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**Building the shield: new insights into
cell envelope biogenesis and
homeostasis in *Pseudomonas aeruginosa***

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Building the shield: new insights into cell envelope biogenesis and homeostasis in *Pseudomonas aeruginosa*

PhD thesis

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SUMMARY

Gram-negative bacteria have evolved a highly specialized cell envelope that allows them to survive in dynamic and often hostile environments by providing both mechanical protection and selective permeability. Unlike Gram-positive bacteria, whose envelope consists of the cytoplasmic membrane surrounded by a thick peptidoglycan layer, Gram-negative bacteria possess a more complex, multilayered envelope composed of the cytoplasmic (or inner) membrane (IM), a thin peptidoglycan layer located within the periplasm, and an outer membrane (OM). The IM is a typical glycerophospholipid (GPL) bilayer, while the OM is a defining feature of Gram-negative bacteria and represents a unique biological membrane with an asymmetric organization. Its inner leaflet is composed of GPLs, whereas the outer leaflet is primarily formed by lipopolysaccharide (LPS), a hallmark molecule of Gram-negative bacteria. The dense packing of LPS molecules, stabilized by divalent cations, confers low membrane fluidity and reinforces the barrier properties of the OM, which are a major determinant of the intrinsic antibiotic resistance of Gram-negative bacteria. In addition to GPLs and LPS, the OM contains β -barrel OMPs that mediate nutrient uptake and regulate membrane permeability, as well as lipoproteins involved in envelope biogenesis, maintenance, and signaling. Together, these components contribute to the structural integrity of the OM and establish a selective permeability barrier that limits the entry of many toxic compounds and antibiotics. Located between the IM and the OM, the periplasm contains the peptidoglycan layer, a rigid macromolecular network consisting of repeating units of N-acetylglucosamine and N-acetylmuramic acid, which are cross-linked by peptide side chains. Peptidoglycan provides structural support, maintains cell shape, and protects the cell from osmotic stress, thus being essential for envelope integrity and cell viability. This structural complexity is essential to ensure envelope integrity and maintain a robust permeability barrier, that underlies the intrinsic antibiotic resistance of Gram-negative bacteria. Thus, dissecting the molecular pathways underlying cell envelope biogenesis is crucial both to advance our understanding of fundamental aspects of Gram-negative bacterial physiology and to uncover new targets for antibacterial intervention. However, despite major advances in recent years, several key aspects of the pathways governing cell envelope biogenesis and homeostasis remain poorly understood. In this context, the aim of this PhD thesis was to investigate unexplored molecular mechanisms involved in the biogenesis of Gram-negative cell envelope, using the human pathogen *Pseudomonas aeruginosa* as a model organism. In particular, this PhD project focused on elucidating the pathways responsible for the anterograde transport of GPLs and characterizing the mechanisms underlying the recycling of the essential lipid carrier undecaprenyl phosphate (Und-P), two central hubs in cell envelope biosynthesis. In parallel, as part of a PRIN-funded project aimed at identifying

novel antibacterial targets, the essential role of the lipoprotein signal peptidase LspA was examined, and its suitability as a candidate target for antibacterial drug development was evaluated.

To understand how GPLs move from the IM to the OM, in the study presented in chapter 3 we systematically investigated the role of AsmA-like family proteins, previously proposed to be implicated in GPL transport in the model organism *Escherichia coli*. Bioinformatic analyses identified seven *asmA*-like genes in *P. aeruginosa*, whose deletion in multiple combinations coupled with conditional mutagenesis demonstrated that four of them are essential but redundant for *P. aeruginosa* growth and OM integrity. Depletion of AsmA-like proteins caused severe defects in the OM permeability barrier, which were partially alleviated by reducing LPS synthesis or transport. Given that a precise balance between GPLs and LPS is critical for OM integrity, these findings support a role for AsmA-like proteins in mediating GPL transport to the OM.

Then, to elucidate the specific features that enable individual AsmA-like proteins to facilitate GPL transport and maintain membrane homeostasis, in the study reported in chapter 4 we investigated the structural and functional determinants of YhdP, the AsmA-like protein that exhibits the highest activity in maintaining OM integrity in both *E. coli* and *P. aeruginosa*. Using structural predictions, molecular dynamics simulations, and genetic complementation assays, we identified two hydrophobic α -helices at the C-terminal region of YhdP that are crucial for its function. Notably, fusing the YhdP C-terminal domain to YdbH, a shorter essential AsmA-like protein with lower activity, was found to enhance its functionality. Specifically, a YdbH-YhdP fusion protein long enough to span the periplasmic space was more effective than the wild-type YdbH protein in promoting growth and OM integrity. We also showed that, while YdbH activity depends on the OM protein partner YnbE, the YdbH-YhdP fusion protein functions independently of YnbE. Overall, these results suggest that the YhdP C-terminus provides OM anchoring and is likely to assist GPL insertion.

In the next study, presented in chapter 5, we explored the functional importance of DedA family proteins, which have been recently proposed as Und-P flippases in some Gram-positive and Gram-negative bacteria. *P. aeruginosa* encodes six DedA proteins, and by combining deletion mutagenesis, phenotypic assays, and biochemical analyses we demonstrated that one of them, *i.e.* PA4029, is involved in Und-P recycling. Indeed, deletion of PA4029 sensitized cells to the Und-P synthesis-targeting antibiotic fosmidomycin and reduced the emergence of spontaneous colistin-resistant mutants, phenotypes consistent with defects in Und-P recycling. Using native mass spectrometry, we demonstrated that PA4029 binds Und-P with high affinity, and that this interaction is disrupted by the Und-P-targeting antibiotic amphomycin. Together, these findings place PA4029 within the

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conserved DedA-mediated lipid carrier pathway and highlight its importance for cell envelope homeostasis and antibiotic resistance in *P. aeruginosa*.

In the manuscript in preparation described in chapter 6, we further investigated the role of DedA proteins in *P. aeruginosa*. Here, we focused on the PA4011 protein which, besides the DedA domain, also contains a PAP2-like domain which is found in the *E. coli* phosphatases YbjG, PgpB, and LpxT. These enzymes, together with UppP, are responsible for Und-PP dephosphorylation, which occurs before Und-P is flipped back to the cytoplasmic side of the IM and is essential for the Und-P recycling process. Through the generation of double deletion mutants and a triple conditional mutant in PA4011 and the genes encoding the homologs of the *E. coli* proteins UppP and LpxT, we provided preliminary evidence suggesting that PA4011 contributes to Und-P(P) recycling as an Und-PP phosphatase. Deletion of *uppP* in the PA4011 mutant increased susceptibility to EDTA and to fosmidomycin, and led to growth arrest at 25°C, a temperature which strongly suppresses *lpxT* expression. Consistently, growth was fully abrogated when LpxT was depleted in Δ PA4011 Δ *uppP* cells. Furthermore, by creating PA4011 variants carrying amino acid substitutions in predicted catalytic residues, as well as a construct expressing only the PAP2-like domain, we showed that the activity of PA4011 primarily depends on its phosphatase domain. Together, these results draw attention to a previously unrecognized role for PA4011 in the Und-P(P) recycling pathway of *P. aeruginosa*, likely mediated through the phosphatase function of its PAP2-like domain.

In chapter 7 we systematically investigated whether DedA proteins are required for AsmA-like protein function. Indeed, based on the observed interaction and cooperation of some DedA and AsmA-like homologs in lipid trafficking in Eukaryotes, DedA proteins were hypothesized to act as functional partners of AsmA-like proteins, possibly assisting them in GPL extraction from the IM. In this work, we deleted each *dedA* gene in four *P. aeruginosa* triple mutants that rely on a single AsmA-like protein for growth. Through growth assays, we demonstrated that loss of individual DedA proteins do not decrease growth rates or yields in any genetic background, implying that DedA proteins are not required for the function of any essential AsmA-like protein. This evidence strongly suggests that DedA-mediated GPL transport is unlikely to underlie GPL extraction from the IM by AsmA-like proteins. This study has been presented as a concise short-format article, reflecting its specific scope and the presentation of a single experimental data set.

Finally, chapter 8 presents the results obtained from the study of the *P. aeruginosa* LspA enzyme, which have been compiled in a manuscript currently under review. LspA is a signal peptidase that catalyzes a critical step in lipoprotein maturation and was predicted to be essential in *P. aeruginosa*. In this study, we generated a conditional *lspA* mutant to investigate the role of LspA in this bacterium. LspA depletion caused complete growth arrest, loss of viability, and severe OM defects, increasing

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susceptibility to antibiotics normally ineffective against this pathogen. Analysis of suppressor mutants revealed a gain-of-function mutation in *amgS*, encoding the sensor kinase of the AmgRS two-component system, which partially restored growth, membrane integrity, and antibiotic resistance under LspA-limiting conditions, without however restoring lipoprotein maturation. Functional assays showed that this compensatory effect requires the AmgRS-regulated IM proteins HtpX and YccA. Overall, this work confirms LspA as essential in *P. aeruginosa*, validates it as a potential antibacterial target, and uncovers a previously unrecognized role of the AmgRS stress response in alleviating defects in lipoprotein biogenesis.

CHAPTER 1. INTRODUCTION

1.1. The cell envelope of Gram-negative bacteria

Unlike the cells of higher organisms, bacteria must face unpredictable, diluted, and often hostile environments. To survive, they have evolved a sophisticated and complex cell envelope that not only provides physical protection but also mediates the selective uptake of nutrients and the release of waste products. In 1884, Christian Gram developed a differential staining procedure that allowed the classification of most bacteria into two major groups: those that retain the Gram's stain were designated as Gram-positive and those that do not as Gram-negative. The distinction reflects fundamental differences in the architecture of the cell envelopes of these two groups of bacteria (Figure 1). The Gram-positive cell envelope consists of a cytoplasmic membrane surrounded by a thick layer of peptidoglycan, which also contains long anionic polymers, called teichoic acids, composed of glycerol phosphate, glucosyl phosphate, or ribitol phosphate repeats. Gram-negative bacteria, by contrast, have a more complex envelope consisting of three principal layers: the cytoplasmic or inner membrane (IM), the outer membrane (OM), and a thin peptidoglycan layer located within the periplasmic space between them. The IM is a typical glycerophospholipid (GPL) bilayer containing proteins essential for energy generation, lipid biosynthesis, signal transduction, nutrient uptake, and protein secretion. The OM, instead, is an asymmetric bilayer composed of GPLs in the inner leaflet, while the outer leaflet contains lipopolysaccharide (LPS), the hallmark molecule of Gram-negative bacteria. In addition, the OM hosts lipoproteins and β -barrel proteins involved in nutrient transport, signal transduction, and the biogenesis and homeostasis of the membrane itself (1).

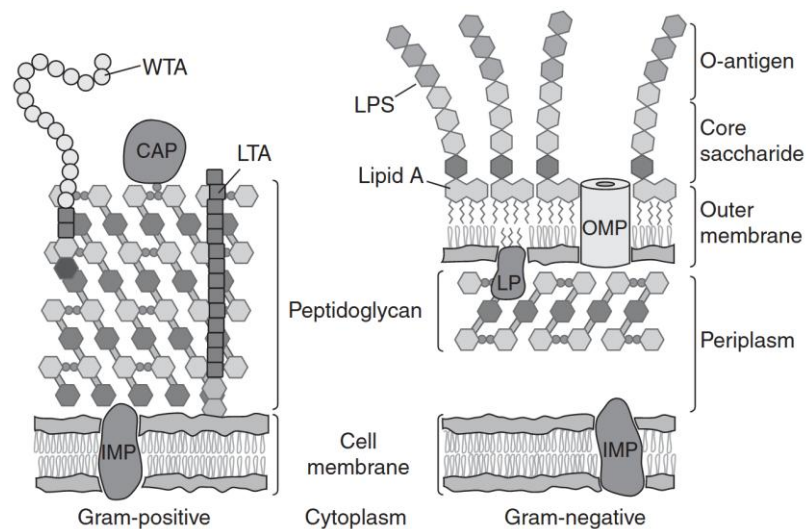


Figure 1. Depiction of Gram-positive and Gram-negative cell envelopes. CAP: covalently attached protein; IMP: integral membrane protein; LP: lipoprotein; LPS: lipopolysaccharide; LTA: lipoteichoic acid; OMP: outer membrane protein; WTA: wall teichoic acid. (1)

1.1.1. The outer membrane: composition, biogenesis and function

The OM is the defining feature of Gram-negative bacteria. Its unique organization provides bacterial cells with mechanical strength and protection from large and/or lipophilic compounds (2, 3), thus rendering many antibiotics which are effective against Gram-positive bacteria inactive against Gram-negative ones (4). The inner leaflet of the OM is composed of GPLs, which slightly vary in composition among bacterial species; the major GPL classes in Gram-negative bacteria are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, the same GPL species that also predominate in the IM (5, 6). The OM also contains proteins that can be broadly divided into two classes: lipoproteins and β -barrel proteins. Lipoproteins are found across all bacterial phyla and are characterized by a lipid moiety covalently attached to the N-terminal cysteine residue. In Gram-negative bacteria, they can be anchored to the periplasmic leaflet of IM or to either leaflet of the OM (1, 7). OM lipoproteins participate in various cellular processes, including OM biogenesis, maintenance of cell shape, and transport of diverse molecules. β -barrel proteins, commonly referred to as OM proteins (OMPs), are integral components of the OM. They consist of 8-26 antiparallel β -strands inserted into the OM, with hydrophobic amino acid residues facing the lipid environment and hydrophilic residues lining a center pore, forming water-filled channels that mediate the passage of hydrophilic molecules across the membrane (8, 9). Some OMPs, such as porins, facilitate the passive diffusion of small solutes with a molecular mass cutoff of approximately 600 Da, whereas others mediate active or energy-coupled transport of specific substrates (1). OMPs thus play a key role in bacterial physiology and environmental adaptation by enabling nutrient uptake essential for metabolism and modulating the permeability of the OM through their distinctive structural and functional properties (8).

What most clearly distinguishes the OM from other biological membranes, however, is the presence of LPS in its outer leaflet, the distinctive feature of most Gram-negative bacteria. LPS is a large, amphipathic and negatively charged molecule whose stability relies on hydrophobic and electrostatic interactions mediated by divalent cations such as Mg^{2+} and Ca^{2+} . It is composed of three major regions: the lipid A, the core oligosaccharide, and the O-antigen (Figure 2) (4, 10). Lipid A is a phosphorylated glycolipid composed of a diphosphorylated glucosamine (GlcN) disaccharide backbone decorated with four to seven acyl chains and serves as the hydrophobic anchor that tethers LPS to the OM. It can undergo several modifications, such as the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (PEtN), which neutralize its negative charge and reduce cell susceptibility to cationic antimicrobial peptides, including polymyxin antibiotics (e.g., colistin) (11, 12). The core oligosaccharide is covalently linked to the GlcN backbone of lipid A and consists of an inner and outer region. The inner core, which is highly conserved among Gram-negative

species, contains 3-deoxy- α -D-manno-octulosonic acid (Kdo), whereas the outer core includes heptose sugars, some of which are phosphorylated, and provides the attachment site for the O-antigen. Owing to its exposure to selective pressures and environmental stress, the outer core is less conserved and varies in sugar composition and arrangement (11, 12). The O-antigen represents the most distal and variable portion of LPS, extending outward from the cell surface. It is an elongated polysaccharide composed of repeating oligosaccharide units (each containing two to eight sugars) and displays extensive variability both across and within species (4, 13).

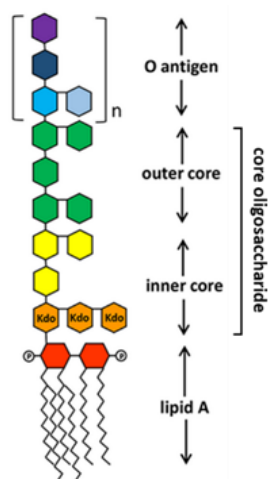


Figure 2. Schematic representation of the core structural components of LPS P: phosphate group; Kdo: 3-deoxy- α -D-manno-octulosonic acid (Adapted from ref. 14).

The length of the O-antigen can vary from complete absence to more than 100 repeating units with branched sugar chains. Gram-negative bacteria that do not produce the O-antigen assemble only lipid A linked to the core oligosaccharide; this truncated form is known as lipooligosaccharide (LOS) (13). The O-antigen is antigenic and can be recognized and neutralized by human antibodies; however, some variants can mimic the composition of host glycans, allowing bacteria to hide from the innate and acquired immune systems of the host (4). LPS plays a crucial role in maintaining the barrier function of the OM. The acyl chains of lipid A contribute to the formation of the hydrophobic region of the membrane, which prevents the diffusion of hydrophilic molecules, whereas the core oligosaccharide and O-antigen create a dense hydrophilic layer that limits the penetration of hydrophobic compounds (13). The barrier properties of the OM are also enhanced by its low fluidity. The acyl chains of lipid A are predominantly saturated, allowing tight packing and extensive intermolecular interactions among LPS molecules. These interactions are further stabilized by divalent cations such as Ca^{2+} and Mg^{2+} , which neutralize the negative charges of phosphate groups and bridge adjacent LPS molecules, reducing membrane fluidity (1). This complex architecture is a

key factor underlying the intrinsic antibiotic resistance of Gram-negative bacteria, as the OM acts as a selective permeability barrier that restricts the entry of many toxic and antimicrobial compounds. Understanding the specific molecular mechanisms involved in OM assembly is therefore fundamental for the development of novel antimicrobial strategies.

The biosynthesis, transport, and insertion of OM components must be tightly coordinated with cell growth and must occur without compromising OM integrity and function (15). All OM components are synthesized in the cytoplasm and must therefore cross the IM and the aqueous periplasmic environment to reach and be properly inserted into the OM. While transport across the IM can exploit the energy sources available in the cytoplasm or in the membrane, translocation across the periplasm and insertion into the OM pose unique challenges, as no utilizable energy sources, like adenosine triphosphate (ATP) or the proton motive force (PMF), are present in the periplasm or the OM (15). Much of our current knowledge about these transport mechanisms comes from studies performed in *Escherichia coli*, the Gram-negative model organism.

Synthesis and transport of lipopolysaccharide

The biosynthesis of LPS, the major component of the OM outer leaflet, is a complex process that requires spatial and temporal coordination of several independent pathways that converge to produce the full-length LPS molecule (Figure 3) (17). The lipid A moiety is synthesized together with the Kdo residues of the core oligosaccharide through a biosynthetic pathway called the Raetz pathway, whereas the O-antigen subunits are assembled independently from LOS on the cytoplasmic side of the IM (13). These intermediates are then independently flipped to the periplasmic leaflet of the IM by dedicated transporters; then, the O-antigen is polymerized and ligated to the core oligosaccharide moiety (13). Finally, the mature LPS is extracted from the IM, transported across the periplasm, and inserted into the OM outer leaflet by the LPS transport (Lpt) complex (16). More in detail, the first step of the Raetz pathway (Figure 3) consists in the fatty-acylation of N-acetylglucosamine (GlcNAc) linked to the nucleotide carrier uridine diphosphate (UDP) by the enzyme LpxA (17). LpxA catalyses the transfer of *R*-3-hydroxymyristate from acyl carrier protein (ACP) to the 3-hydroxyl group of UDP-GlcNAc, generating UDP-3-O-(3-OH-acyl)-GlcNAc (17). This intermediate is then irreversibly deacetylated by the zinc-dependent enzyme LpxC to form UDP-3-O-(acyl)-GlcN, which is further acylated by LpxD to yield UDP-2,3-diacyl-GlcN (13). The pyrophosphatase LpxH then cleaves the pyrophosphate bond of UDP-2,3-diacyl-GlcN to generate 2,3-diacyl-GlcN-1-phosphate, known as lipid X (17). Lipid X is condensed with 2,3-diacyl-GlcN by LpxB, forming a lipid A disaccharide that is inserted in the inner leaflet of the IM (13). The lipid A disaccharide is then phosphorylated at the fourth position by the kinase LpxK to produce lipid IVA, which is subsequently linked to two

activated Kdo molecules (CMP-Kdo) by the enzyme WaaA (13). Two further acylation reactions, catalyzed sequentially by the LpxL and LpxM acyltransferases, complete the lipid A-Kdo moiety, to which additional sugars of the core oligosaccharide are added by specific glycosyltransferases (13). Finally, the lipid A-core moiety is flipped to the periplasmic leaflet of the IM by the ATP binding cassette (ABC) transporter MsbA, which couples this translocation with ATP hydrolysis, since the transfer of amphipathic molecules across GPL membranes is energetically unfavorable (18).

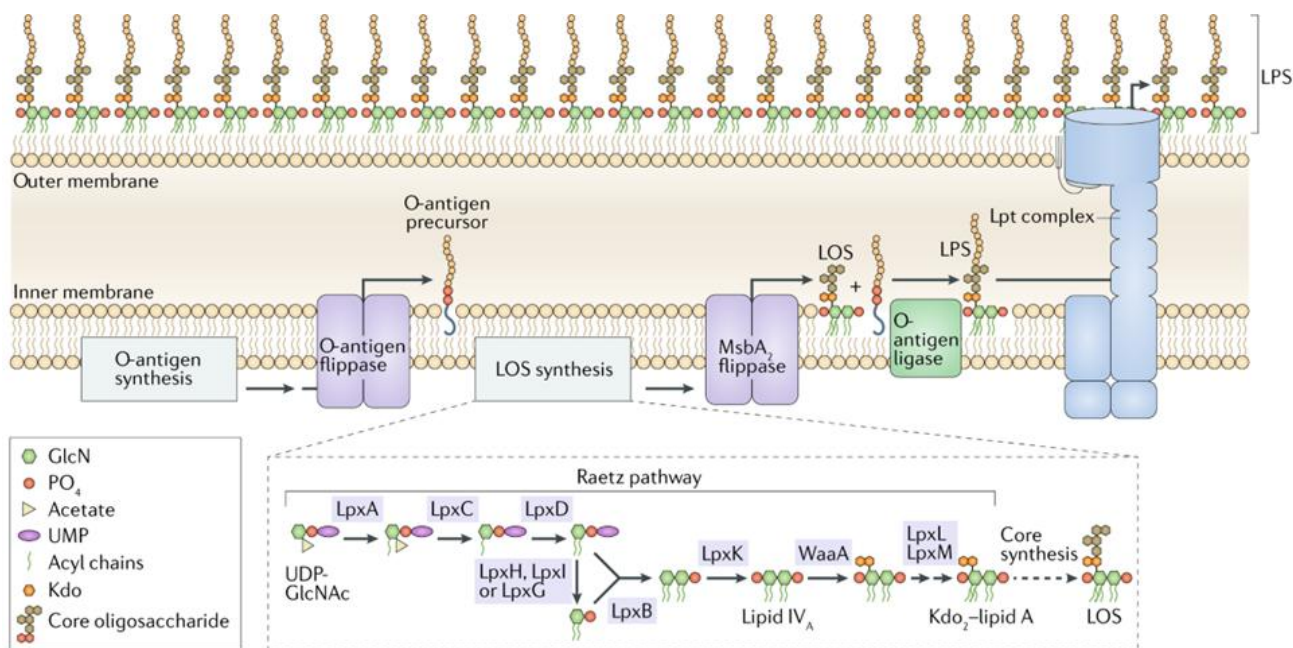


Figure 3. Schematic representation of LPS biogenesis and transport. Lipid A and core oligosaccharide synthesis occurs on the cytoplasmic side of the IM via the Raetz pathway, whereas O-antigen subunits are produced separately on the lipid carrier undecaprenyl pyrophosphate (Und-PP). Specific ABC transporters independently flip the lipid A-core moiety (LOS) and O-antigen subunits across the IM. In the periplasm, O-antigen is polymerized and subsequently ligated to LOS. Finally, the Lpt complex transports mature LPS from the IM to the outer leaflet of the OM (19).

The O-antigen is synthesized through a distinct biochemical pathway. Despite the variable nature of O-antigens, the first step is well conserved among Gram-negative bacteria and involves the transfer of a sugar monophosphate to the lipid carrier molecule undecaprenyl phosphate (Und-P) on the cytoplasmic side of the IM. This reaction generates sugar-pyrophosphate-undecaprenol (sugar-Und-PP), which serves as the acceptor for further glycosylation reactions that vary among species and strains (13). The O-antigen subunits are then flipped to the periplasmic leaflet of the IM by the integral membrane protein Wzx, where the Wzy polymerase catalyzes the block-wise polymerization of O-antigen repeat units, with chain length regulated by Wzz. Once assembled, the mature O-antigen is ligated to the lipid A-core oligosaccharide moiety of the nascent LPS molecule by the WaaL ligase on the periplasmic side of the IM (20, 21).

At this point, LPS molecules are anchored to the periplasmic leaflet of the IM. To reach their final destination in the OM, they must be extracted from IM, transported across the periplasm, and finally inserted into the outer leaflet of the OM. All these steps are mediated by the Lpt complex (Figure 4), which harnesses the energy of ATP hydrolysis in the cytoplasm to drive LPS transport from the IM to the OM. The Lpt system is highly conserved among Gram-negative bacteria and typically consists of seven proteins spanning all compartments of the cell envelope: LptB on the cytoplasmic side of the IM; LptF, LptG, and LptC within the IM; LptA in the periplasm; LptD and LptE in the OM (13). The engine of the system is constituted by the tetramer LptB₂FG, an ABC transporter that couples the energy from ATP hydrolysis in the cytoplasm to the extraction of LPS from the periplasmic leaflet of the IM (17). LptB provides the nucleotide-binding domains (NBDs) responsible for ATP binding and hydrolysis, while LptF and LptG are transmembrane proteins, each formed by six transmembrane helices (TMHs) and a large periplasmic domain folded as a β -jellyroll (17). LptF and LptG interact with each other through their TM1 and TM5 helices, forming an outward facing V-shaped hydrophobic cavity that spans the IM and protrudes slightly into the periplasm (17). The LptB₂FG complex cycles through three conformational states: a nucleotide-free state with an open conformation of the LptB dimer when ATP is not bound; an ATP-binding state in which LptB monomers move close together, with this conformational change being transmitted to LptF and LptG, inducing the opening of a lateral gate between one of the TM1-TM5 interfaces and allowing LPS to enter the cavity; last, an ATP-hydrolyzed state in which ADP release induces the conformational switch back to the nucleotide-free state, closing the lateral gate and expelling LPS toward the periplasmic domains of LptF and LptG (17). Once extracted, LPS is transferred to a periplasmic protein bridge formed by LptC, LptA, and the periplasmic domain of LptD. All three proteins share a β -jellyroll fold characterized by a hydrophobic pocket that accommodates the acyl chains of LPS. LptC is anchored to the IM via a single transmembrane domain and is stably associated with the LptB₂FG complex. LptA is a soluble periplasmic protein that oligomerizes to connect the IM and OM by binding to the periplasmic domains of LptC and LptD. During LPS transport, the acyl chains of the lipid A moiety are shielded by this hydrophobic bridge, while the hydrophilic oligosaccharide portion remains exposed to the periplasm. Finally, LPS transport across the OM and its assembly at the cell surface is mediated by the OM translocon, formed by the β -barrel protein LptD and the tightly associated lipoprotein LptE, which helps LPS insertion and stabilizes LptD, ensuring correct assembly of the translocon (22, 23). When LPS reaches the periplasmic β -jellyroll domain of LptD, its acyl chains are deposited directly into the hydrophobic OM environment, while the hydrophilic portion of LPS is translocated through the lumen of the LptD β -barrel (13).

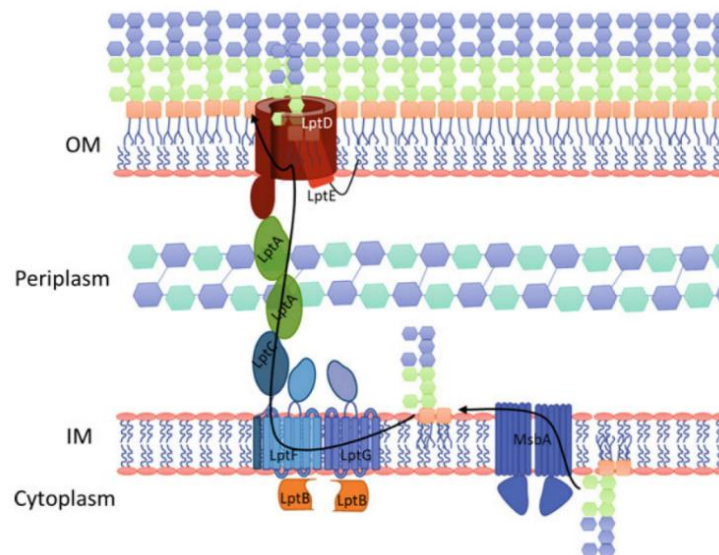


Figure 4. The LPS transport pathway. The lipid A-core moiety synthesized at the inner leaflet of the IM is flipped across the membrane by the ABC transporter MsbA and ligated to the O-antigen (not shown in the figure). The LPS molecule is then extracted from the IM, translocated across the periplasm, and assembled into the outer leaflet of the OM by the transenvelope Lpt complex (17).

Synthesis and transport of integral outer membrane proteins

All proteins are synthesized by ribosomes in the cytoplasm and then sorted to their appropriate cellular localizations. Proteins destined for the periplasm, the OM, or the extracellular milieu are synthesized as precursors carrying an N-terminal cleavable signal sequence, known as a type I signal peptide, which directs them for translocation across the IM (1). Although the sequence of the signal peptide varies among proteins, it exhibits conserved structural features: a positively charged N-terminal region, a central hydrophobic core, and a hydrophilic C-terminal region containing the signal peptidase cleavage site (24). Exported proteins are recognized by the heterotrimeric SecYEG translocon, an essential and universally conserved protein transport channel that mediates translocation of proteins across the cytoplasmic membrane (25) (Figure 5). The SecYEG translocon constitutes only a passive pore that connects the cytoplasm to the periplasm, and therefore its activity relies on the coordinated action of multiple partner proteins that select potential Sec substrates, provide the driving force for their transport, regulate substrate release from the SecYEG channel, and interact with components of the proteostasis network (25). Because folded polypeptides cannot pass through the SecYEG channel, the substrates need to be maintained in an unfolded form. In the phylum Pseudomonadota (formerly known as Proteobacteria), this function is mainly carried out by the cytoplasmic chaperone SecB, which forms a tetramer with a hydrophobic surface involved in substrate binding (Figure 5) (25). Sec substrates are translocated in a post-translational phase,

meaning that the energy for transport is not provided by polypeptide ribosomal elongation but instead by the ATPase SecA (1). SecB delivers the preprotein to SecA by binding its C-terminal domain, while SecA recognizes the hydrophobic region of N-terminal signal peptides via its preprotein-binding domain (25). SecA then loads the preprotein onto the Sec translocon by binding with high affinity the cytosolic loops of SecY. During secretion, the signal peptide is proteolytically removed by a Type I Signal Peptidase (SPase-I) (25).

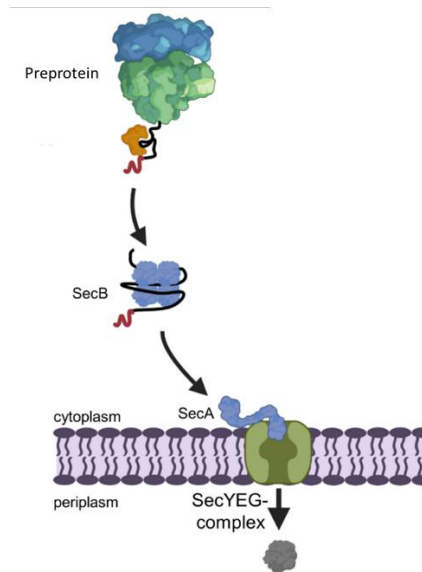


Figure 5. The general secretory (Sec) pathway in Pseudomonadota (Proteobacteria). The chaperone SecB binds preproteins in the cytoplasm and maintains them in an unfolded state. SecA recognizes the signal peptide of secretory proteins (in red) and interacts with the SecYEG translocon, which functions as the protein-conducting channel. ATP hydrolysis by SecA pushes the polypeptide through the SecYEG channel (Adapted from ref. 25)

After translocation through the Sec pathway, the proteins located into the periplasm follow different routes depending on their final destination and/or their chemical composition. The OMPs are inserted into the OM through the β -barrel assembly machinery (Bam). The Bam system of *E. coli*, the best characterized and widely used as a model, is composed of five proteins. BamA, itself a β -barrel OMP, contains five periplasmic polypeptide transport-associated domains (POTRA, P1-P5), and interacts with four OM-anchored lipoproteins, namely BamB, BamC, BamD, and BamE (Figure 6) (15). BamB and BamD bind directly to BamA via the P3 and P5 domains, respectively, whereas BamC and BamE associate with BamD to stabilize the complex (8). While BamA and, to a lesser extent, BamD are essential in *E. coli* and highly conserved among Gram-negative bacteria, the other components are present only in certain species and are nonessential in *E. coli*, indicating that they play accessory roles in the β -barrel assembly pathway (15).

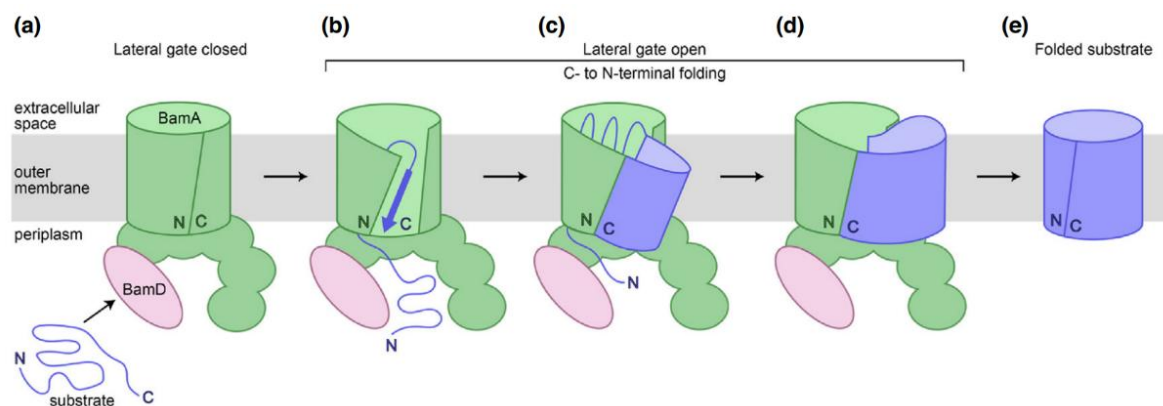


Figure 6. Model of β -barrel OMP assembly by the Bam complex. (a) In its closed conformation, BamA prevents substrate access to its lumen. (b) Substrate engagement with BamD triggers opening of the BamA lateral gate. (c–d) The substrate then enters the BamA lumen, where it is progressively folded in a C-to-N-terminal direction. (e) The fully folded β -barrel is subsequently released into the OM (26)

To reach the Bam complex, OMPs, which are rich in hydrophobic residues, must traverse the aqueous periplasmic environment without (mis)folding. To accomplish this, Gram-negative bacteria rely on periplasmic chaperones such as SurA and Skp, which prevent misfolding and aggregation of OMPs during their transit toward the OM (8). Although in *E. coli* both chaperones participate in OMP transport, most OMPs are escorted primarily by SurA, whereas Skp appears to play an additional role in rescuing OMPs under stress conditions, when the normal assembly pathway is compromised (8). SurA interacts directly with BamA via the P1 domain, whereas BamD binds unfolded OMPs (15). BamD not only recognizes substrates but also regulates BamA conformational cycling through its interaction with the P5 domain (15). BamA initially adopts a closed conformation that blocks access to the barrel lumen. Upon substrate delivery, BamD triggers a conformational change in the BamA β -barrel, allowing capture of the OMP C-terminus by the N-terminal β -strand of BamA. The substrate then enters the BamA β -barrel, where folding occurs, and is ultimately inserted into the OM through the opening of a lateral gate in BamA (8, 26).

Synthesis and transport of outer membrane lipoproteins

Lipoproteins are synthesized as non-acylated precursors, named prelipoproteins. These are translocated across the cytoplasmic membrane by the Sec pathway and remain anchored to the membrane via their N-terminal type II signal peptide, which contains at its C-terminus a conserved four-amino acid motif known as the lipobox, typically composed of leucine, alanine, glycine, and cysteine. The cysteine residue provides the acylation site during lipoprotein maturation and becomes the N-terminus of the mature lipoprotein (27).

After translocation across the cytoplasmic membrane, prelipoproteins undergo a series of sequential modifications catalyzed by three transmembrane enzymes, namely Lgt, LspA, and Lnt (Figure 7) (28). First, Lgt transfers a diacylglycerol moiety from phosphatidylglycerol to the sulfhydryl group of the lipobox cysteine, generating a prelipoprotein. Next, the type II signal peptidase (SPase-II), named Lsp or LspA in many bacteria, cleaves the lipobox, leaving the diacylglyceryl-modified cysteine as the N-terminus of the protein; at this stage, the lipoprotein remains anchored to the cytoplasmic membrane via the diacylated cysteine residue. Finally, the acyltransferase Lnt adds a third acyl chain from phosphatidylethanolamine to the amino group of the N-terminal cysteine, producing a mature triacylated lipoprotein (28). In Gram-negative bacteria, lipoproteins destined for the OM are extracted from the IM and transported by the lipoprotein OM localization (Lol) system, which in most Gram-negatives consists of five proteins: LolA, LolB, LolC, LolD, and LolE (Figure 7).

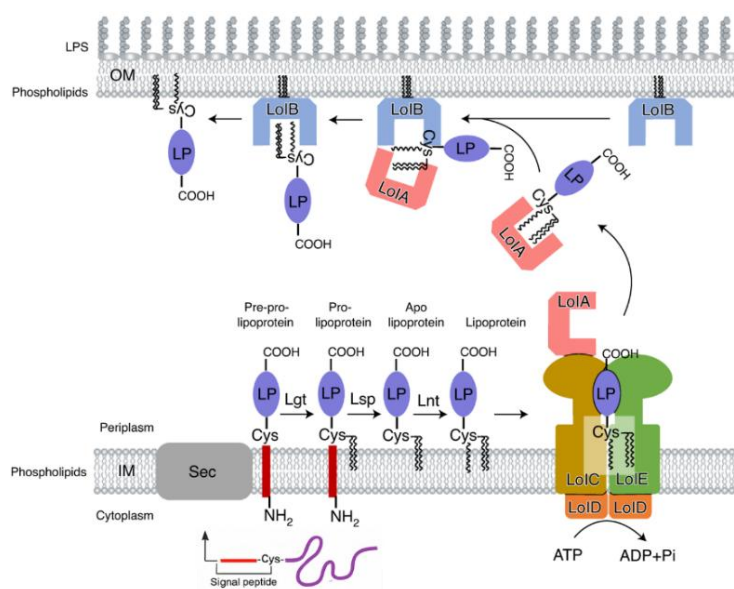


Figure 7. Lipoprotein maturation and transport in Gram-negative bacteria. The N-terminal signal peptide (in red) directs cytoplasmic prelipoproteins to the Sec translocon. After translocation across the IM, Lgt transfers a diacylglycerol moiety to the conserved cysteine within the lipobox. LspA then cleaves the signal peptide, and Lnt adds a third acyl chain to the amino group of the newly exposed N-terminal cysteine. The mature triacylated lipoprotein is subsequently extracted from the IM by the LolCDE ABC transporter in an ATP-dependent manner, shuttled across the periplasm by LolA, and finally inserted into the OM by LolB (Adapted from ref. 29).

Due to their N-terminal lipid moiety, lipoproteins are highly hydrophobic and must therefore overcome the energetically unfavorable processes of detachment from the IM and transport across the hydrophilic periplasmic space to reach the OM (18). This is made possible by the hydrolysis of ATP mediated by an ABC transporter, the LolCD₂E complex, composed of two integral membrane

proteins, LolC and LolE, and two copies of the ATPase LolD (18). LolC and LolE are homologous in structure, each containing four TMHs, but functionally distinct: LolE binds the lipoprotein, whereas LolC recruits the periplasmic chaperone LolA (30). After extraction of the mature OM lipoprotein from the IM by LolCD₂E, LolA receives the lipoprotein from LolC and shields its hydrophobic acyl chains from the aqueous periplasm. Finally, lipoproteins are directionally delivered from LolA to LolB in a mouth-to-mouth manner, due to the higher affinity of LolB for the lipid moiety compared to LolA (28).

Synthesis and transport of glycerophospholipids

In Gram-negative bacteria, GPL biosynthesis takes place in the inner leaflet of the IM and begins with the stepwise acylation of glycerol-3-phosphate by PlsB and PlsC to generate phosphatidic acid (PA). PA is then converted into CDP-diacylglycerol (CDP-DAG), the central precursor for the major GPLs. From CDP-DAG, phosphatidylserine synthase (PssA) and phosphatidylserine decarboxylase (Psd) produce phosphatidylethanolamine, while phosphatidylglycerophosphate synthase (PgsA) together with specific phosphatases generates phosphatidylglycerol. Two phosphatidylglycerol molecules can be condensed by cardiolipin synthases (ClsA/B/C) to form cardiolipin, releasing glycerol (31).

In contrast to other OM components, the mechanism mediating the anterograde transport of GPLs from the IM to the OM remained uncertain for many years. Decades ago, it was proposed that GPLs could flow bidirectionally between the IM and OM (32), in contrast to the unidirectional transport of LPS mediated by the Lpt system. One possible explanation is that GPLs move via diffusive flow, either at zones of hemifusion between the IM and OM, referred to as Bayer's junctions (33), or through protein channels connecting the two membranes. Although the hemifusion sites were initially suggested based on electron microscopy images of *E. coli* cells, later studies indicated that they likely were artifacts (34). More recently, proteins containing the Mammalian Cell Entry (MCE) domain and AsmA-like proteins, which are implicated in lipid trafficking in eukaryotes, have been proposed to play a role in the anterograde transport of GPLs from the IM to the OM.

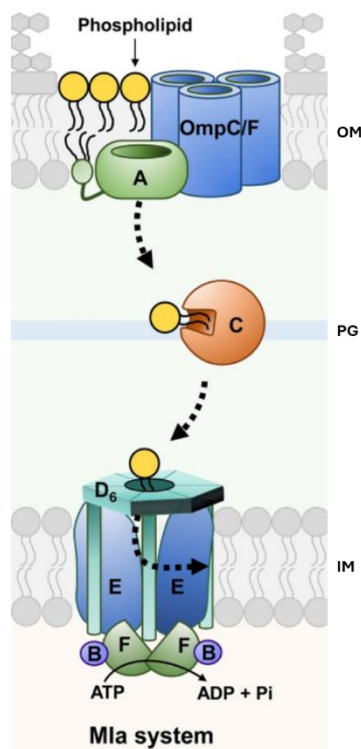


Figure 8. The retrograde transport of GPLs. The Mla proteins (MlaA–F), together with their trimeric β -barrel partner OmpC (or OmpF), mediate the retrograde unidirectional transport of GPLs from the OM back to the IM in an ATP-dependent manner (35).

The first GPL transport system to be characterized, however, was the one responsible for retrograde movement from the OM to the IM, identified several years ago (36). This system, named OmpC-Mla, consists of the OM porin OmpC and several Mla proteins that are localized in the OM, the periplasm, or the IM (Figure 8). The OmpC-MlaA complex specifically recognizes mislocalized GPLs in the outer leaflet of the OM and delivers them to the periplasmic lipid chaperone MlaC, which possesses a deep hydrophobic pocket to bind the GPL molecule and shuttle it to the IM protein MlaD. MlaD contains a periplasmic MCE domain, and six of these domains have been shown to oligomerize into a donut-shaped structure with a hydrophobic central pore capable of hosting GPLs. MlaD is part of the ABC transporter MlaFEDB, which utilizes ATP to extract GPLs from MlaC and finally insert them into the IM (37).

The MCE domain capable of hosting GPLs is also present in other protein complexes conserved in some Gram-negative bacteria, namely PqiABC and YebST, which have been proposed to play a role in lipid transport between the IM and OM (38). The PqiABC system consists of three components: the IM integral protein PqiA, the single-pass IM protein PqiB, and the OM protein PqiC. PqiB contains three MCE domains and a long C-terminal helix, and, like MlaD, it can oligomerize into a

hexamer, forming a structure with the MCE rings stacked near the IM and connected to a more constricted lipophilic tunnel, possibly interacting with PqiC in the OM to bridge the two membranes. The YebST system works in a similar manner, with YebS as the integral IM protein and YebT containing seven MCE domains. YebT forms a hexameric assembly of MCE rings that form a long hydrophobic tunnel which is predicted to connect the IM and OM (37). Both PqiB and YebT have been shown to be capable of interacting with GPLs and possess structural and biochemical features compatible with lipid transport across the periplasmic space (37, 40). However, the simultaneous deletion of PqiB, YebT, and the other MCE protein MlaD in *E. coli* only causes minor defects in OM integrity without affecting bacterial growth (38). This strongly suggests that MCE proteins are not responsible for the essential anterograde transport of GPLs in Gram-negative bacteria. However, it cannot be excluded that they may play a role under stress conditions, since the expression of both PqiABC and YebST is regulated by pathways activated in response to membrane stress (40-42).

Only recently, studies performed in *E. coli* provided evidence that members of the AsmA-like protein family are likely responsible for the transport of GPLs from the IM to the OM. The AsmA-like protein clan is a family of proteins conserved in Gram-negative bacteria, anchored to the IM by an N-terminal TMH, and characterized by a large periplasmic region containing a Chorein-N domain and an AsmA-like domain (Figure 9). The 100–150-residue Chorein-N domain is also present in eukaryotic lipid-transfer proteins (43), whereas the AsmA-like domain varies in length and is predicted to be composed of β -taco repeats, forming a structure that resembles the Lpt bridge transporting LPS across the periplasm (35). YhdP was the first AsmA-like protein proposed to participate in anterograde GPL transport (44). Grimm and colleagues identified YhdP while studying a *mlaA* gain-of-function mutant (*mlaA**), which expresses an MlaA variant that aberrantly translocates GPLs to the outer leaflet of the OM, opposite to the normal retrograde function of wild-type MlaA. Mislocated GPLs activate the OM phospholipase PldA, which generates free fatty acids from mislocalized GLPs (45). The cell perceives this as a sign of lipid imbalance, triggering increased LPS synthesis (46). Normally, this response restores OM lipid balance, but in *mlaA** cells the constant GPL mislocalization drives chronic LPS overproduction, OM destabilization and loss of OM material through blebbing. This triggers an excessive compensatory GPL flow from the IM, ultimately compromising IM integrity and leading to cell lysis (44). In this context, YhdP was identified in a transposon mutant screen, as *yhdP* inactivation suppressed *mlaA**-induced lysis, likely by slowing GPL flow to the OM. This hypothesis was supported by the observation that the double mutant $\Delta yhdP$ *mlaA** showed a reduced anterograde GPL flux, confirming that YhdP contributes to GPL transport across the periplasm. However, the viability of $\Delta yhdP$ cells indicates that YhdP is not essential, suggesting redundancy in GPL transport pathways (44).

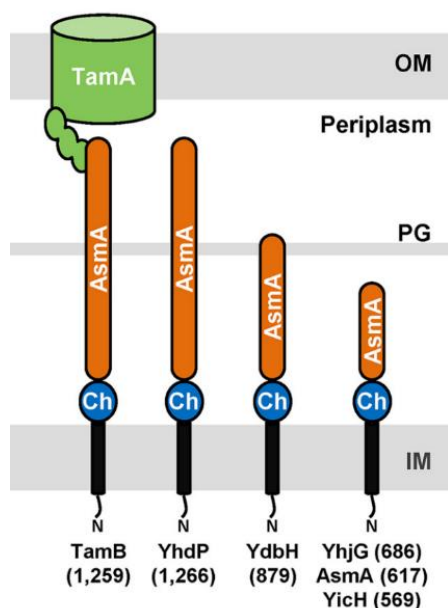


Figure 9. Cellular localization and predicted domain organization of the six AsmA-like proteins of *E. coli*. Each protein is anchored to the IM by an N-terminal TMH. In the periplasm, they contain a region homologous to the eukaryotic chorein-N domain, implicated in intermembrane lipid transport, followed by a large C-terminal region predicted to include multiple AsmA domains. Among them, TamB has been experimentally shown to interact with the OM β -barrel protein TamA (47).

