Functional categorization of these genes reveals that they impinge upon a diverse array of cellular processes, highlighting the interconnectedness of envelope biogenesis pathways. A significant subset of the identified genes has direct roles in LPS, PL, or FAB synthesis and sensing. This group includes *lapD*, *acpP*, *acpT*, *accB*, and *pldA*. Other suppressors encode transcriptional regulators, such as *marA*, *dksA*, and *srrA*. The screen also identified genes such as *yceJ*, *yfgM*, *gnsA*, and *ymgG,* which are involved in different processes. The identification of this wide array of suppressors underscores the complexity of the regulatory network governing LpxC turnover and cell envelope homeostasis.

4.2.2. *lapD* overexpression suppresses the Ts phenotype of *lapC190* mutant bacteria at 43°C (results from[293])

We aimed to further check the ability of the *lapD* gene to restore the growth deficiency of the *lapC190* bacteria after it was discovered to be one of the multicopy suppressors. The *lapC190* bacteria were transformed with DNA from either the empty pCA24N vector as a control or the same vector harbouring the *lapD* gene, whose expression is inducible from an IPTG-regulated promoter.

Cultures of these transformants were grown under permissive conditions, serially diluted, and spotted onto LA plates at the permissive (30°C) and the non-permissive temperature (43°C) with the inducer, IPTG. As anticipated, the *lapC190* strain carrying the empty vector displayed Ts growth at 43°C. In striking contrast, the *lapC190* strain overexpressing the *lapD* gene exhibited a full restoration of growth at the non-permissive temperature of 43°C, with a colony-forming efficiency comparable to that observed at 30°C (**Figure 37**).

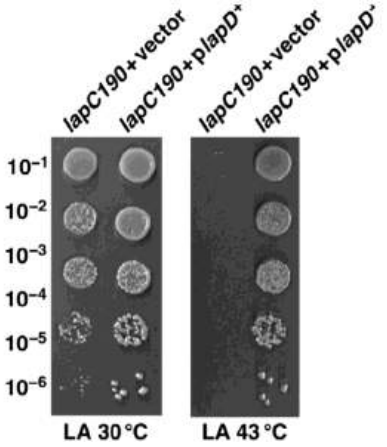

Figure 37: Overexpression of *lapD* gene can restore growth of *lapC190* bacteria at 43°C[293].
Growth of isogenic cultures of *lapC190* bacteria transformed with either plasmid DNA of the vector alone or when the *lapD* gene is present on the plasmid was quantified by spot dilution on LA 30°C and 43°C. The relevant genotype and temperature of incubation are indicated. All experiments were performed with three biological repeats and a representative picture from each set is shown.

The fact that increased LapD dosage can suppress Ts phenotype of *lapC190* bacteria suggests that these two components of the Lap system operate in a closely related functional pathway. While LapC is required for the regulation of LpxC amounts, our finding indicates that LapD, potentially through its interactions with other envelope biogenesis machinery, can functionally substitute for or stabilize the system when LapC function is compromised.

4.2.3. LapD-mediated suppression of a *lapC190* mutant bacteria requires LapD N- and C-terminal domains and identification of some of the critical amino acid residues required for LapD function (results from[293])

From the above result, it is clear that overexpression of the full-length *lapD* gene can suppress the Ts growth defect of the *lapC190* bacteria. Therefore, our next goal was to delineate the specific structural domains and identify the amino acid residues essential for this function. The LapD protein is predicted to carry a single N-terminal transmembrane domain that anchors it to the IM, coupled with a largely cytosolic C-terminal domain that is highly conserved across species[293]. Structural insights from a homologous LapD protein in *Haemophilus ducreyi* revealed a unique tetrameric α-helical coiled-coil architecture within this cytosolic region, which is strongly predicted to serve as a scaffold for critical protein-protein interactions[292]. To dissect the functional importance of these regions, we used a structure-guided mutagenesis approach. A series of mutations in the *lapD* gene were constructed. This consisted of deletion derivatives by truncating the last 32 amino acids of the C-terminal domain and removing the N-terminal membrane anchor (amino acids 1-20).

Also, alanine-substituted mutations in conserved amino acid clusters within the cytoplasmic domain with alanine residues were constructed. Specifically, we generated single, double and triple-alanine substitutions for the conserved motifs Asp69-Tyr70-Arg71 (D69A/Y70A/R71A), Leu84-Leu85-Pro86 (L84A/L85A/P86A), and Asn93-Pro94-Phe95 (N93A/P94A/F95A) (**Figure 38 B**). The resultant plasmid DNA were transformed into isogenic *lapC190* and Δ*lapD* genetic backgrounds. The functionality of each mutation was assessed by its ability to rescue the Ts phenotype on LA media at the restrictive temperature upon induction with 75 μM IPTG.

As anticipated, the *lapC190* bacteria's growth at high temperatures was restored by the plasmid expressing the wild-type *lapD* gene, which functioned as a positive control. On the other hand, under the same inducing conditions, none of the deletion or alanine-substitution mutations could suppress the Ts phenotype (**Figure 38 A**).

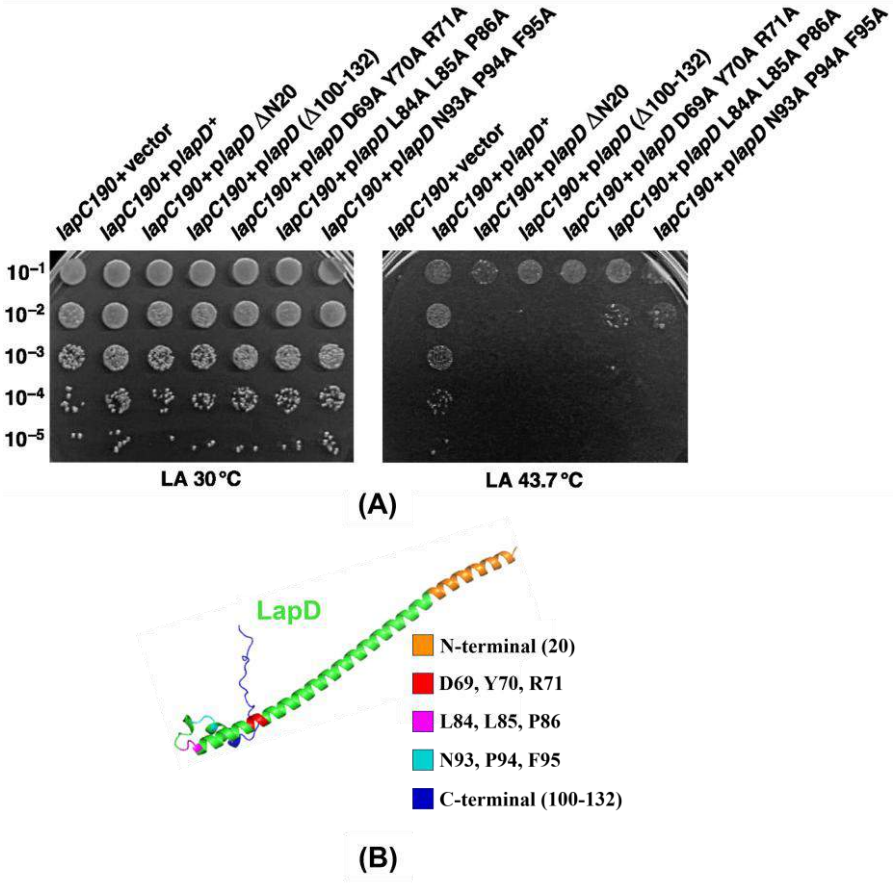

Figure 38: The N-terminal membrane anchor of LapD and its conserved amino acid residues in the C-terminal domain are essential for the restoration of the growth of *lapC190* bacteria at 43.7°C[293].

(**A**) In-frame deletion of N-terminal 20 amino acid residues, ΔN20, deletion of last 32 amino acid residues Δ100-132 and a triple Ala replacement of conserved amino acid were constructed by gene synthesis and cloned in expression vector. Plasmid DNA with the wild-type *lapD* gene and plasmids DNAs with various substitutions or deletions in the *lapD* gene were used to transform *lapC190* bacteria. Growth of such isogenic cultures of *lapC190* was quantified by serial spot dilution assay on LA 30°C and 43.7°C. The relevant genotype and temperature of incubation are indicated. All experiments were performed with three biological repeats and a representative picture from each set is shown. (**B**) The structure of LapD predicted by AlphaFold (AF-P0ADW3-F1-v6). The positions of mutations introduced in this study are shown in different colours.

Thus, we conclude that the structural integrity of LapD is required for its function and for IM localization and context-dependent interactions, the N-terminal transmembrane anchor seems to play an essential role. Additionally, the integrity of the 32 amino acid residues at the extreme C-terminal is crucial, indicating that this region is involved in important interactions or structural stability rather than just being a flexible tail. Besides, the loss-of-function phenotype associated with the triple-alanine substitutions pinpoints specific, conserved residues within the cytoplasmic coiled-coil domain that are vital for the activity of LapD. These residues may form part of an interaction surface required for engaging with partner proteins necessary for its role in regulating envelope biogenesis and compensating for the loss of LapC function.

4.2.4. Multicopy suppressors which restore LpxC and LPS levels in *lapC190* bacteria (results from[293])

We further examined the effects of the identified multicopy suppressors on the cellular levels of the crucial LPS biosynthesis enzyme LpxC in order to clarify the molecular mechanism by which they suppress the Ts growth phenotype of *lapC190* bacteria.

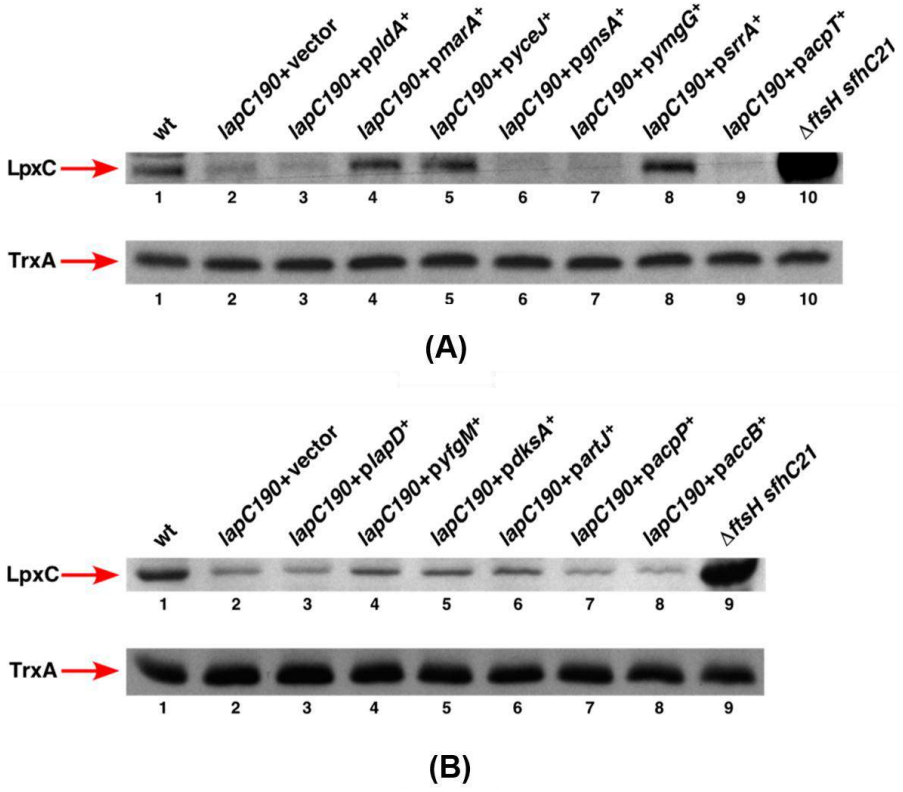

Figure 39: Restoration of LpxC amounts to different extents upon the overexpression of various multicopy suppressor encoding genes in *lapC190* bacteria[293].

Immunoblots of whole cell lysates obtained from isogenic strains grown at 30°C, followed by the addition of 75 µM IPTG and a temperature shift for 2 h at 43°C (**A**, **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. As controls, lane 2 in panel (**A**) and panel (**B**) samples from *lapC190* bacteria are loaded, which have reduced LpxC amounts. Additional control involves samples from Δ*ftsH sfhC21* which expectedly reveal higher amounts of LpxC due to its stabilization lane 10 [panel (**A**)] and lane 9 [panel (**B**)]. As loading control, the same samples were probed with anti-TrxA antibodies.

The main idea was that if these suppressors work to stabilize LpxC, the primary defect in the *lapC190* bacteria, which is hyperdegradation of LpxC resulting in decreased LPS, could be fixed. Therefore, we examined the status of LpxC in whole-cell lysates obtained from isogenic strains, including the wild type and the *lapC190* derivatives carrying either an empty vector or plasmids for the inducible expression of individual suppressor encoding genes. The induction of *srrA*, *yceJ*, and *marA* in the *lapC190* background restored LpxC abundance to levels comparable to that in the wild type, as revealed by immunoblotting using LpxC-specific antibodies (**Figure 39 A, B**). Overexpression of *yfgM*, *dksA,* and *artJ* also resulted in a slight increase in LpxC levels. However, under the same circumstances, a different set of suppressors, such as *pldA*, *gnsA*, *ymgG*, *acpT*, *lapD*, *acpP*, and *accB*, did not increase LpxC levels, suggesting that their suppression mechanisms are separate from directly stabilizing LpxC.

After demonstrating that a subset of suppressors works by reestablishing LpxC stability, we evaluated cellular LPS levels to ascertain the physiological result.

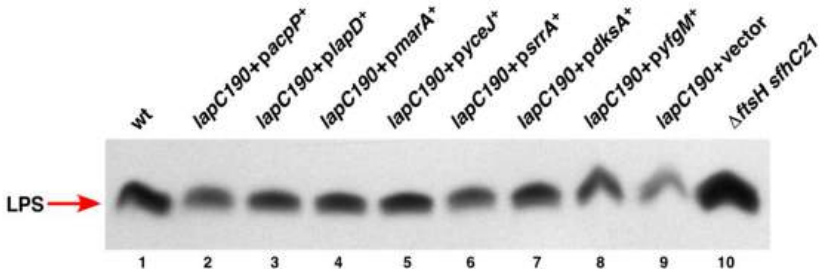

Figure 40: Restoration of LPS amounts by overexpression of *marA*, *yceJ*, *srrA*, and *dksA* to different extents in *lapC190* bacteria[293].

Immunoblots of whole cell lysates obtained from isogenic strains grown at 30°C, following a temperature shift for 2 h at 43°C. Expression of the specific gene was induced by the addition of IPTG. An equivalent portion of whole cell lysates were applied and samples were resolved on a 15.5% SDS-Tricine gel and LPS was transferred by Western blotting. Amounts of LPS were revealed using a LPS-specific WN1 222-5 monoclonal antibody.

The induction of *marA*, *yceJ*, *srrA*, and *dksA* expression in *lapC190* bacteria led to a significant recovery of LPS quantities to near wild-type levels, which is consistent with the LpxC results (**Figure 40**). Overexpression of the *acpP* gene did not increase LPS amount.

4.2.5. *srrA*, *marA*, *lapD*, and *pldA* genes are indispensable in the *lapC190* mutant background (results from[293])

We expanded our analysis to look into genetic interactions in order to obtain a deeper mechanistic understanding of how multicopy suppressors mitigate the Ts phenotype of the *lapC190* bacteria. To find out whether any of the suppressor genes are necessary for the viability of *lapC190* bacteria, we performed systematic bacteriophage P1-mediated transductions. This approach aimed to identify potential synthetic lethal interactions, which would indicate that the corresponding gene products function in parallel, compensatory pathways that become essential when the LapC periplasmic domain is absent. Our strategy was two-fold. Initially, we modified the *lapC190* genetic background by introducing null alleles of the potential suppressor genes. Second, we transduced

the *lapC190* mutation into several single-gene deletion contexts in order to carry out the reciprocal experiment. Even at the permissible temperature of 30°C, *lapC190* bacteria cannot survive without the presence of *srrA*, *marA*, *pldA*, and *lapD* genes. The inability to produce viable transductants for the Δ*srrA lapC190* and Δ*lapD lapC190* combination served as proof of this (**Table 12**). Since their deletion was readily tolerated in the parental wild-type strain, the essential nature of these genes was confirmed to be specific to the compromised *lapC190* background. To definitively prove that the observed lethality was due to the loss of the specific gene and not an unlinked secondary mutation, we performed complementation assays. The associated mutants' viability was completely restored by introducing a wild-type copy of either the *srrA* gene or the *lapD* gene in trans on a plasmid, enabling their isolation at frequencies similar to those of control transductions (**Table 12**)

Table 12: Synthetic lethality of *marA*, *srrA,* and *lapD* in the *lapC190* background.

Numbers of transductants on LA 30°C		
Donor	**wt**	***lapC190***
Δ*marA*	1274	92[1]
Δ*srrA*	650	2
Δ*dksA*	>2000	163[2]
Δ*pldA*	1460	34 tiny
Δ*lapD*	733	25

[1]not viable. [2]viable but heterogenous

This confirms a direct, synthetic lethality between *lapC190* and the absence of these multicopy suppressor encoding genes. The genetic interactions for other suppressors were more nuanced. A Δ*dksA lapC190* derivative showed heterogenous colony growth, indicating a synthetic sickness, despite being viable up to 37°C. Additionally, it was possible to introduce *marA* or *pldA* deletions into the *lapC190* strain, but only at a highly reduced frequency, and most transductants failed to proliferate (**Table 12**). When taken as a whole, these genetic interaction data enable us to determine that SrrA, MarA, LapD, and PldA are fundamental elements of a crucial genetic network that sustains cell viability in the absence of a functional LapC periplasmic domain.

4.2.6. MarA-mediated suppression requires the presence of *mla* genes (results from[293])

Since overexpression of the *marA* gene restored the growth of the *lapC190* bacteria at higher temperatures, we sought to elucidate the underlying molecular mechanism of this suppression. To achieve this, a saturated transposon mutagenesis approach was employed. The strain SR23660 (*lapC190* carrying *pmarA*$^+$) was used as a host to generate a saturated Tn*10* insertion library under permissive growth conditions of 30°C. Approximately 50,000 independent

Tn*10* insertion mutants were obtained and subsequently screened for growth on LA medium at 43°C in the presence of 75 µM IPTG, which induces *marA* expression. Growth at 30°C without IPTG induction served as a control. Mutants that exhibited normal growth at 30°C but failed to grow at 43°C despite *marA* induction were retained as candidates potentially defective in *marA*-dependent suppression. These Tn*10* insertion mutants were backcrossed into the original SR23660 strain using bacteriophage P1-mediated transductions to confirm the loss of *marA*-mediated suppression of the *lapC190* Ts phenotype. From this screening, 54 independent Tn*10* insertions were isolated that were unable to sustain growth at elevated temperature despite *marA* induction. To exclude insertions conferring general Ts unrelated to the *lapC190* background, these Tn*10*Kan insertions were transduced into the wild-type strain using bacteriophage P1. Twenty-two mutations conferred a Ts phenotype even in the wild-type background, whereas the remaining insertions were found to specifically affect the *lapC190* strain.

Mapping and characterization of the remaining Tn*10* insertions revealed that 12 out of 15 mutations that prevented *marA*-mediated multicopy suppression carried disruptions in genes of the *mla* operon. The majority of the Tn*10* insertions were confirmed to reside within the *mla* locus. Inverse PCR followed by DNA sequencing of the chromosomal regions flanking the Tn*10* insertions identified four mutations each in the *mlaC* and *mlaD*, three in *mlaF*, and one in *mlaA*. The Mla pathway is a critical system responsible for preserving the lipid asymmetry of the OM by facilitating retrograde and/or anterograde PL transport between the IM and OM[315].

To confirm whether the *mla* genes are directly required for the MarA mediated suppression of the *lapC190* Ts phenotype, we employed well-characterized, in-frame deletion mutations of *mlaC* and *mlaD* genes, along with a newly constructed derivative, Δ(*mlaC mlaD*)[88]. This analysis was necessary because the Tn*10* transposon insertions identified in the earlier screen could exert polar effects on the transcription of downstream genes within the *mla* operon, potentially complicating the interpretation of results. The in-frame deletions of *mlaC*, *mlaD* and the combined Δ(*mlaC mlaD*) mutation were individually introduced into strain SR23660 (*lapC190* harbouring an inducible p*marA*$^+$ plasmid) via bacteriophage P1-mediated transduction. Transductants were selected and subsequently plated on LA medium at both permissive (30°C) and non-permissive (43°C) temperatures in the presence of 75 µM IPTG to induce the *marA* expression. As controls, the same deletion mutations were introduced into the wild-type strain and the chromosomal *lapC190* bacteria lacking the *marA*-expressing plasmid. Under permissive conditions (30°C), the in-frame deletions of *mlaC*, *mlaD* and Δ(*mlaC mlaD*) could be successfully introduced into the wild type, *lapC190* and SR23660 (*lapC190* + p*marA*$^+$) strains at comparable frequencies. However, under non-permissive conditions (43°C), none of the *mla* deletion combinations could be introduced into the SR23660 strain even when *marA* expression was induced by IPTG (**Table 13**).

Table 13: Requirement of the Mla system for MarA-mediated suppression.

Recipient	Donor					
	30°C			43°C		
	Δ*mlaC*	**Δ*mlaD***	**Δ*mlaCD***	**Δ*mlaC***	**Δ*mlaD***	**Δ*mlaCD***
wt BW25113	1260	1380	1429	1412	1274	980
lapC190	623	710	690	12	14	9
lapC190 + p*marA*$^{+}$	654	740	565	9	12	8

In contrast, control transductions into the wild-type background were viable under the same conditions, confirming that the deletions themselves were not inherently lethal. These results demonstrate that the functional *mlaC* and *mlaD* genes are indispensable for the *marA* mediated multicopy suppression of the *lapC190* bacteria.

4.2.7 Absence of *mla* genes does not alter LPS composition (results from[293])

To date, the impact of an impaired Mla system on the structural composition of LPS in *E. coli* has not been characterized. To determine whether the deletion of *mla* genes induces significant changes in LPS composition or its non-stoichiometric modifications, we analyzed purified LPS from the wild-type strain and its isogenic Δ*mlaC* and Δ*mlaD* derivatives using mass spectrometry, which was performed at Research Center Borstel by Professors Klein and Raina. Bacteria were cultured in phosphate-limiting medium as described previously[93,97]. The mass spectrometry profiles obtained from all three strains were comparable (**Figure 41**). In each case, spectra revealed the presence of the complete glycoform I (observed at 3936.7 Da) along with its derivatized variants, which included the addition of *P*-EtN, L-Ara4N, and GlcUA. Peaks corresponding to larger additions, such as GlcNAc and GlcUA (at 4489.9 Da and 4612.9 Da), were also common to all samples. Furthermore, all strains exhibited prominent peaks representing glycoforms IV and V (ranging from 3948.7 to 4298.9 Da), which are characterized by the incorporation of a third Kdo and rhamnose. Variations within these glycoforms were consistent across strains and attributable to the same non-stoichiometric substitutions.

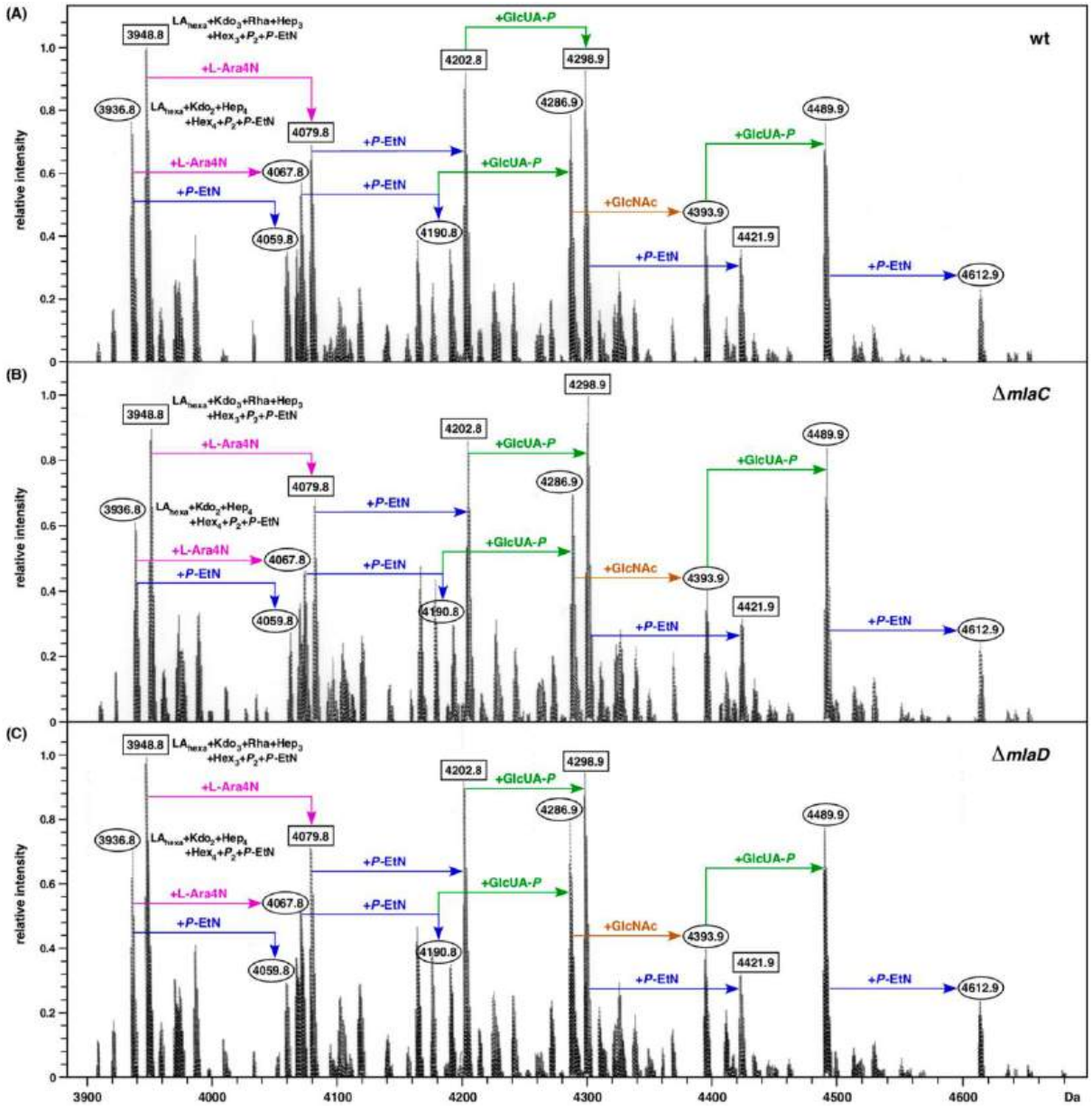

Figure 41: In the absence of either the *mlaC* or *mlaD* gene, LPS structure is similar to that of parental wild type[293].

Charge-deconvoluted mass spectra in the negative ion mode of LPS from the wild type (**A**) and its Δ*mlaC* (**B**) and Δ*mlaD* (**C**) derivatives. Cultures were grown in phosphate-limiting medium at 30°C. Mass numbers refer to monoisotopic peaks. Mass peaks with rectangular boxes correspond to the glycoform containing the third Kdo. Mass peaks marked with an oval box correspond to predicted LPS containing two Kdo residues.

Collectively, these data indicate that under the phosphate-limiting conditions tested, the absence of the *mlaC* or *mlaD* genes does not result in any major alterations to the core or lipid A structure of the LPS.

5. DISCUSSION

The fundamental imperative for any Gram-negative bacterium is the maintenance of its OM, a robust yet dynamic asymmetric barrier that is essential for its viability. The synthesis of its two key cell envelope components, LPS and PL, must be exquisitely coordinated. An imbalance, whether an excess or a deficit of LPS relative to PL, disrupts OM integrity, leading to increased permeability, induction of stress responses, and ultimately cell death[1]. The regulation of the first committed enzyme of lipid A biosynthesis, LpxC, has been recognized as the central control point for this balancing act[114]. The discovery of the LapB-FtsH proteolytic axis provided the first mechanistic insight into how LpxC turnover is achieved, with LapB delivering LpxC for degradation[24]. The subsequent identification of LapC as an antagonist to LapB revealed a push-pull system, where LapB drives degradation and LapC provides a stabilizing counteract, potentially in response to the status of LPS accumulation[113].

The research presented in this thesis expands this paradigm by introducing and characterizing LapD as a link to balance LPS and PL amounts. Our data demonstrate that LapD is not a peripheral player but a core component that links the LapB/C-regulated LpxC degradation machinery with the broader metabolic networks of FA and PL biosynthesis. Our findings allow us to present a holistic model of envelope homeostasis, in which LapD ensures the coordinated flux of fatty acid precursors into their respective pathways, thereby safeguarding OM asymmetry under both standard and stressful conditions.

Our initial proteomic analysis provided the foundational insight for this study. LapD co-purifies with a specific set of proteins. This interactome includes partners like LapA, LapB, FtsH and also a certain enzymes dedicated to LPS biosynthesis (LpxM and GmhA), LPS transport (LptB/C/D), and PL/FAB (PssA, FabB/F/H/Y, and AccD) biosynthesis. This physical association is not trivial, it positions LapD at the juncture of the two major biosynthetic pathways. In this context, LapD could act by organizing a complex that ensures the efficient flux from the FA synthesis machinery to the LPS biosynthesis enzymes (LpxA and LpxD) and the PL biosynthesis pathway.

The functional consequences of disrupting this hub are severe and pleiotropic. The reduction in LpxC levels in Δ*lapD* bacteria is a pivotal finding at high temperature. This suggests that in the absence of LapD, the regulatory circuit is biased towards LpxC degradation at high temperatures. This could occur through several non-mutually exclusive mechanisms. LapD's absence might constitutively activate LapB, perhaps by failing to sequester it, or the metabolic dysregulation caused by LapD's loss could be sensed by the LapB/C system as a signal for reduced LPS synthesis, triggering LpxC proteolysis. This phenotype aligns Δ*lapD* more closely with mutations in *lapC* rather than with those in the *lapB gene*, genetically placing LapD's function on the stabilizing side of the regulatory balance, akin to LapC. Furthermore, Δ*lapD* bacteria exhibit a profound defect in LPS translocation, with a significant accumulation of LPS, including immature forms, in the IM. This reveals a role for LapD that extends beyond the mere regulation of synthesis into the realm of transport. We propose that the LapD-containing complex is not only a biosynthetic

centre but also a control checkpoint. By associating with both biosynthetic enzymes (LpxM), the complex could facilitate the direct handoff of newly synthesized LPS to the Lpt machinery by MsbA, ensuring that only properly assembled molecules are committed to transport. In the absence of LapD, this coordination fails, leading to the observed IM accumulation and explaining the synthetic lethality with mutations that further compromise LPS structure. The genetic interaction profiles of Δ*lapD* provide powerful *in vivo* validation of its central role. The conditional synthetic lethality observed with mutations in *waaC* (LPS core biosynthesis) and *clsA* (cardiolipin synthesis) is particularly illuminating. Removing WaaC in the Δ*lapD* background compromises OM integrity. Similarly, disrupting cardiolipin synthesis alters the physical properties of the membrane, and Δ*lapD* bacteria cannot compensate for this additional perturbation. These genetic interactions underscore that LapD's function becomes critical when the cell envelope is challenged. The suppressor analysis of the Δ(*lapD waaC*) conditional synthetic lethality further deepens our understanding. The isolation of suppressor mutations in *lptD* and *nlpI* genes reveals the specific pathways that are likely failing. LptD is essential for bacterial growth, is a member of the RpoE regulon, and is required for the assembly of LPS in the OM. Even more interesting is the suppressor mutation in the *nlpI* gene encoding an OM lipoprotein that regulates PGN hydrolysis by recruiting the Prc protease to degrade hydrolases like MepS[316,317,318]. This finding shows a functional link between LapD and PGN metabolism.

The identification of suppressor mutations within the core PL biosynthetic genes *pgsA* and *cdsA* in the Δ(*lapD clsA*) background provides critical genetic evidence that profoundly expands the functional scope of the LapD regulatory network. The isolation of these specific suppressors demonstrates that the lethal envelope defect in Δ(*lapD clsA*) bacteria originates from a dysregulation of anionic PL homeostasis, rather than merely an additive loss of two unrelated functions. This finding positions LapD as an essential node within a broader metabolic circuit that coordinates fatty acid flux with the balanced synthesis of specific PL classes. In the absence of cardiolipin synthase, the cell appears to rely on LapD to manage the consequent metabolic perturbation potentially a toxic accumulation of its precursor PG or an altered membrane surface charge. The suppression of this lethality by mutations that cripple PgsA (the enzyme committing metabolism to the PG/CL branch) or reduce flux through CdsA (the gateway to all major PLs) indicates that rescuing viability requires a fundamental re-wiring of the entire PL biosynthetic pathway. Also, the mapping of one suppressor to the *murD* gene links to the PGN as well. This genetic interaction elegantly demonstrates that LapD’s role is not limited to interfacing with LPS biogenesis but is intrinsically linked to the metabolic decisions governing acyl chain utilization for membrane PL production. Thus, LapD emerges as a key integrator that dynamically synchronizes the biosynthesis of all three major envelope macromolecules: LPS, PGN, and PL.

The multicopy suppressors of the Δ*lapD* phenotypes provided a direct window into the metabolic limitations that arise in its absence. The most potent suppressor, *acpP*, is highly significant. AcpP is important in shuttling growing acyl chains between all the Fab enzymes and delivering them to the PL pathway and the LPS pathway (via LpxA, LpxD, LpxL and LpxM)[319]. The

fact that overproducing AcpP suppresses temperature sensitivity, vancomycin hypersensitivity, filamentation and even the RpoE stress response indicates that a primary defect in Δ*lapD* is dysfunctional FASII system. Overexpression of the *accB* gene, which encodes a subunit of acetyl-CoA carboxylase (the first and rate-limiting enzyme in FA synthesis), could reduce the activity of the ACC enzyme, which is required for the first step in fatty acid biosynthesis to produce malonyl-CoA. Inhibition of the ACC enzyme will reduce overall fatty acid synthesis. Consistent with this hypothesis, concurrent complementary studies in the laboratory of bacterial genetics isolated extragenic suppressors that relieve the lethal phenotype of Δ(*lapD lpxM*) bacteria. These suppressor mutations specifically act at the level of inhibition of acetyl-CoA carboxylase, thereby effectively overcoming this toxicity[96]. The suppression by MenI, the cytoplasmically localized TesA', and the newly identified thioesterase TesD offers a complementary strategy[199]. They can potentially hydrolyse acyl-ACP or acyl-CoA, dampening an FA synthesis pathway that, in the unbalanced Δ*lapD* background, might be producing an inappropriate mix of fatty acids. By hydrolysis of acyl chains, these thioesterases could reduce fatty acid synthesis to rebalance LPS vs PL amounts to restore homeostasis in the cell envelope. The ultimate proof of this metabolic rebalancing is seen in the PL composition of the suppressed strains. The suppressors that rescue morphology (*acpP*, *accB*, and *tesA'*) consistently reduce the cellular levels of the anionic PLs, PG, and CL. This is a logical compensatory adjustment. Since Δ*lapD* bacteria have less LPS in their OM, reducing the synthesis of the major IM PLs helps to rebalance the overall cellular lipid ratio, moving the system back towards homeostasis. The fact that different suppressors achieve this through distinct mechanisms, increasing precursor flux versus redirecting it, highlights the robustness and flexibility of the cellular response to lipid dysregulation.

The interplay between LapC and LapD represents one of the most significant findings of this thesis, revealing both functional overlap and specialization within the envelope regulatory network. The identification of the *lapD* gene as a multicopy suppressor of the *lapC190* Ts phenotype provides evidence that these two proteins operate in a closely related functional pathway. This suppression is not merely a trivial bypass, it demonstrates that overexpression of the *lapD* gene can compensate for the dysfunctional LapC, suggesting that LapD can either substitute for or stabilize the compromised system. The molecular basis for this genetic interaction likely lies in their respective positions within the regulatory complex. We propose that LapC and the LapD represent two complementary input streams into the core LapB/FtsH regulatory module. Under normal conditions, LapC senses the periplasmic accumulation of LPS and transduces signals across the membrane to modulate LpxC degradation, while the LapD is likely to act via pathway that integrates signals of PL and fatty acid metabolism.

Certain phenotypic similarities between *lapC* and *lapD* mutant bacteria are another interesting finding. Both exhibit reduced cellular levels of LpxC although to different extents, increased OM permeability, and activation of the RpoE stress response. However, there are critical differences between them. The synthetic lethality between the *lapC190* mutation and null mutations in *lapD*, *srrA*, *marA,* and *pldA* reveals the non-redundant essentiality of these genes in the absence

of a functional LapC periplasmic domain. This points that an essential genetic network where the loss of one node creates an absolute dependency on the others. The fact that the deletion of the *lapD* gene is synthetically lethal with the *lapC190* mutation indicates that the products of these two genes function in parallel, compensatory pathways that both feed into the essential process of maintaining LpxC homeostasis. When both are compromised, the system collapses. The relationship with lipid A acyltransferases further illustrates this point. The synthetic lethality between Δ*lapD* and deletion derivatives of *lpxL* or *lpxM* genes supports these findings[188]. In both cases, the common suppressors map to different subunits of the ACC complex. LpxM and LpxL are also required for the viability of *lapC190* bacteria. This recurrent theme underscores that both LapC and LapD are critical for managing the cellular consequences of defective LPS, and their loss makes the cell exquisitely sensitive to any further perturbation in LPS.

The structure-function analysis of LapD provides additional insights into the molecular determinants of its function. The demonstration that both the N-terminal transmembrane anchor and the C-terminal 32 amino acid residues are absolutely essential for LapD's suppressor function confirms the specific requirement of LapD. More revealing is the alanine-substituted mutagenesis results. The complete loss of function upon substitution of conserved residues in the cytoplasmic domain (D69/Y70/R71, L84/L85/P86, and N93/P94/F95) suggests that these amino acid residues may form critical interaction surfaces. Given the predicted tetrameric α-helical coiled-coil structure of the LapD C-terminal domain, these clusters likely represent key points of contact with other components of the regulatory complex. The D69/Y70/R71 motif, with its combination of charged and aromatic residues, might mediate specific protein-protein interactions. The L84/L85/P86 and N93/P94/F95 clusters, rich in hydrophobic and polar residues, could be involved in either intra- or inter-molecular contacts that stabilize the coiled-coil structure or mediate interactions with other partners. The failure of these mutations in the *lapD* gene to suppress *lapC190* bacteria indicates that LapD's function is not merely dependent on its presence but also on the importance of the amino acid residues required for its function. This reinforces the concept of LapD as a molecular scaffold whose value lies in bringing together the right partners at the right time and place. Disrupting these precise interactions, even while maintaining the overall protein structure, is sufficient to abolish function. These findings provide a foundation for future biochemical studies aimed at mapping these interaction surfaces precisely which could reveal new targets for disrupting this essential regulatory complex.

The comprehensive multicopy suppressor selection approach in the *lapC190* background revealed two fundamentally distinct classes of suppressors, illuminating the multiple layers of regulation that maintain envelope integrity. This classification provides a powerful framework for understanding how bacteria can bypass defects in a central regulator like LapC. Class I suppressors that operate through LpxC-dependent mechanisms. These genes, when overexpressed, restore LpxC levels and consequently LPS abundance in *lapC190* bacteria. The transcriptional regulators MarA and SrrA are particularly interesting in this context. Their ability to suppress *lapC190* bacteria implies that the regulation of LpxC may be connected with broader cellular physiology. They may

achieve this by modulating the expression of components of the Lap complex or by influencing the activity of the FtsH protease or may be through an unknown mechanism. Similarly, YfgM is a substrate for FtsH. Overproduction of YfgM might titrate the FtsH protease, thereby reducing its availability for LpxC degradation. This diverse set of LpxC-dependent suppressors reveals that the bacteria have multiple regulatory inputs that can fine-tune LpxC stability beyond the core Lap proteins. Class II suppressors operate through LpxC-independent mechanisms. These genes suppress the Ts phenotype of *lapC190* bacteria without significantly increasing LpxC or LPS levels. This demonstrates that viability can be restored by addressing the consequences of reduced LPS levels, rather than correcting the primary defect. As discussed previously, AcpP and AccB likely function by reducing the fatty acid synthesis. The identification of PldA (outer membrane phospholipase A) in this class is interesting. PldA degrades PLs in the outer leaflet of the OM, and its overexpression in the *lapC190* background could help maintain OM asymmetry by its mislocalisation[320]. Similarly, *acpT*, which encodes a phosphopantetheinyl transferase that activates AcpP, would enhance the functionality of the acyl carrier protein pool. The presence of LapD in this class, despite its physical association with the Lap complex, suggests that its overexpression may enhance the efficiency of LPS utilization or transport or through other mechanism rather than directly affecting LpxC stability in the *lapC190* background. The existence of these two parallel suppression strategies highlights the remarkable plasticity of the bacterial cell in maintaining envelope homeostasis. When the primary regulatory circuit fails, cells can either attempt to fix the circuit itself (Class I) or develop workarounds that mitigate the downstream consequences (Class II). This redundancy provides robustness to the system and represents an important consideration for antimicrobial strategies that target this pathway.

The discovery that MarA mediated suppression of the *lapC190* bacteria requires a functional Mla system represents one of the most interesting findings of this work. This genetic connection forges a novel bridge between a well-characterized transcriptional regulator of multidrug resistance and a dedicated machinery for maintaining PL asymmetry. Using saturated transposon mutagenesis, we identified genes required for MarA to restore the growth of the *lapC190* bacteria at high temperature. Most insertions that blocked MarA's suppression mapped to the *mla* genes.

The integrated model of envelope homeostasis that emerges from this study has significant implications for both our fundamental understanding of bacterial cell biology and the development of novel antimicrobial strategies. The LapB/C/D complex represents a sophisticated regulatory node that continuously monitors and adjusts the synthesis and maintenance of the OM asymmetry, a task essential for bacterial survival in diverse environments. From a physiological perspective, our work reveals the remarkable complexity and robustness of the systems that maintain envelope integrity. The connections we have revealed between LPS regulation, FA metabolism, PL asymmetry, and PGN remodelling suggest an integrated "envelope network" rather than a collection of independent pathways. The conservation of these regulatory components across Gram-negative pathogens suggests that they represent fundamental aspects of Gram-negative cell biology. LapB, LapC, and LapD homologs are found in many clinically relevant species, including *Klebsiella pneumoniae* and

Salmonella enterica. This conservation indicates that the regulatory principles we have elucidated in *E. coli* likely apply broadly across Gram-negative bacteria, making this system an attractive target for novel antimicrobial development.

Several aspects of the Lap system present particularly promising targeting opportunities. First, the essential nature of these regulators means that their disruption is likely to be bactericidal. Second, the extensive protein-protein interactions within the complex offer potential sites for interference using small molecules or peptides. Third, the synthetic lethal interactions we have identified, such as between *lapD* and *waaC* or *clsA* genes, suggest combination therapy approaches where Lap function is compromised in parallel with other envelope targets. A drug that partially inhibits LapD might not be effective alone but could become highly effective when combined with inhibitors of LPS core biosynthesis or cardiolipin synthesis. From an evolutionary perspective, the complex regulation of LpxC reflects the fundamental challenge of balancing the synthesis of two essential, but chemically distinct, membrane components that share common precursors. The LapB/C/D system appears to have evolved as a sophisticated solution to this problem, integrating multiple inputs to maintain the homeostasis of cell envelope. Understanding how this system varies across species and environments may provide insights into bacterial adaptation and the evolution of resistance mechanisms.

6. CONCLUSION

The intricate architecture of the Gram-negative bacterial envelope, characterized by its asymmetric OM, represents one of the most formidable barriers in the biological world. The preservation of this structure is not a passive event but an active, energy-dependent process requiring the exquisite coordination of synthesis, assembly, and regulation. This thesis has endeavoured to deconstruct the complex regulatory circuitry governing this process, with a particular focus on the enigmatic proteins LapD and LapC. The culmination of this work positions LapD and LapC not as a peripheral accessory, but as a central integrator within the essential Lap regulatory network, fundamentally advancing our understanding of how *E. coli* maintains cell envelope homeostasis.

The foundational discovery of this thesis, revealed through co-purification studies, is that LapD resides at the heart of a multi-protein complex that bridges the major envelope biogenesis pathways. Its physical association with proteins involved in LPS biosynthesis, its transport, FA synthesis, and PL metabolism provided the first crucial clue that its function transcends a single pathway. This interactome, where LapD acts as the structural framework, ensures the efficient channelling of substrates and information. This role was functionally confirmed by the pleiotropic consequences of its absence, such as temperature sensitivity, increased OM permeability, aberrant cell morphology, and a specific reduction in the cellular level of the rate-limiting enzyme LpxC. The genetic interaction profiles of Δ*lapD* bacteria provided powerful *in vivo* validation of their critical role. The conditional synthetic lethal phenotype observed with mutations in LPS core biosynthesis and cardiolipin synthesis demonstrated that LapD function becomes indispensable at elevated temperatures. Perhaps the most mechanistically illuminating findings came from the suppressor analyses. The isolation of extragenic suppressors mapping to *lptD* and *nlpI* genes in the Δ(*lapD waaC*) background revealed the specific pathways that were pushed to failure. LptD is involved in the LPS transport machinery. In contrast, the *nlpI* suppressor is linked to PGN. Suppressor mutations in the PL biosynthesis genes *pgsA* and *cdsA* reveal that the lethality of a Δ(*lapD clsA*) bacteria stems from dysregulated anionic PL homeostasis. This genetic evidence expands the role of LapD, in coordinating fatty acid flux with balanced PL synthesis. Specifically, in the absence of cardiolipin synthase, LapD is required to manage the resulting metabolic imbalance. The suppression of lethality by reducing flux into PG or overall PL synthesis demonstrates that viability requires a wholesale rewiring of fatty acid production. Also, identification of the *murD* gene as a suppressor links LapD to PGN. Consequently, LapD emerges as a central integrator, dynamically synchronizing the biosynthesis of LPS, PGN, and PLs in response to envelope stress. The identification of the *acpP* gene as a potent multicopy suppressor of the Δ*lapD* phenotypes pointed directly to a primary metabolic correlation. The LapD could optimize the concentration and delivery of acyl-AcpP to its diverse partner enzymes. In its absence, a kinetic bottleneck arises, limiting the flux of FAs into one or more critical pathways, most notably the rapid initiation of LPS molecules. The additional suppression by *tesA'* and *menI* further supports a model of metabolic rewiring, where

the bacteria act by reducing fatty acid synthesis to correct the imbalance created by LapD's absence.

A parallel and equally significant contribution of this study was the elucidation of the functional relationship between LapD and the essential regulator LapC. The identification of the *lapD* gene as a multicopy suppressor of *lapC190* bacteria (lacking the LapC's periplasmic domain) was a pivotal discovery. This demonstrated functional overlap, indicating that increased LapD dosage can compensate for the impaired LapC function. The comprehensive multicopy suppressor selection in the *lapC190* background revealed a masterclass in cellular resilience, revealing two fundamental strategies for maintaining viability in the face of a compromised central regulator. The classification of suppressors into LpxC-dependent and LpxC-independent categories illustrates the hierarchical nature of cell envelope regulation. Class I suppressors, which restore LpxC levels, represent attempts to fix the broken regulatory circuit. In contrast, Class II suppressors exemplify bypass and compensation mechanisms. They acknowledge the broken circuit and address the downstream consequences instead.

Integrating all these findings, we propose a refined, dynamic model for the regulation of cell envelope biogenesis in *E. coli*. The LapB/C/D complex forms an essential regulatory hub. LapB serves as the primary driver of LpxC degradation via FtsH. LapC acts by sensing LPS imbalances whereas LapD could act by tethering this regulatory core to the biosynthetic machineries for FAs, PLs, LPS, and PGN. It facilitates the channelling of the common precursor regulating the accumulation of varying amounts of different acyl-AcpP chain lengths, to the divergent pathways of LPS and PL synthesis. The accumulation of long-chain acyl-ACPs is known to inhibit ACC enzyme activity. This is consistent with the identified vast number of suppressors of Δ(*lapD lpxM*) lethality mapping to genes encoding various subunits of ACC[96]. The relative activity of LapB and LapC is potentially influenced by the flux of intermediates through the complex, which determines the allocation of this precious resource, thereby maintaining the LPS:PL balance. Under optimal conditions, this system maintains steady-state homeostasis. However, under envelope stress, such as the presence of underacylated LPS, altered PL composition, or elevated temperature, the role of Lap proteins becomes critical. This ensures that the complex can adapt and maintain coordination, preventing a catastrophic failure of OM integrity. When the components of this core system fail, the cell deploys backup strategies.