Later, Ruiz and colleagues systematically investigated the involvement of AsmA-like proteins in GPL transport and OM homeostasis (47). To this aim, they generated single and multiple mutants in the six *asmA*-like genes of *E. coli* (*tamB*, *yhdP*, *ydbH*, *asmA*, *yicH*, and *yhjG*). While single deletions did not cause major growth defects, the double mutant $\Delta tamB \Delta yhdP$ displayed severe envelope-associated phenotypes, including increased OM permeability, altered cell morphology, and a strong tendency to lyse in stationary phase, suggesting functional redundancy between these two AsmA-like proteins. Notably, inactivation of the Mla system or of PldA fully suppressed the lysis phenotype of the $\Delta tamB \Delta yhdP$ mutant, further supporting a link between TamB/YhdP and GPL transport. Remarkably, the simultaneous loss of a third AsmA-like protein, YdbH, together with TamB and YhdP resulted in a lethal phenotype, indicating that these three proteins are essential but functionally redundant in *E. coli* (47). Concurrently, another research group reached similar conclusions and additionally demonstrated that a $\Delta tamB \Delta ydbH$ mutant with reduced YhdP levels shows decreased amounts of GPLs in the OM, further supporting the involvement of AsmA-like proteins in intermembrane GPL transport (48).

The model proposed for GPL transport mediated by AsmA-like proteins suggests that they form bridge-like structures reminiscent of the Lpt system, thereby connecting the IM and OM to enable GPL delivery to the OM. For such a mechanism to function, their structures would need to extend

approximately ~ 250 Å to span the periplasmic space. Among these proteins, YhdP is the only one predicted to be long enough to traverse the entire periplasm. YdbH, in contrast, is considerably shorter, implying that it likely requires an OM partner to reach the OM (35). Consistent with this idea, *ydbH* is part of an operon that also encodes the OM lipoprotein YnbE. Structural predictions indicate that these proteins can interact, with the C-terminal region of YdbH engaging a β -strand periplasmic domain of YnbE multimers positioned near the OM. Deletion of *ynbE* in a $\Delta tamB \Delta yhdP$ background resulted in a lethal phenotype, mirroring the effect of removing *ydbH* (47), supporting the hypothesis that YdbH requires YnbE to fulfill its function in GPL transport (49). TamB and YhdP are similar in size, each containing roughly 1,260 residues. However, TamB is predicted to have a shorter periplasmic domain of ~ 160 Å due to its higher degree of superhelicity (50). Nevertheless, TamB is known to interact with the OM protein TamA, a component of the translocation and assembly module (TAM), which was first proposed to mediate the transport or insertion of specific OMP subclasses, such as autotransporters (51). This interaction enables TamB to reach the OM by forming a stable intermembrane-spanning complex, providing a plausible route for delivering TamB-transported GPLs to the OM. Indeed, TamB function in GPL transport in *E. coli* has been shown to depend on TamA (47). Nonetheless, TamA is unlikely to constitute a universal OM partner for AsmA-like proteins, as it is generally nonessential and is absent from many Gram-negative bacteria (52). In contrast to TamB and YdbH, YhdP is predicted to be able to reach the OM autonomously, suggesting that it may insert into the IM via its N-terminal TMH and into the OM through its C-terminal domain. This hypothesis is supported by the presence of two highly hydrophobic α -helices near the YhdP C-terminus, which could mediate interactions with the OM. Consistently, a very recent study demonstrated that these hydrophobic helices are essential for the functioning of *E. coli* YhdP (53). To date, it remains unclear whether AsmA-like proteins involved in GPL transport require IM partners to extract their substrates from the membrane. While the extraction of LPS and GPLs by the Lpt and Mla systems depends on ATP hydrolysis, preliminary evidence suggests that YhdP-mediated GPL movement across the cell envelope may occur without ATP consumption (44). One possibility is that GPL transfer through AsmA-like proteins is driven solely by the concentration gradient of GPLs across the envelope, given that their synthesis takes place in the IM.

1.1.2. The cell wall: structural organization and assembly

The bacterial cell wall contains peptidoglycan, a rigid exoskeleton that provides structural support to the cell, protects it from osmotic lysis, and maintains cell shape. Peptidoglycan consists of repeating units of GlcNAc and N-acetylmuramic acid (MurNAc), which are cross-linked by peptide side chains (1). Each MurNAc residue carries a peptide chain linked to its D-lactoyl group, most commonly

composed of L-alanyl- γ -D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-alanine in nascent peptidoglycan monomers, with the terminal D-Ala residue being removed during maturation of the macromolecule. Cross-linking of glycan strands generally occurs between the carboxyl group of the D-Ala at position 4 and the amino group of the diamino acid (meso-diaminopimelic acid or L-lysine) at position 3, either directly or via a short peptide bridge (54).

The biosynthesis of peptidoglycan is a complex, multistep process that begins in the cytoplasm, where the precursors are synthesized, and continues on the inner and outer sides of the cytoplasmic membrane, where lipid-linked intermediates are assembled and polymerization reactions take place, respectively (Figure 10) (55). Cytoplasmic precursors are synthesized through independent pathways: (i) UDP-GlcNAc is produced from fructose-6-phosphate by the Glm enzymes; (ii) UDP-MurNAc is derived from UDP-GlcNAc through the activity of MurA and MurB; (iii) UDP-MurNAc-pentapeptide (UDP-Mpp) is formed by the sequential addition of amino acids composing the peptide chain to the D-lactoyl group of UDP-MurNAc by the Mur ligases MurC, MurD, MurE, and MurF; (iv) D-Glu is synthesized either from racemization of L-Glu or through a D-amino-acid aminotransferase that converts D-Ala and α -ketoglutarate into D-Glu and pyruvate; and (v) the D-Ala-D-Ala dipeptide is formed by condensation of two molecules of D-Ala, previously generated from L-Ala by alanine racemase (55). Then, the integral membrane phospho-MurNAc-pentapeptide transferase MraY catalyzes the first membrane-associated step of peptidoglycan synthesis by transferring the phospho-MurNAc-pentapeptide moiety from UDP-Mpp to Und-P, leading to uridine-monophosphate (UMP) and undecaprenyl-pyrophosphoryl-MurNAc-pentapeptide, commonly referred to as Lipid I (56). Subsequently, the glycosyltransferase MurG transfers a GlcNAc moiety from UDP-GlcNAc to Lipid I to form undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc, known as Lipid II. Lipid II is then flipped across the cytoplasmic membrane by a dedicated flippase, identified as either MurJ (57) or FtsW (58), delivering peptidoglycan precursors to the outer leaflet of the membrane, where polymerization takes place. The final steps of peptidoglycan synthesis are catalyzed by a set of well characterized enzymes, including peptidoglycan glycosyltransferases and transpeptidases as well as the bifunctional penicillin binding proteins (PBPs), which harbor both enzymatic activities within distinct transglycosylase and transpeptidase domains (56, 59).

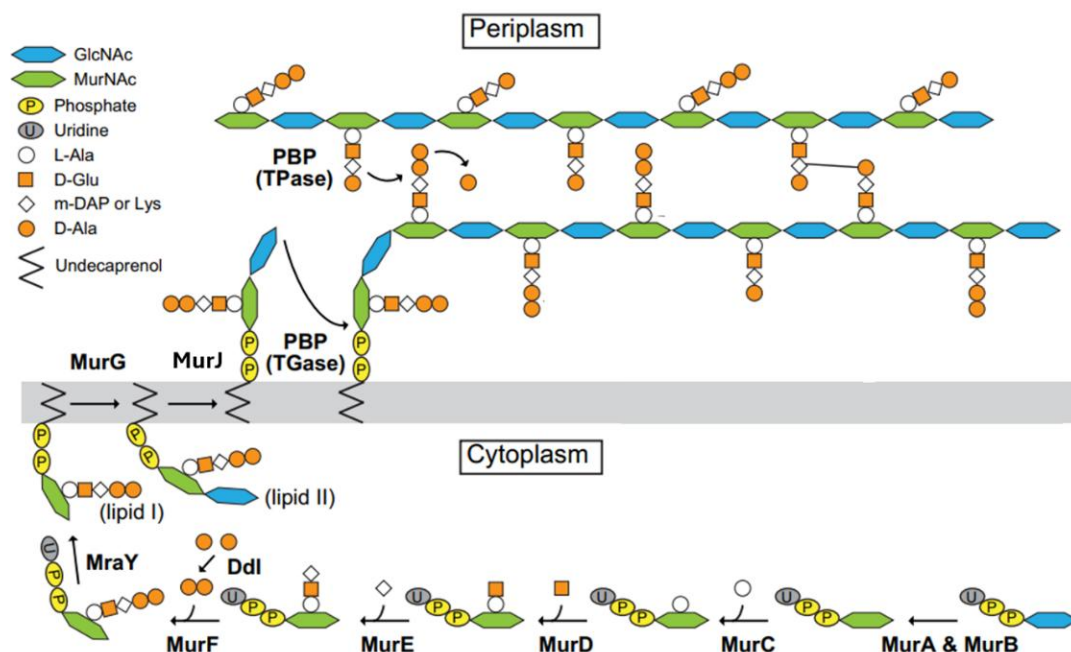


Figure 10. Peptidoglycan biosynthesis. Mur enzymes generate UDP-MurNAc-pentapeptide (UDP-Mpp), and MraY transfers this phospho-MurNAc-pentapeptide to Und-P, forming lipid I. MurG then adds GlcNAc to produce Lipid II, which is flipped to the outer side of the cytoplasmic membrane by MurJ. At the cell surface, PBPs or dedicated transglycosylases (TGases) and transpeptidases (TPases) polymerize glycan strands and form peptide crosslinks, ultimately assembling the three-dimensional peptidoglycan network. Ddl: D-Ala-D-Ala ligase; PBP: penicillin-binding proteins; TGase: transglycosylase; TPase: transpeptidase. (Adapted from ref. 60).

The transglycosylase domain polymerizes the glycan strands by repetitively adding disaccharide-peptide units to either the reducing or the nonreducing end of the growing chain. The transpeptidase domain catalyze peptide crosslinking through a three-step mechanism: (i) the rapid, reversible, and non-covalent formation of a complex between the enzyme and the pentapeptide, (ii) the cleavage of the C-terminal D-Ala-D-Ala peptide bond, leading to the release of a D-Ala, and (iii) the transfer of the D-Ala residue to the stem on the diamino acid in the third position of another peptide (61, 62). Together, these reactions ultimately result in the formation of the peptidoglycan layer.

The lipid carrier undecaprenyl phosphate: synthesis, recycling and functions

A key role in the peptidoglycan synthesis is played by Und-P, an essential lipid carrier belonging to the polyprenyl phosphate family, which includes carriers of sugars and glycopolymers across biological membranes in all domains of life (63). Und-P is a long-chain isoprenoid composed of 11 isopentenyl-diphosphate units forming a 55-carbon lipid that serves as the membrane-embedded scaffold for assembling and flipping lipid-linked intermediates required for cell wall biogenesis (64). The role of Und-P is to facilitate translocation of a polar head group (mono- or oligosaccharides)

across the highly hydrophobic environment of the cytoplasmic membrane (Figure 11) (64). Besides peptidoglycan precursors, it also mediates the transport of the monomers of other glycopolymers, including O-antigens, teichoic acids, and capsular polysaccharides, and carries sugars and oligosaccharides that are used to glycosylate lipid A, teichoic acids, and surface proteins (63).

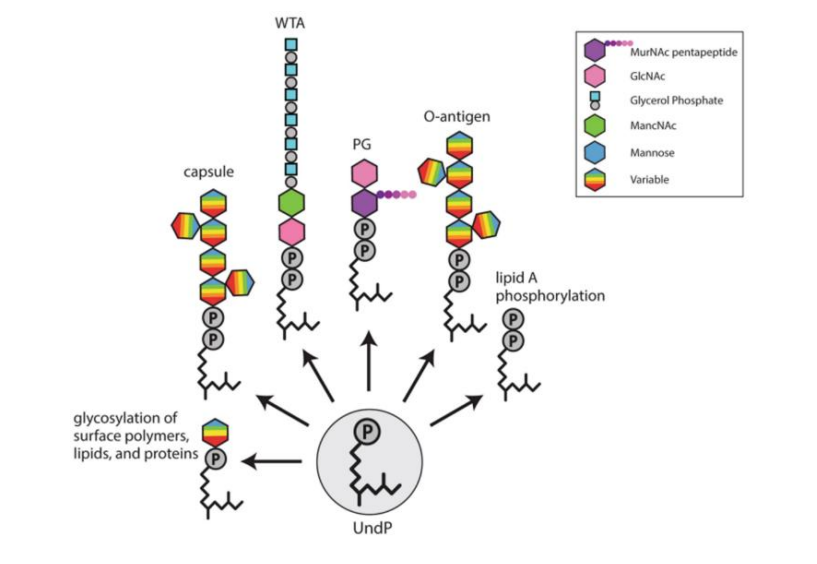


Figure 11. Glycopolymer substrates transported by Und-P. Schematic representation of glycopolymers and sugar modifications whose translocation and assembly in bacteria depend on the lipid carrier Und-P (Adapted from ref. 63).

Und-P is produced by both *de novo* synthesis and recycling pathways (64). The *de novo* synthesis is performed by Und-PP synthase (UppS), that catalyzes consecutive condensation reactions in which eight isopentenyl pyrophosphates are added to a farnesyl pyrophosphate, which is a 15-carbon long chain composed of three units of isopentenyl diphosphate previously synthesized by a farnesyl-diphosphate synthase. The resulting Und-PP is then dephosphorylated to produce Und-P (56). This final reaction was originally attributed to the Und-P phosphatase (UppP), but this was shown to be incorrect when the membrane topology of the *E. coli* UppP enzyme was determined, which revealed that its catalytic residues face the periplasmic leaflet of the IM. This finding suggested that there is either an as yet unidentified Und-PP phosphatase specifically acting on the cytoplasmic face of the membrane, or that *de novo* synthesized Und-PP undergoes a “flip-flop” mechanism, being flipped to the periplasmic leaflet of the IM for UppP-dependent dephosphorylation before being flipped back to the cytoplasmic side for use in cell envelope polysaccharide biosynthesis (65).

The recycling of Und-P(P), on the other hand, depends on membrane associated phosphatases, that in *E. coli* are the above-mentioned phosphatase UppP and the PAP2 (Phosphatidic Acid Phosphatase type 2)-domain proteins PgpB, YbjG (Figure 12) and LpxT (64-67). These enzymes remove a phosphate group from Und-PP to regenerate Und-P, which is then translocated back to the

cytoplasmic leaflet of the membrane. However, how Und-P is flipped back to the cytoplasmic side of the IM during the last step of recycling has remained unclear for a long time.

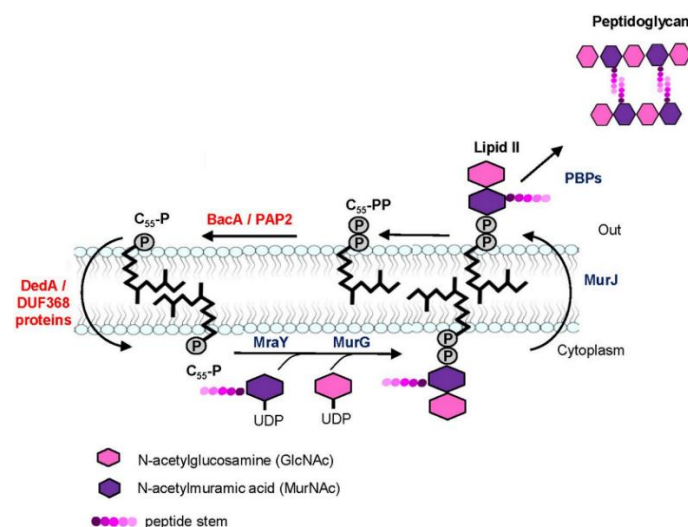


Figure 12. Schematic representation of Und-P(P) recycling process. Und-P(P) is recycled through a dephosphorylation reaction catalyzed by BacA (recently renamed UppP) or a member of the PAP2 superfamily (e.g., PgpB and YbjG in *E. coli*), followed by flipping back to the cytoplasmic side of the cytoplasmic membrane, likely mediated by DedA proteins or DUF368-containing proteins (Adapted from ref. 68)

Recent studies, performed in the Gram-negative bacterium *Vibrio cholerae* and the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (63, 66), provided evidence that Und-P translocation from the outer to the inner side of the cytoplasmic membrane is mediated by proteins containing the DUF368 domain and/or belonging to the DedA family, a highly conserved family of integral membrane proteins found across all domains of life, which vary in number among bacterial species (69-71). Sit and colleagues were studying the *V. cholerae* gene *vca0040*, which encodes for a multi-pass IM protein containing the widely conserved domain DUF368 and a large putative cleft, typical of ligand-binding or transport domains. A *V. cholerae* mutant strain lacking *VCA0040* ($\Delta vca0040$) exhibited alkaline sensitivity and cell shape defects, reduced peptidoglycan levels with concurrent accumulation of the peptidoglycan precursor UDP-Mpp, suggestive of a peptidoglycan biosynthesis defect. Similar defects were observed in a *S. aureus* deletion mutant in the SAOUHSC_00846 gene, encoding a DUF368 protein. This mutant also displayed increased sensitivity to amphomycin, a Ca^{2+} -dependent lipopeptide antibiotic that specifically binds Und-P in the outer leaflet of the cytoplasmic membrane, indicative of a defective Und-P recycling and suggesting that DUF368 proteins could act as Und-P flippases. Transposon mutagenesis in *V. cholerae* further showed that simultaneous inactivation of *vca0040* and *vca0534*, encoding a homologue of the *E. coli* DedA protein YghB, caused a lethal phenotype, suggesting that VCA0534

(YghB) is also involved in Und-P translocation. Accordingly, overexpression of VCA0534 (YghB) was able to rescue the growth defect of the *V. cholerae* $\Delta vca0040$ mutant under alkaline conditions (66). These findings were confirmed by another study conducted in *B. subtilis* and *S. aureus* (63). Using an amphomycin derivative conjugated with a fluorescent dye (MX2401-FL), it was observed that mutants lacking the *dedA* genes *yngC* in *B. subtilis* and *SAOUHSC_02816* in *S. aureus* had higher membrane-associated fluorescence than wild-type strains, and overexpression of those genes drastically reduced the MX2401-FL signal. Cross-species complementation also demonstrated that *yngC* and *SAOUHSC_02816* homologs from *E. coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Bacillus cereus* conferred MX2401 resistance to a *B. subtilis* double mutant lacking *yngC* and its paralog *ykoX*. However, since *B. subtilis* $\Delta yngC \Delta ykoX$ had no discernable growth or morphological defects, it was also hypothesized that Und-P could at least partially flip-flop spontaneously across the membrane or through additional transporters involved in Und-P recycling (63).

The mechanism underlying Und-P function appears to be highly conserved: the sugar/glycan is first transferred from a nucleotide-diphosphate activated donor to the lipid carrier, generating a membrane-bound polyprenyl-(mono- or di-) phosphate-glycan intermediate. However, the linkage of the sugar to the lipid carrier is not sufficient to overcome the thermodynamic barrier represented by the translocation of a polar group across a lipid bilayer; therefore, this step requires a dedicated flippase (64), such as MurJ or FtsW for Lipid II translocation or ArnE and ArnF for the translocation of Und-P-L-Ara4N. Indeed, Und-P is also involved in the modification of lipid A with L-Ara4N, which is the main mechanism of polymyxin resistance in several Gram-negative bacteria, as it reduces the negative charge of lipid A and thereby decreases its affinity for polymyxins. L-Ara4N biosynthesis starts with the conversion of UDP-glucose to UDP-glucuronic acid (Figure 13). ArnA then catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate a novel UDP-4-ketopentose. This is then transaminated by ArnB, using glutamic acid as the amine donor, to form UDP- β -L-Ara4N, which is N-formylated by the N-terminal domain of ArnA. ArnC then transfers the N-formylated L-Ara4N moiety to Und-P, with the product being rapidly deformylated by ArnD. After being transported to the periplasmic leaflet of the IM by the flippases ArnE and ArnF, the L-Ara4N group is transferred from Und-P-L-Ara4N to lipid A by ArnT (Figure 13) (72).

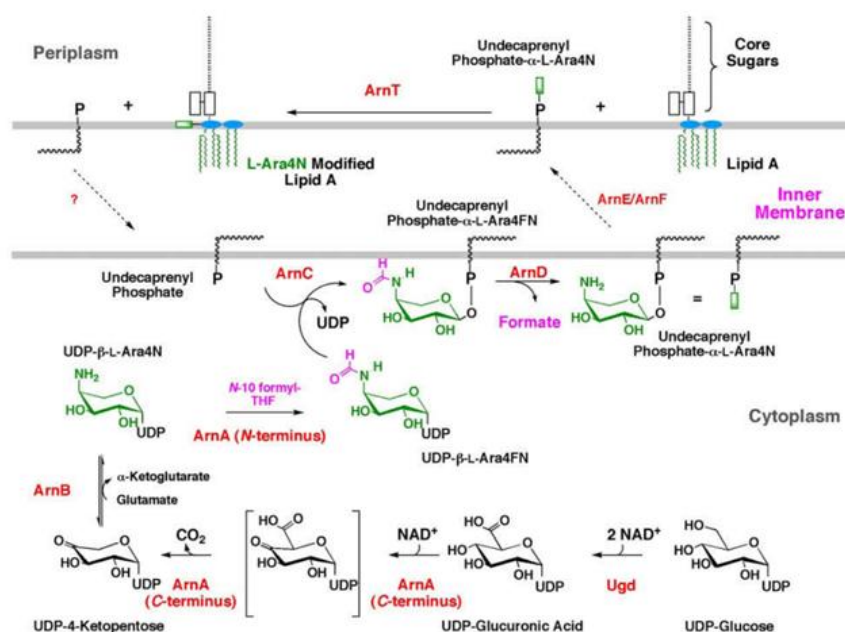


Figure 13. Biosynthesis transfer of L-Ara4N to lipid A. ArnA, ArnB, ArnC, and ArnD catalyze the synthesis of Und-P-L-Ara4N. Then, ArnE and ArnF mediate the transport of Und-P-L-Ara4N to the periplasmic leaflet of the IM. Finally, the membrane enzyme ArnT transfers L-Ara4N to lipid A (72)

1.2. The cell envelope as target for antibiotics: state of the art and perspectives

Antibiotic resistance remains a major global public-health challenge. According to a recent report by World Health Organization (WHO), antimicrobial resistance is directly responsible for more than 1 million deaths worldwide each year and contributes to a total of about 5 million deaths annually when including indirect effects (73). The European Centre for Disease Prevention and Control (ECDC) estimates that more than 35,000 people die each year in Europe because of infections caused by antimicrobial-resistant bacteria (74). These numbers underscore the severity of the crisis, a situation that some experts describe as a return to a “pre-antibiotic era,” in which even common infections and minor injuries may again become lethal (75). Recent global projections from the Global Research on Antimicrobial Resistance (GRAM) further emphasize the urgency of this problem: according to updated analyses, drug-resistant infections could be responsible for over 39 million deaths by 2050 if no effective countermeasures are implemented (75). This scenario highlights the pressing need for robust antibiotic stewardship, improved infection-prevention strategies, better diagnostics, and the development of new antimicrobials. In 2024, the WHO included in its *Bacterial Priority Pathogens List* Gram-negative species and strains all sharing a common feature: they are protected by complex cell envelopes that most antibiotics cannot penetrate, which reduce drug efficacy contributing to antibiotic resistance (76). However, since the discovery of penicillin, some of the most successful antibiotics have targeted the bacterial cell envelope. One clear advantage of targeting the envelope is

that these drugs do not need to enter the cytoplasm (76). Other attractive aspects of cell envelope-targeting antibacterials include the fact that cell envelope biogenesis relies on specialized enzymatic machineries and essential precursors that represent vulnerable points that antibiotics can exploit. In addition, antibiotics that act on non-proteinaceous targets, such as lipid components of the bacterial cell envelope, are usually less prone to resistance development, as modifications in these targets require extensive changes in the bacterial biosynthetic machinery rather than simple genetic mutation (77, 78). Furthermore, as some envelope components only exist in bacteria, the risk for unwanted off-target effects in human cells is reduced. Together, these considerations make the cell envelope of bacterial pathogens an attractive target for the development of new and potentially safe antibacterial drugs (76).

1.2.1. Mechanisms of action and resistance of envelope-targeting antibiotics

When Alexander Fleming deliberately left his plates on the bench to capture airborne agents that might kill staphylococci, his success exceeded anything he could have anticipated. In his original publication, he described a substance that was unstable in aqueous solution but that could act as an antiseptic or as a selective agent for isolating Gram-negative bacteria from mixed cultures of staphylococci and streptococci. This substance, later recognized as penicillin G, became the first antibiotic ever discovered, revolutionizing the treatment of bacterial infections (79).

Beta-lactam antibiotics

As the potential utility of penicillin G as a therapeutic agent became more evident, Fleming and a consortium of scientists optimized its isolation and identification (79, 80). These efforts set the stage for the launch of the most successful class of antibiotics in history, the β -lactams, currently the most widely used antibacterial agents for treating infectious diseases (81). β -lactams exert bactericidal activity by interrupting bacterial cell-wall synthesis through covalent binding to essential PBPs, thereby disrupting peptidoglycan cross-linking and ultimately causing cell lysis (82). Penicillin G represents the archetype of all β -lactam antibiotics and remains a benchmark for understanding the structural and functional principles of this antibiotic class. Originally isolated from *Penicillium chrysogenum* (79, 80), the molecular architecture of penicillin G is defined by the penam nucleus, composed of a β -lactam ring fused to a thiazolidine ring, to which a benzyl side chain is attached (83). It is highly effective against many Gram-positive bacteria, including streptococci, non- β -lactamase-producing staphylococci, and certain anaerobic species, while showing limited activity against most Gram-negative bacteria, due to permeability barriers and β -lactamase-mediated hydrolysis (81). Indeed, the vulnerability of penicillin G to enzymatic degradation by β -lactamases

has historically restricted its clinical utility (84). This limitation prompted the development of semi-synthetic penicillins with enhanced properties, including ampicillin and amoxicillin with broader Gram-negative coverage, penicillinase-resistant penicillins such as methicillin and nafcillin, designed to overcome staphylococcal resistance, and extended-spectrum penicillins such as carbenicillin and piperacillin, which are active against *P. aeruginosa* and certain enteric pathogens. Furthermore, the combination of penicillins with β -lactamase inhibitors (e.g., amoxicillin-clavulanate and piperacillin-tazobactam) has become a widely adopted strategy to restore activity against resistant strains. Despite these advancements, the continuous emergence of β -lactamase-producing organisms, including extended-spectrum β -lactamases (ESBLs) and other resistance mechanisms, has gradually reduced the clinical efficacy of penicillin G and many of its derivatives (81, 84).

Cephalosporins are a major subclass of β -lactam antibiotics, derived from the natural compound cephalosporin C, originally isolated from *Cephalosporium acremonium* (85), later renamed *Acremonium chrysogenum*. Their bicyclic β -lactam structure resembles that of penicillins, but chemical modifications on side chains have yielded numerous semi-synthetic derivatives with expanded antibacterial spectrum (86). First generation cephalosporins were primarily effective against Gram-positive bacteria (e.g., staphylococci and streptococci) and some non- β -lactamase-producing enteric species. Later generations of cephalosporins with “expanded-spectrum” (e.g., cefotaxime, ceftriaxone, ceftazidime, and cefepime) exhibited increased stability against common β -lactamases (e.g., TEM-1 and SHV-1), and greater potency against Gram-negative bacteria. Some newer cephalosporins (e.g. ceftaroline and ceftobiprole) were designed to bind PBP2a of methicillin-resistant *S. aureus* (MRSA), thereby restoring activity against resistant staphylococci. However, the emergence and global spread of ESBLs have significantly compromised the efficacy of most cephalosporins (81).

Carbapenems represent the broadest-spectrum β -lactams. Structurally, they consist of a classic β -lactam ring fused to a five-membered ring in which a carbon atom replaces the sulfur found in penicillins (87), a modification that confers remarkable stability against many β -lactamases (84). The prototype, imipenem, retains activity against a wide range of Gram-positive, Gram-negative, and anaerobic bacteria. Later carbapenems such as meropenem, doripenem, and ertapenem are characterized by improved chemical stability and pharmacokinetics properties, broadening their clinical utility. Carbapenems act by binding multiple PBPs, interfering with cell-wall synthesis at several steps, a multi-target mechanism that reduces the likelihood of resistance emerging from a single PBP mutation (81). Their major limitation today is the rise of carbapenemases (both serine-type and metallo- β -lactamases) which can efficiently hydrolyze carbapenems and severely compromise their efficacy (81, 84).

Monobactams, clinically represented by aztreonam, differ structurally from penicillins and cephalosporins because they contain a single β -lactam ring without a fused second ring (88). Aztreonam shows selective activity mainly against aerobic Gram-negative rods, especially *Pseudomonas* spp., by binding tightly to PBP3, leading to filamentation and cell lysis. When first introduced, it was resistant to hydrolysis by all common β -lactamases, but the subsequent spread of ESBLs and carbapenemases has reduced its effectiveness against multi-resistant organisms. Nevertheless, because its monocyclic core is a poor substrate for many metallo- β -lactamases, there remains interest in combining aztreonam with β -lactamase inhibitors (or other β -lactams) to treat infections caused by bacteria expressing multiple β -lactamases (81, 84).

Building on the therapeutic potential of β -lactam antibiotics, β -lactamase inhibitors were developed to counteract one of the major resistance mechanisms which limits their efficacy, i.e. enzymatic degradation by β -lactamases. These molecules, including clavulanic acid, sulbactam, and tazobactam, resemble the β -lactam core of penicillins but possess little intrinsic antibacterial activity. By forming a stable complex with β -lactamases, they prevent the hydrolysis of co-administered β -lactam antibiotics, allowing these drugs to reach their targets. Widely used in combination therapies, such as amoxicillin-clavulanate or piperacillin-tazobactam, β -lactamase inhibitors have significantly expanded the clinical utility of older β -lactams, providing an effective strategy against both Gram-positive and Gram-negative β -lactamase-producing pathogens (81, 84).

Polymyxins

A small number of antibiotics can directly target LPS with high affinity. Beyond not requiring entry into the cell, a key advantage is that these antibiotics also permeabilize the OM, enabling the internalization of agents that would normally be excluded, thus opening additional therapeutic avenues (89, 90). Polymyxins are the most clinically important antibiotics that bind LPS (91). They are produced by the Gram-positive spore-forming soil bacterium *Paenibacillus polymyxa* (previously known as *Bacillus polymyxa*) via non-ribosomal peptide synthesis (92, 93). Historically, five variants (polymyxin A-E) were described, all with similar antibacterial spectra and properties. Today, polymyxin B and polymyxin E (also known as colistin), are the agents used in clinical settings because of their relatively lower nephrotoxicity (94, 95). Additional polymyxins produced by different have been identified, including polymyxin M (96), polymyxin S (97), polymyxin T (98) and polymyxin F (99).

Colistin consists of a cyclic heptapeptide with a tripeptide side chain acylated at the N-terminus by a fatty acid tail (100, 101), and differs from polymyxin B only in the amino acid residue at position 6, which is a D-leucine in colistin and a D-phenylalanine in polymyxin B (101, 102). The remaining

residues in polymyxins consist of various L-leucine and L-threonine residues, along with six conserved L- α - γ -diaminobutyric acid (DAB) residues at positions 1, 3, 4, 5, 8, and 9. These DAB residues play a crucial role in imparting a net positive charge to the polymyxin ring at physiological pH. The cationic, hydrophilic nature of this macrocycle, together with the presence of the hydrophobic lipid tail, is essential for the antimicrobial properties of polymyxins (101, 102).

The mode of action of colistin involves binding to LPS molecules, which explains their exclusive activity against Gram-negative bacteria (101, 103, 104). The most recent proposed mechanism of action involves the interaction of the antibiotic with the phosphate groups of lipid A moiety of LPS, which leads to the displacement of divalent cations such as Mg^{2+} and Ca^{2+} , resulting in the destabilization of the OM. Consequently, the weakened bonds in the LPS monolayer trigger the release of LPS from the bacterial surface, allowing colistin to access the periplasm. Here, it interacts with newly synthesized LPS molecules transiently present in the periplasmic leaflet of the IM, awaiting translocation to the OM by the Lpt system. Colistin binding to LPS in the IM destabilizes the membrane bilayer, ultimately causing membrane permeabilization, loss of cytoplasmic content, and cell lysis (105). Colistin has a narrow antibacterial spectrum, being active against some clinically relevant Gram-negative pathogens, including *P. aeruginosa*, *Acinetobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *E. coli*, *Salmonella* spp., *Shigella* spp., and *Haemophilus influenzae* (101, 103, 106). Conversely, it is also noteworthy that some Gram-negative species, such as *Proteus* spp., *Serratia* spp., *Neisseria* spp., *Brucella* spp., *Helicobacter pylori*, and the *Burkholderia cepacia* complex, are intrinsically resistant to colistin (101, 103, 106, 107). Colistin resistance in Gram-negative bacteria arises through intrinsic, acquired, and plasmid-mediated mechanisms, all converging on lipid A modification. Intrinsically resistant bacteria, such as *Serratia*, *Neisseria*, and *Proteus*, constitutively express lipid A-modifying enzymes that add cationic groups such as L-Ara4N or PEtN to the phosphate group of lipid A, reducing the affinity of colistin for its target and ultimately leading to resistance (19, 101, 108). In *Serratia*, resistance is mediated by the expression of the *arnBCADTEF* (*arn*) operon, which encodes the enzymes for L-Ara4N-dependent modification of lipid A, whereas in *Neisseria* and *Proteus* it depends on the expression of *eptA/eptB/eptC* (*ept*) genes, which encode PEtN transferases enzymes responsible for the addition of PEtN (109-111).

In most Gram-negative bacteria such as *P. aeruginosa* and *A. baumannii*, acquired colistin resistance results from mutations in the two-component systems PhoPQ and/or PmrAB, which lead to constitutive activation of the *arn* operon or *ept* genes, thereby promoting L-Ara4N or PEtN modification of lipid A (112, 113). Additional resistance arises from *mgrB* loss-of-function mutations in *K. pneumoniae*, *E. coli*, and *S. enterica*, causing PhoPQ hyperactivation (114, 115).

A unique mechanism occurs in *A. baumannii*, where loss-of-function mutations in lipid A biosynthesis genes (*lpxA/lpxC/lpxD*) lead to complete LPS loss and high-level colistin resistance, albeit with major fitness costs (108, 116). More recently, plasmid-mediated resistance has emerged through *mcr* genes, first identified in China in 2015 (117). *mcr* encodes a PEtN transferase that modifies lipid A analogously to the products of chromosomal *ept* genes. Since then, *mcr-1* to *mcr-10* and over 30 *mcr-1* variants have been detected globally in Enterobacteriaceae (118, 119). The co-occurrence of *mcr* with ESBL or carbapenemase genes on the same plasmid is of major clinical concern, prompting ECDC recommendations for surveillance (74).

Other cell wall- and membranes-targeting antibiotics

The peptidoglycan precursor molecules of the Lipid II cycle, anchored in the cytoplasmic membrane by Und-P, also represent antibiotic targets. As these precursors are not directly genetically encoded, resistance against antibiotics targeting them typically develops very slowly (76, 78). The most prominent class of clinically used Lipid II-binding antibiotics are the glycopeptides, with vancomycin representing the prototypical agent (120). Vancomycin exerts its bactericidal effect by binding the D-Ala–D-Ala dimer of Lipid II, disrupting transglycosylation and thus peptidoglycan polymerization, similarly to β -lactams. Although vancomycin resistance via substitution of D-Ala–D-Ala with D-Ala–D-Lac/Ser emerged decades after its introduction (121), semi-synthetic analogues have restored potency against resistant strains (122). Teicoplanin, for example, is a lipoglycopeptide which similarly binds Lipid II but also forms supramolecular assemblies on bacterial membranes, enhancing membrane residence time and target specificity (123). Another strategy is to target the pyrophosphate moiety of cell wall precursors, which further lowers down resistance development. An example is teixobactin, which sequesters Lipid II by self-assembling into supramolecular fibrils that block peptidoglycan synthesis and compromise membrane integrity showing potent activity against resistant bacteria *in vitro* and *in vivo*, with a very low frequency of resistance (124–127). Bacitracin, although structurally distinct, also targets the Lipid II cycle by forming a zinc-mediated complex with Und-PP, preventing its dephosphorylation and recycling (128, 129). Moreover, the calcium-dependent lipopeptide daptomycin targets phosphatidylglycerol-rich membranes, where its Ca^{2+} -dependent insertion perturbs membrane organization and ultimately compromises membrane integrity (130, 131). Finally, fosfomycin acts by irreversibly inhibiting the cytoplasmic enzyme MurA, thereby blocking the first step of peptidoglycan synthesis and exerting bactericidal activity against a broad spectrum of Gram-positive and also some Gram-negative bacteria, as its small size and hydrophilic nature allows it to cross the OM of Gram-negatives enabling access to its intracellular target (132, 133). In contrast, large antibiotics such as vancomycin and other glycopeptides or

polypeptides retain potent activity against Gram-positive pathogens but are largely ineffective against Gram-negative species, as their OM serves as a permeability barrier that prevents these compounds from reaching their targets (2).

1.2.2. Emerging targets and novel therapeutic strategies

As discussed above, although numerous antibiotics targeting the bacterial cell envelope are currently available and active against some Gram-negative pathogens, the widespread and increasing resistance to many of these agents underscores the urgent need not only to discover new antibiotics but also to identify novel and more effective therapeutic targets for drug development. Given that most of the OM assembly pathways are essential in many Gram-negative bacteria, it is reasonable to consider that directly inhibiting these pathways might be a compelling strategy for developing antibiotic compounds.

The LPS biosynthesis and transport pathways can be targeted at various stages as part of an antibiotic drug discovery approach (134). LpxC, a key enzyme in the Raetz pathway for lipid A biosynthesis, has long been considered an attractive antimicrobial target because it is essential and conserved across Gram-negative pathogens (135). LpxC inhibitors were the first and only LPS-biosynthesis inhibitors to enter clinical development. The first compound, L-161,240, showed potent activity against *E. coli* but lacked efficacy against *P. aeruginosa* (135, 136). The most advanced candidate, ACHN-975, reached phase I trials but was discontinued due to hydroxamate-related off-target toxicity (135, 136). Although challenges remain, such as poor solubility and toxicity of currently available LpxC inhibitors, LpxC persists as a promising, conserved, and human-homologue-free target. Another promising strategy focuses on the IM flippase MsbA: compounds such as G907 block its activity by wedging into a transmembrane pocket and stabilizing the transporter in a cytoplasmic-facing conformation, thereby preventing LPS translocation toward the periplasm (137)

Additional therapeutic opportunities arise from targeting components of the Lpt machinery, particularly the β -barrel protein LptD responsible for inserting LPS into the OM. The first LptD inhibitors emerged from peptidomimetic libraries modeled on protegrin-1, yielding compounds with nanomolar potency and remarkable specificity for *P. aeruginosa*, as they target the N-terminal region of LptD, which is distinct in this species compared to other Gram-negative species (135). Further optimization led to POL7080, also known as murepavadin (138), which advanced to phase III trials for ventilator-associated pneumonia caused by *P. aeruginosa* (135). Another strategy focuses on inhibiting the Bam complex, responsible for inserting β -barrel OMPs into the OM. The macrocyclic β -hairpin peptidomimetic JB-96 was proposed to interfere with β -barrel assembly, as it disrupts OM integrity, rapidly depletes OM β -barrel proteins, and photolabels both BamA and the structurally

similar LptD (139). However, its comparable activity against the Gram-positive bacterium *S. aureus* suggests additional targets beyond BamA (139). A more specific BamA-targeting antibiotic is darobactin, a ribosomally synthesized and post-translationally modified heptapeptide produced by some species of the genus *Photorhabdus*, which acts by stabilizing the closed lateral gate of BamA, thus blocking OMP insertion (140). Darobactin shows potent activity against major Gram-negative pathogens, including *E. coli* and *P. aeruginosa*, and efficacy in protecting mice from lethal infections. These results further highlight surface-exposed β -barrel proteins like BamA and LptD as highly promising targets for OM-acting antibiotics.

The Sec machinery, essential for protein translocation across the cytoplasmic membrane, also represents a potential drug target. Virtual screening for inhibitors of *E. coli* SecA led to the identification of SCA-21, a bis-triazole pyrimidine compound, and of the more potent analogues SCA-107 and SCA-112 (141). Another target is the SPase-I LepB, which is inhibited by arylomycins; however, their activity against Gram-negative is limited due to amino acid substitutions in the LepB protein that vary among species, reducing the binding affinity of these compounds (142). Nevertheless, optimized analogues, such as G0775, are highly active against multi-drug resistant (MDR) *E. coli* and *K. pneumoniae* and show efficacy in murine models of Gram-negative infections (142).

Finally, also lipoprotein biogenesis represents attractive antibacterial targets as lipoproteins are essential for bacterial physiology, and the enzymes responsible for their maturation are conserved across Gram-negative pathogens. One strategy involves inhibiting LspA, the SPase-II that cleaves the signal peptide of prolipoproteins. Globomycin, an amphiphilic antibiotic produced by *Streptomyces*, and its analogues display antibacterial activity against some Gram-negative bacteria (143), by inhibiting LspA and blocking its access to the prolipoprotein substrate (144). Another approach is targeting the Lol system that shuttles lipoproteins to the OM. The periplasmic chaperone LolA was initially considered promising after its identification as the target of the antibacterial compound MAC13243 (145), but later studies demonstrated that MAC13243 antibacterial activity likely results from inhibition of the cytoskeletal protein MreB (28). Thus, despite its potency, MAC13243 requires careful reassessment due to off-target effects.

To date, no inhibitors of GPL transport from the IM to the OM have been identified, as the underlying mechanisms have only been partially elucidated in recent years and remain poorly understood. However, this pathway may represent a promising antibacterial target for future research.

1.3. The Gram-negative pathogen *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped bacterium belonging to the class Gammaproteobacteria and the family Pseudomonadaceae. It was first isolated and described by Carle Gessard in 1882 in his study “On the blue and green coloration of bandages”. The distinctive blue-green pigmentation of *P. aeruginosa* cultures is due to the production of metabolites such as pyocyanin, a phenazine compound with both antimicrobial and cytotoxic properties (146). This bacterium is characterized by a large circular chromosome of approximately 6-7 Mbp. The first genome to be sequenced was that of the laboratory-adapted strain PAO1, originally isolated from a chronic wound in the 1950s (147, 148). The PAO1 genome, consisting of 6.3 Mbp and 5,570 predicted open reading frames, represented one of the largest bacterial genomes sequenced at the time (147).

Recent bioinformatic analyses have revealed the existence of a core genome and an extremely large pangenome in *P. aeruginosa*. A study analyzing over 1,300 strains identified a pangenome of 54,272 genes, consisting of core, flexible, and unique genes, accounting for approximately 1.2%, 48.7%, and 50.1% of the total genes, respectively (149). Core genes are conserved across all isolates, flexible genes are shared among multiple but not all isolates, and unique genes are specific to a single isolate. This extensive genetic variability translates into remarkable phenotypic diversity, supporting metabolic versatility and the ability to thrive in diverse environmental niches, including soil, water, and marine habitats, as well as in multiple host species such as plants, animals, and humans (150-152).

Clinically, *P. aeruginosa* is responsible for a wide range of potentially life-threatening acute and chronic infections, particularly in hospitalized or immunocompromised patients and individuals with traumatic wounds or burns. It is also a major cause of chronic lung infections in people with cystic fibrosis (150-152). Its persistence in healthcare settings is facilitated by multiple adaptive, survival, and resistance mechanisms, providing tolerance to disinfectants and antibiotics, as well as the ability to colonize moist surfaces of medical devices (151, 153-155). These factors, together with the high adaptability and virulence potential, have led the WHO to classify *P. aeruginosa* as a high priority pathogen, highlighting the urgent need for new therapeutic strategies and continued research (156). *P. aeruginosa* exhibits multiple traits that significantly contribute to its pathogenicity, including the production of several cell-associated factors, such as LPS, flagellum, type IV pili, and the type III secretion system (T3SS) (157, 158), and secreted virulence factors, including pyocyanin, which induces oxidative stress and promotes extracellular DNA release, contributing to biofilm formation (159, 160), and the siderophores pyoverdine and pyochelin which are crucial for iron acquisition, promoting growth and biofilm formation in iron-limited environments such as human tissues (161, 162). Collectively, these cell-associated and secreted virulence factors, along with the strong host

inflammatory response they trigger, make acute *P. aeruginosa* infections severe and potentially life-threatening (163).

Given the high prevalence of hospital-acquired infections caused by *P. aeruginosa*, prevention of patient colonization is critical. Strategies include strict hygiene practices, such as regular disinfection and replacement of moisture-prone medical devices (e.g., catheters, tubing), and frequent handwashing by healthcare providers (164). In recent years, several trials have been initiated to develop vaccines against *P. aeruginosa*, but none of them has progressed to clinical stages. So, the use of antibiotics continues to be essential in the fight against *P. aeruginosa* infections (165). While the variety of β -lactam antibiotics exhibiting antipseudomonal activity is limited, they remain commonly prescribed as the primary treatment for *P. aeruginosa* infections. Non-carbapenem β -lactam antibiotics, such as ceftazidime, cefepime, piperacillin-tazobactam, carbenicillin and aztreonam are the first-line therapy against *P. aeruginosa* susceptible clinical isolates (166, 167), while carbapenems, including imipenem, doripenem, and meropenem are reserved to eliminate infections caused by *P. aeruginosa* resistant to traditional β -lactams (152, 166, 167). Moreover, fluoroquinolones such as ciprofloxacin and levofloxacin also serve as broad-spectrum antibiotics employed in the treatment of *P. aeruginosa* infections. They exert their bactericidal effect by inhibiting DNA gyrase and topoisomerase IV, essential enzymes for DNA synthesis in bacteria (168). In addition to β -lactam antibiotics and fluoroquinolones, aminoglycosides, including amikacin, gentamicin, and tobramycin, play a crucial role as broad-spectrum bactericidal antibiotic targeting the 30S subunit of bacterial ribosomes, inhibiting protein synthesis (169). In particular, tobramycin is extensively utilized to address exacerbations of chronic pneumonia caused by *P. aeruginosa* in individuals with CF (170, 171).

Combination therapy, typically a β -lactam with an aminoglycoside or fluoroquinolone, is commonly employed to exploit distinct mechanisms of action and reduce the risk of inadequate empirical treatment (152, 172, 173).

Unfortunately, *P. aeruginosa* has developed multiple resistance mechanisms that can be classified as intrinsic, arising from inherent bacterial traits, and acquired, through chromosomal mutations or horizontal gene transfer (HGT) (174, 175). Intrinsic resistance is mainly due to: (i) the low permeability of its OM, which restricts antibiotic entry; (ii) the activity of chromosomally encoded efflux pumps, which expel β -lactams, fluoroquinolones, and aminoglycosides (176, 177); and (iii) the expression of antibiotic-modifying enzymes (155, 178). Beyond intrinsic resistance, *P. aeruginosa* readily acquires resistance via HGT or mutations in genes encoding antibiotic targets or regulatory systems (179).