In summary, this thesis has provided complementary pathways of LpxC regulation beyond those governed by LapB and FtsH. This study revealed the existence of a sophisticated, multi-layered network centred on the Lap protein complex. By integrating proteomic, genetic, biochemical, and cell biological approaches, we have demonstrated that Lap proteins are the critical regulators where metabolic flux, proteolytic control, and transport efficiency converge to safeguard the asymmetric OM. One of the major findings concerning LapD is how it integrates signals between fatty acid and LPS biosynthesis. LapD more specifically interacts physically with several enzymes

involved in FAS II metabolism (**Figure 42**). Some of these interactions were validated by genetic interactions.

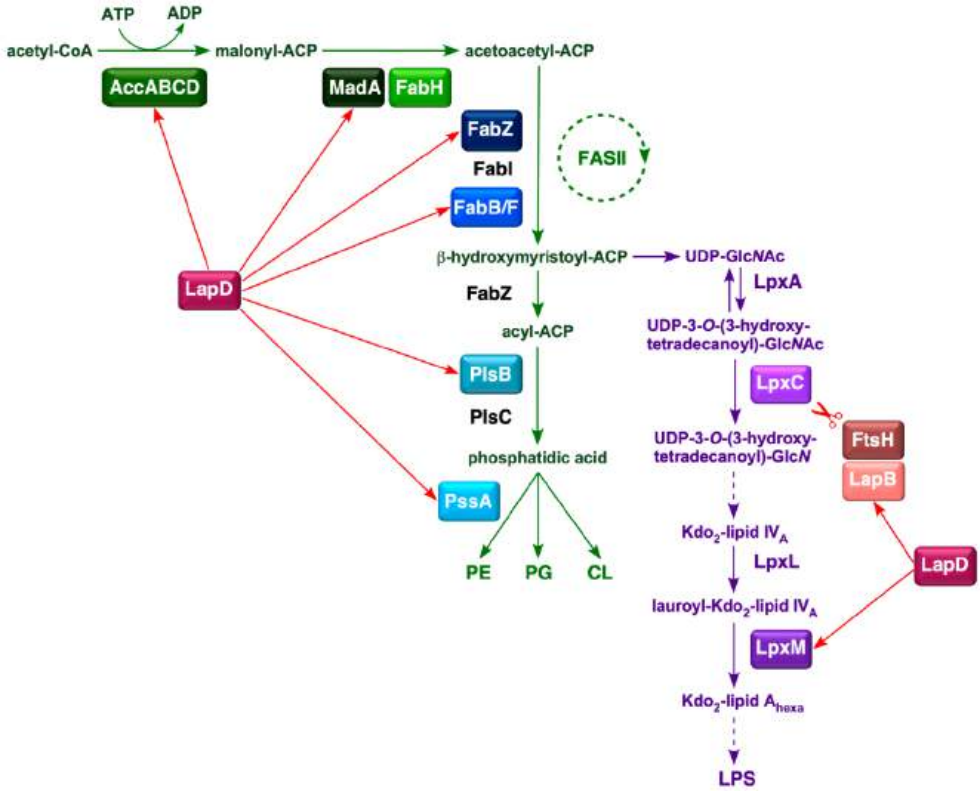

Figure 42: Schematic drawing of LapD-mediated coupled PL and LPS biosynthesis in *E. coli*[96].

Various steps in fatty acid biosynthesis, with initiation catalyzed by the acetyl-CoA carboxylase enzyme and entry into the FASII cycle, are shown. The intermediate *β*-hydroxymyristoyl-ACP serves as a precursor in the subsequent steps of PL and LPS biosynthesis. Red arrows depict the role of LapD based on its co-purification with specific proteins involved in fatty acid and LPS biosynthesis, and genetic interactions based on the synthetic lethality with Δ*lapD* in mutational combinations.

The regulatory principles uncovered, including metabolic scaffolding, synthetic lethal interactions, and compensatory network rewiring, provide a new framework for understanding bacterial envelope biology. This work not only deepens our fundamental knowledge of a quintessential Gram-negative structure but also illuminates a landscape of new vulnerabilities that can be strategically targeted to overcome the growing crisis of antimicrobial resistance. In the broader context of bacterial cell biology, this study highlights the remarkable integration of cellular processes required to build and maintain the complex envelope architecture of Gram-negative bacteria. The connections we have revealed between metabolic flux, protein degradation, and membrane transport demonstrate that envelope biogenesis is not merely an assembly process but a dynamically regulated system that continuously monitors and adjusts its components in response to internal and external cues. As we continue to unravel these complex relationships, we move closer to a comprehensive understanding of one of the most fundamental structures in biology, the bacterial cell envelope.

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Essential Role of LapD in the Absence of Cardiolipins

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Abstract

To maintain the integrity of the outer membrane of Gram-negative bacteria, such as *Escherichia coli*, the levels of two essential components, phospholipids (PL) and lipopolysaccharide (LPS), are tightly regulated, although the underlying molecular mechanisms are unclear. *E. coli* synthesizes three main PLs, including essential phosphatidylethanolamine and phosphatidylglycerol and nonessential cardiolipin (CL). We showed that CL synthesis is conditionally essential in Δ*lapD* bacteria. Using this synthetic lethal phenotype, we isolated suppressors that rescued growth at elevated temperatures. We showed that loss-of-function mutations in *cdsA* encoding CDP-diglyceride synthetase, and *pgsA*, which encodes phosphatidylglycerophosphate synthase, bypass this lethality. Such mutations reduce the relative abundance of acidic phospholipids, which are otherwise elevated in Δ(*lapD clsA*) bacteria, and increase the amounts of *cis*-vaccenic acid without altering amounts of LpxC mediating the first committed step in LPS biosynthesis. Interestingly, overexpression of genes, including *accC* and *glnB*, whose products can inhibit fatty acid/PL synthesis, overcame the lethality of Δ(*lapD clsA*) bacteria. We demonstrated that PgsA co-purifies with LapB, which regulates LpxC stability and acts as a hub for proteins involved in PL and LPS biosynthesis, including LapD. Overall, our results reveal that LapD is positioned at the regulatory nexus between LPS assembly and fatty acid/PL synthesis.

Keywords: lipopolysaccharide (LPS); phospholipids; LapD (YhcB); cardiolipin; phosphatidylglycerol; thioesterase; LapB; PgsA; acetyl-CoA carboxylase (ACC)

1. Introduction

The cell envelope of Gram-negative bacteria, including *Escherichia coli*, contains two distinct membranes, an inner (IM) and an outer (OM) membrane, separated by the periplasm, which includes a thin layer of peptidoglycan. The defining feature of the OM of Gram-negative bacteria is its asymmetry due to the presence of phospholipids (PLs) in the inner leaflet and the location of lipopolysaccharide (LPS) in the outer leaflet [1]. LPS constitutes the major component of the OM, and its essentiality is due to its role in providing a permeability barrier and structural integrity of the OM [2]. Similarly, PLs are integral in regulating membrane integrity and its fluidity [3]. Both of these components of OM are essential for bacterial viability, and their amounts are tightly regulated, as these two essential components use the same β-hydroxymyristoyl-ACP as a common metabolic precursor [4,5]. Hence, any imbalance between LPS and PLs leads to a loss of bacterial viability. Thus, bacteria, such as *E. coli*, hold a constant ratio of 0.15:1.0 for these two essential components, respectively [6]. However, the mechanisms by which bacteria maintain homeostatic control are poorly understood. One of the most studied regulatory controls that has received

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recent attention is the regulated turnover of LpxC, which catalyzes the first committed step in LPS biosynthesis [7]. This involves the positive regulation of LpxC proteolysis by the FtsH-LapB complex and its negative control by LapC [5,8,9]. PLs are derived from fatty acids (FA). Bacteria, such as *E. coli*, have devised controls for the biosynthesis of new fatty acids and the modification of the structure of existing fatty acids [3]. Similarly, LPS can undergo structural non-stoichiometric modifications in its lipid A and core parts, which impart resistance to cationic antibiotics [10–12]. Together, these processes allow bacteria to adjust LPS amounts according to their demand and control membrane viscosity to match environmental requirements. Another player, LapD, has recently been implicated in the regulation of LPS and fatty acid balance, although the precise mechanism of its involvement remains unknown [13,14].

In *E. coli* LPS biosynthesis, a tetraacylated lipid A precursor is first synthesized via a pathway that requires six essential enzymes [2,15]. Two Kdo residues are added to lipid IV_A by the WaaA enzyme, followed by the addition of secondary lauroyl and myristoyl chains, resulting in the synthesis of hexaacylated lipid A. Additional sugars are added to this key intermediate, leading to the LPS core formation. In smooth bacteria, a distal *O*-antigen is further attached after flipping LPS across the IM on the periplasmic side [15]. The synthesis of hexaacylated LPS is crucial because LPS that is either tetraacylated or pentaacylated is poorly translocated to the OM [16,17]. Furthermore, the absence of myristoyl acyltransferase LpxM causes synthetic lethality when either LPS or PL synthesis is compromised [13,14,18,19]. The fatty acid biosynthesis pathway in *E. coli* is of the type FASII system that comprises four steps using both acetyl coenzyme A (acetyl-CoA) and malonyl-ACP as starter units and malonyl-ACP as the extender unit [3]. In the initiation of FA biosynthesis, acetyl-CoA carboxylase (ACC) catalyzes the first committed step in fatty acid biosynthesis: the conversion of acetyl-CoA to malonyl-CoA [20,21]. Each turn of the FA biosynthesis cycle adds two carbon atoms to the growing acyl chain. The long-chain acyl-ACP thioesters generated during FA synthesis serve as acyl donors for PLs synthesis [22]. Phosphatidic acid (PA) serves as a universal precursor for membrane PL formation [23]. In *E. coli*, PA is generated by the acylation of *sn*-glycerol-3-phosphate (G3P) to 1-acyl-G3P, followed by a second acylation, requiring PlsB and PlsC acyltransferases, respectively [23,24]. PA is then converted to the central precursor of all phospholipids, cytidine diphosphate-diacylglycerol (CDP-DAG), by CDP-DAG synthase encoded by the *cdsA* gene (Figure 1). The primary PLs in *E. coli* are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). PE and PG are essential for bacterial viability, whereas CL is dispensable. CDP-DAG is positioned at a branch point in PL biosynthesis, where it reacts with *sn*-glycerol 3-phosphate to form PG or with L-serine to synthesize phosphatidylserine (Figure 1) [25]. CL is produced via the condensation of two PG molecules or a single PE and PG molecule [22]. The balance of zwitterionic (PE) and acidic phospholipids (PG and CL) in *E. coli* stems from the continued synthesis of PG by the PG phosphate synthase–phosphatase (PgsA–PgpA/B) system, which is coupled with the tight regulation of PE synthesis through the PssA–Psd pathway [3] (Figure 1).

E. coli has three CL synthases, with ClsA being the major contributor for producing CL. Although nonessential for bacterial viability, CL plays a crucial role in maintaining structural integrity, cell size, and regulating the activity of various proteins, such as the polar localization of ProP, assembly of LacY, and protein transport through the Sec apparatus [26–28]. More importantly, it was shown that CL presence is essential for the viability of strains lacking either WaaA Kdo transferase or LpxM myristoyl acyltransferase [19]. As suppressors that overcome the synthetic lethality of Δ(*clsA lpxM*) mapped to MsbA LPS flippase, it was proposed that CL participates in LPS translocation [18,19].

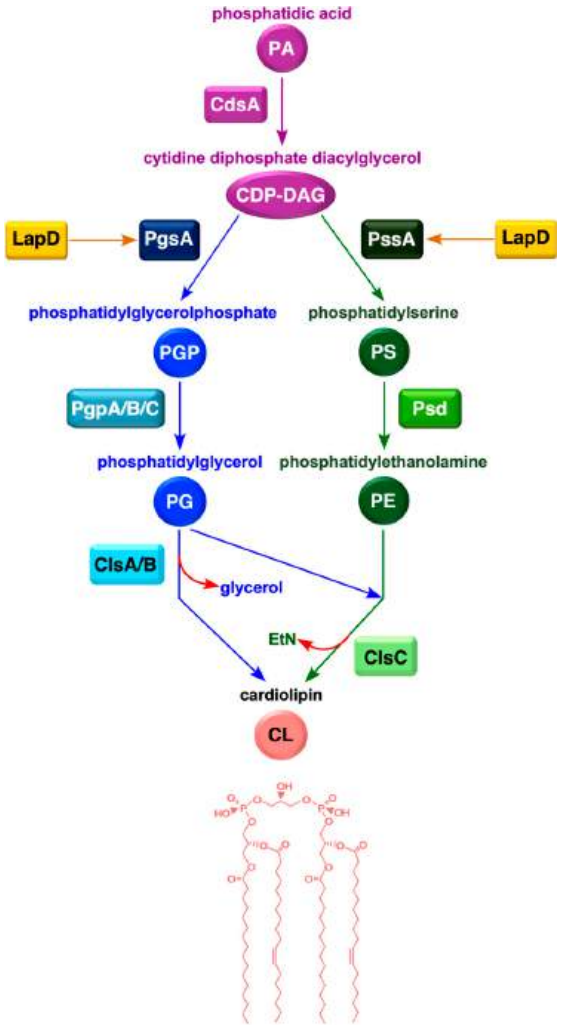

Figure 1. Schematic drawing of the phospholipid biosynthetic pathway in *E. coli*. The key intermediate in PL synthesis is cytosine diphosphate-diacylglycerol (CDP-DAG), which is synthesized by CdsA from phosphatidic acid. The major PL and PE are synthesized in two steps, as shown. PG molecules are also synthesized from CDP-DAG using PgsA to form phosphatidylglycerolphosphate, which is then dephosphorylated by Pgp enzymes. CL molecules are synthesized by the condensation of either two PG molecules or a single PE and PG molecule, as indicated. Co-purification of LapD with PgsA and PssA are indicated by arrows.

Biochemical characterization of LapD revealed that it co-purifies with enzymes involved in PL, FA, and LPS biosynthesis. Although LapD is non-essential, its absence confers synthetic lethality when lipid A is underacylated or when LPS is composed only of Kdo_2-lipid A [13,14]. Δ(*lapD lpxM*) bacteria were found to contain elevated amounts of acidic phospholipids and significant alterations in FA composition, supporting a role of LapD in regulation of FA composition. Consistent with this hypothesis, suppressor mutations that overcome the lethal phenotype of Δ(*lapD lpxM*) were found to map to genes encoding various subunits of the ACC complex, which restored PL amounts. However, the absence of LapD also causes retention of LPS in the IM, and suppressors that relieve the conditional lethal phenotype of Δ(*lapD waaC*) bacteria map to either genes involved in LPS assembly in the OM (*lptD*) or in the *nlpI* gene, whose product regulates proteolysis of peptidoglycan hydrolase [14]. Thus, LapD is an enigmatic protein, and significant gaps remain in our understanding of the pleiotropic phenotypes associated with its absence.

In this study, we showed that Δ(*lapD clsA*) bacteria exhibited conditional lethality. Thus, we used this synthetic lethal phenotype of Δ(*lapD clsA*) to isolate single-copy chro-

mosomal and multicopy suppressors that overcame this lethality and addressed their cell morphology defects and PL/fatty acid composition (Figure 2). Most single-copy extragenic suppressors mapped to genes involved in PL biosynthesis. These include single amino acid exchanges in the *pgsA* gene, whose product catalyzes the condensation of CDP-DAG with G3P to form the intermediate PG phosphate (PGP), which is then dephosphorylated by Pgp phosphatases to produce PG (Figure 1). Another set of suppressors carried a mutation mapping to the essential *cdsA* gene, which encodes CDP-DAG synthetase that catalyzes the synthesis of CDP-DAG [29]. Mutations in *pgsA* and *cdsA* genes reduced PL levels, which are otherwise elevated in Δ(*lapD clsA*) bacteria, and restored the fatty acid composition. Identification and characterization of multicopy suppressors revealed that overexpression of genes (*accC* and *glnB*), whose products inhibit fatty acid biosynthesis at the level of the ACC enzyme, or the *tesD* gene encoding a putative thioesterase, which causes a decrease in the overall PL content, overcomes the lethality of Δ(*lapD clsA*) bacteria. We further showed that PgsA co-purifies with LapB/D complex and that Δ(*lapD clsA*) bacteria exhibit gross alterations in cellular morphology, which are suppressed by loss-of-function mutations in the *pgsA* gene. These genetic and biochemical studies revealed that the lethality of Δ(*lapD clsA*) is due to the toxic accumulation of PL moieties and that LapD acts as a nodal point in the homeostatic control of FA and LPS biosynthesis.

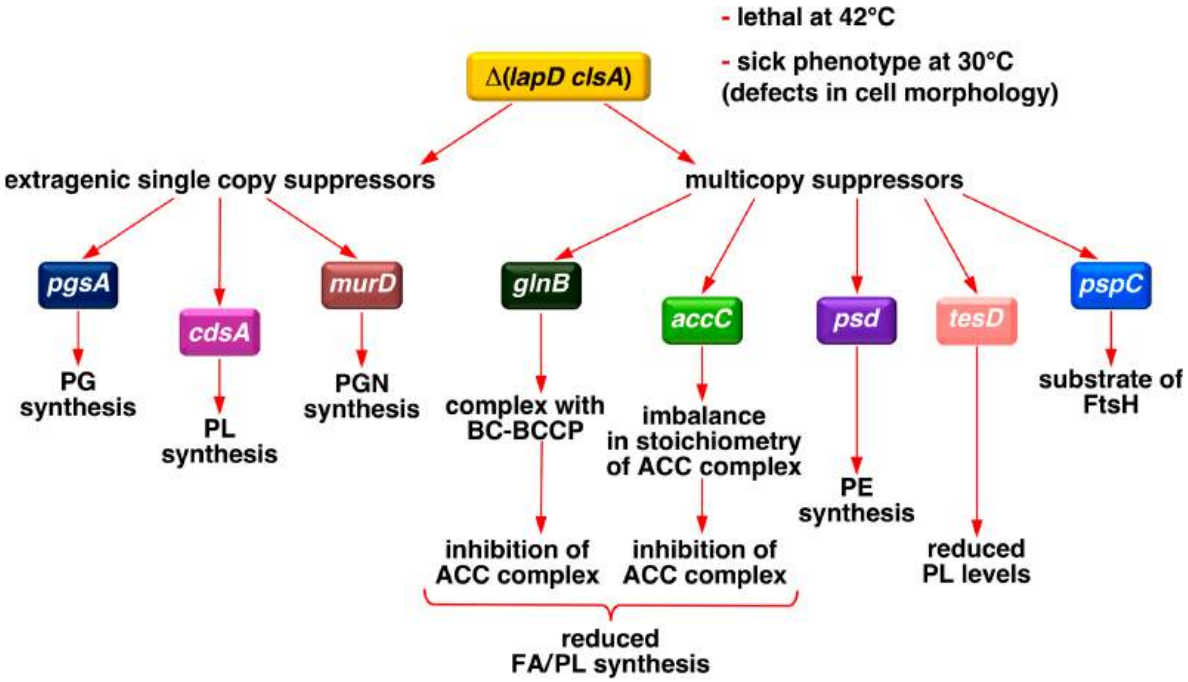

Figure 2. Schematic drawing of the approaches used to address the molecular basis of the lethality of Δ(*lapD clsA*) bacteria. Extragenic single-copy suppressors that restore growth at elevated temperatures were mapped to either PL biosynthetic (*pgsA* and *cdsA*) genes or those involved in peptidoglycan (PGN) formation (*murD*). Identification of multicopy suppressors revealed that either the inhibition of FA/PL biosynthesis (*glnB* and *accC*), reduction in PL amounts (*tesD*), or titration of the FtsH protease (*pspC*) can overcome this lethality. GlnB forms a complex with biotin carboxylase/biotin carboxyl carrier protein (BC-BCCP), which inhibits the acetyl-CoA carboxylase (ACC) enzyme.

2. Results

2.1. Suppressor-Free Δ(lapD clsA) Bacteria Exhibit Conditional Synthetic Lethality

The function of LapD is not well understood but has been implicated in cell envelope-related functions, including a role in maintaining a balance between LPS and PLs. Thus, we undertook several experiments to address the role of LapD when PL biosynthesis is impaired. One of the non-essential PL components is CL, but its cellular requirement

is not well understood. The major enzyme required for cardiolipin formation under exponential growth conditions is ClsA [30,31]. Individually, *lapD* and *clsA* genes are dispensable for bacterial viability under laboratory growth conditions. However, as shown earlier, the *clsA* gene becomes essential when LPS is underacylated and exhibits a sick phenotype in the absence of LapD [13]. We reconstructed Δ(*lapD clsA*) strains in the W3110 background, which has been extensively used for LPS and PL analysis, because some other background strains, such as BW25113, carry a non-functional *fabR* gene [32]. Thus, reciprocal bacteriophage P1-mediated transductions were carried out in the wild type and its isogenic derivatives lacking either *lapD* or *clsA* genes with the vector alone or with a covering plasmid. Parallel transductants were plated on LA plates at 30, 37, and 42 °C. The results from these experiments revealed that Δ*lapD* and Δ*clsA* can be introduced into the wild type at the same frequency at the three temperatures. However, Δ(*lapD clsA*) or reciprocal Δ(*clsA lapD*) viable transductants could be obtained at 30 °C with or without covering the plasmid, although the transductants were obtained at a lower frequency (Table 1). These transductants exhibited a small colony morphology. At 42 °C, no viable Δ(*lapD clsA*) and Δ(*clsA lapD*) transductants could be obtained without covering the plasmid (Table 1). At 37 °C, transductants could be obtained but at a highly reduced frequency. These results allow us to conclude that the Δ(*lapD clsA*) combination is synthetically lethal at 42 °C and, although viable at 30 °C, exhibits a sick phenotype.

Table 1. Δ(*lapD clsA*) bacteria exhibit synthetic lethality at 42 °C.

Genotype of Recipient	Number of Transductants					
	P1 Δ*clsA*			P1 Δ*lapD*		
	30 °C	**37 °C**	**42 °C**	**30 °C**	**37 °C**	**42 °C**
wt	1290	1363	1180	1406	1512	955
Δ*lapD*	633	455 [a]	6	ND	ND	ND
Δ*lapD* + p*clsA*$^+$	1234	1340	1185	ND	ND	ND
Δ*clsA*	ND	ND	ND	430	84 [a]	3
Δ*clsA* + p*lapD*$^+$	ND	ND	ND	1293	1365	1196

[a] small colonies, ND denotes not determined.

2.2. Suppressors That Allow Δ(lapD clsA) Bacterial Growth at Elevated Temperatures Map to Phospholipid Biosynthetic Genes pgsA, cdsA, and Those Involved in Peptidoglycan Biosynthesis

To address the molecular basis of the conditional synthetic lethality of Δ(*lapD clsA*) bacteria, extragenic suppressors that allow growth at 42 °C were sought by plating cultures at elevated temperatures. Temperature-resistant (Ts$^+$) survivors were obtained at a frequency of approximately 10^{-8}. Ts$^+$ independently obtained suppressors were marked with the Tn*10* transposon, as described previously [14]. Linked Ts$^+$ mutations were verified by retransducing the suppressor mutation into the parental Δ(*lapD clsA*) strain SR25209. Fourteen independent suppressors with transposon linked >90% were retained after verification. To map the suppressor mutations, Tn*10* insertions were recombined onto cosmids. The identity of the suppressor mutation was determined by sequencing the PCR products of the complementing cosmids. These mutations could be grouped into three complementation groups, with 10 out of 14 linked to the *uvrC* gene. The second complementation group of three strains with the suppressor mutation was linked to the *skp* gene. The third complementation group had Tn*10* mapping to the *mraZ* gene, located next to the gene cluster carrying genes whose products are involved in cell division and peptidoglycan biosynthesis. DNA sequencing of the region next to *uvrC*, *skp*, and *mraZ* genes revealed single amino acid exchanges in the essential *pgsA*, *cdsA*, and *murD* genes, respectively. These results further showed that five independently isolated suppressors had the suppressor mutation in the *pgsA* gene encoding phosphatidylglycerophosphate synthase, causing alteration of aa T60.

Four out of five had a single amino acid exchange of T60A (ACC to GCC) in the *pgsA* gene (Table 2). Another suppressor-carrying strain had a single amino acid alteration, T60P, in the coding region of the *pgsA* gene. Significantly, the T60A mutation results in the alteration of aa 60, which is the same residue with a substitution to be the first reported and very well characterized *pgsA3* mutation [33–35]. However, the original *pgsA* has an exchange of T60P, as opposed to our T60A substitution, which was found in four out of five independently obtained Ts^+ suppressors. Significantly, it is encouraging that we also obtained one suppressor mutation that is identical to the original *pgsA3* mutation. The *pgsA3* mutation in the *pgsA* gene lacks the potential to synthesize phosphatidylglycerolphosphate, a precursor of PG [36]. The original *pgsA3* was identified as a Ts^+ suppressor mutation of the (*pssA-1 clsA*) strain [36]. Similarly to the *pgsA3* mutation, *pgsA* T60A was found to be recessive, as it could be complemented by a cosmid clone carrying the *pgsA* gene. Further DNA sequence analysis revealed that three strains carried a single amino acid substitution of T7A (ACG to GCG) in the coding region of the *pgsA* gene, and the two remaining strains carried a single amino acid substitution of I99N at aa position 99 due to ATC to AAC nucleotide alteration (Table 2). All three amino acid residues, T60, T7, and I99, are highly conserved in PgsA orthologs, and substitution of T60 has been shown to cause a loss of enzymatic activity (Figure 3).

Table 2. Mapping of extragenic chromosomal suppressors of Δ(*lapD clsA*) bacteria.

Single-Copy Chromosomal Suppressors of Δ(*lapD clsA*)			
Gene	**Function**	**Mutation Position**	**Number of Isolates**
pgsA	phosphatidylglycerophosphate synthase	T60A (ACC → GCC)	4
		T60P (ACC → CCC)	1
		T7A (ACG → GCG)	3
		I99N (ATC → AAC)	2
cdsA	CDP-diglyceride synthetase	D249G (GAC → GGC)	3
murD	UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase	M407V (ATG → GCG)	1

The complementation group linked to the *skp* gene, comprising three Ts^+ suppressor-bearing strains, had a single amino acid substitution at aa position 249 due to GAC to GCC alteration in the coding region of the *cdsA* gene, resulting in the D249G substitution. The *cdsA* gene encodes CDP-diglyceride synthetase, which catalyzes the synthesis of CDP-diacylglycerol (CDP-DAG) using CTP and phosphatidic acid (PA) as substrates [29,37]. As CDP-DAG serves as a precursor of all PLs in *E. coli*, we expect that the suppressor mutation could act by reducing the overall PL amounts. DNA sequencing analysis of the adjoining regions of the Tn*10* mutation revealed that the last Ts suppressor of Δ(*lapD clsA*) carries a suppressor mutation in the essential *murD* gene due to the exchange of M407V (ATG to GTG). The *murD* gene encodes UDP-*N*-acetylmuramoyl-L-alanine:D-glutamate ligase, which catalyzes the addition of the second amino acid to the peptide moiety of the monomer unit of peptidoglycan [38]. The amino acid residue 407 is located in the conserved C-terminal domain, which could be important for the stability and specificity of the MurD enzyme [38].

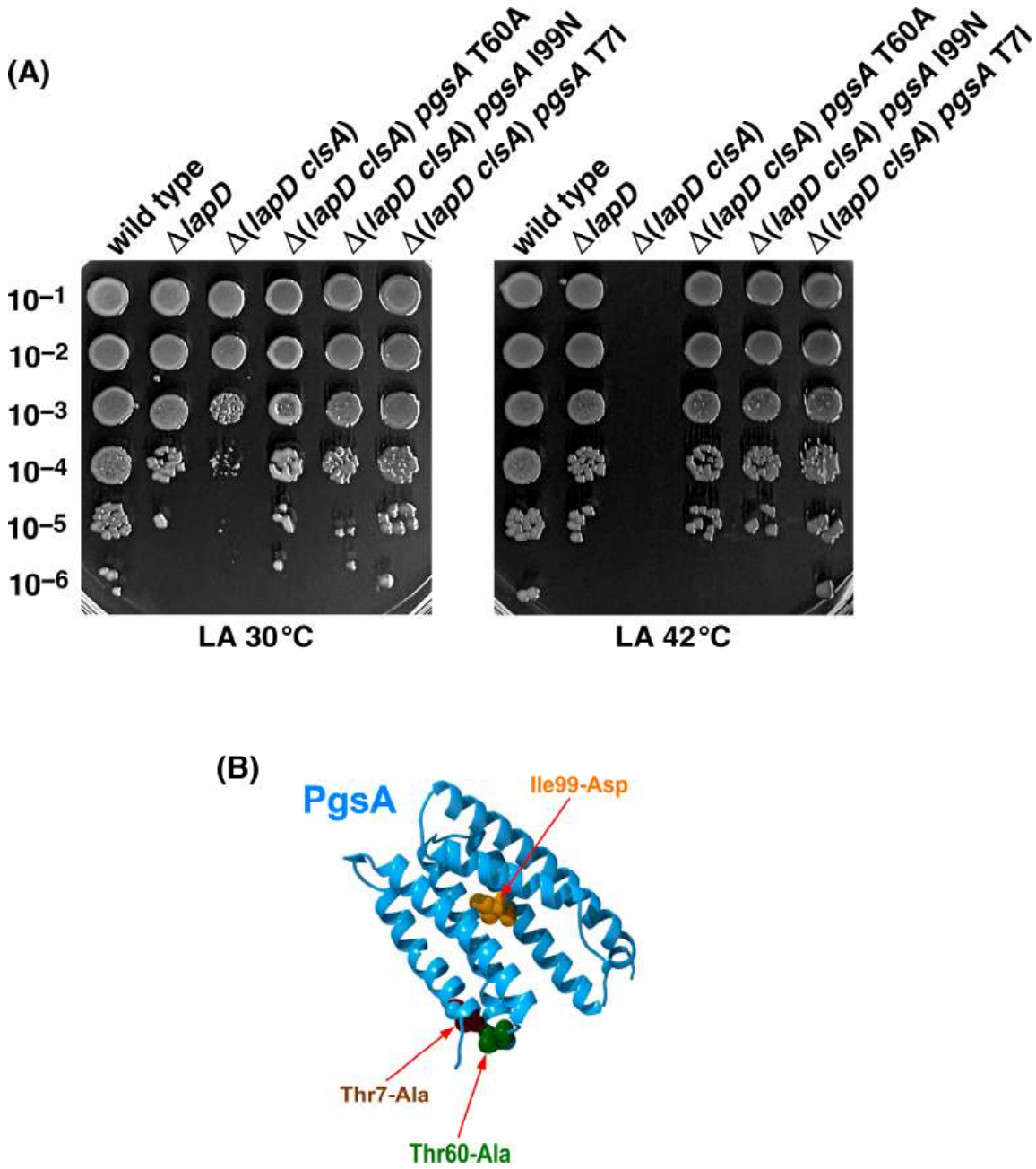

Figure 3. Mutations mapping to the *pgsA* gene encoding phosphatidylglycerophosphate synthase overcome the synthetic lethality of Δ(*lapD clsA*) bacteria. (**A**) Exponentially grown cultures of the wild type, Δ*lapD*, and Δ(*lapD clsA*) derivatives with and without suppressor mutations mapping to the *pgsA* gene grown at 30 °C. The cultures were adjusted to an OD_{595} of 0.1 and serially diluted. Bacterial growth was estimated by spot dilution on LA media at 30 and 42 °C. The relevant genotypes and incubation temperatures are indicated. (**B**) Positions of various single amino acid substitutions in the structure of PgsA AlphaFold AF-P0ABF8-F1 are shown.

2.3. Evaluation of Growth Properties of Suppressors That Overcome Synthetic Lethality of Δ(lapD clsA) Bacteria at Elevated Temperatures

As the majority of single-copy suppressor mutations mapped to the *pgsA* gene that overcame the synthetic lethality of Δ(*lapD clsA*) bacteria, we estimated their growth properties using a spot-dilution assay at 30 and 42 °C. The wild type, Δ*lapD*, and isogenic strains with suppressor mutations in the *pgsA* gene showed normal growth at 30 and 42 °C. However, Δ(*lapD clsA*) bacteria exhibited a 10-fold reduction in colony-forming ability, even at 30 °C, and lethality at 42 °C (Figure 3). Thus, the Δ(*lapD clsA*) combination conferred a sick

phenotype at 30 °C, with a severe reduction in the colony size. Furthermore, Δ(*lapD clsA*) bacteria are unable to grow at elevated temperatures and is synthetically lethal at 42 °C. In parallel, we quantified the growth properties of Δ(*lapD clsA*) derivatives with suppressor mutations mapping to *cdsA* and *murD* genes at 30 and 42 °C. The spot-dilution assay again revealed the sick phenotype of Δ(*lapD clsA*) bacteria at 30 °C and the lethal phenotype at 42 °C. Importantly, suppressor mutations in either *cdsA* or *murD* genes restored nearly wild-type-like growth at 42 °C, validating their isolation (Figure 4).

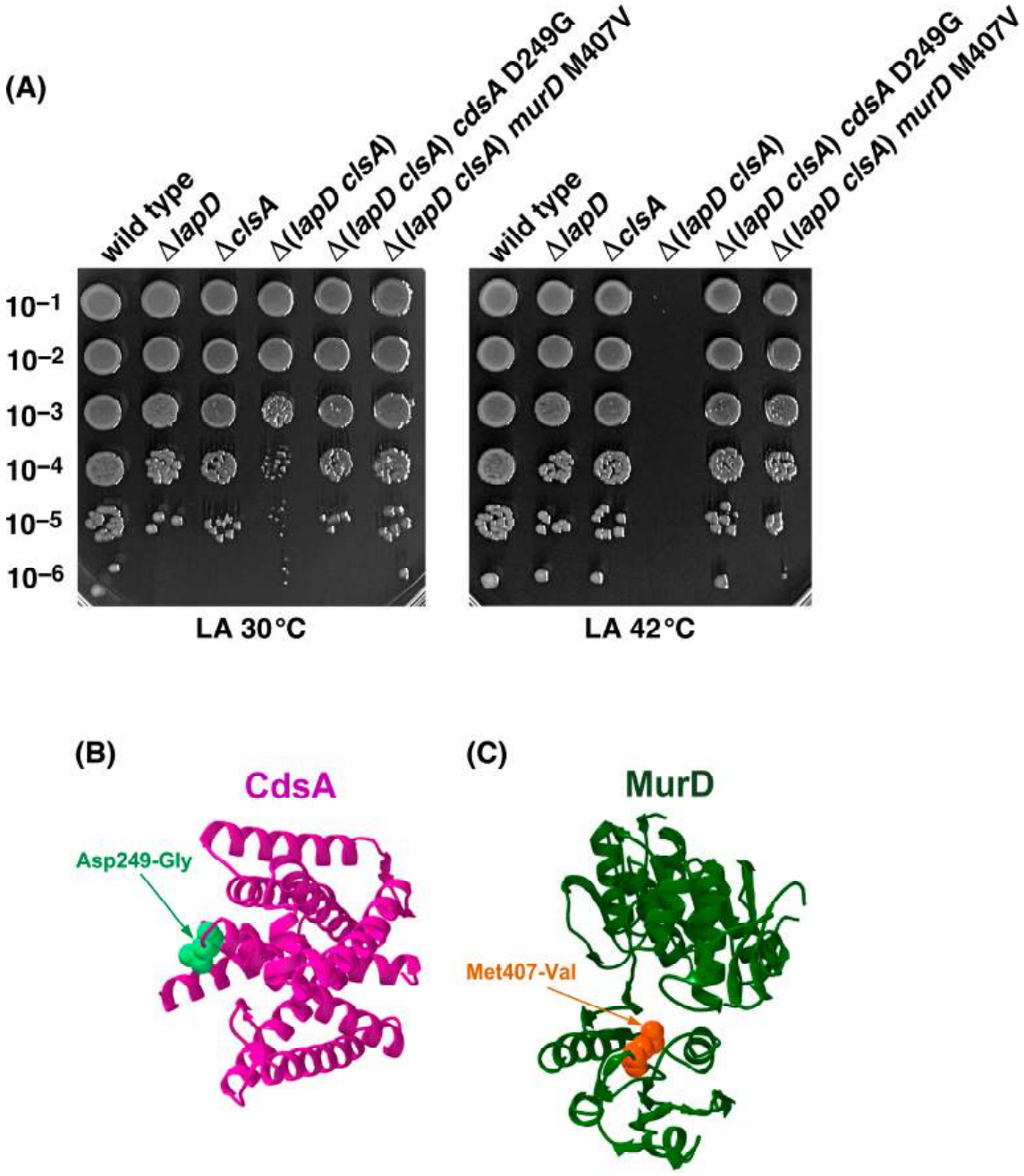

Figure 4. Mutations mapping to *cdsA* and *murD* genes overcome the synthetic lethality of Δ(*lapD clsA*) bacteria at 42 °C. (**A**) Growth of isogenic cultures of the wild type, Δ*lapD*, Δ*clsA*, and Δ(*lapD clsA*) derivatives with and without suppressor mutations mapping to various *cdsA* and *murD* genes was quantified by spot dilution on LA media at 30 and 42 °C. The relevant genotypes and incubation temperatures are indicated. (**B**) Positions of various single amino acid substitutions in the structure of CdsA AlphaFold AF-P0ABG1-F1, (**C**) MurD PDB 1E0D are shown.

2.4. Restoration of Phosphatidylglycerol Levels in Δ(*lapD clsA*) Bacteria by Suppressor Mutations in PgsA

To address the molecular basis of the synthetic lethality of Δ(*lapD clsA*) bacteria, the PL composition of panels of strains with and without suppressor mutations in *pgsA*, *cdsA*, and *murD* genes was analyzed using thin-layer chromatography (TLC). PL were extracted as a control from the strain RU857, which cannot synthesize cardiolipin (CL) and phosphatidylglycerol (PG) species and serves as an internal marker for various PL species. The cultures were labeled with inorganic phosphate, and PL was extracted as described previously [14]. Equivalent amounts of total radiolabeled PL were applied after adjusting for the total protein content. PL species were visualized using phosphorimaging analysis and quantified by densitometry. Densitometric quantification of different PL species revealed that the wild-type strain contained approximately 74.30% ± 3.9 PE, followed by 19.15% ± 0.8 PG and 6.54% ± 0.5 CL (Figure 5, lane 1). These values are consistent with those of the three known PL species in *E. coli* [39]. No major differences in the composition of PL species were observed between the extracts obtained from wild-type and Δ*lapD* bacteria, except for the minor accumulation of lysophosphatidylethanolamine (LPE) in Δ*lapD* and in some of its derivatives (Figure 5, lane 2). However, most significantly, Δ(*lapD clsA*) contained approximately a 4.2-fold increase in PG and a 1.5-fold increase in PE compared to the wild type (Figure 5, lane 3). Interestingly, the isogenic strain Δ(*lapD clsA*) with the *pgsA* T60A suppressor mutation repressed this vast accumulation of PG, reducing it by 5.9-fold compared to the wild-type level (Figure 5, lane 4). Other suppressor mutations, *pgsA* I99N and *pgsA* T7A, also reduced the elevated accumulation of PG amounts (Figure 5, lanes 5 and 6), although not as dramatically as observed with the *pgsA* T60A suppressor mutation. The suppressor mutation *pgsA* I99N reduced PG levels by nearly 1.8-fold compared to those in the parental Δ(*lapD clsA*) strain. The suppressor mutation *cdsA* D249G reduced elevated PE levels by more than 15% compared to that in the wild type (Figure 5, lane 8). However, no major differences were observed with the suppressor mutation in the *murD* gene, which is not expected to directly regulate PL amounts and would likely only regulate PGN. Thus, we can conclude that suppressor mutations in either *pgsA* or *cdsA* genes relieve the lethality of Δ(*lapD clsA*) by reducing the toxic accumulation of elevated PG and PE levels.

2.5. Δ(*lapD clsA*) Bacteria Exhibit Gross Alterations in Fatty Acid Composition, Which Are Suppressed by Mutations in PgsA

It is well established that fatty acid and PL synthesis are intricately linked. In the above sections, we showed that Δ(*lapD clsA*) bacteria have significantly altered amounts of PL species, particularly with hyperelevated amounts of PG. Thus, we further analyzed the fatty acid composition of Δ(*lapD clsA*) bacteria and their isogenic suppressors that overcome their lethal phenotype. Cultures were grown in LB medium, and free fatty acids (FFA) were extracted from an isogenic panel of strains grown at 28 and 37 °C and analyzed using gas chromatography (GC).

GC quantification of FFA extracted from cultures grown at 28 °C revealed that most significantly Δ(*lapD clsA*) had approximately 20% less unsaturated fatty acid (UFA) species 18:1 *cis*-vaccenic acid than the wild type (Table 3). The most robust suppressor mutation in Δ(*lapD clsA*) *pgsA* T60A reversed this composition, leading to an increase in more than 23% in 18:1 *cis*-vaccenic acid content compared to that in the wild type. Interestingly, the suppressor mutations *pgsA* T7A and *cdsA* D249G restored 18:1 levels to nearly wild-type levels (Table 3). Furthermore, the Δ(*lapD clsA*) strain accumulated 3-fold more amounts of saturated fatty acid 18:0 as compared to the parental strain, which was partly reversed by the *pgsA* T60A mutation. Significantly, Δ(*lapD clsA*) *pgsA* T60A bacteria accumulated only 58% myristic acid (14:0). Another major difference is an approximately 30% decrease

compared to the wild type in the amount of 16:1 palmitoleic acid UFA when *pgsA* T60A is present in Δ(*lapD clsA*) bacteria at 28 °C.

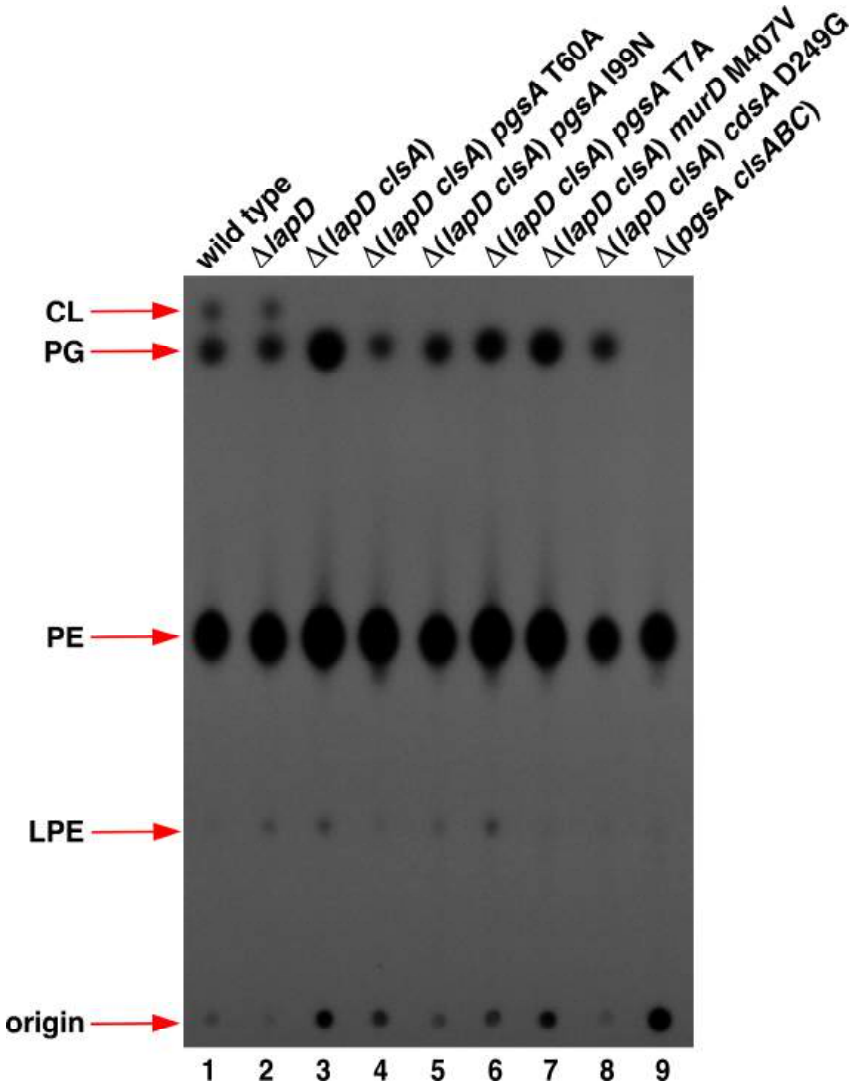

Figure 5. Δ(*lapD clsA*) bacteria have excess PG and PE species of phospholipids and suppressor mutations in *pgsA* and *cdsA* genes, whose products are involved in PL biosynthesis, which overcome their synthetic lethality by decreasing PG and/or PE synthesis. Thin-layer chromatography of ^{32}P-labeled PLs extracted from isogenic strains derived from Δ(*lapD clsA*) bacteria carrying suppressor mutations in *pgsA*, *murD*, and *cdsA* genes. In parallel, ^{32}P-labeled PLs were extracted from the parental wild-type and Δ*lapD* bacteria, with samples from the RU857 strain serving as a marker for the migration of PL species. A representative image from three biological replicates is shown. The arrows indicate the positions of the major PL species.

Analysis of GC data from FFA extracted from bacteria grown at 37 °C further reinforces the results from 28 °C that the severe growth defects of Δ(*lapD clsA*) bacteria are due to gross alterations in the fatty acid composition. The amount of UFA *cis*-vaccenic acid 18:1 species was even more reduced, being approximately 64% of that in the wild type (Table 3). This was reversed by suppressor mutations in *pgsA* and *cdsA* genes. The suppressor mutation *pgsA* T60A caused more than a 32% increase in the 18:1 UFA content compared to the wild type. Notably, Δ(*lapD clsA*) bacteria exhibited an increased presence of 12:0 and 14:0 saturated fatty acids, which were repressed by some of the suppressor mutations, particularly for myristic acid (14:0). Taken together, the results from the GC experiments allow us to conclude that the absence of CL in Δ*lapD* bacteria results in large-scale changes in

the biosynthesis of fatty acids, some of which are reversed by suppressor mutations in *pgsA* and *cdsA* genes. Thus, these results can explain the molecular basis of the synthetic lethality of the Δ(*lapD clsA*) combination and provide a rationale for the isolation of suppressor mutations in the PL biosynthetic pathway. These results are consistent with the known bacterial requirement to maintain a balance between saturated and unsaturated fatty acids, which is critical for membrane fluidity, and in this process, LapD and ClsA functions are critical.

Table 3. Measurement of relative fatty acid composition using gas chromatography. The data are presented for three biological replicates grown at 28 and 37 °C, and the average with S.E is shown.