The spread of MDR *P. aeruginosa* is a major public health concern. In Europe, about 32.2 % of invasive *P. aeruginosa* isolates were resistant to at least one of the antimicrobial groups under surveillance (74). MDR *P. aeruginosa* is defined as nonsusceptible to ≥ 1 agent in ≥ 3 antimicrobial classes, β -lactams, fluoroquinolones, and aminoglycosides (180). More recently, the category of “difficult-to-treat resistance” (DTR) was established, designating strains nonsusceptible to multiple key antipseudomonal agents, including piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, carbapenems, and fluoroquinolones (166). Given the rise of MDR and DTR isolates, polymyxins such as colistin and polymyxin B have re-emerged as last-resort treatments. However, as discussed above, *P. aeruginosa* can also acquire resistance to colistin through multiple mechanisms, most commonly chromosomal mutations leading to constitutive induction of the *arn* operon and subsequent addition of L-Ara4N to lipid A, which reduces its negative charge and impairs colistin binding. (181-183). *P. aeruginosa* also carries an endogenous gene, *eptA*, which encodes a functional PEtN transferase capable of modifying lipid A with PEtN and thereby reducing the efficacy of colistin (184, 185). However, in vitro evolution studies and genetic analysis of clinical isolates have shown that, although *eptA* is functional, lipid A aminoarabinosylation remains the predominant mechanism of colistin resistance in *P. aeruginosa* (183, 186, 187). In addition, although less frequently reported in *P. aeruginosa* as compared to Enterobacteriaceae, HGT of *mcr* genes can similarly mediate lipid A phosphoethanolamination and colistin resistance (117, 185). These mechanisms collectively limit the efficacy of colistin and highlight the urgent need for novel therapeutic options.

1.4. References

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CHAPTER 2. AIM OF THE THESIS

The Gram-negative cell envelope functions as an integrated and highly coordinated system whose structural complexity is central to bacterial viability, environmental fitness, and pathogenicity. Understanding the molecular mechanisms governing cell envelope biogenesis is essential not only for elucidating core principles of Gram-negative bacterial cell biology, but also for identifying novel vulnerabilities that can be exploited for the development of innovative antimicrobial strategies targeting the bacterial cell envelope.

Despite continuous advances in the characterization of Gram-negative cell envelope biogenesis, some fundamental processes remain poorly understood. In particular, at the outset of this PhD project, little was known about the molecular mechanisms of GPL transport from the IM to the OM. Only recently, studies performed in *E. coli* proposed a role for some AsmA-like proteins in this pathway (47, 48). Similarly, the mechanisms governing the recycling of the essential lipid carrier Und-P were poorly understood until latest studies implicated members of the DedA family as proteins involved in this process (63, 66). Addressing these gaps is critical for achieving a comprehensive understanding of envelope homeostasis and for identifying novel targets for antimicrobial intervention.

Based on this, the overall aim of this PhD thesis was to further investigate fundamental mechanisms underlying the biogenesis and maintenance of the Gram-negative bacterial cell envelope, with a particular focus on GPL anterograde transport and Und-P recycling process, using the opportunistic human pathogen *P. aeruginosa* as a model organism. Specifically, my studies focused on (i) confirming the possible role of AsmA-like proteins in GPL transport and identifying the molecular determinants by which these proteins contribute to OM biogenesis, and (ii) investigating the functional importance of DedA proteins in Und-P recycling and/or other functions related to cell envelope homeostasis. Additionally, within the framework of a PRIN project focused on the characterization of potential antibacterial drug targets, I also investigated the essentiality of the *P. aeruginosa* LspA enzyme, involved in lipoprotein maturation, and its potential as a target for the development of new antibacterial compounds.

Each of the following chapters is preceded by a short preface introducing (i) the published work on AsmA-like proteins and a DedA protein responsible for Und-P transport (chapters 3-5), (ii) two manuscripts in preparation on a DedA protein involved in Und-PP dephosphorylation (chapter 6) or the possible functional interaction between AsmA-like and DedA proteins (chapter 7), and (iii) a manuscript currently under review on LspA (chapter 8). Together, these chapters summarize the results collected during this PhD journey. Finally, the thesis includes an appendix with two manuscripts I co-authored that are not directly related to the main focus of my PhD research.

CHAPTER 3. Redundant essentiality of AsmA-like proteins in *Pseudomonas aeruginosa*

PREFACE

The OM of Gram-negative bacteria represent a crucial permeability barrier and is a major determinant of intrinsic resistance to antibiotics. While dedicated pathways for LPS and OMP assembly have been characterized in detail in the last decades, how GPLs are delivered and inserted into the inner leaflet of the OM has long remained elusive until studies performed in *E. coli* proposed a role for AsmA-like proteins in this process. However, their functional relevance and essentiality in other bacteria were unknown.

In the study described in this chapter, we investigated the role of AsmA-like proteins in the opportunistic human pathogen *P. aeruginosa*. Through a combination of genetic, molecular, and phenotypic analyses, this work confirmed that multiple AsmA-like proteins are essential but redundant for bacterial viability and OM integrity also in *P. aeruginosa*. Notably, the observation that perturbations in LPS synthesis or transport partially suppressed the defects associated with AsmA-like protein deficiency highlighted the critical importance of maintaining a finely tuned balance between GPLs and LPS in the OM. These findings are consistent with the proposed role for AsmA-like proteins in GPL transport toward the OM. Finally, we demonstrated that *P. aeruginosa* possesses an additional essential AsmA-like protein that is not present in *E. coli*, thereby expanding the range of AsmA-like proteins known to play key roles in Gram-negative bacteria.

This work has been published in mSphere in 2024. The Supplementary material has not been included in this chapter, but is available at

<https://pmc.ncbi.nlm.nih.gov/articles/instance/10900882/bin/msphere.00677-23-s0001.pdf>



Bacteriology | Research Article

Redundant essentiality of AsmA-like proteins in *Pseudomonas aeruginosa*

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ABSTRACT The outer membrane (OM) is an essential structure of Gram-negative bacteria that provides mechanical strength and protection from large and/or hydrophobic toxic molecules, including many antibiotics. The OM is composed of glycerophospholipids (GPLs) and lipopolysaccharide (LPS) in the inner and outer leaflets, respectively, and hosts integral β -barrel proteins and lipoproteins. While the systems responsible for translocation and insertion of LPS and OM proteins have been elucidated, the mechanism(s) mediating transport of GPLs from the inner membrane to the OM has remained elusive for decades. Very recently, studies performed in *Escherichia coli* proposed a role in this process for AsmA-like proteins that are predicted to share structural features with eukaryotic lipid transporters. In this study, we provide the first systematic investigation of AsmA-like proteins in a bacterium other than *E. coli*, the opportunistic human pathogen *Pseudomonas aeruginosa*. Bioinformatic analyses revealed that *P. aeruginosa* possesses seven AsmA-like proteins. Deletion of *asmA*-like genes in many different combinations, coupled with conditional mutagenesis, revealed that four AsmA-like proteins are redundantly essential for growth and OM integrity in *P. aeruginosa*, including a novel AsmA-like protein (PA4735) that is not present in *E. coli*. Cells depleted of AsmA-like proteins showed severe defects in the OM permeability barrier that were partially rescued by lowering the synthesis or transport of LPS. Since fine balancing of GPL and LPS levels is crucial for OM integrity, this evidence supports the role of AsmA-like proteins in GPL transport toward the OM.

IMPORTANCE Given the importance of the outer membrane (OM) for viability and antibiotic resistance in Gram-negative bacteria, in the last decades, several studies have focused on the characterization of the systems involved in OM biogenesis, which have also been explored as targets for antibacterial drug development. However, the mechanism mediating translocation of glycerophospholipids (GPLs) to the OM remained unknown until recent studies provided evidence that AsmA-like proteins could be responsible for this process. Here, we demonstrate for the first time that AsmA-like proteins are essential and redundant for growth and OM integrity in a Gram-negative bacterium other than the model organism *Escherichia coli* and demonstrate that the human pathogen *Pseudomonas aeruginosa* has an additional essential AsmA-like protein that is not present in *E. coli*, thus expanding the range of AsmA-like proteins that play key functions in Gram-negative bacteria.

KEYWORDS *epaF*, glycerophospholipid transport, outer membrane, PA2708, PA4735, TamA, TamB, YhdP, YdbH

The cell envelope of diderm (Gram-negative) bacteria is composed of two lipid bilayers, separated by an aqueous periplasmic space that contains a thin peptidoglycan cell wall (1). While the cytoplasmic inner membrane (IM) is a bilayer

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of glycerophospholipids (GPLs), the outer membrane (OM) is an asymmetric bilayer composed of GPLs in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, which hosts integral β -barrel proteins (OMPs) and lipoproteins (2). The OM is an essential structure of Gram-negative bacteria, and the asymmetric assembly of its lipid components provides a selective permeability barrier that protects the cell from large and/or hydrophobic toxic molecules such as many antibiotics, partially accounting for the high intrinsic drug resistance of most Gram-negative pathogens (3). Recent studies uncovered that the OM also provides mechanical strength to compensate for the thin cell wall of Gram-negative bacteria (4).

Since the OM is essential and represents a major hurdle to antibiotic success against Gram-negative bacteria, the molecular machineries involved in its biogenesis have been investigated as targets for drug development (5–7). Interfering with OM biogenesis can directly kill bacterial cells or sensitize them to antimicrobials unable to cross a functional OM barrier (8, 9).

OM biogenesis is a complex process that poses several challenges. The biosynthesis of the major OM components takes place in the cytoplasm or at the IM and their transport to and assembly into the OM must be coordinated with cell growth and accomplished in an environment, the periplasm, that is devoid of obvious energy sources (1). Progress has been made in the past two decades in elucidating the molecular mechanisms that enable Gram-negative bacteria to overcome these challenges, leading to the discovery and characterization of dedicated protein machineries that mediate the assembly of individual OM components. OM proteins (OMPs and lipoproteins) are predominantly translocated across the IM in an unfolded state through the general secretory (Sec) system (10, 11). In the periplasm, OMPs are assisted by chaperones and delivered to the β -barrel assembly machinery (Bam), which promotes their final folding and integration into the OM (12, 13). Lipoproteins are matured on the periplasmic side of the IM through the addition of the lipid moiety which anchors them to the IM. Then, OM lipoproteins are detached from the IM and shuttled to the OM by the Lol (localization of lipoproteins) system, which involves a soluble periplasmic protein (LolA) that masks the hydrophobic acyl chains from the aqueous periplasmic environment (14, 15). Intermembrane transport of LPS occurs through the seven-component LPS transport (Lpt) trans-envelope system, which forms a continuous protein bridge that connects the IM and OM and mediates LPS detachment from the IM, transit across the periplasm, and insertion into the outer leaflet of the OM (16, 17).

Despite this wealth of information on OM biogenesis, the mechanism mediating transport across the periplasm of the GPLs necessary for building the inner leaflet of the OM has remained elusive for decades. Several hypotheses have been formulated, including the presence of (i) hemifusion structures formed by the fusion of the IM and OM periplasmic leaflets into a contiguous bilayer crossing the periplasm, (ii) soluble periplasmic carriers, or (iii) proteinaceous tunnels connecting the IM and OM, but none of them has been experimentally confirmed yet (18, 19). This is mainly due to the inability to identify essential genes involved in this process, while it is logical to assume that GPL transport to the OM should be crucial for cell survival. In contrast, a system called Mla (maintenance of OM lipid asymmetry), that is devoted to maintaining OM asymmetry by removing mislocalized GPLs from the outer leaflet of the OM and transporting them back to the IM (retrograde transport), has been identified and characterized in several Gram-negative bacteria (18, 20, 21).

Recently, two independent studies performed in the model organism *Escherichia coli* demonstrated that three members of the AsmA-like protein clan (i.e., TamB, YhdP, and YdbH) are critical for OM integrity and proposed that they could provide a hydrophobic proteinaceous tunnel for GPL transport to the OM (22, 23). Indeed, single or double deletion mutants in these AsmA-like genes displayed impaired growth, cell morphology alterations, enhanced vesiculation, and/or increased antibiotic susceptibility, due to defects in OM integrity, while the triple deletion of *yhdP*, *tamB*, and *ydbH* was synthetically lethal, indicating that these genes are essential but redundant in *E. coli* (22, 23).

Notably, *E. coli* cells impaired in the expression of these essential AsmA-like proteins were also found to accumulate GPLs in the IM (23), suggesting that these proteins are involved in GPL transport toward the OM. This is in line with the results of a previous study showing that the inactivation of the *yhdP* gene was able to reduce the flow of GPLs to the OM and, thus, attenuate the membrane defects of an *E. coli* mutant (*mlaA*^{*}) in which the Mla system works in reverse, leading to GPL accumulation in the outer leaflet of the OM, increased flow of GPLs from the IM, and consequently, IM shrinkage (24). Notably, AsmA-like proteins are predicted to have an IM-anchoring N-terminal transmembrane helix (TMH) and a large periplasmic domain with a structure resembling that of the eukaryotic proteins (e.g., Vps13 and Atg2) involved in GPL transport between organelles (25–27).

Overall, this body of evidence strongly supports the role of AsmA-like proteins in GPL transport. However, up to date, the involvement of AsmA-like proteins in GPL transport and OM homeostasis has been systematically investigated only in *E. coli*. To provide further support to the possible role of AsmA-like proteins in the biogenesis of the Gram-negative cell envelope, we analyzed the effect of AsmA-like protein depletion in the opportunistic human pathogen *Pseudomonas aeruginosa*. Here, we confirm that AsmA-like proteins are essential and redundant for growth and OM integrity also in *P. aeruginosa* and provide indirect evidence that supports their role in the transport of GPLs to the OM. We also demonstrate that *P. aeruginosa* has an additional essential AsmA-like protein that is not present in *E. coli*, thus expanding the range of AsmA-like proteins that might play essential role(s) in diderm bacteria.

RESULTS

P. aeruginosa has seven AsmA-like proteins

The possible role of AsmA-like proteins in GPL transport and OM homeostasis has only been investigated in the model organism *E. coli*. This bacterium possesses six AsmA-like proteins (i.e., AsmA, TamB, YhdP, YdbH, YhjG, and YicH), of which TamB, YhdP, and YdbH were found to be essential but redundant for growth and OM integrity (22, 23).

To verify the role of AsmA-like proteins in *P. aeruginosa*, we first searched for homologs of *E. coli* AsmA-like proteins in the *Pseudomonas* Genome Database (<https://pseudomonas.com/>) and for *P. aeruginosa* proteins in the Pfam database that belong to families within the AsmA-like clan. Seven genes encoding AsmA-like proteins were identified in the reference strain *P. aeruginosa* PAO1, all of which are conserved in the *P. aeruginosa* complete genomes deposited in the *Pseudomonas* Genome Database (Table 1). Five of these proteins have homology with *E. coli* proteins, and hereafter, they have been renamed as their putative *E. coli* orthologs, while a homolog of the *E. coli* protein YicH was not identified in *P. aeruginosa*. In addition, *P. aeruginosa* has two other AsmA-like proteins, PA4735 and PA2708, which are not present in *E. coli* (Table 1). The

TABLE 1 AsmA-like proteins in *P. aeruginosa*

<i>P. aeruginosa</i> PAO1 AsmA-like protein (aa)	<i>E. coli</i> homolog (aa)	Identity (%)	Alignment length (aa)	E value	Pfam	% of <i>P. aeruginosa</i> genomes with ortholog ^a
PA2542 (1,221)	TamB (1,259)	25.81	1,259	0.0	PF04357	100 ^b
PA4476 (1,276)	YhdP (1,266)	21.62	1,309	0.0	PF13502/PF13116	100 ^c
PA4879 (688)	YhjG (686)	53.00	666	0.0	PF05170	100
PA5146 (750)	AsmA (617)	17.81	320	1.15E–6	PF05170	100
PA5307 (855)	YdbH (879)	24.49	147	7.13E–5	PF11739	100
PA4735 (1,088)	– ^e	–	–	–	PF05359	100
PA2708 (361)	–	–	–	–	PF05359	100 ^d

^aPutative orthologs were retrieved from 257 complete genomes according to the *Pseudomonas* Ortholog Groups of the *Pseudomonas* Genome Database (<https://pseudomonas.com/>).

^bThe ortholog of the *P. aeruginosa* strain PADK2_CFS10 is probably a pseudogene.

^cThe ortholog of the *P. aeruginosa* strain 2192 is probably a pseudogene.

^dThe orthologs of the *P. aeruginosa* strains 2192; BWH034 and MRSN 20176 are probably pseudogenes.

^eThe symbol "–" indicates the absence of a homolog in *E. coli*.

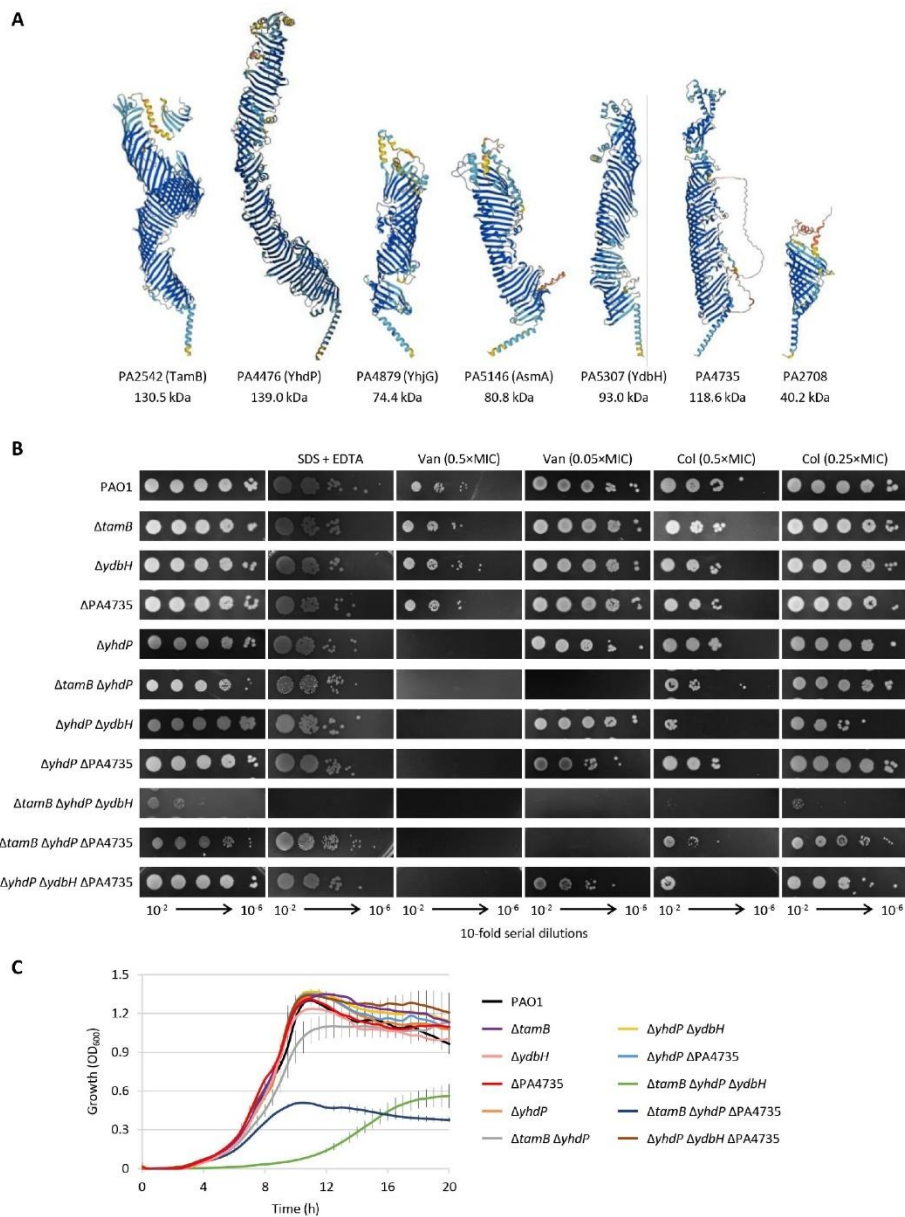


FIG 1 (A) Three-dimensional structures of *P. aeruginosa* AsmA-like proteins predicted by AlphaFold (<https://alphafold.ebi.ac.uk/>). The predicted molecular weights are also shown. (B) Plating efficiency of *P. aeruginosa* PAO1 and selected isogenic mutants with single, double, or triple deletions in *asmA*-like genes on MH agar plates supplemented or not with SDS and EDTA (0.25% and 0.25 mM, respectively), vancomycin (Van), or colistin (Col) at concentrations corresponding to 0.05, 0.25, or 0.5× MIC for the wild-type strain (as indicated). Images were taken after 24 h of incubation at 37°C and are representative of at least three independent experiments. The single mutants $\Delta tamB$, $\Delta ydbH$, and $\Delta PA4735$ were included as controls. (C) Planktonic growth of *P. aeruginosa* PAO1 and selected isogenic mutants with single, double, or triple deletions in *asmA*-like genes in Mueller-Hinton (MH) broth in microtiter plates at 37°C. Values are the mean (±standard deviation) of three independent experiments performed in triplicate.

predicted three-dimensional structure of the *P. aeruginosa* AsmA-like proteins has been retrieved from the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>) (28). All *P. aeruginosa* AsmA-like proteins are predicted to share an N-terminal TMH and an elongated tube-like structure rich in β -strands with a lateral opening along the long axis (Fig. 1A). This structure resembles the periplasmic bridge made by Lpt proteins responsible for LPS transport across the periplasm (29, 30). Notably, the *P. aeruginosa* protein PA2708 is much smaller than the other AsmA-like proteins of both *P. aeruginosa* and *E. coli* (Table 1), and the length of its periplasmic domain is likely not sufficient to span the entire periplasmic space (Fig. 1A).

Four AsmA-like proteins are redundantly essential in *P. aeruginosa*

In order to investigate the role of AsmA-like proteins in *P. aeruginosa*, we attempted to construct single, double, and triple in-frame deletion mutants in *asmA*-like genes in the reference strain *P. aeruginosa* PAO1. To reduce the number of possible combinations, at this stage, we decided to exclude PA2708 from the analysis, as its length and structure do not appear to be compatible with GPL transport across the cell envelope (Fig. 1A). However, the possible role of this protein has been investigated later in this study (see below). Forty-two mutants, corresponding to single, double, and triple mutants in *asmA*, *tamB*, *yhdP*, *ydbH*, *yhjG*, and PA4735 in all possible combinations, were generated (Table S1). Notably, although the concomitant inactivation of *tamB*, *yhdP*, and *ydbH* was found to be lethal in *E. coli* (22, 23), the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ was successfully obtained in *P. aeruginosa*.

To preliminary screen all these mutants for defects in growth and/or OM integrity, we performed a qualitative spot plating assay on MH agar plates supplemented or not with the membrane-perturbing agents SDS and EDTA or sub-inhibitory concentrations of vancomycin (poorly active against Gram-negative cells with an intact OM) or colistin (that primarily targets the OM) (31–34). Several mutants displayed impaired growth and/or OM integrity defects with respect to the wild-type strain PAO1 (Fig. S1), thus confirming that AsmA-like proteins play important role(s) also in *P. aeruginosa*. The mutants that show an individual effect of each *asmA*-like gene on *P. aeruginosa* growth and OM integrity are shown in Fig. 1B. Single deletion of any *asmA*-like genes did not affect *P. aeruginosa* growth, even if the absence of YhdP made the cells more sensitive to vancomycin (no growth at 0.5 $\times$ MIC for PAO1, corresponding to 1 mg/mL) (Fig. 1B; Fig. S1). The concomitant lack of YhdP and TamB ($\Delta tamB \Delta yhdP$ mutant) slightly impaired growth (smaller colonies on MH agar) and hugely increased vancomycin sensitivity (no growth at 0.05 $\times$ MIC for PAO1). The absence of YdbH in YhdP-deficient cells ($\Delta yhdP \Delta ydbH$ mutant) slightly increased colistin sensitivity at both 0.5 $\times$ and 0.25 $\times$ MIC for PAO1 (corresponding to 0.25 and 0.125 μ g/mL, respectively). Although viable, the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ was strongly defective in growth and more sensitive to the membrane-destabilizing agents SDS and EDTA compared with the other strains (Fig. 1B). The deletion of *asmA* and/or *yhjG* in combination with all other mutations did not further impair growth under any conditions tested (Fig. S1), implying that these genes have no relevant effects on the phenotypes investigated in this assay. Interestingly, the inactivation of PA4735 in the $\Delta tamB \Delta yhdP$ and $\Delta yhdP \Delta ydbH$ mutants reduced the bacterial colony size under some conditions tested (Fig. 1B), suggesting that the lack of this protein might have a detrimental effect on *P. aeruginosa* cells defective in other important AsmA-like proteins.

To quantitatively assess the impact of AsmA-like proteins on *P. aeruginosa* growth, planktonic growth assays were performed in MH broth for all mutants. The growth curves of the single mutants (including $\Delta yhdP$) and the wild-type strain were almost identical (Fig. 1C and data not shown), corroborating the results of the spot plating assay. A slight decrease in growth yields was observed for the double mutant $\Delta tamB \Delta yhdP$ and, to a greater extent, for the triple mutant $\Delta tamB \Delta yhdP \Delta PA4735$, while both growth rates and yields were drastically reduced in the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ (Fig. 1C).

These results demonstrate that YhdP, TamB, and YdbH are redundant and important for *P. aeruginosa* growth and OM integrity, in line with what was previously observed in *E. coli* (22, 23). However, the viability of the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ in *P. aeruginosa* strongly suggests the presence of additional protein(s) in this bacterium that may be involved in the same physiological process, thus sustaining the low level of growth observed in the absence of YhdP, TamB, and YdbH. The detrimental effect of PA4735 inactivation on cells deficient in other AsmA-like proteins (Fig. 1) prompted us to investigate whether PA4735 could be responsible for the residual growth of $\Delta tamB \Delta yhdP \Delta ydbH$ cells. Several attempts to obtain a quadruple deletion mutant in *tamB*, *yhdP*, *ydbH*, and PA4735 failed, corroborating the hypothesis that PA4735 could be essential in YhdP-, TamB-, and YdbH-deficient cells. Therefore, we generated a conditional mutant carrying a rhamnose-dependent copy of *tamB* in a neutral site of the chromosome and deleted the endogenous copies of *tamB*, *yhdP*, and *ydbH* ($\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB$; Table S1). As expected, the growth of this conditional mutant was promoted by rhamnose (Fig. 2), although basal *tamB* expression in the absence of the inducer alleviated the growth defect relative to the triple-deletion mutant $\Delta tamB \Delta yhdP \Delta ydbH$ (Fig. 1 and 2). Then, *asmA*, *yhjG*, PA4735, and PA2708 genes were individually deleted in this conditional mutant. Growth assays on agar plates and in liquid medium revealed that *P. aeruginosa* growth was abrogated by the concomitant inactivation or depletion of YhdP, YdbH, TamB, and PA4735 (Fig. 2). In contrast, the deletion of *asmA*, *yhjG*, or PA2708 in the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB$ did not exacerbate the growth defects of this strain under non-inducing conditions (Fig. 2A). This result demonstrates that PA2708, which was not investigated in the initial genetic screening, is not required for *P. aeruginosa* growth, and confirms that, compared with *E. coli*, *P. aeruginosa* has an additional AsmA-like protein (i.e., PA4735) that cooperates with YhdP, TamB, and YdbH to support bacterial growth.

To further investigate this issue, we introduced a plasmid for the IPTG-inducible overexpression of PA4735 (pMEPA4735; Table S2) into the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$. For comparison, YhdP- or YdbH-expressing plasmids (i.e., pMEyhdP and pMEydbH; Table S2) were also generated and tested. Plasmids carrying *yhdP* or *ydbH* were able to rescue the growth of the conditional mutant under non-permissive conditions (absence of rhamnose) both in the presence and in the absence of IPTG (Fig. 3A and B), indicating that the basal level of expression from

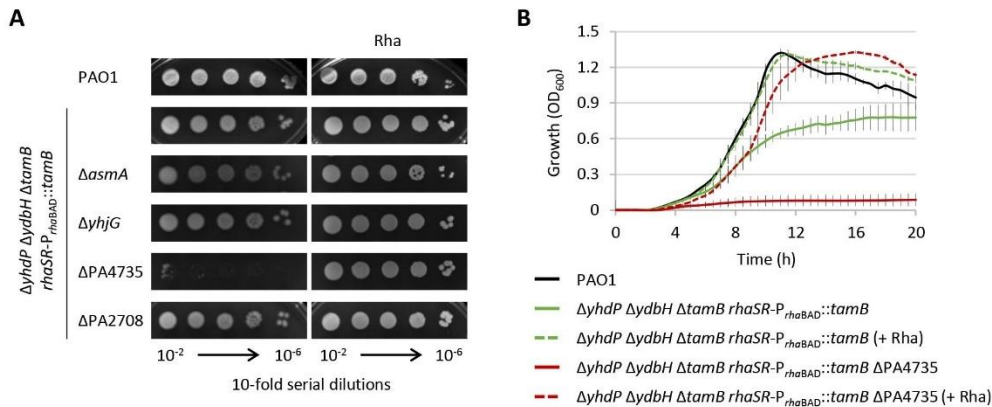


FIG 2 (A) Plating efficiency of *P. aeruginosa* PAO1, the rhamnose-dependent conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB$, and its derivatives deleted of *yhjG*, *asmA*, PA4735, or PA2708 on MH agar plates supplemented or not 0.01% rhamnose (Rha). Images were taken after 24 h of incubation at 37°C and are representative of three independent experiments. (B) Planktonic growth of the wild-type strain and the conditional mutants $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB$ and $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ in MH broth in microtiter plates at 37°C. Values are the mean ($\pm$ standard deviation) of three independent experiments performed in triplicate.

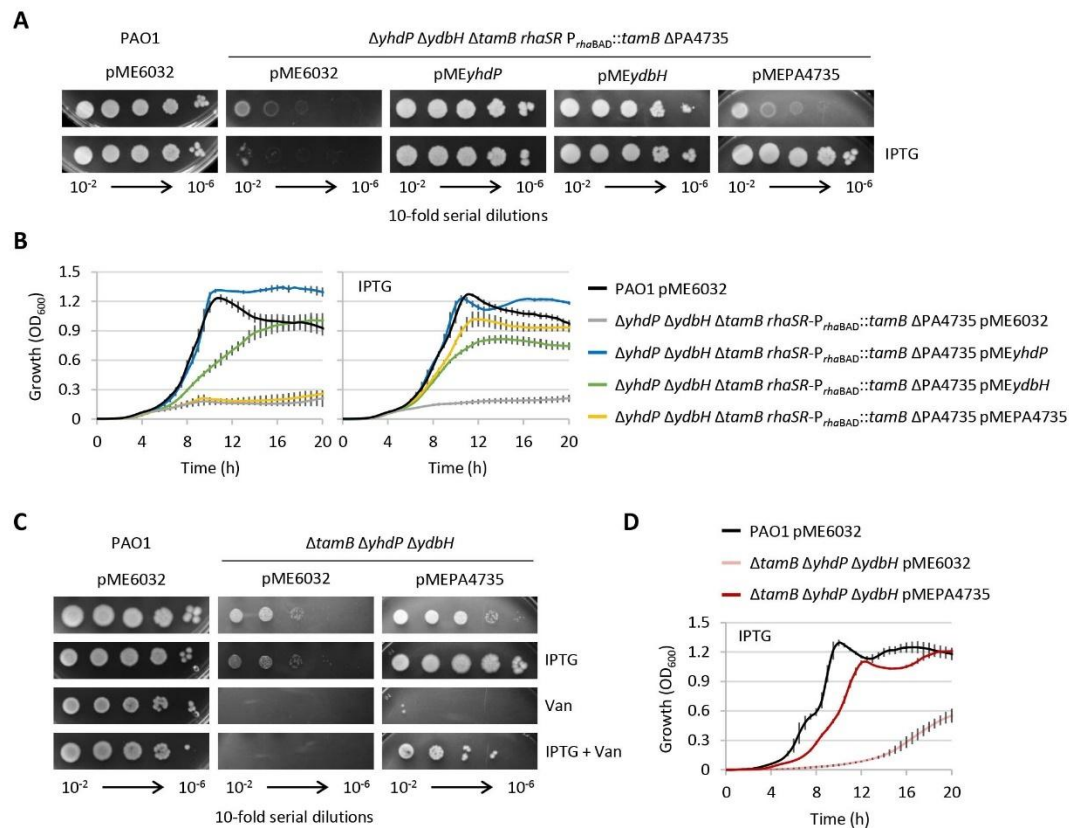


FIG 3 (A) Plating efficiency and (B) planktonic growth of the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ complemented with the IPTG-inducible construct pMEyhdP, pMEydbH, or pMEPA4735 or the empty plasmid pME6032 as negative control, cultured in the presence or absence of 0.5 mM IPTG. (C) Plating efficiency and (D) planktonic growth of the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ carrying pME6032 or pMEPA4735, cultured in the presence and/or absence of 0.5 mM IPTG and/or vancomycin at 100 μ g/mL. In all experiments, *P. aeruginosa* PAO1 carrying pME6032 was used as the positive control of growth. Data are the mean ($\pm$ standard deviation) or are representative of at least three independent experiments.

the IPTG-dependent promoter in *P. aeruginosa* is sufficient to restore YhdP and YdbH functionality. Interestingly, ectopic expression of YdbH at both basal and induced levels did not restore growth at wild-type levels or at levels achieved upon YhdP (over)expression (Fig. 3B). This suggests that YdbH could be less effective than YhdP in performing the essential function(s) that AsmA-like proteins provide in *P. aeruginosa*. The introduction of pMEPA4735 in the conditional mutant did not restore growth unless IPTG was added to the medium (Fig. 3A and B), implying that high levels of PA4735 expression are required to support growth. This was confirmed by introducing the pMEPA4735 plasmid into the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$, which expresses PA4735 at physiological levels. Upon induction with IPTG, the mutant carrying pMEPA4735 showed growth and vancomycin resistance profiles almost comparable to those of the wild-type strain (Fig. 3C and D), indicating that, when overexpressed, PA4735 is able to sustain growth and OM integrity even in the absence of the other essential AsmA-like proteins YhdP, TamB, and YdbH.

In conclusion, these results demonstrate that four AsmA-like proteins are essential but redundant in *P. aeruginosa*, including one (PA4735) that is not present in the model organism *E. coli*. A search for orthologs in the *Pseudomonas* Genome Database revealed

that PA4735 is not restricted to *P. aeruginosa*, as putative PA4735 orthologs are present in many *Pseudomonas* species (<https://pseudomonas.com/>). While BLASTP search in the NCBI database did not identify any putative PA4735 homologs outside the *Pseudomonas* genus, structure-based comparisons in the AlphaFold database retrieved putative structural homologs of PA4735 in some distantly related bacteria belonging to different classes or phyla of diderm bacteria (Table S3). Alignment of the predicted 3D structures of PA4735 and some of these putative structural homologs confirmed that the overall fold of PA4735 is highly conserved in these proteins (Fig. S2). Even if this analysis is only based on structural predictions and might not be comprehensive, it strongly suggests that PA4735-like proteins are not exclusive to the *Pseudomonas* genus.

AsmA-like proteins are important for cell morphology and membrane integrity

In *E. coli*, depletion of essential AsmA-like proteins causes significant defects in cell morphology and membrane permeability (22, 23). Confocal microscopy imaging of cells stained with the membrane-labeling dye FM4-64 showed that cells of the double mutant $\Delta tamB \Delta yhdP$ are smaller (reduced cell area) and rounder (lower length/width ratio) than wild-type cells (Fig. 4 and S3), while all other single or double mutants did not show any apparent alteration of cell morphology (data not shown). Comparable phenotypes were observed for the triple mutants $\Delta tamB \Delta yhdP \Delta PA4735$ and $\Delta tamB \Delta yhdP \Delta ydbH$, as well as for cells of the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$, whose growth was arrested by progressively reducing the concentration of rhamnose in the medium (Fig. 4 and S4). This suggests that the lack of TamB and YhdP proteins is mainly responsible for the observed cell elongation defects, even if growth is only marginally affected in TamB- and YhdP-depleted cells (Fig. 1). Notably, under non-permissive conditions, the growth of the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ was arrested without a relevant decrease in the optical density at 600 nm (OD₆₀₀), suggesting that depletion of essential AsmA-like proteins does not cause lysis in *P. aeruginosa*, different from what was observed in *E. coli* (22).

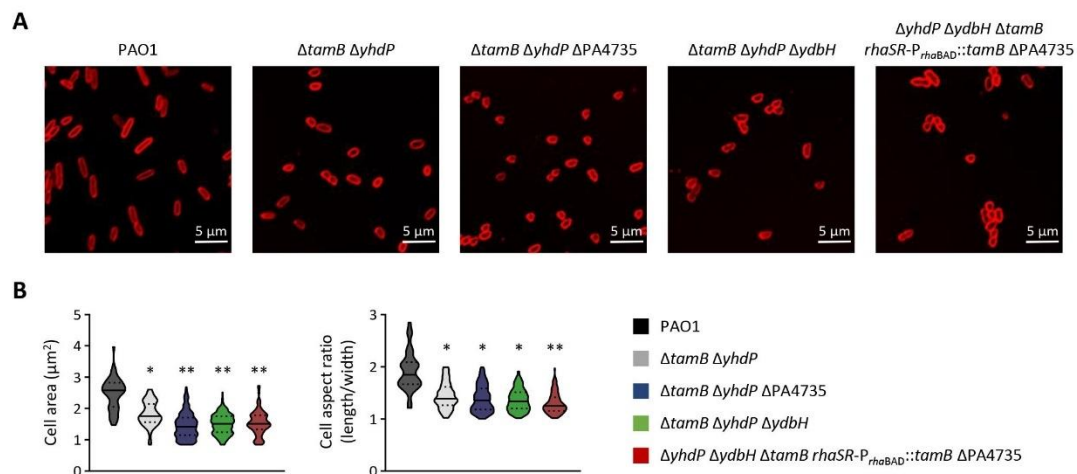


FIG 4 (A) Confocal microscopy images of *P. aeruginosa* PAO1 and the mutants $\Delta tamB \Delta yhdP$, $\Delta tamB \Delta yhdP \Delta ydbH$, $\Delta tamB \Delta yhdP \Delta PA4735$, and $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ stained with the membrane-labeling dye FM4-64. Strains were cultured in MH until the mid-exponential phase, except for the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ that was progressively depleted of TamB using the culturing strategy shown in Fig. S4. Images are representative of two independent experiments and several fields of view showing the same results. (B) Violin plots showing cell area (μm^2) and aspect ratio (length/width) for 100 cells of the same strains shown in panel A, calculated with the ImageJ software. Asterisks indicate a statistically significant difference with respect to the wild-type strain (* $P < 0.05$ and ** $P < 0.01$; unpaired t test). Confocal microscopy images and analysis of the single mutants $\Delta tamB$, $\Delta yhdP$, $\Delta ydbH$, and $\Delta PA4735$ are shown in Fig. S3.

The introduction of a plasmid expressing an FtsZ-GFP fusion protein (pMEftsZ-GFP; Table S2) (35) allowed demonstrating that the Z-ring is assembled and correctly localized at mid-cell in double and triple *asmA*-like gene mutants (Fig. S5), leading to hypothesizing that depletion of essential AsmA-like proteins does not directly affect the divisome assembly and, plausibly, cell constriction.

The importance of AsmA-like proteins for membrane functionality was investigated through cell permeability assays with the fluorescent probes 1-N-phenylnaphthylamine (NPN) and propidium iodide (PI), on strains that showed growth or antibiotic resistance defects (Fig. 1 and 2). NPN is a hydrophobic probe whose fluorescence is strongly increased upon binding to membrane lipids and is commonly used to measure OM destabilization, while PI only enters cells with damaged membranes and increases its fluorescence upon binding to nucleic acids (36, 37). Cells lacking TamB and YhdP were found to be 20-fold more permeable to NPN than wild-type cells (Fig. 5), indicating that the permeability barrier of the OM is significantly compromised. Permeability to NPN was further increased in TamB- and YhdP-deficient cells also lacking PA4735 or YdbH (Fig. 5), indicating that all these AsmA-like proteins contribute to some extent to OM homeostasis. Accordingly, the OM of *P. aeruginosa* cells was extremely permeable to NPN when the four AsmA-like proteins were concomitantly depleted (conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ in Fig. 5). The PI permeability profiles of all these mutants substantially mirrored those of NPN (Fig. 5), confirming that AsmA-like protein depletion causes relevant defects in membrane functionality.

Lowering LPS synthesis or transport partially rescues the OM defects caused by AsmA-like protein depletion

Reduced biosynthesis or transport of GPLs is expected to affect bacterial cell size and growth rate by hampering the ability of the bacterium to create biomass for cell elongation and division (38, 39). However, the integrity and functionality of the OM as a permeability barrier mainly rely on the balance between the LPS and GPL content in the outer and inner leaflets, respectively (23, 38, 40, 41). Thus, if AsmA-like proteins are indeed responsible for GPL transport from the IM to the OM, one might expect the OM defects of cells depleted of AsmA-like proteins to be rescued by reducing the amount of LPS in the OM. This has been demonstrated in *E. coli*, where the replacement of the LPS biosynthetic gene *lpxC* with the less functional allele *lpxC101*, resulting in reduced LPS synthesis, partially restored OM functionality in cells deficient in essential AsmA-like proteins (23). We attempted to demonstrate if the same occurs in *P. aeruginosa* by using two complementary approaches, based on (i) chemical inhibition of LpxC activity or (ii)

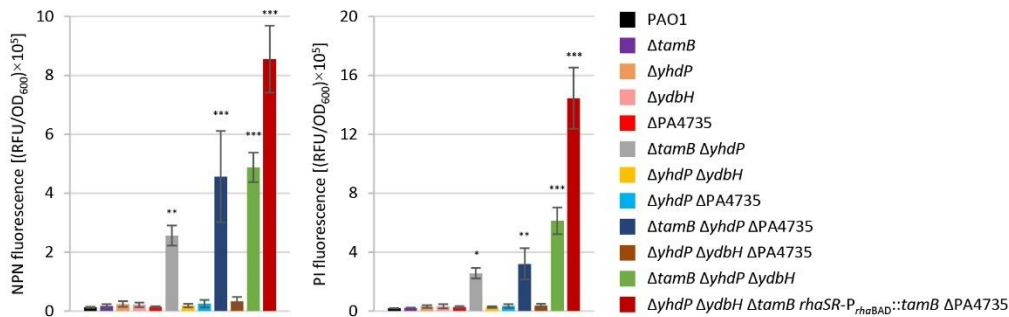


FIG 5 Uptake of NPN (left panel) and PI (right panel) by *P. aeruginosa* PAO1 and selected isogenic mutants, measured as relative fluorescence units (RFU) normalized to the OD₆₀₀ of the bacterial suspension. Strains were cultured in MH until the mid-exponential phase, except for the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ that was progressively depleted of TamB using the culturing strategy shown in Fig. S4. Values are the mean ($\pm$ standard deviation) of three independent experiments, and asterisks indicate a statistically significant difference with respect to the wild-type strain (* P < 0.05, ** P < 0.01, and *** P < 0.001; ANOVA).

conditional mutagenesis to lower the expression of the *lptB* gene, which encodes the ATPase essential to energize LPS transport through the Lpt machinery (42).

For chemical inhibition, we used the LpxC inhibitor PF-5081090 previously demonstrated to be active against many Gram-negative bacteria including *P. aeruginosa* (43). Preliminary growth assays in the presence of different concentrations of PF-5081090 were performed for the wild-type strain PAO1 and the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ to select the highest concentration that did not affect growth (Fig. 6A). Then, strains were cultured in the presence of the selected sub-inhibitory PF-5081090 concentration (0.031× MIC) and the NPN permeability assay was performed to monitor OM functionality. In wild-type cells, PF-5081090 treatment caused a mild (1.6-fold) increase in OM permeability (Fig. 6B), in line with what was previously observed in *P. aeruginosa* cells impaired in LPS transport (44). Conversely, treatment with an equivalent amount of PF-5081090 led to a significant twofold reduction of OM permeability in the $\Delta tamB \Delta yhdP \Delta ydbH$ mutant (Fig. 6B), though this was not sufficient to restore growth and OM integrity at wild-type levels (Fig. 6A and B).

Almost identical results were obtained by placing the LPS transport gene *lptB* under the control of a rhamnose-dependent promoter in PAO1 and in the $\Delta tamB \Delta yhdP \Delta ydbH$

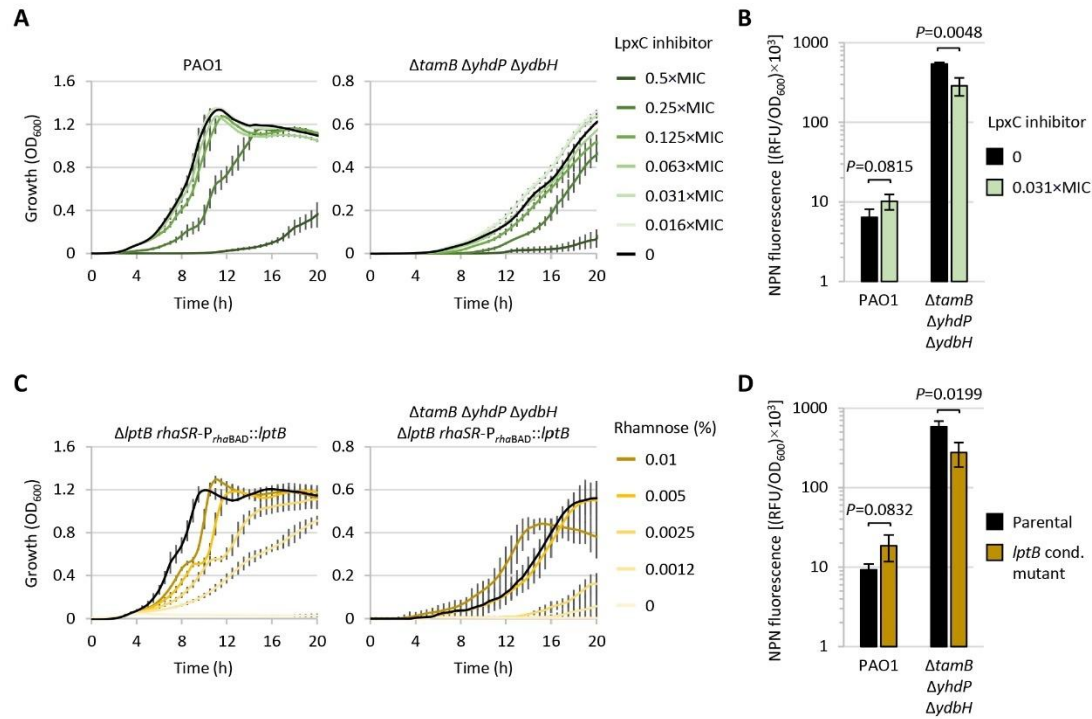


FIG 6 (A) Planktonic growth of *P. aeruginosa* PAO1 and the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ cultured in MH broth in the presence or absence of different sub-inhibitory concentrations of the LpxC inhibitor PF-5081090. The MIC of PF-5081090 was 0.25 µg/mL for PAO1 and 0.125 µg/mL for $\Delta tamB \Delta yhdP \Delta ydbH$. (B) Uptake of NPN by the same strains described in panel A cultured until mid-exponential phase in the presence or absence of 0.031× MIC of PF-5081090, measured as RFU normalized to the OD₆₀₀ of the bacterial suspension. (C) Planktonic growth of the conditional mutants $\Delta lptB rhaSR-P_{rhaBAD}::lptB$ and $\Delta tamB \Delta yhdP \Delta ydbH \Delta lptB rhaSR-P_{rhaBAD}::lptB$ cultured in MH broth in the presence or absence of different concentrations of rhamnose as indicated. The parental strains PAO1 (left panel) and $\Delta tamB \Delta yhdP \Delta ydbH$ (right panel) cultured in the absence of rhamnose were included as controls (black lines). (D) Uptake of NPN by the same strains described in panel C cultured until mid-exponential phase in the presence of rhamnose at 0.01% ($\Delta lptB rhaSR-P_{rhaBAD}::lptB$) or 0.005% ($\Delta tamB \Delta yhdP \Delta ydbH \Delta lptB rhaSR-P_{rhaBAD}::lptB$), measured as RFU normalized to the OD₆₀₀ of the bacterial suspension. Values are the mean (±standard deviation) of three independent experiments. The unpaired t test was used to assess statistically significant differences between treated and untreated samples in panel B and between parental strains and the cognate *lptB* conditional mutants in panel D.

mutant. As expected, the growth of both conditional mutants ($\Delta lptB$ *rhaSR*-*P_{rhaBAD}::lptB* and $\Delta tamB$ $\Delta yhdP$ $\Delta ydbH$ $\Delta lptB$ *rhaSR*-*P_{rhaBAD}::lptB*; Table S1) was promoted by rhamnose in a dose-dependent manner (Fig. 6C), confirming the essentiality of the Lpt system in *P. aeruginosa* (45). By performing the NPN permeability assay on cells cultured in the presence of sub-optimal concentrations of rhamnose, again, we observed that the OM was twofold more permeable in the AsmA-like protein-proficient strain and twofold less permeable in the strain deficient in AsmA-like proteins (Fig. 6D).