Fatty Acid Species	wt	Δ*lapD*	Δ*clsA*	Δ(*lapD clsA*)	Δ(*lapD clsA*) *pgsA*T60A	Δ(*lapD clsA*) *pgsA*I99N	Δ(*lapD clsA*) *pgsA*T7A	Δ(*lapDclsA*) *cdsA*D249G
LB 28 °C								
12:0	0.15 ± 0.01	0.33 ± 0.02	0.24 ± 0.01	0.18 ± 0.01	0.16 ± 0.02	0.19 ± 0.01	0.17 ± 0.01	0.241 ± 0.01
14:0	2.61 ± 0.03	2.73 ± 0.03	3.19 ± 0.04	2.51 ± 0.02	1.52 ± 0.01	3.36 ± 0.03	2.36 ± 0.01	2.16 ± 0.02
16:0	34.80 ± 0.07	38.44 ± 0.09	36.81 ± 0.17	38.48 ± 0.12	37.36 ± 0.20	37.43 ± 0.23	38.08 ± 0.29	38.77 ± 0.31
18:0	0.75 ± 0.02	1.16 ± 0.01	0.54 ± 0.01	2.30 ± 0.03	1.34 ± 0.03	2.63 ± 0.04	0.86 ± 0.01	0.91 ± 0.02
16:1	31.89 ± 0.31	31.49 ± 0.26	34.45 ± 0.23	31.68 ± 0.40	23.15 ± 0.021	31.97 ± 0.39	28.52 ± 0.41	28.66 ± 0.24
cyclopropane	1.65 ± 0.01	1.17 ± 0.01	2.32 ± 0.02	1.52 ± 0.01	1.95 ± 0.02	2.63 ± 0.03	2.37 ± 0.02	2.21 ± 0.02
18:1	28.15 ± 1.91	23.80 ± 1.01	22.19 ± 1.93	22.40 ± 1.03	34.53 ± 0.89	23.61 ± 1.19	27.64 ± 0.18	27.04 ± 0.15
LB 37 °C								
12:0	0.18 ± 0.01	0.290 ±0.02	0.28 ± 0.01	0.28 ± 0.01	0.21 ± 0.02	0.28 ± 0.01	0.26 ± 0.02	0.25 ±0.01
14:0	3.55 ± 0.06	3.730 ± 0.04	3.72 ± 0.03	5.30 ± 0.07	1.88 ± 0.02	4.15 ± 0.03	2.84 ± 0.02	2.47 ± 0.03
16:0	41.53 ± 2.21	44.338 ± 1.90	41.67 ± 0.89	45.53 ± 0.15	40.94 ± 1.03	44.24 ± 1.14	43.67 ± 0.09	43.67 ± 0.08
18:0	0.68 ± 0.01	3.412 ± 0.06	0.65 ± 0.01	0.66 ± 0.02	1.51 ± 0.07	0.70 ± 0.02	0.80 ± 0.03	0.83 ± 0.02
16:1	32.20 ± 1.04	30.80 ± 0.09	34.14 ± 1.03	32.59 ± 0.08	27.57 ± 0.05	32.46 ± 0.08	30.06 ± 0.05	28.70 ± 0.06
cyclopropane	2.38 ± 0.02	2.49 ± 0.03	2.80 ± 0.01	2.78 ± 0.02	2.19 ± 0.07	2.43 ± 0.06	2.19 ± 0.04	2.83 ± 0.01
18:1	19.48 ± 0.06	14.94 ± 0.05	16.45 ± 0.09	12.42 ± 0.04	25.72 ± 0.09	15.38 ± 0.08	20.18 ± 1.01	21.25 ± 0.09

2.6. Δ(lapD clsA) Bacteria Have Severe Defects in Cell Morphology, Which Are Suppressed by Mutations in PgsA

Fatty acid composition is known to control the cell length [40]. Increased fatty acid synthesis can lead to abnormal cell expansion, resulting in filamentous cell morphology. Δ*lapD* bacteria also exhibit cell morphology defects but are viable under normal laboratory growth conditions [14]. As Δ(*lapD clsA*) bacteria exhibit a sick phenotype even at 30 °C and have elevated PG levels with alterations in fatty acid composition, the cell morphology of such bacteria was examined in parallel with isogenic parental strains and derivatives with single amino acid substitutions that overcome their lethal phenotype. Exponentially grown bacteria were analyzed using fluorescence microscopy after staining with DAPI. Δ*lapD* bacteria exhibited a high proportion of filamentous cells. However, this phenotype was exacerbated in Δ(*lapD clsA*) bacteria (Figure 6). Most of the cells of Δ(*lapD clsA*) bacteria exhibited abnormally long cellular morphology. Quite significantly, suppressor mutations in the *pgsA* gene suppressed this severe long filamentous phenotype (Figure 6). The more robust suppressor mutation *pgsA* T60A virtually restored wild-type- like cell morphology. Notably, *pgsA* T7A also effectively suppressed the filamentous morphology of Δ(*lapD clsA*) bacteria (Figure 6). These data are consistent with the restoration of PL levels and fatty acid composition by such suppressor mutations. Taken together, the bacterial synthetic lethality of Δ(*lapD clsA*) and the sick phenotypes under permissive growth conditions are due to changes in fatty acid composition concomitant with severe cell morphology defects.

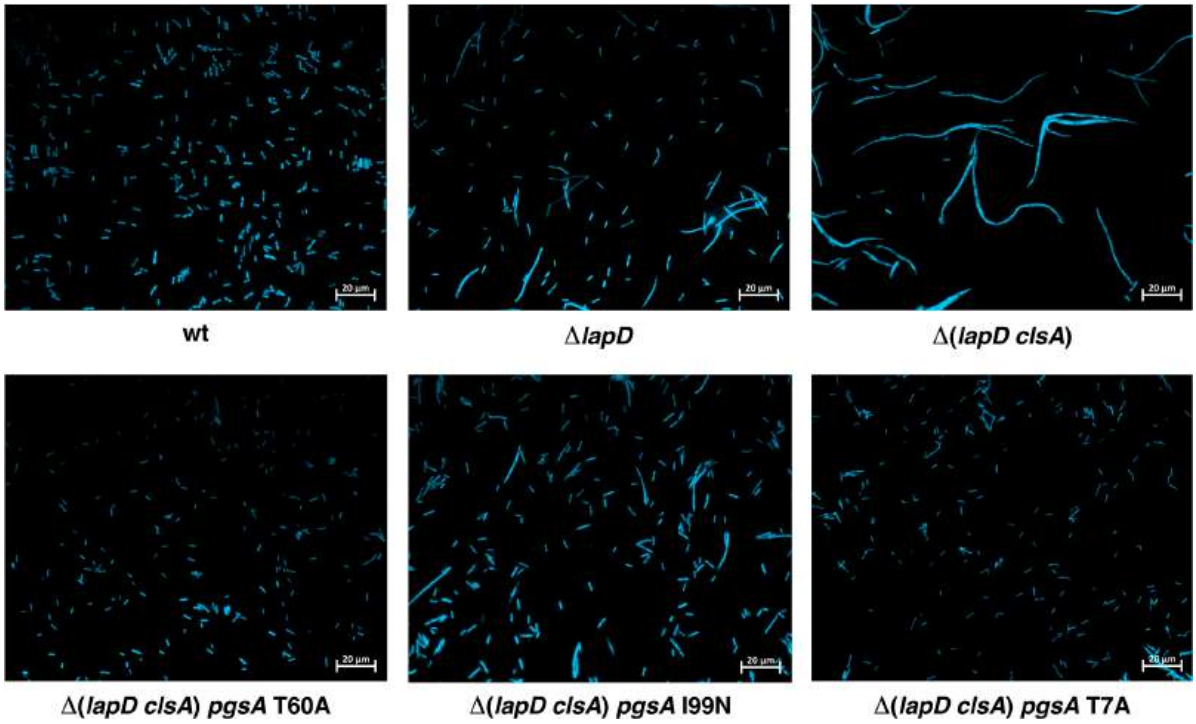

Figure 6. Loss-of-function suppressor mutations in the *pgsA* gene rescues the cell morphology defects of Δ(*lapD clsA*) bacteria. Microscopic images of the wild type, its isogenic Δ*lapD*, Δ(*lapD clsA*), and its derivatives with single-copy suppressor mutations in the *pgsA* gene. Bacterial cultures were grown overnight at 30 °C, diluted 1:100 in fresh LB, and grown until an OD_{600} of 0.6 at 30 °C. Cultures were harvested by centrifugation, and bacterial cells were stained with DAPI and imaged using fluorescence microscopy at ×1000 with a 20 μm scale. The relevant genotypes are indicated.

2.7. Suppressors of Δ(lapD clsA) Bacteria Do Not Alter LpxC Levels

As PL and LPS biosynthesis utilize (*R*)-3-hydroxymyristate-ACP as a common metabolic precursor, it is possible that altered PL and fatty acid synthesis due to suppressor mutations in *pgsA* and *cdsA* might cause disturbances in LPS synthesis at the level of LpxC. LpxC is a key enzyme involved in regulating the balance between the LPS and PL biosynthetic pathways [4,5]. Thus, we examined LpxC levels using Western blotting. Isogenic cultures of wild type, Δ*lapD*, Δ*clsA*, Δ(*lapD clsA*), and its derivatives with suppressor mutations in *pgsA*, *cdsA*, and *murD* genes were grown under permissive growth conditions. As a control, cell lysates were prepared from isogenic Δ*ftsH sfhC21* bacteria that are known to have elevated levels of LpxC [4]. Equivalent amounts of proteins were transferred by Western blotting and immunoblotted with anti-LpxC antibodies. Estimation of LpxC levels revealed that none of the suppressor mutations in the *pgsA* gene caused any significant alteration in its amount (Figure 7, panel A). As expected, *ftsH* mutant bacteria showed increased LpxC levels. No impact of the *clsA* gene deletion was observed. Similarly, no significant changes in LpxC levels of Δ(*lapD clsA*) derivatives with the suppressor mutation in either *cdsA* or *murD* genes were observed (Figure 7, panel B). These results allow us to conclude that the single-copy extragenic suppressor mutation in either *pgsA* or *cdsA* or *murD* genes that overcome the lethal phenotype of the Δ(*lapD clsA*) combination do not operate by changing LpxC levels and are unlikely to impact LPS biosynthesis.

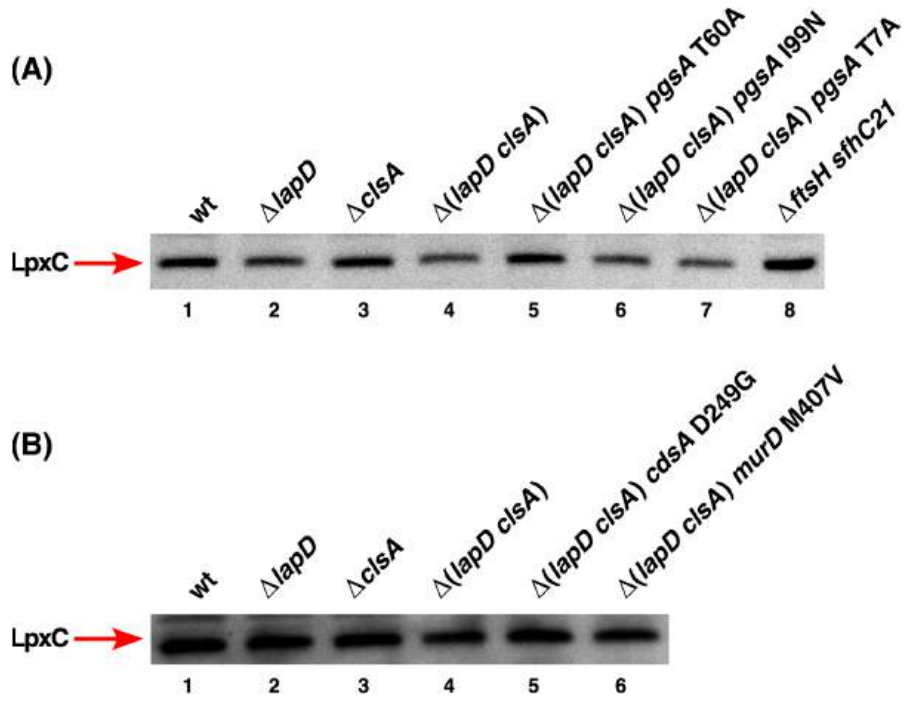

Figure 7. Suppression mutations in *pgsA*, *cdsA*, and *murD* genes do not act by altering LpxC levels. Immunoblots of whole-cell lysates obtained from isogenic strains grown at 30 °C in LB medium. An equivalent amount of total protein was resolved by 12% SDS-PAGE and transferred by Western blotting. Membranes were immunoblotted with LpxC-specific antibodies and revealed by chemiluminescence. Images from a representative experiment with the relevant genotypes are shown in panels (**A**,**B**). As an internal control, a cell lysate from the isogenic Δ*ftsH sfhC21* strain was applied ((**A**), lane 8). The arrow indicates the position of LpxC.

2.8. PgsA Co-Purifies with the Essential LapB Protein

Previously, we showed that the inner membrane essential protein LapD co-purifies with several proteins involved in LPS biosynthesis or its assembly (LapC, LapA, WaaC, and LapD) and the initiation of PL synthesis (FabZ) [5,8,13]. Hence, it was proposed that, in addition to regulating LpxC stability via interaction with FtsH, LapB could act as a scaffold for recruiting various enzymes in PL and LPS to coordinate their synthesis using its conserved tetratricopeptide repeats (TPR) [5]. Thus, we wondered whether any of the proteins examined in this study are also part of the LapB interactome. To achieve this, pull-down experiments were performed using the affinity purification of C-terminally His-tagged LapB, as described previously [5]. His-tagged LapB co-eluted with several proteins (Figure 8). MALDI-TOF analyses of co-purifying proteins revealed that the LapB complex, in addition to including known interacting partners such as LapA, LapC, LapD, and FabZ, also includes PgsA and PlsB (Figure 8). PlsB glycerol-3-phosphate acyltransferase catalyzes the first committed step in phospholipid biosynthesis [23]. Co-purification of PgsA and PlsB suggests that LapB-interacting partners are dedicated to PL and LPS biosynthesis.

2.9. Multicopy Suppressors of Δ(lapD clsA) Identify Factors That Can Inhibit Fatty Acid Biosynthesis or Regulate Cell Envelope Homeostasis

To further understand the physiological limiting factors that cause the lethality of Δ(*lapD clsA*) bacteria, multicopy suppressor analysis was performed. Two genomic plasmid DNA libraries were used. One of these was based on the ordered ASKA genomic library of cloned ORFs, where the expression of individual genes is under the control of an inducible, tightly regulated P_{T5}-*lac* promoter [41]. Transformants of Δ(*lapD clsA*) with this library were selected at 42 °C in the presence of 75 μM IPTG. The second library was generated

using the mini-Mu system [42]. In this case, the first deletion derivatives of *clsA* and *lapD* genes were transduced into the p15A mini-Mu lysogenic strain, and lysates prepared from such strain were used to generate plasmid libraries. Transductants in the Δ(*lapD clsA*) background were plated to isolate Ts^+ colonies. Plasmid DNAs from such Ts^+ strains were isolated, verified by retransformation, and used to identify the minimal coding sequence by DNA sequencing that could restore the growth of Δ(*lapD clsA*) bacteria. The usage of mini-Mu-based libraries was necessitated to prevent recloning of *lapD* and *clsA* genes, which was the case with the ASKA library. These multicopy suppressors identified a set of genes involved in fatty acid/PL synthesis or regulation of cell envelope-related functions (Table 4).

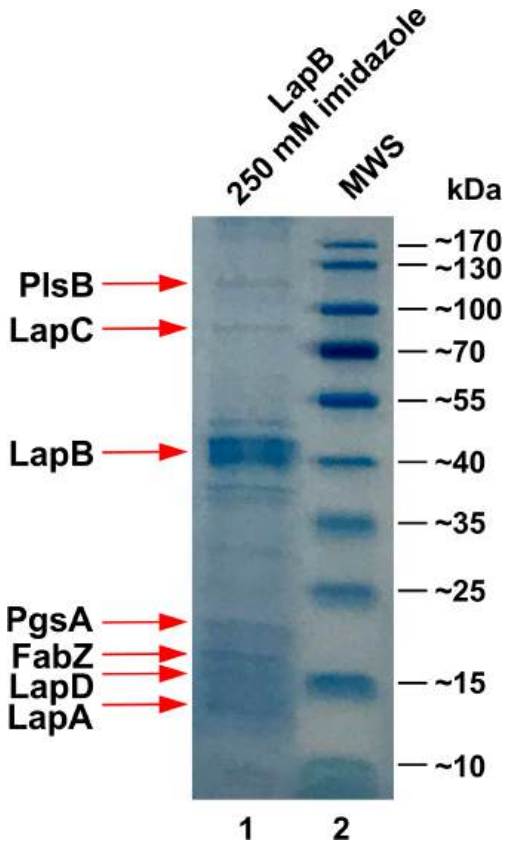

Figure 8. PgsA and PlsB are part of the LapB interactome. Purification profile of co-eluting proteins with His_6-LapB (lane 1). Proteins were eluted using 250 mM imidazole. Proteins were resolved on a 12% SDS-PAGE. The identites of the co-eluting proteins are depicted by arrows.

Table 4. Multicopy suppressors of Δ(*lapD clsA*) bacteria identify genes whose products can repress fatty acid biosynthesis or titrate elevated PG levels.

Gene	Function
accC	biotin carboxylase subunit of the ACC enzyme
glnB	the PII-1 protein-regulator of nitrogen metabolism and fatty acid via its interaction with the biotin carboxylase/biotin carboxyl carrier protein
psd	phosphatidylserine decarboxylase required for the formation of phosphatidylethanolamine
tesD	putative thioesterase with a hot-dog fold
pspC	lipoprotein, positive regulator of the *pspABCDE* operon, substrate of FtsH
metQ	lipoprotein, required for methionine uptake
mliC	lipoprotein, multicopy suppressor of Δ*lapB*, inhibitor of c-type lysozyme

To validate these results, the suppression ability was analyzed using a spot-dilution assay of Δ(*lapD clsA*) derivatives carrying these genes expressed from the P_{T5}-*lac* promoter (Figure 9). These results revealed a variable degree of restoration of bacterial growth at 43 °C in the presence of 75 μM IPTG, although all the analyzed strains performed better than the parental strain with the vector alone. The most robust suppression was observed with the gene encoding putative thioesterase *tesD*, followed by *pspC* and *accC* genes when mildly overexpressed (Figure 9). Noticeable restoration of bacterial growth at 43 °C was also observed when *glnB*, *psd*, and *metQ* genes were overexpressed.

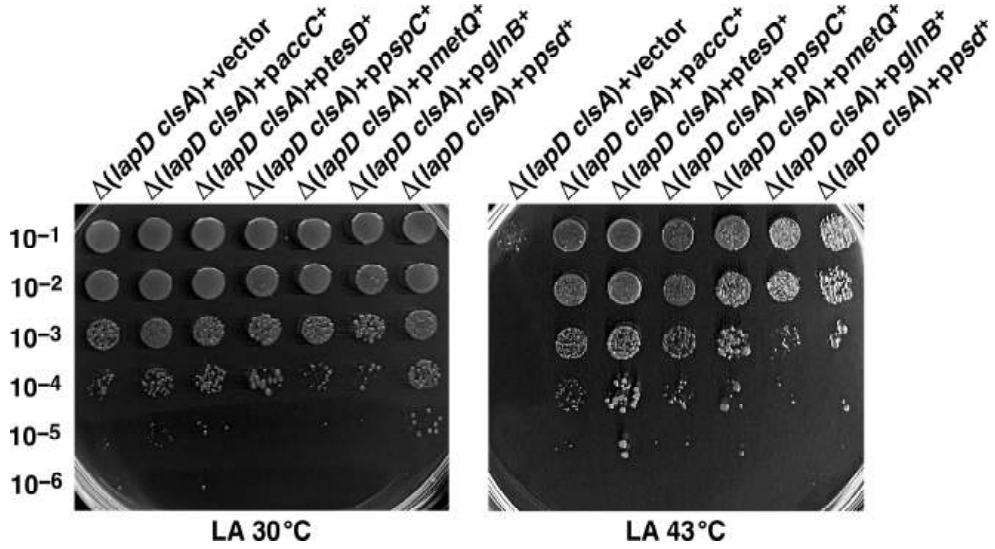

Figure 9. Overexpression of specific multicopy suppressors that can reduce fatty acid biosynthesis (*accC*, *tesD*, and *glnB*) can overcome the synthetic lethality of Δ(*lapD clsA*) bacteria. Growth of isogenic cultures of Δ(*lapD clsA)* with the vector alone or its isogenic derivatives carrying plasmids expressing various multicopy suppressors from the P_{T5}-*lac* promoter was quantified using spot dilution on LA at 30 °C and LA media supplemented with 75 μM IPTG at 43 °C. The plates were incubated for 24 h. Data from a representative experiment with the indicated genotypes are shown.

The products of some genes can inhibit fatty acid biosynthesis or affect PL biosynthesis. The most prominent of these was the repeated cloning of the *accC* gene, which encodes the biotin carboxyl carrier protein, one of the four subunits of the ACC complex [21]. AccC catalyzes the first step of the acetyl-CoA carboxylase reaction mediated by the ACC enzyme. It has been demonstrated that for the enzymatic activity of the ACC complex, all four subunits must be maintained in stoichiometric amounts, and any imbalance can inhibit the ACC enzymatic activity [14]. These results are consistent with the demonstration of a reduction in the amount of PL when one of the subunits of the ACC complex is overproduced or mutated [14]. Another interesting multicopy suppressor identified is the *glnB* gene (Table 4). The *glnB* gene encodes the PII-1 protein, which is involved in the regulation of nitrogen metabolism by controlling the activity of glutamine synthetase [43–45]. However, more importantly, in the context of this study, GlnB also acts as a regulator of fatty acid synthesis via its interaction with the biotin carboxylase/biotin carboxyl carrier protein (BC-BCCP) subcomplex of the ACC enzyme [46]. GlnB interacts with BC-BCCP to form a ternary complex that inhibits the ACC enzyme activity [46]. Another gene whose overexpression rescues the lethality of Δ(*lapD clsA*) is the *psd* gene, encoding phosphatidylserine decarboxylase. Psd catalyzes the formation of the most abundant PL species, phosphatidylethanolamine. The isolation of PspC as a dosage-dependent suppressor can be rationalized, as it has been shown to be a substrate of FtsH protease [47]. MetQ is a lipoprotein and its overproduction could cause diversion of PG pools that can overcome toxic accumulation of PG and thereby overcome lethality. Thus, multicopy suppressor analysis further reinforces our

results that LapD and ClsA absence leads to dysfunctional fatty acid/PL biosynthesis, causing an increase in PG amounts. Currently, the precise function of TesD is unknown; however, its predicted polypeptide has a characteristic hot-dog fold observed in the family of thioesterases and could act by hydrolyzing acyl chains.

2.10. Overexpression of accC and tesD Suppresses Elevated Accumulation of PL Species in Δ(lapD clsA) Bacteria

To address the molecular basis of the restoration of growth of Δ(*lapD clsA*) bacteria by overexpression of various multicopy suppressor-encoding genes, PL analysis was undertaken using TLC. We specifically analyzed those, which are predicted to inhibit fatty acid biosynthesis and alter PL composition. Thus, isogenic cultures of derivatives of Δ(*lapD clsA*) with the vector alone or expressing plasmid-born *accC*, *psd*, and *tesD* were labelled with inorganic phosphate in the presence of 75 μM IPTG to induce gene expression. Samples from such cultures were used to extract PLs and analyzed for their composition using TLC. As is evident, Δ(*lapD clsA*) had more than 33% elevation of PG amounts compared to the parental wild type, which was significantly suppressed by overexpression of either *tesD* or *accC* gene (Figure 10, lanes 3 vs. 4 and lanes 3 vs. 5). Densitometric quantification of PL species when the *tesD* gene was overexpressed showed PG to be approximately 47% and PE nearly 63% compared to the parental wild type, which was taken as 100%. An even more severe reduction in PG and PE amounts was observed when the *accC* gene was overexpressed. Thus, the PG amounts were approximately 24% and PE only 46% compared to the parental wild type upon *accC* overexpression. As mentioned above, overproduction of any of the subunits of the ACC complex can disturb the balance in the stoichiometry of various components, leading to the inhibition of ACC enzymatic activity that catalyzes the first committed step in the initiation of fatty acid synthesis. If TesD is proven to be a thioesterase, it could impact by releasing acyl chains by hydrolysis that can reduce PL synthesis. No significant alterations were observed with overexpression of the *psd* gene, consistent with the observation of homeostatic control of fatty acid biosynthetic enzymes and the lack of changes in PL composition due to the amplification of Psd enzymes [48].

2.11. Overexpression of pspC Elevates LpxC Levels

One of the multicopy suppressors that restores the growth of Δ(*lapD clsA*) at high temperatures is the *pspC* gene. PspC is a positive regulator of the *psp* operon and has been suggested to be a substrate of FtsH protease [47]. The FtsH-LapB complex catalyzes the proteolysis of LpxC. Thus, we rationalized that excess of PspC could titrate FtsH, which could potentially stabilize LpxC. To test this hypothesis, we examined LpxC levels using immunoblotting. Total cell lysates were prepared from isogenic wild type, Δ(*lapD clsA*), and its derivative expressing the *pspC* gene under the control of the inducible P_{T5}-*lac* promoter and analyzed by Western blotting. Estimation of LpxC levels revealed that overexpression of the *pspC* gene led to an increase in LpxC amounts compared to those observed in the parental wild type and its Δ(*lapD clsA*) derivative (Figure 11). These results provide a rationale explanation for the isolation of *pspC* gene as a multicopy suppressor of the Ts phenotype of Δ(*lapD clsA*) bacteria by stabilization of LpxC.

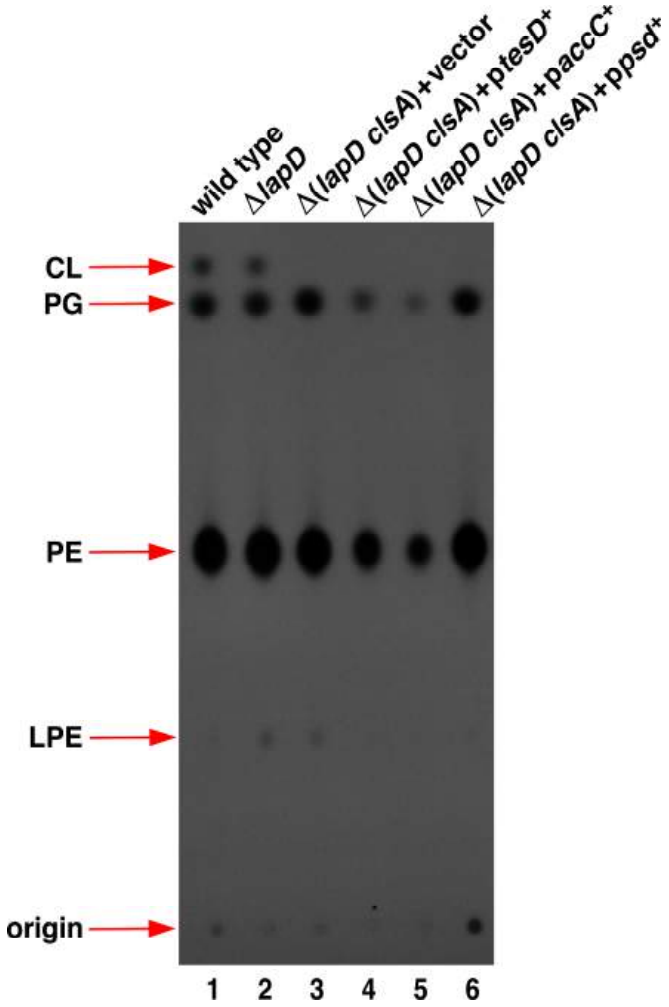

Figure 10. Overexpression of either *tesD* or *accC* genes in Δ(*lapD clsA*) can overcome their synthetic lethality by decreasing phospholipid synthesis. Thin-layer chromatography of ^{32}P-labeled PLs extracted obtained from isogenic wild type, Δ*lapD*, Δ(*lapD clsA*) with empty vector and its derivatives carrying plasmids expressing either t*esD* or *accC* or *psd* genes from P_{T5}-*lac* promoter. A representative image from three biological replicates is shown. The arrows indicate the positions of major PL species.

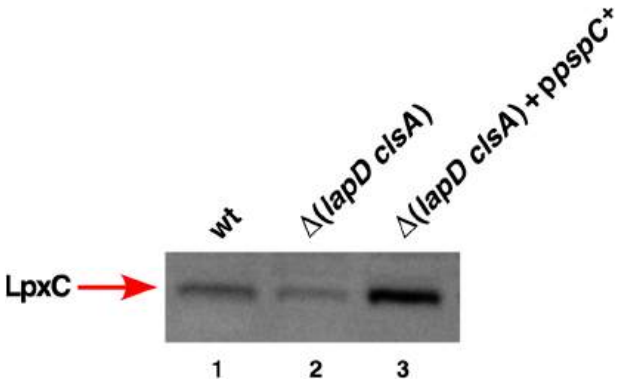

Figure 11. Overexpression of the *pspC* gene in Δ(*lapD clsA*) stabilizes LpxC. Immunoblot of whole cell lysates obtained from isogenic wild type, Δ(*lapD clsA*) with empty vector, and its derivative carrying plasmid expressing the *pspC g*ene from P_{T5}-*lac* promoter. Cultures were grown at 30 °C and shifted to 42 °C until an OD_{595} of 0.6. An equivalent amount of total protein was resolved on a 12% SDS-PAGE and transferred by Western blotting. Membranes were immunoblotted with LpxC-specific antibodies and revealed by chemiluminescence. Images from a representative experiment with the relevant genotypes are shown.

3. Discussion

The outer membrane of Gram-negative bacteria is primarily composed of lipopolysaccharides and phospholipids. In *E. coli*, FASII generates two products, acyl-ACP and β-hydroxyacyl-ACP, which are components of LPS and PLs biosynthesis. Acyl-ACPs are used by PlsB and PlsC enzymes to generate phosphatidic acid, which is a precursor of all cytoplasmic membrane phospho- and glycolipids [3]. β-Hydroxyacyl-ACP molecules serve as substrates for the acyltransferases catalyzing the initial steps in the biosynthesis of the essential conserved lipid A component of LPS. We previously showed that LapD is one of the factors that links the LPS and PL biosynthetic pathways, as it co-purifies with various components of PL and LPS biosynthesis [13]. Genetic and biochemical studies have shown that although LapD is non-essential for bacterial growth, it becomes indispensable when the lipid A is underacylated (absence of LpxM myristoyl acyltransferase) or when LPS is composed of only Kdo_2-lipid A due to the lack of heptosyltransferase WaaC [13,14]. We showed that suppressors that bypass the lethality of Δ(*lapD lpxM*) and Δ(*lapD waaC*) map to either various subunits of the essential enzyme acetyl-CoA carboxylase transferase or components of LPS transport and peptidoglycan biosynthesis [14]. Here, we show that LapD also becomes conditionally essential in the absence of the major cardiolipin biosynthesis pathway. CLs are the only PLs that are non-essential for bacterial viability. Thus, it is intriguing that cardiolipins are required for bacterial viability in the absence of LapD. However, CL synthesis has been shown to be required for viability of bacteria lacking LpxM [18,19]. Although the Δ(*lapD clsA*) mutational combination has been shown to confer lethality, its molecular basis has not been addressed [13,49]. In this study, we showed that Δ(*lapD clsA*) bacteria exhibited severe morphological defects. We used this conditional synthetic lethal phenotype to isolate extragenic chromosomal and multicopy suppressors to investigate its molecular basis.

Characterization of these suppressors showed that the majority of single-copy suppressor mutations mapped to the *pgsA* gene, encoding phosphatidylglycerophosphate synthase, whose product is required for PG synthesis (Figure 1). PG synthesis occurs via the condensation of CDP-DAG with G3P mediated by PgsA to form the intermediate PG phosphate, which is then dephosphorylated by Pgp phosphatase to form PG [50]. Another set of suppressor mutations was mapped to the *cdsA* gene encoding CDP-diglyceride synthetase. CDP-DAG is the central precursor of all PL in *E. coli*. Interestingly, the most frequent single amino acid exchange observed in the Ts^+ suppressors of Δ(*lapD clsA*) was in the amino acid residue Thr 60 in PgsA. Alteration of this highly conserved amino acid residue impairs PgsA activity [36,51]. To our knowledge, this is the first study to identify suppressor mutations in the *pgsA* gene in the absence of LapD. Analysis of the PL content of Δ(*lapD clsA*) bacteria revealed elevated levels of PG accumulation, which can contribute to the toxicity and severe defects in cell division observed in these bacteria. Importantly, loss-of-function *pgsA* suppressor mutations restored the wild-type-like cell morphology and significantly reduced PG abundance. Notably, suppressor mutations in the *pgsA* gene were shown to repress highly elevated PL levels, particularly in the presence of the PgsA T60A amino acid exchange. Thus, Δ*lapD* bacteria are sensitized due to an imbalance in either PL composition, or underacylation of lipid A, or when LPS is composed of only Kdo_2-lipid A. While extragenic suppressors of Δ(*lpxM clsA*) were mapped to the *msbA* gene encoding LPS flippase [16], suppressors of Δ(*lapD clsA*) were mapped to PL biosynthetic genes, implying that CLs have a dual function. Thus, CLs can assist MsbA in LPS translocation and cooperate with LapD to maintain a balanced fatty acid composition.

Acidic phospholipids, including CL, localize to cell poles and interact with key players such as RodZ [52,53]. The absence of CL in Δ*lapD* could be one of the reasons for the severe cell morphology defects in Δ(*lapD clsA*) bacteria. Consistent with this notion, *rodZ*

has been shown to be essential in the absence of LapD, although no explanation for this lethality has been provided [54]. Furthermore, it is well established that *E. coli* size is dependent on fatty acid/PL levels [40,55,56]. The suppression of cell morphology defects of Δ(*lapD clsA*) bacteria by loss-of-function mutations in the *pgsA* gene is consistent with the prominent role of anionic PLs in cell morphology. Anionic lipids have been shown to be required for the assembly of the Z-ring and for DNA replication [57–59]. In this study, we observed an abnormally high accumulation of PG in Δ(*lapD clsA*) bacteria, which was relieved by *pgsA* suppressor mutations. Consistent with these results, we previously showed restoration of normal cell morphology when fatty acid biosynthesis was repressed by overexpression of either the *tesA'* thioesterase gene, induction of *acpP*, or inhibition of ACC complex activity [14].

Interestingly, gross alterations in Δ(*lapD clsA*) bacteria were observed in FFA composition with an increased presence of saturated vs. unsaturated fatty acids, which was particularly reflected by a 3-fold increase in 18:0 and a 20–40% decrease in the presence of *cis*-vaccenic acid (18:1). Increased accumulation of *cis*-vaccenic unsaturated fatty acid can lead to a decrease in LPS biosynthesis, which can reset the balance between LPS and PL amounts, as observed in Δ(*lapD lpxM*) bacteria [14]. Significantly, *cis*-vaccenic acid amounts were restored in the presence of suppressor mutations in either *cdsA* or *pgsA* genes. Overall, these results imply that the Δ(*lapD clsA*) lethal phenotype stems from alterations in the fatty acid and PL composition. Another interesting suppressor mutation was mapped to the essential *murD* gene, which connects PGN synthesis and LapD function, consistent with our earlier findings [14].

In support of the results revealing excess PG and changes in fatty acid composition, the multicopy suppressor approach revealed that reducing fatty acid synthesis by inhibiting acetyl-CoA carboxylase activity, which catalyzes the rate-limiting step in the initiation of fatty acid synthesis, can overcome the lethality at elevated temperatures. This approach revealed that overexpression of either *accC*, encoding the subunit of the ACC enzyme, or *glnB* effectively restored the growth of Δ(*lapD clsA*) bacteria. The ACC complex has four subunits, and all components are held in stoichiometric amounts [60]. Overexpression of any of the subunits reduces PL and fatty acid content by decreasing the ACC enzyme activity [14]. We showed a significant decrease in PG molecules, which are otherwise elevated in Δ(*lapD clsA*) bacteria when the AccC subunit is overproduced, providing a genetic and biochemical link to the reasons for the lethality and mode of growth restoration of Δ(*lapD clsA*) bacteria. Another multicopy suppressor is encoded by the *glnB* gene, whose product belongs to the PII signal transduction family and plays a key role in nitrogen metabolism [43–45]. However, GlnB proteins from Proteobacteria have also been shown to interact with the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (ACC) [46,61]. In *E. coli*, this GlnB interaction reduces the k_{cat} of acetyl-CoA carboxylation [46]. This overproduction of GlnB should result in the reduced ACC enzyme activity, which can provide a rational explanation for its role as a dosage-dependent suppressor. The isolation of *metQ* as a multicopy suppressor is interesting because the encoded protein is a predicted lipoprotein that is part of the methionine transport system. Prosite annotation suggests lipidation at amino acid position 23, which could be a PG moiety. MetQ is included in the TIGRFAM lipoprotein TIGR00363/NlpI family (http://ca.expasy.org/prosite (accessed on 9 October 2025)). PspC, whose overproduction also suppresses the Ts phenotype, acts as a positive regulator of the *psp* operon [62]. We speculate that overproduction of MetQ could titrate out PG molecules, which are present in toxic amounts in Δ(*lapD clsA*) bacteria. However, MetQ is not well characterized, and further studies are required to validate its lipidation and its impact on PL pools. However, it has parallels, as the absence of the most abundant lipoprotein, Lpp, allows the growth of *pgsA* mutant bacteria [63]. The rationale for Δ*lpp* to

relive the lethality of *pgsA* mutants is based on the known PG attachment to this highly abundant lipoprotein, which can lead to the availability of PG molecules that are very limiting in *pgsA* mutant bacteria [64]. The isolation of *pspC* as a multicopy suppressor is interesting and can be explained by the observed stabilization of LpxC upon its overexpression in Δ(*lapD clsA*) bacteria. This is consistent with PspC being a substrate of FtsH [47]. We explain these results because titration of FtsH by excess of PspC can lead to the stabilization of LpxC. This can reset the LPS vs. PL balance, which is disturbed in Δ(*lapD clsA*) bacteria. These results are further supported by our previous findings showing that LpxC stabilization can overcome the growth defects of Δ*lapD* bacteria [13]. Thus, increasing either the stability of LpxC or repressing PL synthesis can overcome the lethality of Δ(*lapD clsA*) bacteria, positioning LapD as the link between LPS and FA synthesis. Another possibility of PspC-mediated suppression is the association of CL with Psp proteins in polar membrane regions, which can affect interactions with the RodZ/MreB cytoskeleton complex and Tat-dependent protein translocation [65]. It is possible that PspC is limiting in Δ(*lapD clsA*) bacteria, which can impact the extracytoplasmic stress response, which is elevated in the absence of LapD [13,14].

Similarly, we can explain the isolation of putative thioesterase TesD, as its overproduction reduced PL synthesis and restored growth at elevated temperatures. We have not yet biochemically established the thioesterase activity of TesD, and this awaits confirmation. However, TesD has a characteristic hot-dog fold, which is present in the thioesterase superfamily [66]. Thioesterases typically catalyze the cleavage of thioester bonds in a wide range of activated fatty acyl coenzyme A (CoA) substrates, acyl carrier proteins (ACPs), and other cellular molecules [66]. Importantly, it has been shown that thioesterase overproduction can alter the degree of saturation of the membrane lipids in *E. coli* [67]. It has been shown that the biosynthetic coupling between fatty acid and phospholipid syntheses could be disrupted by the high-level expression of thioesterases [68]. The isolation of *tesD* is also supported by our earlier results with overexpression in the cytoplasm of *tesA'* lacking its signal sequence, which effectively suppressed PL accumulation in Δ*lapD* derivatives [14]. Isolation of *psd* as a multicopy suppressor is also logical, as its product encodes phosphatidylserine decarboxylase catalyzing the synthesis of PE.

Thus, based on our results of the synthetic lethality of the concomitant absence of LapD and CL, we can conclude that they together constitute essential components required to maintain the homeostasis of essential cell envelope components. In such bacteria, several key components, particularly the composition of PLs, fatty acids, and cellular morphology, are severely affected. Thus, Δ(*lapD clsA*) bacteria accumulated toxic amounts of PG species of PLs, causing gross alterations in the fatty acid composition, which resulted in defects in cellular morphology. Our results show that lethality can be overcome by reducing PG synthesis, as shown by the preponderance of suppressor mutations that relieve this lethality mapping to the *pgsA* gene. Furthermore, Δ(*lapD clsA*) bacteria accumulated less unsaturated fatty acids, particularly *cis*-vaccenic acid, with a significant increase in saturated fatty acids. This imbalance can cause alterations in the regulation of membrane fluidity and hence, Δ(*lapD clsA*) bacteria cannot withstand temperatures above 37 °C, which contributes to the synthetic lethal phenotype. Suppressor mutations in the *pgsA* gene, which are recessive, not only reduced the hyperelevated toxic accumulation of PG but also corrected cellular morphological defects. These findings were supported by the isolation of multicopy suppressors, whose overexpression can inhibit the initiation of fatty acid at the level of the acetyl coenzyme A carboxylase enzyme. Notably, we identified a new putative thioesterase, TesD, whose overproduction effectively prevents the synthetic lethality of Δ(*lapD clsA*) bacteria and significantly reduces the accumulation of elevated PL species. Currently, efforts are underway to establish the thioesterase activity of TesD and

its specificity for the acyl chain length. Thus, we can conclude that the synthetic lethality of Δ(*lapD clsA*) is due to the increased toxic accumulation of PG species and an altered ratio of saturated and unsaturated fatty acids. Hence, consistent with our present results and previous findings, LapD is a multitasking protein that connects LPS, PL, and PGN synthesis, and cardiolipins play a pivotal role in this process. This is further supported by the finding that PgsA is part of the large protein complex of LapB/LapD, which can lead to the coordination of LPS and PL biosynthetic pathways.

4. Materials and Methods

4.1. Bacterial Strains, Plasmids and Media

The bacterial strains and plasmids used in this study are described in Table 5.

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

Strains	Genotype	Reference
W3110	λ$^-$, *IN* (*rrnD-rrnE*)*1*, *rph-1*	CGSC, Yale
GK1111	W3110 Δ*lac*	[17]
SR7106	GK1111 *clsA*<>*aph*	This study
SR25204	GK1111 *lapD*<>*frt*	[14]
SR25209	SR25204 *lapD*<>*frt clsA*<>*aph*	This study
SR25533	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* I99N	This study
SR25534	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* T7A	This study
SR25537	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* T60A	This study
SR25535	SR25204 *lapD*<>*frt clsA*<>*aph cdsA* D249G	This study
SR25532	SR25204 *lapD*<>*frt clsA*<>*aph murD* M407V	This study
SR25538	SR25204 *lapD*<>*frt clsA*<>*aph pgsA* T60P	This study
RU857	Δ*pgsA* Δ(*clsABC*) *lpp*2	[69]
SR25228	SR25209 *lapD*<>*frt clsA*<>*aph* + p*accC*$^+$	This study
SR25229	SR25209 *lapD*<>*frt clsA*<>*aph* + p*psd*$^+$	This study
SR25230	SR25209 *lapD*<>*frt clsA*<>*aph* + p*metQ*$^+$	This study
SR25231	SR25209 *lapD*<>*frt clsA*<>*aph* + p*glnB*$^+$	This study
SR25232	SR25209 *lapD*<>*frt clsA*<>*aph* + p*pspC*$^+$	This study
SR25233	SR25209 *lapD*<>*frt clsA*<>*aph* + p*tesD*$^+$	This study
SR25540	W3110 *pgsA* T60A Tn*10*	This study
SR25548	W3110 *cdsA* D249A Tn*10*	This study
SR25874	W3110 + pCA24N	[14]
SR23799	SR25204 *lapD*<>*frt* + pCA24N	[14]
SR10488	MG1655 Muc ts62 Mud 5005	[70]
SR23769	SR10488 *lapD*<>*frt clsA*<>*aph*	This study
Plasmids	**Genotype**	**Reference**
pCA24N	IPTG-inducible expression vector cm^R	[41]
pKD13	*oriR6K*$_g$, *bla*(Amp^R), *kan*, *rgnB*(Ter)	[71]
pKD46	*araBp-gam-bet-exo*, *bla*(Amp^R), *repA101*(ts)	[71]
pCP20	ts replicon with inducible FLP recombinase	[72]
pMBL18	p15A replicon amp^R	[73]
pSR23502	*tesD*$^+$ in pMBL18 amp^R	This study
pSR23505	*tesD*$^+$ in pCA24N cm^R	This study
JW3224	*accC*$^+$ in pCA24N cm^R	[41]
JW0193	*metQ*$^+$ in pCA24N cm^R	[41]
JW2537	*glnB*$^+$ in pCA24N cm^R	[41]
JW1299	*pspC*$^+$ in pCA24N cm^R	[41]
JW4121	*psd*$^+$ in pCA24N cm^R	[41]
pREG	cosmid cloning vector	[74]
pSR25577	*pgsA*$^+$ in pREG amp^R	This study
pSR25590	*cdsA*$^+$ in pREG amp^R	This study
pSR25596	*murD*$^+$ in pREG amp^R	This study

Luria–Bertani (LB) broth and agar (LA) (Difco, Franklin Lakes, NJ, USA) were prepared as described earlier [75]. As per the experimental requirements, the media were supplemented with ampicillin (100 μg/mL), kanamycin (50 μg/mL), tetracycline (10 μg/mL), or chloramphenicol (20 or 30 μg/mL). The expression of various genes in the pCA24N expression vector was induced by adding 75 μM IPTG to the growth medium.

4.2. Strain Construction and Isolation of Extragenic Chromosomal Suppressors

All strains used in this study were derived from W3110, serving as the parental wild-type strain, unless otherwise stated. The construction of a non-polar antibiotic-free deletion derivative of *lapD* SR25204 in W3110 has been previously described [14]. Bacteriophage P1-mediated transductions were performed as previously described [76]. The Δ*lapD* derivative SR25204 served further as a recipient to construct the Δ(*lapD clsA*) derivative SR25209 by bacteriophage P1-mediated transduction. To construct this strain, the Δ*clsA* non-polar deletion was first constructed by replacing the coding sequence of the *clsA* gene by the *aph* cassette from the pKD13 plasmid using the λ recombinase system, as described [17]. The *aph* antibiotic cassette was removed by transformation with pCP20 plasmid DNA, which expresses a site-specific recombination flippase [72]. To construct Δ(*lapD clsA*), bacteriophage P1-mediated transductions were performed in reciprocal order and plated on LA agar plates at 30 °C, which was found to be a permissive condition for such derivatives. As a control, each recipient in the transduction experiments was transformed with a covering plasmid expressing either *lapD* or *clsA* genes. To isolate extragenic chromosomal suppressor mutants, cultures of several independently obtained suppressor-free Δ(*lapD clsA*) transductants grown at 30 °C were plated at 42 °C. The Ts^+ properties were verified by re-streaking. Cultures from these Δ(*lapD clsA*) Ts^+ isolates, 65 putative suppressor-containing strains, were retained. Suppressor mutations were marked with mini-Tn*10* Tet [77], and verified that the Tn*10*-linked suppressor mutation breeds true. To identify the suppressor mutation, linked Tn*10* mutations were recombined onto single-copy cosmid clones using a previously described DNA library [14,74]. To identify the candidate gene with a suppressor mutation, DNA from recombinant cosmids was subcloned, selecting for Tn*10* Tet and Amp markers or by inverse PCR using chromosomal DNA to identify Tn*10* junctions, as described [78]. DNA from the recombinant plasmid was used to sequence the Tn*10* ends and flanking regions using inverse PCR, as previously described [8,78]. This allowed us to place 14 suppressor mutations in three complementation groups. The same cosmid DNAs were then used to sequence the candidate genes using specific oligonucleotides.

4.3. Identification of Multicopy Suppressors, Whose Overexpression Overcomes Lethal Phenotype at 42 °C

DNA from plasmid pools obtained from the ordered genomic library cloned in pCA24N carrying all predicted ORFs of *E. coli* (ASKA collection) [41] was used to transform the Δ(*lapD clsA*) strain SR25209, selecting directly for Ts^+ survivors at 42 °C in the presence of 75 μM IPTG. Cultures for Ts^+ colonies were grown to isolate plasmid DNAs, which were used to retransform the Δ(*lapD clsA*) strain SR25209 for the validation of growth restoration at 42 °C. The identity of the cloned gene, whose overexpression conferred suppressing ability, was obtained by DNA sequencing. However, this method gave a lot of background due to the recloning of *lapD* and *clsA* genes, although several *bona fide* multicopy suppressors were also isolated. To overcome this drawback, the mini-Mu in vivo cloning approach was used [42,70]. To achieve this, a Δ(*lapD clsA*) derivative was constructed in a derivative with Muc ts62 Mud5005, resulting in the construction of SR23769. Mini-Mu lysates of SR23769 were prepared by thermal induction and used to transduce plasmids into Δ(*lapD clsA*) derivative SR25209, selecting for Ts^+ colonies at 42 °C. Plasmid DNAs from such Ts^+ derivatives were subcloned into a p15A-based medium copy plasmid vector pMBL18 [73]

to identify the minimal coding region that could suppress the lethality of Δ(*lapD clsA*) at 42 °C. Plasmids that bred true in suppressing ability were used for DNA sequencing.

4.4. Isolation and Analysis of ^{32}P-Labeled Phospholipid Species

Overnight cultures of isogenic strains of wild type, Δ*lapD*, Δ(*lapD clsA*), and its derivatives with suppressor mutations in *pgsA*, *cdsA*, and *murD* were grown in LB medium under permissive growth conditions at 30 °C. The cultures were diluted, adjusted to an OD_{595} of 0.05, and grown for another 45 min and labelled with 2.5 μCi/mL of $^{32}P_i$ in 2.5 mL of LB medium until an OD_{595} of 1.0. For the growth of strains Δ(*lapD clsA*) derivatives carrying multicopy suppressors, during the labelling period, 75 μM IPTG was added for the expression of P_{T5}-*lac* promoter-inducible *accC*, *psd*, and *tesD* genes. Cultures were harvested by centrifugation at 4300× *g* for 10 min. The pellets were dispersed in water. Phospholipids were extracted using the procedure described by Bligh and Dyer, and Ames [79,80]. The PLs were collected from the lower organic phase and dried under a stream of nitrogen. Dried samples containing phospholipids were reconstituted in 50 μL of a 2:1 chloroform/methanol mixture. Before the extraction of PLs, an aliquot of the samples was used to measure the total protein concentration using a Pierce BCA protein assay kit (ThermoScientific, Warsaw, Poland). Equivalent amounts (10,000 cpm/lane) of ^{32}P-labeled phospholipids after adjusting for protein concentration were separated on TLC Silica gel 60 F254, aluminum sheets, 20 cm × 20 cm plates (Merck, Mississauga, ON, Canada). Lipids were separated using a solvent system, as described [14]. The TLC plates were dried and exposed to CL-Xposure^{Tm} Film (ThermoScientific, Rockford, IL, USA) or exposed to a PhosphorImager screen to visualize and quantify the labeled PLs using the Amersham Typhoon Biomolecular Imager (Cytiva, Uppsala, Sweden). Densitometry analysis of the autoradiograms was performed using the ImageJ software (rsb.info.nih.gov). Three biological replicates were used for quantification.