Overall, these results demonstrate that the OM of cells defective in AsmA-like protein function(s) can be partially stabilized by reducing LPS levels in the OM, thus corroborating the proposed role of AsmA-like proteins in GPL transport to the OM (22–24, 26).

TamA is only required for TamB activity

In *E. coli*, TamB interacts with the OM β -barrel protein TamA to form the so-called TAM complex (46), which was originally proposed to be involved in the insertion into the OM of a subset of β -barrel proteins, such as autotransporters and fimbrial ushers (47, 48). More recently, genetic evidence highlighted that TamA is required for the function of TamB in maintaining OM homeostasis in *E. coli* (22). Since a *tamA* ortholog is present in *P. aeruginosa* (PA2543 in PAO1) and appears to be operonic with *tamB* (<https://pseudomonas.com/>), we decided to verify whether TamA is required for TamB activity also in this bacterium. To this aim, we generated an in-frame deletion in *tamA* in the wild-type and $\Delta yhdP$ $\Delta ydbH$ backgrounds. The single mutant $\Delta tamA$ did not show any growth defect, in line with what was observed for the $\Delta tamB$ mutant (Fig. 7). In contrast, deletion of *tamA* in the mutant lacking YhdP and YdbH drastically reduced growth, both on agar plates and in liquid medium. Notably, the growth profile of the $\Delta yhdP$ $\Delta ydbH$ $\Delta tamA$ mutant was almost identical to that of the $\Delta tamB$ $\Delta yhdP$ $\Delta ydbH$ mutant (Fig. 7), strongly suggesting that the absence of TamA makes TamB inactive and, thus, that TamB-TamA interaction is likely important for TamB functionality also in *P. aeruginosa*. Notably, the $\Delta tamA$ mutant also showed no increase in NPN permeability or vancomycin sensitivity (data not shown), indicating that the integrity of the OM is not (or only marginally) compromised in TamA-deficient *P. aeruginosa* cells. Since OM integrity is very sensitive to the absence of YhdP, YdbH, and/or PA4735 (Fig. 1 and 5), this finding leads us to propose that the other essential AsmA-like proteins do not rely on TamA for their function(s).

DISCUSSION

The anterograde transport of GPLs from the IM to the OM of Gram-negative bacteria is one of the most elusive essential physiological processes in bacteria. Recently, independent studies performed in the model organism *E. coli* provided genetic and biochemical evidence that proteins belonging to the AsmA-like clan could account for this task

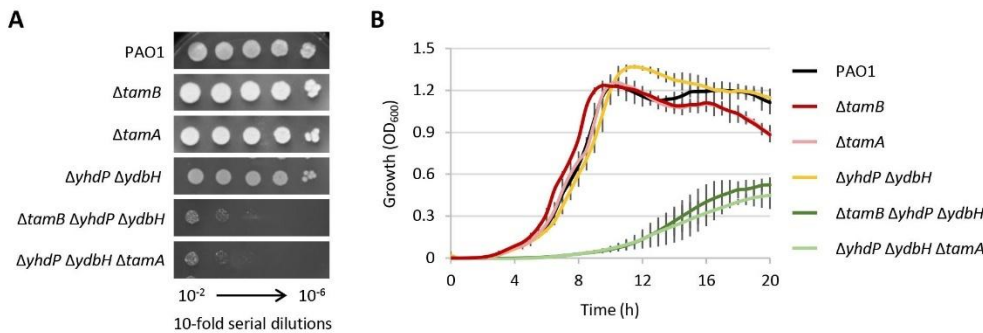


FIG 7 Comparison of (A) plating efficiency and (B) planktonic growth between selected *tamA* and *tamB* mutants on MH agar plates or in MH broth. The wild-type strain PAO1 and the double mutant $\Delta yhdP$ $\Delta ydbH$ were used as controls. Data are the mean ($\pm$ standard deviation) or are representative of at least three independent experiments.

(22–24). Although a direct demonstration that AsmA-like proteins are able to bind and transport GPLs still lacks, it has been shown that *E. coli* cells depleted of the AsmA-like proteins YhdP, TamB, and/or YdbH (i) have a reduced flux of GPLs toward the OM (24); (ii) are defective in growth, cell elongation, OM integrity, and antibiotic resistance, all phenotypes reminiscent of impaired OM biogenesis (22, 23); and (iii) accumulate GPLs in the IM (23). These findings, together with the predicted subcellular localization and tube-like structure of AsmA-like proteins, resembling eukaryotic inter-organelle GPL transporters and Lpt proteins responsible for LPS transport toward the OM (26, 27, 29, 30), strongly support the hypothesis that these proteins may represent the long sought-after mechanism for GPL translocation from the IM to the OM (22, 23, 26).

Since GPL transport is expected to be essential for OM biogenesis in all diderm bacteria, proving that AsmA-like proteins play a key role in cell envelope biogenesis and cell viability also in other species is pivotal to propose these proteins as the universal GPL transport mechanism of Gram-negative bacteria. Although the possible role of AsmA-like proteins in GPL transport has only been recently proposed (22, 23), the effect of the lack of specific AsmA-like protein(s) has been occasionally investigated in diderm bacteria other than *E. coli*. Most of the studies were focused on TamB orthologs, probably because of the originally proposed role of this AsmA-like protein in the maturation of a subset of OM β -barrel proteins (46–48). Similar to what was observed in *E. coli*, the *Brucella suis* TamB homolog MapB is required for proper assembly of an autotransporter adhesin in the OM, although MapB-deficient cells also display anomalies in cell morphology and division (49). The lack of the TamB homolog MorC in *Aggregatibacter actinomycetemcomitans* does not affect autotransporter levels in the OM (50) but changes OM morphology from rugose to flat, decreases cell size, and induces autoaggregation (51). Likewise, the TamB homolog DR_146T of *Deinococcus radiodurans* is important for the maintenance of cell envelope integrity and for resistance to shear and osmotic stresses (52), while the lack of TamB in the intracellular pathogen *Edwardsiella tarda* leads to pleiotropic defects in membrane integrity under stresses, motility, cell invasion, and intracellular replication (53). In *Salmonella*, the Tam system does not appear to be involved in the assembly of autotransporters but affects OM homeostasis and shows some functional interplay with the Bam machinery (54). Finally, it has been recently found that *tamB* inactivation alleviates the OM lipid asymmetry defects of a Mla-defective mutant of *Veillonella parvula*, a diderm member of the Gram-positive phylum Firmicutes (55), suggesting that the role of TamB in OM lipid homeostasis may be widely conserved.

In this study, we provide the first systematic investigation of AsmA-like proteins in a Gram-negative bacterium different from *E. coli*. Our results revealed that, in line with what was previously demonstrated in *E. coli*, *P. aeruginosa* has multiple highly conserved AsmA-like proteins, some of which are essential but redundant for growth and OM integrity. The three essential AsmA-like proteins of *E. coli* (i.e., YhdP, TamB, and YdbH) seem to be functionally conserved in *P. aeruginosa*, as well as their relative contribution to OM homeostasis, with YhdP playing a more critical role than TamB and YdbH (Fig. 1) (22). However, we also found that an additional AsmA-like protein absent in *E. coli*, PA4735, compensates for the lack of YhdP, TamB, and YdbH, thus supporting the growth of the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ in *P. aeruginosa* (Fig. 2 and 3). We therefore propose to name the PA4735 gene *epaF* (for essential Pseudomonas Asma-like protein four). While sequence homology search suggested that this protein may be specific to the *Pseudomonas* genes, we identified putative structural homologs in several diderm bacteria (Table S3 and Fig. S2), implying that this protein might be involved in cell envelope biogenesis also in other species. Although we did not provide direct evidence of GPL translocation through AsmA-like proteins, we confirmed that also in *P. aeruginosa*, the defects in the OM permeability barrier caused by AsmA-like protein deficiency can be partially rescued by reducing the synthesis or transport of LPS (Fig. 6), as previously observed in *E. coli* (23). This is consistent with the notion that the balance between LPS and GPL in the OM is crucial for maintaining OM asymmetry and a functional permeabil-

ity barrier (23, 38, 40, 41), thus indirectly supporting the proposed role of AsmA-like proteins in GPL anterograde transport (22, 23, 26).

The *P. aeruginosa* genomes have three other *asmA*-like genes that, based on the assays performed in this study, do not seem to play a major role in OM biogenesis or homeostasis. Two of them encode putative homologs of the *E. coli* proteins AsmA and YhjG. While inactivation of *yhjG*, alone or in combination with other *asmA*-like genes, has no or very low impact on phenotypes related to OM integrity also in *E. coli* (22, 23), the effect of AsmA deficiency on *P. aeruginosa* markedly differs from that observed in the model organism *E. coli*. Indeed, the importance of AsmA for *E. coli* cell envelope homeostasis has been known for years (56) and has been recently confirmed (22). In contrast, we did not observe any effects of *asmA* deletion on *P. aeruginosa*, even when combined with the inactivation of multiple *asmA*-like genes (Fig. S1 and S2). Notably, the *P. aeruginosa* and *E. coli* AsmA proteins are highly divergent in terms of sequence homology and size, with the *P. aeruginosa* protein being 20% longer than the *E. coli* counterpart (Table 1), suggesting that either they are not actually orthologs or they diverged toward different functions in these two bacteria. However, the possibility that *asmA* in *P. aeruginosa* is only expressed in response to specific stimuli cannot be ruled out. The third non-essential AsmA-like protein of *P. aeruginosa* (PA2708) is much shorter than all the other AsmA-like proteins characterized so far (Table 1 and Fig. 1) (22, 23, 49–55), and its size is not expected to allow to cross the periplasmic space unless PA2708 can interact with OM partners protruding into the periplasm. Interestingly, this protein is conserved in different *Pseudomonas* species (<https://pseudomonas.com/>), indicating that it has been maintained during *Pseudomonas* evolution. Further studies are required to uncover its role in *Pseudomonas* physiology.

Overall, this work supports the recently proposed role of AsmA-like proteins in GPL transport, by demonstrating that a subset of AsmA-like proteins is redundantly essential for growth and OM biogenesis also in *P. aeruginosa*, and provides evidence that the repertoire of essential AsmA-like proteins of Gram-negative bacteria might be more variegated than that described in the model organism *E. coli*. However, there are still many open issues that need to be addressed to confirm the involvement of AsmA-like proteins in GPL anterograde transport and to decipher their mechanism of action. First, there is no biochemical and structural evidence that confirms the capability of AsmA-like proteins to interact with GPLs. Second, no data are available on the mechanism(s) by which AsmA-like proteins could extract GPLs from the IM and insert them into the OM. The OM β -barrel protein TamA is required for TamB function in OM biogenesis, both in *E. coli* and *P. aeruginosa* (22) (Fig. 7). Moreover, TamA possesses a lateral gate in the barrel domain similar to the one present in BamA that allows insertion of OM β -barrel proteins (57, 58). Thus, TamA could provide a possible route for inserting GPLs into the OM. However, since TamA is generally not essential in Gram-negative bacteria (59), it is unlikely that TamA represents a universal OM partner of AsmA-like proteins for GPL insertion into the OM. This would also be in line with phylogenomic evidence suggesting that many diderm bacteria have TamB but not TamA orthologs (60). Thus, either the other OM partner(s) of AsmA-like proteins have yet to be identified or, alternatively, some AsmA-like proteins could have intrinsic insertase activity, as it has been recently demonstrated for lipoprotein insertion by LolA in Gram-negative bacteria lacking the OM component LolB of the Lol system (61). Regarding the pathway(s) and mechanism(s) responsible for GPL extraction from the IM, if AsmA-like proteins require a dedicated IM protein machinery for GPL extraction, this should be essential for growth. However, it is hard to believe that it has not yet been identified after decades of research on cell envelope biogenesis in Gram-negative bacteria, unless redundant systems exist (as for AsmA-like proteins themselves) or protein machineries responsible for other essential transport processes are involved. The most obvious candidates could be the LptBFG or the LolCDE complexes, responsible for ATP-dependent extraction from the IM of LPS and lipoproteins, respectively (14–17). However, the first amino acids of mature OM lipoproteins appear to play a prominent role in substrate binding by LolCDE (62), and

biochemical and structural studies strongly suggest that LptBFG specifically binds LPS (63–65). Thus, unless their specificity is somehow affected by AsmA-like proteins or other still unidentified modulators, these ABC transporters are unlikely to account for GPL extraction from the IM and transfer to the AsmA-like proteins. Alternatively, the GPL flux toward the periplasmic groove of AsmA-like proteins could be directly driven by GPL synthesis at the IM, a mechanism that would not require the involvement of a specific energy-dependent transporter and that has been hypothesized for eukaryotic inter-organelle GPL transporters (27, 66). The high-flux diffusive flow of GPLs from the IM to the OM that has been observed in *E. coli* (24) would however require the presence of spots of very intensive GPL synthesis or local lipid packing perturbations that could facilitate an efficient loading of AsmA-like proteins with GPLs. In the coming years, we expect much exciting research to clarify these issues and finally unravel the mechanism of anterograde GPL transport in Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Bacteria were routinely cultured in Lysogeny broth, Lennox formulation (LB) for genetic manipulation. MH broth was used for plate efficiency and growth assays, permeability assays, and confocal microscopy studies. When required, antibiotics were added at the following concentration for *E. coli* (the concentrations used for *P. aeruginosa* are shown in brackets): ampicillin, 100 µg/mL; tetracycline, 12.5 µg/mL (50–100 µg/mL); carbenicillin (500 µg/mL); nalidixic acid, 15 µg/mL; and chloramphenicol, 30 µg/mL (375 µg/mL).

Generation of plasmids and recombinant strains

To obtain the constructs for the in-frame deletion of the *P. aeruginosa* *tamB*, *yhdP*, *yhjG*, *asmA*, *ydbH*, PA4735, and PA2708 genes, two DNA fragments of approximately 500 bp each, encompassing the upstream and downstream region of each gene of interest, were PCR amplified, directionally cloned into the *sacB*-based suicide vector pDM4 (67), and verified by DNA sequencing. Primers and restriction enzymes used for PCR and cloning are listed in Table S4. The resulting pDM4 derivatives were transferred into *P. aeruginosa* by conjugation, and transconjugants were selected on LB agar plates containing 15 µg/mL nalidixic acid and 375 µg/mL chloramphenicol. Deletion mutations were obtained by recombination and sucrose-based selection as previously described (68). Gene deletions were verified by PCR. To generate the *tamB* and the *lptB* conditional mutants, the *tamB* and the *lptB* coding sequences were individually PCR amplified and directionally cloned into the mini-CTX1 derivative pJM253 (69) downstream of the *rhaSR*-*P_{rhaBAD}* regulatory element. The resulting pJM253 derivatives were transferred into *P. aeruginosa* by conjugation, and transconjugants were selected on LB agar plates containing 15 µg/mL nalidixic acid and 100 µg/mL tetracycline. The plasmid backbone was removed using the *sacB*-based suicide vector pFLP2 (70) as previously described (68), and pFLP2 was cured by plating onto LB agar plates supplemented with 10% sucrose. Carbenicillin-sensitive clones were analyzed by colony PCR to verify the insertion of the *rhaSR*-*P_{rhaBAD}*::*tamB* or *rhaSR*-*P_{rhaBAD}*::*lptB* construct into the chromosome. Then, in-frame deletion of the endogenous copies of *tamB* or *lptB* was obtained by recombination and sucrose-based selection using the pDM4*tamB* and pDM4*lptB* constructs under permissive condition (i.e. growth in the presence of 0.01% rhamnose). The complementing plasmids pMEyhdP, pMEydbH, and pMEPA4735 (Table S2) were generated by individually cloning the PCR-amplified coding sequence of *yhdP*, *ydbH*, or PA4735 into the shuttle vector pME6032, under the control of an IPTG-inducible promoter (71). All constructs were verified by DNA sequencing.

Plating efficiency and growth assays

For plating efficiency assays, bacterial strains were precultured in MH, supplemented with 0.01% rhamnose for the conditional mutants, harvested by centrifugation, and resuspended in sterile saline solution at $OD_{600} = 1$. Serial 10-fold dilutions were prepared, and 5- μ L aliquots of selected dilutions were spotted onto MH agar plates containing or not 0.25% SDS and 0.25 mM EDTA, 100 or 1,000 μ g/mL vancomycin (corresponding to 0.05 $\times$ or 0.5 $\times$ MIC for the PAO1 wild-type strain, respectively), or 0.125 or 0.25 μ g/mL colistin (corresponding to 0.25 $\times$ and 0.5 $\times$ MIC for the PAO1 wild-type strain, respectively) in the presence or not of 0.01% rhamnose or 0.5 mM IPTG, when appropriate.

For planktonic growth assays, bacterial strains were precultured in MH, supplemented with 0.01% rhamnose for the conditional mutants, and then refreshed 1:1,000 in MH in the absence or presence of 0.01% rhamnose, 0.5 mM IPTG, or increasing concentrations of the LpxC inhibitor PF-5081090 (43), when appropriate. Bacterial cultures were incubated in 96-well microtiter plates (200 μ L in each well) at 37°C in a Tecan Spark 10M microtiter plate reader, and growth was measured over time as the OD_{600} of the bacterial cultures.

TamB-depleted cells of the conditional mutant $\Delta yhdP \Delta ydbH \Delta tamB rhaSR-P_{rhaBAD::tamB} \Delta PA4735$ (Table S1), which were used for microscopy and cell envelope stability assays, were obtained through a previously described dual-refresh culturing strategy (72). Briefly, cells were cultured overnight in the presence of 0.01% rhamnose and then refreshed at high cell density (1:30 dilution) in the absence of rhamnose, cultured for 2 h, and then refreshed again (1:50 dilution) in the same medium. Cells were collected as soon as a growth defect was observed in the conditional mutant with respect to the wild-type strain (Fig. S4).

Confocal microscopy

Bacterial strains carrying the constructs pMEftsZ-GFP were cultured in flask in MH, supplemented or not with 0.01% rhamnose, in the presence of 0.003 mM IPTG to induce non-toxic expression levels of the FtsZ-GFP fusion protein (35). Two hundred microliters of late-exponential phase cultures were collected, the fluorescent dye FM4-64 (73) was added at 5 μ g/mL, and the samples were incubated at 37°C for 30 min. Then, 5 μ L of the bacterial cell suspensions were spotted on a microscope glass slide overlaid with 0.5% agarose and imaged with a Nikon A1R HD25 confocal laser scanning microscope equipped with an Apo TIRF 100 $\times$ oil immersion objective (NA 1.49). The 488- and 561-nm laser lines were employed for the GFP and FM4-64 excitation, respectively. Emission bandwidths at 500–540 nm and 600–720 nm were used for GFP and FM4-64 detection, respectively. Images were acquired at a sampling dimension of 512 $\times$ 512 pixels and were deconvoluted using the NIS-Elements software (Nikon), using default parameters. Image processing to determine cell morphological parameters was performed according to a previously established pipeline (74). Cell area and aspect ratio were calculated using ImageJ v.1.53c for 100 cells of each bacterial strain (75).

Membrane permeability assays

Bacterial strains were cultured in MH, supplemented or not with 0.01% rhamnose, in the absence or presence of the LpxC inhibitor PF-5081090 at 0.031 $\times$ MIC (corresponding to 0.008 μ g/mL for PAO1 and 0.004 μ g/mL for $\Delta tamB \Delta yhdP \Delta ydbH$). Exponential phase cells were harvested by centrifugation and resuspended in 5 mM HEPES (pH 7.2) at $OD_{600} = 3$. Equal volumes (150 μ L) of HEPES solution containing or not PI (40 μ g/mL) or NPN (20 μ M) and the bacterial suspensions were mixed, and 100 μ L of each sample was aliquoted on a black flat-bottom 96-well plate. OD_{600} and fluorescence were measured in a Tecan Spark 10M microtiter plate reader (excitation at 350 nm and emission at 420 nm for NPN; excitation at 580 nm and emission at 620 nm for PI) after 2 min at room temperature,

subtracted of the background values of samples without NPN or PI, and normalized to the OD₆₀₀ of the cell suspension (76).

MIC assays

The MIC of antibiotics (vancomycin and colistin) or the LpxC inhibitor PF-5081090 were determined through the broth microdilution method. *P. aeruginosa* strains precultured in MH were refreshed in the same medium at ca. 5×10^5 cells/mL in the presence of increasing concentrations of each antibiotic/compound. MIC was recorded after 20 h at 37°C. At least three independent experiments were performed for each strain/compound.

Bioinformatic analyses

TMHs were predicted using the online tool TMHMM 2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) (77). Predicted 3D structures were retrieved from the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>) (28). Orthologs within the *Pseudomonas* genus were identified using the Ortholog Group Members function of the *Pseudomonas* Genome Database (<https://pseudomonas.com/>) (78). Putative structural homologs of PA4735 were searched with Foldseek (<https://search.foldseek.com/search>) (79), by using the PDB downloaded by the AlphaFold Database as query.

Statistical analysis

Statistical analysis was performed with the software GraphPad Instat, using the unpaired t test or the ANOVA test as indicated.

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ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental figures and tables (mSphere00677-23-S0001.pdf). Tables S1 to S4 and Figures S1 to S5.

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CHAPTER 4. Structure-function relationship of the *Pseudomonas aeruginosa* AsmA-like proteins YhdP and YdbH involved in outer membrane biogenesis

PREFACE

While the previous study identified AsmA-like proteins as key contributors to OM integrity in *P. aeruginosa* (see chapter 3), the structural determinants underlying their function remained unexplored. In particular, the specific features that enable individual proteins to facilitate GPL transport and maintain membrane homeostasis were unknown.

In the study described in this chapter, we focused on two AsmA-like proteins, YhdP and YdbH, to dissect the structural elements required for their function. Structural modeling, molecular dynamics simulations, and genetic complementation assays revealed that the C-terminal domain of YhdP contains two hydrophobic α -helices that are crucial for its function in maintaining OM integrity. To further explore the modularity of AsmA-like proteins and functional significance of these hydrophobic α -helices, we constructed a YdbH-YhdP hybrid protein by fusing the C-terminal domain of YhdP to YdbH. This hybrid protein markedly enhanced YdbH function, promoting bacterial growth and OM stability more effectively than wild-type YdbH. Interestingly, while we confirmed that YdbH normally requires the OM partner protein YnbE for its activity also in *P. aeruginosa*, the YdbH-YhdP hybrid operates independently of this partner, suggesting that the C-terminal domain of YhdP can act autonomously as a modular element capable of anchoring and/or facilitating GPL insertion into the OM. Overall, this work provided new insights into the molecular determinants of GPL transport and established a framework for investigating the mechanisms by which AsmA-like proteins contribute to OM biogenesis.

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RESEARCH ARTICLE



Structure–function relationship of the *Pseudomonas aeruginosa* AsmA-like proteins YhdP and YdbH involved in outer membrane biogenesis

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Abstract

The outer membrane (OM) of Gram-negative bacteria is an asymmetric bilayer composed of glycerophospholipids (GPLs) in the inner leaflet and lipopolysaccharide in the outer leaflet, which is critical for viability and antibiotic resistance. While the mechanisms for lipopolysaccharide and OM protein transport across the periplasm are well characterized, it has only recently been proposed that AsmA-like proteins are likely involved in the anterograde transport of GPLs. Here, we investigated the structural and functional features of *Pseudomonas aeruginosa* YhdP, an AsmA-like protein that exhibits higher activity in maintaining OM integrity than its paralogs. Through structural predictions, molecular dynamics simulations, and genetic complementation assays, we demonstrated that two hydrophobic α -helices at the C-terminal region of YhdP are crucial for its function. Moreover, we found that fusing the YhdP C-terminal domain to YdbH enhances the functionality of this shorter and lower-activity AsmA-like protein. Specifically, a YdbH-YhdP fusion protein long enough to potentially span the periplasmic space was more active than the wild-type YdbH protein in promoting growth and OM integrity. Furthermore, while YdbH activity requires the OM protein partner YnbE, the functionality of the YdbH-YhdP fusion protein is YnbE-independent. These findings support the hypothesis that the YhdP C-terminus provides OM anchoring and likely assists GPL insertion, and prove that it can be used as a modular element for improving the activity of other AsmA-like proteins. Our work sheds light on the molecular determinants of GPL transport and offers a framework for studying the mode of action of AsmA-like proteins in OM biogenesis.

KEYWORDS

anterograde transport, *Escherichia coli*, glycerophospholipids, Gram-negative bacteria, hybrid proteins, molecular dynamics simulations, outer membrane integrity, periplasm, *Pseudomonas aeruginosa*, structure–function relationship

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1 | INTRODUCTION

The tripartite cell envelope of Gram-negative bacteria consists of the cytoplasmic/inner membrane (IM), a periplasmic space containing a thin peptidoglycan cell wall, and the outer membrane (OM) (Silhavy et al., 2010). While the IM is a typical glycerophospholipid (GPL) bilayer, the OM is an asymmetric bilayer made of GPLs in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, which also accommodates numerous integral OM proteins (OMPs) and lipoproteins (Henderson et al., 2016).

The OM is an essential structure of Gram-negative bacteria that provides mechanical strength and protection from large and/or hydrophobic toxic molecules, including many antibiotics, partly explaining the intrinsic drug resistance of Gram-negative pathogens (Nikaido, 2003; Sun et al., 2022). Understanding the composition and biogenesis of the OM remains a crucial area of research, as it holds potential for identifying novel drug targets and developing new antibacterials (Choi & Lee, 2019; Sperandio et al., 2023; Walker & Black, 2021). Disrupting OM biogenesis can indeed directly lead to bacterial cell death or increase susceptibility to antimicrobials that would otherwise be ineffective due to the OM permeability barrier (Lehman & Grabowicz, 2019; Wesseling & Martin, 2022).

The assembly of the OM requires the transport of several hydrophobic molecules across the aqueous periplasmic space, which occurs through specialized protein transport systems. Lipoproteins and OMPs are translocated and inserted into the OM by the Lol and Bam systems, respectively, which use soluble carrier proteins to shuttle their substrates across the periplasm (Plummer & Fleming, 2016; Zückert, 2014). LPS is transported to the OM as a continuous stream through the Lpt system, a periplasm-spanning protein complex that bridges the IM and the OM (Okuda et al., 2016; Sperandio et al., 2019). On the other hand, the mechanism by which GPLs are translocated across the periplasm and inserted into the OM (anterograde GPL transport) remained elusive for decades. However, recent studies performed in *Escherichia coli* and *Pseudomonas aeruginosa* provided indirect genetic and biochemical evidence that members of the AsmA-like protein family are likely responsible for this process (Cooper et al., 2025; Douglass et al., 2022; Grimm et al., 2020; Rai et al., 2024; Ruiz et al., 2021; Sposato et al., 2024). Specifically, three AsmA-like proteins (YhdP, TamB, and YdbH) were found to be essential yet redundant in *E. coli* (Ruiz et al., 2021), and four in *P. aeruginosa* (YhdP, TamB, YdbH, and PA4735/EpaF) (Sposato et al., 2024) (Figure 1a). Moreover, the depletion of these proteins resulted in defective phenotypes consistent with impaired GPL transport from the IM to the OM, which could be partly rescued by restoring the asymmetry of the OM through chemical or genetic inhibition of LPS transport (Douglass et al., 2022; Ruiz

et al., 2021; Sposato et al., 2024). Very recently, Cooper and collaborators provided the first evidence that one of these proteins, *E. coli* YhdP, interacts in vivo with phosphate-containing molecules, likely GPLs (Cooper et al., 2025).

Bacterial AsmA-like proteins are characterized by an N-terminal transmembrane α -helix (TMH), which anchors them to the IM, and a large soluble domain predicted to span into the periplasm, which contains a Chorein-N domain followed by an AsmA-like domain of variable length (Kumar & Ruiz, 2023; Levine, 2019). These proteins share structural similarity with eukaryotic proteins, such as Vps13 and Atg2, that mediate lipid transfer between organelles (Kumar and Ruiz 2023; Lees & Reinisch, 2020; Levine, 2019; Melia & Reinisch, 2022), suggesting a conserved role in intermembrane lipid transport. Structural predictions from AlphaFold indicate that AsmA-like proteins form extended, tube-like structures consisting of stacked β -strands, which create a continuous hydrophobic groove along the long axis (Cooper et al., 2025; Douglass et al., 2022; Ruiz et al., 2021; Sposato et al., 2024). Such a groove is reminiscent of that formed by the β -jellyroll domains of Lpt proteins in the periplasm, which accommodate LPS during transport from the IM to the OM (Dong et al., 2017; Hicks & Jia, 2018), supporting the hypothesis that AsmA-like proteins may work as protein bridges for GPL translocation across the periplasm.

While a single AsmA-like protein associated with GPL transfer is sufficient to support bacterial growth, highlighting redundant functions, in both *E. coli* and *P. aeruginosa*, the absence of YhdP causes more relevant defects in OM integrity as compared to the lack of other AsmA-like proteins (Ruiz et al., 2021; Sposato et al., 2024). YhdP is the only AsmA-like protein whose length seems sufficient to span the periplasm (Cooper et al., 2025; Ruiz et al., 2021; Sposato et al., 2024), and molecular dynamics simulations suggested that its C-terminus may interact with the OM, thus supporting its possible envelope spanning nature (Cooper et al., 2025). In contrast, other AsmA-like proteins are predicted to be either too twisted (TamB) or too short (YdbH and PA4735) to cross the entire periplasmic space (Kumar & Ruiz, 2023; Ruiz et al., 2021; Sposato et al., 2024). Accordingly, TamB interacts with the OM β -barrel protein TamA (Selkrig et al., 2015), and this interaction was found to be required for the function of TamB in maintaining OM homeostasis in both *E. coli* and *P. aeruginosa* (Ruiz et al., 2021; Sposato et al., 2024). Recently, it has also been shown that the *E. coli* protein YdbH forms a functional complex with the OM lipoprotein YnbE, which extends the continuous hydrophobic groove of YdbH and is important for its functionality (Kumar et al., 2024).

Using *P. aeruginosa* AsmA-like proteins as a model system, in this study we aimed to elucidate the structural/biochemical features of YhdP that account for its apparently higher efficiency in supporting OM functionality and,

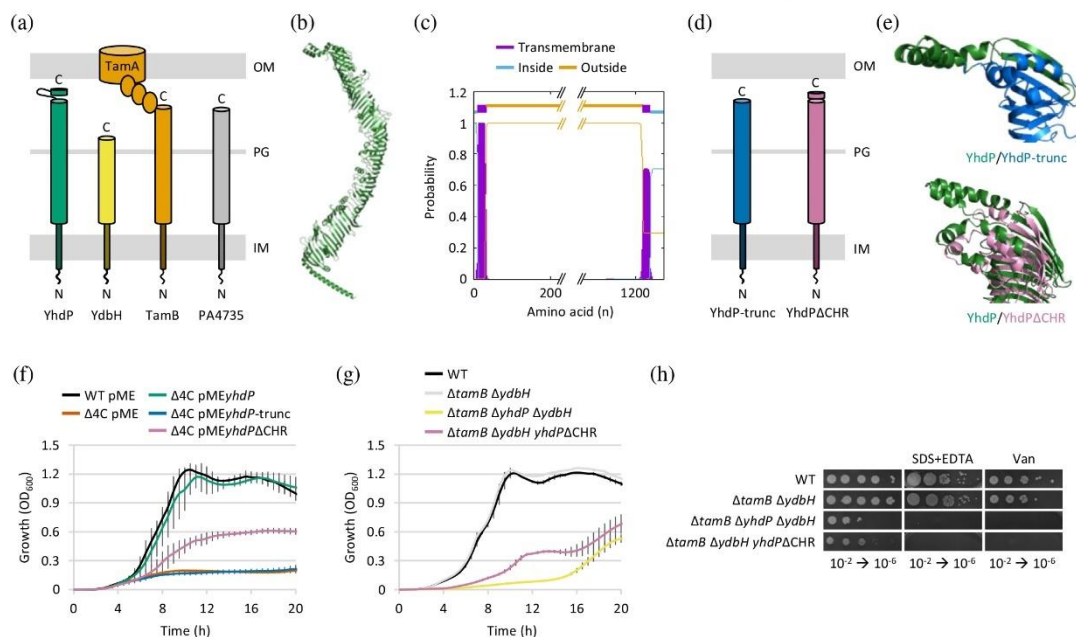


FIGURE 1 (a) Schematic representation of the *Pseudomonas aeruginosa* AsmA-like proteins that are essential but redundant for growth and OM integrity. (b) Predicted 3D structure of *P. aeruginosa* YhdP. (c) Putative TMHs in *P. aeruginosa* YhdP predicted by TMHMM 2.0. (d) Schematic representation of the *P. aeruginosa* YhdP variants lacking the C-terminal domain (YhdP-trunc) or only the C-terminal hydrophobic residues (YhdPΔCHR). (e) Superimposition of the predicted structures of the C-terminal domain of *P. aeruginosa* YhdP and the variant YhdP-trunc (upper panel) or YhdPΔCHR (lower panel). (f) Growth curves of the wild-type strain *P. aeruginosa* PAO1 (WT) and the rhamnose-dependent conditional mutant $\Delta tamB \Delta yhdP \Delta ydbH rhaSR-P_{rhaBAD::tamB} \Delta PA4735$ ($\Delta 4C$) carrying the empty plasmid pME6032 (pME) or its derivatives for IPTG-inducible expression of YdbH, YhdP-trunc, or YhdPΔCHR, cultured in Mueller Hinton broth (MH) supplemented with 125 μ M IPTG. Growth curves of the same strains at different IPTG concentrations are shown in Figure S2. (g) Growth curves of PAO1 (WT) and the isogenic mutants $\Delta tamB \Delta ydbH$, $\Delta tamB \Delta yhdP \Delta ydbH$, and $\Delta tamB \Delta ydbH yhdP\Delta CHR$ cultured in MH. (h) Plating efficiency of the same strains described in panel G on MH agar plates supplemented or not with 0.25% SDS and 0.25 mM EDTA (SDS + EDTA) or 100 μ g/mL vancomycin (Van). Data in panels f–g are the mean ($\pm$ standard deviation) of three independent experiments. Images in panel H are representative of three independent experiments. IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

presumably, GPL transport or insertion into the OM. By generating and expressing mutant YhdP variants and YdbH-YhdP fusion proteins, we demonstrated that two hydrophobic α -helices in the C-terminal region of YhdP are crucial for its function. Moreover, the YhdP C-terminal domain can confer to YdbH higher activity and the ability to function independently of its OM protein partner YnbE, provided that the fusion protein is long enough to potentially span the periplasmic space.

2 | RESULTS AND DISCUSSION

2.1 | Hydrophobic α -helices at the C-terminus are crucial for *P. aeruginosa* YhdP function

Alpha-fold structural prediction suggested the presence of two putative α -helices in the C-terminal region of

P. aeruginosa YhdP (Figure 1b). These α -helices contain several hydrophobic residues and are indeed predicted as a possible TMH by the transmembrane prediction software TMHMM 2.0, spanning residues 1220–1239 (Figure 1c). Similar C-terminal hydrophobic regions are not present in any other *P. aeruginosa* AsmA-like protein (data not shown), suggesting that this may represent a unique feature of YhdP. Notably, analogous C-terminal hydrophobic α -helices were recently identified in the YhdP ortholog of *E. coli* and shown to be required for its function (Cooper et al., 2025).

To confirm that such C-terminal region is also important for the *P. aeruginosa* YhdP protein, we generated plasmid constructs to express two YhdP variants lacking either the entire C-terminal region (residues 1220–1276) or only the two predicted α -helices (residues 1216–1241). These variants were named YhdP-trunc and YhdPΔCHR (C-terminal hydrophobic residues),

respectively (Figure 1d; Table S1). Structural predictions suggested that these deletions are unlikely to disrupt the overall folding of the YhdP tubular domain (Figure 1e). To assess their structural stability and confirm that they retain proper folding and domain organization, we performed extensive coarse-grained molecular dynamics simulations (Joshi & Deshmukh, 2021), comparing their trajectories to those of the wild-type protein (Figure S1). Structural stability was evaluated using three key metrics: (i) the radius of gyration, to monitor global compactness and folding consistency; (ii) the fraction of native contacts (Best et al., 2013), to assess preservation of the native tertiary structure; (iii) the level of frustration, as a measure of the energetic conflict in the folded proteins (Onuchic et al., 1997; Vannimenus & Toulouse, 1977). All three metrics indicated that the studied proteins remained structurally stable throughout the simulations. Radius of gyration values remained consistent over time, showing no significant fluctuations. The fraction of native contacts remained high ($Q > 0.9$) for all models, confirming retention of overall structural integrity. Analysis of local frustration revealed comparable energetic profiles between the chimeric variants and the wild-type protein, with no increase in destabilizing interactions (Figure S1). Together, these results suggest that the chimeric proteins are intrinsically stable, supporting the accuracy of the modeled structures and their potential to maintain native-like functional behavior.

These YhdP variants were expressed in the *P. aeruginosa* conditional mutant $\Delta tamB \Delta yhdP \Delta ydbH rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ (Sposato et al., 2024), which lacks *yhdP*, *ydbH*, and *PA4735*, and expresses *tamB* from a rhamnose-inducible promoter. As expected, ectopic expression of wild-type YhdP restored the growth of the conditional mutant to wild-type levels in the absence of rhamnose (Figure 1f), confirming that YhdP alone is sufficient to efficiently support *P. aeruginosa* growth (Sposato et al., 2024). In contrast, expression of YhdP-trunc had no effect on the growth profile of the conditional mutant, while expression of YhdP Δ CHR partially promoted the growth of the conditional mutant; however, it did not allow to reach the levels of the wild-type control strain (Figure 1f).

In order to assess the growth-promoting effect of YhdP Δ CHR when expressed at physiological levels, we compared the growth profile of the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ to that of the double mutant $\Delta tamB \Delta ydbH$ carrying a mutated *yhdP* allele for expression of the YhdP Δ CHR variant. The double mutant expressing YhdP Δ CHR exhibited minimal growth improvement compared to the triple mutant lacking YhdP (Figure 1g). Moreover, both the absence of YhdP and the expression of YhdP Δ CHR in *TamB*- and *YdbH*-deficient cells caused extreme sensitivity to agents that disrupt the membranes (SDS and EDTA) or to an antibiotic that is poorly active against Gram-negative cells with an intact OM (vancomycin)

(Figure 1h), indicating that YhdP Δ CHR is not able to restore OM integrity of cells lacking essential AsmA-like proteins.

Overall, these results demonstrate that the C-terminal hydrophobic α -helices of *P. aeruginosa* YhdP are crucial for its function, in line with what was previously reported for *E. coli* YhdP (Cooper et al., 2025).

2.2 | Fusion with YhdP C-terminal region enhances YdbH activity

Molecular dynamics simulations with the *E. coli* YhdP protein suggested that its C-terminus may interact with the OM and that the C-terminal hydrophobic helices can insert into the inner leaflet of the bilayer (Cooper et al., 2025). Based on this prediction, one could hypothesize that the role of the C-terminal hydrophobic region could be to anchor YhdP to the OM and/or facilitate GPL insertion into the membrane by creating a local perturbation of the inner leaflet. We therefore wondered whether the C-terminal domain of YhdP could provide new functional properties to other AsmA-like proteins and possibly enhance their activity. To this aim, we generated two constructs that express the first 808 residues of YdbH (out of 855) fused to either the YhdP C-terminal domain only (residues 1161–1276) or a longer YhdP C-terminal region (residues 933–1276), resulting in the fusion proteins YdbH-YhdP_{short} and YdbH-YhdP_{long}, respectively (Figure 2a,b). The length of YdbH-YhdP_{short} and YdbH-YhdP_{long} is comparable to that of YdbH or YhdP, respectively (Table S1). YdbH was chosen for the generation of fusion proteins since its absence has the least impact on OM integrity among AsmA-like proteins likely involved in GPL transport (Ruiz et al., 2021; Sposato et al., 2024), suggesting that it may be less active than other AsmA-like proteins, at least under the conditions tested in previous studies. Moreover, the structure of the AsmA-like domain of YdbH enabled the fusion of the regions of interest of YhdP in a way that preserves a continuity between heterologous β -sheets and maintains the integrity of the hydrophobic groove, according to structural predictions (Figure 2c). Based on the results of molecular dynamics simulations, the two fusion proteins are not expected to experience unfolding or significant alteration in stability or domain organization (Figure S3). Indeed, the radius of gyration and the fraction of native contacts were constant during the simulation time. Also, the number of residues with minimal frustration was much higher than that of highly frustrated residues, suggesting overall stability of the hybrid proteins. Furthermore, the residues close to the fusion point ($-10 < 808 < +10$ residues) were classified as minimally or neutrally frustrated (Figure S3).

To verify their *in vivo* activity, growth kinetics and vancomycin resistance, used as a proxy for OM

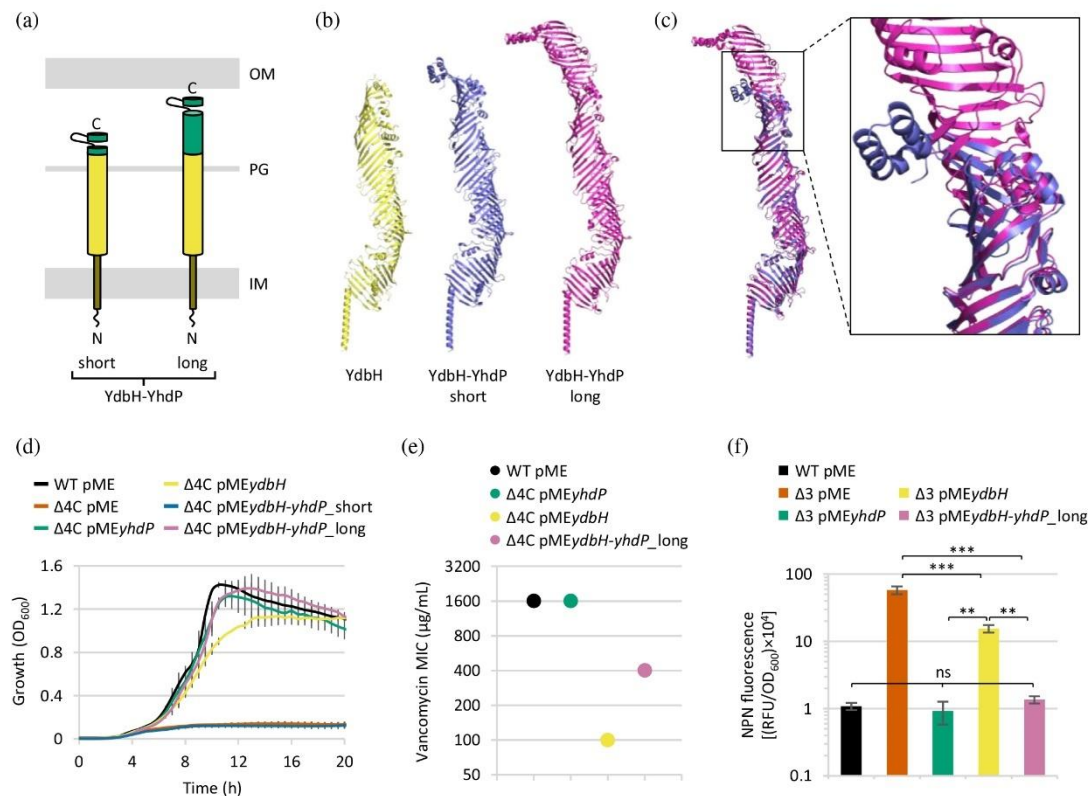


FIGURE 2 (a) Schematic representation of the recombinant fusion proteins YdbH-YhdP_{short} and YdbH-YhdP_{long}. (b) Predicted 3D structure of *Pseudomonas aeruginosa* YdbH, YdbH-YhdP_{short}, and YdbH-YhdP_{long}. (c) Superimposition of the predicted structures of YdbH-YhdP_{short} (blue) and YdbH-YhdP_{long} (purple). The inset highlights the fusion region between YdbH and YhdP domains. (d) Growth curves of *P. aeruginosa* PAO1 (WT) and the conditional mutant $\Delta tamB \Delta yhdP \Delta ydbH rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ ($\Delta 4C$) carrying the empty plasmid pME6032 (pME) or its derivatives for IPTG-inducible expression of YhdP, YdbH, YdbH-YhdP_{short}, or YdbH-YhdP_{long}, cultured in MH supplemented with 16 μM IPTG. Growth curves of the same strains at different IPTG concentrations are shown in Figure S4. (e) Vancomycin MIC for the conditional mutant $\Delta 4C$ expressing YhdP, YdbH, or YdbH-YhdP_{long}, cultured in MH supplemented with 16 μM IPTG. The WT strain carrying the empty plasmid was used as positive control. (f) NPN-mediated fluorescence of WT and triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ ($\Delta 3$) cells carrying pME or its derivatives for expression of YhdP, YdbH, or YdbH-YhdP_{long}, cultured in MH supplemented with 16 μM IPTG. Data in panels d and f are the mean ($\pm$ standard deviation) of three independent experiments. The asterisks indicate the statistically significant differences between strains of interest (** $p < 0.01$; *** $p < 0.001$; ns, not significant) (ANOVA). Values in panel E represent the mode of at least three independent experiments. IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

integrity, were compared in the rhamnose-dependent conditional mutant $\Delta tamB \Delta yhdP \Delta ydbH rhaSR-P_{rhaBAD}::tamB \Delta PA4735$ alternatively expressing the fusion proteins YdbH-YhdP_{short} or YdbH-YhdP_{long}, or the wild-type proteins YhdP or YdbH from the same backbone plasmid. As shown in Figure 2d, YdbH-YhdP_{short} did not restore growth in the absence of rhamnose, while YdbH-YhdP_{long} promoted growth at levels comparable to the wild-type protein YhdP. Moreover, both YhdP and YdbH-YhdP_{long} appeared more efficient than YdbH in growth promotion (Figure 2d), suggesting that the fusion with the YhdP C-terminal

domain enhanced the ability of YdbH to carry out the essential function exerted by AsmA-like proteins, plausibly the transport of GPLs to the OM. This was confirmed by vancomycin minimum inhibitory concentration (MIC) assay, which revealed that the expression of YdbH-YhdP_{long} conferred fourfold higher vancomycin resistance than wild-type YdbH, even if it was not able to restore resistance at the level of the wild-type strain or the YhdP expressing conditional mutant (Figure 2e). The lack of functional complementation by YdbH-YhdP_{short} is consistent with structural predictions suggesting that it cannot span the periplasmic

space. Nonetheless, since we were unable to evaluate the expression levels of the different constructs, the possibility that reduced expression or stability *in vivo* may have contributed to this outcome cannot be excluded.

To further investigate the effect of fusion proteins on OM integrity, the different proteins were expressed in the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$ and an *N*-phenyl-1-naphthylamine (NPN) permeability assay was performed. NPN is a hydrophobic probe commonly used to measure OM destabilization, as its fluorescence strongly increases upon binding to membrane lipids (Kwon et al., 2019; Sposato et al., 2024). The triple mutant was used for this assay because it carries the PA4735 gene and can therefore grow even without ectopically expressed proteins, thus allowing OM integrity to be assessed relative to the empty plasmid control. Expression of YhdP and YdbH-YhdP_{long} completely restored OM integrity in the $\Delta tamB \Delta yhdP \Delta ydbH$ mutant, as NPN fluorescence was comparable to that of the wild-type strain (Figure 2f). Although YdbH expression significantly reduced NPN permeability with respect to the empty plasmid control, the OM of YdbH-expressing cells was still about 10-fold more permeable than that of cells expressing YdbH-YhdP_{long} (Figure 2f). This result confirmed that YdbH-YhdP_{long} is more efficient than the wild-type protein YdbH in maintaining OM integrity. Notably, no significant difference was observed in this assay between cells expressing YhdP or YdbH-YhdP_{long}, in contrast to what was previously noted for vancomycin resistance in the conditional mutant (Figure 2e). This is likely due to the presence of PA4735 in the triple mutant, which could compensate for the slightly lower activity of YdbH-YhdP_{long} compared to YhdP.

2.3 | Fusion with YhdP C-terminal region makes YdbH independent of its protein partner YnbE

Recently, the OM lipoprotein YnbE was found to be required for YdbH activity in *E. coli*, by interacting with the last β strand of YdbH and extending its hydrophobic groove (Kumar et al., 2024). We hypothesized that fusing YdbH to the C-terminal domain of YhdP could enable YdbH to function independently of YnbE. To prove this hypothesis, we first verified whether YnbE is also crucial for YdbH functionality in *P. aeruginosa*. The YnbE orthologue of *P. aeruginosa* PAO1 is encoded by the PA5306 gene, adjacent to *ydbH* (PA5307), and shares 49.1% identity with *E. coli* YnbE. Thus, we deleted PA5306 in the double mutant $\Delta tamB \Delta yhdP$ and compared the growth profile of the resulting strain ($\Delta tamB \Delta yhdP \Delta ynbE$) to that of the triple mutant $\Delta tamB \Delta yhdP \Delta ydbH$. If *ynbE* is essential for YdbH functionality also in *P. aeruginosa*, then the absence of YnbE should impair growth and OM integrity to a similar

extent as the absence of YdbH. As expected, the deletion of *ynbE* in cells lacking TamB and YhdP caused marked growth defects and increased SDS/EDTA and vancomycin sensitivity (Figure 3a,b), suggestive of relevant OM destabilization. However, *ydbH* deletion had more severe effects on growth and OM integrity than *ynbE* deletion, leading to the hypothesis that YdbH may retain some residual activity in YnbE-deficient cells. Notably, *ynbE* deletion in the wild-type strain did not affect growth and OM functionality (Figure 3a,b). This implies that YnbE function is specifically required in cells that strongly rely on YdbH for growth, as previously observed in *E. coli* (Kumar et al., 2024). Overall, this preliminary experiment confirmed that YdbH needs YnbE for its function also in *P. aeruginosa*.

Then, we ectopically expressed the fusion protein YdbH-YhdP_{long} in the triple mutant $\Delta tamB \Delta yhdP \Delta ynbE$ to verify whether it was able to promote growth also in YnbE-deficient cells. YdbH-YhdP_{long} fully restored the growth of $\Delta tamB \Delta yhdP \Delta ynbE$ cells to wild-type levels (Figure 3c), indicating that this fusion protein does not require YnbE to accomplish its essential function. In contrast, the expression of YdbH-YhdP_{short} in the same mutant was unable to promote growth (Figure 3c), further suggesting that this fusion protein is inactive.

Remarkably, also ectopic expression of wild-type YdbH failed to support growth of the mutant $\Delta tamB \Delta yhdP \Delta ynbE$, which already expresses YdbH at physiological levels from the chromosomal gene (Figure 3c). This result was unexpected, as deletion experiments suggested that YdbH likely retains some activity in the absence of YnbE (Figure 3a,b), leading us to assume that YdbH overexpression could have provided some benefit. The lack of any growth promoting effect upon YdbH overexpression might be explained by the involvement of additional OM partner(s) beyond YnbE, which may be already saturated by the YdbH levels expressed by the chromosomal gene. This hypothesis would align with structural predictions indicating that the YdbH-YnbE complex is still too short to bridge the entire periplasm (Kumar et al., 2024). At present, the only AsmA-like protein for which an integral OM partner that could assist GPL insertion into the OM has been identified is TamB (Kumar & Ruiz, 2023; Ruiz et al., 2021; Sposato et al., 2024), which interacts with TamA, a β -barrel protein of the Omp85 family (Selkig et al., 2015). Although recently proposed to participate in GPL insertion into the OM (Douglass et al., 2022; Rai et al., 2024; Ruiz et al., 2021; Sposato et al., 2024), the TamB-TamA complex was originally implicated in the assembly of a subset of OM β -barrel proteins (Stubenrauch & Lithgow, 2019), and its possible dual role in protein and/or GPL transport remains under debate (Goh et al., 2024; Wang et al., 2024). We have previously shown that *tamA* deletion has no effect on *P. aeruginosa* growth, suggesting that TamA is not required for the activity of all essential AsmA-like

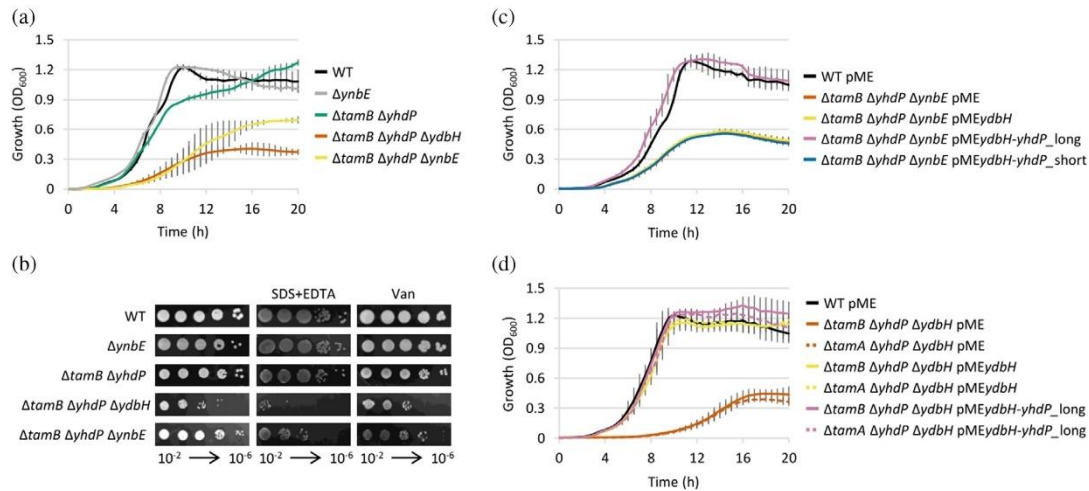


FIGURE 3 (a) Growth curves of the wild-type strain *Pseudomonas aeruginosa* PAO1 (WT) and the isogenic mutants $\Delta ynbE$, $\Delta tamB \Delta yhdP$, $\Delta tamB \Delta yhdP \Delta ydbH$, and $\Delta tamB \Delta yhdP \Delta ynbE$, cultured in MH. (b) Plating efficiency of the same strains described in panel A on MH agar plates supplemented or not with 0.25% SDS and 0.25 mM EDTA (SDS + EDTA) or 10 μ g/mL vancomycin (Van). (c) Growth curves of the WT strain and the triple mutant $\Delta tamB \Delta yhdP \Delta ynbE$ carrying the empty plasmid pME6032 (pME) or its derivatives for IPTG-inducible expression of YdbH, YdbH-YhdP_{long}, or YdbH-YhdP_{short}, cultured in MH supplemented with 16 μ M IPTG. Growth curves of the same strains at different IPTG concentrations are shown in Figure S5. (d) Growth curves of the WT strain and the triple mutants $\Delta tamB \Delta yhdP \Delta ydbH$ and $\Delta tamB \Delta yhdP \Delta ydbH$ carrying the empty plasmid pME or its derivatives for IPTG-inducible expression of YdbH or YdbH-YhdP_{long}, cultured in MH supplemented with 16 μ M IPTG. Growth curves of the same strains at different IPTG concentrations are shown in Figure S6. Data in panels a, c, and d are the mean ($\pm$ standard deviation) of three independent experiments. Images in panel B are representative of three independent experiments.

proteins (Sposato et al., 2024). However, this evidence does not rule out a possible contribution of TamA in YdbH function, as YdbH inactivation per se has negligible effects on growth and OM integrity (Sposato et al., 2024). To conclusively determine whether TamA contributes to GPL insertion mediated by YdbH, here we ectopically expressed YdbH, and the fusion protein YdbH-YhdP_{long} as control, in the triple mutant $\Delta tamA \Delta yhdP \Delta ydbH$, which lacks TamA and displays severely compromised growth due to the simultaneous inactivation of TamB and absence of YhdP and YdbH (Figure 3d) (Sposato et al., 2024). Notably, YdbH expression fully restored the growth of the mutant even in the absence of TamA, and the same result was obtained with YdbH-YhdP_{long} (Figure 3d). Such results rule out that TamA is required for YdbH function and, thus, that it may represent an additional component of the YhdP-YnbE complex.

2.4 | YhdP and YdbH fused to the YhdP C-terminal region function in a heterologous host

Our data demonstrated that fusing YdbH to the C-terminal region of YhdP bypasses the requirement for YdbH protein partner(s) (Figure 3) and enhances

the protein ability to promote growth and support OM biogenesis compared to the YdbH native protein (Figure 2). Based on molecular dynamics simulations, Cooper et al. proposed that the C-terminal hydrophobic helices of *E. coli* YhdP insert into the periplasmic leaflet of the OM (Cooper et al., 2025). However, it remains unclear whether these helices could promote GPL insertion into the membrane, possibly through perturbation of the GPL leaflet continuity, or if this process requires a yet-unidentified YhdP OM partner.

We reasoned that initial insights to address this issue could be gained by examining protein behavior in a heterologous host. Indeed, the YhdP orthologues from *E. coli* and *P. aeruginosa* share very low amino acid identity (Sposato et al., 2024), with only 26.7% identity in the final 90 residues including the hydrophobic C-terminal region. This is suggestive of poor conservation of putative interacting residues and, consequently, of only weak or inefficient interaction between the *P. aeruginosa* protein and potential non-native partners in *E. coli* (Lewis et al., 2012; Mika & Rost, 2006). We therefore ectopically expressed all *P. aeruginosa* protein variants investigated in this study in the *E. coli* mutant $\Delta tamB \Delta yhdP$, which is characterized by impaired growth and hypersensitivity to vancomycin (Figure 4, Figure S7), indicative of OM integrity defects (Ruiz et al., 2021).

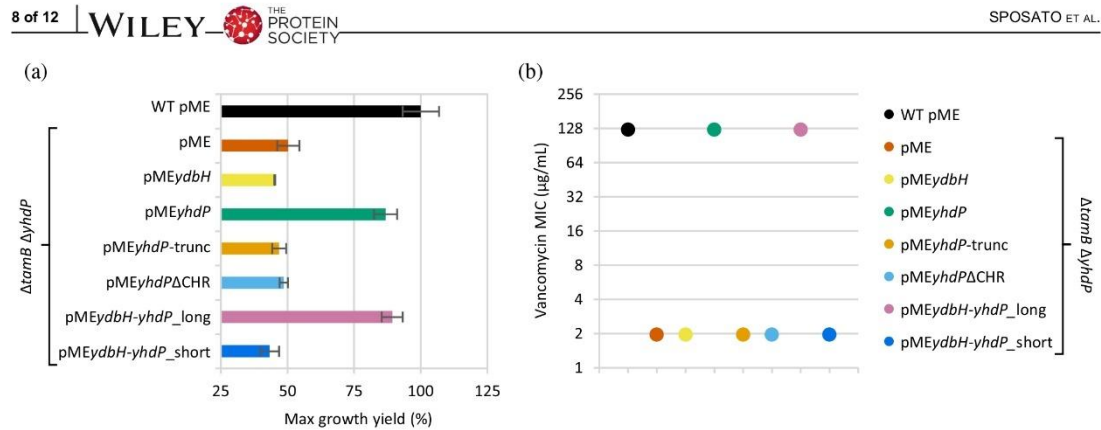


FIGURE 4 (a) Maximum growth yields and (b) vancomycin MIC for the *Escherichia coli* wild-type strain MG1655 (WT) and the isogenic mutant $\Delta tamB \Delta yhdP$ carrying the empty plasmid pME6032 (pME) or its derivatives for IPTG-inducible expression of the *Pseudomonas aeruginosa* proteins YdbH, YhdP, or their variants YhdP-trunc, YhdP Δ CHR, YdbH-YhdP_{long}, or YdbH-YhdP_{short}, cultured in MH supplemented with an optimal concentration of IPTG (depending on the strain, see Figure S7 for details). Values in panel a are expressed as percentages with respect to the maximum growth yields of WT pME and correspond to the mean ($\pm$ standard deviation) of three independent experiments. Growth curves of the same strains at different IPTG concentrations are shown in Figure S7. Values in panel b represent the mode of at least three independent experiments.

Interestingly, both *P. aeruginosa* YhdP and the fusion protein YdbH-YhdP_{long} restored growth and vancomycin resistance to (almost) wild-type levels, while all other proteins did not display any rescue effect (Figure 4, Figure S7). The inability of *P. aeruginosa* YdbH to complement the *E. coli* mutant can be attributed either to inefficient interaction with the *E. coli* YdbH partner(s), as YdbH orthologues also share very low homology (24.4% identity over only one sixth of the proteins) (Sposato et al., 2024), or to the fact that $\Delta tamB \Delta yhdP$ cells express the endogenous YdbH protein, which might saturate YnbE and thereby render the *P. aeruginosa* orthologue inactive. Either way, these results further confirm that the C-terminal region of YhdP confers new functions to YdbH, including the ability to work in the heterologous host *E. coli*. While this interspecies complementation assay supports the hypothesis that the YhdP C-terminal region may have intrinsic properties that allow OM interaction and GPL insertion, this remains to be confirmed through biochemical and biophysical analyses.

3 | CONCLUSIONS

In this study we demonstrated that the C-terminal hydrophobic α -helices of *P. aeruginosa* YhdP are crucial for its function in maintaining OM integrity, likely by enabling membrane interaction and/or facilitating GPL insertion. This aligns with what was recently reported for the *E. coli* orthologue (Cooper et al., 2025), suggesting that this structural and functional feature of YhdP may be conserved across different species. In addition, we found that also in *P. aeruginosa* the function of

YdbH relies on the OM lipoprotein YnbE, recently identified as a YdbH protein partner in *E. coli* (Kumar et al., 2024). Most importantly, not only does the deletion of the C-terminal hydrophobic region impair YhdP functionality, but its fusion to YdbH also enhances YdbH activity and makes it independent of its OM partner YnbE, provided that the fusion protein is long enough to span the entire periplasmic space. Furthermore, both *P. aeruginosa* YhdP and the fusion protein YdbH-YhdP_{long} proved to be active in the heterologous host *E. coli*, reinforcing the notion that the C-terminal hydrophobic region of YhdP may possess intrinsic, transferable properties that support the essential function played by AsmA-like proteins in Gram-negative bacteria. Overall, these results are consistent with a model in which the hydrophobic C-terminus of YhdP facilitates interaction with the OM, possibly enabling GPL delivery in the absence of additional protein partners. The presence of this hydrophobic C-terminal region, along with the protein length compatible with a transmembrane periplasmic bridge (Cooper et al., 2025; Kumar & Ruiz, 2023), could therefore explain the more prominent role of YhdP in maintaining OM homeostasis compared to other AsmA-like proteins (Douglass et al., 2022; Ruiz et al., 2021; Sposato et al., 2024). However, structural and biophysical studies will be needed to confirm this hypothesis and dissect the molecular basis of GPL insertion into the OM mediated by YhdP and other AsmA-like proteins.

Our findings also offer insights into the modularity and mechanistic versatility of AsmA-like proteins, as evidenced by the finding that the expression of the YdbH-YhdP_{long} fusion protein essentially phenocopied the

wild-type protein YhdP, both in *P. aeruginosa* and in the heterologous host *E. coli*. Recently, Rai and colleagues reported genetic evidence suggesting that different AsmA-like proteins may have different GPL substrate transport preferences (Rai et al., 2024), presumably due to the N-terminal regions that interact with the IM. However, we observed that the expression of YhdP or YdbH-YhdP_{long} alone can restore wild-type levels of growth and OM integrity in a mutant deficient in all other essential AsmA-like proteins. While this result does not exclude the possibility of different substrate preferences, it implies that a single protein, either YhdP or YdbH fused to the C-terminal YhdP domain, is sufficient to supply the OM with all GPL species required for normal membrane functionality. This suggests that at least some AsmA-like proteins may be rather promiscuous, accommodating and transporting different GPL types. In vitro binding assays with purified proteins will be crucial not only to validate this hypothesis but also to uncover the molecular determinants of substrate specificity in this fascinating class of bacterial transport proteins.

4 | MATERIALS AND METHODS

4.1 | Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Tables S2 and S3, respectively. Bacteria were routinely cultured in Lysogeny broth, Lennox formulation (LB) for genetic manipulation. Mueller Hinton broth (MH) was used for plate efficiency, growth, MIC, and permeability assays. When required, antibiotics were added at the following concentrations for *E. coli* (the concentrations used for *P. aeruginosa* are shown in brackets): ampicillin, 100 µg/mL; tetracycline, 10 µg/mL (25–100 µg/mL); nalidixic acid, 15 µg/mL; chloramphenicol, 30 µg/mL (375 µg/mL).

4.2 | Generation of plasmids and mutant strains

To obtain the constructs for the in-frame deletion of a 78-bp region in the *yhdP* coding sequence encoding the C-terminal hydrophobic residues (aa 1216–1241) of YhdP, two DNA fragments of approximately 500 bp each, encompassing the upstream (↑) and downstream (↓) regions of the *yhdP* CHR coding sequence, were PCR-amplified, directionally cloned into pBluescript II KS (pBS; Table S3), and verified by DNA sequencing. Then, the ↑↓ fragment was excised from pBS and sub-cloned into the *sacB*-based suicide vector pDM4 (Milton et al., 1996). The resulting construct pDM4yhdPCHR↑↓ was transferred into the *P. aeruginosa* mutant $\Delta tamB \Delta ydbH$ by conjugation, and transconjugants were

selected on LB agar plates containing 15 µg/mL nalidixic acid and 375 µg/mL chloramphenicol. The deletion mutant was obtained by homologous recombination and sucrose-based selection as previously described (Lo Sciuto et al., 2022), and verified by PCR and DNA sequencing.

The same strategy was employed to obtain deletion mutants in the *ynbE* (PA5306) gene, by generating the construct pDM4ynbE↑↓ (Table S3), introducing it into the *P. aeruginosa* strains PAO1 and $\Delta tamB \Delta ydbH$, and selecting the *ynbE* deletion mutants as described above.

To generate the plasmid pMEyhdP-trunc, the coding sequence of *yhdP* corresponding to residues 1–1219 was amplified by PCR, using the genomic DNA of the PAO1 as the template. To generate the plasmid pMEyhdP Δ CHR, the entire coding sequence of the *yhdP* Δ CHR allele was amplified by PCR, using the genomic DNA of the $\Delta tamB \Delta ydbH$ *yhdP* Δ CHR mutant as the template. The amplicons were individually cloned into the shuttle vector pME6032 (Heeb et al., 2002), under the control of an IPTG-inducible promoter, and verified by DNA sequencing.

For the construction of the plasmids for the expression of the YdbH-YhdP fusion proteins, the DNA region encoding the residues 1–808 of YdbH was amplified by PCR from the genomic DNA of PAO1 and cloned into pBS, yielding pBSydbH₁₋₈₀₈ (Table S3). Then, two DNA regions encoding either the residues 1161–1276 or the residues 933–1276 of YhdP were PCR-amplified from the genomic DNA of PAO1 and individually cloned into pBSydbH₁₋₈₀₈, resulting in pBSydbH-yhdP_{short} and pBSydbH *yhdP*_{long}, respectively (Table S3), which were verified by DNA sequencing. Finally, the DNA fragments *ydbH-yhdP*_{short} and pBSydbH *yhdP*_{long} were excised from the pBS derivatives and subcloned into pME6032 under the control of the IPTG-inducible promoter, yielding pMEydbH-yhdP_{short} and pMEydbH *yhdP*_{long}, respectively (Table S3).

All primers and restriction enzymes used for PCR and cloning are listed in Table S4.

4.3 | Plating efficiency and growth assays

For plating efficiency assays, bacterial strains were cultured in MH, supplemented with 0.01% rhamnose for the conditional mutants, harvested by centrifugation, and resuspended in sterile saline solution at OD₆₀₀ = 1. Serial 10-fold dilutions were prepared and 5 µL aliquots of selected dilutions were spotted onto MH agar plates supplemented or not with either 0.25% SDS and 0.25 mM EDTA or vancomycin at 100 or 10 µg/mL, as indicated.

For planktonic growth assays, bacterial strains were precultured in MH, supplemented with 0.01% rhamnose for the conditional mutants, and then refreshed 1:1000

in MH in the absence or presence of rhamnose or IPTG at the indicated concentrations. Bacterial cultures were incubated in 96-well microtiter plates (200 μ L in each well) at 37°C in a Tecan Spark 10 M microtiter plate reader, and growth was measured over time as the optical density at 600 nm (OD_{600}) of the bacterial cultures. Maximum growth yields were determined by calculating the ratio of the maximum OD_{600} values, expressed as a percentage relative to the control strain, as indicated in the figure legends.

4.4 | Membrane permeability assays

Bacterial strains were cultured in MH, and exponential phase cells were harvested by centrifugation and resuspended in 5 mM HEPES (pH 7.2) at $OD_{600} = 3$. Equal volumes (150 μ L) of HEPES solution, containing or not 20 μ M NPN, and bacterial suspensions were mixed, and 100 μ L of each sample was aliquoted on a black flat bottom 96-well plate. OD_{600} and fluorescence (excitation at 350 nm and emission at 420 nm) were measured in a Tecan Spark 10 M microtiter plate reader after a 2-min incubation at room temperature, subtracted of the background values of samples without NPN, and normalized to the OD_{600} of the cell suspension (Cervoni et al., 2021).

4.5 | MIC assays

The MIC of vancomycin was determined through the broth microdilution method. Strains carrying pME6032 or its derivatives were precultured in MH, supplemented with tetracycline at 10 or 25 μ g/mL (for *E. coli* and *P. aeruginosa*, respectively) and 0.01% rhamnose for the *P. aeruginosa* conditional mutants, and refreshed at ca. 5×10^5 cells/mL in MH in the presence of the indicated IPTG concentrations and increasing concentrations of vancomycin. MIC was recorded after 24 h at 37°C.

4.6 | Bioinformatic analyses and molecular dynamics simulations

TMHs were predicted using the online tool TMHMM 2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>) (Krogh et al., 2001). The predicted structures of YhdP (PA4476) and YdbH (PA5307) were downloaded from AlphaFold (Q9HVU5 YhdP 9828 atoms; Q9HTP7 YdbH 6579 atoms). Classical molecular dynamics simulations of 500 ns were performed to relax the systems; then the last frame was used as the initial structure for the following molecular dynamics simulations. The models were built with Maestro Schrodinger

(Schrödinger Release 2023–4: Maestro, Schrödinger, LLC, New York, NY, 2023), then translated into coarse grain using the martinize script (de Jong et al., 2013) and embedded in a membrane bilayer simulating the IM composed of 70% of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 30% of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC). The insane.py script (Wassenaar et al., 2015) was used to create a membrane environment around these structures. YhdP and YdbH-YhdP_long were modeled with the inner leaflet of the OM at the C-terminus and the outer leaflet of the IM at the N-terminus; all the other studied proteins were modeled only with the outer leaflet of the IM. All simulations were performed within a charge neutralized system with the addition of ions corresponding to a 0.15 M NaCl solution. The models featured around 3 hundred thousand atoms including water and ions. All molecular dynamics simulations were performed using GROMACS 2024.1 (Abraham et al., 2015). Martini v3.0 (Souza et al., 2021) was used as the force field with an elastic network. The system was minimized using a steep integrator with a maximum number of 500,000 steps to perform. An npt equilibration of 5,000,000 nsteps was performed after minimization, with constant temperature of 300 K using a velocity rescale thermostat; also, the pressure was held at 1 bar by using a Berendsen barostat with a relaxation time of 4 ps. Then all systems were subjected to simulations for 1 μ s each and replicated three times to ensure reproducibility. The simulations were converted into all-atomistic simulations using the cg2all script (Heo & Feig, 2024) to gain insights into the structural differences induced by the chimeric modifications and to obtain all atomistic structure for the frustrator analysis. MDAnalysis was used for the calculation of the radius of gyration and the fraction of native contacts (Michaud-Agrawal et al., 2011). Graphs were generated with Matplotlib (Hunter, 2007) and pictures were created with the PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC). The Frustrator package was used to calculate the frustration of the protein structures during the simulations (Rausch et al., 2021). This method of measuring frustration assumes that the residues are not only changed in identity but also displaced in position. A local frustration index is defined as the Z-score (0.78) of the free energy of parts of the native structure with respect to the distribution of the energy of rearranged decoys. If a native pair of interacting residues has an energy that lies in the most favorable end of the distribution (>0.78), the interaction is labeled as minimally frustrated; if the energy of the residues pair lies in the most unfavorable end of the distribution (<-1), the interaction is labeled as highly frustrated; if the energy of the residues pair lies in the range between 0.78 and -1 , it is labeled as neutral (Ferreiro et al., 2018).

4.7 | Statistical analysis

Statistical analysis was performed with the software GraphPad Instat, using the ANOVA with Tukey's multiple comparisons test.

AUTHOR CONTRIBUTIONS

Davide Sposato: Conceptualization; methodology; data curation; investigation; validation; visualization; writing – original draft; writing – review and editing. **Camilla Pederzoli:** Investigation; validation; writing – review and editing. **Marianna Bufano:** Methodology; investigation; validation; visualization; writing – original draft; data curation; writing – review and editing. **Pietro Sciò:** Investigation; validation; writing – review and editing. **Ludovica Rossi:** Investigation; validation; writing – review and editing. **Livia Leoni:** Funding acquisition; writing – review and editing; visualization. **Giordano Rampioni:** Writing – review and editing; data curation; visualization; funding acquisition. **Paolo Visca:** Visualization; writing – review and editing; project administration. **Antonio Coluccia:** Supervision; conceptualization; methodology; data curation; visualization; funding acquisition; writing – original draft; writing – review and editing; resources. **Francesco Imperi:** Writing – original draft; writing – review and editing; funding acquisition; supervision; resources; project administration; conceptualization; methodology; data curation.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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CHAPTER 5. The *Pseudomonas aeruginosa* DedA protein PA4029 is an undecaprenyl phosphate flippase important for polymyxin resistance

PREFACE

Peptidoglycan is an essential component of the bacterial cell envelope, providing structural support and protection against osmotic lysis. Its biosynthesis is a complex, multistep process, in which the lipid carrier Und-P (also named C55-P) plays a crucial role by transporting cell wall precursors across the cytoplasmic (inner) membrane. The C55-P pool is maintained by *de novo* synthesis or through a recycling pathway in which the C55-P(P), generated at the periplasmic leaflet of the cytoplasmic membrane after peptidoglycan monomer polymerization, is dephosphorylated and then flipped back to the cytoplasmic side of the membrane. However, the molecular players responsible for this process remained largely undefined until recent studies implicated DedA family proteins and DUF368-domain proteins as C55-P flippases in the Gram-negative bacterium *V. cholerae* and the Gram-positive bacteria *B. subtilis* and *S. aureus*.

In this study, we investigated the functional role of the six DedA proteins of *P. aeruginosa*. Through an integrated approach combining deletion mutagenesis, phenotypic assays, and biochemical analyses, we demonstrated that one of them, *i.e.* PA4029, is critical for C55-P recycling. Deletion of PA4029 sensitized cells to fosmidomycin, which inhibits C55-P *de novo* synthesis, and impaired the ability of *P. aeruginosa* to develop L-Ara4N-mediated colistin resistance, indicating defects in lipid carrier recycling and downstream cell envelope modification. Native mass spectrometry revealed that PA4029 binds C55-P with high specificity and affinity, and that this interaction can be disrupted by the C55-P-targeting antibiotic amphomycin. Genomic analyses showed that some *Pseudomonas* species lack a PA4029 ortholog but have a DUF368-family protein. When ectopically expressed in PA4029-deficient *P. aeruginosa* mutants, the DUF368 protein from *Pseudomonas stutzeri* can functionally compensate for the loss of PA4029, restoring both fosmidomycin resistance and the ability to acquire colistin resistance, suggesting divergent strategies for C55-P recycling across the *Pseudomonas* genus. Altogether, this work identified PA4029 as the main DedA protein involved in C55-P recycling in *P. aeruginosa* and revealed an evolutionary divergence within the *Pseudomonas* genus whereby a DUF368 protein can likely substitute for PA4029 as the main C55-P flippase in certain species.

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| Bacteriology | Research Article

The *Pseudomonas aeruginosa* DedA protein PA4029 is an undecaprenyl phosphate flippase important for polymyxin resistance

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ABSTRACT Undecaprenyl phosphate (C55-P) is a critical lipid carrier required for the transport of cell envelope precursors across the cytoplasmic membrane in bacteria. Recent studies have identified proteins of the DedA family and DUF368 domain family as C55-P flippases in both Gram-positive and Gram-negative organisms. However, their roles remain undefined in many clinically relevant pathogens. Here, we screened for DedA and DUF368 proteins in *Pseudomonas aeruginosa* and assessed their functional importance. We show that PA4029, a DedA family membrane protein, is involved in C55-P recycling. Deletion of PA4029 sensitizes cells to fosmidomycin and limits the emergence of spontaneous colistin-resistant mutants. Using native mass spectrometry, we demonstrate that PA4029 binds C55-P with high affinity and selectivity over membrane phospholipids, and that this interaction is disrupted by the C55-P targeting antibiotic amphotericin. We also show that a DUF368 protein, found in some *Pseudomonas* species lacking PA4029 orthologs, can functionally substitute for PA4029 in *P. aeruginosa*, suggesting divergent strategies for C55-P recycling in this genus. Together, these findings position PA4029 within the conserved DedA-mediated lipid carrier pathway and highlight its importance for cell envelope homeostasis and antibiotic resistance in *P. aeruginosa*.

IMPORTANCE Bacteria use lipid carrier undecaprenyl phosphate (C55-P) to build and maintain their cell envelope, which is necessary for survival and is the target of many antibiotics. Recent studies have implicated DedA family proteins in C55-P transport, but how these proteins function in important pathogens like *Pseudomonas aeruginosa* remains uncharacterized. In this work, we uncover a specific DedA protein, PA4029, and show its involvement in C55-P recycling and importance for bacteria's ability to develop resistance to the last-resort antibiotic colistin. These findings extend the relevance of DedA-mediated lipid transport to one of the most dreaded human pathogens.

KEYWORDS aminoarabinose, colistin, DedA, DUF368, native mass spectrometry, peptidoglycan, transporter, undecaprenyl phosphate

The bacterial cell wall is a defining feature of most prokaryotic organisms and plays a crucial role in maintaining cellular integrity, shape, and viability. The cell wall allows bacteria to withstand internal turgor pressure and provides protection against environmental stressors. The structural rigidity of the cell wall is largely attributed to peptidoglycan, an essential, mesh-like polymer composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid, crosslinked via short peptide stems (1, 2). The peptidoglycan is the target of many clinically relevant antibiotics, including β -lactams, glycopeptides, and fosfomycin (3).

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The biosynthesis of peptidoglycan is a complex process that involves (i) synthesis of precursors in the cytoplasm, (ii) membrane-associated steps where precursors are assembled onto a lipid carrier, and (iii) polymerization and crosslinking of precursors on the outer side of the cytoplasmic membrane (CM) (2). A critical component of the membrane-associated steps is undecaprenyl phosphate (C55-P), a long-chain polyisoprenoid lipid that serves as the carrier for peptidoglycan monomers (4). The disaccharide-pentapeptide precursor is assembled and linked to C55-P to form the so-called lipid II, which is flipped to the outer leaflet of the CM by the membrane protein MurJ (5) and finally polymerized into the peptidoglycan meshwork by transglycosylase and transpeptidase enzymes (6). Monomer release generates C55-PP, which is dephosphorylated to C55-P and flipped to the inner leaflet of the CM to complete the cycle (7). Besides peptidoglycan precursors, C55-P also mediates the transport of monomers for other glycopolymers, such as O-antigens, teichoic acids, and extracellular polysaccharides, as well as sugars used in the glycosylation of lipid A of lipopolysaccharides or surface proteins (8). These processes depend on specific transporters that flip C55-P-(oligo)saccharide intermediates toward the outer leaflet of the CM (9–11).

Given its central role in the biosynthesis of peptidoglycan and other cell envelope structures, C55-P(P) is an essential resource that must be efficiently recycled. Therefore, it is not surprising that some antibiotics exert their activity by sequestering intermediates of the lipid II cycle, such as lipid II (nisin), C55-PP (bacitracin), or C55-P (amphotycin) (12, 13). These are, however, only active against Gram-positive bacteria, as their size prevents diffusion across the outer membrane of Gram-negatives (12). Moreover, several enzymes involved in C55-P metabolism have been investigated as targets for novel antibacterial drugs, such as the C55-P synthase UppS (14), C55-PP phosphatase BacA/UppP (15), and lipid II flippase MurJ (16).

While the transporter of lipid II was identified more than a decade ago (5, 17), the retrograde flipping of C55-P across the CM remained elusive for years. Recently, two independent studies provided compelling evidence that proteins belonging to the DedA and DUF368 families are responsible for flipping back C55-P in representatives of both Gram-negative (*Vibrio cholerae*) and Gram-positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) (18, 19). DedA proteins are highly conserved and nearly ubiquitous across bacteria (20), while DUF368 proteins are present in less than 30% of bacterial species (18). Most bacteria have multiple DedA proteins (e.g., eight in *Escherichia coli* and six in *B. subtilis*), but only some of them exhibit C55-P flippase activity or are important for cell viability (19–22), implying that DedA proteins can also perform additional functions. For instance, some bacterial DedA proteins resemble eukaryotic phospholipid scramblases (20) and, accordingly, the DedA protein PetA of *B. subtilis* functions as a phosphatidylethanolamine (PE) transporter (23). Moreover, some DedA proteins have been implicated in resistance to cationic compounds and/or polymyxins in various bacteria (24–28), although the underlying molecular mechanisms remained unclear.

In this study, we investigated the DedA proteins of the Gram-negative human pathogen *Pseudomonas aeruginosa*, where their functions have not yet been explored. By integrating genetic, phenotypic, biochemical, and biophysical assays, we provide evidence supporting PA4029 as the main *P. aeruginosa* DedA protein involved in flipping back C55-P, as its inactivation results in defects consistent with impaired C55-P recycling, and the purified protein specifically binds C55-P. Notably, the lack of PA4029 compromises colistin resistance and limits the emergence of spontaneous colistin-resistant mutants. While PA4029 is conserved across most *Pseudomonas* species, certain pseudomonads instead have a DUF368 protein which can functionally replace PA4029 in *P. aeruginosa*, suggesting divergent evolutionary paths for C55-P flippases within the *Pseudomonas* genus.

RESULTS

P. aeruginosa has six DedA proteins

To perform a phylogenetic analysis of the bacterial DedA proteins, Todor et al. recently searched for DedA homologs in the proteomes derived from 6,000 bacterial genomes, by using the PF09335.14 motif characteristic of DedA family proteins as the query. This led to the identification of more than 16,000 bacterial DedA proteins, five of which were identified in *P. aeruginosa* (20). BLAST analysis revealed that these proteins correspond to the genes/proteins PA1209, PA4011, PA4029, PA5244, and PA5517 in the reference strain *P. aeruginosa* PAO1, all described as hypothetical proteins in the *Pseudomonas* Genome Database (<https://pseudomonas.com/>). Notably, one of these proteins (PA5517) is not annotated as a member of the PF09335 family (<https://pseudomonas.com/>) (Table 1).

To verify whether other DedA-like proteins are present in *P. aeruginosa*, we performed a BLAST analysis to search the proteome of *P. aeruginosa* PAO1 for additional homologs of (i) the eight DedA proteins of *E. coli* (22), (ii) the five DedA proteins identified in *P. aeruginosa* by Todor et al. (20), and (iii) DedA proteins recently demonstrated to function as lipid flippases in other bacteria (18, 19, 23). The results of this analysis, reported in Table S1, revealed that another member of the DedA family is present in *P. aeruginosa* PAO1 (PA2752) (Table 1). Orthologs of the six *P. aeruginosa* PAO1 *dedA*-like genes are well conserved in almost all *P. aeruginosa* strains, while other *Pseudomonas* species mainly harbor orthologs of PA2752, PA4011, PA4029, and PA5244, with PA1209 and PA5517 being present in only a few species (Table 1; Table S2).

The putative 3D structure and transmembrane helices (TMHs) of the *P. aeruginosa* DedA proteins are shown in Fig. S1. PA2752, PA4029, PA5244, and PA5517 only contain the DedA-like domain, whilst PA1209 and PA4011 are predicted to be bifunctional proteins with an N-terminal DedA-like domain and a C-terminal rhodanese-like (PF00581) or PAP2-like (PF01569) domain, respectively (Table 1 and Fig. S1). Bacterial rhodanases catalyze sulfur transfer reactions and are generally involved in cyanide detoxification and/or sulfur metabolism (29). The PAP2 family contains both soluble and integral membrane proteins, including nonspecific acid phosphatases, lipid phosphatases, and other uncharacterized enzymes. Some bacterial membrane PAP2 proteins have been reported to play a role in the metabolism of glycerophospholipids, C55-P, or lipopolysaccharides (8, 30).

TABLE 1 DedA-like proteins identified in *P. aeruginosa*

<i>P. aeruginosa</i> PAO1 DedA-like protein (aa) ^a	Pfam	<i>P. aeruginosa</i> genomes with ortholog ^b	<i>Pseudomonas</i> genomes (other than <i>P. aeruginosa</i>) with ortholog ^c
PA1209 (311)	PF09335 and PF00581	297/297 (100%)	10/236 (4.2%)
PA2752 (147)	PF09335	297/297 (100%)	209/236 (88.6%)
PA4011 (437)	PF09335 and PF01569	296/297 (99.7%) ^d	235/236 (99.6%)
PA4029 (221)	PF09335	297/297 (100%)	201/236 (85.2%)
PA5244 (196)	PF09335	297/297 (100%)	179/236 (75.8%)
PA5517 (194)	–	294/297 (99%) ^e	4/236 (1.7%)

^aAccording to the results of Todor et al. (20) and BLASTP analysis (see Table S1).
^bPutative orthologs were retrieved from 297 genomes according to the *Pseudomonas* Ortholog Groups of the *Pseudomonas* Genome Database (<https://pseudomonas.com/>) and individually checked.
^cPutative orthologs were retrieved from 236 genomes according to the *Pseudomonas* Ortholog Groups of the *Pseudomonas* Genome Database (<https://pseudomonas.com/>).
^dThe ortholog of the *P. aeruginosa* strain MRW44.1 (A542_RS0128425) is annotated as a pseudogene.
^eThe orthologs of the *P. aeruginosa* strains BWHPSA037 (Q042_RS13290) and AZPAE15065 (NT81_RS08910) are annotated as pseudogenes, while the ortholog appears to be absent from the strain PA7.

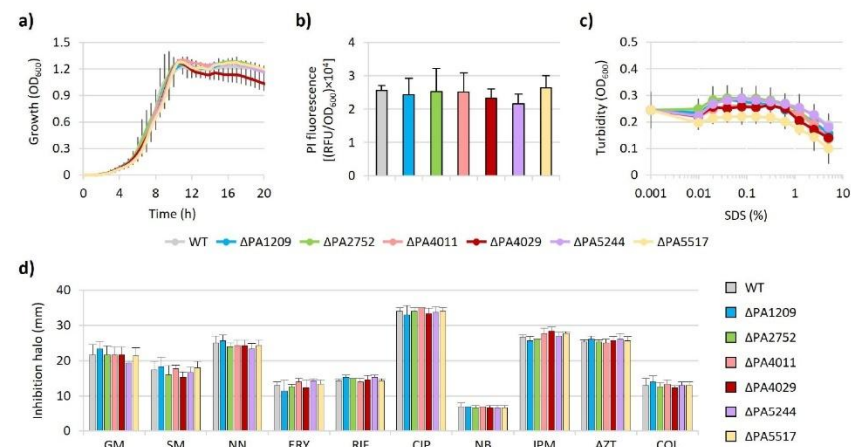


FIG 1 Effect of DedA proteins on *P. aeruginosa* growth and cell envelope integrity. (a) Growth curves of *P. aeruginosa* PAO1 (WT) and isogenic mutants in *dedA*-like genes cultured in MH. (b) Permeability to PI, measured as relative fluorescence units (RFU) normalized to the OD₆₀₀ of the bacterial suspension, of WT and *dedA* mutants. (c) Sensitivity to the lytic effect of different concentrations of SDS (0%–5%), measured as a decrease in cell suspension turbidity (OD₆₀₀), of WT and *dedA* mutants. (d) Antibiotic sensitivity profile of WT and *dedA* mutants determined through the Kirby-Bauer disk diffusion test on MH agar plates. GM, gentamycin; SM, streptomycin; NN, tobramycin; ERY, erythromycin; RIF, rifampicin; CIP, ciprofloxacin; NB, novobiocin; IPM, imipenem; AZT, aztreonam; COL, colistin. Values are the mean (±standard deviation) of three independent experiments. No statistically significant differences were observed between the WT and each deletion mutant ($P > 0.05$; one-way analysis of variance [ANOVA], Kruskal-Wallis test).

Individual DedA deletions do not compromise growth or cell envelope integrity

To investigate the role of DedA proteins in *P. aeruginosa*, we generated six deletion mutants in the reference strain PAO1, each lacking one *dedA* gene. These mutants were preliminarily tested for the ability to grow in a rich medium. As shown in Fig. 1a, the growth curves of the mutants were comparable to that of the parental strain, implying that individual DedA proteins are not required for *P. aeruginosa* cell viability and growth.

To assess whether the absence of DedA proteins might affect cell envelope functionality in *P. aeruginosa*, we first measured the uptake of propidium iodide (PI). This fluorescent probe is commonly used to assess membrane integrity, as it only enters cells with damaged membranes and its fluorescence increases upon binding to DNA (31, 32). None of the *dedA* mutants showed any increase in fluorescence with respect to the parental strain (Fig. 1b), ruling out relevant defects in the membrane permeability barrier. Then, we evaluated the sensitivity of the mutants to the lytic effect of the detergent SDS, as an indicator of cell envelope damage (33, 34). Also in this case, deletion of any individual *dedA* gene did not result in increased susceptibility to SDS (Fig. 1c). Finally, to further verify the functionality of the cell envelope as a permeability barrier, we also performed a Kirby-Bauer disk diffusion assay to assess the resistance profile of *dedA* mutants to 10 antibiotics belonging to different classes and having different mechanisms of action. Once again, no differences were observed between *dedA* mutants and the parental strain (Fig. 1d), indicating that the absence of each individual DedA protein does not promote antibiotic entry and, thus, confirming that the permeability barrier of the cell envelope is not compromised. Overall, these assays rule out that individual DedA proteins are essential for cell viability and cell envelope integrity in *P. aeruginosa*.

PA4029 is likely involved in undecaprenyl phosphate recycling

Some DedA proteins have been proposed to act as flippases in the recycling of C55-P in *V. cholerae*, *B. subtilis*, and *S. aureus* (18, 19). To verify whether any *P. aeruginosa* DedA protein might perform a similar function, we evaluated the sensitivity of *dedA* mutants to the antibiotic fosmidomycin, which inhibits the 1-deoxy-D-xylulose-5-phosphate reductoisomerase enzyme that produces isopentenyl diphosphate, a precursor of C55-P in the *de novo* synthesis pathway (8, 35). Previous studies in *E. coli* showed that mutants impaired in C55-P recycling are more susceptible to fosmidomycin (36), confirming that fosmidomycin sensitivity can be used as a proxy to assess defects in C55-P recycling in Gram-negative bacteria. As shown in Fig. 2a, the mutant lacking PA4029 displayed a fourfold decrease in fosmidomycin MIC with respect to the parental strain, while the deletion of the other *dedA* genes did not affect fosmidomycin susceptibility. Complementation of the Δ PA4029 mutant through ectopic expression of PA4029 restored fosmidomycin resistance to the levels of the parental strain carrying the empty plasmid (Fig. 2b), confirming that the increase in fosmidomycin sensitivity was specifically caused by the lack of PA4029. This result points to PA4029 as the main DedA protein involved in C55-P recycling in *P. aeruginosa*.

Since C55-P is also used as a carrier for flipping aminoarabinose (L-Ara4N) across the CM (9), whose addition to lipid A is responsible for colistin resistance in *P. aeruginosa* (37–39), we hypothesized that defects in C55-P recycling could compromise the ability to acquire colistin resistance. No differences were observed in colistin MIC between the six *dedA* mutants and the parental strain (1 μ g/mL for all strains). This was, however, expected, as the lipid A of colistin-sensitive *P. aeruginosa* isolates (including PAO1) is not modified with L-Ara4N (40) and, accordingly, mutants defective in L-Ara4N biosynthesis show the same colistin MIC as their colistin-sensitive parental strains (37). Thus, we performed a mutant selection assay on agar plates supplemented with colistin (10 $\times$ MIC) to assess whether the lack of DedA proteins can affect the emergence of colistin resistance, which in *P. aeruginosa* is generally associated with mutations, inducing lipid A aminoarabinylation (38, 39). Notably, the Δ PA4029 and Δ PA4011 mutants displayed a frequency of colistin-resistant spontaneous mutants significantly lower than the parental strain, comparable to the L-Ara4N-deficient mutant Δ arnBCA used as a control (Fig. 2c) (37). In contrast, no significant differences were observed between the other *dedA* mutants and PAO1 (Fig. 2c). Complementation assays confirmed that the ectopic expression of PA4029 or PA4011 in the corresponding mutants effectively restored their ability to evolve colistin resistance (Fig. 2c). To verify whether the reduced ability of the Δ PA4029 and Δ PA4011 mutants to develop colistin resistance was specifically due to impaired lipid A aminoarabinylation, we individually deleted the PA4011 or PA4029 gene in a recombinant strain (PAO1 *PrpsA::arn*) which has higher colistin resistance because of constitutive expression of the *arn* operon and the consequent lipid A modification with L-Ara4N (40). Remarkably, deletion of PA4029 in PAO1 *PrpsA::arn* resensitized this strain to colistin, restoring susceptibility comparable to the wild-type strain PAO1 (Fig. 2d), and reduced the aminoarabinylation of lipid A species to undetectable levels (Fig. S2). Conversely, PA4011 deletion had no effect on colistin resistance in the recombinant strain (Fig. 2d). Again, ectopic expression of PA4029 restored colistin resistance to the level of PAO1 *PrpsA::arn* carrying the empty vector (Fig. 2e). Although indirect, these results further support the hypothesis that PA4029 is involved in the flipping of C55-P during the recycling process.