4.5. Isolation of Fatty Acids and Their Analysis Using Gas Chromatography

Isogenic bacterial cultures were grown at 28 and 37 °C as described for phospholipid extraction, without adding either IPTG or $^{32}P_i$. Fifteen mL cultures at an OD_{595} of 1.0 were harvested by centrifugation at 4300× *g* for 10 min, and the pellets were frozen. The pellets were thoroughly resuspended in 2.5 mL of H_2O with vortexing, followed by the addition of 5 μL of 10 mg/mL heptadecanoic acid (C17:0) (Merck, Warsaw, Poland) dissolved in ethanol, as an internal standard. Free fatty acids were extracted as per the procedure described [14,81]. Fatty acid methyl esters were obtained by treating the dried extract with 0.5 mL of 1.25 M HCl in methanol and incubating at 50 °C for 15 h. Fatty acids were extracted twice in 500 μL of hexane. FFA were analyzed by gas chromatography using a Shimadzu GC-2010 Pro (Shimadzu, Kyoto, Japan) with a 30 m × 0.25 mm capillary column under the experimental conditions described [14]. One μL of each sample was injected using a 1:100 split ratio of the helium carrier gas. For calibration, a fatty acid methyl ester mixture C8-C22 (CRM18920 Merck, Warsaw, Poland) was used to establish the retention time of various peaks corresponding to different fatty acids. Each experiment was repeated with three independent biological repeats. The data were analyzed using the software provided by Shimadzu (Kyoto, Japan).

4.6. Estimation of LpxC Amounts by Immunoblotting

Isogenic strains of wild type, Δ(*lapD clsA*), and its derivatives with either chromosomal extragenic suppressor mutation or carrying plasmid born multicopy suppressors were routinely grown at 30 °C in 5 mL LB medium up to an OD_{595} of 1.0. The cultures were harvested by centrifugation at 7000× *g* for 10 min. The cell pellets were resuspended in SDS lysis buffer. As an internal control, cell lysates were prepared from the Δ*ftsH sfhC21*

derivative grown under similar conditions. Protein concentrations were measured using the Pierce BCA protein assay kit (ThermoScientific, Warsaw, Poland). An equivalent amount of protein was applied to a 12% SDS-PAGE for resolution. After electrophoresis, the proteins were blotted to a PVDF membrane. Membranes were immunoblotted with LpxC-specific antibodies and revealed using a chemiluminescence kit (ThermoScientific, Warsaw, Poland) according to the manufacturer's instructions.

4.7. Examination of Cellular Morphology and Measurement of Bacterial Growth

For quantification of bacterial growth, isogenic bacterial cultures were grown overnight in LB medium at 30 °C. Exponentially grown cultures were adjusted to an OD_{595} of 0.1, serially diluted, and bacterial growth was measured using spot-dilution assay as described [82]. To examine cellular morphology, cultures of wild type, Δ*lapD*, Δ(*lapD clsA*), and its three isogenic derivatives with independent chromosomal suppressor mutations in the *pgsA* gene were grown in LB medium. Overnight cultures were diluted 1:100 and allowed to grow to an OD_{595} of 0.6 under permissive conditions at 30 °C in LB medium. Aliquots of cultures were centrifuged at 4300× *g* for 5 min and incubated for 10 min in TBS supplemented with 10 µg/mL of 4′,6-diamidino-2-phenylindole (DAPI) stain. Samples were immobilized on agarose pads, and cell morphology was examined using epifluorescence and differential interference contrast (DIC) microscopy, as described [14]. Cellular morphology was observed using a Zeiss apotome microscope (Carl Zeiss, Jena, Germany).

4.8. Purification of LapB

The production of C-terminal His_6-tagged LapB was induced with 0.3 mM IPTG, using expression system as described [5]. Inner membrane proteins were extracted in binding buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (buffer A) supplemented with 1% octyl-β-D-glucoside. After centrifugation, the supernatant was purified by FPLC (AKTA Pure, Cytiva, Warsaw, Poland) using a 1 mL HisTrap FF column. The column was washed with a binding buffer containing 20 mM imidazole to remove non-tagged proteins. Proteins were eluted with buffer A using a step gradient ranging from 50, 100, 250, and 500 mM imidazole and analyzed on 12% SDS-PAGE.

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Coordinated Biosynthesis of Essential Cell Envelope Components: Lipopolysaccharide and Fatty Acids Requires LapD, Acyl Carrier Protein, and Fully Hexaacylated Lipid A

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Abstract

Lipopolysaccharide (LPS) is an essential component of the outer membrane (OM) of Gram-negative bacteria, and its levels are tightly co-regulated with phospholipid (PL) amounts. This homeostatic regulation necessitates the involvement of numerous genes, including *lapD* in a poorly defined manner. To understand the function of LapD, we took advantage of the synthetic lethal phenotype conferred by the concomitant absence of LapD and myristoyltransferase LpxM or heptosyltransferase WaaC and isolated extragenic suppressors that could bypass this lethality. Suppressor analyses of Δ(*lapD lpxM*) bacteria identified five single amino acid exchanges in AccA and two in each of AccC and AccD. These proteins comprise different subunits of the acetyl-CoA carboxylase complex, which catalyzes the rate-limiting step in the initiation of fatty acid synthesis, mediating the conversion of acetyl-CoA to malonyl-CoA. Fatty acid analysis revealed that these mutations restored the ratio of saturated to unsaturated fatty acids and repressed elevated PL levels. Suppressor analyses of Δ(*lapD waaC*) identified a single amino acid substitution in LptD, which is required for LPS assembly in the OM, and in NlpI, which regulates the amount of peptidoglycan hydrolase MepS. These results posit LapD as the point of critical regulation of homeostatic control of three essential cell envelope components.

Keywords: lipopolysaccharide (LPS); fatty acid biosynthesis FASII; myristoyltransferase; heptosyltransferase; acetyl-CoA carboxylase; LapD; LapB; phospholipids

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1. Introduction

The cell envelope of Gram-negative bacteria, including *Escherichia coli*, contains two distinct membranes, an inner (IM) and an outer (OM) membrane, separated by the periplasm, a hydrophilic compartment that includes a layer of peptidoglycan (PGN). The OM is essential for the viability of Gram-negative bacteria. This OM is asymmetric in nature due to the location of lipopolysaccharide (LPS) in its outer leaflet and phospholipids (PLs) directed inward [1]. The maintenance of this asymmetry is crucial for the integrity of the bacterial cell envelope. LPS is the major component of OM, covering nearly 70% of OM, and is the key virulence factor and causative agent of sepsis caused by Gram-negative bacteria. LPSs are highly heterogeneous in composition [2]. However, they share a common architecture composed of a membrane-anchored phosphorylated and acylated $\beta(1\longrightarrow6)$-linked GlcN disaccharide, termed lipid A, to which a carbohydrate moiety of varying size is attached [3]. In *E. coli*, the precursor lipid IV_A acts as an acceptor for the transfer of two 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) residues by WaaA, generating the key Kdo_2-lipid IV_A

intermediate, which serves as a substrate for the transfer of two additional fatty acids that require LpxL and LpxM enzymes [4,5]. All four lipid A acyltransferases, LpxA, LpxD, LpxL, and LpxM, are dependent on acyl chain activation by the acyl carrier protein AcpP, as coenzyme A thioesters do not serve as substrates [4,6]. AcpP also participates in several steps of fatty acid biosynthesis. Thus, a balanced cell envelope composition involves inbuilt mechanisms to sustain a balance between the LPS and glycerophospholipids biosynthetic pathways, as excess of either component causes lethality.

Bacteria maintain a tight balance between the amounts of LPS and PL because they use (*R*)-3-hydroxymyristate-ACP as a common metabolic precursor [7]. In *E. coli*, this balance is achieved by regulating the amount of LpxC, which catalyzes the first committed step in LPS biosynthesis [8]. The LpxC enzyme is unstable, and its amount is determined by FtsH-mediated proteolysis and several poorly defined signals, such as bacterial growth rate, ppGpp levels, fatty acid composition, FabZ, FabI, FabK activities, and the accumulation of lipid A precursors [8–12]. However, FtsH requires another essential protein, LapB, to catalyze LpxC proteolysis [11]. The proteolysis of LpxC is counteracted by LapC, which is thought to be adjusted depending on the accumulation and demand for LPS synthesis [13–19]. LapC and LapB interact genetically and physically [13,15]. Current models suggest that LapC senses the accumulation of LPS in the IM and can bind to either LPS or LapB [7,19]. Binding of LapC to LPS, instead of LapB, promotes LapB–FtsH interaction to degrade LpxC to overcome the lethal accumulation of LPS in the IM. However, the mechanism by which bacteria couple LPS and phospholipid synthesis remains unclear. In this process, LapD, another protein that interacts with LapB, could be a potential link in coupled fatty acid and LPS biosynthesis, as it forms a complex with several enzymes involved in fatty acid and phospholipid biosynthesis (Figure 1) [20].

The fatty acid biosynthesis pathway in *E. coli* is a type II FAS system [21]. During this process, acyl intermediates are covalently conjugated to ACP via a thioester linkage. *E. coli* synthesizes four major fatty acids, $C_{14:0}$(myristate), $C_{16:0}$(palmitate), $C_{16:1}$(palmitoleate), and $C_{18:1}$(*cis*-vaccenate), and two minor components, laurate ($C_{12:0}$) and 3-hydroxymyristate. The latter two are components of lipid A, whereas palmitate, palmitoleate, and *cis*-vaccenate are phospholipid components. Fatty acids form the acyl chains found in all PLs. *E. coli* can also take up exogenous fatty acids and convert them into acyl-CoA. Acyl-CoA molecules constitute a separate pool from endogenously synthesized acyl-ACPs [22]. Acyl-CoA can be used for phospholipid synthesis or broken down by *β*-oxidation. Fatty acid synthesis is also the primary biosynthetic determinant of *E. coli* size [23]. Regulation of the first step in PL synthesis, catalyzed by the PlsB enzyme, which synthesizes lysophosphatidic acid from long-chain ACP and *sn*-glycerol-3 phosphate, is another point of coupling between PL and LPS biosynthesis [24] (Figure 1).

Currently, the precise function of LapD remains unknown. One study implicated LapD in cell division [25]. Another study suggested that LapD plays a role in diverse cellular envelope-related functions, including PGN biosynthesis [26]. However, no direct proof has been provided concerning the involvement of LapD in these processes. Prior to the above-mentioned studies, our laboratory has shown that LapD is required for growth at critical high temperatures [27]. Subsequently, we presented evidence suggesting a role in LPS and fatty acid biosynthesis [20] (Figure 1). First, purification of LapD revealed that it co-purifies with several enzymes that participate in the initiation of fatty acid biosynthesis, AccD, MadA (FabY), and those mediating committed steps of FASII (FabH, FabF, and FabB) [20]. Second, the temperature sensitivity (Ts) of Δ*lapD* bacteria can be effectively suppressed by overexpression of the *acpP* gene or by an imbalance in the synthesis of acetyl coenzyme A (acetyl-CoA) carboxylase (ACC) complex (due to overexpression of one of the subunits of the ACC complex) [20]. ACC enzyme catalyzes the first committed step in fatty

acid biosynthesis, which is the conversion of acetyl-CoA to malonyl-CoA [28]. Curiously, overexpression of the *acpP* gene or one of the genes encoding ACC subunits suppresses Δ*lapD* phenotypic defects without increasing LpxC levels, suggesting an alternative role in linking fatty acid and LPS coordination [20]. In support of our findings, it has been suggested that the absence of LapD causes the activation of fatty acid biosynthesis [29]. As Δ*lapD* bacteria have reduced LPS levels, an increase in LPS amounts by the introduction of either stable variants of LpxC or inactivation of the *lapB* gene can relieve some of the phenotypic defects of Δ*lapD* bacteria [20].

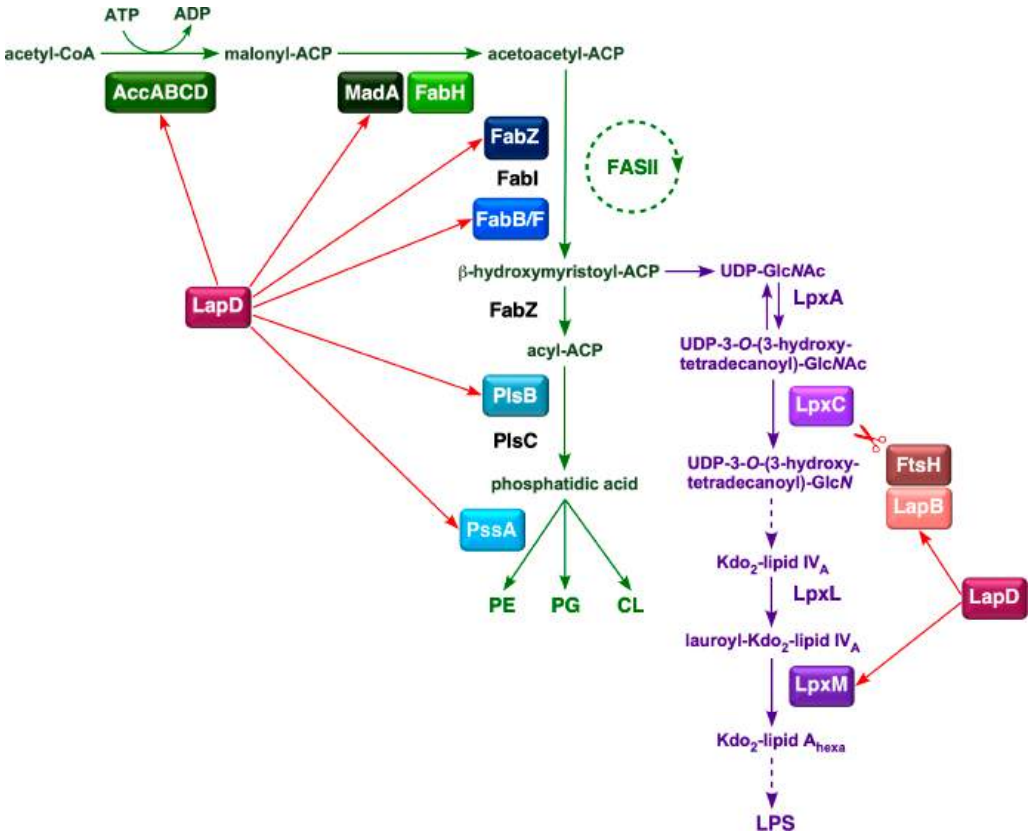

Figure 1. Schematic drawing of LapD-mediated coupled PL and LPS biosynthesis in *E. coli*. Various steps in fatty acid biosynthesis, with initiation catalyzed by the acetyl-CoA carboxylase enzyme and entry into the FASII cycle, are shown. The intermediate β-hydroxymyristoyl-ACP serves as a precursor in the subsequent steps of PL and LPS biosynthesis. Red arrows depict the role of LapD based on its co-purification with specific proteins involved in fatty acid and LPS biosynthesis, and genetic interactions based on the synthetic lethality with Δ*lapD* in mutational combinations.

Here, we used multipronged approaches to unravel the physiological function of LapD. LapD by itself is non-essential for bacterial viability, except at temperatures above 44.5 °C or when the growth medium is supplemented with vancomycin [20,26]. However, the lack of LapD causes a reduction in LpxC at elevated temperatures and reduces the amount of LPS in the OM due to the retention of significant amounts in the IM [20]. Interestingly, we recently showed that LapD becomes essential in the absence of myristoyltransferase LpxM, and Δ*lapD* bacteria also exhibit synthetic growth defects in the absence of heptosyltransferase WaaC [20]. These phenotypes were used to isolate extragenic suppressors that bypass this lethality. Single-copy chromosomal suppressors of Δ(*lapD lpxM*) mapped mostly to genes encoding different subunits of the acetyl-CoA carboxylase enzyme, which mediates the first step in fatty acid biosynthesis. These suppressors significantly altered fatty acid levels and reduced elevated PL levels. Similarly, suppressors of Δ(*lapD waaC*) synthetic lethality were isolated and shown to map to the *lptD* gene, whose product is required for

LPS assembly in the OM, and to the *nlpI* gene. NlpI regulates the amount of peptidoglycan hydrolases, such as MepS, by acting as an adaptor for Prc protease. We further show that overexpression of *acpP* and *accB* genes overcomes cell morphology defects of Δ*lapD* bacteria and a reduction in PL amounts. These results allow us to conclude that LapD in concert with LpxM is required to regulate balanced amounts of fatty acids and to couple LPS, PL, and PGN synthesis.

2. Results

2.1. Suppressor-Free Δ(lapD lpxM) Bacteria Are Viable in Minimal Medium Under Slow Growth Conditions but Require Extragenic Suppressors for the Growth in Rich Medium

We previously showed that the Δ(*lapD lpxM*) mutational combination is lethal and Δ(*lapD waaC*) bacteria exhibit conditional lethality [20]. To understand the molecular basis of this lethality, we initiated several approaches based on the isolation and characterization of extragenic chromosomal suppressors that overcome this synthetic lethality in the W3110 background, which is wild type for the *fabR* gene, unlike BW25113, which has a non-functional *fabR* gene [30]. Viable suppressor-free Δ(*lapD lpxM*) transductants could not be obtained on LA medium at either 30 or 37 °C unless a wild-type copy of either the *lapD* gene or the *lpxM* gene was provided from a plasmid (Table 1). Because strains synthesizing LPS composed of only lipid IV_A are viable on M9 minimal medium at low temperatures [31], we used the same approach to construct Δ(*lapD lpxM*) derivatives in the wild-type W3110 background. Δ(*lapD lpxM*) transductants could be obtained readily on the minimal medium at 30 °C at the same frequency, when only an empty vector was present in the recipient or with the ectopic presence of either *lapD* or *lpxM* on the plasmid (Table 1). However, when Δ(*lapD lpxM*) derivatives grown in M9 medium were analyzed for bacterial growth on LB medium at either 30 or 37 °C, they exhibited severe growth defects and were unable to form colonies (Figure 2). These results imply that under slow growth conditions of minimal medium, viable suppressor-free Δ(*lapD lpxM*) can be constructed at 30 °C; however, they cannot be propagated in rich medium even at 30 °C. The construction and viability of suppressor-free Δ(*lapD lpxM*) on minimal medium at 30 °C provided an excellent tool to study their properties and isolate suppressors that overcome their synthetic lethality on rich medium.

2.2. Suppressor Mutations Mapping to Genes Encoding Different Subunits of Acetyl Coenzyme A Carboxylase Can Bypass the Lethal Phenotype of Δ(lapD lpxM) Bacteria

To address the reasons for the lethality of Δ(*lapD lpxM*) bacteria under fast-growing conditions, suppressor-free, independently isolated transductants of such bacteria were grown in M9 minimal medium (permissive growth conditions) and plated on LA medium at 37 and 42 °C. Marking and mapping of suppressor mutations with Tn*10* and further transduction of individual suppressor mutations allowed the growth of the suppressor-free Δ(*lapD lpxM*) GK6173 strain on LA medium at 30, 37, and 42 °C in 16 out of 23 strains, and thus were not strain-specific suppressor mutations constituting six complementation groups with three covering genes encoding subunits of the ACC complex. DNA sequence analysis of PCR products from one complementation group revealed that they have a single amino acid exchange in the *accA* gene (K31E, R175L) (Table 2). In the second complementation group, which included six strains, DNA sequence analysis revealed five single amino acid suppressor mutations in the *accC* gene (K40T, V42I, R253H, V299A, and I309F). A similar strategy revealed that the third complementation group contained single amino acid substitutions in the *accD* gene (L39P and S173P). The amino acid exchanges of V299A in *accC*, and K31E and R175L in *accA* genes were recovered in two independently isolated Ts^+ derivatives of Δ(*lapD lpxM*) (Table 2).

Table 1. LapD is essential in the absence of myristoyl acyltransferase LpxM, and exhibits conditional synthetic lethality in the absence of heptosyltransferase I WaaC.

Genotype	Number of Transformants		
	P1 Δ*lapD* LA 30 °C	P1 Δ*lapD* M9 30 °C	P1 Δ*lapD* LA 42 °C
Wt	1020	923	ND [1]
Δ*lpxM*	8	875	ND
Δ*lpxM* p(*lpxM*$^+$)	768	901	ND
Δ*waaC*	676	810	17
Δ*waaC* p(*waaC*$^+$)	740	720	830

[1] ND denotes not determined.

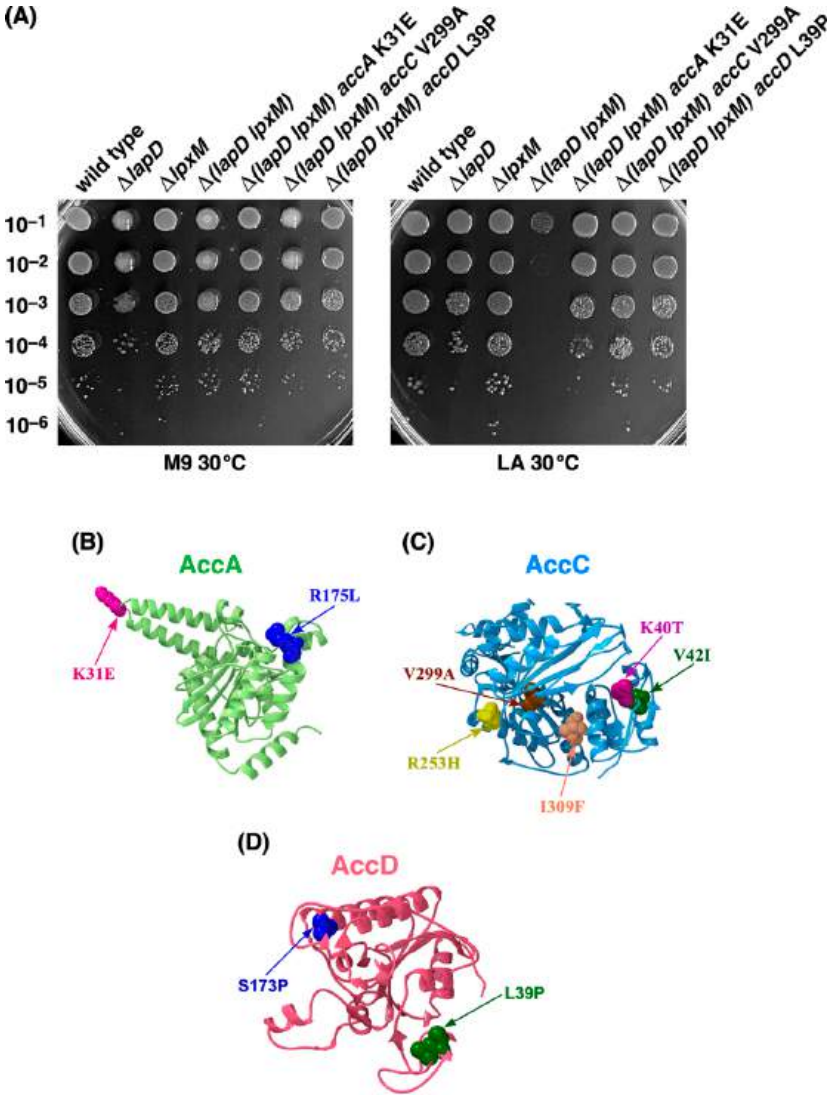

Figure 2. Mutations mapping to *accA*, *accC*, and *accD* genes encoding various subunits of the acetyl-CoA carboxylase enzyme overcome the synthetic lethality of Δ(*lapD lpxM*) bacteria on LA medium. (**A**) Growth of isogenic cultures of the wild type, its Δ*lapD*, Δ*lpxM*, and Δ(*lapD lpxM*) derivatives with and without suppressor mutations mapping to various *acc* genes was quantified by spot dilution on M9 minimal and LA media. The relevant genotypes and incubation temperature are indicated. (**B**) Positions of various single amino acid substitutions in the structure of AccA PDB 8UZ2, (**C**) AccC PDB 1BNC and (**D**) AccD PDB 2F9Y are shown.

Table 2. Suppressors of Δ(*lapD lpxM*) and Δ(*lapD waaC*) map to genes involved in fatty acid biosynthesis, peptidoglycan remodeling, and OM assembly of LPS.

Gene	Function	Mutation Position	Amounts of Isolates
Suppressors of Δ(*lapD lpxM*)			
accA	acetyl-CoA carboxyltransferase subunit α	K31E (AAA ⟶ GAA)	2
		R175L (CGT ⟶ CTT)	2
accC	biotin carboxylase	K40T (AAA ⟶ ACA)	1
		V42I (GTA ⟶ ATA)	1
		R253H (CGT ⟶ CAT)	1
		V299A (GTT ⟶ GCT)	2
		I309F (ATC ⟶ TTC)	1
accD	acetyl-CoA carboxyltransferase subunit β	L39P (CTG ⟶ CCG)	1
		S173P (TCG ⟶ CCG)	1
opgG	osmoregulated periplasmic glucans biosynthesis protein G	A100T (GCC ⟶ ACC)	2
aceK	isocitrate dehydrogenase kinase	Y474S (TAT ⟶ TCT)	1
mukE	chromosome partitioning	A122P (GCA ⟶ CCA)	1
Suppressors of Δ(*lapD waaC*)			
lptD	lipopolysaccharide assembly	L651P (CTG ⟶ CCG)	1
nlpI	lipoprotein	Y243H (TAC ⟶ CAC)	1
lptD and *bamC*	outer membrane protein assembly factor	*lptD* L651P (CTG ⟶ CCG)	1
		bamC F248L (TTC ⟶ CTC)	

The ability to restore the growth of Δ(*lapD lpxM*) bacterial strains in the presence of various extragenic suppressor mutations mapping to the ACC complex was quantified using spot-dilution experiments. These analyses revealed that suppressor mutations mapping to either *accA*, *accC*, or *accD* in the Δ(*lapD lpxM*) background restored colony-forming ability on LA medium to nearly wild-type levels (Figure 2). These conditions are lethal to the parental Δ(*lapD lpxM*) strain, which, without suppressors, can grow only on M9 minimal medium at 30 °C (Figure 2). Genomic DNA of the remaining seven Ts^+ suppressor strains was subjected to whole-genome sequencing but could not be followed because of the presence of more than one mutation.

In *E. coli*, the acetyl-CoA carboxylase complex is heteromultimeric and comprises multiple subunits of AccA, AccB, AccC, and AccD. In the initiation of fatty acid biosynthesis, the major regulation is exerted at the level of the acetyl-CoA carboxylase complex, as it constitutes a rate-limiting step and is required to produce malonyl-CoA by the carboxylation of acetyl-CoA [28]. The ACC complex is subjected to feedback inhibition because its activity is inhibited by acylated derivatives of AcpP [32,33]. Hence, the loss-of-function mutations that we obtained as suppressors of Δ(*lapD lpxM*) bacteria are expected to cause an overall reduction in the biosynthesis of fatty acids/PL.

2.3. Suppressor Mutations in Acetyl-CoA Carboxylase Complex That Bypass the Lethal Phenotype of Δ(lapD lpxM) Bacteria Accumulate Reduced Amounts of Phospholipid Species

Fatty acid and phospholipid biosynthesis are intricately linked. Thus, we analyzed the PL pattern of isogenic parental wild-type W3110 and its Δ*lapD*, Δ*lpxM*, Δ(*lapD lpxM*),

and Δ(*lapD lpxM*) derivatives with chromosomal single amino acid substitutions in *accA*, *accC*, and *accD* genes using thin-layer chromatography (TLC). As an additional control and to serve as a marker for PL species, we labeled cultures of strain RU857, which cannot synthesize cardiolipin (CL) and phosphatidylglycerol (PG) species. PL species were visualized by phosphorimaging analysis and densitometry. These experiments did not show any significant differences in the total PL amounts of the three PL species between isogenic wild-type and Δ*lapD* bacteria. However, densitometric analysis of the PL species of Δ*lpxM* bacteria revealed an approximately 24% increase in the abundance of CL species. Most significantly, there was an overall higher accumulation of PL species in Δ(*lapD lpxM*) bacteria, with a nearly 64% increase in PG and approximately 3-fold elevated levels of CL species as compared to those in Δ*lapD* or wild-type bacteria (Figure 3 lane 4). Significantly, all three suppressor-containing strains of Δ(*lapD lpxM*) with mutations in the ACC complex exhibited a remarkable reduction in the incorporation of ^{32}Pi in PE, PG, and CL species compared to the parental Δ(*lapD lpxM*) strain (Figure 3 lanes 5 to 7). This reduction in the accumulation of PLs in ACC suppressor-containing strains is particularly evident for PG and CL species, which are otherwise elevated in Δ(*lapD lpxM*) bacteria. The most noticeable results were from the strain SR25219 Δ(*lapD lpxM*) with *accC* V299A exchange, which showed a reduction in PG amounts of more than 60% compared to the parental strains, with only trace amounts of CL species (only 3% of the wild-type level) (Figure 3 lane 6) and nearly unaffected levels of PE. Interestingly, SR25218 Δ(*lapD lpxM*) *accA* K31E exhibited a nearly 30% reduction in PE and approximately 55% reduction in PG levels. Examination of PL amounts in SR25217 Δ(*lapD lpxM*) *accD* L39P also revealed a nearly 48% decrease in PG levels (Figure 3). Thus, these suppressor mutations in the ACC complex that relieve toxicity associated with Δ(*lapD lpxM*) act by reducing PL synthesis.

2.4. Suppressor Mutations in Acetyl-CoA Carboxylase Complex That Bypass the Lethal Phenotype of Δ(lapD lpxM) Bacteria Are Recessive

It became imperative to verify whether the suppressor mutations identified in *acc* genes were loss-of-function mutations. To address this, mutations in *accA*, *accC*, and *accD* were transduced using the linked Tn*10* Cm marker. The same cosmid clones (single-copy) that were used to recombine the Tn*10* marker were used for complementation and analyzed for PL content using TLC. This analysis revealed restoration of the abundance of PG, and CL species, which were diminished in *accA* and *accC* mutants using complementing cosmid clones for *accA* K31E and *accC* V299A (Figure 4, lanes 1 vs. 2 and lanes 3 vs. 4).

These data allow us to conclude that mutations in genes encoding the ACC complex are recessive, which lead to the reduction in PL species.

2.5. Suppressor Mutations in Acetyl-CoA Carboxylase Complex That Overcome the Lethal Phenotype of Δ(lapD lpxM) Bacteria Do Not Alter LpxC Levels

To rule out that suppressor mutations mapping to genes encoding the ACC complex do not alter LpxC amounts, immunoblot analysis was performed. Isogenic cultures of wild type, Δ*lapD*, Δ(*lapD lpxM*), and its derivatives with suppressor mutations in *accA*, *accC*, and *accD* genes were grown under permissive growth conditions. Equivalent amounts of proteins were transferred by Western blotting and immunoblotted with anti-LpxC antibodies. Estimation of LpxC levels revealed that they were nearly unaltered by the presence of suppressor mutations in *acc* genes (Figure 5). As a control for LpxC levels, equivalent amounts of proteins from the cell lysate of Δ*ftsH sfhC21* bacteria were applied, which had expectedly elevated amounts of LpxC due to its stabilization (Figure 5 lane 7).

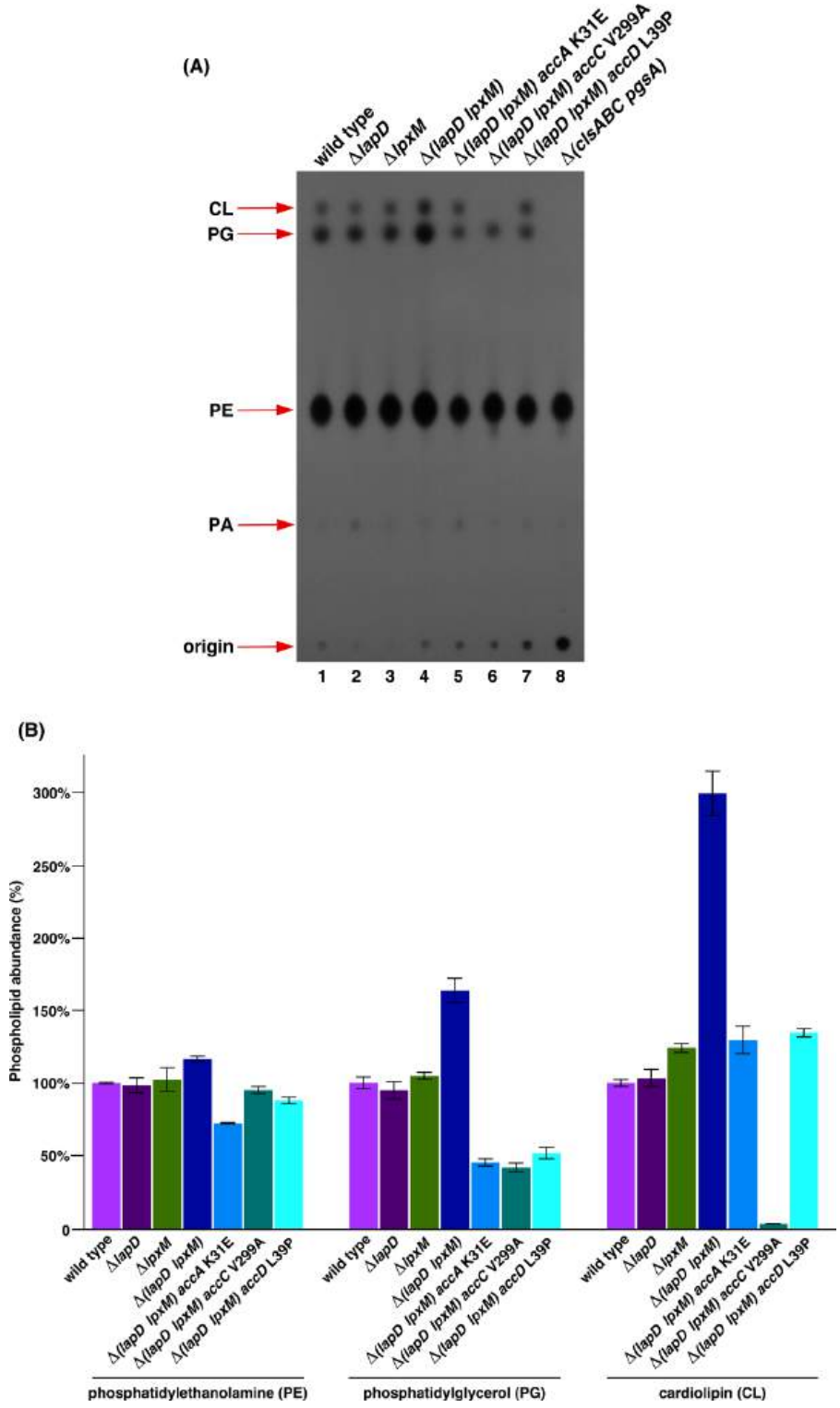

Figure 3. Δ(*lapD lpxM*) bacteria have excess of phospholipids and suppressor mutations in genes encoding various subunits of acetyl-CoA carboxylase enzyme that overcome their synthetic lethality act by decreasing phospholipid synthesis. (**A**) Thin-layer chromatography of ^{32}P-labeled PLs extracted from isogenic strains derived from Δ(*lapD lpxM*) bacteria carrying suppressor mutations in *accA*, *accC*, and *accD* genes. In parallel, ^{32}P-labeled PLs were extracted from the parental wild-type, Δ*lapD*, and Δ*lpxM* bacteria, with samples from the RU857 strain serving as a marker for the migration of PL species. A representative image of from three biological replicates is shown. The arrows indicate the positions of major PL species. (**B**) Bar graph representing the relative amounts of CL, PG, and PE species quantified using densitometry and quantification by phosphorimaging analysis from triplicate biological repeats are presented. The S.E and relevant genotype are indicated.

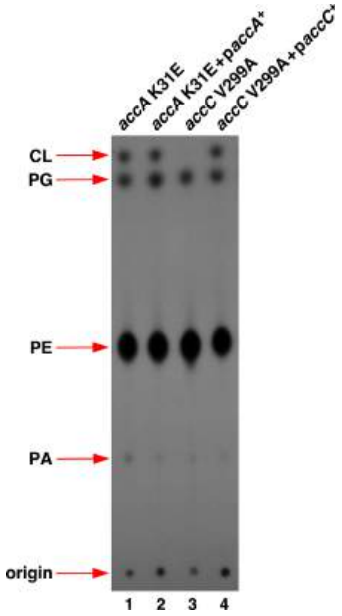

Figure 4. Suppressor mutations in *accA* and *accC* genes that overcome the lethal phenotype of Δ(*lapD lpxM)* bacteria are recessive. Thin-layer chromatography of ^{32}P-labeled PLs extracted from isogenic strains derived from Δ(*lapD lpxM*) bacteria carrying suppressor mutations in *accA* and *accC* genes, and their isogenic derivatives transformed with single-copy complementing cosmid clones. A photographic image of a representative autoradiogram from three biological replicates is shown. The arrows indicate the positions of major PL species.

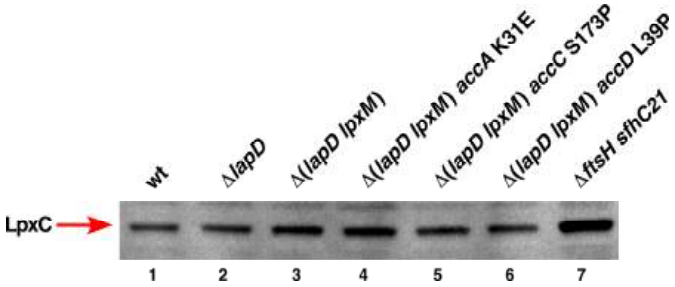

Figure 5. LpxC levels are unaltered by suppression mutations in *accA* and *accC* genes. Immunoblot of whole-cell lysates obtained from isogenic strains grown at 30 °C in M9 minimal medium. An equivalent amount of total protein was resolved by 12% SDS-PAGE prior to immunoblotting. For immunoblotting, LpxC-specific antibodies were used, and the relevant genotype is indicated. As an internal control, a cell lysate from the isogenic Δ*ftsH sfhC21* strain was applied (lane 7).

2.6. Lethality of Δ(lapD lpxM) Bacteria Due to Gross Changes in the Amounts of Free Fatty Acids That Can Be Bypassed by Mutations in the ACC Complex

As shown in the above sections, the concomitant absence of LapD and myristoyl acyltransferase LpxM causes significant changes in the phospholipid content. Thus, it became imperative to examine the fatty acid composition of Δ(*lapD lpxM*) bacteria and their isogenic suppressors that overcome the lethal phenotype. Thus, free fatty acids (FFAs) were extracted in hexane and examined using gas chromatography. Two sets of growth conditions were used, with one condition as described in the PL compositional analysis, with a brief shift to LB medium, and another when bacterial cultures were grown only in minimal medium, since in these experiments, bacteria were not labeled with ^{32}Pi. These experiments revealed that Δ(*lapD lpxM*) bacteria have nearly a 2-fold increase in myristic acid (C14:0), and the levels are restored by suppressor mutations in *acc* genes under M9 minimal medium growth conditions. This is best exemplified by the restoration of wild-type-like amounts of myristic acid, particularly by the *accC* V299A

mutation. Under the same growth conditions, the amounts of unsaturated fatty acids (UFAs) C16:1 palmitoleic acid increased by approximately 2-fold in Δ(*lapD lpxM*) bacteria. This phenotype is repressed by suppressor mutations in either *accA* or *accC* genes. This analysis further showed that the amounts of *cis*-vaccenic acid C18:1 were reduced in Δ(*lapD lpxM*) bacteria by approximately 22% compared to those measured in the isogenic wild-type strain when grown at 30 °C, but not at 37 °C. Interestingly, suppressor mutation mapping to the *accC* gene caused a significant increase in the amount of *cis*-vaccenic acid (more than 1.6-fold) compared to that in the wild type and a 2-fold increase over that measured in Δ(*lapD lpxM*) bacteria at 30 °C. Consistent with these results, even when FFAs were extracted from bacteria grown at 37 °C, the amount of *cis*-vaccenic acid significantly increased (Table 3). These results suggest that the absence of myristoyl acyltransferase in Δ*lapD* bacteria results in large-scale changes in the biosynthesis of fatty acids, some of which are reversed by suppressor mutations in genes encoding different subunits of the ACC complex.

Table 3. Measurement of relative fatty acid composition by gas chromatography. Data are presented for three biological replicates for both conditions, and the average with S.E is shown.

	M9 30 °C					
	wt	**Δ*lapD***	**Δ(*lapD lpxM*)**	**Δ(*lapD lpxM*) *accA* K31E**	**Δ(*lapD lpxM*) *accC* V299A**	**Δ(*lapD lpxM*) *accD* L39P**
C14:0	1.49 ± 0.13	1.88 ± 0.05	4.07 ± 0.04	3.11 ± 0.07	1.03 ± 0.07	2.62 ± 0.04
C16:0	45.01 ± 1.04	45.35 ± 0.24	41.86 ± 0.07	43.17 ± 0.02	41.35 ± 0.06	45.94 ± 0.04
C18:0	0.72 ± 0.03	0.95 ± 0.10	0.39 ± 0.04	0.58 ± 0.01	2.01 ± 0.10	0.61 ± 0.08
C16:1	15.91 ± 0.40	19.32 ± 0.04	30.13 ± 0.20	30.12 ± 0.06	19.68 ± 0.08	20.99 ± 0.01
cyclopropane	17.93 ± 0.02	14.14 ± 0.07	7.89 ± 0.11	6.25 ± 0.12	5.39 ± 0.15	15.26 ± 0.11
C18:1	18.94 ± 1.66	18.36 ± 0.19	15.66 ± 0.06	16.78 ± 0.23	30.54 ± 0.16	14.58 ± 0.20
	LB 37 °C					
C14:0	3.21 ± 0.03	4.03 ± 0.04	3.85 ± 0.10	3.43 ± 0.03	2.14 ± 0.01	3.71 ± 0.11
C16:0	42.60 ± 1.11	45.17 ± 0.14	43.40 ± 0.16	45.29 ± 0.07	44.58 ± 0.21	44.11 ± 1.94
C18:0	3.21 ± 0.09	3.31 ± 0.08	0.87 ± 0.20	0.77 ± 0.06	1.35 ± 0.04	0.74 ± 0.05
C16:1	31.41 ± 0.73	31.51 ± 0.02	28.78 ± 0.05	31.01 ± 0.14	25.09 ± 0.17	31.63 ± 1.86
cyclopropane	2.18 ± 0.35	2.53 ± 0.08	4.79 ± 0.06	2.85 ± 0.01	2.00 ± 0.04	2.37 ± 0.21
C18:1	17.37 ± 0.629	13.45 ± 0.05	18.31 ± 0.03	16.66 ± 0.17	24.83 ± 0.08	17.43 ± 0.75

2.7. Inhibition of Fatty Acid Biosynthesis by Exogenous Addition of Either Triclosan or Cerulenin Overcomes Lethality of Δ(lapD lpxM) Bacteria

As Δ(*lapD lpxM*) bacteria exhibit an increase in the abundance of PL species, indicating excessive fatty acid synthesis contributing to lethality, we hypothesized that the inhibition of fatty acid synthesis could overcome the synthetic lethal phenotype. Thus, we supplemented the growth medium with sublethal doses of inhibitors, such as triclosan and cerulenin. Triclosan is a well-characterized inhibitor of FabI enzyme, the enoyl-ACP (acyl-carrier protein) reductase component of FASII [34]. Cerulenin inhibits the elongation steps of fatty acid biosynthesis by irreversibly binding to FabB and FabF enzymes [35]. After titration experiments, we supplemented M9 minimal and LA growth medium with 0.01 and 0.05 μg/mL of triclosan and 25 μg/mL of cerulenin, the concentration tolerated by the wild-type bacteria, although the colony size was reduced and viability was reduced by a factor of 10-fold (Figure 6). Using spot dilution assays, we found that supplementation of LA medium with either triclosan or cerulenin slightly promoted the growth of Δ(*lapD lpxM*) bacteria at 30 °C (Figure 6). As shown earlier and in the control experiments, Δ(*lapD lpxM*) bacteria did not grow on LA medium. Interestingly, on M9 and LA media, supplementation with 0.05 μg/mL of triclosan was tolerated by Δ(*lapD lpxM*) bacteria but not by isogenic

Δ*lapD* bacteria, indicating that excess fatty acid biosynthesis is one of the reasons for the lethal phenotype of Δ(*lapD lpxM*) bacteria.

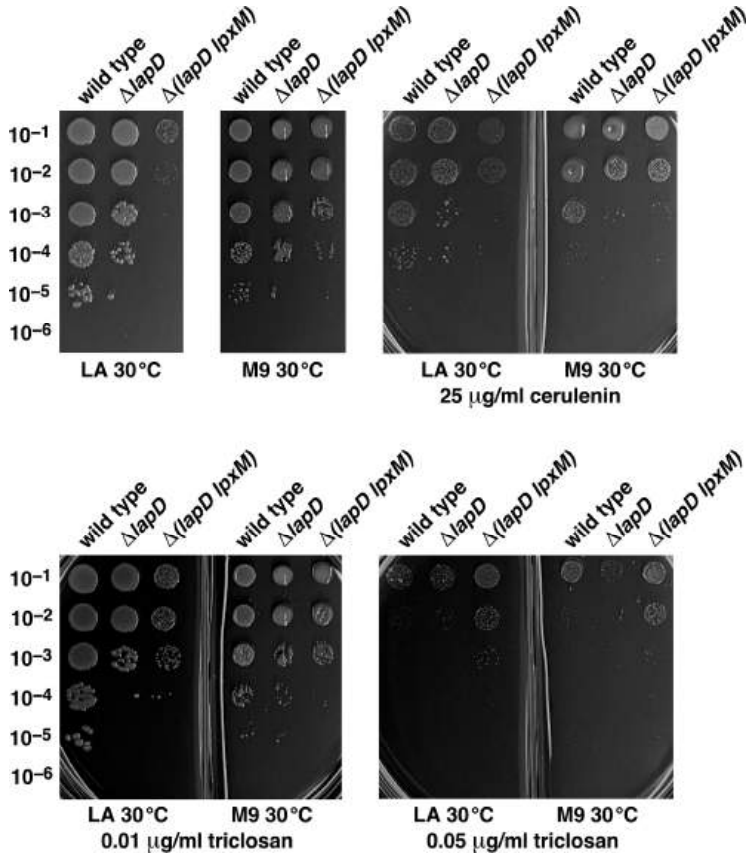

Figure 6. Inhibition of fatty acid biosynthesis by the addition of either cerulenin or triclosan can overcome the lethal phenotype of Δ(*lapD lpxM*) bacteria. Growth of isogenic cultures of wild type, its Δ*lapD*, and Δ(*lapD lpxM*) derivatives was quantified by spot dilution on M9 minimal medium and LA alone or when supplemented with either 25 μg/mL of cerulenin (upper panel) or with 0.01 or 0.05 μg/mL of triclosan. The relevant genotypes and incubation temperature are indicated. Split plates with either M9 or LA were used for spot dilution. The concentrations of cerulenin or triclosan and relevant genotypes are indicated.

2.8. Suppressor Mutations Mapping to opgG, aceK and mukE Genes Can Bypass the Lethal Phenotype of Δ(lapD lpxM) Bacteria at 37 °C

As mentioned above, Tn*10*-linked suppressor mutation of Δ(*lapD lpxM*) bacteria fell into six complementation groups. Using the same strategy after marking with Tn*10*, mapping of three remaining sets and DNA sequence of relevant PCR products revealed that two strains, SR25437 and SR25443, contained a single amino acid exchange of A100T in the *opgG* gene (Table 2). DNA sequencing of PCR products of relevant genomic regions identified two additional Δ(*lapD lpxM*) suppressor-carrying strains, SR25452 and SRSR25458, with single amino acid exchanges in *aceK* (Y474S) and *mukE* (A122P) genes, respectively. The restoration of growth of Δ(*lapD lpxM*) bacteria by suppressor mutations mapping to *opgG*, *mukE*, and *aceK* was quantified by growth measurement using spot-dilution assays (Figure 7). The *aceK* gene encodes isocitrate dehydrogenase kinase/phosphatase (AceK), which controls the branch point between two pathways of central metabolism: the TCA cycle and the glyoxylate cycle [36]. Mutation in the *aceK* gene could potentially alter the levels of precursor acetyl-CoA used in these cycles and also feeds into fatty acid biosynthesis. Significantly, amino acid residue Y474 is located next to the catalytically essential amino acid residue D475, whose inactivation abolishes the kinase activity of AceK [37]. The active

site of AceK contains a highly conserved VVF**Y**DYDEI motif (Y474 is in bold) [38]. OpgG is a periplasmic protein required for the synthesis of branched periplasmic oligosaccharides, which requires UDP-glucose and the AcpP [39,40].

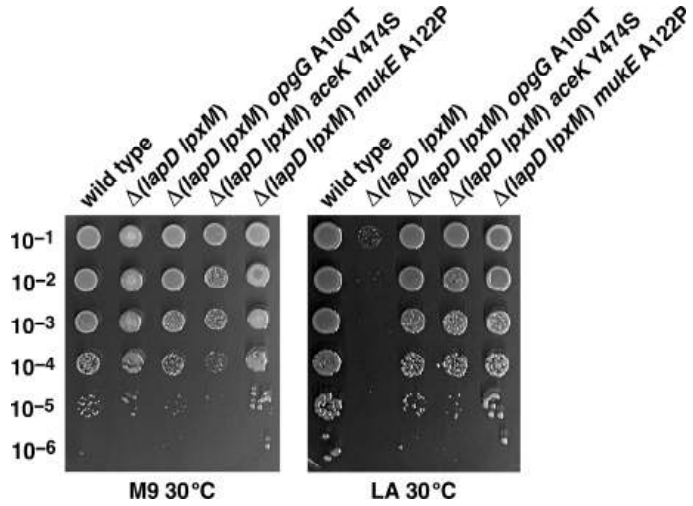

Figure 7. Single amino acid substitutions in *opgG*, *aceK* and the *mukE* gene can overcome the synthetic lethality of Δ(*lapD lpxM*) bacteria. Growth of isogenic cultures of wild type, Δ(*lapD lpxM)*, its isogenic derivatives with various suppressor mutations was quantified by spot dilution on M9 and LA 30 °C. Plates were incubated for 24 h. Data from a representative experiment with indicated genotype are shown.

MukE is involved in chromosome partitioning, and its loss affects the focal localization of the MukBEF complex [41–43]. MukB, MukE, and MukF are also interacting partners of LapD [20]. Taken together, suppressors mapping to either the ACC complex or in *aceK* or *mukE* genes could regulate fatty acid amounts, the amount of acyl-CoA precursor and cell division, providing a reasonable explanation for their isolation and the molecular basis of the synthetic lethal phenotype of Δ(*lapD lpxM*) bacteria.

2.9. Suppressor Mutations Mapping to lptD and nlpI Genes Can Bypass the Synthetic Lethal Phenotype of Δ(lapD waaC) Bacteria at 42 °C

We previously showed that Δ*lapD* bacteria exhibit a synthetic lethal phenotype in the absence of WaaC heptosyltransferase at temperatures above 42 °C, which are otherwise permissive for deletions in either *lapD* or *waaC* genes [20,31]. However, the molecular basis of this synthetic lethal phenotype remains unknown. To address this issue, we isolated extragenic Ts^+ chromosomal suppressor mutations. Ts^+ suppressors were obtained at a frequency of 10^{-6} to 10^{-7} and analyzed further after marking with Tn*10*. Three Ts^+ suppressors were retained. DNA sequence analysis demonstrated that the SR25560 Δ(*lapD waaC*) Ts^+ suppressor-carrying strain had a single amino acid Leu-to-Pro substitution at amino acid position 651 of LptD (Table 2). Another isogenic Ts^+ suppressor-carrying SR25561 was found to have a single amino acid substitution, Tyr 243 to His, in the coding sequence of the *nlpI* gene (Table 2). However, the third Ts^+ suppressor-carrying strain SR25562 identified two Tn*10* linked mutations, LptD L651P and BamC F248L, and was not analyzed further. LptD is an outer membrane protein that assembles LPS in the OM, together with the OM lipoprotein LptE [44]. NlpI is an OM lipoprotein that interacts with the tail-specific periplasmic protease Prc and acts as an adapter to mediate the proteolysis of MepS hydrolase by Prc [45–47]. NlpI is suggested to bring endopeptidases close to the complexes involved in PGN synthesis, thereby spatially connecting peptidoglycan hydrolysis to PGN expansion [46,47]. Transduction of the F248L *nlpI* mutation into the wild-type W3110 conferred an osmosensitive phenotype, which could be suppressed by

the *nlpI*-expressing plasmid, as is known for the Δ*nlpI* phenotype [48], and hence is a recessive mutation.

The restoration of Δ(*lapD waaC*) growth at 42 °C by suppressor mutation mapping to *lptD* and *nlpI* was quantified using spot-dilution assays. Thus, isogenic cultures of wild-type, Δ*lapD*, Δ(*lapD waaC*), and two suppressor-carrying strains, SR25560 and SR25561, were grown under permissive growth conditions at 30 °C in LB medium and examined for growth at 30 °C and 42 °C on LA plates using spot-dilution assays. The results of these experiments revealed more than a 10^4-fold increase in colony-forming ability at 42 °C in Δ(*lapD waaC*) *lptD* L651P and Δ(*lapD waaC*) *nlpI* Y243H strains compared to the parental Δ(*lapD waaC*) strain (Figure 8). As expected, the Δ(*lapD waaC*) strain was unable to propagate at 42 °C and formed mucoid colonies at 30 °C, similar to the parental Δ*waaC* strain. Interestingly, the Δ(*lapD waaC*) *lptD* L651P strain SR25560 exhibited a more robust (10-fold) suppression at 42 °C than the Δ(*lapD waaC*) *nlpI* Y243H strain SR25561. The LptD L651P suppressor mutation also abolished the mucoid phenotype at 30 °C (Figure 8). The mucoid phenotype is a known phenotype of deep rough mutants lacking heptose I due to the induction of capsular polysaccharide synthesis [49]. Isolation of suppressor mutation mapping to the *nlpI* gene links LapD to PGN biosynthesis, and the suppressor mutation in the *lptD* gene could probably improve LPS translocation to the OM, which we have previously shown to be impaired in Δ*lapD* bacteria [20]. Thus, the function of LapD is not only linked to the regulation of fatty acid biosynthesis but also to ensure coordination with PGN and LPS biosynthesis.

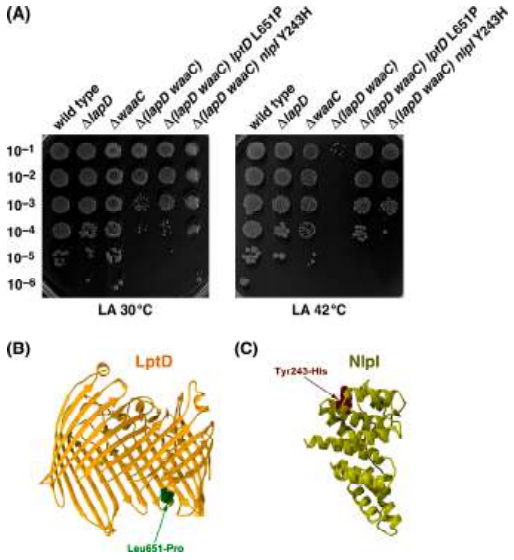

Figure 8. Single amino acid substitutions in either *lptD* or *nlpI* can overcome the synthetic lethality of Δ(*lapD waaC*) bacteria. (**A**) The growth of isogenic cultures of the wild type, its Δ*lapD*, Δ(*lapD waaC*), Δ(*lapD waaC*) *lptD* L615P, and Δ(*lapD waaC*) *nlpI* Y243H derivatives was quantified by spot dilution on LA medium at 30 and 42 °C. The relevant genotypes and incubation temperatures are indicated. A representative image of the spot dilution assay after 24 h of incubation is shown. (**B**) Positions of single amino acid substitutions in the structures of LptD PDB 4RHB and (**C**) NlpI PDB 1XNF are shown.

2.10. Overexpression of Either the acpP Gene or the accB Gene Overcome Cell Morphology Defects of ΔlapD Bacteria

Δ*lapD* bacteria are known to exhibit morphological defects; however, the underlying reasons remain unknown [25]. As cell size is significantly correlated with fatty acid synthesis [23], we rationalized that multicopy suppressors of Δ*lapD* bacteria such as *acpP* and *accB* could act by restoring fatty acid levels. Therefore, we examined their effects on cell

morphology. These experiments revealed that Δ*lapD* bacteria in the W3110 background exhibited a filamentous phenotype, which was repressed by overexpression of either *acpP* or *accB* genes (Figure 9). Thus, these experiments allow us to postulate that acyl-ACPs generated by AcpP might be a limiting factor in Δ*lapD* mutants for cellular processes involved in either cell division directly or regulation of fatty acid synthesis, as AcpP interacts with several proteins in these two pathways. The suppression of cell morphology defects by overexpression of the *accB* gene can be explained by disturbing stoichiometry between different subunits of ACC enzyme, which can lead to loss of activity of the enzyme and consequent reduction in fatty acid biosynthesis.

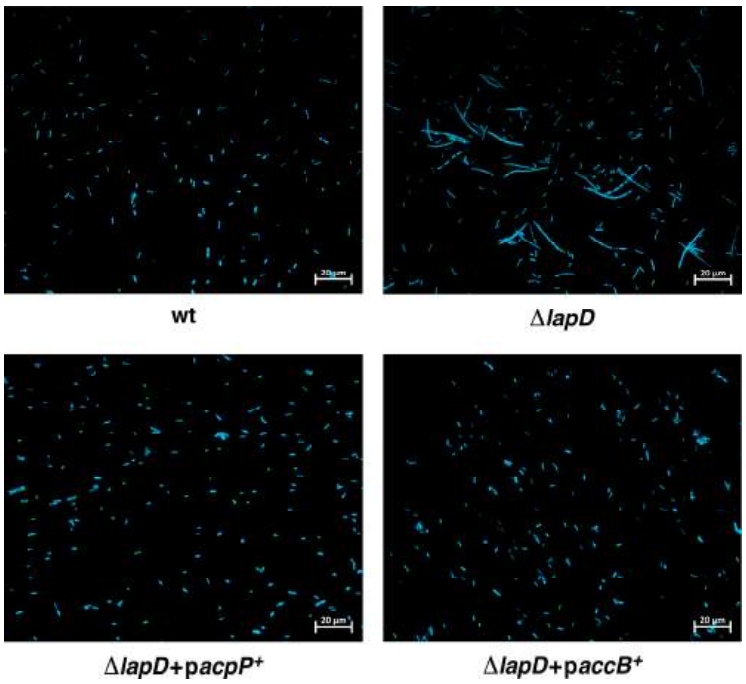

Figure 9. Overexpression of either *acpP* or *accB* genes rescues the cell morphology defects of Δ*lapD* bacteria. Microscopic images of the wild type, its isogenic Δ*lapD* with the empty vector, and Δ*lapD* expressing either *acpP* or *accB* genes. Bacterial cultures were grown overnight at 37 °C, diluted 1:100 into fresh LB, and grown until an OD_{600} of 0.1 at 37 °C. IPTG (75 μM) was added, and the cultures were grown for another 2 h. Cultures were pelleted, and bacterial cells were stained with DAPI and imaged using fluorescence microscopy at ×1000 with a 20 μm scale.

2.11. Overexpression of Either acpP or menI Overcomes Vancomycin Sensitivity of ΔlapD Bacteria

We previously reported that *acpP* overexpression effectively suppresses the Ts phenotype of Δ*lapD* bacteria. However, Δ*lapD* bacteria also exhibit sensitivity to antibiotics, such as vancomycin [20]. Nevertheless, whether *acpP* overexpression or that of some other gene can restore vancomycin resistance has not been investigated in the W3110 background. Here, we performed a multicopy screen to identify genes whose overexpression confers resistance to vancomycin in Δ*lapD* bacteria. DNA sequence analysis and retransformation identified *acpP* and *menI* genes. This suppression was further quantified by growth analysis using a spot-dilution assay in a growth medium supplemented with 125 μg/mL vancomycin and 75 μM IPTG. The data presented demonstrate that the induction of *menI* expression restores vancomycin resistance in Δ*lapD* bacteria, similar to that observed with *acpP* overexpression (Figure 10). MenI belongs to the 4-hydroxybenzoyl-CoA thioesterase subfamily of the hot-dog fold superfamily of proteins [50,51]. MenI has been reported to exhibit activity toward a wide range of acyl-CoA thioesters, and its role in the menaquinone biosynthetic pathway has been confirmed [51]. However, as MenI overproduction restores vancomycin resistance, it is likely that moderate induction of thioesterase, due to its de-

generate activity, could dampen increased fatty acid synthesis in the absence of LapD and hence is relevant to the identification of MenI as a dosage-dependent suppressor of Δ*lapD* bacteria. Furthermore, although acyl carrier protein is a highly abundant protein, it might be limiting in Δ*lapD* bacteria because of its multiple roles in LPS, fatty acid biosynthesis, and potentially cell division.

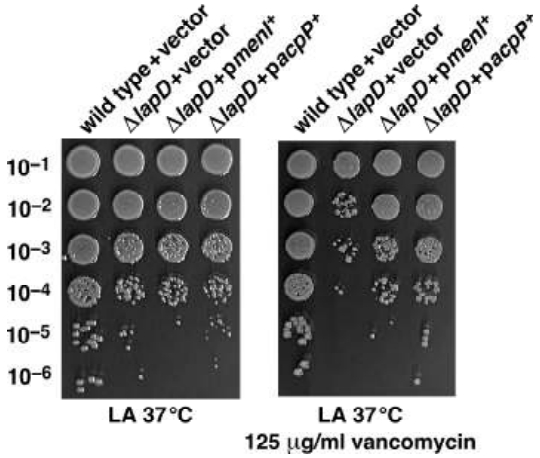

Figure 10. Overexpression of either *menI* or *acpP* genes can suppress vancomycin sensitivity of Δ*lapD* bacteria. The growth of isogenic cultures of wild type with vector alone, its Δ*lapD* derivative with empty vector, and Δ*lapD* bacteria carrying a plasmid expressing either *menI* or *acpP* genes was quantified by spot dilution on LA with and without vancomycin supplementation. The relevant genotypes and vancomycin concentration are indicated.

2.12. Overexpression Signal Sequence-Less TesA (TesA′) Overcome Cell Morphology Defects of ΔlapD Bacteria

As MenI with degenerate thioesterase activity can suppress some of the defects of Δ*lapD* bacteria, we reasoned that overexpression of a well-characterized thioesterase, such as TesA, would suppress some phenotypic defects of such bacteria; hence, we expressed the *tesA* gene in the cytoplasm. Overexpression of *tesA* has been reported to change the fatty acid composition when expressed in the cytoplasm of *E. coli* [52]. As TesA is a periplasmic protein, we cloned the *tesA* gene lacking its signal sequence (*tesA′*) downstream of the tightly regulated p*tac* promoter using the pTTQ18 vector. The *tesA′*-expressing plasmid DNA and the empty vector were used to transform Δ*lapD* to evaluate the impact on cell morphology and *tesA′* in the presence of 50 μM IPTG as an inducer of gene expression. These experiments revealed that *tesA′* overexpression suppressed the filamentous morpholgy of Δ*lapD* bacteria (Figure 11).

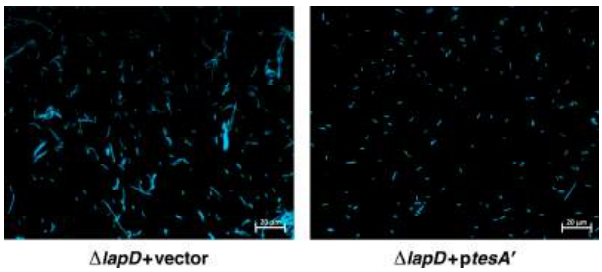

Figure 11. Induction of signal sequence-less TesA′ restores normal cellular morphology of Δ*lapD* bacteria. Microscopic images of the wild type, its isogenic Δ*lapD* with the empty vector, and Δ*lapD* expressing the *tesA′* were obtained as described in legend to Figure 6. bacterial cells were stained with DAPI and imaged using fluorescence microscopy at ×1000 with a 20 μm scale.

2.13. Induction of Either the Acyl-Carrier Protein or AccB Subunit of the Acetyl-CoA Carboxylase Enzyme or TesA′ Represses Phospholipid Biosynthesis in ΔlapD Bacteria

As shown in the above sections, overexpression of *acpP*, *accB* and *tesA′* can restore normal cell morphology and *menI* overexpression can restore vancomycin resistance of Δ*lapD* bacteria, so we further explored their impact on PL amounts by TLC analysis. Densitometric quantification of different PL species revealed that the wild-type strain contained approximately 77.69% ± 3.6 PE, followed by 17.93% ± 0.8 PG and 4.2% ± 0.4 CL (Figure 12 lane 1). These values are consistent with the known abundance of PLs in wild-type *E. coli* K-12 strains [53]. Quantification of different PL species further revealed that overexpression of either *acpP* or *accB* genes reduced the overall amounts of acidic phospholipid species, PG and CL (Figure 12). The impact of *accB* overexpression caused a more significant decrease in CL and PG amounts, with nearly 24% and 54% compared to the wild type, respectively (Figure 12). The induction of *accB* gene expression also led to a decrease in phosphatidylethanolamine (PE) presence. These experiments also revealed a drastic reduction in the PE and PG species of PL when the expression of *tesA′* was induced (Figure 12). Overexpression of *accB* can disturb the stoichiometric balance of ACC enzyme subunits, leading to the inhibition of ACC enzyme activity [32,33]. However, the induction of *menI* gene expression did not significantly alter PE and PG amounts. The main impact of MenI induction was the reduction in CL species, which was 56% compared to that of the isogenic wild type (Figure 12 Lane 6). These experiments also revealed that Δ*lapD* bacteria were not impaired in the regulation of PL, as no significant differences were observed in the abundance of PE, PG, or CL (Figure 12 Lane 2). However, Δ*lapD* bacteria do accumulate phosphatidic acid (PA) species, although less than 1%, which were not observed in PL extracted from the wild type (Figure 12 Lane 2 vs. Lane 1).

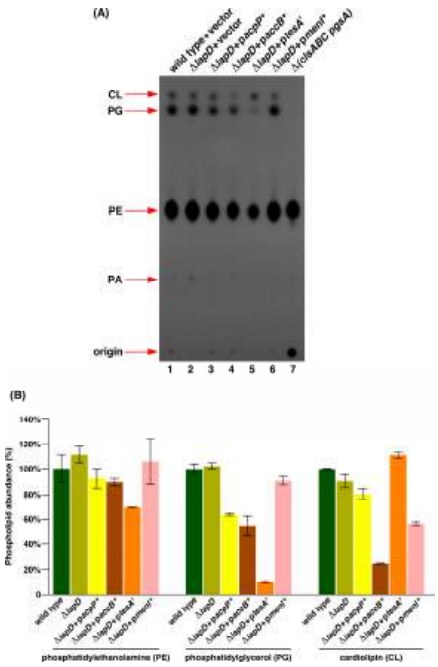

Figure 12. Induction of expression of either *acpP*, *accB* or *tesA′* genes repress phospholipid biosynthesis in Δ*lapD* bacteria. (**A**) Thin-layer chromatography of ^{32}P-labeled PLs extracted from isogenic strains carrying either vector alone or individually expressing *acpP*, *accB*, *tesA′*, and *menI* genes. As a control, ^{32}P-labeled PLs were extracted from the RU857 strain, which cannot produce CL and PG and synthesizes only PE, serving as a marker for three major PL species. A representative autoradiogram is presented. The arrows indicate the positions of major PL species. (**B**) Bar graph representing the relative amounts of CL, PG, and PE species quantified using densitometry from three biological replicates. Error bars represent a S.E. of three independent measurements.

Thus, the reduction in the amount of PL by the overproduction of either AcpP or AccB can adjust the balance between LPS and PL amounts, since Δ*lapD* bacteria have less LPS in the OM, which helps explain the mechanism of suppression and the need to achieve homeostatic control of these essential elements of the cell envelope.

2.14. Overexpression of the acpP Gene Suppresses Elevated RpoE-Regulated Envelope Stress Response of ΔlapD Bacteria

It is well established that RpoE responds to cell envelope defects in the periplasm, misfolding of OMPs, and defects in LPS and PL regulation [11,15,54,55]. Consequently, deletion of the *lapD* gene, quite like when LPS biosynthesis is dysfunctional in *lapB* or *lapC* mutants, causes elevated RpoE induction [20]. As mild induction of *acpP* gene was found to overcome cell morphology and vancomycin sensitivity defects, we examined whether its overexpression affected RpoE activity. This was measured by estimation of *rpoE*P6-*lacZ* activity in the wild type, Δ*lapD*, and its derivative carrying the inducible *acpP* gene. The *rpoEP6* promoter is well characterized, is positively autoregulated by the RpoE sigma factor [56] and is induced approximately by a 4-fold in Δ*lapD* derivative (Figure 13). Mild induction of *acpP* gene reduced RpoE activity by nearly 50% (Figure 13). These results are consistent with *acpP* acting as a multicopy suppressor of various phenotypic defects, including the reduction in elevated PL amounts and overcoming cell morphology defects in Δ*lapD* bacteria.

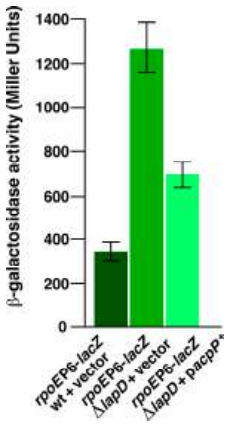

Figure 13. Overexpression of the *acpP* gene represses elevated envelope stress-responsive *rpoE* promoter activity. Exponentially grown wild type, its isogenic Δ*lapD* derivative with the vector alone, and its derivative expressing the *acpP* gene, carrying the single-copy chromosomal *rpoE*P6-*lacZ* fusion, were analyzed for the *β*-galactosidase activity. The cultures were adjusted to an OD_{595} of 0.05. IPTG (75 μM) was added to induce *acpP* expression, and the cultures were allowed to grow in LB medium at 37 °C. Aliquots of samples were drawn after different time intervals and used to measure the *β*-galactosidase activity. Error bars represent a S.E. of three independent measurements.

2.15. Essentiality of the mepS Gene in ΔlapD Bacteria

As suppressor mutation mapping to the *nlpI* gene can overcome the synthetic lethality of Δ(*lapD waaC*) bacteria, we reasoned that it could be at the level of regulation of *mepS*, since NlpI is required for MepS proteolysis by Prc protease. It has been shown that the hydrolytic activity of MepS is associated with murein incorporation during cell growth [46,47]. Thus, we examined whether MepS is required in the absence of LapD. To address this possibility, a Δ*mepS* derivative was constructed in the wild type and used in transduction experiments. Hence, parallel bacteriophage P1-mediated transductions were performed in wild-type W3110 and its Δ*lapD* derivative SR25204. A deletion *mepS* mutation could be readily introduced in the wild-type strain but not in the Δ*lapD* background, allowing us to conclude that MepS is essential in the Δ*lapD* background (Figure 14). Similar synthetic lethality

results were obtained when reciprocal transductions were performed by attempting to introduce the Δ*lapD* mutation in a Δ*mepS* background. These results confirm the essential role of murein endopeptidase MepS in the absence of LapD. These findings further support the role of LapD in coordinating LPS, PL, and PGN biosynthesis to maintain the homeostasis of these three essential components of the cell envelope in *E. coli*.

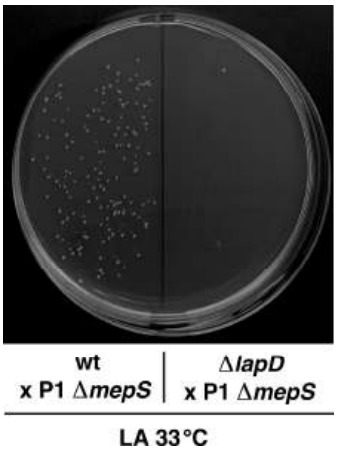

Figure 14. Peptidoglycan hydrolase MepS is essential in the absence of LapD. A representative picture of colonies obtained by bacteriophage P1-mediated transduction, when the Δ*mepS* mutation was introduced into the wild type and its isogenic Δ*lapD* derivative. LA plates were incubated for 24 h at 33 °C.

3. Discussion

The biosynthesis of two essential cell envelope components, LPS and PLs, is coupled because they share a common metabolic precursor and are maintained in a tight balance, with excess of either component leading to lethality [8,57]. However, the mechanisms underlying this phenomenon are not sufficiently clear. In this direction, it was shown that LapD physically interacts with several Fab enzymes, components of the acetyl-CoA carboxylase enzyme, and those involved in phospholipid and LPS biosynthesis [20]. However, the precise function of LapD remains elusive. Furthermore, the molecular basis of the lethality of Δ(*lapD lpxM*) and Δ(*lapD waaC*) mutational combinations, purportedly with involvement in cell division or PGN synthesis, has not been elucidated. Notably, these studies used BW25113 as the parental strain, which has a nonfunctional fatty acid regulator FabR [20,25,26,30,58], which can confound the results. Here, we reconstructed Δ*lapD* derivatives in W3110, which is FabR$^+$, and showed that suppressor-free Δ(*lapD lpxM*) derivatives can be constructed in minimal medium at 30 °C, but not in LA medium. This paved the way to address the molecular basis of their lethality on LA medium by isolating single-copy extragenic chromosomal suppressor mutations and analyzing changes in phospholipid and fatty acid composition. Additionally, the Δ(*lapD waaC*) combination in W3110, which exhibited conditional synthetic lethality at temperatures above 42 °C, was used to isolate extragenic suppressors. Furthermore, dosage-dependent suppressors that overcame the cell morphology defects, and vancomycin sensitivity of Δ*lapD* bacteria were characterized.

Mapping of extragenic chromosomal suppressors that bypassed the lethality of Δ(*lapD lpxM*) bacteria in rich medium identified that the majority of suppressor mutations mapped to *accA*, *accC*, and *accD* genes, which encode various subunits of the essential enzyme acetyl-CoA carboxylase transferase. In fatty acid biosynthesis, the first committed step of fatty acid synthesis is the carboxylation of acetyl-CoA by the acetyl-CoA carboxylase enzyme complex to produce malonyl-CoA [28]. This initiation step, catalyzed by the ACC enzyme, is a rate-limiting step that is subjected to growth phase regulation, and its activity

is inhibited by acylated derivatives of AcpP [32,33,59]. Loss-of-function mutations in the ACC complex will lead to a reduced rate of fatty acid synthesis and changes in phospholipid amounts. Indeed, complementation of *acc* mutants by single-copy cosmids carrying wild-type genes established that our suppressor mutations were loss-of-function mutations. The identification of suppressor mutations in the ACC complex is not unique to Δ(*lapD lpxM*) but reflects that late acyltransferases required for the completion of lipid A synthesis are critical for balanced LPS and phospholipid biosynthesis. More than three decades ago, work in our group identified the extragenic suppressors of Δ*lpxL* (then *htrB*), which overcame its lethal phenotype at elevated temperatures, to map to the ACC complex [60]. LpxL is required for the incorporation of the lauroyl acyl chain; hence, Δ*lpxL* strains have a majority of tetraacylated species in the lipid A part [31]. These results are also supported by the recent independent isolation of suppressor mutations of *lapD* mutant bacteria mapping to the ACC complex [29].

Analyses of the FFA composition of Δ(*lapD lpxM*) bacteria showed that they accumulated nearly 2-fold excess of unsaturated C16:1 palmitoleic acid compared to the wild type as well as more than 2.5-fold increased amount of myristic acid, which were reversed by suppressor mutations mapping to *accA* and *accC* genes when grown at 30 °C. Furthermore, a single amino acid exchange of AccC Val299 to Ala in Δ(*lapD lpxM*) bacteria resulted in more than a 2-fold increase in C18:1 *cis*-vaccenic acid amounts compared to those in the suppressor-free parental Δ(*lapD lpxM*) derivative. These large-scale changes in fatty acid composition in Δ(*lapD lpxM*) bacteria with suppressor mutations in the ACC complex could help maintain a balance between LPS and PL amounts. It has been shown that acyl-ACP with *cis*-vaccinate can be a potent inhibitor of the ACC complex; hence, feedback inhibition could result in a reduction in the rate of fatty acid balance [33]. This can be a physiologically relevant manner of overcoming synthetic lethality associated with the Δ(*lapD lpxM*) mutational combination. Notably, no major differences in the ratio of either saturated versus unsaturated fatty acids or in terms of the overall pattern of fatty acid composition were discernible between isogenic wild-type and Δ*lapD* bacteria, and the main differences were only observed when comparing Δ(*lapD lpxM*) bacteria with and without suppressor mutations.

Analysis of phospholipid composition revealed the most striking differences in composition and relative abundance, shedding further light on the potential reasons for the lethality of the Δ(*lapD lpxM*) combination. Even under permissive growth conditions, Δ(*lapD lpxM*) bacteria contained more acidic PLs than the parental strains, resulting in nearly a 3-fold increase in CL levels and approximately 60% increase in PG amounts. These results allow us to conclude that the accumulation of increased species of acidic PLs could be one of the major contributors to the lethality associated with the Δ(*lapD lpxM*) mutational combination. In support of this model, suppressor mutations in various subunits of the ACC complex significantly reversed the increase in the acidic phospholipid levels. Although no major differences in the relative abundance of PE, PG, and CL species between the wild type and its isogenic Δ*lapD* derivative were observed, a significant phosphatidic acid (PA) accumulation was found in these bacteria. PA serves as a universal precursor of glycerophospholipids (GLPs) [22], and its accumulation in Δ*lapD* bacteria suggests a requirement for LapD in controlling the amount of this precursor for PL synthesis, probably by controlling the activity of CDP-diglyceride synthetase encoded by the *cdsA* gene. Indeed, mutations in *cdsA* are known to lead to the accumulation of increased amounts of PA [61]. CDP-diacylglycerol is positioned at a branch point in the lipid biosynthetic pathway, where it reacts with *sn*-glycerol 3-phosphate to form phosphatidylglycerophosphate, or with L-serine for the synthesis of phosphatidylserine [62]. It is the source of the phosphatidyl group for all major PLs in *E. coli*.

This study also showed that LapD is involved in coordinating LPS, PL, and PGN synthesis (Figure 15). It is based on the isolation of suppressor mutations of conditional synthetic lethality of Δ(*lapD waaC*) mutants, which allow growth at temperatures above 42 °C. These suppressors were mapped to *lptD*, *nlpI*, and *bamC* genes. LptD is essential for bacterial growth, is a member of the RpoE regulon, and is required for the assembly of LPS in the OM [7,63]. Suppressor mutation in the *lptD* gene could potentiate better assembly of LPS in the OM, as a large fraction of LPS is retained in the IM in Δ*lapD* bacteria, and truncation of LPS due to the concomitant absence of WaaC could further limit its translocation, in accordance with our earlier results [20]. Consistent with the isolation of suppressor mutation in the gene encoding the LPS transporter LptD in the Δ(*lapD waaC*) background, we previously showed that increasing LPS amounts by introducing either *lapB* loss-of-function mutations or stable variants of LpxC can overcome vancomycin sensitivity of Δ*lapD* bacteria [20]. In contrast, NlpI is known to regulate the turnover of PGN hydrolases by the periplasmic protease Prc [45–47]. NlpI has been shown to bind to Prc and MepS PGN hydrolase by acting as an adaptor [46,47]. Consistent with these results, we showed that MepS becomes essential in the absence of LapD. These findings are supported by the inability to isolate transposon insertion mutations in *mepS* in the whole-genome saturated mutagenesis of a Δ*lapD* strain [26].

We further speculate that the lethality of the Δ(*lapD lpxM*) combination could also arise from dysfunctional central metabolism and cell division machinery. This notion is supported by the isolation of suppressor mutations mapping to *aceK* and *mukE* genes that allow the growth of Δ(*lapD lpxM*) bacteria. Isocitrate dehydrogenase kinase/phosphatase (AceK) controls the branch point between two pathways of central metabolism, namely the TCA cycle and glyoxylate cycle, by controlling the activity of isocitrate dehydrogenase (ICDH) [64]. Its function is central to the successful adaptation and growth of *E. coli* and related genera on acetate and fatty acids. Suppressor mutation in the *aceK* gene is located next to the catalytically essential amino acid residue D475, whose inactivation abolishes the kinase activity of AceK [37]. If the Y474S alteration abolishes AceK kinase activity, it would prevent the inactivation of IDH and hence, its diversion to the glyoxylate cycle. *E. coli* can utilize exogenous fatty acids, and growth on fatty acids requires a glyoxylate shunt to permit the entry of the acetyl-CoA products of the β-oxidation into the TCA cycle [65]. Thus, the suppressor mutation in the *aceK* gene reveals a link between LapD and the generation of acetyl-CoA. This, in turn, can affect the ability of Δ(*lapD lpxM*) bacteria to use exogenous fatty acids in the fatty acid degradation pathway to generate acetyl-CoA. Thus, LapD is a multitasking protein involved in the coordination of central metabolism, LPS, PL, and PGN synthesis (Figure 15).

It is well known that *E. coli* size is dependent on fatty acid/PL levels [23,66,67]. Consequently, increased fatty acid/PL availability results in larger cell size and cell expansion. In support of LapD involvement in cell elongation, four major observations were made: first, increased expression of the central regulator of fatty acid/PL synthesis AcpP suppresses cell morphology defects, and the isolation of the suppressor mutation of Δ(*lapD lpxM*) bacteria to the *mukE* gene and co-purification of several Muk proteins with LapD [20]. The second reason for the cell morphology defect could be due to interaction with the essential MukB protein component of the structural maintenance of the chromosome complex, which interacts with the AcpP protein, which is required for its ATPase activity and MukBEF function in vivo [68]. The third reason for the cell morphology defect could be the requirement for MepS in Δ*lapD* bacteria. MepS plays an essential role in cell expansion. As discussed above, MepS, in addition to promoting cell elongation, links LapD to PL, LPS, and PGN homeostasis. Furthermore, it has been shown that elevated MepS activity can interfere with the activation of septal PG biogenesis by the divisome [69]. In support of

these observations, MepS levels were shown to be stabilized in Δ*lapD* bacteria [70]. The fourth reason comes from the measurement of PL amounts in Δ*lapD* bacteria when AccB or AcpP are overproduced, that showed a reduction in PL species, which is simultaneously associated with the restoration of normal cell morphology.

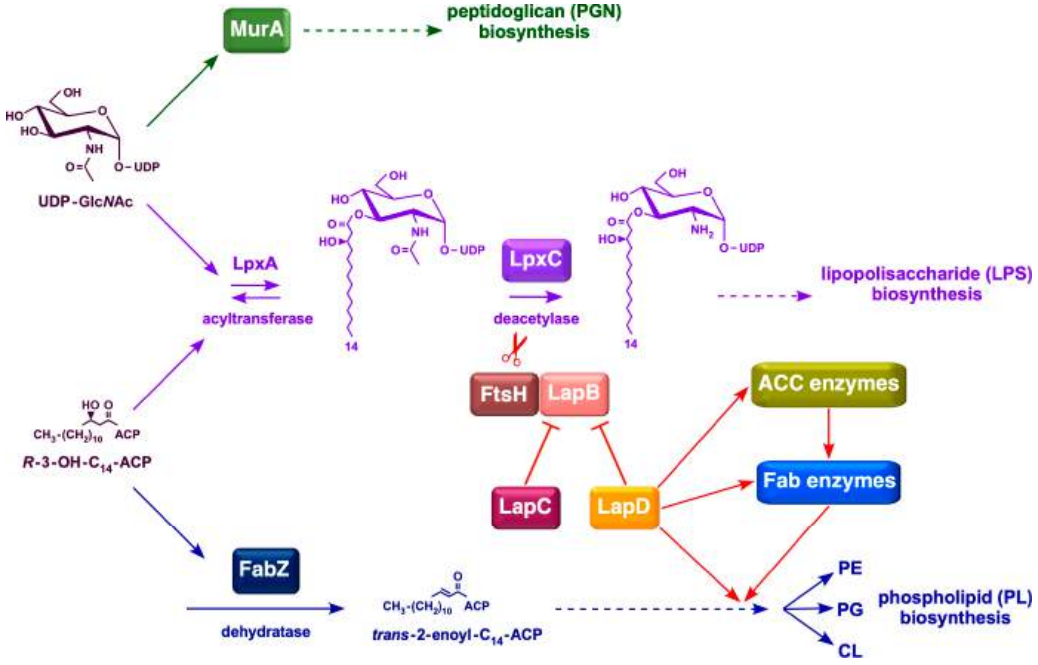

Figure 15. Model of coupled biosynthesis of three essential components of the cell envelope of *E. coli*. The shared precursors and initial enzymes of the three biosynthetic pathways are depicted. The importance of LapD at the interface of LPS and phospholipid/fatty acid synthesis is indicated by the arrows.

Finally, in this study, we addressed the molecular basis of the synthetic lethality of Δ(*lapD lpxM*) and the conditional lethality of the Δ(*lapD waaC*) combination using genetic and biochemical assays. The PL and fatty acid composition suggested that Δ(*lapD lpxM*) lethality could be due to an excess of PL, and this can be overcome by suppressor mutations in *accA*, *accC*, and *accD* genes thereby, bypassing this lethality. In support of these results, we also showed that the inhibition of fatty acid biosynthesis by the FabI inhibitor triclosan restored the growth of Δ(*lapD lpxM*) bacteria. Suppressor analysis of Δ(*lapD waaC*) mapping to the *nlpI* gene, whose product regulates MepS murein hydrolase, further demonstrated that LapD coordinates LPS and PGN synthesis. Consistent with this conclusion, we showed that MepS murein endopeptidase is essential in the absence of LapD. Furthermore, the requirement for hexaacylated lipid A in Δ*lapD* could be critical for its survival, since the absence of LpxM renders it pentaacylated and could impede LPS translocation. Consistent with the function of LpxM, it is required for the viability of CL-deficient strains [71,72]. This is further supported by the significant alteration of CL amounts in Δ(*lapD lpxM*) bacteria and their suppressors. However, further studies are required to directly measure alterations in acyl-ACP pools in Δ(*lapD lpxM*) bacteria and their suppressors and measure the impact of suppressor mutations in *acc* genes on the biochemical activity of the ACC complex.

4. Materials and Methods

4.1. Bacterial Strains, Plasmids and Media

The bacterial strains and plasmids used in this study are described in Table 4. Luria–Bertani (LB) broth and M9 minimal medium (Difco, Franklin Lakes, NJ, USA) were prepared as described earlier [73]. When required, the media were supplemented with ampi-

cillin (100 μg/mL), kanamycin (50 μg/mL), tetracycline (10 μg/mL), or chloramphenicol (20 μg/mL). When needed, vancomycin (125 μg/mL), cerulenin (25 μg/mL), or triclosan (0.01 and 0.05 μg/mL) were added to the growth medium. To induce the expression of various genes, 50 or 75 μM IPTG was supplemented as per the experimental requirements.

Table 4. Bacterial strains and plasmids used in this study.

Strains	Genotype	Reference
W3110	λ^-, *IN* (*rrnD-rrnE*)*1*, *rph-1*	CGSC, Yale
GK1275	W3110 *lpxM<>aph*	[31]
SR7336	GK1275 *lpxM<>frt*	This study
SR8035	W3110 *waaC<>aph*	[31]
SR25196	W3110 *lapD<>aph*	This study
SR25204	SR25196 *lapD<>frt*	This study
GK6173	SR25204 *lapD<>frt lpxM<>aph*	This study
SR25236	SR7336 *lpxM<>frt lapD<>aph*	This study
SR25217	SR25204 *lapD<>frt lpxM<>aph accD* L39P	This study
SR25218	SR25204 *lapD<>frt lpxM<>aph accA* K31E	This study
SR25219	SR25204 *lapD<>frt lpxM<>aph accC* V299A	This study
SR25220	SR25204 *lapD<>frt lpxM<>aph accD* S173P	This study
SR25224	SR25204 *lapD<>frt lpxM<>aph accA* R175L	This study
SR25225	SR25204 *lapD<>frt lpxM<>aph accC* R253H	This study
SR25235	SR25204 *lapD<>frt lpxM<>aph accA* K31E	This study
SR25240	SR25204 *lapD<>frt lpxM<>aph accC* V299A	This study
SR25241	SR7336 *lpxM<>frt lapD<>aph accC* K40T	This study
SR25307	SR25204 *lapD<>frt lpxM<>aph accA* R175L	This study
SR25438	SR7336 *lpxM<>frt lapD<>aph accC* I309F	This study
SR25437	SR7336 *lpxM<>frt lapD<>aph opgG* A100T	This study
SR25443	SR7336 *lpxM<>frt lapD<>aph opgG* A100T	This study
SR25454	SR7336 *lpxM<>frt lapD<>aph accC* V42I	This study
SR25452	SR7336 *lpxM<>frt lapD<>aph aceK* Y474S	This study
SR25458	SR7336 *lpxM<>frt lapD<>aph mukE* A122P	This study
SR25651	SR25204 *lapD<>frt waaC<>aph*	This study
SR25660	SR25204 *lapD<>frt waaC<>aph lptD* L651P	This study
SR25661	SR25204 *lapD<>frt waaC<>aph nlpI* Y243H	This study
SR25662	SR25204 *lapD<>frt waaC<>aph lptD* L651P *bamC* F248L	This study
RU857	Δ*pgsA* Δ(*clsABC*) *lpp*2	[74]
SR25199	SR25204 *lapD<>frt* + p*acpP*$^+$	This study
SR25203	SR25204 *lapD<>frt* + p*accB*$^+$	This study
SR25870	SR25204 *lapD<>frt* + p*tesA′*	This study
SR25872	SR25204 *lapD<>frt* + p*menI*	This study
SR25467	W3110 *accA* K31E Tn*10*	This study
SR25468	W3110 *accC* V299A Tn*10*	This study
SR25874	W3110 + pCA24N	This study
SR23799	SR25204 *lapD<>frt* + pCA24N	This study
SR25878	W3110 + pTTQ18	This study
SR25880	SR25204 *lapD<>frt* + pTTQ18	This study
Plasmids	**Genotype**	**Reference**
pCA24N	IPTG-inducible expression vector cm^R	[75]
pKD3	*oriR6K$_g$*, *bla*(Amp^R), *kan*, *rgnB*(Ter), *cat*	[76]
pKD13	*oriR6K$_g$*, *bla*(Amp^R), *kan*, *rgnB*(Ter)	[76]
pKD46	*araBp-gam-bet-exo*, *bla*(Amp^R), *repA101*(ts)	[76]
pCP20	ts replicon with inducible FLP recombinase	[76]
pTTQ18	expression vector with LacI represor	[77]
pSR25845	*tesA′* in pTTQ18	This study
JW1080	*acpP*$^+$ in pCA24N cm^R	[75]
JW3223	*accB*$^+$ in pCA24N cm^R	[75]
JW1676	*menI*$^+$ in pCA24N cm^R	[75]
JW5539	*lapD*$^+$ in pCA24N cm^R	[75]
pREG	cosmid cloning vector	[78]
pSR25460	*accA*$^+$ in pREG amp^R	This study

Table 4. *Cont.*

Plasmids	Genotype	Reference
pSR25461	*accC*$^{+}$ in pREG amp^{R}	This study
pSR25469	*accD*$^{+}$ in pREG amp^{R}	This study

4.2. Strain Construction and the Isolation of Extragenic Chromosomal Suppressors

Constructions of non-polar deletion derivatives of *lpxM*, *lapD*, and *waaC* have been previously described [20,31]. In this study, W3110 served as the parental wild-type strain, as it has been widely used as the *E. coli* K-12 wild-type strain in LPS structural analysis. Hence, deletion mutations of *lapD*, *waaC*, and *lpxM* were transduced by bacteriophage P1- or T4-mediated transductions into W3110, as described earlier [79,80]. The non-polar deletion derivative of the *mepS* gene was constructed by replacing its coding sequence by the *aph* cassette from the pKD13 plasmid using the λ recombinase system, as described previously [11,76]. To construct deletion combinations of Δ(*lapD lpxM*), Δ(*lapD waaC*), and Δ(*lapD mepS*), bacteriophage-mediated transductions were performed. In the case of Δ(*lapD lpxM*) construction, thusly obtained transductants were plated on M9 minimal agar plates at 30 °C, which was found to provide permissive conditions. As a control, each recipient in the transduction experiments was transformed with a covering plasmid expressing either *lpxM*, *lapD* or *waaC* genes. As Δ(*lapD lpxM*) transductants were only viable on M9 minimal medium, to identify extragenic chromosomal suppressor mutants, several independently obtained suppressor-free cultures grown under permissive growth conditions were plated on LA plates at 37 or 42 °C. From these Δ(*lapD lpxM*) Ts^{+} isolates, 150 putative suppressor-containing strains were retained. Suppressor mutations were marked with mini-Tn*10* Cm [81], and verified that the Tn*10*-linked suppressor mutation breeds true. A previously described cosmid library [15,78] in a single-copy vector was used to identify cosmid clones that could recombine linked Tn*10* Cm, which were used to group 16 out of 23 suppressors in six complementation groups. To identify the position of the linked transposon, Tn*10* and its flanking regions were subcloned from recombinant cosmid clones, and their DNA was sequenced. The same cosmids sets were used for complementation studies. To identify the mutation in a specific gene, chromosomal DNA of 16 suppressor strains was isolated and used to PCR amplify candidate genes, which were then subjected to DNA sequencing. The chromosomal DNA of the remaining seven Ts^{+} Δ(*lapD lpxM*) strains was used for whole-genome sequencing performed by Novogene Europe, as their suppressor mutation could not be marked by a single Tn*10*. To isolate Ts+ suppressors of Δ(*lapD waaC*) strains, cultures were plated at 42 °C on LA medium. The same approach of mapping the position of suppressor mutation of Δ(*lapD waaC*) was used after marking with Tn*10*. Two of the five Ts^{+} suppressor-carrying strains yielded Tn*10* recombinant cosmids, defining regions covering *lptD* and *nlpI* genes. The third suppressor strain yielded cosmids covering two different genomic regions. To identity the gene with the suppressor mutation, the chromosomal region spanning the candidate genes was PCR-amplified and subjected to DNA sequencing.

4.3. The Identification of Multicopy Suppressors, Whose Overexpression Overcomes Vancomycin Sensitivity of ΔlapD Bacteria

The complete genomic library of all predicted ORFs of *E. coli* cloned in pCA24N (ASKA collection) [75] was used to transform the Δ*lapD* strain SR25204. As Δ*lapD* bacteria exhibit sensitivity to vancomycin, multicopy suppressors were directly selected for the restoration of growth on LA medium supplemented with 125 μg/mL of vancomycin and 75 μM IPTG to induce the gene expression from the P_{T5}-*lac* promoter of the pCA24N vector. Vancomycin-resistant colonies were purified and used to isolate plasmid DNA. DNA from such plasmids was used to retransform the Δ*lapD* strain SR25204 on vancomycin-

supplemented plates in the presence of 75 μM IPTG. Plasmids that allowed the growth of Δ*lapD* bacteria on vancomycin were retained, and their DNA was sequenced.

4.4. The Construction of Plasmid Expressing Signal Sequence-Less tesA Gene

The *tesA* gene lacking its signal sequence (*tesA′*), with Shine–Dalgarno sequences appended at the 5′ end with oligo nucleotides *tesA*_For: 5′-CATTAGgaattcTAAGGAGGACGT ACATGGCGGACACGTTATTGATTCTGGGTG-3′ and *tesA*_Rev: 5′-CATTAGggatccTTATG AGTCATGATTTACTAAAGGC-3′, was PCR amplified. PCR products were digested with EcoRI and BamHI and cloned into the pTTQ18 expression vector [77] using the same restriction sites.

4.5. Isolation and Analysis of ^{32}P-Labeled Phospholipids Species

Overnight cultures, grown in M9 minimal medium at 30 °C [permissive conditions for Δ(*lapD lpxM*) bacteria], were harvested by centrifugation at 4300× *g* for 10 min. Pellets were resuspended in M9 medium at an OD_{595} of ≈0.05 and allowed to grow up to an OD_{595} of 0.25. This was necessitated by the fact that suppressor-free Δ(*lapD lpxM*) bacteria can be propagated on minimal medium and survive only a few doublings in rich medium at 37 °C. Cultures were centrifuged and washed 3X in LB medium and labeled with 2.5 μCi/mL of $^{32}P_i$ in 2.5 mL of LB medium until an OD_{595} of 1.0. These growth conditions were necessary because ^{32}Pi cannot be efficiently incorporated into the phosphate-rich M9 minimal medium. When required, during the labeling period, 50 μM IPTG was added to induce the expression from the p*tac*-regulated *tesA′* gene and 75 μM IPTG was added for the expression of P_{T5}-*lac* promoter-inducible *menI*, *acpP*, and *accB* genes. The cultures were harvested by centrifugation, and PLs were extracted using the procedure described by Bligh and Dyer, and Ames [82,83]. PL were collected from the lower organic phase and dried under a stream of nitrogen. Dried samples containing PLs were reconstituted in 50 μL of a 2:1 chloroform/methanol mixture. In all these experiments, before the extraction of PLs, an aliquot of the samples was used to measure the total protein concentration using the Pierce BCA protein assay kit (ThermoScientific Poland, Warsaw, Poland). Equivalent amounts (10,000 cpm/lane) of ^{32}P-labeled PLs after adjusting for protein concentration were separated on TLC Silica gel 60 F254, aluminum sheets, 20 cm × 20 cm plates (Merck, Mississauga, ON, Canada). The lipids were separated on plates using a mobile phase of chloroform/ethanol/water/tri-ethylamine 30:35:7:35 (*v*/*v*/*v*/*v*) [84]. The plate was dried and exposed to Kodak BioMax XAR X-ray film or exposed to a PhosphorImager screen to visualize and quantify the labeled PLs using the Amersham Typhoon Biomolecular Imager (Cytiva, Uppsala, Sweden). Densitometry analysis of the autoradiograms was performed using the software from Bio-Rad, Warsaw Poland.