In contrast, the function of PA4011 remains elusive. As discussed above, its absence impaired the acquisition of colistin resistance but did not affect the colistin resistance level conferred by lipid A aminoarabinylation. Notably, the deletion of PA4011 in the Δ PA4029 mutant did not exacerbate fosmidomycin sensitivity (Fig. 2a), and plasmid-mediated overexpression of PA4011 in PA4029-deficient cells failed to rescue the defects associated with PA4029 deficiency (Fig. 2b and e). Altogether, this body of evidence strongly suggests that the two proteins fulfill distinct roles in *P. aeruginosa*, implying that PA4011 is likely not involved in the flip-back of C55-P.

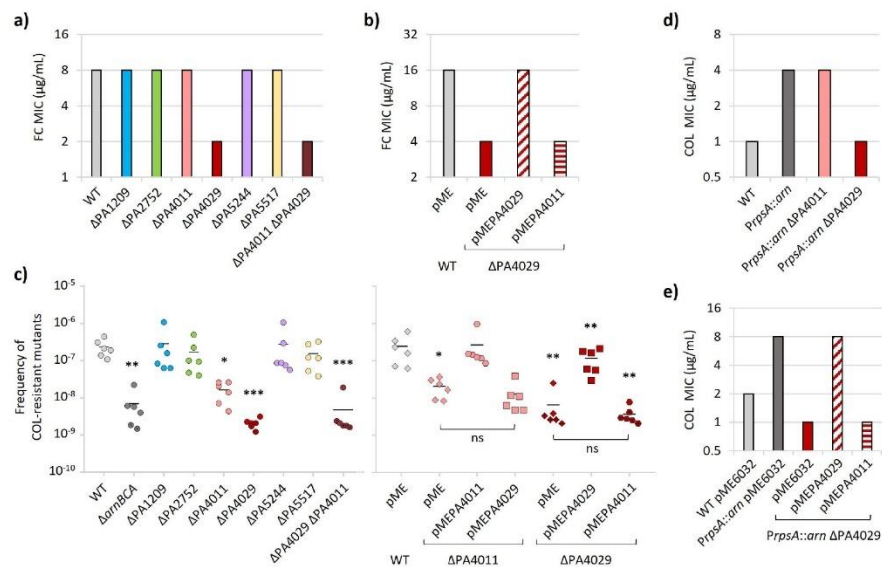


FIG 2 Role of PA4029 in C55-P recycling *in vivo*. (a) Fosmidomycin (FC) MIC for *P. aeruginosa* PAO1 (WT), the single mutants in *dedA*-like genes, and the double mutant ΔPA4011 ΔPA4029. (b) FC MIC for the WT and ΔPA4029 strains carrying the empty plasmid pME6032 (pME) or its derivatives for ectopic expression of PA4011 or PA4029. (c) Frequency of colistin-resistant spontaneous mutants for the WT strain and *dedA* mutants (left panel), and the mutants ΔPA4011 and ΔPA4029 ectopically expressing PA4011 or PA4029 (right panel). The ΔarnBCA mutant (left panel) and the strains carrying the empty plasmid pME (right panel) were used as controls. Each symbol corresponds to an individual experiment. Crossed symbols correspond to half the limit of detection of the individual experiment in which no colonies appeared on selective plates. (d) Colistin (COL) MIC for the WT, the recombinant strain *PrpsA::arn*, and its ΔPA4011 and ΔPA4029 isogenic deletion mutants. (e) Colistin MIC for the recombinant strain *PrpsA::arn* and its deletion mutants ΔPA4011 and ΔPA4029 carrying the empty plasmid pME or its derivatives for ectopic expression of PA4011 or PA4029. Values in panels a, b, c, and d are the mode of at least three independent experiments. Asterisks in panel c indicate a statistically significant difference with respect to WT or WT pME (**P* < 0.05, ***P* < 0.01, *** *P* < 0.001; ANOVA, Kruskal-Wallis test).

Purified PA4029 binds C55-P

To investigate whether PA4029 interacts directly with C55-P, we purified *P. aeruginosa* PA4029. The protein was then analyzed by native mass spectrometry (MS) in a buffer containing 200 mM ammonium acetate (pH 7.0) and 0.05% LDAO. The resulting spectrum revealed two main charge state distributions corresponding to monomeric (25,477.93 ± 0.1 Da) and dimeric (50,958.21 ± 0.23 Da) forms of PA4029 (Fig. S3). Notably, the observed monomeric mass was ~27 Da higher than the theoretical sequence mass (25,451.12 Da), a difference consistent with formylation of the N-terminal methionine, a modification also observed in other bacterial DedA proteins, such as UptA from *B. subtilis* (41).

To determine whether C55-P binds to PA4029, we conducted binding experiments by incubating 5 μM PA4029 with increasing concentrations of C55-P (0–20 μM) and recording native MS data. Mass spectra revealed peaks for PA4029 in both its apo form and C55-P-bound form (Fig. 3). We observed that C55-P binds both monomeric and dimeric PA4029 (Fig. S4), with a higher affinity for dimeric PA4029, consistent with previous findings on UptA (41). The apparently lower level of C55-P binding to the monomer is likely due, at least partly, to the loss of bound lipid during the collisional activation needed to dissociate the detergent micelles. Overall, the dimeric data align with the trends seen in the monomer. Next, we deconvoluted the spectra to determine the mean relative intensities of C55-P-bound and unbound forms of PA4029 at all concentration points. The data showed that the intensity of the PA4029:C55-P complex

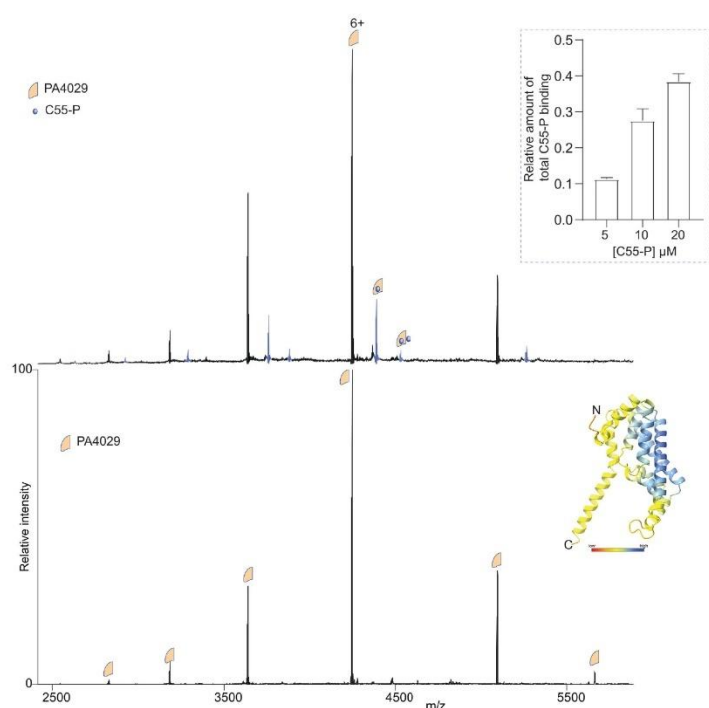


FIG 3 Binding of C55-P to PA4029. Native mass spectra of purified PA4029 (5 μ M) with (top spectrum) and without (bottom spectrum) C55-P (5 μ M). The data reveal adduct peaks corresponding to C55-P binding to PA4029 (blue series), with their intensity increasing as the C55-P concentration rises. Quantification of this data is displayed in the inset. Data are expressed as mean $\pm$ standard deviation (SD) of three independent replicates. Ribbon diagram is the model structure of PA4029 predicted by AlphaFold, colored by pLDDT (orange, 0–50; yellow, 50–70; cyan, 70–90; and blue, 90–100).

increases as a function of C55-P concentration (Fig. 3). These results suggest a concentration-dependent interaction, further confirming that PA4029 binds to C55-P.

Amphomycin disrupts the PA4029:C55-P complex

To further confirm whether the interaction observed above is specific, we have focused on lipopeptide antibiotics that target cell wall synthesis. A recent study on *B. subtilis* UptA established a methodology to assess the effects of lipopeptide antibiotics on C55-P binding to DedA proteins and found that C55-P targeting antibiotics, such as amphomycin, bind to UptA and induce dissociation of the UptA:C55-P complex (41). To test whether amphomycin has a similar effect on the PA4029:C55-P complex, we performed binding studies with amphomycin. As a control, we included vancomycin, a glycopeptide antibiotic that targets lipid II but not C55-P. We recorded spectra for solutions containing 5 μ M PA4029 and 10 μ M C55-P, then equilibrated with 10 μ M of each antibacterial compound. In the presence of amphomycin, the mass spectrum showed distinct peaks corresponding to amphomycin-PA4029 binding and a significant reduction in the peak intensity of the PA4029:C55-P complex (Fig. 4). In contrast, vancomycin had no appreciable effect on the C55-P complex. These results indicate that amphomycin destabilizes the ternary complex, most likely by sequestering C55-P or impairing binding through competitive binding effects or conformational effects.

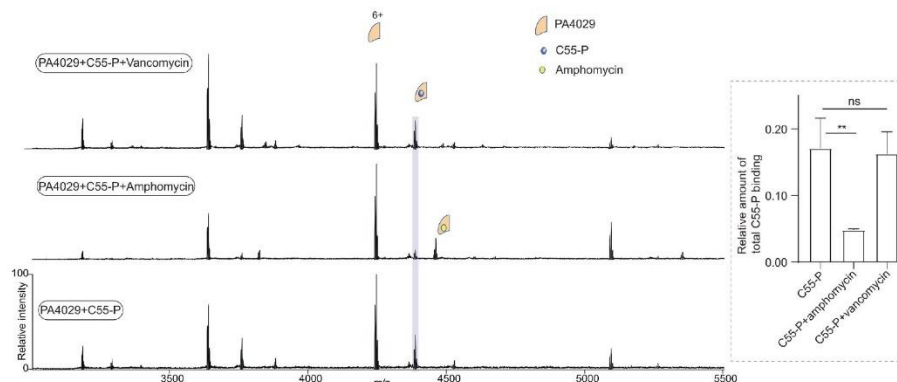


FIG 4 Amphomycin disrupts PA4029:C55-P interaction. Spectra of PA4029 with C55-P (bottom spectrum), C55-P and amphomycin (middle spectrum), and C55-P and vancomycin (top spectrum). The bar charts (inset) show the quantification of the amount of C55-P binding and suggest that the amount of PA4029:C55-P binding is significantly reduced in the presence of amphomycin. In contrast, the difference with vancomycin is not significant. Data are expressed as mean $\pm$ standard deviation (SD) of three independent replicates. Statistical significance was assessed using multiple *t*-tests, two-sided and unpaired (***P* = 0.0017, ns = not significant).

PA4029 preferentially binds C55-P over phospholipids

Recent studies have highlighted the role of some DedA family transporters in phospholipid translocation (23). To determine whether PA4029 recognizes phospholipids and to evaluate their potential role in influencing C55-P interaction, we examined the binding of PA4029 to phosphatidylglycerol (PG) and PE. We obtained mass spectra of solutions with 5 μ M PA4029 incubated with 10 μ M PE (16:0–18:1) and 10 μ M PG (16:0–18:1). In both cases, the spectra displayed peaks corresponding to PA4029 in both apo and lipid-bound forms (Fig. 5a and Fig. S5). We detected the formation of 1:1 and 1:2 protein-lipid complexes, with the mean relative intensity of the PA4029:PE complex exceeding that of the PA4029:PG complex (Fig. 5a), indicating that PA4029 interacts more favorably with PE than PG.

We also examined the relative affinity of C55-P binding compared to PE and PG (Fig. S5 and Fig. 5b). To achieve this, we recorded the spectra of 5 μ M PA4029 in the presence of 10 μ M C55-P, 10 μ M PE, and 10 μ M PG. We observed the formation of ternary complexes, indicating that the binding sites for C55-P and phospholipids are distinct. More importantly, we noted that the intensity of the PA4029:C55-P complex was significantly higher than that of the phospholipid-bound species (Fig. 5b). This supports the hypothesis that PA4029 binds more strongly to C55-P than to phospholipids, further suggesting its likely physiological role in recognizing and potentially recycling C55-P.

Distribution of putative *Pseudomonas* undecaprenyl phosphate flippases

Our *in vivo* results pointed out PA4029 as the primary DedA protein responsible for C55-P flip-back in *P. aeruginosa*, and *in vitro* data confirmed the capability of PA4029 to bind C55-P. However, while this protein is conserved in all *P. aeruginosa* strains analyzed in this study (Table 1), the search for orthologous genes in other *Pseudomonas* species revealed some species that lack PA4029 orthologs (Table 1; Table S2). In *V. cholerae*, *B. subtilis*, and *S. aureus*, proteins containing the DUF368 domain were also found to be involved in C55-P transport across the CM (18, 19). While DUF368 proteins were not identified in *P. aeruginosa* (Table S1; <https://pseudomonas.com/>), BLAST analysis revealed a homolog of the *V. cholerae* and *S. aureus* DUF368 proteins (VCA0040 and SAOUHSC_00846, respectively) in *Pseudomonas stutzeri* ATCC 17588 (Table S3), which was used as an example of a *Pseudomonas* strain lacking a PA4029 ortholog (Table S2). Interestingly, orthologs of the *P. stutzeri* ATCC 17588 DUF368 protein (PSTAB_1476) were

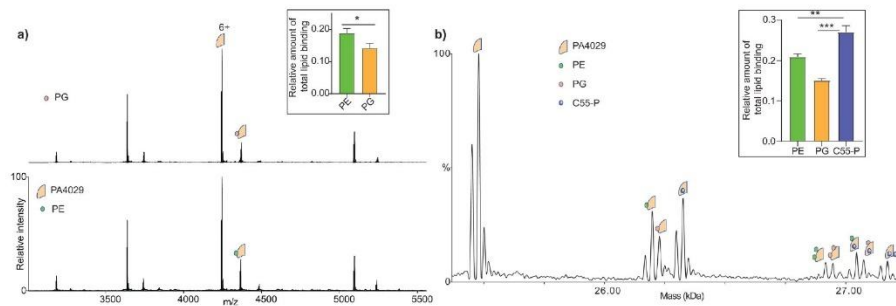


FIG 5 Phospholipid binding to PA4029 and its effect on C55-P binding. (a) Spectra of PA4029 in the presence of equal quantities of POPE and POPG. The relative intensity of lipid binding is higher in the case of PE than PG (insert). Data are expressed as mean $\pm$ standard deviation (SD) of three independent replicates. Statistical significance was assessed using multiple *t*-tests, two-sided and unpaired ($*P = 0.02$). (b) Deconvoluted spectrum of PA4029 with equal quantities of C55-P, POPE, and POPG. The bar charts (in inset) show the quantification of the amount of lipid binding and suggest that the amount of PA4029:C55-P binding is significantly higher than PA4029:POPE and PA4029:POPG. Additionally, ternary complexes are observed. Data are expressed as mean $\pm$ standard deviation (SD) of three independent replicates. Statistical significance was assessed using multiple *t*-tests, two-sided and unpaired ($**P = 0.0041$ and $***P = 0.0002$).

identified in 33 out of 35 of the *Pseudomonas* strains without PA4029 orthologs, but only in 3 out of 201 strains carrying PA4029 orthologs (Table S2 and Fig. 6a), indicating that PA4029 orthologs and DUF368 proteins are mutually exclusive in *Pseudomonas*. This pattern suggests that the two proteins may serve a similar function, making the presence of both in the same strain redundant or unnecessary. To verify whether the DUF368 protein identified in some *Pseudomonas* species is involved in C55-P recycling, we ectopically expressed the ortholog of *P. stutzeri* KC (ATCC 55595) in *P. aeruginosa* cells lacking PA4029 and verified whether it can rescue the defects associated with impaired C55-P recycling. Notably, expression of the DUF368 protein restored both fosmidomycin resistance in cells lacking PA4029 (Fig. 6b) and the ability to acquire colistin resistance through lipid A aminoarabinylation in the PAO1 *PrpsA::am* Δ PA4029 recombinant strain (Fig. 6c), at levels identical to those obtained upon ectopic expression of PA4029 (Fig. 2e).

DISCUSSION

Our study explores the role of DedA-family proteins in *P. aeruginosa*. Using bioinformatics, we screened for DedA family and DUF368 domain family members and characterized them functionally. We discovered that individual deletions of each DedA family member did not compromise cell growth and cell envelope integrity but that PA4029 is probably the major protein involved in C55-P recycling. Deleting PA4029 made cells more sensitive to fosmidomycin and made them less likely to acquire mutations conferring colistin resistance. These phenotypes are consistent with defects in C55-P recycling and suggest that PA4029 plays a role in this process. Notably, while this work was under revision, a manuscript was published reporting that PA4029 deletion causes accumulation of peptidoglycan precursors in the cytoplasm (42), consistent with the proposed role of PA4029 in C55-P recycling.

Native MS confirms that PA4029 binds C55-P directly, in a concentration-dependent manner. The complex is disrupted by C55-P targeting antibiotic amphotycin but not by vancomycin. This is consistent with recent biochemical work with *B. subtilis* UptA (41). We also tested whether PA4029 binds other phospholipids (PE or PG). Although the protein formed complexes with both, C55-P binding was clearly favored in mixed-lipid assays, supporting the idea that PA4029 is not a general phospholipid transporter, but instead has a distinct affinity for C55-P. Unlike some other DedA proteins that may act as lipid scramblases or contribute to membrane homeostasis, PA4029 appears specific for C55-P.

In *P. aeruginosa*, colistin resistance is usually linked to the addition of L-Ara4N to lipid A, a process that requires C55-P as a lipid carrier for precursor transport. When

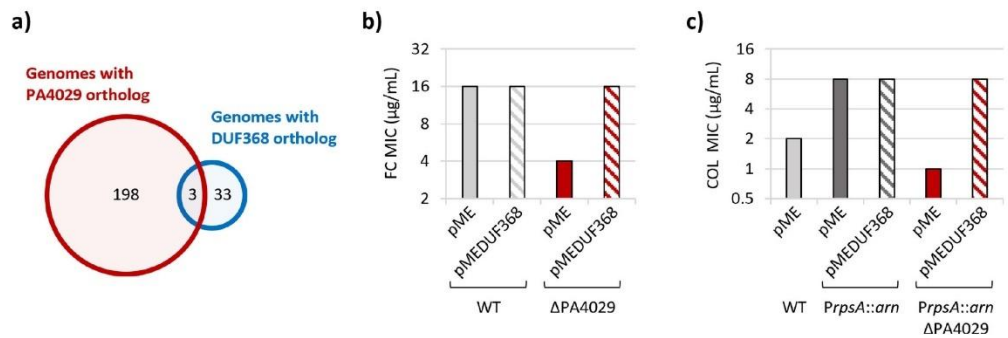


FIG 6 Distribution of PA4029 orthologs or DUF368 proteins in *Pseudomonas*. (a) Venn diagram showing the distribution of orthologs of PA4029 or DUF368 domain-containing protein across 236 genomes from different *Pseudomonas* species and strains. Note that two strains (*P. stutzeri* SLG510A3-8 and *Pseudomonas resinovorans* DSM 21078) apparently do not carry any PA4029 or DUF368 ortholog (Table S2). (b) Fosmidomycin (FC) MIC of *P. aeruginosa* PAO1 (WT) and the ΔPA4029 mutant carrying the empty plasmid pME6032 (pME) or its derivative for ectopic expression of the DUF368 domain-containing protein CXXK92_RS12370 of *P. stutzeri* KC (pMEDUF368). (c) Colistin (COL) MIC of the WT, the recombinant strain *PrpsA::arn*, and its ΔPA4029 mutant carrying the empty plasmid pME or its derivative pMEDUF368. Values in panels b and c are the mode of at least three independent experiments.

C55-P recycling is disrupted, the absence of PA4029 likely reduces precursor flux, and the supply of lipid-linked intermediates becomes limited, which likely prevents L-Ara4N lipid A modification in favor of essential processes requiring C55-P, such as cell wall biogenesis (Fig. 7). This would explain why ΔPA4029 mutants show impaired emergence of colistin-resistant mutants and why PA4029 loss reverses colistin resistance and inhibits lipid A aminoarabinylation under constitutive *arn* gene expression. Notably, defects in lipid A aminoarabinylation were observed in *Burkholderia thailandensis* and *Klebsiella pneumoniae* mutants lacking specific DedA family proteins (26, 28), providing support to this hypothesis.

PA4011, another DedA homolog harboring a PAP2-like domain, displays a partial phenotype, as its absence reduces colistin resistance emergence but does not affect colistin MIC under constitutive *arn* expression. While functional overlap is possible, the inability of ectopically expressed PA4011 to compensate for PA4029 absence, along with the fact that PA4011 deletion does not worsen the defects caused by PA4029 deficiency, strongly suggests PA4011 plays a distinct or accessory role, perhaps in phosphoregulation or membrane lipid remodeling (43).

Interestingly, most *Pseudomonas* species lacking PA4029 possess a DUF368-family protein, and ectopic expression of one such DUF368 homolog from *P. stutzeri* fully rescues defects in fosmidomycin and colistin resistance in PA4029-deficient *P. aeruginosa* strains. This mutual distribution and functional complementation support the proposal that DedA and DUF368 proteins provide alternative solutions for C55-P flipping in the *Pseudomonas* genus. Future work should focus on direct reconstitution of flipping activity *in vitro*, high-resolution structural studies of PA4029:C55-P binding, and probing how DUF368 proteins fulfill this role in other *Pseudomonas* species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media

Strains and plasmids used in this work are listed in Tables S4 and S5, respectively. Bacteria were routinely cultured in Lysogeny broth, Lennox formulation (LB) for genetic manipulation, while growth and antibiotic susceptibility assays were performed in Mueller-Hinton broth (MH) and/or cation-adjusted MH (CAMH), as indicated.

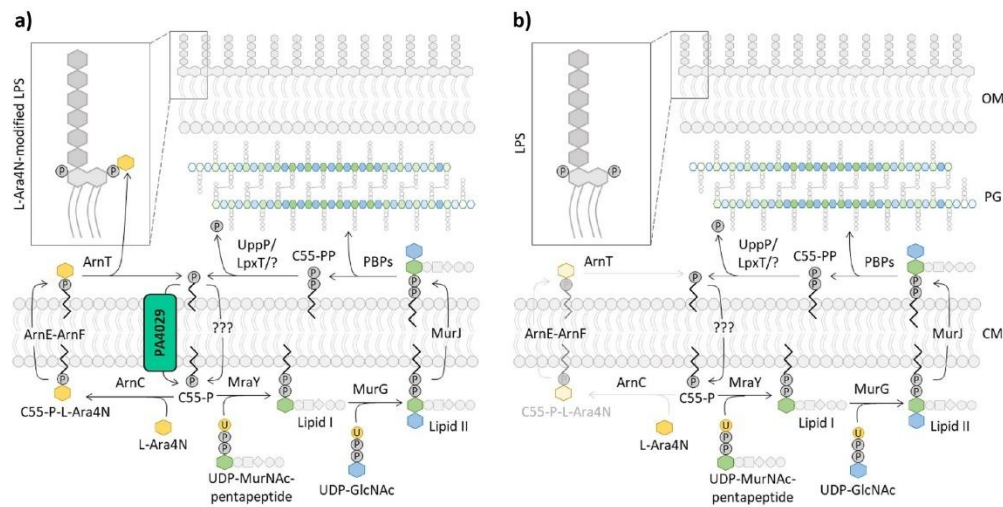


FIG 7 Schematic of PA4029 function. (a) PA4029 ensures proper recycling of C55-P for essential processes, such as cell wall biogenesis, and non-essential processes, such as lipid A aminoarabinylation, mediated by Arn proteins. (b) PA4029 deficiency disrupts C55-P recycling, likely reducing the pool of C55-P available on the cytoplasmic side of the CM, which is mainly directed toward peptidoglycan biosynthesis to support growth, at the expense of lipid A aminoarabinylation, thus impairing colistin resistance acquisition. GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; LPS, lipopolysaccharide; PG, peptidoglycan; PBPs, penicillin binding proteins; OM, outer membrane.

Generation of plasmids and mutants

To obtain the constructs for the generation of *P. aeruginosa* deletion mutants, two DNA fragments of approximately 500 bp encompassing the upstream and downstream regions of each gene of interest were amplified by PCR, directionally cloned into pBluescript II (pBS), and verified by DNA sequencing. Primers and restriction enzymes used for PCR and cloning or DNA sequencing are listed in Table S6. Then, the DNA fragment encompassing the upstream and downstream regions of each gene was excised from pBS and sub-cloned into the *sacB*-based suicide vector pDM4 (44). The resulting pDM4 derivatives (Table S5) were transferred into *P. aeruginosa* by conjugation, and the transconjugants were selected on plates containing 15 µg/mL nalidixic acid and 375 µg/mL chloramphenicol. Deletion mutations were obtained by recombination and sucrose-based selection as previously described (45), identified by PCR with the primer pairs UP_FW and DOWN_RV of each gene (Table S6), and confirmed by DNA sequencing.

The complementing plasmids pMEPA4011 and pMEPA4029 (Table S4) were generated by individually cloning the PCR-amplified coding sequence of PA4011 or PA4029 into the shuttle vector pME6032, downstream of the IPTG-inducible promoter (46). The plasmid pMEDUF368_{ps} was generated by cloning the coding sequence of the gene CXK92_RS12370, encoding the DUF368 protein of *P. stutzeri* KC, into pME6032 downstream of the IPTG-inducible promoter. All inserts were verified by DNA sequencing. Primers and restriction enzymes used for PCR and cloning or DNA sequencing are listed in Table S6.

Growth assay

Strains were precultured in MH, containing 50 µg/mL tetracycline in the case of strains carrying pME6032 or its derivatives, until late exponential phase and then refreshed 1:1,000 in fresh MH medium, supplemented with 30 µM IPTG for strains carrying pME6032 or its derivatives. Cultures were aliquoted in flat 96-well microtiter plates (200

μL in each well) and growth was measured as the optical density at 600 nm (OD_{600}) of the bacterial cultures every 30 min in a Tecan Spark 10M microtiter plate reader.

Membrane permeability assay

Exponential phase cells cultured in MH were harvested by centrifugation and resuspended in 5 mM HEPES (pH 7.2) at $OD_{600} = 3$. Equal volumes (150 μL) of bacterial suspensions and HEPES solution containing or not PI (40 μg/mL) were mixed, and 100 μL of each sample was aliquoted on a black flat-bottom 96-well plate. OD_{600} and fluorescence were measured in a Tecan Spark 10M microtiter plate reader (excitation at 580 nm and emission at 620 nm) after 2 min at room temperature, subtracted from the background values of samples without PI, and normalized to the OD_{600} of the cell suspension (47).

Detergent sensitivity assay

Sensitivity to the lytic effect of SDS was assessed by determining the turbidity (OD_{600}) of bacterial cell suspensions in saline after 5-min incubation at room temperature in the presence of increasing concentrations of SDS (0%–5%, wt/vol) (33).

Antibiotic sensitivity assay

The resistance profile of bacterial strains to several antibiotics was assessed by the disc diffusion test. Strains were cultured in MH until late exponential phase, centrifuged, resuspended at a theoretical $OD_{600} = 0.08$ in sterile saline solution, and swabbed onto MH agar plates. Disks containing rifampicin (30 μg) (Liofilchem), ciprofloxacin (5 μg), colistin (10 μg), aztreonam (30 μg), streptomycin (10 μg), gentamycin (10 μg), imipenem (10 μg), novobiocin (30 μg), tobramycin (10 μg), and erythromycin (15 μg) (Becton Dickinson) were placed on the agar surface before incubation of plates. The diameters of the growth inhibition halos were measured after 24 h of incubation at 37°C.

MIC assays

The MIC of antibiotics was determined through the broth microdilution method. *P. aeruginosa* strains were precultured in MH for fosmidomycin MIC assay or CAMH for colistin MIC assay and then refreshed in the same medium at ca. 5×10^5 cells/mL in the presence of increasing concentrations of each antibiotic. MIC was defined as the lowest antibiotic concentration for which no visible growth was observed after 24 h of incubation at 37°C under static conditions.

Frequency of colistin-resistant mutants

To evaluate the frequency of emergence of colistin-resistant spontaneous mutants, strains were cultured in MH at 37°C until the late exponential phase, harvested by centrifugation, and resuspended in sterile saline solution at a theoretical $OD_{600} = 1$. Serial 10-fold dilutions in saline were prepared and spotted onto LB agar plates to measure the number of colony-forming units (CFUs). Mutants were selected by plating 200 μL aliquots of the $OD_{600} = 1$ suspensions onto MH agar plates supplemented with 10 μg/mL colistin (and 0.031 mM IPTG for strains carrying pME6032 or its derivatives) and incubated at 37°C for 72 h. Frequency of colistin-resistant spontaneous mutants was calculated as the ratio between the number of colonies that appeared on MH agar plates supplemented with colistin and the total number of CFUs plated.

Protein expression and purification

A codon-optimized version of *P. aeruginosa* PA4029 (Integrated DNA Technologies) was inserted into NdeI and NheI restriction sites (by In-Fusion HD cloning kit, Takara BioTM) of a modified pET15b vector that adds a TEV-GFP-6×His tag at the end of the protein (41) and verified by sequencing. The resulting plasmid was inserted into *E. coli* C43 (DE3) cells (Lucigen) by transformation. For large-scale protein expression, 10 mL of

overnight culture was transferred into 1 L of LB containing 100 µg/mL ampicillin, and cells were allowed to grow at 37°C until the OD₆₀₀ reached 0.6–0.8, after which the temperature was lowered to 18°C, and the expression was induced with 0.5 mM IPTG for 16 h. Cells were harvested by centrifugation, pellets were resuspended in resuspension buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol) and lysed immediately or N₂ flash-frozen and stored at –80°C until required.

Cells were thawed on ice and resuspended in a buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol) containing lysozyme and protease-inhibitor cocktail tablets and then lysed using sonication (total processing time = 10 min, pulse-on:pulse-off = 3:6, amplitude = 65 with a Mili-tip). Non-lysed cells and debris were pelleted by centrifugation at 20,000 × *g* for 20 min at 4°C, and the clarified lysate was retained. The membrane fraction (supernatant) was then pelleted by ultracentrifugation at 160,000 × *g* for 1 h at 4°C and resuspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20% glycerol. Membrane aliquots were solubilized immediately or flash-frozen and stored at –80°C. The membrane was thawed and made up to a volume of 25 mL with resuspension buffer and solubilized by incubation with 2% DDM for 1 h at 4°C. The solubilized fraction was centrifuged at 20,000 × *g* for 20 min at 4°C to remove non-solubilized aggregates. The supernatant containing the membrane proteins was loaded onto a 5-mL HisTrap nickel nitriloacetic acid (Ni-NTA) agarose resin column pre-equilibrated in a buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.03% DDM, 20 mM imidazole). Ni-NTA beads were taken out into a Falcon tube and placed on a rotor for 1 h at 4°C. After binding of the His-tagged protein, the mixture was loaded back to the column and washed with 100 mL buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.03% DDM, 20 mM imidazole), and then with 50 mL of another buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.03% DDM, 80 mM imidazole). PA4029-GFP was eluted from the column with elution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.03% DDM, 200 mM imidazole). To remove imidazole and to cleave the GFP fusion, the eluted protein was mixed with TEV proteases and dialyzed against dialysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.03% DDM) at 4°C for 20 h. The digested mixture was subsequently incubated with 3 mL of Ni-NTA agarose resin to remove His-tagged GFP and TEV protease. Tag-free PA4029 was collected as the flowthrough, concentrated using a Sartorius™ Vivaspin centrifugal concentrator with a nominal molecular weight cut-off of 50 kDa, and finally purified by size-exclusion chromatography (SEC) using a 24-mL Superdex 200 Increase column. The SEC buffer was 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.03% DDM. After SEC purification, PA4029 was concentrated to 200 µM, aliquoted (50 µM each), flash-frozen in N₂, and stored at –80°C until required. Concentration was measured using a Nanodrop UV spectrophotometer at 280 nm.

Native MS

Protein was buffer exchanged into 200 mM ammonium acetate (pH 7.0) and 0.05% LDAO using BioSpin-6 (BIORAD) columns. About 3 µL protein sample was introduced directly into the mass spectrometer, Q-Exactive UHMR (Thermo Fisher Scientific, Bremen, Germany), using a gold-coated borosilicate capillary (prepared in-house, originally from Harvard Apparatus). The instrument settings were as follows: capillary voltage 1.2 kV, S-lens RF 100%, quadrupole selection from 1,000 to 20,000 *m/z* range, collisional activation in the HCD cell 120 V, trapping gas pressure setting 7.5, temperature 200°C, and resolution of the instrument was set to 12,500. The noise level was set at 3 rather than the default value of 4.64. No in-source dissociation was applied. Data were exported for processing using the Qual browser of Xcalibur 4.2 (Thermo Scientific), and spectral deconvolution was performed using UniDec software packages. Relative binding affinities were obtained from deconvoluted spectra by dividing the intensity of ligand-bound protein peaks by the sum of the intensities of ligand-bound and ligand-free protein peaks.

Lipid A analysis by matrix-assisted laser desorption/ionization–time of flight

Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS spectra were acquired in resolution mode and negative ion polarity on a Waters SYNAPT XS mass spectrometer (Waters, Manchester, UK) equipped with an 8 kDa quadrupole and a MALDI source. Lipid A was extracted as previously described (48) from dried bacterial pellets obtained from early stationary phase bacterial cultures in MH, and then dissolved in chloroform/methanol (50:50, vol:vol), while the matrix solution was 2,4,6-trihydroxyacetophenone (Sigma Aldrich) dissolved in methanol/0.1% trifluoroacetic acid/acetonitrile (7:2:1, vol:vol:vol) at concentration of 75 mg/mL. A volume of 0.5 μ L of the sample and 0.5 μ L of the matrix solution were applied to the MALDI plate and allowed to air dry at room temperature. Spots were randomly but evenly sampled, and experiments were executed in triplicate.

Bioinformatic analyses

TMHs were predicted using DeepTMHMM (<https://dtu.biolib.com/DeepTMHMM>) (49). Predicted 3D structures were retrieved from the AlphaFold database (<https://alpha-fold.ebi.ac.uk/>) (50). Homologous proteins were identified using BLASTP searches in the *Pseudomonas* Genome Database (<https://pseudomonas.com/>) and the amino acid sequence of proteins of interest as the query. Orthologs within the *Pseudomonas* genus were identified using the Ortholog Group Members function of the *Pseudomonas* Genome Database (51).

Statistics

Statistical analysis was performed with the software GraphPad Instat, using the one-way analysis of variance (ANOVA), followed by the Kruskal-Wallis multiple comparisons test.

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DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available in the article and its supplementary material.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental Material (mBio02408-25-s0001.pdf). Tables S1 to S6 and Fig. S1 to S5.

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CHAPTER 6. The *Pseudomonas aeruginosa* DedA protein PA4011 functions as a C55-PP phosphatase via a PAP2-like domain

PREFACE

During its recycling process, C55-PP must be dephosphorylated before being flipped back to the cytoplasmic side of the cytoplasmic membrane. In the previous work on DedA proteins, we have identified PA4029 as the main flippase of C55-P in *P. aeruginosa*. However, loss of another DedA protein (*i.e.*, PA4011) also affected relevant phenotypes, such as growth in the presence of the cation-chelating agent EDTA and acquisition of colistin resistance, even if in a manner independent of lipid A aminoarabinylation (see chapter 5). Notably, besides the DedA domain, PA4011 also contains a PAP2-like domain which is found in the *E. coli* phosphatases YbjG, PgpB, and LpxT. These proteins, together with UppP, are involved in C55-PP dephosphorylation.

In this chapter, through the generation of double deletion mutants and a triple conditional mutant in PA4011 and the genes encoding the homologs of the *E. coli* phosphatases UppP and LpxT, we provided preliminary evidence suggesting that the DedA protein PA4011 contributes to C55-P(P) recycling by acting as a C55-PP phosphatase. Deletion of *uppP* in the PA4011 mutant increased sensitivity to the cation-chelating agent EDTA and to the antibiotic fosmidomycin, and caused growth arrest at 25°C, a temperature which strongly reduces *lpxT* gene expression. Accordingly, growth was completely inhibited upon LpxT depletion in Δ PA4011 Δ *uppP* cells. Moreover, by generating PA4011 variants with amino acid substitutions in putative catalytic residues and constructs individually expressing only the DedA or PAP2-like domain, we demonstrated that PA4011 activity mainly relies on its phosphatase domain. Overall, these findings highlighted a previously unrecognized role of PA4011 in the C55-P(P) recycling pathway of *P. aeruginosa*, likely mediated by the phosphatase activity of its PAP2-like domain.

The following text represents a draft of the manuscript describing our genetic and phenotypic findings, which will be complemented by the biochemical analyses carried out by our collaborator Prof. Jani Bolla (University of Oxford, UK).

The *Pseudomonas aeruginosa* DedA protein PA4011 functions as a C55-PP phosphatase via a PAP2-like domain

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INTRODUCTION

The bacterial cell wall is a hallmark of almost all prokaryotic organisms and is essential for preserving cell integrity, morphology, and survival. By counteracting internal turgor pressure, the cell wall enables bacteria to resist mechanical stress and persist in diverse environmental conditions. Its mechanical strength primarily derives from peptidoglycan, a three-dimensional polymer composed of repeating N-acetylglucosamine and N-acetylmuramic acid units that are interconnected by short peptide cross-links (1, 2). Due to its essential role in cell wall homeostasis, peptidoglycan biosynthesis represents a major target for numerous clinically important antibiotics, including β -lactams, glycopeptides, and fosfomycin (3). Peptidoglycan biosynthesis is a highly coordinated, multistep process that spans multiple cellular compartments. The initial stages occur in the cytoplasm, where a disaccharide-pentapeptide precursor (the glycan pentapeptide) is synthesized on the membrane-associated lipid carrier undecaprenyl phosphate (C55-P), forming the so-called Lipid II (2, 4). This intermediate is translocated to the outer leaflet of the cytoplasmic membrane by the flippase MurJ (5) and subsequently the precursor is inserted into the growing peptidoglycan network through the coordinated action of transglycosylase and transpeptidase enzymes, which polymerize the disaccharide moiety and cross-link the resulting glycan chains through peptide bonds, respectively (6). Notably, C55-P is also responsible for the translocation across the cytoplasmic membrane of monomers for other glycopolymers, such as teichoic acids, O-antigens, and exopolysaccharides, as well as sugars for glycosylation of lipopolysaccharides or surface proteins (7).

After monomer polymerization, the lipid carrier is released as undecaprenyl pyrophosphate (C55-PP), which needs to be subsequently dephosphorylated back to C55-P by dedicated phosphatases. In *Escherichia coli* this process is mediated by the undecaprenyl pyrophosphate phosphatase UppP and the PAP2-like domain-containing proteins PgpB and YbjG (7). *E. coli* has another PAP2 phosphatase,

named LpxT, that can dephosphorylate C55-PP (8), transferring the phosphate group to the lipid A moiety of lipopolysaccharide (9). However, this enzyme does not appear to contribute significantly to C55-P recycling, as the loss or depletion of the three phosphatases UppP, PgpB, and YbjG is lethal in *E. coli* (7, 8).

Once dephosphorylated, C55-P is flipped back to the inner leaflet of the cytoplasmic membrane to sustain successive rounds of cell wall biosynthesis (10). Recent studies demonstrated that this process is mediated by C55-P flippases belonging to the DedA protein family or having a DUF368 domain (11, 12). Notably, we recently showed that, among the six DedA proteins encoded by the *Pseudomonas aeruginosa* genome, only one (PA4029) plays a major role as C55-P flippase, while the function of the other five remains unknown (13). However, the loss of the DedA protein PA4011 was found to affect relevant phenotypes, such as acquisition of colistin resistance and growth in the presence of the cation-chelating agent EDTA. Notably, besides the DedA domain, PA4011 also contains a PAP2-like domain (13). In this study, we provide genetic evidence indicating that *P. aeruginosa* PA4011 participates in C55-P(P) recycling by acting as a C55-PP phosphatase thanks to its PAP2-like domain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Strains and plasmids used in this work are listed in Tables S1 and S2, respectively. Bacteria were routinely cultured in Lysogeny broth, Lennox formulation (LB) for genetic manipulation, while growth assays were performed in Mueller-Hinton broth (MH) supplemented with IPTG or rhamnose at the indicated concentration when appropriate. Antibiotics were used at the following concentrations for *E. coli* (the concentrations used for *P. aeruginosa* are shown in brackets): ampicillin, 100 µg/mL; tetracycline, 12.5 µg/mL (50-100 µg/mL); carbenicillin (500 µg/mL); nalidixic acid, 15 µg/mL; chloramphenicol, 30 µg/mL (375 µg/mL).

Generation of plasmids and mutants. Primers and restriction enzymes used for PCR, cloning, and DNA sequencing are listed in Table S3. To obtain the constructs for the generation of *P. aeruginosa* deletion mutants, two DNA fragments of approximately 500 bp, encompassing the upstream and downstream regions of each gene of interest, were amplified by PCR using the genomic DNA of the wild-type strain PAO1 as the template, directionally cloned into pBluescript II (pBS), and verified by DNA sequencing. Then, the DNA fragment encompassing the upstream and downstream regions of each gene was excised from pBS and sub-cloned into the *sacB*-based suicide plasmid pDM4 (14). The resulting pDM4 derivatives (Table S2) were transferred into *P. aeruginosa* by conjugation, and the transconjugants were selected on plates containing 15 µg/mL nalidixic acid and 375 µg/mL

chloramphenicol. Gene deletions were obtained by recombination and sucrose-based selection as previously described (15) and identified by PCR with the primer pairs UP_FW and DOWN_RV of each gene (Table S3). Each deletion was further confirmed by DNA sequencing.

To generate the *lpxT* conditional mutant, the coding sequence of *lpxT* was PCR amplified, directionally cloned into the mini-CTX1 derivative pJM253 (16) downstream of the *rhaSR* P_{rha} regulatory element, and verified by DNA sequencing. The resulting plasmid was introduced into *P. aeruginosa* by conjugation and transconjugants were selected on LB agar plates containing 15 µg/mL nalidixic acid and 100 µg/mL tetracycline. The plasmid backbone was removed using the *sacB*-based suicide vector pFLP2 (17) as previously described (15) and pFLP2 was cured by plating onto LB agar plates supplemented with 10% sucrose. Carbenicillin-sensitive clones were analyzed by colony PCR to verify the insertion of the *rhaSR* P_{rha}::*lpxT* construct into the chromosome. Then, in-frame deletion of the endogenous copy of *lpxT* was obtained by recombination and sucrose-based counterselection, as described above, using the pDM4Δ*lpxT* construct (Table S2) and culturing bacteria under permissive conditions (*i.e.*, in the presence of 0.04% rhamnose). The deletion was verified by PCR and DNA sequencing.

To generate the pMEPA4011_{DedA} and pMEPA4011_{PAP2} plasmids (Table S2), the sequences encoding the DedA-like domain or the PAP2-like domain of PA4011, corresponding to residues 1-255 and 226-437, respectively, were amplified by PCR from PAO1. Amplicons were then individually cloned into the shuttle vector pME6032 (18), under the control of an IPTG-inducible promoter, and verified by DNA sequencing.

Site-specific mutagenesis was performed using the Q5[®] Site-Directed Mutagenesis Kit (New England Biolabs). To this aim, the coding sequence of PA4011 was PCR amplified from PAO1, cloned into pBS, and verified by DNA sequencing. The resulting plasmid, named pBSPA4011, was PCR amplified using the primer pairs PA4011_H323A_FW/PA4011_H323A_RV and PA4011_H371A_FW/PA4011_H371A_RV (Table S3), to introduce each codon substitution of interest. The PCR products were then treated with the Kinase-Ligase-DpnI (KLD) enzyme mix and used to transform *E. coli* DH5αF' competent cells. The clones carrying the PA4011^{H323A} or PA4011^{H371A} alleles were identified by PCR using the primer pairs H323A_check_FW/PA4011_pME6032_RV or PA4011_pME6032_FW/H371A_check_RV (Table S3), respectively, and confirmed by DNA sequencing. The PA4011^{H323A} and PA4011^{H371A} alleles were then excised from pBS and subcloned into pME6032 downstream of the IPTG-inducible promoter, yielding pMEPA4011^{H323A} and pMEPA4011^{H371A} (Table S2).

Planktonic growth and plating efficiency and assays. For planktonic growth assays, bacteria were precultured in MH, supplemented with 0.04% rhamnose for the conditional mutant, until late

exponential phase and then refreshed 1:1,000 in fresh medium, supplemented or not with increasing concentrations of rhamnose or IPTG, when indicated, in the presence or not of 0.125 mM EDTA or 4 µg/mL fosmidomycin. Cultures were aliquoted in flat-bottom 96-well microtiter plates (200 µL per well in the presence of EDTA, 100 µL per well in the presence of fosmidomycin) and plates were incubated at 25°C or 37°C in a Tecan Spark 10M microtiter plate reader. Growth was measured as the optical density at 600 nm (OD₆₀₀) of the bacterial cultures. The area under the growth curve (AUC) was calculated using the GraphPad Prism software and the trapezoidal method.

For plating efficiency assays, bacterial strains were precultured in MH, supplemented with 0.04% rhamnose for the conditional mutant, harvested by centrifugation, and resuspended in sterile saline solution at OD₆₀₀ = 1. Serial ten-fold dilutions were prepared in saline solution and 5-µL aliquots of selected dilutions were spotted onto MH agar plates in the presence or not of 0.04% rhamnose. Plates were incubated at 37°C for 48 h.

Bioinformatic and statistical analyses. Homologues proteins were identified using BLASTP searches in the *Pseudomonas* Genome Database (<https://pseudomonas.com>) and the amino acid sequence of proteins of interest as the query. Alignments were generated with the software MEGA (<https://megasoftware.net/>).

Statistical analysis was performed with the GraphPad Prism software, using the One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test.

RESULTS AND DISCUSSION

PA4011 likely contributes to C55-P(P) recycling

In the attempt to confirm the possible function of DedA proteins as C55-P flippases in *P. aeruginosa*, we recently investigated the effect of the loss of each of the six DedA proteins of this bacterium. This analysis led to the identification of PA4029 as the main C55-P flippase, while the role of the other five proteins remained unclear (13). Notably, one of these proteins (PA4011) has an N-terminal DedA domain fused to a C-terminal PAP2-like domain, which is also present in phosphatases involved in C55-PP dephosphorylation (7, 8). While bifunctional DedA-PAP2 proteins account for approximately 2% of the DedA proteins identified in bacteria (11, 12, 19), none of them has yet been experimentally investigated.

We previously observed that PA4011 inactivation does not affect *P. aeruginosa* growth in rich media and does not increase susceptibility to fosmidomycin (13), an antibiotic which inhibits C55-P *de novo* synthesis (20) and is more effective against mutants impaired in C55-P(P) recycling (13, 21). This evidence implies that, even if PA4011 is involved in C55-PP dephosphorylation, this function is likely

carried out by redundant proteins, as is the case in *E. coli* (7, 8). Thus, we performed a BLAST analysis to search the proteome of *P. aeruginosa* PAO1 for homologs of the C55-PP phosphatases of *E. coli*, namely UppP, PgpB, and YbjG. The *P. aeruginosa* ortholog of *E. coli* LpxT was already described and confirmed to function as a lipid A kinase (22). This analysis revealed that *P. aeruginosa* has an ortholog of *E. coli* UppP (PA1959, hereafter renamed UppP) and that the PAP2-like domain of PA4011 is the only homolog of both PgpB and YbjG (Table 1). To rule out the presence of other potential C55-PP phosphatases, we also searched for homologs of *P. aeruginosa* UppP and the PA4011 PAP2-like domain; however, no further hits were identified (Table 1).