4.6. Isolation of Fatty Acids and Their Analysis by Gas Chromatography

Free fatty acids were extracted according to an established procedure [85]. Briefly, isogenic bacterial cultures were grown as described for phospholipid extraction without adding $^{32}P_i$. 15 mL cultures were harvested by centrifugation, and the frozen pellets were resuspended in 2.5 mL of H_2O and vortexed. To this mixture, 5 μL of 10 mg/mL heptadecanoic acid (C17:0) (Merck) dissolved in ethanol was added as an internal standard. Fatty acid methyl esters were obtained by treating the dried extract with 0.5 mL of 1.25 M HCl in methanol and incubated at 50 °C for 15 h. Fatty acids were extracted in 1 mL of hexane and analyzed by gas chromatography using a Shimadzu GC-2010 Pro (Kyoto, Japan) with a 30 m × 0.25 mm capillary column. Using an autosampler, a 1 μL sample was injected using a 1:100 split ratio of the helium carrier gas. The column temperature was maintained at 100 °C with an SPL1 temperature of 250 °C and an SPL1 pressure. For calibration, a commercial fatty acid methyl ester mix C8-C22 (CRM18920 Merck, Warsaw Poland) was

used to establish the retention time of various peaks, serving as the reference material, and the data were analyzed using the software provided by Shimadzu (Kyoto, Japan).

4.7. Examination of Cellular Morphology

For 4′,6-diamidino-2-phenylindole (DAPI) staining, cultures of the wild-type strain, its isogenic Δ*lapD* transformed with the vector alone, or with the plasmids carrying either *acpP*, *accB*, or *tesA*′, were grown in LB medium at 37 °C in the presence of 0.3% glucose, supplemented with the appropriate antibiotic. Overnight cultures were washed and diluted 1:100, and when the OD_{595} reached 0.1, they were supplemented with 75 μM IPTG to induce gene expression from the P_{T5}-*lac* promoter. In the case of *tesA*′, the expression was induced with 50 μM IPTG. Cultures were grown up to an OD_{595} of 0.6 under permissive conditions at 37 °C, and aliquots of cultures were centrifuged at 4300× *g* for 5 min, incubated for 10 min in TBS with 10 μg/mL DAPI solution, and immobilized on agarose pads prior to microscopic observation. The cell morphology was examined using epifluorescence and differential interference contrast (DIC) microscopy. Filters used for DAPI: excitation wavelength 370–410 nm, emission wavelength 430–470 nm. Cells were observed using a Zeiss apotome microscope (Carl Zeiss, Jena, Germany).

4.8. Immunoblotting and Measurement of β-Galactosidase Activity

To measure the *β*-galactosidase activity, isogenic cultures of the wild type and its Δ*lapD* derivative with the vector alone or the plasmid expressing the *acpP* gene carrying *rpoE-lacZ* promoter fusions were grown overnight under permissive growth conditions. Cultures were adjusted to an optical density OD_{595} of 0.05 and allowed to grow at 30 °C for another 30 min. IPTG (75 μM) was added to induce the *acpP* gene expression. Aliquots of cultures were taken after different intervals and analyzed for *β*-galactosidase activity as described previously [11]. For immunoblotting, isogenic bacterial cultures of wild type and its Δ*lap*D, Δ(*lapD lpxM*), and its derivatives with extragenic suppressor mutations in *acc* genes and as a control Δ*ftsH sfhC21* were grown in M9 minimal medium at 30 °C to an OD_{595} of 0.5. Equivalent amounts of proteins were applied to a 12% SDS-PAGE and transferred onto a membrane for Western blotting. The blots were probed with polyclonal antibodies against LpxC and revealed using a chemiluminescence kit (Thermo Scientific, Warsaw, Poland) according to the manufacturer's instructions.

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Suppressors of *lapC* Mutation Identify New Regulators of LpxC, Which Mediates the First Committed Step in Lipopolysaccharide Biosynthesis

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Abstract: Gram-negative bacteria, such as *Escherichia coli*, are characterized by an asymmetric outer membrane (OM) with lipopolysaccharide (LPS) located in the outer leaflet and phospholipids facing the inner leaflet. *E. coli* recruits LPS assembly proteins LapB, LapC and LapD in concert with FtsH protease to ensure a balanced biosynthesis of LPS and phospholipids. We recently reported that bacteria either lacking the periplasmic domain of the essential LapC protein (*lapC190*) or in the absence of LapD exhibit an elevated degradation of LpxC, which catalyzes the first committed step in LPS biosynthesis. To further understand the functions of LapC and LapD in regulating LPS biosynthesis, we show that the overproduction of the intact LapD suppresses the temperature sensitivity (Ts) of *lapC190*, but not when either its N-terminal transmembrane anchor or specific conserved amino acids in the C-terminal domain are mutated. Moreover, overexpression of *srrA, marA, yceJ* and *yfgM* genes can rescue the Ts phenotype of *lapC190* bacteria by restoring LpxC amounts. We further show that MarA-mediated suppression requires the expression of *mla* genes, whose products participate in the maintenance of OM asymmetry, and the SrrA-mediated suppression requires the presence of cardiolipin synthase A.

Keywords: lipopolysaccharide (LPS); LPS assembly proteins LapB; LapC and LapD; MarA; LpxC; acyltransferases LpxL and LpxM

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1. Introduction

The most distinguishing feature of Gram-negative bacteria, such as *Escherichia coli*, is the presence of an asymmetric outer membrane (OM) [1]. This asymmetry is due to the characteristic location of lipopolysaccharide (LPS) in the outer leaflet of the cell envelope, with phospholipids facing its inner leaflet [1,2]. The maintenance of this OM asymmetry is essential to impart a permeability barrier, which prevents the entry of bulky toxic molecules, such as antibiotics and bile salts, inside the cells. Thus, with few exceptions, LPS is essential for bacterial viability and is one of the major virulence factors in pathogenic Gram-negative bacteria. LPS is a complex glycolipid and, in general, they share a common architecture and can be, for convenience, divided into three parts. The most conserved component is the hydrophobic membrane-anchored lipid A part, which constitutes the endotoxin principal. To the lipid A part is attached a core oligosaccharide, to which an oligosaccharide of variable length called the *O*-antigen is attached in bacteria with smooth LPS [3]. The biosynthesis of LPS at the genetic and biochemical levels is well established, although how LPS amounts are regulated and its final assembly in the OM is not well understood. This is particularly important because bacteria must maintain a strict balance of 1:0.15 between phospholipids and LPS, the two essential components of the cell envelope [4]. In *E. coli*, this is achieved by

regulating the first committed step in LPS biosynthesis, catalyzed by the essential enzyme LpxC, because LPS and phospholipids use the same (*R*)-3-hydroxymyristate as the common metabolic precursor [5]. However, the regulation of LpxC as we understand now turns out to be more complex than it was thought a few years ago, as it responds to multiple physiological cues and a regulated turnover by FtsH and HslVU proteases (Figure 1) [5,6].

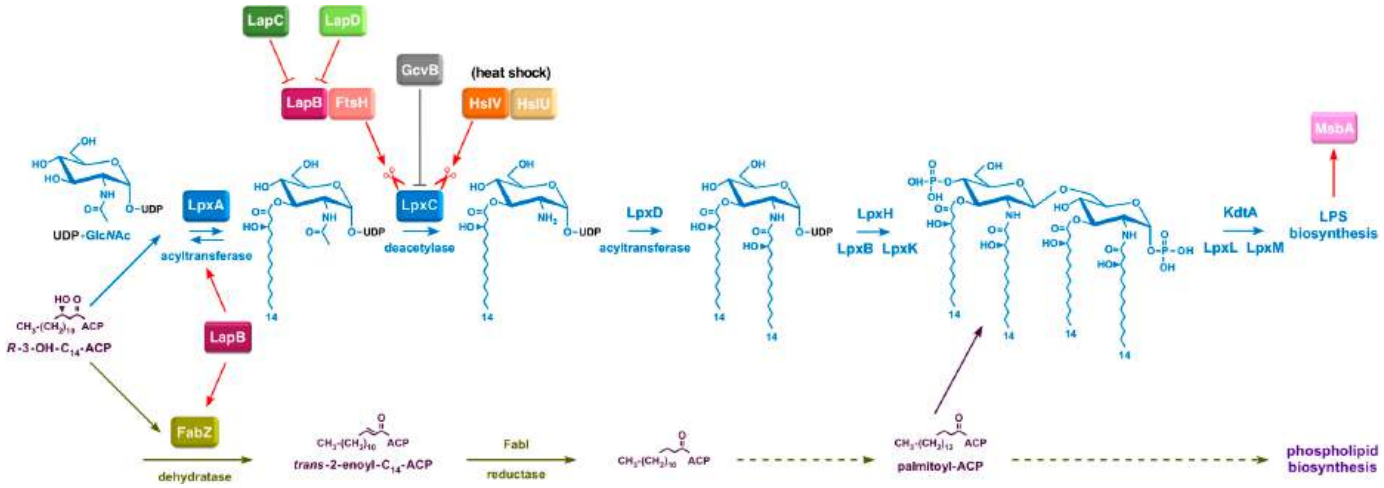

Figure 1. Schematic drawing depicting various players that regulate the first committed step in LPS biosynthesis catalyzed by LpxC and maintain a balance between LPS and phospholipids. Shown is the utilization of the common metabolic precursor (*R*)-3-hydroxymyristate by LpxA and by FabZ in LPS and phospholipid biosynthesis, respectively. LpxC amounts are regulated by its turnover by the FtsH-LapB complex and at high temperature by HslVU protease. LapC and LapD act as antagonist of LapB to regulate LPS biosynthesis as per its demand. Also shown is LapB interaction with LpxA and FabZ, thus acting as a central hub. LpxC amounts can also be fine-tuned by GcvB sRNA.

In *E. coli*, the biosynthesis and transport of LPS require products of more than thirty genes and many of them are essential for bacterial viability [2]. Briefly, LPS biosynthesis occurs on the inner leaflet of the inner membrane and begins with the acylation of UDP-GlcNAc, catalyzed by LpxA. In *E. coli*, LpxA is selective for β-hydroxymyristoyl acyl carrier protein [7]. This is followed by deacetylation of UDP-3-*O*-(acyl)-GlcNAc by the Zn^{2+}-dependent LpxC enzyme [8]. As the equilibrium constant for LpxA-mediated acylation is unfavorable, LpxC-catalyzed deacetylation becomes the first committed step in lipid A biosynthesis [9]. Following the deacetylation step, a second β-hydroxymyristoyl chain is incorporated by *E. coli* LpxD, leading to the synthesis of UDP-2,3-diacyl-GlcN [10]. Three additional enzymes, LpxH, LpxB and LpxK, act successively to generate the intermediate lipid IV_A [11]. This intermediate serves as an acceptor for the incorporation of two 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) residues by WaaA, with CMP-Kdo as the donor, generating Kdo_2-lipid IV_A [12]. In *E. coli*, up to the step of Kdo_2-lipid IV_A biosynthesis, all enzymes are essential for bacterial viability, although strains synthesizing only a lipid IV_A precursor or Kdo_2-lipid IV_A can be constructed [13]. The incorporation of Kdo residues serves as a key intermediate to generate hexaacylated lipid A via sequential acylation by lauroyl (LpxL) and myristoyl (LpxM) transferases and further incorporation of sugar residues using specific glycosyltransferases to produce a core oligosaccharide linked to the lipid A part [13,14]. Strains synthesizing tetraacylated lipid A can only grow on minimal medium and exhibit an elevated cell envelope stress response [13,14]. Interestingly, suppressors mapping to the *msbA* gene encoding LPS flippase can allow the growth of strains devoid of LpxL, LpxM and LpxP on rich medium and at elevated temperatures. These observations are in agreement with the known higher selectivity exerted by MsbA for hexaacylated lipid A as compared to the less favored underacylated species. Individually, all three late acyltransferases are nonessential, although Δ*lpxL*

exhibits a temperature-sensitive growth phenotype. However, LpxL and LpxM become essential when cardiolipin synthase A, encoded by the *clsA* gene, is absent, suggesting that MsbA and ClsA could cooperate in LPS trafficking, although the precise mechanism of their interaction remains unknown [15,16]. Thus, besides the regulatory control exerted by the regulation of LpxC amounts, the preferential selection of hexaacylated lipid A species by MsbA constitutes early checkpoints in controlling the proper amounts of LPS and its translocation across the inner membrane [17].

Initial studies on the regulation of LpxC deacetylase revealed that its levels are controlled in a 20-fold range in relation to the lipid A content without any increase in the corresponding mRNA levels of the encoded gene [18]. Subsequently, it was shown that LpxC is a substrate of FtsH protease and this proteolytic activity might be affected by alterations in acyl-ACP pools [5]. Such a model of FtsH-mediated proteolysis of LpxC could, in turn, provide a balance between biosynthesis of phospholipids and lipid A since both derive their fatty acyl chains from the same (*R*)-3-hydroxyacyl-ACP pool (Figure 1) [5]. In an important breakthrough, a new essential inner membrane protein was identified and shown to co-purify with FtsH and several proteins involved in either LPS and phospholipid biosynthesis or LPS translocation, and hence designated the LPS assembly protein LapB [19]. The absence of LapB could be tolerated either in the presence of a hyperactive *fabZ* variant that allowed an *ftsH* deletion or when the biosynthesis of LPS was reduced [19,20]. The co-purification of LapB with FtsH, FabZ, WaaC and Lpt proteins led to the proposal that LapB could act as a hub for LPS assembly in the IM and further cooperate with FtsH to regulate LpxC turnover [19]. More recent studies have validated these earlier observations and indeed, LapB was found to interact with LpxA, LpxC, LpxD and FabZ [21]. Similarly, recent structural and biochemical studies have shown that LpxC degradation by FtsH requires LapB [22]. However, how such LpxC degradation by FtsH-LapB is adjusted to the cellular demand for LPS remained unknown till the more recent discovery of LapC [2]. We and other independent research groups have shown that LapC acts as an antagonist of the proteolytic pathway of LpxC mediated by FtsH-LapB [6,22–26]. Our results were further supported by an examination of the properties of strains with chromosomal mutations that cause truncations in the periplasmic domain of LapC [6]. Such mutant bacteria exhibited a temperature-sensitive (Ts) phenotype, hypersensitivity to the LpxC inhibitor CHIR090, hyperdegradation of LpxC and consequently reduced levels of LPS (Figure 1). Thus, not only our group, but parallel independent studies, identified extragenic suppressors of *lapC* mutant bacteria mapping to the *lapA/B* operon, *lpxC* and *ftsH* genes [6,23,24,26,27]. Furthermore, an additional partner, LapD, which also co-purifies with LapB, was identified [28]. Δ*lapD* bacteria were found to exhibit a Ts phenotype and membrane permeability defects with a synthetic lethality in the absence of either cardiolipin synthase A, or when LpxL or LpxM acyltransferase was absent or when bacteria synthesize LPS composed of only Kdo_2-lipid A due to the absence of WaaC heptosyltransferase [28,29]. Moreover, extragenic suppressors that overcome defects of *lapC* mutant bacteria were also found to rescue the Ts phenotype or vancomycin sensitivity of Δ*lapD* bacteria [28].

However, how LapB, LapC and LapD sense LPS biosynthetic demand and whether there are additional factors that either inhibit or enhance LpxC degradation remain unknown. Adding to this complexity, LpxC can be degraded in the absence of LapB by HslVU, and levels of LpxC can also be regulated by regulatory small RNAs, although the precise mechanism remains unknown (Figure 1) [6]. LPS synthesis can also be stimulated by additional signals that may involve systems that respond to lipid asymmetry, including the activation of outer membrane phospholipase PldA, the Mla system that facilitates retrograde lipid trafficking, and PagP palmitoyltransferase [30]. LpxC stability can further respond to changes in levels of acyl-CoA, ppGpp and alterations in the levels of saturated fatty acids as compared to unsaturated [17,31]. Thus, the regulation of LpxC and, in turn, that of LPS involves several factors. Hence, in this study, we identified additional genes whose products might regulate LpxC or LPS levels by selecting for suppressors whose

overexpression can overcome the Ts phenotype of *lapC* mutant bacteria (Figure 2). We show that overexpression of the *lapD* gene and some other potential new regulatory factors (MarA, SrrA, DksA and YceJ) can suppress the Ts phenotype of *lapC190* mutant bacteria and, among these, some act by increasing the amount of LpxC. MarA is a well-characterized transcription factor that regulates the expression of several genes involved in regulating antibiotic resistance [32]. Using transposon mutagenesis, gene(s) whose products are required for MarA- and SrrA-mediated suppression were identified to gain a better understanding of that factors that limit bacterial growth when LapC is dysfunctional and additional players that regulate LpxC amounts (Figure 2).

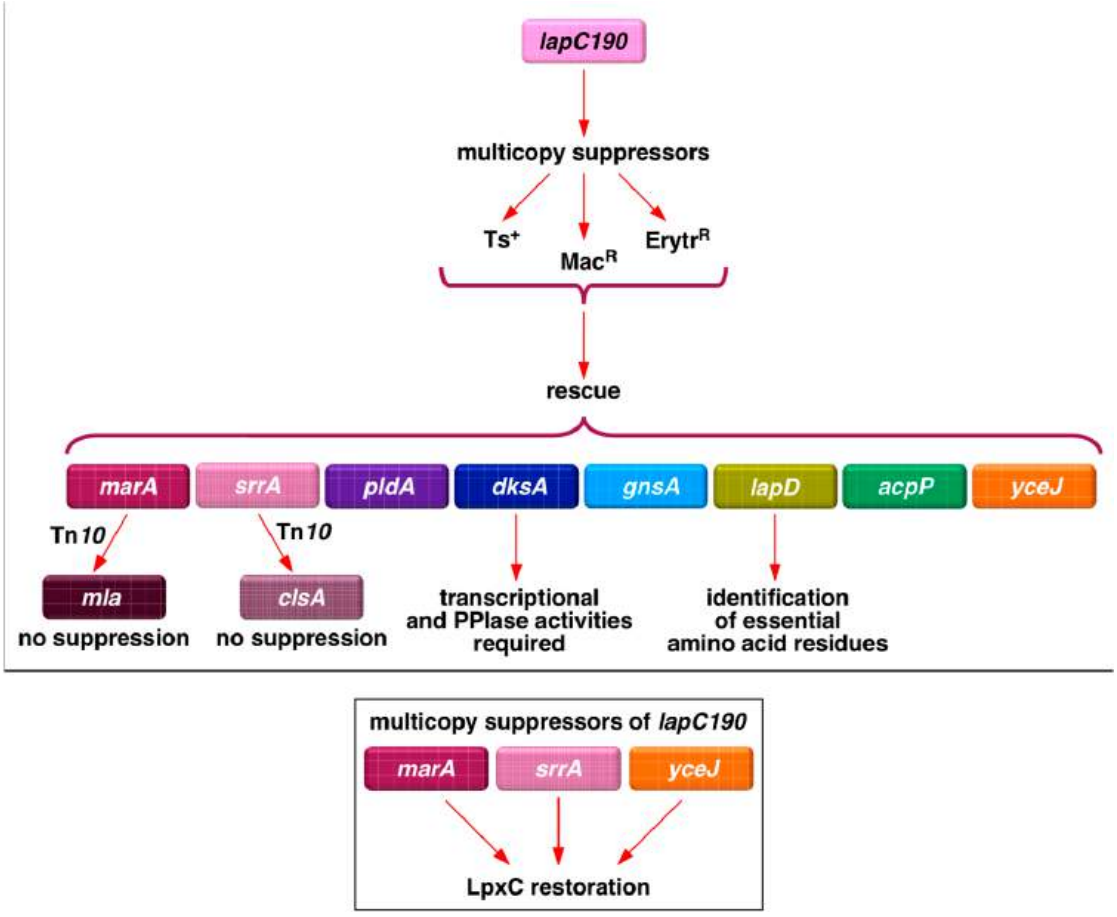

Figure 2. Schematic depiction of various approaches that identify new regulators of LpxC. Multicopy suppressors of sensitivity to high temperature, growth on either MacConkey agar or erythromycin of *lapC190* identified several genes, some of which act by increasing LpxC and LPS amounts (bottom panel). Transposon mutagenesis showed that while as MarA-mediated suppression requires the functional presence of Mla system, SrrA-dependent suppression needs the presence of cardiolipin synthase A.

2. Results

2.1. Genes Whose Overexpression Overcomes the Temperature Sensitivity (Ts) of lapC Mutant Bacteria with a Truncation of LapC Periplasmic Domain Identify New Players That Can Regulate LpxC Amounts

We previously reported the isolation of mutations in the essential gene encoding the LapC protein. Such mutations cause truncation in the periplasmic domain of LapC and confer a Ts phenotype, permeability defects and a reduced amount of LPS due to the hyperdegradation of LpxC [6]. Some of these phenotypic defects could be suppressed by chromosomal single-copy mutations mapping to *lpxC*, *ftsH* and the *lapA/B* operon [6]. This suppression was attributed to the stabilization of LpxC. However, since several

factors can contribute towards the regulation of LpxC, here we asked if the overexpression of any gene can also suppress growth defects of *lapC* mutant bacteria. Thus, we used *lapC190* mutant bacteria, which lack the periplasmic domain of LapC on the chromosome, to isolate multicopy suppressors, using a complete genomic library. To achieve this goal, plasmid DNA pools from the ASKA collection of single ORFs expressed from the tightly regulated IPTG-inducible P_{T5}-*lac* promoter in the vector pCA24N [33] were used to transform competent cells of *lapC190* mutant bacteria. Transformants were selected for the restoration of growth on LA medium supplemented with 75 μM IPTG at 43.5 °C, a condition that is non-permissive for *lapC190* mutant bacteria. This concentration of inducer of gene expression has been optimized as described earlier [19]. Cultures from identified Ts^+ colonies were used to extract plasmid DNA to retransform *lapC190* bacteria. Plasmids that bred true were retained and their DNA was sequenced, which identified 13 unique genes, whose mild overexpression can rescue the Ts phenotype of *lapC190* mutant bacteria (Table 1, Figure 3). Based on their known or predicted function, these genes can be grouped into various classes: those with a role in LPS, phospholipid/fatty acid biosynthesis or sensing (*lapD*, *acpP, acpT*, *accB*, *pldA*) (Figures 3 and 4), transcription factors (*marA*, *dksA*, *srrA*), an IM-anchored heat shock protein-encoding gene with an unknown function (*yceJ*) and others whose products could either titrate the FtsH protease responsible for LpxC degradation (*yfgM*) or modulate saturated versus unsaturated fatty acid abundance (*gnsA*) or related with the stress response (*ymgG*), whose product is the part of toxin–antitoxin system.

Table 1. Identification of the multicopy suppressors of *lapC190* bacteria that restore the growth at high temperatures and on MacConkey agar.

	Growth Conditions			
Gene	**LA 43 °C**	**MacConkey Agar 37 °C**	**Δ*lapD***	**Function**
yfgM	+	+	+	FtsH substrate, ancillary SecYEG translocon subunit
dksA	+	+	+	RNA polymerase-binding transcription factor
artJ	+	+	+	L-arginine ABC transporter periplasmic binding protein
acpP	+	+	+	acyl carrier protein
accB	+	-	+	biotin carboxyl carrier protein
lapD	+	ND	+	LPS assembly protein
pldA	+	-	-	outer membrane phospholipase A
marA	+	±	-	DNA-binding transcriptional dual regulator
yceJ	+	-	-	putative cytochrome b561
gnsA	+	-	-	putative phosphatidylethanolamine synthesis regulator
ymgG	+	-	±	PF13436 family protein toxin-antitoxin system
srrA	+	+	+	transcription (stress response regulator A)
acpT	+	+	-	*holo*-(acyl carrier protein) synthase 2

ND denotes not determined. Sign + indicates restoration of growth, - indicates lack of growth and ± refers to partial restoration of growth.

Next, we examined the suppression of permeability and growth defects. For such studies, we investigated the degree of restoration of bacterial growth/viability. Thus, spot dilution assays were performed on LA medium and on MacConkey agar supplemented with 75 μM IPTG or when supplemented with 15 μg/mL of erythromycin. Data are presented for growth on MacConkey agar as a similar pattern was observed with resistance to erythromycin. These experiments revealed that although most of the multicopy suppressors restored bacterial growth on LA agar at 43.5 °C, the permeability defect was suppressed to a variable extent (Figure 3). Even on LA medium, the colony-forming ability and the colony size indicated the degree of suppression of Ts phenotype was variable. For example, the overexpression of the *acpT* gene conferred only a partial suppression (Figure 3). Thus, on MacConkey agar, only overexpression of *srrA* and *yfgM* genes restored growth to a near-wild-type level. On this medium, which reflects a permeability

phenotype, a modest suppression is also conferred by the overexpression of *dksA*, *acpP* and *acpT* genes (Figure 3). Noteworthy of these suppressor-encoding genes is the isolation of a set of genes (*srrA*, *dksA*, *acpP*, *yfgM*, *ymgG*, *artJ* and *accB*) whose overexpression was earlier shown to suppress the Ts phenotype of Δ*lapD* bacteria [28]. Such results suggest that some common defects in bacteria that are either lacking the periplasmic domain of LapC or when LapD is absent, can be rescued by overexpression of this set of genes (Table 1). However, the identification of *marA* and *pldA* genes is interesting, and their overexpression only suppresses the Ts phenotype of *lapC190* bacteria but not of Δ*lapD* (Table 1). MarA (multiple antibiotic resistance) is a well-characterized transcriptional regulator, known to regulate the expression of several genes whose products are required for antibiotic resistance, oxidative stress and heavy metals tolerance [32,34,35]. Similarly, overexpression of the *acpT* gene only suppresses the Ts phenotype of *lapC190* bacteria but not of the Δ*lapD* bacteria. Thus, although some suppressors identified in this work are common to Δ*lapD* and *lapC190*, the overexpression of some genes such as *marA*, *pldA* and *acpT* is unique for overcoming the Ts phenotype of only *lapC190* bacteria, suggesting a partial overlap in the function of LapC and LapD and not all interacting partners could be the same. These conclusions draw further support from results showing that while in multicopy the *lapD* gene can suppress the Ts phenotype of *lapC190* bacteria (Figure 4), but in converse, overexpression of the *lapC* gene does not rescue the Ts phenotype of Δ*lapD* bacteria.

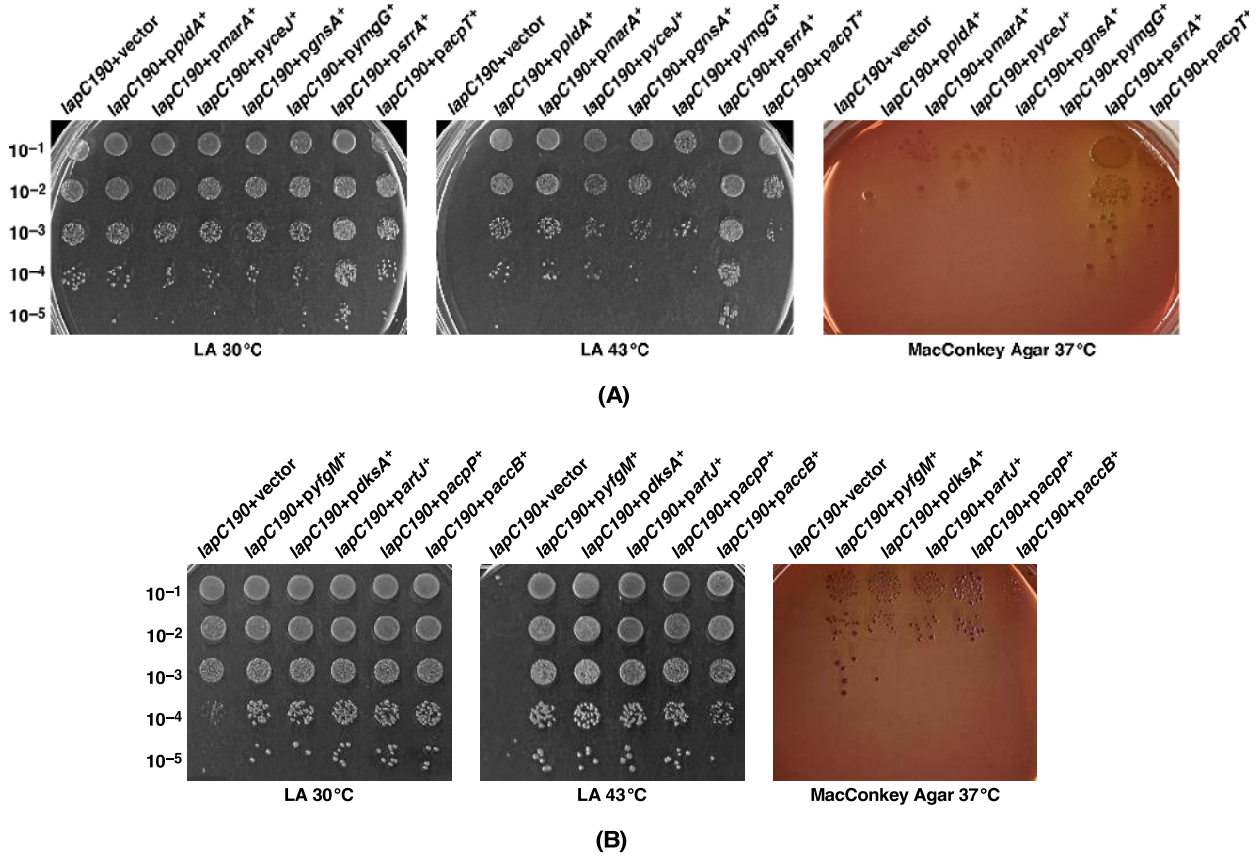

Figure 3. Overexpression of specific genes, including *marA*, *srrA* and *acpP* genes, restore the growth of *lap190* bacteria at 43 °C. Overexpression of some of them, such as *srrA* (**A**) and *yfgM* (**B**), also restore growth on MacConkey agar. Growth of isogenic cultures of *lap190* bacteria transformed 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 30 °C and 43 °C. For the restoration of growth on MacConkey agar, plates were incubated at 37 °C (**A**,**B**). The relevant genotype and temperature of incubation are indicated.

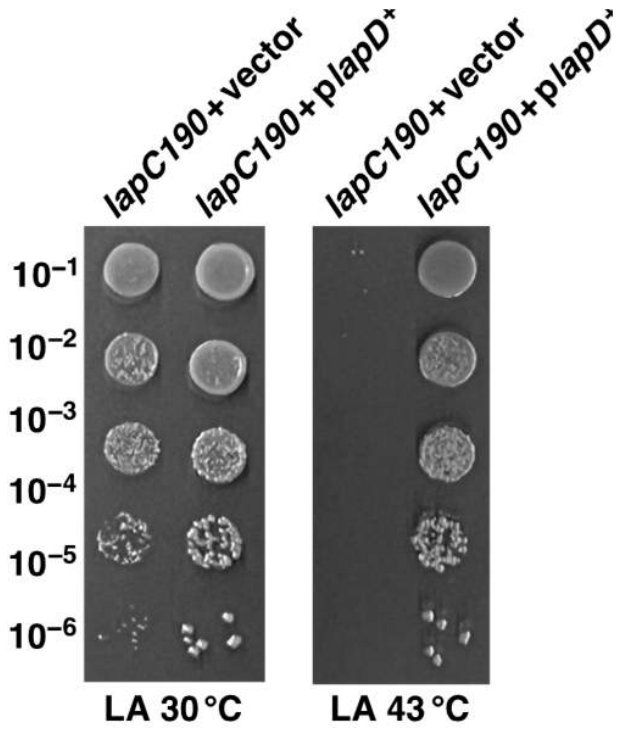

Figure 4. Overexpression of the *lapD* gene can restore the growth of *lap190* bacteria at 43 °C. Growth of isogenic cultures of *lap190* bacteria transformed with either plasmid DNA of the vector alone or when the *lapD* gene is present on the plasmid was quantified by spot dilution on LA 30 °C and 43 °C. The relevant genotype and temperature of incubation are indicated.

2.2. Overexpression of srrA, marA, yceJ and yfgM Restore LpxC Amounts in lapC Mutant Bacteria

To address the molecular basis of the suppression of growth defects at elevated temperatures by overexpression of the above-mentioned multicopy suppressor-encoding genes, experiments were undertaken to examine the amount of LpxC upon their mild induction. The rationale for these studies is to verify if the major defect reflected by the reduction in LpxC amounts associated with the absence of the periplasmic domain of LapC can be overcome by overexpression of any of these genes. Thus, total cell extracts were obtained from the wild type and panels of isogenic Δ*lap190* derivatives either with an empty vector or when the overexpressing plasmid was present under conditions described in the legend to Figure 5. The equivalent amount of proteins was resolved on a 12% SDS-PAGE, and LpxC was detected by immunoblotting with LpxC-specific antibodies. The estimation of relative amounts of LpxC in such experiments prominently shows that induction of expression of *marA*, *yceJ* and *srrA* restore LpxC to near wild-type levels (Figure 5A, lanes 4, 5 and 8). We applied the same amounts of total proteins from the Δ*ftsH sfhC21* strain since, in the absence of FtsH, LpxC amounts are increased to serve as a marker for LpxC and as a control. In parallel, the same samples were immunoblotted with anti-TrxA antibodies serving as an additional loading control. Among the remaining genes, whose overexpression also suppresses the Ts phenotype of *lapC190* bacteria, a modest increase in LpxC abundance can be observed in strains with induced expression of either *yfgM* or *dksA* or *artJ* genes in the *lapC190* background (Figure 5B, lanes 4, 5 and 6). These results suggest that the induction of *marA*, *yceJ* and *srrA* genes and, to a certain extent, of *yfgM*, *dksA* and *artJ* can suppress the growth defect of *lapC190* bacteria by restoring LpxC amounts. Surprisingly, the overexpression of *pldA*, *gnsA*, *ymgG* and *acpT* genes (Figure 5A) as well as *lapD*, *acpP* and *accB* (Figure 5B) did not cause any noticeable increase in LpxC amounts and remained similar to when extracts from *lapC190* with the vector alone were applied. It is possible that in the case of expression of such multicopy-encoding suppressor genes, a mild

induction of their expression may not be sufficient to significantly increase LpxC amounts, although Ts of *lapC190* bacteria can be suppressed. Overall, we can conclude that a mild overproduction of SrrA, MarA, YceJ and YfgM suppress the Ts phenotype and also lead to the restoration of LpxC amounts, explaining the molecular basis of their contribution in the regulation of LpxC.

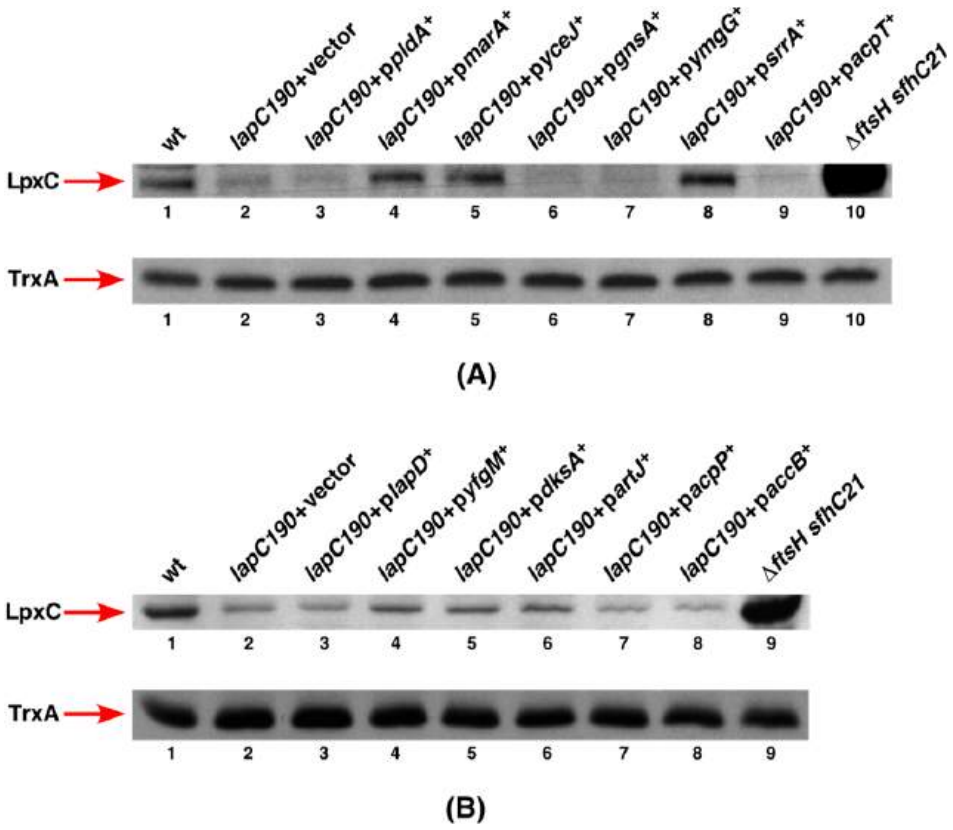

Figure 5. Restoration of LpxC amounts to different extents upon the overexpression of various multicopy suppressor encoding genes in *lap190* bacteria. Immunoblots of whole cell lysates obtained from isogenic strains grown at 30 °C, followed by the addition of 75 μM IPTG and a temperature shift for 2 h at 43 °C (**A**,**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. As controls, lane 2 in panel (**A**) and panel (**B**) samples from *lapC190* bacteria are loaded, which have reduced LpxC amounts. Additional control involves samples from Δ*ftsH sfhC21* which expectedly reveal higher amounts of LpxC due to its stabilization lane 10 (panel (**A**)) and lane 9 (panel (**B**)). As loading control, the same samples were probed with anti-TrxA antibodies.

2.3. Overexpression of srrA, marA, yceJ and dksA also Restore LPS Amounts in lapC190 Bacteria

We previously showed that *lapC190* bacteria have reduced amounts of LPS due to the enhanced degradation of LpxC as compared to the wild type. Thus, to address the mechanism of suppression by overexpression of specific genes that provide a robust restoration of growth at elevated temperatures, we estimated levels of LPS from total cell extracts. For such experiments, we measured levels of LPS from cellular extracts obtained from exponentially grown cultures of the isogenic wild type, its *lapC190* derivative with the vector alone and *lapC190* bacteria carrying plasmid that expresses a specific suppressor encoding gene under an inducible P_{T5}-*lac* promoter. Bacterial cultures were grown under permissive growth conditions at 30 °C in LB medium, the gene expression was induced with the addition of 75 μM IPTG at OD_{595} 0.1 and samples obtained under the same conditions when LpxC amounts were measured as described in Figure 5. Total cell extracts were also applied from isogenic Δ*ftsH sfhC21* derivative as a control. Equivalent

amount of whole cell lysates was treated with Proteinase K. Samples were resolved on an SDS-Tricine gel and LPS was transferred by Western blotting. Amounts of LPS were revealed using an LPS-specific WN1 222-5 monoclonal antibody as described in the Methods section.

The estimation of relative amounts of LPS in such experiments show that *lapC190* bacteria have highly diminished amounts of LPS (Figure 6 lane 9) as compared to the wild type and the Δ*ftsH sfhC21* derivative contains elevated amounts of LPS. Most importantly, results from such immunoblots reveal that extracts from the *lapC190* derivative overexpressing either *marA*, or *yceJ*, or *srrA* or the *dksA* gene show the restoration of LPS amounts to near wild-type levels explaining the mechanism of their suppression (Figure 6). As with the estimation of LpxC amounts, overexpression of the *acpP* gene did not cause any increase in the amounts of LPS and remained low as observed with *lapC190* bacteria. Similarly, overexpression of the *lapD* gene only marginally enhances LPS amounts. These results allow us to conclude that some of the multicopy suppressors such as *marA*, *dksA*, *srrA* and *yceJ* contribute via increasing LpxC and LPS amounts, providing a reasonable explanation for their identification. However, the mode of restoration of growth of *lapC* mutant bacteria by an overexpression of the *acpP* gene could involve the LpxC independent mode.

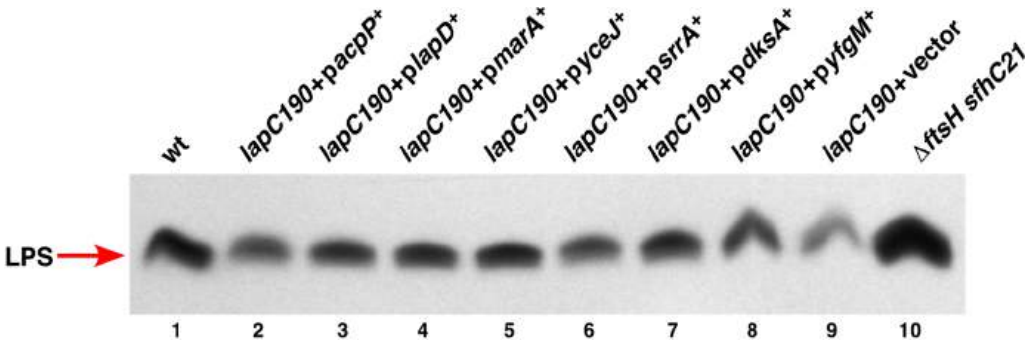

Figure 6. Restoration of LPS amounts by overexpression of *marA*, *yceJ*, *srrA* and *dksA* to different extents in *lap190* bacteria. Immunoblots of whole cell lysates obtained from isogenic strains grown at 30 °C, following a temperature shift for 2 h at 43 °C. Expression of the specific gene was induced by the addition of IPTG. An equivalent portion of whole cell lysates were applied and samples were resolved on a 15.5% SDS-Tricine gel and LPS was transferred by Western blotting. Amounts of LPS were revealed using a LPS-specific WN1 222-5 monoclonal antibody. 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 using chemiluminescence detection kit.

2.4. srrA, marA, lapD and pldA Are Indispensable in the lapC190 Mutant Background

To further understand the mechanism of suppression by various genes whose increased expression mitigates the Ts phenotype of *lapC190* bacteria, a systematic series of bacteriophage-mediated transduction was executed. This was to ascertain if there are any genetic interactions and if any of such genes are essential for the growth and viability of *lapC190* bacteria. We focused on those genes, whose overexpression also restores LpxC levels. Thus, null alleles of such candidates were introduced in *lapC190* bacteria. In parallel, converse reverse transductions were also carried out by attempting to bring in a *lapC190* mutation in deletion derivatives of genes whose overexpression results in the restoration of growth. Such transductions revealed that the presence of SrrA, MarA, LapD and PldA are absolutely required for the viability of *lapC190* bacteria even at 30 °C but not in the wild-type parental strain (Table 2). Thus, no viable transductional Δ*srrA lapC190* and Δ*lapD lapC190* combinations could be obtained. However, when the wild-type copy of the *srrA* or *lapD* gene was provided on a plasmid, viable chromosomal Δ*srrA lapC190* and Δ*lapD lapC190* mutational combinations were obtained at the same frequency when

the wild-type parental strain was used as a recipient to bring in either *srrA* or *lapD* null mutations (Tables 2 and 3). In contrast, the Δ*dksA lapC190* mutational combination was found to be viable up to 37 °C, although the colony size was heterogeneous. A deletion of either the *marA* gene or the *pldA* gene could be introduced in *lapC190* bacteria, albeit at a highly reduced frequency, and the majority of such transductants were found to be not viable (Table 2). These genetic interactions allow us to conclude that SrrA, MarA, LapD and PldA are essential for the viability of strains lacking the chromosome of the DNA sequence encoding the periplasmic domain of LapC.

Table 2. Synthetic lethal combinations reveal essentiality of *marA*, *srrA* and *lapD* genes in *lapC190* bacteria.

Numbers of Transductants on LA 30 °C		
Donor	**Wild Type**	***lapC190***
Δ*marA*	1274	92 [1]
Δ*srrA*	650	2
Δ*dksA*	>2000	163 [2]
Δ*pldA*	1460	34 tiny
Δ*lpxL*	742	6
Δ*lpxM*	960	12
Δ*lapD*	733	25

[1] not viable. [2] viable but heterogenous.

Table 3. Synthetic lethal combinations reveal essentiality of *lpxL* and *lpxM* genes in *lapC190* bacteria, which can be overcome in the presence of ectopic plasmid containing a wild-type copy of either of the genes.

Numbers of Transductants on LA 30 °C		
Donor	**Wild Type**	***lapC190***
P1 Δ*lpxL*	wild type + p*lpxL* 1234	*lapC190* + p*lpxL* 1164
P1 Δ*lpxM*	wild type + p*lpxM* 968	*lapC190* + p*lpxM* 1005
P1 Δ*lapD*	wild type + p*lapD* 811	*lapC190* + p*lapD* 840
P1 Δ*srrA*	wild type + p*srrA* 794	*lapC190* + p*srrA* 736

2.5. Lauroyl and Myristoyl Transferases Are Required for the Viability of lapC190 Mutant Bacteria

Next, we examined if acyltransferases that mediate the transfer of lauroyl (LpxL) and myristoyl (LpxM) acyl chains to Kdo_2-lipid IV_A are essential for the growth of *lapC190* mutant bacteria. Bacteriophage P1-mediated transfer of *lapC190* or an introduction of Δ*lpxL* or Δ*lpxM* revealed that Δ*lpxL lapC190* and Δ*lpxM lapC190* mutational combinations are only possible in the ectopic presence of covering plasmid containing the wild-type gene (Tables 2 and 3). However, at a very low frequency, viable suppressors could be obtained after an incubation of more than 48 h when Δ*lpxL lapC190* and Δ*lpxM lapC190* transductants were plated. Further characterization of two suppressors of each such mutational combinations identified a single amino acid exchange, L412P, in the *msbA* gene. Interestingly, the same *msbA* suppressor mutation was found to overcome the lethality of Δ(*lpxL lapD*) and Δ(*lpxM lapD*) combinations [28]. Thus, we can conclude that truncation of the *lapC* gene, resulting in the expression of only the transmembrane anchor, requires complete acylation of the lipid A. Since underacylated LPS (lack of LpxL or LpxM) is poorly translocated by MsbA as compared to hexaacylated lipid A, this could lead to a further reduction in the amount of LPS in the OM in *lapC190* bacteria and hence the lethality. Taken

together, *lapC190* derivatives synthesizing either tetraacylated or pentaacylated lipid A, cannot be constructed unless a suppressor mutation in the *msbA* gene, which encodes lipid A flippase, is present.

2.6. MarA-Mediated Suppression Requires the Presence of mla Genes

As the induction of the *marA* gene expression restored the growth of *lapC190* mutant bacteria at an elevated temperature, we further investigated its possible mechanism of suppression. Thus, strain SR23660 (*lapC190* + p*marA*$^+$) served as a host to generate a saturated Tn*10* Kan transposon library at 30 °C. Nearly 50,000 such transposon mutants were screened for the growth on LA medium at 43 °C in the presence of 75 μM IPTG and as a control at 30 °C without the presence of an inducer. This is the strategy that we earlier successfully employed to identify genes whose products are required for the multicopy suppressor phenotype of the *dksA* gene [36]. Thus, we retained those Tn*10* insertion mutants that grew at 30 °C but were unable to propagate at 43 °C, even with the induction of the *marA* gene expression. Such transposon mutations were backcrossed into SR23660 and verified for lack of suppression of *lapC190* mutant bacteria in the presence of the inducible *marA* gene. This resulted in the identification of fifty-four independent Tn*10* mutants. Next, such Tn*10* Kan transposon insertions were introduced into the wild type by bacteriophage P1-mediated transductions to eliminate mutations that conferred a Ts phenotype at 43 °C, even in the wild-type background. Such experiments identified 22 Tn*10* insertions that were found to confer the Ts phenotype in the wild type, while the remaining were specific to the *lapC190* strain. Most of the Ts mutants were found to have the Tn*10* insertion in the *degP* gene, whose product is known to be required for bacterial growth at temperatures above 42 °C [37,38]. This is consistent with a similar spectrum of Ts insertions obtained in an identical strategy described to unravel the requirement of specific genes for DksA-mediated multicopy suppression [36]. The mapping and characterization of the remaining Tn*10* insertions that prevent the multicopy suppression by *marA* identified 12 out of 15 mapping to genes whose products are part of the Mla pathway. To facilitate the mapping of Tn*10* insertions, we used a previously described linked Δ*rpoN* [39] to find how many can cross out the Tn*10* insertion mutation. Such transductions revealed that the majority of Tn*10* insertion mutants had a disruption of the *mla* operon. DNA sequence analysis of the Tn*10* junction from chromosomal DNA of *mla* Tn*10* insertions was obtained by inverse PCR, which identified four insertions each in *mlaC* and *mlaD*, three insertions in *mlaF* and one insertion in the *mlaA* gene. The Mla system is known to be required for the maintenance of outer membrane lipid asymmetry and the inactivation of *mla* genes confers sensitivity to SDS+EDTA [40]. The *mla* operon contains *mlaF*, *mlaE*, *mlaD*, *mlaC* and *mlaB* genes, out of which the *mlaF* gene encodes the ATP-binding protein of an inner membrane complex (MlaFEDB). The Mla pathway functions as part of the retrograde and/or anterograde intermembrane phospholipid trafficking system [40,41]. The Mla system also contains MlaA, whose encoded gene is located outside the *mla* operon. The Mla system can function by removing mislocalized outer leaflet phospholipids and transporting them back to the inner membrane. Thus, the isolation of Tn*10* insertion in genes encoding the Mla pathway that prevent MarA-mediated suppression of *lapC190* bacteria is consistent with LapC function in regulating LPS amounts and a balance with phospholipid amounts.

Thus, to further support these results, we used previously constructed in-frame deletion derivatives of *mlaC* and *mlaD* [42] and a new construct Δ(*mlaC mlaD*) to verify if such mutations abolish *marA* multicopy suppression. This was required because Tn*10* insertions are expected to be polar on the expression of downstream genes. These in-frame deletions in *mla* genes were introduced in SR23660 carrying chromosomal *lapC190* and a plasmid with an inducible expression of the *marA* gene by bacteriophage P1-mediated transduction. Transductants were plated on LA agar at 30 °C and at 43 °C in the presence of 75 μM IPTG to induce the expression of the *marA* gene (Table 4). As a control, *mlaC*, *mlaD* and (*mlaC mlaD*) in-frame deletions were introduced in the wild-type and *lapC190*

bacteria. While such deletions could be introduced into the wild type, *lapC190* and SR23660 *lapC190* + p*marA*$^{+}$ at 30 °C at a similar frequency; however, at 43 °C, deletion sets of *mla* genes could not be introduced in SR23660 even with the induction of the *marA* gene expression (Table 4). In control transductions, all three deletions of *mla* genes could be introduced in the wild type at 43 °C (Table 4). These results allow us to unambiguously conclude that *marA*-mediated multicopy suppression of *lapC190* bacteria requires the expression of *mla* genes.

Table 4. MarA-mediated multicopy suppression of *lapC190* bacteria requires the presence of Mla system.

Recipient	P1 Donor					
	30 °C			43 °C		
	Δ*mlaC*	**Δ*mlaD***	**Δ*mlaCD***	**Δ*mlaC***	**Δ*mlaD***	**Δ*mlaCD***
wt BW25113	1260	1380	1429	1412	1274	980
lapC190 (SR23583)	623	710	690	12	14	9
lapC190 + p*marA* (SR23660)	654	740	565	9	12	8

2.7. Absence of mla Genes Does Not Alter LPS Composition

In *E. coli*, up to now, no structural/compositional analysis of LPS has been reported when the Mla system is impaired. To investigate if the deletion derivatives of *mla* genes cause any major alterations in the LPS composition or in the incorporation of non-stoichiometric modifications, purified LPS obtained from the wild type and its isogenic derivatives were examined using mass spectrometric analysis. For such experiments, LPS was extracted after growth in phosphate-limiting growth medium as described earlier, since such growth conditions are inducive for lipid A and LPS core modifications due to the induction of BasS/R and PhoB/R two-component systems [13,43]. Examination of mass spectra of LPS obtained from the wild type and isogenic Δ*mlaC* and Δ*mlaD* derivatives shows similar mass peaks corresponding to the presence of complete glycoform I represented by mass peaks at 3936.7 Da and its derivatives with predicted incorporation of additional 1 or 2 P-EtN residues and also modification by L-Ara4N and GlcUA moieties as indicated. These mass peaks can be explained as LA_{hexa} ($Kdo_2Hep_4Hex_4P_2$) accompanied by additional substitutions with P-EtN and L-Ara4N, as shown (Figure 7). The mass peaks at 4489.9 Da and 4612.9 Da can be attributed to the addition of GlcNAc and GlcUA to glycoform I (Figure 7). These spectra from LPS obtained from either the wild type or its Δ*mlaC* and Δ*mlaD* derivatives also reveal a prominent presence of mass peaks corresponding to glycoforms IV and V, which are represented by mass peaks at 3948.7, 4079.8, 4202 and 4298.9 Da. These mass peaks are explained by the incorporation of a third Kdo and Rha linked to the Kdo disaccharide with a concomitant truncation of the outer core with a predicted composition of LA_{hexa} ($Kdo_3RhaHep_3Hex_3P_2$). The mass differences in these derivatives of glycoforms with a third Kdo and Rha are explained by the additional incorporation of P-EtN, L-Ara4N and GlcUA, as shown (Figure 7). The main findings from mass spectrometry data are that a deletion of *mla* genes does not alter LPS core and lipid A structural properties under these defined growth conditions.

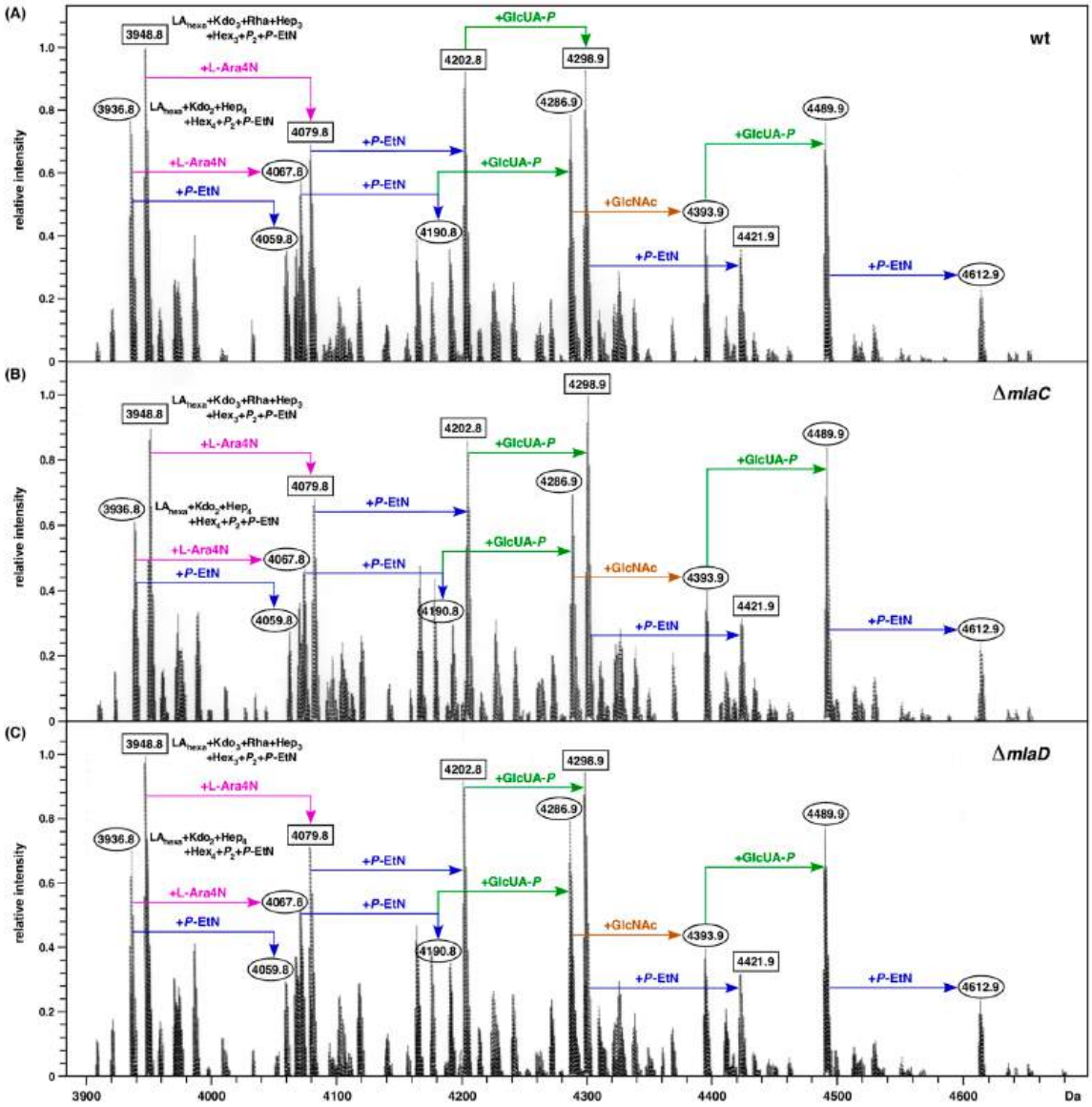

Figure 7. In the absence of either the *mlaC* or *mlaD* gene, LPS structure is similar to that of parental wild type. Charge-deconvoluted mass spectra in the negative ion mode of LPS from the wild type (**A**) and its Δ*mlaC* (**B**) and Δ*mlaD* (**C**) derivatives. Cultures were grown in phosphate-limiting medium at 30 °C. Mass numbers refer to monoisotopic peaks. Mass peaks with rectangular boxes correspond to the glycoform containing the third Kdo. Mass peaks marked with an oval box correspond to predicted LPS containing two Kdo residues.

2.8. SrrA-Mediated Suppression of lapC190 Mutant Bacteria Requires the Presence of Cardiolipin Synthase A and ClsA Is Essential for lapC190

Among various multicopy suppressors that were identified, SrrA has a distinguishing property that the corresponding gene in high dosage can suppress the Ts phenotype of strains lacking six cytoplasmic peptidyl-prolyl *cis*/*trans* isomerases, Δ*lapD* bacteria and is again reisolated as a suppressor of the Ts phenotype of *lapC190* bacteria [28,36]. Since a modest overproduction of SrrA was found to restore LpxC amounts (Figure 5), we further investigated a possible pathway that can explain this suppression. The same strategy of isolating transposon insertion mutants was used as shown above with the *marA* gene. Thus, the strain SR23951 carrying the chromosomal *lapC190* mutation and expressing the *srrA* gene from a tightly regulated P_{T5}-*lac* promoter was used to serve as a host to generate a saturated library of Tn*10* Kan insertion mutants under permissive growth conditions at

LA 30 °C. This library was next screened on LA agar supplemented with 75 μM IPTG to induce the expression of the *srrA* gene at 43.5 °C. Transposon mutants that exhibit a Ts phenotype even with the induction of the *srrA* gene were retained. Following bacteriophage P1-mediated transductions of such Tn*10* Kan mutants in the wild type, those conferring per se a Ts phenotype in the wild type were not followed. The identity of the Tn*10* insertion was obtained after DNA sequencing of inverse PCR products obtained from the template chromosomal DNA of the remaining Tn*10* insertion-carrying mutations, which specifically abrogated the multicopy suppression of the *srrA* gene in *lapC190* mutant bacteria. Analysis of DNA sequencing revealed that the majority of strains (8 out of 11) had Tn*10* insertion in the coding region of the *clsA* gene encoding cardiolipin synthase A. Two other strains had a mutation in the *cpxR* gene. CpxR acts as the DNA-binding response regulator and works with CpxA IM kinase, constituting one of the main two-component systems that responds to protein folding defects in the periplasm and severe defects in LPS biosynthesis [13,44–47]. One Tn*10* insertion mutant that did not allow the multicopy suppression by the *srrA* gene has an identical Tn*10* insertion in the heat shock promoter of the *groSL* operon at nt position -101, which was earlier found to abolish DksA-mediated suppression of Δ6*ppi* and Δ*dnaK/J* strains [36]. GroSL chaperonins constitute the major player in protein folding and are indispensable for bacterial viability [48,49]. This Tn*10* insertion in the *groSL* operon is located 3 nt downstream of the -35 heat shock promoter element, which is 101 nt upstream of the translational initiation codon as described previously with a reduced amount of GroL with its impaired induction at a high temperature [36]. Since the majority of Tn*10* insertion mutations mapped to the *clsA* gene, this suggests that the presence of cardiolipin is essential for SrrA-mediated restoration of the growth of *lapC190* mutant bacteria at elevated temperatures.

To further understand the requirement of the *clsA* gene when LapC function is impaired, a Δ*clsA* mutation was introduced in the wild type, *lapC190* with vector alone and *lapC190* + p*srrA*$^+$ by bacteriophage P1-mediated transductions. Such transductants were subsequently verified for their growth at 43 °C. Results from such experiments show that Δ*clsA* is viable in the wild type under such growth conditions but cannot be introduced into *lapC190* bacteria with a vector alone (Table 5). However, transductants at a highly reduced frequency with a smaller colony size were obtained in SR23951 *lapC190* + p*srrA*$^+$ in the presence of IPTG at only 30 °C as compared to a wild-type background. Furthermore, Δ*clsA* derivatives in SR23951 were unable to propagate at temperatures above 42 °C (Table 5). Thus, these results show that ClsA presence is required for SrrA to act as a multicopy suppressor to allow the growth of *lapC190* bacteria at high temperatures and presumably ClsA and LapC cooperate in the maintenance of phospholipid homeostasis in the cell envelope.

Table 5. The *srrA*-mediated multicopy suppression of *lapC190* bacteria requires the presence of cardiolipin synthase A encoded by the *clsA* gene.

Recipient	P1 Donor Δ*clsA*	
	30 °C	**43 °C**
wt	840	759
lapC190 + vector alone	3	-
lapC190 + p*srrA*$^+$ with IPTG	331 small colonies	38

2.9. SrrA Does Not Regulate Transcription of the clsA Gene

SrrA is predicted to act as a transcriptional regulator since it contains an N-terminal signal recognition Per-Arnt-Sim (PAS) domain and a C-terminal helix-turn-helix motif [36]. However, the genes whose expression is regulated by SrrA are unknown. As described above, the overexpression of the *srrA* gene efficiently suppresses the Ts phenotype of *lapC190* bacteria and also either of the strains collectively lacking six cytoplasmic peptidyl-prolyl *cis/trans* isomerases or of Δ*lapD* bacteria [28,36]. Since SrrA-mediated suppression

requires the presence of a functional *clsA* gene, we undertook studies to address if SrrA acts by regulating transcription of the encoding gene. Thus, total RNA was extracted from the wild type and its isogenic derivative Δ*srrA* from cultures grown at 37 °C. In parallel, total RNA was also extracted from wild-type bacteria carrying either an empty vector or expressing the *srrA* gene from an inducible promoter. For such experiments, cultures were grown at 30 °C. Prior to heat shock, 75 μM IPTG was added to induce transcription of the *srrA* gene, and cultures were shifted to 43 °C for 15 min. The equivalent amount of total RNA from these four sets of RNAs was next subjected to q-RT-PCR analysis using *clsA*-specific oligonucleotides for the synthesis and quantification of cDNA. The quantification of the *clsA* transcription pattern showed nearly similar levels of *clsA* transcripts between the wild-type and Δ*srrA* bacteria at 37 °C (Figure 8). Similarly, when the *srrA* gene expression was induced and when RNA was extracted from 43 °C and the relative abundance of transcripts were estimated by q-RT-PCR, no significant differences were observed in the *clsA* expression with or without *srrA* induction (Figure 8). Although a minor increase in *clsA* mRNA can be observed upon shift to 43 °C as compared to the relative abundance at 37 °C, such an increase is not comparable to that of heat shock genes. Thus, results from q-RT-PCR allow us to conclude that the *clsA* transcription is not regulated by SrrA either at 43 °C or at 37 °C, and overexpression of the *srrA* gene or its absence does not alter the abundance of *clsA* transcripts.

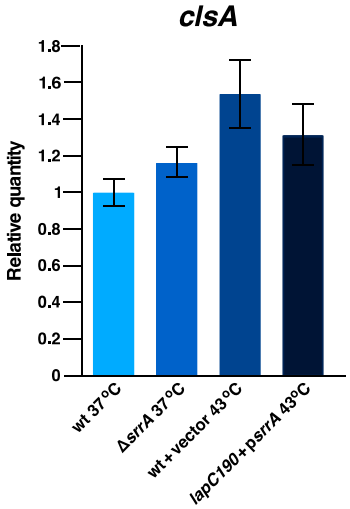

Figure 8. Transcription of the *clsA* gene is not regulated by SrrA. qRT-PCR analysis of mRNA isolated from wild-type bacteria, its isogenic derivative Δ*srrA*, grown up to an OD_{595} of 0.2 in LB medium at 37 °C. For heat shock experiments isogenic cultures of wild type with empty vector and *lapC190* bacteria carrying the inducible *srrA* gene on a plasmid were grown at 30 °C in M9 minimal medium up to an OD_{595} of 0.2 and supplemented with 75 μM IPTG prior to a 15-min shift to 43 °C. Data presented are from RNA isolated from three biological replicates and error bars are indicated.

2.10. LapD-Mediated Suppression of a lapC190 Mutant Bacteria Requires LapD N- and C-Terminal Domains and Identification of Some of the Critical Amino Acid Residues Required for LapD Function

As overexpression of the *lapD* gene restored the growth of *lapC190* mutant bacteria at a high temperature, we sought to address if specific domains of LapD are required for its function. LapD has a single N-terminal transmembrane anchor and a highly conserved C-terminal domain. The crystal structure of the LapD counterpart in *Haemophilus ducreyi* shows a unique tetrameric α-helical coiled-coil structure in the cytosolic domain, which is predicted to mediate protein–protein interactions [50]. Thus, a series of point mutations (substitution of conserved residues by Ala), deletion of the N-terminal membrane anchor (1–20 amino acids) and deletion of the C-terminal last 32 amino acids were constructed. Among the Ala substitutions, we replaced conserved amino acids D69, Y70 and R71 with

three Ala residues. Similarly, L84, L85 and P86 and N93, P94 and F95 were also substituted with three Ala residues in each case. All generated constructs with in-frame mutations were expressed under a tightly regulated *ptac* promoter and, when required, in-frame with an N-terminal hexa-His tag, where the expression is driven from a controlled T7 promoter. Plasmids where the expression is driven from the *ptac* promoter were transformed either into the Δ*lapD* strain or in *lapC190* bacteria and evaluated for the ability to suppress their Ts phenotype. Results from such experiments show that while the control plasmid overexpressing the wild-type *lapD* gene restores growth of *lapC190* at elevated temperature, neither the N-terminal nor the C-terminal deletions were able to suppress the Ts phenotype when the expression was induced by the addition of 75 μM IPTG (Figure 9). Similarly, three plasmid sets expressing replacement by Ala substitutions of amino acids (69–71), (84–86) and (93–95) were also unable to restore the growth of *lapC190* bacteria at elevated temperatures (Figure 9). These results allow us to conclude that specific conserved residues in the cytoplasmic domain are essential for LapD function. Furthermore, the single N-terminal membrane anchor and last 32 amino acid residues of the cytoplasmic domain are required for the *lapD* gene to act as a multicopy suppressor.

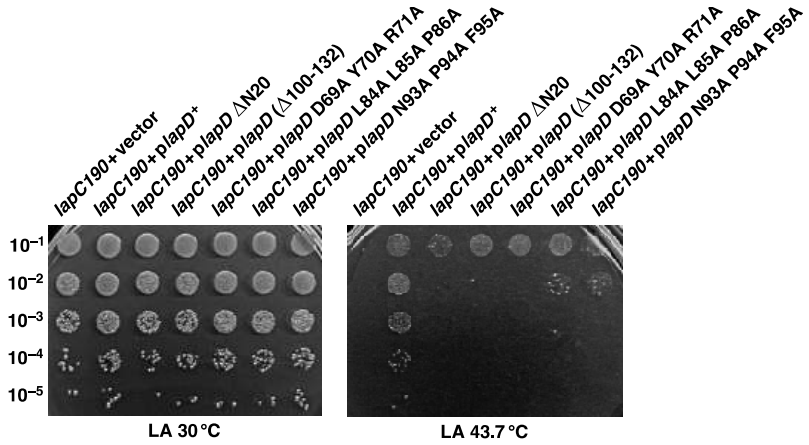

Figure 9. The N-terminal membrane anchor of LapD and its conserved amino acid residues in the C-terminal domain are essential for the restoration of the growth of *lap190* bacteria at 43.7 °C. In-frame deletion of N-terminal 20 amino acid residues, ΔN20, deletion of last 32 amino acid residues Δ100-132 and a triple Ala replacement of conserved amino acid were constructed by gene synthesis and cloned in expression vector. Plasmid DNA with the wild-type *lapD* gene and plasmids DNAs with various substitutions or deletions in the *lapD* gene were used to transform *lapC190* bacteria. Growth of such isogenic cultures of *lap190* was quantified by serial spot dilution assay on LA 30 °C and 43.7 °C. The relevant genotype and temperature of incubation are indicated.

2.11. Multicopy Suppression of lapC190 Bacterial Defects Requires DksA's PPIase and Transcriptional Activities

The results presented in the above sections have shown that overexpression of the global transcriptional regulator encoded by the *dksA* gene can restore the bacterial growth of *lapC190* bacteria at elevated temperatures and also suppress LpxC degradation defects. We have earlier shown that DksA also suppresses the Ts phenotype of Δ6*ppi* bacteria and Δ*lapD* bacteria [28,36]. Characterization of the biochemical properties of DksA further revealed that besides acting as a global transcriptional regulator, it also exhibits the PPIase activity, which can be inhibited by the FK506 macrolide [36]. Several studies have led to the conclusion that the coiled-coil domain of DksA inserts into the secondary channel of RNAP and that residues at the tip of the coiled-coil domain of DksA are important for its activity [51–53]. More recent studies suggest that amino acid residues in this coiled-coil domain play an essential role for the transcriptional and PPIase activities of DksA [36]. Thus, to examine which activity of DksA is required for the suppression of growth defects of *lapC190* bacteria, cloned DNA of D74N, F82Y, S83A, L84A and E85A variants of the

dksA gene were transformed into *lapC190* bacteria, using wild-type p*dksA*$^{+}$ as a control. It is important to mention that D74, F84, S83 and E85 amino acid residues are important for DksA-mediated transcriptional regulation of reporter genes such as the *rrnB*P1 promoter [36]. However, the replacement of the F82 amino acid residue by the Tyr residue abolishes the PPIase activity of DksA, although such a variant behaves like wild type concerning the regulation of the *rrnB*P1 promoter [36]. Our complementation experiments reveal that cloned D74N, F82Y, S83A, L84A and E85A mutants of the *dksA* gene have all lost the ability to fully restore the growth of *lapC190* bacteria at high temperatures upon their overexpression as compared to the complete suppression of Ts phenotype by the wild-type *dksA* gene (Figure 10). Viable colonies at all dilutions, albeit with smaller colony size, are observed when D74N, S83A, L84A and E85A mutants of the *dksA* gene were overexpressed in a *lapC190* strain (Figure 10). However, replacement of F82 by Y, which is a critically important residue for DksA's PPIase activity, completely abrogates the DksA-mediated multicopy suppression of *lapC190* bacteria (Figure 10). These results suggest that the PPIase activity of DksA is most critical for DksA to function as a multicopy suppressor to suppress the Ts phenotype of *lapC* mutant bacteria, and the transcriptional role of DksA, although important, could only play a minor role. This is particularly striking due to the abrogation of suppression by overexpression of the DksA F82Y variant in comparison to only a partial loss of function with other DksA mutants concerning the LPS-related function.

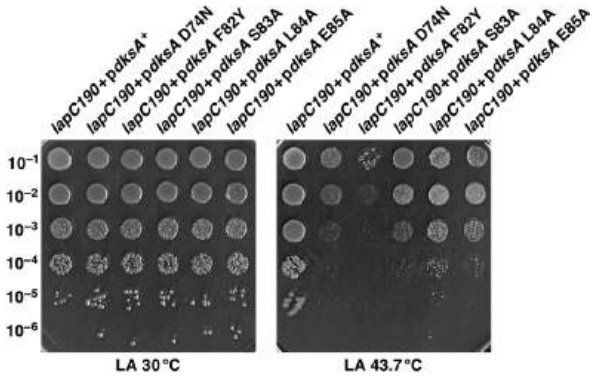

Figure 10. Amino acid residues that are essential for DksA's transcriptional activity and those required for its peptidyl-prolyl *cis/trans* isomerase activity are required for its multicopy suppressing ability of *lapC190* bacteria. Competent cells of *lapC190* bacteria were transformed by plasmid DNA carrying either the wild-type *dksA* gene or with specific single-amino amino acid replacement in the tip domain of the coiled-coil domain of DksA. Cultures of such isogenic cultures of *lap190* bacteria were grown at 30 °C in LB medium and bacterial growth was quantified by serial spot dilution assay on LA 30 °C and 43.7 °C. The relevant genotype and temperature of incubation are indicated.

3. Discussion

In this study, we identified new additional factors that regulate LpxC levels. Regulation of LpxC is critical for our understanding of the mechanism of balancing biosynthesis of LPS and phospholipids, since they both share the same (*R*)-3-hydroxymyristate-ACP as the common metabolic precursor. Recent studies have shown that LpxC stability is regulated by the FtsH-LapB complex, wherein FtsH degrades LpxC, which requires LapB function for its proteolytic activity towards LpxC [19,22]. However, this activity is counteracted by the essential LapC (YejM) protein [6,23,25]. In addition to FtsH-LapB and LapC, many additional factors participate in the regulation of LpxC amounts and, hence, the overall LPS content. Mutations in either the *ftsH* or *lapB* genes lead to an increased abundance of LPS due to the stabilization of LpxC, whereas loss-of-function mutations in the *lapC* gene exhibit enhanced LpxC degradation with a concomitant reduction in the overall LPS content. During earlier work, we isolated temperature-sensitive *lapC190*, *lapC377fs* and *lapC* F349S mutant bacteria [6]. Because *lapC190* mutant bacteria exhibit a tight Ts phenotype, we exploited such a property to isolate multicopy suppressors that restore growth at elevated

temperatures to identify additional factors that regulate LpxC amounts. Such an approach should also reveal what are the limiting factors that confer the Ts phenotype to *lapC190* mutant bacteria. Using such a procedure, thirteen genes were identified whose mild induction overcomes the Ts phenotype. As *lapC190* bacteria exhibit permeability defects and reduced LpxC and LPS levels, we examined whether overexpression of any of these genes also suppress such defects. Quite satisfactorily, a major proportion of such genes can be implicated either directly or indirectly in functions related to LPS, phospholipoid/fatty acid biosynthesis and regulation of LpxC protease FtsH amount (*lapD*, *pldA*, *acpP*, *acpT*, *accB*, *gnsA*, *yfgM*, *marA*) or envelope stress response (*srrA*, *dksA*, *ymgG*). These thirteen genes were thus grouped into different categories based on their known or predicted functions. Three out of them (*marA*, *dksA* and *srrA*) act as transcriptional factors and should contribute by their regulation of downstream target gene(s), whose gene products either directly regulate LPS or phospholipid biosynthesis. Another group of suppressors include genes encoding the LPS assembly protein LapD, the essential AcpP protein that shuttles fatty acid chains in the lipid A and fatty acid/phospholipid biosynthesis, the outer membrane phospholipase A encoded by the *pldA* gene. Isolation of these genes is interesting since this shows that *lapC* mutant bacteria have imbalanced phospholipid and LPS biosynthesis. Importantly, PldA degrades phospholipids that have been mislocalized to the outer leaflet in the OM, which signal stabilization of LpxC and an increased production of LPS [29], explaining its isolation as a multicopy suppressor. However, it should be noted that overexpression of the *pldA* gene does not overcome the permeability defects of *lapC190* bacteria, as reflected by an inability to restore growth on bile-salt-containing MacConkey agar. Interestingly, up to now, the sole dosage-dependent suppressor of *lapC* mutant bacteria expressing only the periplasmic domain has been the *acpT* gene, whose product encodes a phosphopantetheinyl transferase [54]. In this work, we also isolated the *acpT* gene as a multicopy suppressor; however, this suppressing phenotype was much weaker than the others. Surprisingly, it has been shown that AcpT-mediated suppression of *lapC* (*yejM*) mutant bacteria for the Ts phenotype does not require its enzymatic activity [54].

The global transcriptomes of MarA and DksA are known [55–57], but only sketchy information about SrrA is available. Thus, in this study, we systematically attempted to identify genes whose presence is required for multicopy suppression by MarA and SrrA. In *E. coli*, MarA acts as a transcriptional activator and is known to activate the expression of *acrAB-tolC*-encoded efflux pump, thereby contributing to resistance against antimicrobial compounds [58–60]. More recently, approximately 30 transcriptional units have been identified that could be regulated by MarA [61]. Here, we used saturated transposon mutagenesis to identify genes whose inactivation abrogates the *marA*- and *srrA*-mediated restoration of the growth of *lapC190* mutant bacteria at high temperatures. The vast majority of transposon mutants that prevented MarA-mediated multicopy suppression mapped to the *mla* operon, whose products are implicated in lipid trafficking, leading to the removal of the mislocalized phospholipids from the OM [29]. Consistent with our results, a MarA-binding motif has been identified in the promoter region of the *mla* operon [61]. Thus, our results provide a rationale for the identification of the *marA* gene as a multicopy suppressor of *lapC190* mutant bacteria, and this suppression requires the functionality of *mla* genes. In accordance with these findings, the *pldA* gene was isolated as a multicopy suppressor since both PldA and Mla pathways sense OM disturbance due to the accumulation of phospholipids in the OM when LPS is limiting.

In a similar manner, we addressed the pathway of SrrA-mediated suppression. Our results show that the overexpression of the *srrA* gene efficiently restores the growth at high temperatures, LpxC levels as well as permeability defects. This suppression was found to be contingent on the presence of the functional copy of the *clsA* gene encoding cardiolipin synthase A and the envelope-responsive two-component system CpxA/R. The CpxA/R system, along with the RpoE sigma factor, responds to severe defects in LPS assembly and biosynthesis as well as protein misfolding in the periplasm [13,19,39,62]. We have previously shown that ClsA is required for the viability of strains synthesizing either penta-

or tetraacylated lipid A, or when LapD assembly protein is missing [15,28]. Since LPS is presumably sensed in the inner membrane by LapC, the absence of ClsA could further accentuate this defect as it is required to assist MsbA-mediated LPS translocation. However, this requires further studies because the *clsA* gene is per se essential in *lapC190* bacteria. Our gene expression studies did not show that SrrA regulates transcription of the *clsA* gene based on q-RT-PCR results. Thus, we are initiating a global transcriptosome study to identify genes that are regulated by SrrA, and initial results, which require further in-depth experimentation, suggest that SrrA acts as a transcriptional repressor of the envelope stress response and fatty acid metabolism in *E. coli*.

Another important finding from this study is the partial overlap between LapD and LapC functions in the regulation of LpxC and LPS levels. This is based on the following results: (i) Some of the multicopy suppressors identified in this work that relieve the Ts phenotype of *lapC190* bacteria were earlier also identified as suppressors of Δ*lapD*. These include *dksA*, *srrA*, *acpP*, *accB* and *yfgM* [28]. (ii) Of significance is the isolation of the *lapD* gene as a multicopy suppressor of *lapC* mutant bacteria for the Ts phenotype. We recently proposed that LapD could also act upstream of LapB-FtsH in a manner similar to LapC, since suppressors mapping to *lapB*, *ftsH* and *lpxC* that stabilize LpxC, can suppress Δ*lapD* and *lapC190* bacteria. (iii) However, some suppressors, like the *marA* gene and the *acpT* gene cannot relieve the Ts phenotype of Δ*lapD* bacteria upon their overexpression and are specific to *lapC* mutant bacteria. Consistent with these results, the overexpression of the *lapC* gene does not relieve the Ts phenotype of Δ*lapD* bacteria. These results are not surprising because, at the biochemical level, despite the LapC and LapD co-purification with LapB, several other interacting partners of LapD are unique to it and were not found to be part of the LapC complex [6,24,28].

To further understand LapD-mediated multicopy suppression of *lapC* mutant bacteria, we constructed a series of mutations in the *lapD* gene to identify the essential domains of the encoded protein. This allowed us to express such mutant versions in a controlled manner from a regulated inducible promoter in *lapC* mutant bacteria. This revealed that the N-terminal membrane anchor and the last 32 conserved C-terminal amino residues are critical for LapD function. Furthermore, several residues that were predicted for LapD to interact with its partners [50] were also found to be essential for its function since their replacement by Ala amino acid residues abolished LapD function (inability to suppress the Ts phenotype of either Δ*lapD* or *lapC190* mutant bacteria). This includes a motif constituted by the N93 P94 F96 triplet, which we show is essential for the LapD function and has been predicted to interact with LapA. This conserved NFP motif is located after the long cytoplasmic helical domain.

Regarding the mechanism of suppression by overproduction of the global transcriptional regulator DksA, we show that, most critically, its PPIase activity is required for this activity. However, the replacement of conserved amino acid residues in the tip region of the coiled-coil domain of DksA which are known to be essential for its role in interaction with RNA polymerase and hence the transcriptional regulation caused a highly attenuated ability to confer suppressing ability of *lapC190* bacteria. However, the most severe phenotype was the replacement of F82 by Y, which resulted in the total abolishment of the multicopy-suppressing ability of DksA. This amino acid residue is important for conferring the PPIase activity of DksA but is not essential for transcriptional function [36]. Hence, the PPIase activity of DksA is critical for its multicopy-suppressing ability, and its transcriptional activity also plays an important role. Further studies are required to determine the mechanism(s) of the restoration of growth at elevated temperatures by other multicopy suppressors that were identified in this study.

It would be interesting to determine whether the suppression by *gnsA* overexpression is due to an increase in the unsaturated fatty acid synthesis that could lead to the stabilization of LpxC. The *gnsA* gene was initially identified as a multicopy suppressor of the *secG* null mutant and was shown to increase the acidic phospholipid content and inhibit phosphatidylethanolamine accumulation via an unknown mechanism [63]. Furthermore,

overexpression of the *gnsA* gene also suppresses the Ts phenotype of *fabA6* mutation and causes an increase in the unsaturated fatty acid content, such as *cis*-vaccenic acid (18:1), at the expense of palmitic acid (16:0), particularly at low temperatures [64]. At present, the molecular basis of the suppression of either *lapC190* bacteria or the *fabA6* Ts mutation or the ascribed increase in the unsaturated fatty acid content is not known. To this end, we have started to measure the impact of *gnsA* overexpression on fatty acid and phospholipid content in various *lapC* mutant bacteria. It is also intriguing why the central cofactor acyl carrier protein (AcpP) is limiting in *lapC190* bacteria. AcpP is the fourth most abundant protein in *E. coli*, its excess is usually toxic in wild-type bacteria [65], and yet its mild induction quite effectively overcomes the various growth defects of *lapC* mutant bacteria without increasing LpxC stability or restoring LPS amounts. AcpP is involved in not only LPS and phospholipid biosynthesis, but its interactome contains more than 35 proteins, some of which are unrelated to LPS and fatty acid synthesis [66]. Since the replacement of catalytically active site residues confers a toxic growth phenotype due to the accumulation of *apo*-ACP [67], we could not address if the catalytic activity of AcpP is required for the suppressing phenotype. However, AcpP overproduction does not enhance either LPS or LpxC amounts, and thus, its action could be either rebalancing fatty acid composition or via some another mechanism. Isolation of other multicopy suppressors, such as the *yfgM* gene, can be rationalized, since its product has been found to be a substrate of FtsH protease [68]. Overexpression of the *yfgM* gene could potentially titrate out FtsH, and this could lead to the stabilization of LpxC. The function of YceJ is currently unknown. However, the expression of the *yceJ* gene is induced by a variety of stress conditions such as heat shock, the induction of extracytoplasmic responsive CpxR regulon and when the Lol system (lipoprotein sorting) is inhibited [69]. Thus, YceJ may be involved in stress response management that could directly or indirectly modulate LpxC levels and hence more studies are required to address its function.

In summary, in this study, we identified new proteins, such as the transcriptional activator MarA, factors that are required to maintain OM asymmetry (PldA, Mla system), and the essentiality of cardiolipins which participate in the regulation of LPS assembly summarized in the Figure 2. To date, MarA has not been shown to directly regulate LpxC stability, which we have now established here. Isolation of the *srrA* gene as a multicopy suppressor is interesting because its overexpression stabilizes LpxC. We also identified essential amino acid residues that are required for LapD function and its partial overlap with LapC in regulating LpxC amounts.

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 6. Luria-Bertani agar (LA), LB broth (Difco, Franklin Lakes, NJ, USA), M9 minimal media and MacConkey agar (Difco) were prepared as described previously [13,19]. Growth medium was supplemented with antibiotics kanamycin (30 or 50 μg/mL), chloramphenicol (10 or 30 μg/mL) and ampicillin (100 μg/mL). The strain carrying the *lapC190* mutation in BW25113 has been previously described and when required was transduced into different genetic backgrounds using bacteriophage P1-mediated transductions at 30 °C [6]. Non-polar Δ*marA* and Δ*mlaCD* mutations were constructed by using the λ Red recombinase mediated recombination system [70]. The kanamycin resistance cassette was amplified using pKD13 as a template [70]. PCR products from such amplification reactions were electroporated into BW25113 derivative GK1942 containing the λ Red recombinase-encoding plasmid pKD46. The *aph* cassette was removed using the plasmid pCP20 expressing FLP recombinase at 30 °C and verified to have lost the antibiotic marker by streaking on LA plates with an appropriate antibiotic. The construction of some of the deletion derivatives used in this study, Δ*dksA*, Δ*srrA*, Δ*clsA*, Δ*lpxL*, Δ*lpxM* and Δ*lapD* has been previously described [13,28,36]. Isogenic deletion combinations were constructed using bacteriophage P1-mediated transductions at 30 °C on either the LA or M9 medium.

Table 6. Bacterial strains and plasmids used in this study.

Strains	Genotype	Reference
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[70]
GK1942	BW25113+pKD46	[19]
W3110	λ^{-}, *IN* (*rrnD-rrnE*)*1*, *rph-1*	CGSC, Yale
GK6075	BW25113 *lapC190*<>*cat*	[6]
SR23529	GK6075 *lapC190*<>*frt*	This study
SR23583	BW25113 *lapC190*<>*aph*	This study
SR23679	BW25113 *lapD*<>*aph*	[28]
SR23743	SR23679 *lapD*<>*frt*	[28]
SR9073	BW25113 *mlaC*<>*aph*	[42]
SR9078	BW25113 *mlaD*<>*aph*	[42]
SR23627	BW25113 *mlaCD*<>*aph*	This study
SR23630	GK6075 *mlaCD*<>*aph*	This study
SR19957	BW25113 *srrA*<>*aph*	[36]
SR23605	BW25113 *marA*<>*aph*	This study
SR7092	BW25113 *clsA*<>*aph*	Our collection
SR23320	BW25113 *clsA*<>*frt*	[28]
SR20066	BW25113 *dksA*<>*aph*	[36]
SR24224	BW25113 *pldA*<>*aph*	This study
GK1275	W3110 *lpxM*<>*aph*	[13]
GK1077	W3110 *lpxL*<>*aph*	[13]
SR23941	GK6075 *lpxL*<>*aph msbA* L412P	This study
SR23946	GK6075 *lpxM*<>*aph msbA* L412P	This study
SR19761	BW25113 + pCA24N	This study
SR23951	SR23529 + p*srrA*$^{+}$	This study
SR23952	SR23951 *clsA*::Tn*10*	This study
SR23992	SR23529 + p*yfgM*$^{+}$	This study
SR23994	SR23529 + p*dksA*$^{+}$	This study
SR23996	SR23529 + p*artJ*$^{+}$	This study
SR23998	SR23529 + p*acpP*$^{+}$	This study
SR24000	SR23529 + p*accB*$^{+}$	This study
SR24027	SR23529 + p*lapD*$^{+}$	This study
SR24097	SR23529 + p*pldA*$^{+}$	This study
SR23660	SR23591 + p*marA*$^{+}$	This study
SR24099	SR23529 + p*marA*$^{+}$ *mlaC*<>*aph*	This study
SR24100	SR23529 + p*marA*$^{+}$ *mlaC*::Tn*10*	This study
SR24101	SR23529 + p*gnsA*$^{+}$	This study
SR24103	SR23529 + p*yceJ*$^{+}$	This study
SR24105	SR23529 + p*acpT*$^{+}$	This study
SR24107	SR23529 + p*ymgG*$^{+}$	This study
GK3592	BW25113 *sfhC21 zad220*::Tn*10* Δ*ftsH3*::Kan	[19]
SR24021	SR23529 + p*dksA*$^{+}$	This study
SR24011	SR23529 + p*dksA* D74N	This study
SR24013	SR23529 + p*dksA* F82Y	This study
SR24015	SR23529 + p*dksA* S83A	This study
SR24017	SR23529 + p*dksA* L84A	This study
SR24019	SR23529 + p*dksA* E85A	This study
SR24056	SR23529 + p*lapD* ΔN20	This study
SR24058	SR23529 + p*lapD* (Δ100-132)	This study
SR24167	SR23529 + p*lapD* D69A Y70A R71A	This study
SR24170	SR23529 + p*lapD* L84A L85A P86A	This study
SR24173	SR23529 + p*lapD* N93A P94A F95A	This study
Plasmids	**Genotype**	**Reference**
pCA24N	IPTG-inducible expression vector cm^{R}	[33]
pDUET	Expression vector	Our collection
pKD3	*oriR6K*$_{g}$, *bla*(Amp^{R}), *kan*, *rgnB*(Ter), *cat*	[70]
pKD13	*oriR6K*$_{g}$, *bla*(Amp^{R}), *kan*, *rgnB*(Ter)	[70]
pKD46	*araBp-gam-bet-exo*, *bla*(Amp^{R}), *repA101*(ts)	[70]

Table 6. *Cont.*

Plasmids	Genotype	Reference
pCP20	ts replicon with inducible FLP recombinase	[70]
pSR23599	*lapD*$^{+}$ in pDUET	This study
pSR22189	*dksA*$^{+}$ in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
pSR22505	*dksA*$^{+}$ D74N in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
pSR22511	*dksA*$^{+}$ F82Y in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
pSR22519	*dksA*$^{+}$ S83A in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
pSR22498	*dksA*$^{+}$ L84A in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
pSR22525	*dksA*$^{+}$ E85A in pBR322 *lacI*^{q} tet^{S} amp^{R}	[36]
JW0141	*dksA*$^{+}$ in pCA24N cm^{R}	[33]
JW0844	*artJ*$^{+}$ in pCA24N cm^{R}	[33]
JW0976	*gnsA*$^{+}$ in pCA24N cm^{R}	[33]
JW1044	*yceJ*$^{+}$ in pCA24N cm^{R}	[33]
JW1080	*acpP*$^{+}$ in pCA24N cm^{R}	[33]
JW2497	*yfgM*$^{+}$ in pCA24N cm^{R}	[33]
JW3223	*accB*$^{+}$ in pCA24N cm^{R}	[33]
JW3440	*acpT*$^{+}$ in pCA24N cm^{R}	[33]
JW3794	*pldA*$^{+}$ in pCA24N cm^{R}	[33]
JW5178	*ymgG*$^{+}$ in pCA24N cm^{R}	[33]
JW5249	*marA*$^{+}$ in pCA24N cm^{R}	[33]
JW5539	*lapD*$^{+}$ in pCA24N cm^{R}	[33]
pSR14857	*srrA*$^{+}$ in pCA24N cm^{R}	This study
pEB797	pBAD-CBP-ACP (S36T)	[71]

4.2. Identification of Multicopy Suppressors Whose Overexpression Suppresses the Temperature-Sensitive Phenotype of lapC190 Bacteria

As *E. coli* K-12 strains carrying the chromosomal *lapC190* mutation exhibit the Ts phenotype (inability to grow above 42 °C), we used such a growth defect to identify genes whose overexpression can allow growth under such non-permissive conditions. To achieve this, competent cells of SR23529 and SR23583 *lapC190* derivatives of BW25113 were transformed with a complete genomic library of all predicted ORFs of *E. coli* cloned in pCA24N (ASKA collection). Transformants were selected at 43 °C in the presence of IPTG (75 μM). In this library, the expression of an individual gene is driven from the tightly regulated IPTG-inducible P_{T5}-*lac* promoter. The concentration of IPTG used in this study has been optimized to provide a mild induction of genes without any toxicity [19]. Cultures of such Ts^{+} colonies were grown to obtain plasmid DNAs and used to retransform parental *lapC190* mutant bacteria. Transformants were plated on growth medium in the presence of IPTG at 43 °C and 43.5 °C, and plasmids which allowed restoration of growth were retained. DNA insert of all relevant plasmids that restored the growth upon retransformation on LA-rich medium at 43.5 °C was sequenced.

4.3. The Isolation of Chromosomal Transposon Insertion Mutations That Prevent the Suppression by Overexpression of Either the marA Gene or the srrA Gene

As overexpression of either the *marA* gene or the *srrA* gene restore the growth of *lapC190* bacteria, chromosomal Tn*10* transposon insertions were isolated to understand the mechanism of their suppression as previously described [36]. Thus, the strains SR23660 and SR23951 *lapC190* derivatives, which can grow at 43.5 °C carrying either the *marA* gene or the *srrA* gene, respectively, were used as a host to isolate Tn*10* insertions. In these strains, suppressing *marA* and *srrA* genes are cloned in pCA24N vector with expression regulated by P_{T5}-*lac* promoter. For the mutagenesis, SR23660 (*lapC190* + p*marA*$^{+}$) and SR23951 (*lapC190* + p*srrA*$^{+}$) isogenic strains served as recipients using λTn*10* kan bacteriophage to obtain random saturated transposon insertions. More than 50,000 transposon insertion mutants were isolated in each strain background in LA medium at 30 °C (permissive growth conditions) as described previously [36]. Transductants were screened for a Ts phenotype on LA medium at 43.5 °C supplemented by 75 μM IPTG to induce the expression

of *marA* and *srrA* genes. Transductants that were unable to grow at 43.5 °C were retained and a bacteriophage P1 was grown on them individually. Such bacteriophage P1 lysates were used to retransduce SR23660 and SR23951 strains. Transductants were tested for their inability to grow at 43.5 °C and those which bred true (Ts phenotype and hence lack suppression) were further used. Such transposon mutants were next transduced into the wild-type strain BW25113 to identify a transposon insertion conferring the Ts phenotype in the wild-type background. Generally, only those Tn insertions that did not allow multicopy suppression by either the *marA* gene or the *srrA* gene were further used to obtain chromosomal DNA to identify the position of Tn*10* insertion. The position of Tn*10* was determined by the inverse PCR with nested primers and sequenced using the Tn*10* primer as described earlier [19].

4.4. Estimation of LpxC Amounts by Immunoblotting

The isogenic bacterial cultures of wild type, its derivative with the *lapC190* mutation with the vector alone and its isogenic cognates carrying multicopy suppressor encoding genes were grown in LB medium at 30 °C. Exponentially grown cultures were adjusted to an OD_{595} of 0.05 and allowed to further grow up to an OD_{595} of 0.2. For the induction of expression of the suppressing gene, IPTG at the final concentration of 75 μM was added and shifted in prewarmed flasks held at 43 °C for 2 h. Cultures were harvested by centrifugation at 10,000 rpm for 15 min and pellets were solubilized in the sample buffer. The protein concentration of each sample was measured using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Equivalent amounts of proteins were applied to a 12% SDS-PAGE and transferred by Western blotting. Blots were probed with polyclonal antibodies against LpxC, or with anti-TrxA as a loading control as described earlier [6]. Blots were revealed by a chemiluminescence kit from Thermo Scientific as per the manufacturer's instructions.

4.5. Site-Directed Mutagenesis of the lapD Gene and In Vivo Complementation Analysis

To identify amino acid residues and specific domains in LapD, PCR and Gibson cloning was used. Firstly, two sets of deletions of the *lapD* gene were constructed using oligonucleotides for PCR amplification to clone only the region encoding either the soluble domain or removal of the coding region for the last 32 C-terminal amino acid residues. PCR products were cloned in the pCA24N vector where the expression is tightly regulated from P_{T5}-*lac* promoter and for the protein induction in T7 promoter-based pIVEX 4.2 vector (Roche diagnostics, Basel, Switzerland). Three specific sets of mutations (replacement of D69, Y70 and R71 with Ala substitutions in the coding region of the *lapD* gene were introduced by using gene synthesis and Gibson cloning. Similarly, L84, L85 and P86 were substituted by three Ala, and in the third set, N93, P94 and F95 were also replaced with Ala residues. In the three resulting plasmid sets, the expression of mutated *lapD* gene is regulated from P_{T5}-*lac* promoter. For in vivo complementation, Δ*lapD* and *lapC190* mutant bacteria were transformed by empty vector and plasmid expressing the wild-type gene and where the *lapD* gene is mutated. Transformants were plated on LA agar at 30 °C. Cultures of such transformed derivatives subsequently were grown to an exponential growth phase and growth properties measured by spot dilution assays on LA medium at 30 °C and 43.5 °C.