Table 1. Homologs of *E. coli* C55-PP phosphatases in *P. aeruginosa* PAO1.^a

Query	PAO1 homolog (PA number)	Annotation	Alignment length (aa)	Identity (%)	E-value
<i>E. coli</i> UppP (AAC76093.1)	PA1959	Bacitracin resistance protein	253	51.38	0
<i>E. coli</i> PgpB (AAC74360.1)	PA4011	Hypothetical protein	113	31.86	1.6×10^{-7}
<i>E. coli</i> YbjG (AAC73928.1)	PA4011	Hypothetical protein	133	33.08	5.3×10^{-6}
<i>E. coli</i> LpxT (AAC75235.2)	PA5194	Hypothetical protein (LpxT)	196	29.59	1.2×10^{-14}
PA1959 (PA UppP)	PA1959	Bacitracin resistance protein	277	100.00	0
PA4011_PAP2 domain (aa 226-437)	PA4011	Hypothetical protein	212	100.00	0

^a Identified by BLASTP analysis with an E-value cutoff of 1×10^{-4} (default value for BLASTP analysis in the *Pseudomonas* Genome Database).

With the aim of assessing whether PA4011 could contribute to C55-PP dephosphorylation, we deleted the *uppP* ortholog (PA1959) in the wild-type strain PAO1 and in the Δ PA4011 mutant and tested the resulting mutants for fosmidomycin sensitivity. While the single mutants Δ PA4011 and Δ *uppP* behaved similarly to the wild-type strain, the double mutant Δ PA4011 Δ *uppP* showed increased fosmidomycin sensitivity, being unable to grow in the presence of 4 μ g/mL fosmidomycin (Figure 1A), corresponding to 0.5×MIC for the wild-type strain and single mutants (Figure S1). Notably, the double mutant also exhibited increased sensitivity to the cation-chelating agent EDTA with respect to the single mutant Δ PA4011 (Figure 1B). In contrast, deletion of *uppP* alone did not affect this phenotype (Figures 1B and S1).

The increased sensitivity of Δ PA4011 Δ *uppP* cells to fosmidomycin, compared with wild-type or single mutant cells, suggests that PA4011 and UppP might contribute redundantly to C55

dephosphorylation in *P. aeruginosa*. On the other hand, the mechanism by which PA4011, alone or together with UppP, influences EDTA resistance remains unclear at this stage.

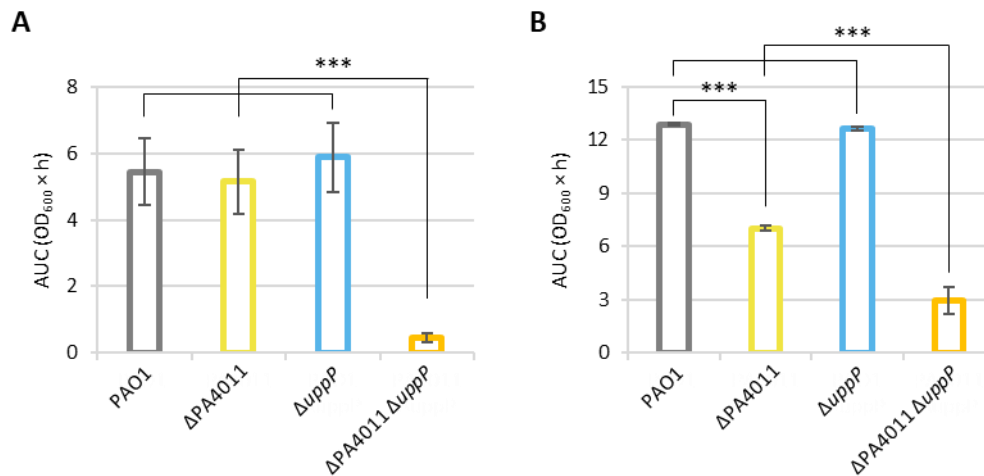


Figure 1. Growth yields, measured as area under the growth curves (AUC), of *P. aeruginosa* PAO1 and isogenic single or double deletion mutants in PA4011 and *uppP* genes cultured at 37°C in MH in the presence of (A) 4 µg/mL fosmidomycin and (B) 0.125 mM EDTA. Values are the mean (± standard deviation) of three independent experiments. Asterisks indicate a statistically significant difference (***) $P < 0.001$; ANOVA). The corresponding growth curves are shown in Figure S1.

The PAP2-like domain is responsible for PA4011 activity and can work independently of the DedA domain

To confirm the role of the PAP2-like domain of PA4011, we investigated whether the inactivation of its predicted catalytic activity can affect protein function. To this aim, we first aligned the amino acid sequence of the PA4011 PAP2-like domain to the amino acid sequence of *E. coli* PgpB, a PAP2 C55-PP phosphatase which has been largely characterized through biochemical, structural, and mutagenesis studies (23-25). Then, we decided to mutagenize two conserved residues, H371 (corresponding to H207 in *E. coli* PgpB), which has been demonstrated to be strictly essential for PgpB catalytic activity (23-25), and H323 (corresponding to H163 in *E. coli* PgpB), which has been proposed to be required for dephosphorylation of phosphatidylglycerol phosphate but not C55-P (25). Mutant alleles were generated by site-directed, alanine substitution mutagenesis and ectopically expressed in the double mutant ΔPA4011 ΔuppP, as well as in the wild-type strain as a control, from an IPTG-inducible promoter. Growth assays in the presence of 0.125 mM EDTA were used to assess protein functionality. Expression of PA4011^{H371A} did not promote the growth of the double mutant at any IPTG concentration, while expression of PA4011^{H323A} efficiently restored EDTA resistance to levels comparable to mutant cells expressing the wild-type protein PA4011 (Figure 2A). This result

confirms that the catalytic activity of the PAP2 domain is crucial for PA4011 function, and suggests that, in line with what was previously reported for *E. coli* PgpB, the H323 residue might be dispensable for the C55-PP phosphatase activity of PA4011. Notably, this assay also revealed that PA4011 overexpression induced by high IPTG concentrations has an inhibitory effect on the growth of the double mutant and, to a lesser extent, of the wild-type strain (Figure 2A). The inhibitory effect of PA4011 overexpression was also observed in the absence of EDTA (Figure 2B). Interestingly, the inactive mutant variant PA4011^{H371A} retained some toxicity in the double mutant, but not in the wild-type strain (Figure 2B).

We also attempted to investigate whether the DedA domain of PA4011 is required for its putative C55-PP phosphatase activity, by ectopically expressing the PA4011 PAP2-like domain alone (PA4011_{PAP2}) in the double mutant Δ PA4011 Δ uppP. Remarkably, expression of PA4011_{PAP2} completely restored the growth of the mutant in the presence of EDTA (Figure 2A), without showing any toxicity even at the highest IPTG concentrations tested, both in the double mutant and in the wild-type strain (Figure 2B). This result clearly shows that the PAP2-like domain of PA4011 does not require the DedA domain to complement the lack of PA4011 and UppP. As expected, expression of the DedA domain alone (PA4011_{DedA}) had no detectable effect under the conditions tested (Figure 2). Based on these results, one might speculate that PA4011 toxicity could be associated with the presence of its DedA domain fused to a functional PAP2-like domain. However, it is important to note that we did not verify whether PA4011_{DedA} is actually expressed and correctly folded in the cytoplasmic membrane; therefore, any hypothesis on its possible toxicity remains too speculative at present. In principle, the same holds true for PA4011_{PAP2} and the mutant variants PA4011^{H323A} and PA4011^{H371A}; however, in these cases, ectopic expression resulted in a detectable phenotype (*i.e.* restoration of EDTA resistance and/or toxicity), strongly suggesting that these proteins are expressed and possibly, at least in part, properly localized and folded.

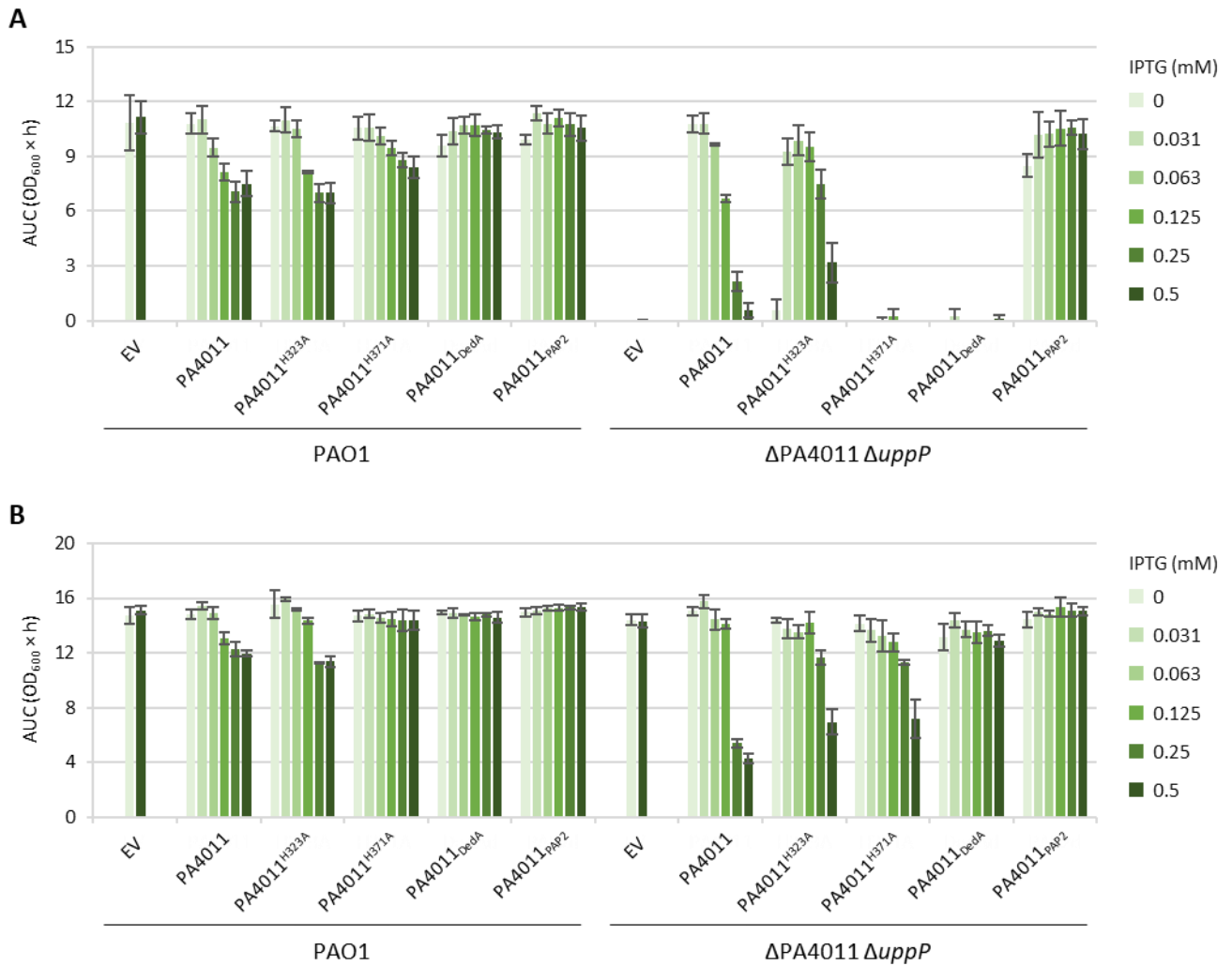


Figure 2. (A) Growth yields, measured as area under the growth curves (AUC), of *P. aeruginosa* PAO1 and the isogenic double mutant Δ PA4011 Δ uppP carrying the empty vector pME6032 (EV) or its derivatives for IPTG-inducible expression of the wild-type protein PA4011, its variants with the H323A or H371A amino acid substitutions (PA4011^{H323A} and PA4011^{H371A}, respectively), or the PA4011 DedA or PAP2-like domains alone (PA4011^{DedA} and PA4011^{PAP2}, respectively) cultured at 37°C in MH in the presence of 0.125 mM EDTA and increasing concentrations of IPTG. (B) AUC of the same strains described in panel A cultured at 37°C in MH in the presence of increasing concentrations of IPTG. Values are the mean ($\pm$ standard deviation) of three independent experiments. The corresponding growth curves are shown in Figure S2.

PA4011, UppP, and LpxT are essential but redundant in *P. aeruginosa*

Since the dephosphorylation of C55-PP is expected to be an essential process in bacteria (7), the capability of the double mutant Δ PA4011 Δ uppP to grow at levels comparable to the wild-type strain (Figures S1 and S2) implies that other C55-PP phosphatases are present in *P. aeruginosa*. Homology search showed that this bacterium has another PAP2 protein, PA5194, potentially involved in C55-PP dephosphorylation (Table 1). This protein was characterized in the *P. aeruginosa* strain PA14 and

confirmed to represent the ortholog of *E. coli* LpxT (22), which transfers a phosphate group from C55-PP to the 1-phosphate group of lipid A (9). Even if the C55-PP phosphatase activity of *E. coli* LpxT has been clearly demonstrated, LpxT expression and/or activity was reported to be insufficient to sustain C55-P(P) recycling in an *E. coli* mutant concomitantly lacking UppP, PgpP, and YbjG (7, 8).

To verify if *P. aeruginosa* LpxT is involved in C55-PP dephosphorylation, we attempted to delete the *lpxT* gene from the double mutant Δ PA4011 Δ uppP. However, all our attempts were unsuccessful, as the transconjugants always reverted to the parental genotype during the selection procedures. In contrast, we were able to delete the *lpxT* gene from the wild-type strain or from single mutants lacking PA4011 or *uppP* (Table S1). Interestingly, the *P. aeruginosa* *lpxT* (PA5194) gene was reported to be repressed at 25°C and induced at higher temperatures ($\geq 37^\circ\text{C}$), due to the presence of a molecular thermosensor (26). This gave us the opportunity to preliminary verify whether LpxT may be responsible for sustaining the growth of PA4011- and UppP-deficient cells, by assessing the growth of the double mutant Δ PA4011 Δ uppP at 25°C, when LpxT is expected to be expressed at low levels. Under these conditions, the growth of the double mutant Δ PA4011 Δ uppP was severely impaired, whereas the mutants lacking PA4011 or *uppP*, alone or in combination with the deletion of *lpxT*, all grew as the wild-type strain (Figure 3A). To confirm this preliminary observation, we generated a rhamnose-dependent *lpxT* conditional mutant in the Δ PA4011 Δ uppP background (Table S1). This mutant showed no or very poor growth unless rhamnose was added to the culture medium (Figures 3B-C), and its growth was promoted by rhamnose in a dose dependent manner (Figure S3). This result demonstrates that LpxT is responsible for the growth of *P. aeruginosa* cells lacking PA4011 and UppP, strongly suggesting that LpxT plays a relevant role in C55-P(P) recycling in *P. aeruginosa*.

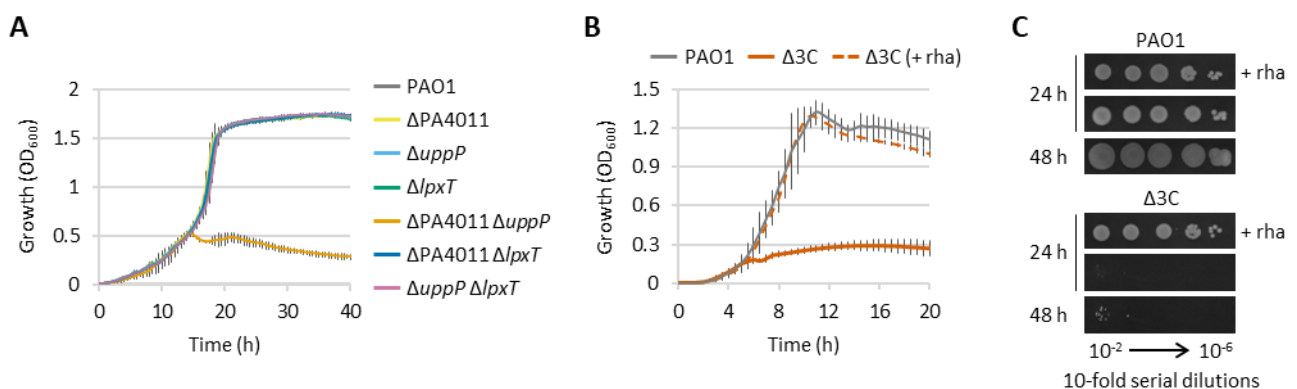


Figure 3. (A) Growth curves of *P. aeruginosa* PAO1 and isogenic single or double deletion mutants in PA4011, *uppP* and *lpxT* genes in different combinations, cultured at 25°C in MH. (B) Growth curves of the wild-type strain *P. aeruginosa* PAO1 and the rhamnose-dependent conditional mutant Δ PA4011 Δ uppP *rhaSR* *P_{rha}* *lpxT* Δ lpxT (Δ 3C) cultured at 37°C in MH supplemented or not with 0.04% rhamnose (+ rha). (C) Plating efficiency of the same strains described in panel B on MH agar

plates supplemented or not with 0.04 % rhamnose, after 24 or 48 h of incubation at 37°C. Values in panels A and B are the mean ($\pm$ standard deviation) of three independent experiments. Images in panel C are representative of three independent experiments.

Notably, it has been reported that the activity of the *P. aeruginosa* LpxT enzyme is inhibited under low-magnesium conditions (22). This evidence could justify the increased EDTA susceptibility of cells lacking PA4011 and/or UppP (Figures 1B and 2A). Indeed, by chelating divalent cations, EDTA could reduce the availability of magnesium required for LpxT activity, ultimately depleting cells of the residual C55-PP phosphatase activity mediated by LpxT. We are currently assessing the susceptibility to EDTA of the different double mutants shown in Figure 3 to verify this hypothesis. Finally, it is interesting to note that expression of the *E. coli* *lpxT* gene has also been shown to be subjected to thermoregulation (27). However, while *P. aeruginosa* LpxT expression is repressed at low temperatures (26), *E. coli* LpxT levels significantly decrease upon a temperature upshift from 28 to 37 or 42°C (27). Since early studies investigating the essentiality of the different C55-PP phosphatases in *E. coli* used a thermosensitive conditional triple mutant $\Delta uppP \Delta ybjG \Delta pgpB$ in which the expression of *uppP* was impaired at 42°C (8), it is plausible that the contribution of LpxT to C55-P(P) recycling in *E. coli* may have been underestimated due to its very low expression at the restrictive temperature (*i.e.*, 42°C).

CONCLUDING REMARKS

In this study, we used a genetic approach to investigate the function of the PA4011 protein in *P. aeruginosa*, a representative of a group of DedA proteins which are fused to a PAP2-like domain predicted to have phosphatase activity (19, 21, 22). Our results strongly suggest that the PAP2-like domain of PA4011 contributes to C55-PP dephosphorylation during the C55-P(P) recycling process, together with the orthologs of *E. coli* UppP (PA1959) and LpxT (PA5194) proteins. We are currently collaborating with Prof. Jani Bolla (University of Oxford, UK) to purify PA4011 and validate its C55-PP phosphatase activity. If biochemical evidence confirms this function, PA4011 would represent the first DedA-PAP2 protein to be experimentally characterized.

Intriguingly, the role of the DedA domain of PA4011 remains unclear. We observed that the PAP2-like domain exerts its function even without the DedA domain, suggesting that this domain is not only dispensable for enzymatic activity, but also for proper folding of the PAP2-like domain in the cytoplasmic membrane. However, while ectopic (over)expression of wild-type (full length) PA4011 is quite toxic, the PAP2-like domain alone is not. Even if this result should be interpreted with caution,

as we did not compare the expression levels, subcellular localization, and stability of the wild-type proteins and PAP2-like domain alone, this evidence suggests that the DedA domain of PA4011 is not simply an evolutionary relic, but may instead exert some biological effect in *P. aeruginosa* cells. Further studies are required to investigate this issue.

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The *Pseudomonas aeruginosa* DedA protein PA4011 functions as a C55-PP phosphatase via a PAP2-like domain

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SUPPLEMENTARY MATERIAL

Table S1. Bacterial strains used in this study.

Table S2. Plasmids used in this study.

Table S3. Primers used in this study.

Figure S1. Effect of PA4011 and UppP on fosmidomycin or EDTA resistance.

Figure S2. Effect of different PA4011 variants on EDTA resistance and growth.

Figure S3. Dose-dependent effect of rhamnose on growth of the conditional mutant Δ PA4011 Δ uppP *rhaSR* *P_{rha}* *lpxT* Δ *lpxT*.

Table S1. Strains used in this study.

Strain	Genotype and/or relevant characteristics	Source/reference
<i>E. coli</i>		
S17.1 λ pir	<i>thi pro hsdR hsdM⁺ recA</i> RP4-2-Tc::Mu-Km::Tn7 λ pir, Gm ^R	Simon <i>et al.</i> , 1983 ^a
DH5 α F'	<i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i> $\Delta(lacZYA-argF)$ U169[ϕ 80 <i>dlacZ</i> Δ M15], Nal ^R	Liss, 1987 ^a
<i>P. aeruginosa</i>		
PAO1 (ATCC15692)	Reference isolate, wild type	American Type Culture Collection
Δ PA4011	PAO1 derivative with an in-frame deletion of the PA4011 coding sequence	Sposato <i>et al.</i> , 2026
Δ uppP	PAO1 derivative with an in-frame deletion of the <i>uppP</i> (PA1959) coding sequence	This work
Δ lpxT	PAO1 derivative with an in-frame deletion of the <i>lpxT</i> (PA5194) coding sequence	This work
Δ PA4011 Δ uppP	Δ PA4011 derivative with an in-frame deletion of the <i>uppP</i> coding sequence	This work
Δ PA4011 Δ lpxT	Δ PA4011 derivative with an in-frame deletion of the <i>lpxT</i> coding sequence	This work
Δ uppP Δ lpxT	Δ uppP derivative with an in-frame deletion of the <i>lpxT</i> coding sequence	This work
Δ PA4011 Δ uppP <i>rhaSRP_{rha}::lpxT</i> Δ lpxT	Δ PA4011 Δ uppP with a rhamnose inducible copy of the <i>lpxT</i> coding sequence inserted into the chromosomal <i>attB</i> site and an in-frame deletion of the endogenous copy	This work

^a References not included in the main text:

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Liss, L. New M13 host: DH5 F' competent cells. *Focus*. 1987; 9-13.

Miroux B, Walker JE. Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol*. 1996; 260:289-98. doi: 10.1006/jmbi.1996.0399.

Table S2. Plasmids used in this study.

Plasmid	Relevant characteristics	Source or reference
pBluescript II	Cloning and sequencing vector; ColE1 replicon; Ap ^R	Stratagene
pDM4	Suicide vector used for deletion mutagenesis in <i>P. aeruginosa</i> ; <i>sacB</i> , <i>oriR6K</i> ; Cm ^R	Milton <i>et al.</i> , 1996
pJM253	mini-CTX1 derivative carrying <i>rhaRS</i> -PrhaBAD, TcR	Meisner and Goldberg, 2016
pFLP2	Plasmid for site specific FLP-mediated recombination; <i>sacB</i> , Ap ^R /Cb ^R	Hoang <i>et al.</i> , 1998
pME6032	IPTG-inducible expression vector; <i>lacI</i> ^Q , Tc ^R	Heeb <i>et al.</i> , 2002
pDM4Δ <i>lpxT</i>	pDM4 derivative for the in-frame deletion of the PA2752 coding sequence	This work
pJM253 <i>lpxT</i>	pJM253 derivative carrying the coding sequence of <i>lpxT</i> downstream of <i>rhaSRP_{rha}</i> regulatory element	This work
pBSPA4011	pBS derivative carrying the PA4011 coding sequence used for site-directed mutagenesis	This work
pMEPA4011	pME6032 derivative carrying the PA4011 coding sequence downstream of the IPTG-inducible promoter	Sposato <i>et al.</i> , 2026
pMEPA4011 _{DedA}	pME6032 derivative carrying the sequence encoding the DedA-like domain of PA4011, approximatively corresponding to residues 1-765, downstream of the IPTG-inducible promoter	This work
pMEPA4011 _{PAP2}	pME6032 derivative carrying the sequence encoding the PAP2-like domain of PA4011, approximatively corresponding to residues 676-1314, downstream of the IPTG-inducible promoter	This work
pMEPA4011 ^{H323A}	pME6032 derivative carrying the PA4011 ^{H323A} allele downstream of the IPTG-inducible promoter	This work
pMEPA4011 ^{H371A}	pME6032 derivative carrying the PA4011 ^{H371A} allele downstream of the IPTG-inducible promoter	This work

Table S3. Primers used in this study^a.

Primer	Sequence (5'-3')	Restriction site ^b	Application
<i>uppP</i> _mut_UP_FW <i>uppP</i> _mut_UP_RV <i>uppP</i> _mut_DOWN_FW <i>uppP</i> _mut_DOWN_RV	<u>cgc</u> <u>ctcga</u> GTCCACTGCCACCAGCCA <u>cggga</u> <u>TCC</u> ACCACTCCATGTTTCTC <u>cggga</u> <u>TCC</u> ACCTGATCGACTGGAG <u>gctct</u> <u>AGAGG</u> ATCAGCAGGACCAG	XhoI BamHI BamHI XbaI	Generation of pDM4Δ <i>uppP</i>
<i>lpxT</i> _mut_UP_FW <i>lpxT</i> _mut_UP_RV <i>lpxT</i> _mut_DOWN_FW <i>lpxT</i> _mut_DOWN_RV	<u>acgcgtcg</u> <u>ACGGT</u> GAGGGTCAGCACG <u>cgggat</u> <u>CCATT</u> GCCAGGCCGCTTG <u>cggga</u> <u>TCCG</u> CGACTCGGCCTGAG <u>gctct</u> <u>AGACG</u> AACCTAGCGGCTGT	Sall BamHI BamHI XbaI	Generation of pDM4Δ <i>lpxT</i>
<i>lpxT</i> _pJM253_FW <i>lpxT</i> _pJM253_RV	<u>ggactagt</u> GCGCCGATAGGGGGAAAC <u>cccaagctt</u> GGCCGAGCAAGAAGCAGC	HindIII EcoRI	Generation of pJM253 <i>lpxT</i>
PA4011_pME6032_FW PA4011_pME6032_RV	<u>cggaatt</u> CAATGAGTCTCGACAGCTTC <u>cccctc</u> GAGCGCCAACCCTCACAG	EcoRI XhoI	Generation of pBSPA4011
PA4011_DedA_pME_RV	<u>cccctcga</u> gtcaGAGCCGGGTGACCACCAC	XhoI	Generation of pMEPA4011 ^{DedA} (coupled with PA4011_pME6032_FW)
PA4011_PAP2_pME_FW	<u>cgggaattc</u> atgCTCCAGGACCTCGACCAG	EcoRI	Generation of pMEPA4011 ^{PAP2} (coupled with PA4011_pME6032_RV)
PA4011_H323A_FW ^c PA4011_H323A_RV ^c	gcgAGCTCGGCGTCTTCGCC TCCGCTGGGGAAGCTGAAG		Generation of pMEPA4011 ^{H323A}
PA4011_H371A_FW ^c PA4011_H371A_RV ^c	gcgTGGCCGTCGGACATCGTC CACGCCAAGGTAGACCCG		Generation of pMEPA4011 ^{H371A}
H323A_check_FW PA4011_pME6032_RV	AGCTTCCCCAGCGGAgcg <u>cccctc</u> GAGCGCCAACCCTCACAG		PCR screening of PA4011 ^{H323A} amino acid substitution
PA4011_RT_FW H371A_check_RV	GCGGTATCCTCCTGCTGAC ATGTCCGACGGCCAacgc		PCR screening of PA4011 ^{H371A} amino acid substitution
M13_FW M13_RV	GTTTTCCCAGTCACGAC AACAGCTATGACCATG		DNA sequencing from pBS
Prha_FW	TCCTGTCAGTAACGAGAAGG		DNA sequencing from pJM253
pME6032_FW pME6032_RV	GCTCTCGGGTAACATCAAG CGGTTCTGGCAAATATTCTG		DNA sequencing from pME6032

^a Unless otherwise stated, the genomic DNA of *P. aeruginosa* PAO1 was used as the template for preparative PCRs for cloning.

^b The restriction site used for cloning is underlined in the primer sequence.

^c The plasmid pBSPA4011 (Table S2) was used as the template for PCR.

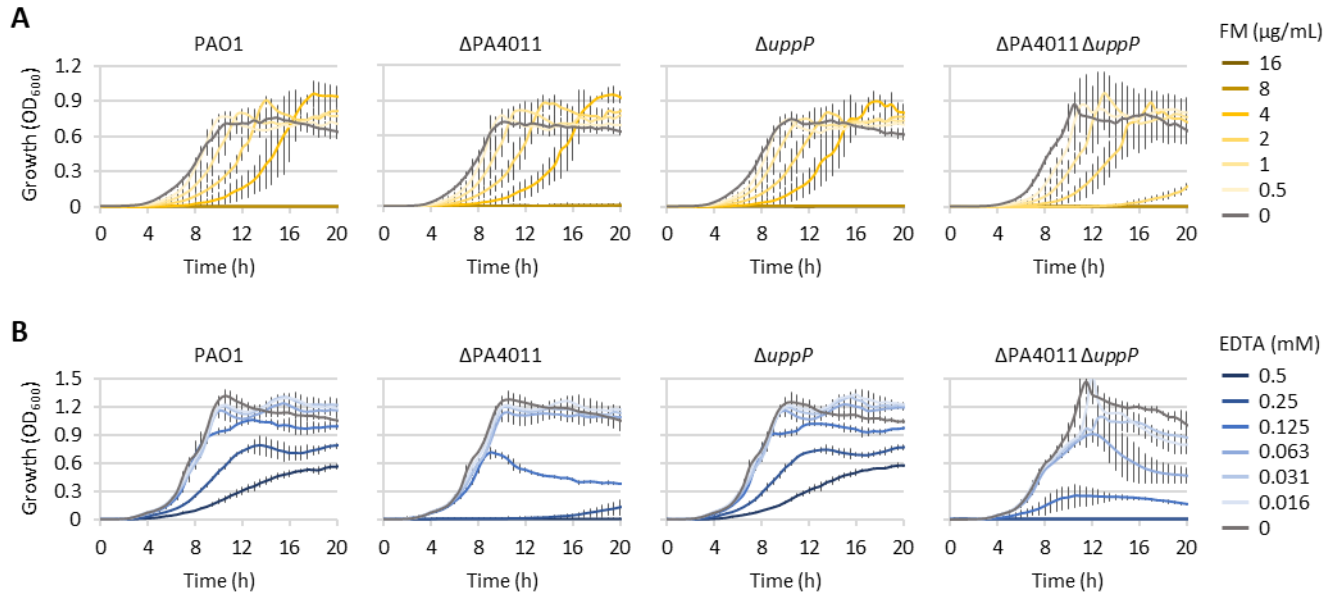


Figure S1. Growth curves of *P. aeruginosa* PAO1 and isogenic single or double deletion mutants in PA4011 and *uppP* genes cultured at 37°C in MH in the presence of increasing concentrations of (A) fosmidomycin (FM) or (B) EDTA. Strains were cultured in flat-bottom 96-well microtiter plates (200 μ L per well for panel A or 100 μ L per well for panel B). Values are the mean ($\pm$ standard deviation) of three independent experiments.

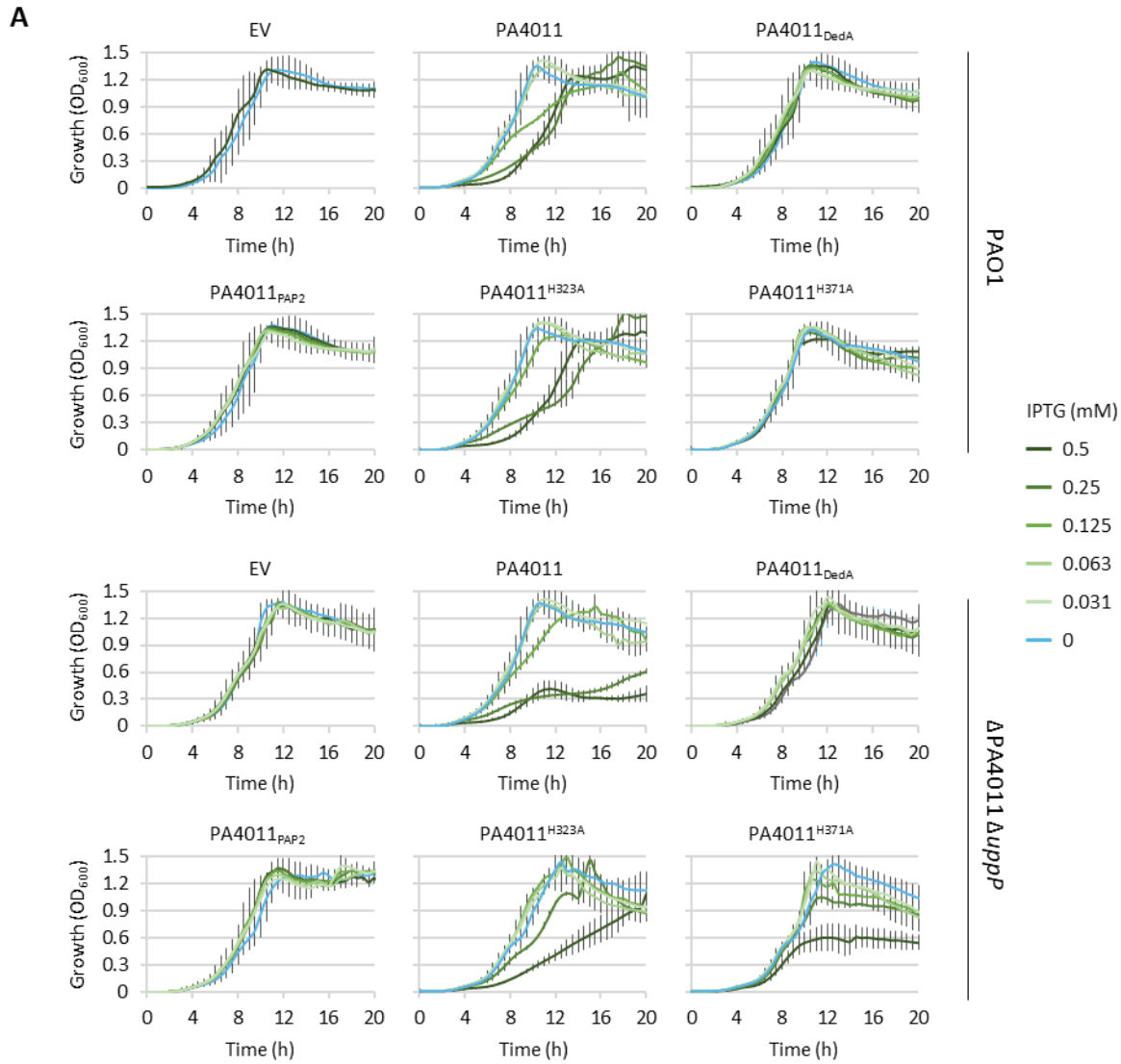


Figure S2. (A) Growth curves of *P. aeruginosa* PAO1 and the isogenic Δ PA4011 Δ uppP mutant carrying either the empty vector pME6032 (EV) or its derivatives expressing the wild-type protein PA4011, its variants with the H323A or H371A amino acid substitutions (PA4011^{H323A} and PA4011^{H371A}, respectively), or the PA4011 DedA or PAP2-like domains alone (PA4011^{DedA} and PA4011^{PAP2}, respectively). Strains were cultured at 37°C in MH in the presence of increasing concentrations of IPTG.

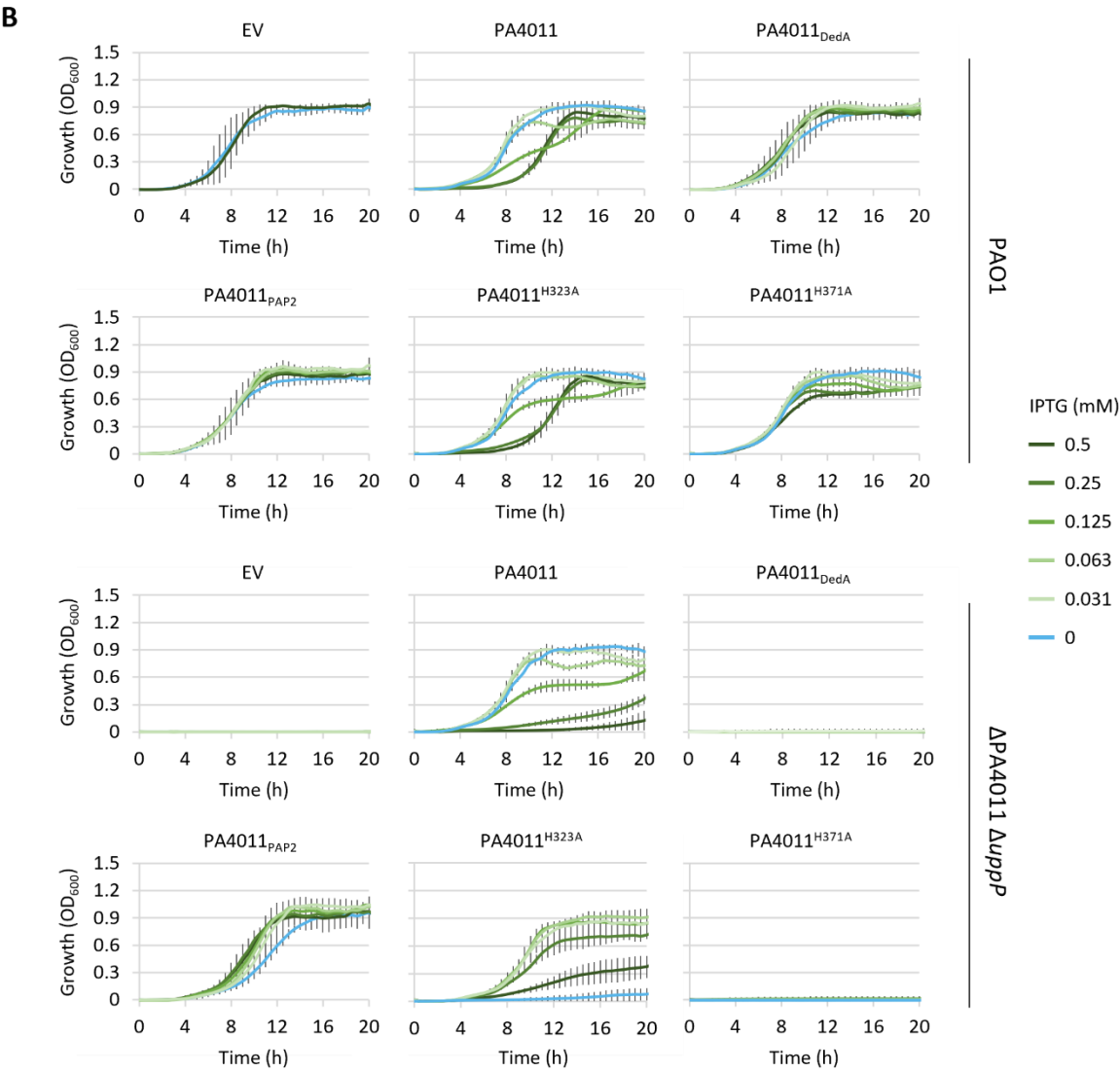


Figure S2. (B) Growth curves of the same strains described in panel A cultured at 37°C in MH in the presence of 0.125 mM EDTA and increasing concentrations of IPTG. Values are the mean ($\pm$ standard deviation) of three independent experiments.

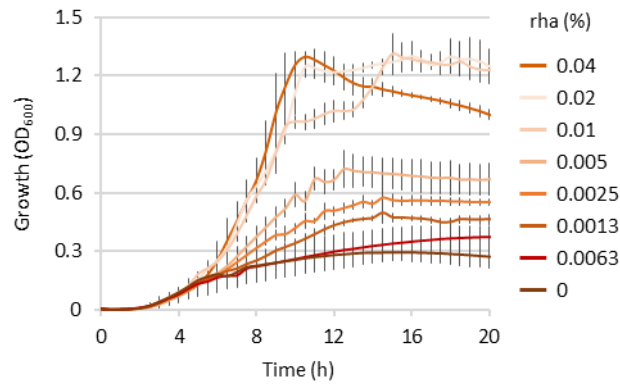


Figure S3. Growth curves of the rhamnose-dependent conditional mutant Δ PA4011 Δ uppP *rhaSR* *P_{rha}* *lpxT* Δ *lpxT* cultured at 37°C in MH in the presence of increasing concentrations of rhamnose (rha). Values are the mean ($\pm$ standard deviation) of three independent experiments.

CHAPTER 7. Bacterial AsmA-like proteins involved in phospholipid transport are independent of DedA family proteins

PREFACE

Based on the functional interaction between some eukaryotic homologs of DedA and AsmA-like proteins in interorganelle lipid trafficking, it has been speculated that DedA proteins may represent possible IM partners of bacterial AsmA-like proteins, assisting them in GPL extraction from the IM. In this chapter we experimentally explored this hypothesis, using *Pseudomonas aeruginosa* as a model and taking advantage of the multiple deletion mutants in *dedA* and *asmA*-like genes generated for previous studies (see chapters 3 and 5). To systematically investigate the requirement of DedA proteins for AsmA-like protein function, I deleted each *dedA* gene in four triple mutants in which a single AsmA-like protein is present and essential for growth. Growth assays revealed that the loss of individual DedA proteins did not impair growth in any genetic background, ruling out that DedA proteins are required for GPL transport mediated by AsmA-like proteins.

The following manuscript has been formatted as a concise short-format article (e.g., Observation, Research Note), reflecting the specific scope of the study and the presentation of a single experimental data set.

Bacterial AsmA-like proteins involved in phospholipid transport are independent of DedA family proteins

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Running title: AsmA-like proteins do not require DedA proteins

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Keywords: DedA, membrane transport, phospholipid, *Pseudomonas*, TamB, YdbH, YhdP, outer membrane.

ABSTRACT

The outer membrane of Gram-negative bacteria requires the transport of glycerophospholipids (GPLs) from the cytoplasmic (inner) membrane, a process recently proposed to be mediated by AsmA-like proteins. Although these proteins are predicted to form periplasm-spanning bridges for GPL diffusion, the mechanisms of GPL extraction from the inner membrane remain unclear. Based on the functional interaction between some DedA and AsmA-like homologs in eukaryotes, DedA family proteins have been hypothesized to assist AsmA-like proteins in GPL extraction. Here, using *Pseudomonas aeruginosa* as a model, we systematically investigated the requirement of DedA proteins for AsmA-like protein function by deleting each *dedA* gene in mutants which rely on a single AsmA-like protein for growth. Loss of individual DedA proteins did not impair growth in any genetic background, indicating that DedA proteins are not required for anterograde GPL transport mediated by AsmA-like-proteins.

MAIN TEXT

The outer membrane (OM) is an essential structure of Gram-negative bacteria that provides mechanical strength and protection from large and/or hydrophobic toxic molecules, including many antibiotics (1). The OM is an asymmetric bilayer composed of glycerophospholipids (GPLs) in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, which contains both integral β -barrel proteins and lipoproteins. While the transport and assembly of LPS, β -barrel proteins, and lipoproteins have been largely characterized (2), how GPLs are translocated from the cytoplasmic (inner) membrane (IM) to the OM has remained elusive for decades, until recent works have provided compelling evidence that some AsmA-like proteins (*e.g.*, YhdP, TamB, and YdbH) may be responsible for this process (3-8). These proteins are anchored to the IM by a single N-terminal transmembrane α -helix (TMH) and have a large soluble domain which contains a Chorein-N domain followed by an AsmA-like domain of variable length (9,10). Structural predictions suggest that AsmA-like proteins form tube-like structures consisting of stacked β -strands, creating a continuous hydrophobic groove that spans the periplasm (4-8, 11), supporting the proposal of AsmA-like proteins working as protein bridges for GPL translocation toward the OM.

While structural predictions and experimental data strongly support the capability of the soluble domain of AsmA-like protein to provide a means for GPL diffusion across the periplasm, the mechanisms underlying GPL extraction from the IM and insertion into the OM are still unclear. TamB and YdbH have OM protein partners that could assist GPL insertion into the OM (4,12), whereas molecular dynamics predictions, site-specific mutagenesis, and chimeric protein engineering have

shown that hydrophobic α -helices in the C-terminal domain of YhdP may facilitate interaction with the OM and likely enable GPL delivery in the absence of protein partners (8,11). In contrast, no experimental evidence has yet been obtained regarding GPL extraction from the IM. However, it has been hypothesized that proteins belonging to the DedA family could represent functional partners of AsmA-like proteins, assisting them in the extraction of GPLs from the IM (8,13). This hypothesis is mainly based on the observation that eukaryotic homologs of DedA and AsmA-like proteins physically interact and cooperate in interorganelle lipid trafficking from the endoplasmic reticulum to the autophagosomal membrane (14,15). DedA proteins belong to a highly conserved family of membrane proteins found in most bacteria and eukaryotes, generally implicated in lipid transport and membrane homeostasis (13). Recent evidence indicates that some bacterial DedA proteins function as proton-dependent transporters involved in flipping undecaprenyl phosphate (Und-P) or GPLs across the cytoplasmic membrane (16-18).

To verify the functional interaction between DedA and AsmA-like proteins, we took advantage of the availability of multiple deletion mutants in *dedA* and *asmaA*-like genes in the Gram-negative bacterium *Pseudomonas aeruginosa* (6,19), to assess whether inactivation of individual DedA proteins can affect the function of AsmA-like proteins likely responsible for GPL transport to the OM. *P. aeruginosa* has four essential, but redundant, AsmA-like proteins, namely YhdP, TamB, YdbH, and EpaF (6), and six non-essential DedA proteins (19). Two DedA proteins (PA4029 and PA4011) were found to contribute to Und-P recycling (19; chapter 6), whereas the function of the others is currently unknown. Notably, a recent study provided indirect evidence suggesting a possible effect of the DedA protein PA5244 on GPL flow across the cell envelope (20).

To assess the potential role of DedA proteins in anterograde GPL transport, we deleted each *dedA* gene in four *P. aeruginosa* triple mutants which rely on a single AsmA-like protein for growth, thus generating 24 quadruple mutants. If any DedA protein is required for the function of one or more AsmA-like proteins, we would expect to observe growth defects in the corresponding *dedA* mutant(s) with respect to the cognate parental triple mutant(s). Remarkably, loss of individual DedA proteins did not decrease growth rates or yields in any genetic background (Figure 1), implying that DedA proteins are not required for the function of any essential AsmA-like protein. This evidence strongly suggests that DedA-mediated GPL transport does not represent the mechanism by which AsmA-like proteins extract GPLs from the IM. While the presence of other IM proteins interacting with AsmA-like proteins and assisting them in GPL uptake cannot be excluded, it is worth noting that molecular dynamics predicted that YhdP is amenable to GPL extraction from model membranes (8). This suggests that GPLs could enter the periplasmic groove of AsmA-like proteins independently of protein partners, possibly driven by GPL concentration gradients present across the cell envelope.

Future biochemical and biophysical studies of AsmA-like proteins will be essential to explore this hypothesis.