4.6. LPS Extraction, Mass Spectrometry and Measurement of LPS Levels

Typically, 400 mL cultures of wild type, its isogenic Δ*mlaC*, Δ*mlaD* and Δ(*mlaC mlaD*) derivatives were grown in phosphate-limiting medium at 37 °C. Cultures were harvested by centrifugation at 10,000 rpm for 30 min and the pellets were lyophilized. LPS was extracted by the phenol/chloroform/petroleum ether procedure [72]. For the LPS analysis, lyophilized material was dispersed in water by sonication and resuspended at a concentration of 2 mg/mL. For mass spectrometric data acquisition, LPS samples were dissolved at a concentration of ~10 ng/μL and analyzed as described previously [13,73]. Electrospray

ionization-Fourier transform ion cyclotron (ESI-FT-ICR)-mass spectrometry was performed on intact LPS in the negative ion mode using an APEX QE (Bruker Daltonics, Breman, Germany) equipped with a 7-tesla actively shielded magnet and dual ESI-MALDI. Mass spectra were charge deconvoluted, and mass numbers given refer to the monoisotopic peaks.

For the estimation of LPS levels, an equivalent amount of bacterial cultures (wild type, its isogenic *lapC190* derivative and its derivatives carrying various multicopy suppressor-encoding genes) were grown up to an OD_{595} of 0.5. Cultures were harvested by centrifugation and pellets were resuspended in 1X sample buffer. To obtain whole cell lysates, samples were boiled for 10 min, followed by digestion with Proteinase K. Equivalent portions of obtained whole cell lysates were applied to a 15.5% SDS-Tricine gel. After the electrophoresis, LPS was transferred by Western blotting. Immunoblots were probed for LPS amounts using the WN1 222-5 monoclonal antibody as described previously [6]. The WN1 222-5 monoclonal antibody [74] was used at a dilution of 1:10,000. Blots were revealed using a chemiluminescence kit from Thermo Scientific as per the manufacturer's instructions.

4.7. RNA Purification and qRT-PCR Analysis

Exponentially grown cultures of the wild-type strain BW25113, its Δ*srrA* derivative SR19957, SR19761 (BW25113+vector pCA24N) and SR14857 (BW25113+p*srrA*$^+$ in pCA24N) were used to extract total RNA. For comparison of wild type and its Δ*srrA* derivative, cultures were grown at 37 °C in LB and adjusted to an optical density OD_{600} of 0.05. Cultures were allowed to grow up to an OD_{595} of 0.2 and were then harvested by centrifugation. To quantify changes in the mRNA abundance when the expression of the *srrA* gene is induced, the bacterial culture of SR19761 and SR14857 were grown at 30 °C in LB-rich medium to an OD_{595} of 0.1. Prior to heat shock, IPTG at the final concentration of 75 μM was added. Aliquots were immediately shifted to a prewarmed medium held at 43 °C and incubated for another 15 min. Equivalent amounts of culture samples were collected from cultures grown at 30 °C and after the heat shock of 43 °C, they were harvested by centrifugation. Total RNA was extracted using hot phenol extraction as described previously [75]. Purified total RNA was treated RQ1 DNase (Promega, Madison, WI, USA) to remove any genomic DNA and ethanol precipitated. Pellets were resuspended in DEPC-treated water. RNA was subjected to another round of purification using the GeneElute universal total RNA isolation kit from Sigma Aldrich, St. Louis, MO, USA. RNA amounts were quantified and their integrity verified using agarose gel electrophoresis. q-RT-PCR was used to quantify changes in the *clsA* gene expression, using gene-specific primers. Two μg of purified mRNA was converted to cDNA using Maxima H-Minus Reverse Transcriptase (Thermo Scientific). Reactions were conducted for 40 cycles using PowerUp SYBR® Green PCR Master Mix (Thermo Scientific), as described previously [6]. 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 2.3.

4.8. Bacterial Growth Analysis

For the measurement of relative restoration of growth by overexpression of different genes identified in this work, growth ability was quantified. For such experiments, exponentially grown cultures grown at 30 °C were adjusted to an optical density OD_{595} of 0.1, and ten-fold dilutions were spot tested on LA agar plates or on MacConkey agar at different temperatures in the presence of 75 μM IPTG to induce the expression of the suppressing gene present on the plasmid. Five μL of each dilution were spotted and the bacterial growth was analyzed after incubation for 24 h at 30 °C and 43 °C or at 43.5 °C as indicated in specific experiments.

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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 Δ*lapD* 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

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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 bisphosphorylated 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-hydroxymyristate derived from (*R*)-3-hydroxymyristoyl-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 IV_A 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 mRNA: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].

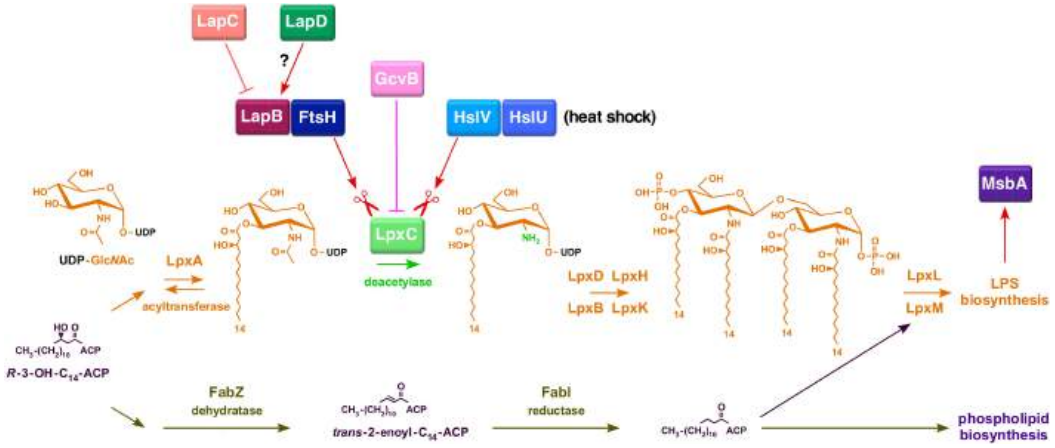

Figure 1. Key steps in the regulation of the first committed step in LPS biosynthesis catalyzed by LpxC and LPS transport mediated by MsbA across the inner membrane. Schematic illustration depicting utilization of the same metabolic precursor (*R*)-3-hydroxymyristate by LpxA and by FabZ in LPS and phospholipid 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 transenvelope 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 under-acylated 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 Δ*waaA* strains synthesizing lipid IV_A 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 Δ(*lpxL lapD*) or Δ(*lpxM lapD*) can be overcome by extragenic suppressor mutations mapping to the essential *msbA* gene (Figure 2B). We further show that Δ*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 Δ*lapD* bacteria to vancomycin (Figure 2B). Consistent with a role in these essential processes, various growth defects of a Δ*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 Δ*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.

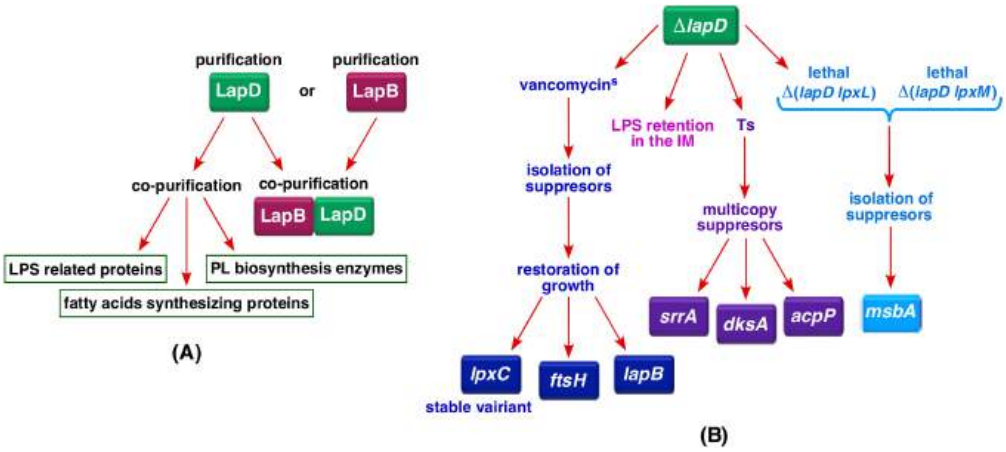

Figure 2. Schematic depiction of various approaches that identify LapD as a regulator of LPS biosynthesis regulating LpxC amounts and assisting MsbA in the LPS transport. Identification of LapD as an IM protein associated with LapB and co-purification of LapD with proteins involved in LPS/phospholipid biosynthesis (**A**). Suppressors of vancomycin sensitivity of Δ*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 LAESEASNDQAPVQMPRDYSEGASGLLR 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, Δ*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].

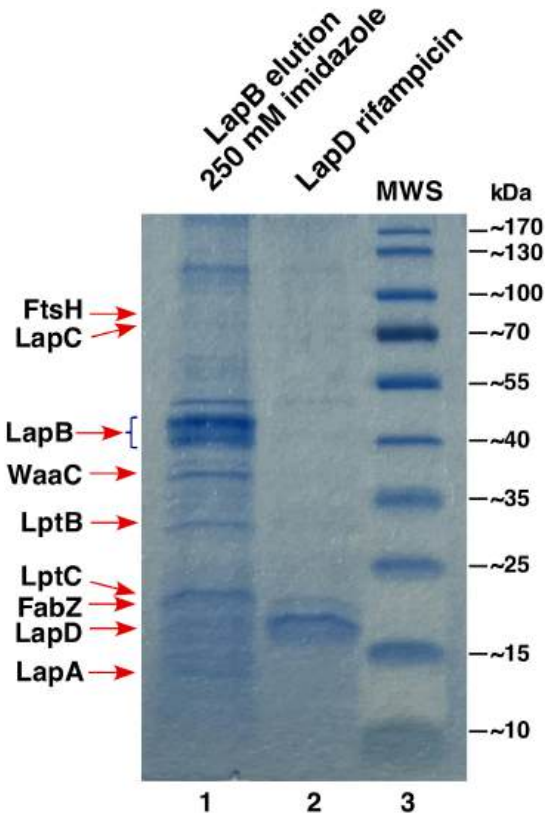

Figure 3. LapD as a new interacting partner of LapB. Purification profile of His_6-tagged LapB protein from the IM fraction after elution with 250 mM imidazole. Lane 1 shows co-purifying proteins with LapB that include LapD. All major co-purifying proteins are indicated by arrows. In lane 2, purified 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.

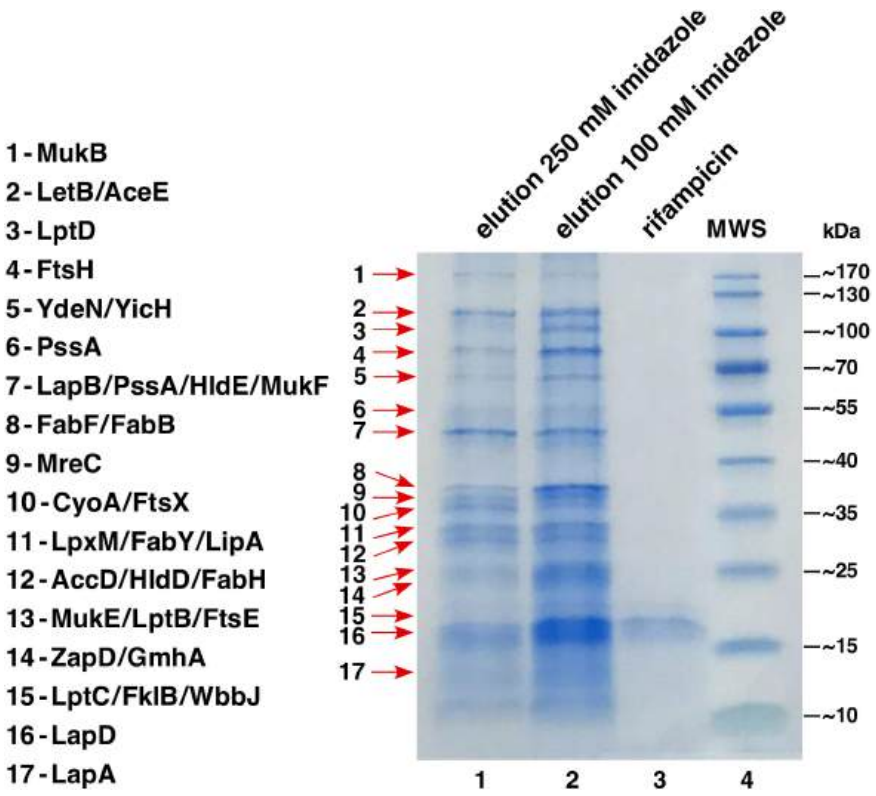

Figure 4. LapD forms a complex in the IM with proteins involved in LPS and phospholipid biosynthesis. Purification profile of His_6-tagged LapD protein from the IM fraction after elution with 250 mM and 100 mM imidazole (lanes 1 and 2, respectively). Lane 3 shows the migration of His_6-tagged LapD protein obtained after rifampicin treatment during the induction of its synthesis. All major co-purifying proteins are indicated by arrows. Proteins were resolved on a 12% SDS-PAGE, stained by Coomassie Brilliant Blue. Lane 4 shows pre-stained molecular weight standards.

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 Δ*lapD* 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, Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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.

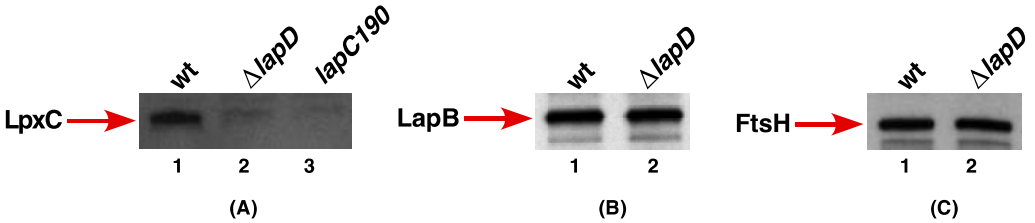

Figure 5. An absence of LapD causes a reduction in the amounts of LpxC. An immunoblot of whole cell lysates obtained from isogenic strains with indicated genotypes using LpxC-specific antibodies (**A**). In parallel, samples from the wild type and Δ*lapD* were immunoblotted with LapB-specific antibodies (**B**) and with FtsH-specific antibodies (**C**). An equivalent amount of total proteins was resolved by SDS-PAGE prior to immunoblotting.

2.3. Suppressor Mutations That Stabilize LpxC Can Restore the Wild-Type-like Growth of ΔlapD Bacteria on Vancomycin-Supplemented Growth Medium

In previous work, we showed that a Ts phenotype and reduced levels of LpxC in *lapC190* bacteria can be rescued by single amino acid suppressor mutations mapping to *lpxC*, *ftsH* and *lapA*/*lapB* operon [21]. This suppression of *lapC190* mutant bacteria by such extragenic suppressors was explained on the basis of increasing LpxC amounts. Thus, we reasoned that the introduction of such extragenic suppressor mutations that stabilize LpxC should also suppress growth defects of Δ*lapD* bacteria. To achieve this, previous *lapC190*::cm^R strains with suppressor mutations in either the *lpxC* gene or the *ftsH* gene [21] were first used as recipients to remove the *lapC190* mutation by the introduction of tightly linked *napA*::Tn*10* 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 *lapC* 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 Δ*lapD* 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 Δ*lapD* bacteria, which are sensitive (Figure 6). However, the restoration of growth of Δ*lapD* 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 Δ*lapD* bacteria can overcome membrane permeability defects.

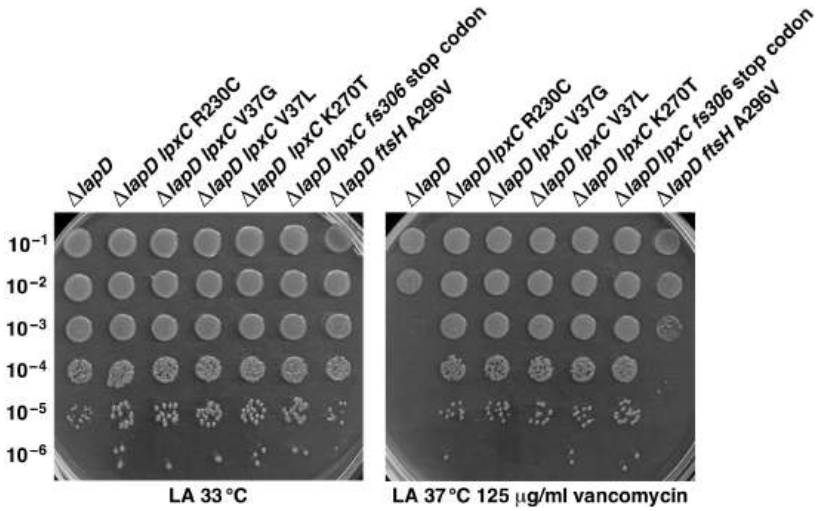

Figure 6. Mutations that stabilize LpxC suppress vancomycin sensitivity of Δ*lapD* bacteria. Growth of isogenic cultures of strains with Δ*lapD* and with suppressor mutations in the *lpxC* gene was quantified by spot dilution on LA with and without supplementation of vancomycin. The relevant genotype 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 Δ*lapD* bacteria have reduced LpxC quite like *lapC190* bacteria, we reasoned that the introduction of such *lapB* mutations should also restore the growth of a Δ*lapD* strain under conditions such as exposure to vancomycin. Thus, as described in the above section, firstly the *lapC190* mutation was removed by introducing *napA*::Tn*10*, selecting for the loss of the Cm^R 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 *lapD* deletion. This resulted in generating strains SR23857 (*lapB* H325P Δ*lapD*), SR23859 (*lapB* A88V Δ*lapD*), SR23861 (*lapB* H181R Δ*lapD*), SR23863 (*lapB* R115H Δ*lapD*), SR23865 (*lapB* D124Y Δ*lapD*) and SR23867 (*lapB* R125L Δ*lapD*) (see Methods section). Such isogenic strains along with parental Δ*lapD* 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 Δ*lapD* bacteria, although to a different extent (Figure 7). Among the tested *lapB* mutants, the introduction of *lapB* R115H, *lapB* D124Y, *lapB* R125L and *lapB* H181R in Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* derivative is not fully suppressed by mutations in the *lapB* gene, which is not the case with *lapC190* mutant bacteria [21].

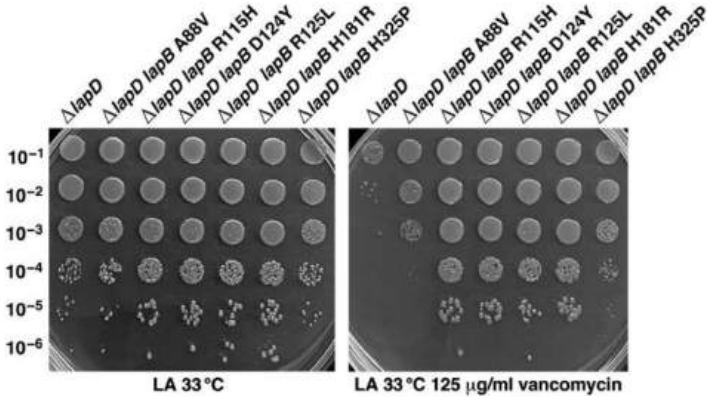

Figure 7. Well-characterized suppressor mutations in the *lapB* gene that prevent LpxC proteolysis suppress vancomycin sensitivity of Δ*lapD* bacteria. Growth of isogenic cultures of strains with Δ*lapD* and with suppressor mutations in the *lapB* gene was quantified by spot dilution on LA with and without supplementation of vancomycin. The relevant genotype and temperature of incubation are indicated.

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 Δ*lapD* 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 Δ*lapD* 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, Δ*lapD* 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 Δ*lapD* background and, in converse, the reduction in the LPS synthesis bypasses the lethality associated with Δ*lapB*. These results support the notion that LapD acts upstream of LapB, acting antagonistically, and has a function similar to LapC.

Table 1. A Δ*lapD* mutation is lethal when the LPS synthesis is impaired, which is the opposite in the case of Δ*lapB* strains.

Genotype	Number of Transductants		Viability in Δ*lapD*
	P1 Δ*lapD* LA 30 °C	P1 Δ*lapB* 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 Δ*lapD* Number of Transductants LA 30 °C	Viability and Colony Size
wt	1243	viable
Δ*lpxM*	6	not viable
Δ*lpxL*	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* I177M	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
Δ*waaC*	433	viable small size not viable at 42 °C
Δ*clsA*	378	viable small size not viable at 42 °C

After the minimal Kdo_2-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 10^3 in terms of colony forming units (cfu) (Figure 8A). At 42 °C, such bacteria exhibit a Ts phenotype, conditions under which Δ*lapD* and Δ*clsA* 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 Δ*waaC* or Δ*lapD* 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 Kdo_2-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*).

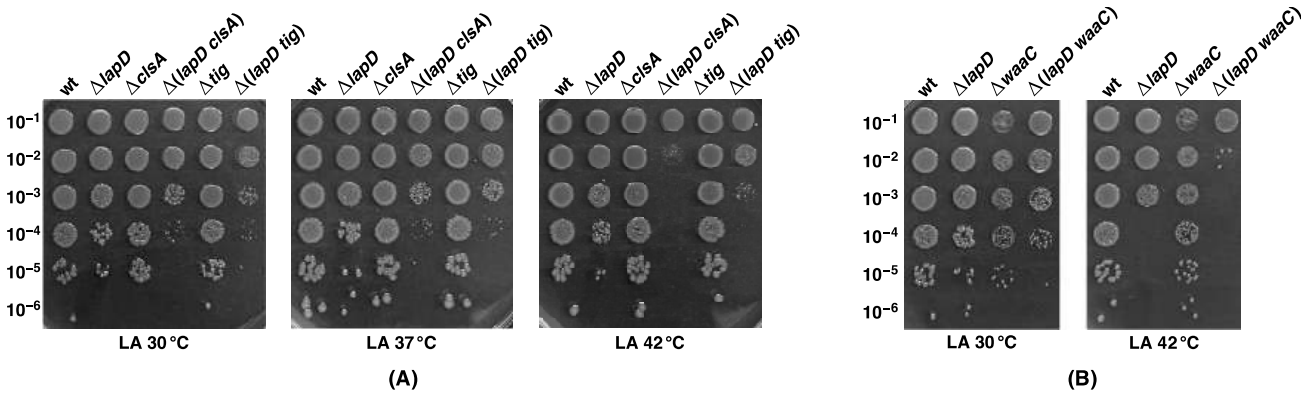

Figure 8. The conditional synthetic lethal phenotype of various mutational combinations reflecting the essentiality of the *lapD* gene when either the LPS is truncated or cardiolipin biosynthesis is disrupted or when the PPIase activity is impaired. Growth of isogenic cultures of strains of wild type, Δ*lapD*, Δ*clsA*, Δ*waaC*, Δ*tig* and various null combinations was quantified by spot dilution on LA at different temperatures (Panels (**A**,**B**)). The relevant genotype and temperature of incubation are indicated.

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 Δ*lpxL* and Δ*lpxM* 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 C**T**G to C**C**G, 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 G**T**T to G**C**T. 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.

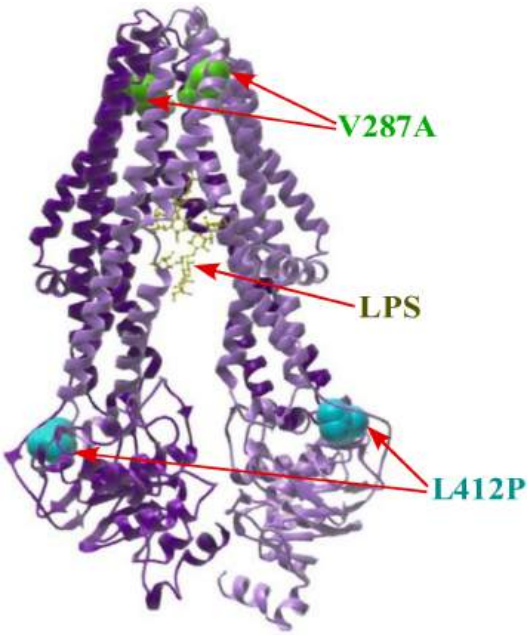

Figure 9. Mutations mapping to the *msbA* gene can bypass the synthetic lethality of Δ(*lpxL lapD*) and Δ(*lpxM lapD*) bacteria. Positions of various single amino acid substitutions in the structure of MsbA (PDB 6BPL) [30] are shown by arrows. The position of the LPS is also indicated and marked by the 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 Δ*lpxM 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 (Δ*lpxM msbA* D498Y), SR23709 (Δ*lpxM msbA* S164C) and SR23707 (Δ*lpxM 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 (Δ*lpxM msbA* D431Y), SR23703 (Δ*lpxM msbA* M160I) and SR23701 (Δ*lpxM msbA* I177M) (Table 2). However, the colony size of transductants (although viable) with SR23699 (Δ*lpxM 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, Δ*lapD*, Δ*waaC*, Δ(*waaC lapD*), Δ*clsA* 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 Δ*clsA* (Figure 10B) or Δ*waaC* strains (Figure 10C). However, a substantial amount of LPS was detected in the IM fraction of either Δ*lapD* 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 Δ*lapD* IM fractions also migrates much faster, resembling LPS of Δ*waaC* 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.

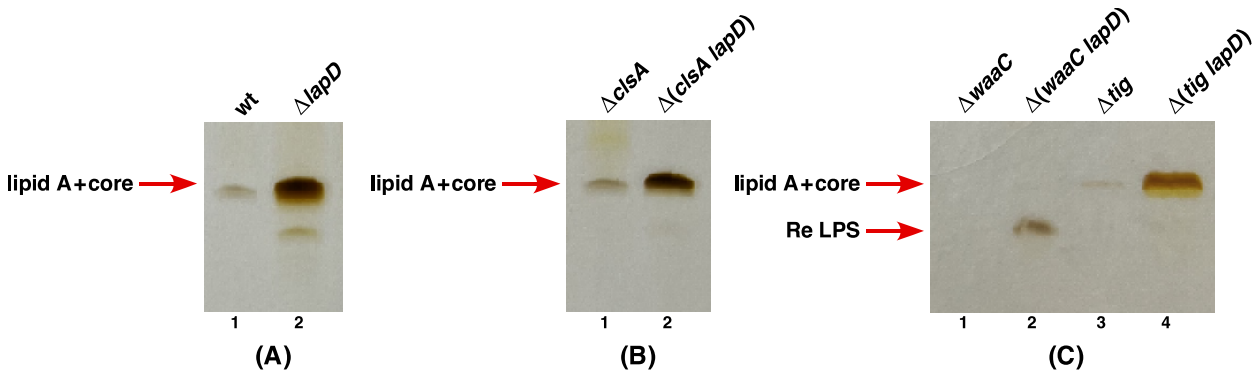

Figure 10. Lack of LapD causes the retention of significant amounts of LPS in the inner membrane. Total cell lysates obtained from isogenic derivatives of wild-type and Δ*lapD* bacteria were subjected to cellular fractionation to obtain the IM. Samples were treated with Proteinase K and resolved on a 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 Δ*lapD* and its derivatives wt vs. Δ*lapD* (**A**), Δ*clsA* vs. Δ(*clsA lapD*) (**B**) and Δ*waaC*, Δ(*waaC lapD*), Δ*tig*, 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 Kdo_2-lipid IV_A or Δ*waaA* 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 *rpoE*P3 promoter [49]. Thus, a *lapD* deletion was transduced in the wild-type strain carrying on the chromosome single-copy *rpoE*P3-*lacZ* fusion. To measure any impact on *rpoE* transcription, the isogenic wild-type strain carrying the *rpoE*P3-*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 *rpoE*P3 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 *rpoE*P3 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.

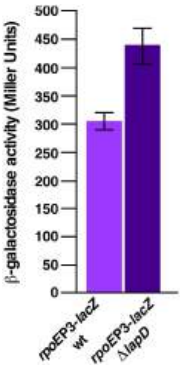

Figure 11. The absence of LapD causes the constitutive induction of the LPS defects responsive *rpoE*P3 promoter, even under permissive growth conditions. Exponentially grown wild type and its Δ*lapD* derivative carrying the single-copy chromosomal *rpoE*P3-*lacZ* fusion were analyzed for the β-galactosidase activity. Bacterial cultures were adjusted to an OD_{595} 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 Δ*lapD* 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 Δ*lapD* 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 repress 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 Δ*lapD*), SR23840 (*lpxC* V37L Δ*lapD*), SR23842 (*lpxC* K270T Δ*lapD*) and SR23844 (*lpxC fs306* stop codon Δ*lapD*) 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 Δ*lapD* bacteria, and increasing the stability of LpxC can rescue the lethal phenotype of the Δ(*lapD micA*) combination.

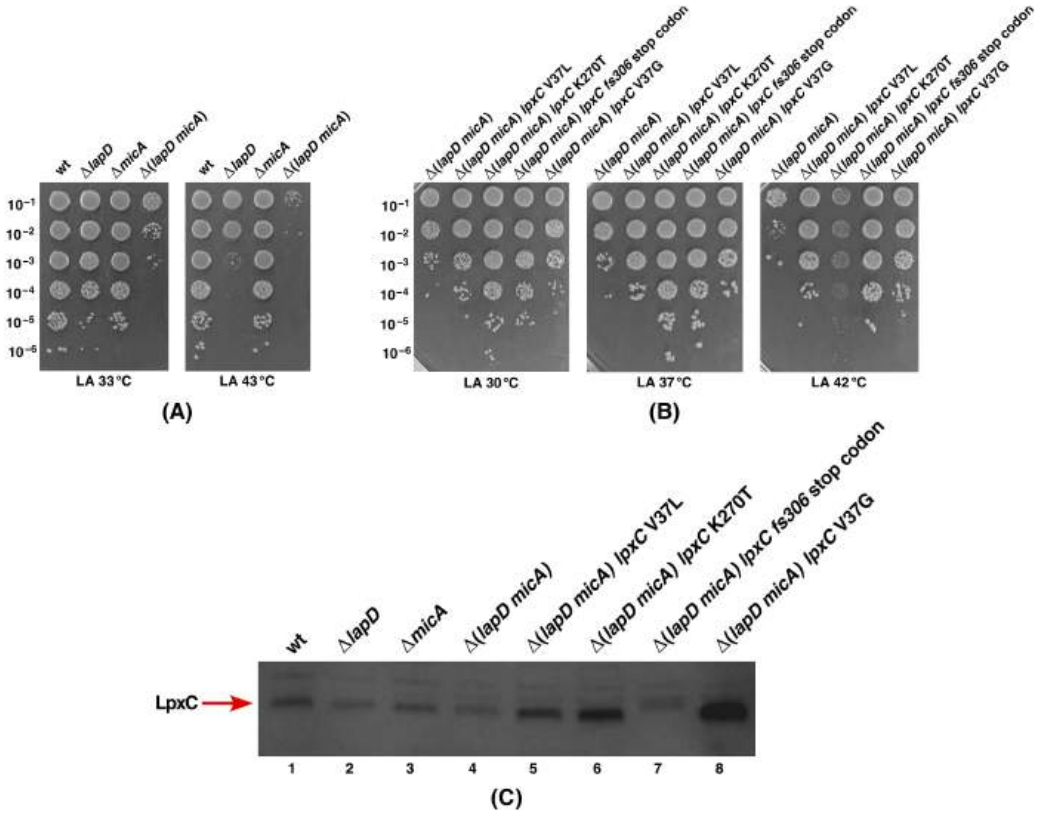

Figure 12. The essentiality of MicA sRNA in the Δ*lapD* background can be bypassed by mutations in the *lpxC* gene. Growth of isogenic cultures of strains of wild type, Δ*lapD*, Δ*micA* and Δ(*micA lapD*) was quantified by spot dilution on LA at 33 and 43 °C (**A**). Growth of isogenic cultures of Δ(*micA 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, Δ*lapD*, Δ*micA*, 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 Δ*lapD* bacteria have reduced amounts of LpxC (Figure 12C, lane 2). Thus, these experiments support a model wherein LapD regulates LpxC stability as Δ*lapD* 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 P_{T5}-*lac* promoter [51]. Plasmid DNA of pooled plasmids from this library was introduced into Δ*lapD* 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]. Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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.

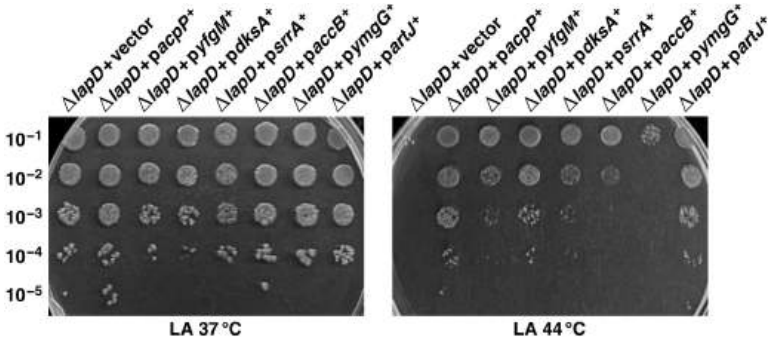

Figure 13. Overexpression of specific genes, including the *acpP* gene, restores the growth of Δ*lapD* bacteria at 44 °C. Growth of isogenic cultures of Δ*lapD* bacteria transformed with either plasmid DNA of the vector alone or when the specific multicopy suppressing gene is present on the plasmid was quantified by spot dilution on LA at 37 and 44 °C. The relevant genotype and temperature of incubation are indicated.

Table 3. Identification of the most relevant multicopy suppressors of Δ*lapD* strains.

Name	Number of Transformants		Function
	LA 44 °C	vancomycin	
vector alone	9 small colonies	6 small colonies	
acpP	750	ND [1]	acyl carrier protein
dksA	486	ND	transcriptional regulator
srrA	520	ND	transcriptional regulator
yfgM	430	ND	inner membrane protein, substrate of FtsH
accB	511	ND	acetylocoenzyme carboxylase
rcsF	ND	701 medium size colonies	colanic acid regulator
artJ	473	ND	L-arginine ABC transporter

[1] ND denotes not determined since suppressors were isolated either at 44 °C or on vancomycin-supplemented growth medium at 37 °C.

*2.12. Impact on LpxC Levels upon Overexpression of Genes That Overcome the Ts Phenotype of Δ*lapD* Bacteria*

As LapD absence results in the Ts phenotype with a concomitant reduction in LpxC levels, we examined levels of LpxC by immunoblotting. Thus, using total cell extracts from the wild type and its isogenic Δ*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 °C and the gene expression was induced with the addition of 75 μM IPTG at OD_{595} 0.1. After 15 min of IPTG addition, an equivalent portion of each culture was shifted to 43 °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 LpxC was detected by immunoblotting with LpxC-specific antibodies. At both 30 and 43 °C, LapD with the vector alone had a reduced amount of LpxC (Figure 14A, lane 2). Most significantly, only overexpression of the *srrA* gene was found to restore LpxC levels to nearly wild-type levels, particularly at 43 °C (Figure 14, lane 6). At 30 °C, also, overexpression of the *srrA* gene shows a modest increase in LpxC amounts. Surprisingly, overexpression of the *acpP* gene, which confers the best suppression at elevated temperatures, did not cause any restoration of LpxC amounts (Figure 14, lane 3). Thus, at least, we can explain that SrrA overproduction can suppress the Ts phenotype by restoring 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 PPIases at high temperature [40].

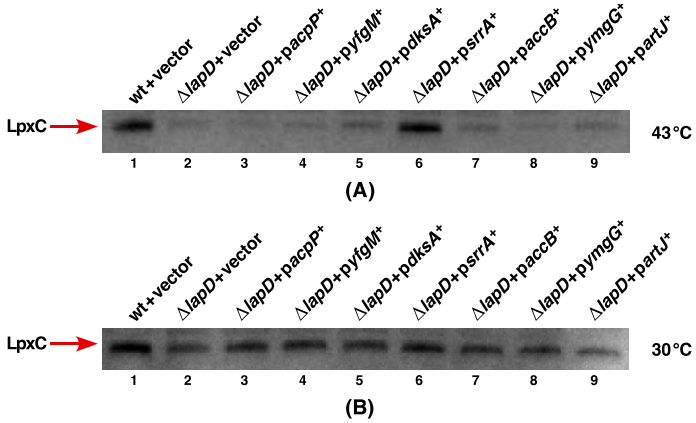

Figure 14. The impact of overexpression of various multicopy suppressor encoding genes in Δ*lapD* bacteria on the levels of LpxC. Immunoblots of whole cell lysates obtained from isogenic strains grown either at 30 °C, following a temperature shift for 2 h at 43 °C (**A**) or when grown at 30 °C (**B**). 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 Δ*srrA* 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 Δ*srrA* 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.

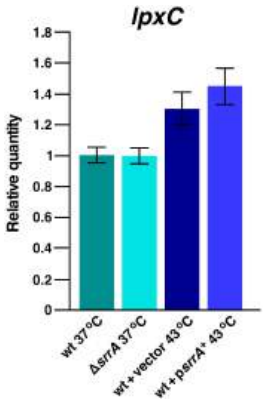

Figure 15. Transcription of the *lpxC* gene is not positively regulated by SrrA. q-RT-PCR analysis of mRNA extracted from wild-type bacteria, its Δ*srrA* derivative and transformed with either the vector DNA alone or when the *srrA* gene expression is induced from the IPTG-inducible promoter present 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 Δ*lapD* 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 Δ*lapD* bacteria by transformation. In parallel, Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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.

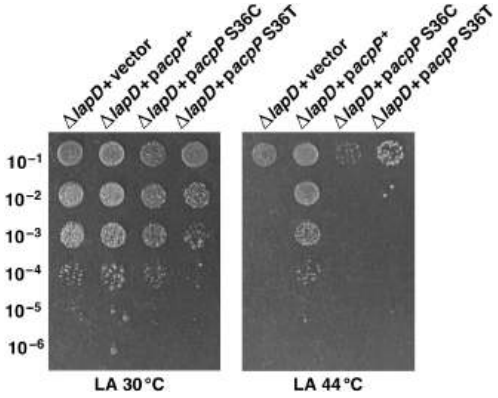

Figure 16. Multicopy suppression of Δ*lapD* bacteria by the *acpP* gene requires its product to retain its active site Ser 36 amino acid residue. Growth of isogenic cultures of Δ*lapD* bacteria transformed with either plasmid DNA of the vector alone or with plasmids carrying the wild-type *acpP* gene 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.

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 Δ6*ppi* and also of Δ*lapD* 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 Δ6*ppi* strains [57]. Thus, a systemic series of bacteriophage P1-mediated transductions were executed using Δ6*ppi* bacteria as recipients. No viable transductants Δ(6*ppi lapD*) were obtained under conditions when Δ6*ppi* 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 10^3-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, Δ*lapD* could be readily introduced when Δ*ppiB* 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 Δ*lapD* Number of Transductants LA 33 °C	Features
wt	1300	viable
Δ6*ppi*	5	not viable
Δ*ppiB*	9	not viable
Δ*ppiB* + p*lpxH*$^+$	920	viable
Δ*ppiC*	1430	viable
Δ*tig*	230 sc [1]	conditional lethality at high temperatures
Δ*fklB*	490 sc	viable but smaller colony size
Δ*fkpB*	735	viable
Δ*slyD*	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 Δ*dksA* 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 Δ*lapD* in Δ6*ppi* 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 Δ*lapD* 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 *ftsH* gene or the *lapB* gene) restore vancomycin resistance in Δ*lapD* bacteria. These very mutations in *lpxC* or *ftsH* or *lapB* genes were earlier shown to suppress the Ts phenotype of *lapC* mutants lacking its periplasmic domain and restore LpxC amounts. Thus, Δ*lapD* 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 Kdo_2-lipid IV_A as a substrate to generate hexa-acylated Kdo_2-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, Δ*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 Kdo_2-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 Δ*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 Δ*lapD* bacteria exhibit a constitutive induction of the *rpoE*P3 promoter even under permissive growth conditions. Transcription of the *rpoE* gene is directed from six promoters, out of which the *rpoE*P3 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*, *fkpA* 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*, *rybB* and *slrA* 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 Δ*lapD* 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 Δ*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 Δ*lapD* could not be introduced into strains lacking six known major cytoplasmic PPIases. This essentiality of LapD in the Δ6*ppi* 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 Δ*lapD* bacteria. As a consequence, Tig not only shows the synthetic lethality in a Δ*dnaK* or Δ*dnaKJ* 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 Δ6*ppi* strains or a Δ*dnaKJ* derivative [40,65] was also found to suppress the Ts phenotype of Δ*lapD* bacteria. Quite interestingly, another multicopy suppressor of Δ6*ppi* bacteria, *srrA* [40], was also found

to suppress the Ts phenotype of Δ*lapD* 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 Δ*lapD* 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 Δ6*ppi*, encoded by the *cmk* gene, becomes indispensable in the Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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, Δ*lapD* bacteria retain significant amounts of LPS. This retention of LPS in the IM in Δ*lapD* bacteria makes it different from ClsA-MsbA assisting LPS transport since Δ*clsA* 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.

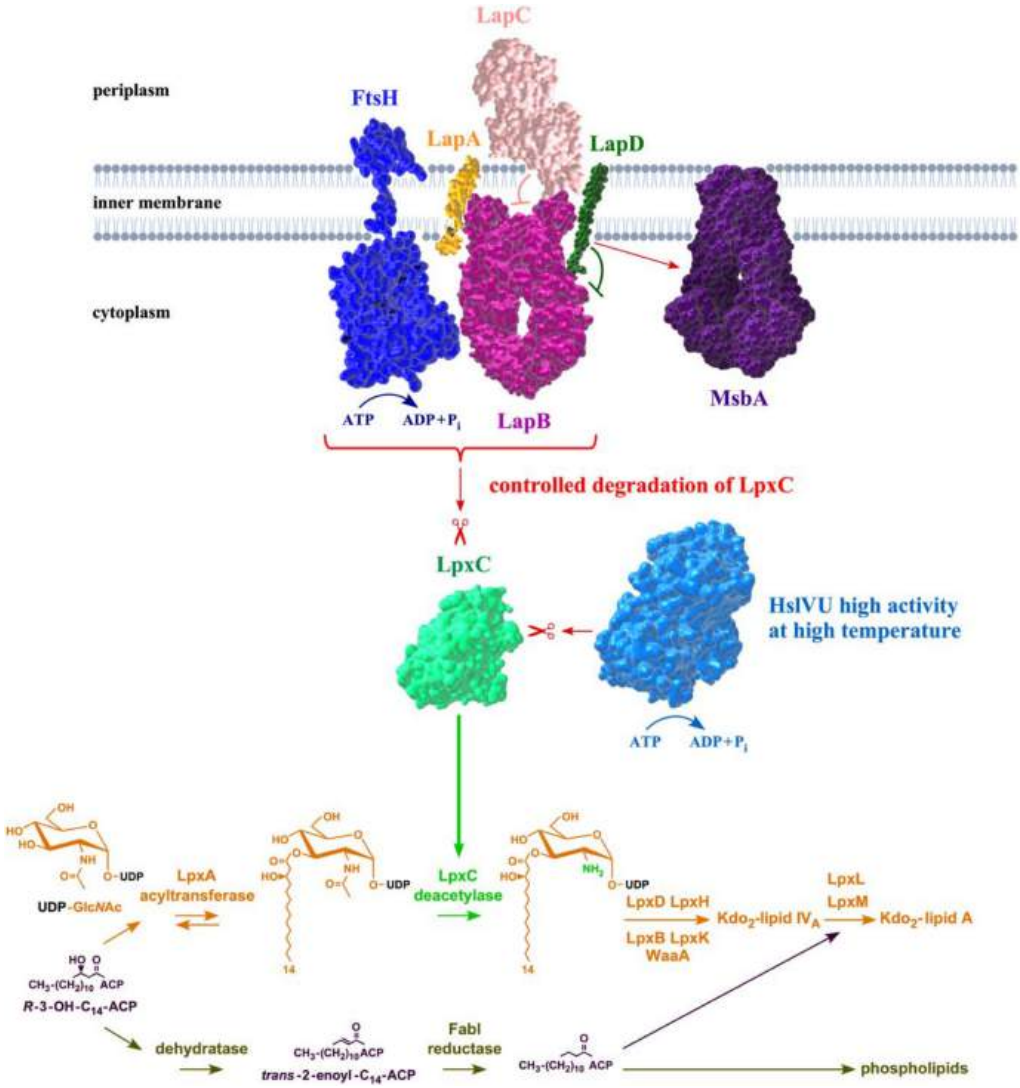

Figure 17. Model of LapD action in regulating LpxC levels and in assisting MsbA-mediated LPS translocation. Based on the co-purification of LapD with the LapA/LapB-FtsH complex and a reduction in LpxC amounts in the absence of LapD, it is proposed that LapD forms a complex in the IM to regulate LpxC amounts. As suppressors mapping to *lapB*, *ftsH* and *lpxC* genes restore growth defects of Δ*lapD* 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, Δ*lapB*, Δ*waaC*, Δ*tig*, Δ*dksA*, Δ*srrA*, Δ*micA*, Δ*cmk*, Δ*clsA*, Δ*lpxL*, Δ*lpxM* and *lapC190*, has been previously described [9,17,21,32,40]. A non-polar Δ*lapD* 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*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[75]
SR23678	BW25113 *lapD*<>*aph*	This study
SR23743	BW25113 *lapD*<>*frt*	This study
SR17532	BW25113 φ(*rpoEP3-lacZ*)	[9]
SR23686	SR17532 *lapD*<>*aph*	This study
SR8233	W3110 *waaC*<>*cat*	[9]
SR23691	SR23678 *waaC*<>*cat*	This study
SR23320	BW25113 *clsA*<>*frt*	This study
SR23769	SR23320 *lapD*<>*aph*	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*<>*aph*	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 ftsH* 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	*ftsH* 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	*ftsH* 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	SR20491 Δ(*lapA lapB*)	This study
SR23138	BW25113 *oppA<>ada*	This study
SM101	*lpxA2*(ts)	CGSC, Yale
MN7	*lpxB1*(ts)	CGSC, Yale
SR23798	SR23678 + pCA24N	This study
SR23790	SR23678 + p*acpP*$^+$	This study
SR23784	SR23678 + p*yfgM*$^+$	This study
SR23786	SR23678 + p*dksA*$^+$	This study
SR23792	SR23678 + p*srrA*$^+$	This study
SR23796	SR23678 + p*accD*$^+$	This study
SR23794	SR23678 + p*ymgG*$^+$	This study
SR23788	SR23678 + p*artJ*$^+$	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**
pCA24N	IPTG-inducible expression vector cm^R	[51]
pDUET	expression vector	Our collection
pKD3	*oriR6K$_g$*, *bla*(Amp^R), *kan*, *rgnB*(Ter), *cat*	[75]
pKD13	*oriR6K$_g$*, *bla*(Amp^R), *kan*, *rgnB*(Ter)	[75]
pKD46	*araBp-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 μM 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 μM 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 NaH_2PO_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, Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 Δ*lapD* 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 P1-mediated transduction. Thus, a bacterio-

phage P1 was grown on a strain (SR9710) carrying a *napA*::Tn*10* 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). Tet^R colonies that lost the Cm cassette were retained. A representative strain from each transduction Tet^R Cm^S 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 Δ*lapD* Kan^R replacement. Transductants were plated at 33 °C and analyzed further. The same strategy of replacing the chromosomal *lapC190* mutation with the wild-type of the *lapC* gene carrying a suppressing mutation mapping to either the *ftsH* gene (GK6095) or in the *lapB* gene (SR22724, SR22726, SR22730, SR22733, and GK6084) using *napA*::Tn*10* as a donor in transductions was adopted. This was followed by introducing the *lapD* null allele.

Concerning 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, $Spec^R$ transductants were selected and those that were Kan^S (replacement of Δ*clsA* 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 Δ*lpxM* background.

4.6. Isolation of Suppressor Mutations That Confer Viability to Δ(lpxL 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 Δ*lpxL* and Δ*lpxM* 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*, *ftsH*, *fabZ* and *msbA*. As both of them had a different single-amino acid substitution in the *msbA*, 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 Δ*srrA* derivative, and strains carrying the inducible *srrA* gene present on a plasmid were grown at 37 °C in LB medium, adjusted to an OD_{595} of 0.05 and allowed to further grow up to an OD_{595} 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 Δ*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 Δ*lapD*, Δ(*waaC lapD*), Δ*clsA* 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 *rpoE*P3-*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.

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