ACKNOWLEDGMENTS

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FIGURE

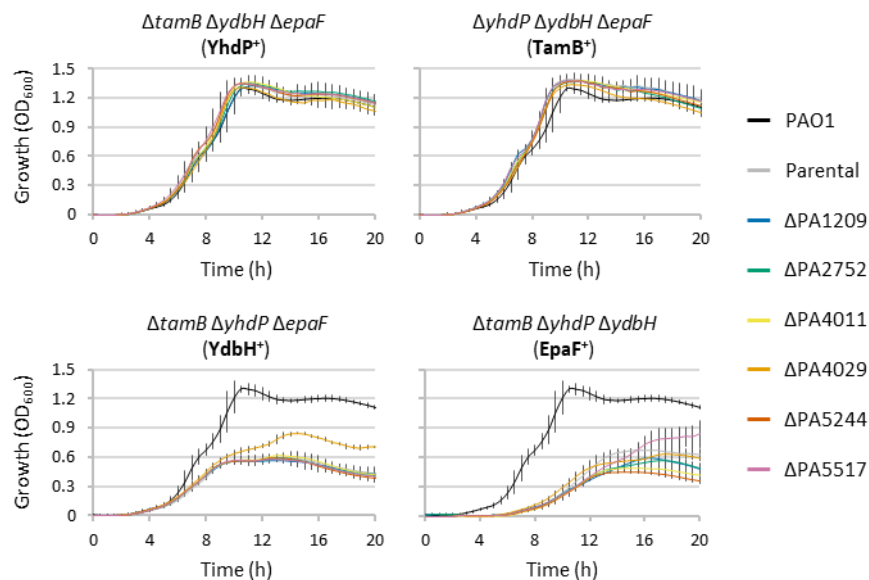


Figure 1. Growth curves of four *P. aeruginosa* triple mutants in the essential *asmaA*-like genes *tamB*, *yhdP*, *ydbH*, and *epaF* in different combinations (parental strains) and the corresponding isogenic mutants in the *dedA* genes PA1209, PA2752, PA4011, PA4029, PA5244, and PA5517. The only essential AsmA-like protein expressed in each mutant is indicated in bold above the graph. The wild type strain *P. aeruginosa* PAO1 was also included as the positive control. Bacteria were cultured in Mueller-Hinton broth at 37°C in microtiter plates, and growth was monitored over time in a microtiter plate reader (Tecan Spark 10M) as the optical density at 600 nm (OD₆₀₀) of the cultures. Data are the mean (± standard deviation) of three independent experiments.

CHAPTER 8. Activation of the envelope stress-responsive two-component system AmgRS compensates for depletion of the essential lipoprotein signal peptidase LspA in *Pseudomonas aeruginosa*

PREFACE

Lipoproteins are indispensable for cell envelope assembly, maintenance, and function in Gram-negative bacteria. For this reason, several enzymes involved in their maturation have emerged as promising antibacterial targets. Among these, the type II signal peptidase LspA catalyzes a critical step in lipoprotein maturation and is predicted to be essential in *P. aeruginosa*. However, despite the identification of potent LspA inhibitors active against other Gram-negative species, these compounds have no or very poor activity against *P. aeruginosa*, leading us to question to what extent LspA is essential in this bacterium and whether this pathogen can tolerate perturbations in lipoprotein processing.

In this study, we investigated the role of LspA in *P. aeruginosa* by generating and characterizing an arabinose-dependent conditional mutant. LspA depletion resulted in complete growth arrest, progressive loss of viability, and severe defects in OM integrity, leading to increased susceptibility to multiple antibiotics that are normally ineffective against this pathogen. Through the isolation and characterization of suppressor mutants, we identified a gain-of-function mutation in *amgS*, encoding the sensor kinase of the cell envelope stress-responsive two-component system AmgRS, capable of partially restoring growth, membrane integrity, and antibiotic resistance under LspA-limiting conditions. Functional analyses further demonstrated that this compensatory effect requires the AmgRS-regulated inner membrane proteins HtpX and YccA, without involving restoration of lipoprotein maturation itself.

Overall, this work confirms the essentiality of LspA in *P. aeruginosa*, establishes it as a promising antibacterial target, and reveals a previously unrecognized role for the AmgRS-mediated stress response in mitigating the consequences of defective lipoprotein biogenesis.

This work has been published in Current Research in Microbial Sciences in 2026. The Supplementary material has not been included in this chapter, but is available at

<https://ars.els-cdn.com/content/image/1-s2.0-S2666517426000209-mmcl1.pdf>



Activation of the envelope stress-responsive two-component system AmgRS compensates for depletion of the essential lipoprotein signal peptidase LspA in *Pseudomonas aeruginosa*

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ABSTRACT

Bacterial lipoproteins play crucial roles in cell envelope biogenesis, signaling, transport, and virulence, making the enzymes responsible for their maturation attractive targets for antibacterial drug development. Among these, the type II signal peptidase LspA is a particularly promising candidate, as several LspA inhibitors have been identified that exert potent antibacterial effects in some Gram-negative species. However, despite predictions that LspA is essential in *Pseudomonas aeruginosa*, these inhibitors show poor or no activity against this Gram-negative pathogen. To assess the essentiality of *P. aeruginosa* LspA and its potential as a drug target, here we generated and characterized an arabinose-dependent *lspA* conditional mutant. LspA depletion completely inhibited bacterial growth, progressively reduced cell viability, and caused severe defects in outer membrane integrity, leading to increased susceptibility to multiple antibiotics, including those that are normally inactive against *P. aeruginosa*. Selection of revertant clones, whole genome sequencing, and allelic replacement mutagenesis revealed that a gain-of-function mutation in *amgS*, encoding the sensor kinase of the envelope stress-responsive two-component system AmgRS, can support growth under LspA-limiting conditions and partially restore membrane integrity and antibiotic resistance. Functional analyses further showed that the AmgRS-regulated inner membrane proteins HtpX and YccA are required for this compensatory effect, although the underlying mechanism remains unclear. Together, these findings confirm the essentiality of LspA in *P. aeruginosa*, establish it as a promising antibacterial target, and uncover a role for the AmgRS-mediated stress response in mitigating the consequences of defective lipoprotein maturation.

1. Introduction

Bacterial lipoproteins are membrane-associated proteins characterized by an N-terminal lipid modification, which anchors them to a membrane. Lipoproteins are predicted to be present in nearly all bacteria (Sutcliffe et al., 2012) and carry out a variety of functions including cell envelope biogenesis, protein secretion, signaling, cell wall

remodeling, antibiotic resistance, biofilm formation, and adhesion (Braun and Hantke, 2019).

Lipoproteins are synthesized as precursors (prolipoproteins) with an N-terminal signal peptide containing the lipobox motif, which directs them to the Sec translocon for translocation across the cytoplasmic membrane. Once translocated, lipoproteins undergo a series of sequential enzymatic modifications (Braun and Hantke, 2019;

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Buddelmeijer, 2015). The first step is catalyzed by the enzyme Lgt (prolipoprotein diacylglycerol transferase), which attaches a diacylglycerol to the thiol group of the invariant cysteine residue contained within the lipobox motif. Then, LspA (lipoprotein signal peptidase II) cleaves the signal peptide immediately upstream of the modified cysteine, thereby exposing the lipidated cysteine at the N-terminus of the nascent lipoprotein. This apolipoprotein is further modified by Lnt (apolipoprotein N-acyl transferase), which transfers a third acyl chain to the free amino group of the N-terminal cysteine, producing a mature triacylated lipoprotein (Braun and Hantke, 2019; Buddelmeijer, 2015).

In monoderm (Gram-positive) bacteria, lipoproteins are restricted to the outer leaflet of the cytoplasmic membrane, with their protein domain extending through the cell wall (Nguyen et al., 2020). In contrast, in diderm (Gram-negative) bacteria, they can remain attached to the periplasmic leaflet of the cytoplasmic (inner) membrane (IM) or be sorted and transported to the outer membrane (OM) by the lipoprotein OM localization (Lol) system. In this system, an ABC transporter (LolCDE) recognizes and extracts OM-targeted lipoproteins from the IM, a periplasmic chaperone (LolA) shuttles them across the periplasmic space, and an OM lipoprotein (LolB) assists their insertion into the periplasmic leaflet of the OM (Okuda and Tokuda, 2011; Grabowicz, 2019). After release from the Lol system, most OM lipoproteins remain oriented toward the periplasm, whereas a subset is flipped to the cell surface. The pathways mediating the translocation of these surface-exposed lipoproteins across the OM are still poorly defined and appear to depend on both the bacterial species and the individual lipoprotein (Konovalova and Silhavy, 2015; Wilson and Bernstein, 2016; Cole et al., 2021). IM lipoproteins escape recognition by the Lol system through a so-called “Lol avoidance” signal, whose nature and mechanism of action remain under discussion (Konovalova and Silhavy, 2015; Lorenz et al., 2019).

Since lipoproteins play central roles in bacterial cell physiology, their maturation is considered a promising target for antibacterial drug development (Lehman et al., 2022). While Lgt and LspA are widely conserved in Bacteria and their function is crucial for proper lipoprotein maturation in most species (Sutcliffe et al., 2012; Nakayama et al., 2012), Lnt is less so. Indeed, N-terminal acylation of apolipoproteins is mediated by different enzymes in some bacterial species (Armbruster and Meredith, 2017; Armbruster et al., 2024), and some Gram-negative bacteria are viable even when this function is inactivated, probably because of the presence of variants of the Lol IM complex that can also recognize diacylated lipoproteins (LoVullo et al., 2015; Gwin et al., 2018).

Several natural and synthetic inhibitors, such as globomycin and myxovirescin, specifically block LspA activity, resulting in bacteriostatic or bactericidal effects in some Gram-negative bacteria (El Arnaout and Soulimane, 2019; Xia et al., 2020). Conversely, Gram-positive bacteria are generally poorly sensitive to LspA inhibitors, consistent with their ability to tolerate LspA depletion, likely because uncleaved lipoproteins remain functional and correctly localized on the membrane surface (Hutchings et al., 2009; Nguyen and Götz 2016).

Notably, while LspA inhibitors have promising activity against *Enterobacteriaceae* and other Gram-negative bacteria, they display poor or no activity against *Pseudomonas aeruginosa* (Gerth et al., 1982; Onishi et al., 1984; Kiho et al., 2003; Garland et al., 2020), even though one of them (globomycin) has been proven to bind and inhibit purified *P. aeruginosa* LspA (Vogelely et al., 2016). While this limited activity could reasonably be ascribed to the poor permeability of the *P. aeruginosa* OM (Breidenstein et al., 2011), it cannot be ruled out that other molecular, genetic, or physiological factors may render this bacterium more tolerant to LspA inhibition. In this regard, it is worth noting that, although *lspA* was predicted as an essential gene in *P. aeruginosa* by

transposon mutagenesis (Tn-seq) studies under different growth conditions (Lee et al., 2015; Turner et al., 2015; Poulsen et al., 2019), a direct assessment of the effects of LspA depletion or inactivation in *P. aeruginosa* has so far been lacking. In this work, through the generation and characterization of an *lspA* conditional mutant, we confirm the essentiality of LspA for *P. aeruginosa* growth and cell envelope integrity and provide evidence that *P. aeruginosa* can withstand the detrimental effects of LspA depletion upon activation of the membrane stress-responsive two-component system (TCS) AmgRS.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Strains and plasmids used in this work are listed in Table S1 and Table S2, respectively. Bacteria were routinely cultured in Lysogeny broth, Lennox formulation (LB) for genetic manipulation, supplemented with 0.5 % arabinose in the case of conditional mutants. Growth and antibiotic susceptibility assays were performed in Mueller-Hinton broth (MH), supplemented with arabinose at the indicated concentration when appropriate. Antibiotics were used at the following concentrations for *Escherichia coli* (the concentrations used for *P. aeruginosa* are shown in brackets): ampicillin, 100 µg/mL; tetracycline, 12.5 µg/mL (50–100 µg/mL); carbenicillin (500 µg/mL); nalidixic acid, 15 µg/mL; chloramphenicol, 30 µg/mL (375 µg/mL).

2.2. Generation of plasmids and mutants

Primers and restriction enzymes used for PCR, cloning, and DNA sequencing are listed in Table S3.

To obtain the constructs for the generation of *P. aeruginosa* deletion mutants, two DNA fragments of approximately 500 bp, encompassing the upstream and downstream regions of each gene of interest, were amplified by PCR, directionally cloned into pBluescript II (pBS), and verified by DNA sequencing. Then, the DNA fragment encompassing the upstream and downstream regions of each gene was excised from pBS and sub-cloned into the *sacB*-based suicide plasmid pDM4 (Milton et al., 1996). The resulting pDM4 derivatives (Table S2) were transferred into *P. aeruginosa* by conjugation, and the transconjugants were selected on plates containing 15 µg/mL nalidixic acid and 375 µg/mL chloramphenicol. Gene deletions were obtained by recombination and sucrose-based selection as previously described (Lo Sciuto et al., 2022) and identified by PCR with the primer pairs UP_FW and DOWN_RV of each gene (Table S3). Each deletion was further confirmed by DNA sequencing.

To generate the *lspA* conditional mutant, the coding sequence of *lspA* was PCR amplified, directionally cloned into the mini-CTX1 *araC* *P_{ara}* plasmid (Lo Sciuto et al., 2014) downstream of the *araC* *P_{ara}* regulatory element, and verified by DNA sequencing. The resulting plasmid was introduced into *P. aeruginosa* by conjugation and transconjugants were selected on LB agar plates containing 15 µg/mL nalidixic acid and 100 µg/mL tetracycline. The plasmid backbone was removed using the *sacB*-based suicide vector pFLP2 (Hoang et al., 1998) as previously described (Lo Sciuto et al., 2022) and pFLP2 was cured by plating onto LB agar plates supplemented with 10 % sucrose. Carbenicillin-sensitive clones were analyzed by colony PCR to verify the insertion of the *araC* *P_{ara}::lspA* construct into the chromosome. Then, in-frame deletion of the endogenous copy of *lspA* was obtained by recombination and sucrose-based counterselection, as described above, using the pDM4Δ*lspA* construct (Table S2) and culturing bacteria under permissive conditions (i.e., in the presence of 0.5 % arabinose). The deletion was verified by PCR and DNA sequencing.

To obtain the construct for *amgS* allelic replacement, a 569-bp DNA fragment containing the mutation of interest was PCR amplified from the *lspA* revertant mutant #4 (Table S4), directionally cloned into pBS for DNA sequencing, and then subcloned into pDM4. Allelic replacement was obtained by recombination and sucrose-based counterselection, as described above. The site-specific mutant was verified by PCR and DNA sequencing.

The plasmids pME*htpX* and pME*yccA* (Table S2) were generated by individually cloning the PCR-amplified coding sequence of *htpX* or *yccA* into the shuttle vector pME6032 (Heeb et al., 2002), downstream of the IPTG-inducible promoter, and inserts were verified by DNA sequencing. Primers and restriction enzymes used for PCR and cloning or DNA sequencing are listed in Table S3.

2.3. Growth assays

For planktonic growth assays, bacteria were precultured in MH, supplemented with 0.5 % arabinose for the conditional mutants, until late exponential phase and then refreshed 1:1000 in fresh MH medium, supplemented or not with 0.5 % arabinose and/or increasing concentrations of fucose. Cultures were aliquoted in flat-bottom 96-well microtiter plates (200 μ L in each well) and plates were incubated at 37 °C in a Tecan Spark 10 M microtiter plate reader. Growth was measured as the optical density at 600 nm (OD₆₀₀) of the bacterial cultures.

For plating efficiency assays, bacterial strains were precultured in MH, supplemented with 0.5 % arabinose for the conditional mutants, harvested by centrifugation, and resuspended in sterile saline solution at OD₆₀₀ = 1. Serial ten-fold dilutions were prepared in saline solution and 5- μ L aliquots of selected dilutions were spotted onto MH agar plates in the presence or not of 0.5 % arabinose and/or 0.5 % fucose. Plates were incubated at 37 °C for 24 or 48 h.

To obtain LspA-depleted cells of the *lspA* and *lspA amgS*^{P381H} conditional mutants, bacteria were cultured overnight in flasks in MH containing 0.5 % arabinose at 37 °C, refreshed at high cell density (1:30 dilution) in MH without arabinose, cultured for 2 h, and then refreshed again (1:3 dilution) in the same medium. At 2-h intervals, the OD₆₀₀ was measured, and aliquots of bacterial cultures were serially 10-fold diluted in saline solution and plated on MH agar containing 0.5 % arabinose to determine the number of colony-forming units (CFUs). Cells for membrane permeability assays, sodium dodecyl sulfate (SDS) sensitivity assays, RNA extraction, and membrane separation (see below) were collected when a growth defect was observed in the conditional mutants with respect to the wild-type or parental strain (Figs. 1C and S2).

2.4. Membrane permeability assay

Exponential-phase cells cultured in MH, or LspA-depleted cells obtained using the dual-refresh strategy described above, were harvested by centrifugation and resuspended in 5 mM HEPES (pH 7.2) at OD₆₀₀ = 3. Equal volumes (150 μ L) of bacterial suspensions and HEPES solution containing or not propidium iodide (PI; 40 μ g/mL) or 1-N-phenylmethylamine (NPN; 20 μ M) were mixed, and 100 μ L of each sample were aliquoted on a black flat-bottom 96-well microtiter plate. OD₆₀₀ and fluorescence were measured in a Tecan Spark 10 M microtiter plate reader (excitation at 580 nm and emission at 620 nm for PI; excitation at 350 nm and emission at 420 nm for NPN) after 2 min at room temperature, subtracted of the background values of samples without PI or NPN, and normalized to the OD₆₀₀ of the cell suspension (Cervoni et al., 2021).

2.5. SDS sensitivity assay

Exponential-phase cells cultured in MH or LspA-depleted cells

obtained using the dual-refresh strategy described above were harvested by centrifugation, and pellets were resuspended in saline solution at OD₆₀₀ = 1. One hundred- μ L aliquots of bacterial suspensions were added to the same volume of saline solution containing decreasing concentrations of SDS (starting from 5 %) (Lo Sciuto et al., 2018). The turbidity (OD₆₀₀) of bacterial suspension was determined after 15 min of incubation at room temperature in a Tecan Spark 10 M microtiter plate reader.

2.6. Antibiotic sensitivity assay

The resistance profile of bacterial strains to several antibiotics was assessed by the Kirby-Bauer disc diffusion assay. Bacteria were cultured in MH until late exponential phase, centrifuged, resuspended at a theoretical OD₆₀₀ = 0.08 in sterile saline solution, and swabbed onto MH agar plates, supplemented with 0.075 % arabinose for the conditional mutants. Disks containing rifampicin (5 μ g), streptomycin (10 μ g), gentamycin (10 μ g), imipenem (10 μ g), novobiocin (30 μ g), tobramycin (10 μ g), or erythromycin (15 μ g) (Becton Dickinson) were placed on the agar surface before incubation of plates. The diameters of the growth inhibition halos were measured after 24 and 48 h of incubation at 37 °C (for the wild type and *lspA* conditional mutants, respectively).

2.7. MIC assays

The MIC of gentamycin, kanamycin, and streptomycin was determined through the broth microdilution method. Bacteria were precultured in MH, supplemented with 0.5 % arabinose for the conditional mutants, and then refreshed in the same medium at ca. 5×10^5 cells/mL in the presence of increasing concentrations of each antibiotic. MIC was defined as the lowest antibiotic concentration for which no visible growth was observed after 24 h of incubation at 37 °C under static conditions.

2.8. Frequency of revertants

To evaluate the frequency of putative revertants, the *lspA*, *lola*, and *lptH* conditional mutants were cultured in MH supplemented with 0.5 % arabinose at 37 °C until early stationary phase, harvested by centrifugation, and resuspended in sterile saline solution at OD₆₀₀ = 1. Serial 10-fold dilutions were prepared in saline and plated onto MH agar plates supplemented with 0.5 % arabinose to measure the number of CFUs. Revertant clones were selected by plating 50 μ L aliquots of the OD₆₀₀ = 1 suspension (or of the 10⁻¹ and 10⁻² dilutions for the *lola* conditional mutant) onto MH agar plates. Colonies obtained in the absence of arabinose were counted after 48 h at 37 °C. Frequency of revertants was calculated as the ratio between the number of revertants (colonies that appeared on MH agar) and the number of total CFUs plated (colonies that appeared on MH agar supplemented with arabinose).

2.9. Whole genome sequencing and comparative genomics

Genomic DNA was extracted with the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) and quantified using the Qubit 2.0 fluorometer (Thermo Fisher Scientific). DNA sequencing (2 $\times$ 150 bp) was performed by BMR genomics S.r.l., Padova, Italy, on an Illumina platform (Illumina, Inc., San Diego, US). Quality control of the reads was assessed with Fastp v0.23.2 (Chen et al., 2018), using parameters `-detect_adapter_for_pe -average_qual 20 -length_required 35 -qualified_quality_phred 20 -thread 60`, and providing the raw sequencing reads as inputs.

Pseudomonas aeruginosa PAO1_107 genomic sequence and annotation data were downloaded from <https://pseudomonas.com/> (last

accessed on June 18th 2023).

Haploid variant calling was performed using the Snippy v4.6.0 pipeline (<https://github.com/tseemann/snippy>) on a Linux kernel, with parameters `-prefix snps -report -unmapped -mapqual 50 -basequal 10 -mincov 8 -minfrac 0.7 -minqual 60 -force`, and providing the polished sequencing reads as well as the reference genome sequence and annotation file (.gbk) as input files. Internally, the pipeline calls freebayes v1.3.6 (Garrison and Marth, 2012), which is a Bayesian variant detection tool that identifies small-scale genetic polymorphisms, such as single-nucleotide polymorphisms, indels and complex variants that are shorter than the length of a typical short-read sequencing alignment.

Nucleotide variant results, including the constructs insertion sites, were manually inspected using Tablet v1.21.02.08 (Milne et al., 2013).

2.10. RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

RNA was extracted from exponential-phase cells cultured in MH supplemented with 0.5 % arabinose or from LspA-depleted cells obtained using the dual-refresh strategy described above. One-ml aliquots of bacterial cultures were mixed with 2 mL of RNA Protect Bacteria Reagent (Qiagen), and RNA was purified using RNeasy Mini Kit (Qiagen). Eluted RNA samples were treated with TURBO DNase (Thermo Fisher Scientific) and SUPERase-In (Ambion) for 1 h at 37 °C. DNase I was removed upon RNA purification with the RNeasy Column Purification Kit (Qiagen). For RT-qPCR, cDNA was reverse transcribed from 0.5 µg of total RNA with Prime Script RT Reagent Kit (Takara). The cDNA was used as the template for RT-qPCR in an AriaMx Real-Time PCR System (Agilent) using TB Green Premier EX Taq master mix (Takara) and the primers listed in Table S3. Relative expression of each gene with respect to the housekeeping gene *rpoD* was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.11. Membrane separation

The IM and OM were separated by sucrose gradient ultracentrifugation according to published procedures (Hancock and Nikaido, 1978), with the following modifications. A total amount of 250 mL of each bacterial culture from late-exponential phase were harvested by centrifugation at 4 °C and $3000 \times g$ (in a Sorvall RC-5B Plus centrifuge) for 30 min. LspA-depleted cells of the *lspA* conditional mutant were obtained using the dual refresh strategy described above and collected when a growth defect was observed with respect to control cultures. Pellets were washed in 50 mL of 10 mM Tris/HCl buffer (pH 7.8) and bacterial suspensions were harvested by centrifugations at 4 °C and $3000 \times g$ for 20 min. Cells were resuspended in 10 mL of 10 mM Tris/HCl buffer containing 20 % sucrose, 50 µg/mL DNase I, 1 mM PMSF, and 100 µg/mL lysozyme. The resuspended cells were lysed by sonication (in a Sonics VCX130 sonicator) on ice for 15 min at 50 % of amplitude (5" on – 10" off). The cell lysate was clarified by centrifugation at $5000 \times g$ for 20 min. Cell membranes were collected by centrifuging the supernatant for 1 h at $210,000 \times g$ (in a Beckman LE-80 ultracentrifuge with a SW41 Ti Swinging-Bucket Rotor). Pellets (corresponding to the total membranes) were resuspended in 1 mL of 10 mM Tris/HCl buffer containing 25 % sucrose and layered on top of the following sucrose gradient: 1 mL of 70 % sucrose, 3 mL of 64 % sucrose, 3 mL of 58 % sucrose, and 3 mL of 52 % sucrose in 30 mM Tris/HCl (pH 7.8). After centrifugation for 18 h at $210,000 \times g$, two fractions corresponding to the layers at the interfaces 0–52 % and 58–64 %, which correspond to the IM and OM, respectively (Hancock and Nikaido, 1978), were collected from the top of the gradient and stored at –20 °C.

2.12. Western blot analyses

Aliquots of the membrane samples were mixed with an equal volume of $2 \times$ SDS-PAGE loading buffer (0.25 M Tris-HCl pH 6.8, 2 % SDS, 10 % β-mercaptoethanol, 20 % glycerol). Five µL of SDS-PAGE samples were loaded onto 15 % polyacrylamide gels. Gels were run, and the proteins were transferred to nitrocellulose membranes using a Turbo-Blot semi-dry transfer apparatus from Bio-Rad (Hercules, CA, USA). Immunoblotting was performed by incubating the membranes with 5 % milk for 1 h, followed by overnight incubation with anti-XcpY, anti-LptC, or anti-LptE polyclonal rabbit antibodies (Michel et al., 1998; Lo Sciuto et al., 2018). Subsequently, incubation with an anti-rabbit IgG HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) was carried out for 1 h. After extensive washes, membranes were incubated with equal amounts of ECL solutions (Bio-Rad, Hercules, CA, USA) for 1 min at room temperature. Finally, the protein bands were detected using a ChemiDoc XRS+ system apparatus from Bio-Rad (Hercules, CA, USA).

2.13. Statistics

Statistical analysis was performed with the software GraphPad Instat, using the unpaired *t*-test or the One-Way Analysis of Variance (ANOVA) followed by Kruskal-Wallis multiple comparisons test.

3. Results

3.1. *LspA* is essential in *P. aeruginosa*

To assess the function and relevance of LspA in *P. aeruginosa*, we generated a conditional mutant carrying an arabinose-inducible copy of the *lspA* coding sequence integrated into a neutral chromosomal locus of the reference strain *P. aeruginosa* PAO1, together with an in-frame deletion of the native *lspA* gene. The resulting mutant, PAO1 *araC* *P_{araC}::lspA ΔlspA*, was evaluated for growth in the presence or absence of arabinose, both in liquid broth and on agar plates. In the absence of arabinose, complete inhibition of both planktonic and colony growth was observed (Fig. 1A-B). As expected, arabinose supplementation fully restored growth, confirming that the growth defects specifically resulted from LspA depletion. To further assess the impact of LspA on cell viability, the conditional mutant was serially refreshed under non-inducing conditions (no arabinose) to progressively decrease LspA levels until growth arrest occurred (Fig. 1C), then plated on agar plates containing arabinose to quantify the CFUs. This analysis revealed a slow but steady decline in cell viability upon LspA depletion (Fig. 1C), indicating that LspA is important not only for growth but also for cell survival. Overall, these results confirm the essentiality of *P. aeruginosa* LspA and its potential as an antibacterial drug target.

3.2. *LspA* depletion compromises cell envelope integrity

LspA is expected to be responsible for the maturation of all lipoproteins, some of which have been reported to play crucial roles in cell envelope biogenesis and maintenance (Remans et al., 2010; Hoang et al., 2011; Lo Sciuto et al., 2018; Paulsson et al., 2021). Thus, we evaluated the effect of LspA depletion on cell envelope integrity and functionality. First, we assessed cell permeability to the fluorescent probes NPN and PI. NPN fluorescence increases upon interaction with membrane lipids, making it a common indicator of OM destabilization in Gram-negative bacteria, while PI fluoresces upon binding nucleic acids and only penetrates cells with compromised membranes (both OM and IM in Gram-negatives) (Ma et al., 2020; Sposato et al., 2024). LspA-depleted

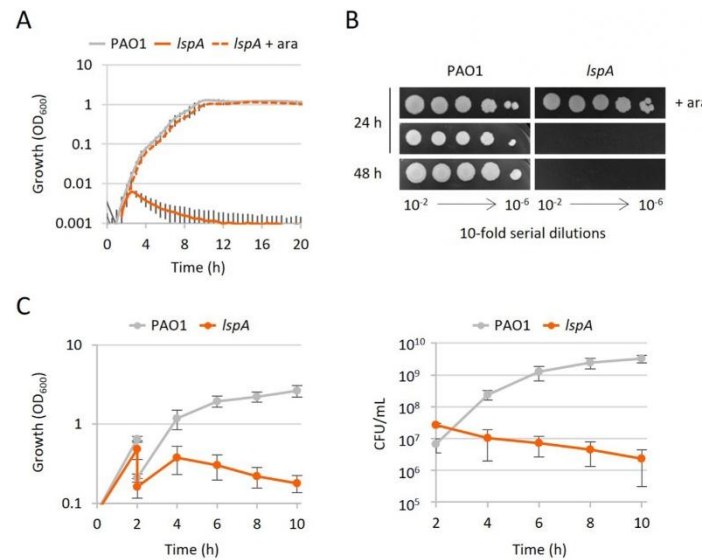


Fig. 1. (A) Growth curves of *P. aeruginosa* PAO1 and the PAO1 *araC* P_{araC}::*lspA* Δ *lspA* conditional mutant (*lspA*) cultured in microtiter plates in MH, supplemented or not with 0.5 % arabinose (+ ara). (B) Plating efficiency of the same strains described in panel A on MH agar plates supplemented or not with 0.5 % arabinose (+ ara). (C) Depletion curves (left panel) and cell viability (right panel) of the same strains described in panel A cultured in flasks. Bacteria were cultured overnight in MH, supplemented with 0.5 % arabinose for the *lspA* conditional mutant, refreshed 1:30 in MH without arabinose, and further diluted 1:3 in fresh medium after 2 h. OD₆₀₀ and cell viability (CFU/mL) were monitored every 2 h. Values in panel A and C are the mean ($\pm$ standard deviation) of three independent experiments, while images in panel B are representative of three independent experiments.

cells, obtained as shown in Fig. 1C, displayed a 15-fold increase in NPN- and PI-associated fluorescence as compared to wild-type cells, indicative of major defects in membrane integrity and permeability barrier (Fig. 2A). Then, we assessed the sensitivity to the detergent SDS, which generally increases in mutants defective in cell envelope stability (Lo Sciuto et al., 2014; Fernández-Piñar et al., 2015). Again, *LspA*-depleted cells were much more sensitive to the lytic activity of SDS than wild-type cells (Fig. 2B). Since defects in the integrity and permeability barrier of the cell envelope are expected to enhance the penetration of antibacterial compounds, we also examined the sensitivity of *LspA*-depleted cells to several antibiotics through the Kirby-Bauer disc diffusion assay. Given that the *lspA* conditional mutant does not grow in the absence of the inducer, we preliminarily determined the lowest arabinose concentration (i.e., 0.075 %) that allows for obtaining a homogeneous bacterial lawn for the conditional mutant (Figure S1). Colony growth assays showed that, under this condition, the conditional mutant

is impaired in growth with respect to the wild-type strain (Figure S1), suggesting that it expresses suboptimal levels of *LspA*. The *lspA* conditional mutant cultured under this condition displayed sensitivity to novobiocin, rifampicin, and erythromycin (Fig. 2C), antibiotics that are poorly active against *P. aeruginosa* due to limited diffusion across the OM, and that are commonly used as indicators of OM integrity defects (Nikaido, 2005; Lo Sciuto et al., 2014, 2018; Wesseling et al., 2021). In addition, the mutant also showed higher susceptibility to tobramycin and imipenem (Fig. 2C). In contrast, the mutant displayed wild-type levels of resistance to streptomycin and gentamycin (Fig. 2C), indicating that the strong increase in sensitivity to most antibiotics is not simply related to poor growth of the mutant under the culture conditions used for the disc diffusion assay. Overall, these results align with those of the NPN/PI permeability and SDS resistance assays, providing evidence that the permeability barrier of *LspA*-depleted cells is strongly compromised.

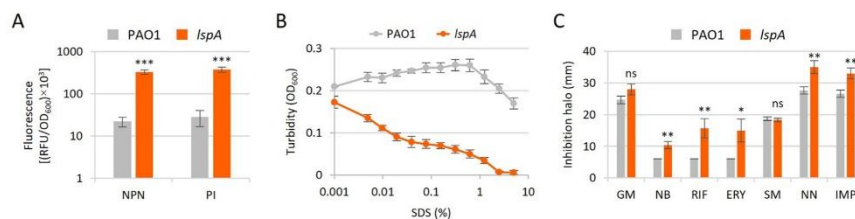


Fig. 2. (A) NPN- and PI-mediated fluorescence, measured as relative fluorescence units (RFU) normalized to the OD₆₀₀ of the bacterial suspension, of *P. aeruginosa* PAO1 and the PAO1 *araC* P_{araC}::*lspA* Δ *lspA* conditional mutant (*lspA*) cultured in MH using the dual-refresh culturing strategy shown in Fig. 1C. (B) Sensitivity to the lytic effect of SDS, measured as a decrease in cell suspension turbidity (OD₆₀₀), of the same strains described in panel A cultured in MH using the dual-refresh culturing strategy shown in Fig. 1C. (C) Antibiotic sensitivity profile of the same strains described in panel A determined through the Kirby-Bauer disk diffusion assay on MH agar plates, supplemented with 0.075 % arabinose for the *lspA* conditional mutant. Abbreviations: GM, gentamicin; NB, novobiocin; RIF, rifampicin; ERY, erythromycin; SM, streptomycin; NN, tobramycin; IMP, imipenem. Values in all panels are the mean ($\pm$ standard deviation) of three independent experiments. The asterisks indicate a statistically significant difference with respect to PAO1 (*** P < 0.001; ** P < 0.01; * P < 0.05; ns, not significant; unpaired *t*-test).

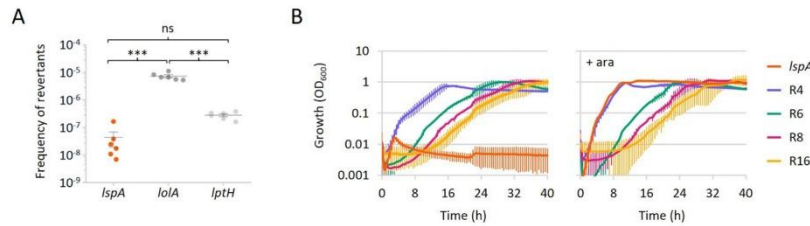


Fig. 3. (A) Frequency of revertant mutants of the PAO1 *araC* *P_{ara}::lspA ΔlspA* (*lspA*), PAO1 *araC* *P_{ara}::lolA ΔlolA* (*lolA*), and PAO1 *araC* *P_{ara}::lptH ΔlptH* (*lptH*) conditional mutants. Six independent experiments were performed for each strain. The asterisks indicate statistically significant differences (***) $P < 0.001$; ns, not significant; ANOVA, Tukey's multiple comparisons test). (B) Growth curves of the *lspA* conditional mutant and selected *lspA* revertant mutants (R#) in MH supplemented (right panel) or not (left panel) with 0.5 % arabinose (+ ara). Values are the mean ($\pm$ standard deviation) of two independent experiments. Several clones selected on MH agar plates (panel A) showed very poor or no growth in MH broth and were not included in the graphs.

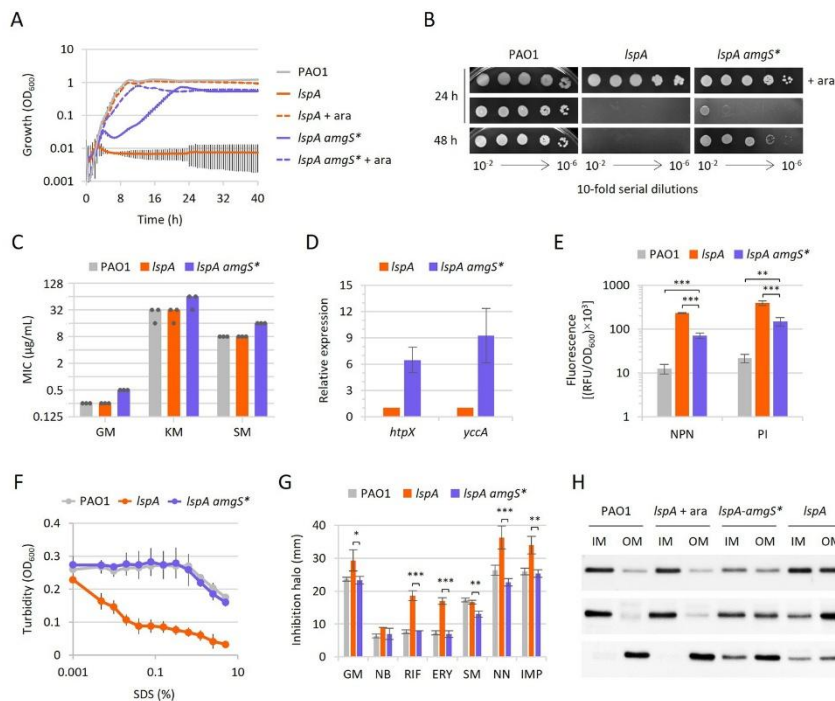


Fig. 4. (A) Growth curves of *P. aeruginosa* PAO1 and the PAO1 *araC* *P_{ara}::lspA ΔlspA* (*lspA*) and PAO1 *araC* *P_{ara}::lspA ΔlspA amgS^{SP381H}* (*lspA amgS**) conditional mutants in MH, supplemented or not with 0.5 % arabinose (+ ara). (B) Plating efficiency of the same strains described in panel A on MH agar plates supplemented or not with 0.5 % arabinose (+ ara). (C) Aminoglycosides resistance levels of the same strains described in panel A cultured in MH, supplemented with 0.5 % arabinose for the conditional mutants, measured as minimum inhibitory concentration (MIC) of gentamicin (GM), kanamycin (KM), and streptomycin (SM). (D) Relative expression levels of the AmgRS-regulated genes *htpX* and *yccA* in the *lspA amgS** conditional mutant with respect to the *lspA* conditional mutant. RNA was extracted from strains cultured in MH supplemented with 0.5 % arabinose. (E) NPN- and PI-mediated fluorescence, measured as relative fluorescence units (RFU) normalized to the OD₆₀₀ of the bacterial suspension, of the same strains described in panel A cultured using the dual-refresh culturing strategy shown in Figure S2. (F) Sensitivity to the lytic effect of SDS, measured as a decrease in cell suspension turbidity (OD₆₀₀), of the same strains described in panel A cultured in MH using the dual-refresh culturing strategy shown in Figure S2. (G) Antibiotic sensitivity profile of the same strains described in panel A, determined through the Kirby-Bauer disk diffusion assay on MH agar plates, supplemented with 0.075 % arabinose for the conditional mutants. Abbreviations: GM, gentamicin; NB, novobiocin; RIF, rifampicin; ERY, erythromycin; SM, streptomycin; NN, tobramycin; IMP, imipenem. (H) Western blot analysis of purified IM and OM fractions, extracted from the strains described in panel A and cultured as shown in Figure S2, with antibodies against the OM lipoprotein LptE or the IM proteins LptC and XcpY. Uncropped western blot images are provided in Figure S5. Values in panel C are the mode from three independent experiments. Images in panels B and H are representative of at least three experiments. All the other values are the mean ($\pm$ standard deviation) of three (panels A, E-G) or two (panel D) independent experiments. The asterisks indicate statistically significant differences (***) $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns, not significant; ANOVA, Tukey's multiple comparisons test).

3.3. AmgRS induction mitigates the effects of LspA depletion

An important aspect to consider in the evaluation of an essential protein as a possible drug target is the likelihood of emergence of bacterial subpopulations or mutants able to tolerate the depletion or inactivation of the essential protein of interest. So, we aimed to investigate the frequency of putative revertants (i.e., cells that regain the ability to grow in the absence of the inducer) by plating cell suspensions of the *lspA* conditional mutant, previously cultured under permissive conditions, on agar plates without arabinose. To take into account the impact of the genetic element *araC* P_{ara} on the emergence of revertants, we included two other arabinose-dependent conditional mutants of *P. aeruginosa* in which the lipoprotein transport gene *lolA* or the LPS transport gene *lptH* is under the control of the same arabinose-dependent regulatory element (Fernandez-Pinar et al., 2015). The frequency of revertants was lower for the *lspA* conditional mutant (about 4×10^{-8}) as compared to the two control strains (7×10^{-6} for the *lolA* conditional mutant and 3×10^{-7} for the *lptH* conditional mutant) (Fig. 3A). The high variability in the frequencies of revertants obtained for the three conditional mutants strongly suggests that the results are only marginally affected by the regulatory element *araC* P_{ara} , which is identical in all mutants (Table S1) (Fernandez-Pinar et al., 2015).

To confirm the capability of the *lspA* revertant clones to grow under non-inducing conditions, they were cultured in MH broth supplemented or not with arabinose. Several clones did not grow in liquid medium (data not shown), suggesting that they have non-inheritable adaptation (s) and/or that the phenotype is very unstable. In contrast, some clones (R6, R8, and R16) grew in liquid medium without arabinose; however, their growth was markedly delayed with respect to the parental strain (i.e., the *lspA* conditional mutant) cultured in the presence of arabinose and was not promoted by arabinose (Fig. 3B). Of note, the clone R4 grew much better than the others, and its growth was restored to almost parental levels by arabinose supplementation (Fig. 3B). Whole genome sequencing and haploid variant calling revealed that clones characterized by delayed and arabinose-independent growth carried mutations in the upstream region of PA3686 (adenylate kinase *Adk*), while clone R4 harbored a missense mutation in the *amgS* (PA5199) coding sequence, causing a proline to histidine substitution at residue 381 (Table S4). The *amgS* gene encodes the sensor kinase of the TCS AmgRS, which controls *P. aeruginosa* response to membrane stress and is involved in aminoglycoside resistance (Lee et al., 2009; Lau et al., 2013).

To assess whether the mutation identified in *amgS* was sufficient to sustain *P. aeruginosa* growth when LspA levels are limiting, we replaced the wild-type *amgS* allele with the mutated one (*amgS*^{P381H}) in the *lspA* conditional mutant. Notably, the resulting strain was able to grow in the absence of arabinose (Fig. 4A-B). We therefore focused our attention on this mutant to gain insight into how *P. aeruginosa* can adapt to LspA-limiting levels.

First, we sought to determine whether the P381H substitution activates or inactivates the TCS AmgRS, which is important for aminoglycoside resistance and whose regulon has been characterized (Lee et al., 2009). Notably, the *lspA* conditional mutant expressing the *AmgS*^{P381H} variant showed less sensitivity to different aminoglycosides and increased expression of two genes (*hpx* and *yccA*) whose transcription is positively regulated by AmgRS (Lee et al., 2009), as determined by MIC assays and RT-qPCR, respectively (Fig. 4C-D). The assays were performed by culturing bacteria in the presence of arabinose, to avoid any indirect effect related to LspA depletion. These results indicate that the P381H substitution activates the AmgRS system, leading to the induction of AmgRS-regulated genes.

Then, we verified the effect of AmgRS induction on the cell envelope integrity of LspA-depleted cells, by comparing NPN and PI permeability, as well as SDS and antibiotic sensitivity, between the wild-type strain and *lspA* conditional mutants carrying or not the *amgS*^{P381H} allele, cultured in the presence of limiting concentrations of arabinose. AmgRS activation caused by the P381H substitution was found to attenuate the

permeability barrier defects of LspA-depleted cells (Fig. 4E) and restore SDS and antibiotic resistance in the *lspA* conditional mutant to almost wild-type levels (Fig. 4F-G). The *amgS*^{P381H} allele did not significantly affect cell envelope integrity or resistance to non-aminoglycoside antibiotics when bacteria were cultured in the presence of arabinose (Figure S3), confirming that its effects are specific to LspA-depleted cells. Overall, these findings suggest that gain-of-function mutations in the AmgRS system can partially rescue growth and cell envelope integrity defects caused by LspA depletion.

Notably, the rescue effect of *AmgS*^{P381H} does not appear to depend on AmgRS-induced expression of *lspA* in the arabinose-dependent conditional mutant. Indeed, as shown in Figure S4, the basal levels of the *lspA* mRNA were not increased in the *lspA amgS*^{P381H} conditional mutant as compared to the *lspA* conditional mutant under non-inducing conditions (no arabinose). Moreover, the growth of the *lspA amgS*^{P381H} mutant was not affected by fucose, which, in contrast, efficiently inhibited arabinose-mediated growth promotion in the *lspA* mutant, in line with its ability to prevent AraC-mediated transcriptional induction (Schleif, 2010). Overall, these findings rule out the possibility that AmgRS can induce *lspA* expression, either directly or through enhanced induction of AraC. This is in line with the observation that the subcellular localization of the OM lipoprotein LptE, used as a proxy for testing the functionality of the lipoprotein maturation pathway, was still largely defective in *lspA amgS*^{P381H} cells, as shown by membrane separation experiments and western blot analysis (Fig. 4H). Moreover, compared to LspA-replete cells, both *lspA* and *lspA amgS*^{P381H} cells grown without arabinose showed a relevant proportion of IM proteins (i.e., LptC and XcpY) in the OM fraction (Fig. 4H). This denotes poor membrane separation, which often occurs in mutants exhibiting some degree of cell envelope instability (Lo Sciuto et al., 2018; Mychack et al., 2019; El Rayes et al., 2021). Overall, these data suggest that the *amgS*^{P381H} allele supports the growth of LspA-depleted cells without directly restoring lipoprotein maturation.

3.4. Growth of LspA-depleted cells relies on the AmgRS-regulated membrane proteins *hpx* and *yccA*

In the attempt to elucidate the mechanism by which AmgRS restores growth and partially rescues cell envelope functionality of LspA-depleted cells, we searched the literature to identify AmgRS-regulated genes that could potentially contribute to alleviating the detrimental effects of defective lipoprotein maturation. In *E. coli*, genetic inactivation or chemical inhibition of lipoprotein maturation enzymes leads to the accumulation of pro/apolipoproteins in the IM (Hussain et al., 1980; Mychack and Janakiraman, 2021; Diao et al., 2021; Legood et al., 2022). Interestingly, a similar effect is observed upon aminoglycoside treatment. Indeed, these antibiotics interfere with translation and increase the frequency of ribosome misreading, leading to the release of aberrant and misfolded proteins that can compromise the cytoplasmic membrane barrier (Davis et al., 1986; Davis, 1987; Lau et al., 2015). It has been shown that the role of AmgRS in aminoglycoside resistance is mainly due to the activity of three IM proteins (*HtpX*, *YccA*, and PA5528), whose expression is increased upon AmgRS activation (Lee et al., 2009; Hinz et al., 2011; Lau et al., 2015) (Fig. 5A). Although none of these proteins have been characterized in *P. aeruginosa*, the *E. coli* orthologs of *hpx* and *yccA* encode an IM protease and a modulator of the IM-anchored protease FtsH, respectively (Kihara et al., 1998; Sakoh et al., 2005). *HtpX* and *FtsH* play major roles in the proteolytic quality control of *E. coli* membrane proteins (Akiyama, 2009). In contrast, the role of PA5528 cannot be predicted, as it is annotated as a hypothetical protein with unknown function (<https://pseudomonas.com>).

To verify if *HtpX*, *YccA*, and PA5528 contribute to AmgRS-mediated rescue of LspA-depleted *P. aeruginosa* cells, we generated single, double, and triple deletion mutants in the corresponding genes in the *lspA amgS*^{P381H} conditional mutant (Table S1), and assessed their ability to grow in the presence or absence of arabinose. All the single mutants and

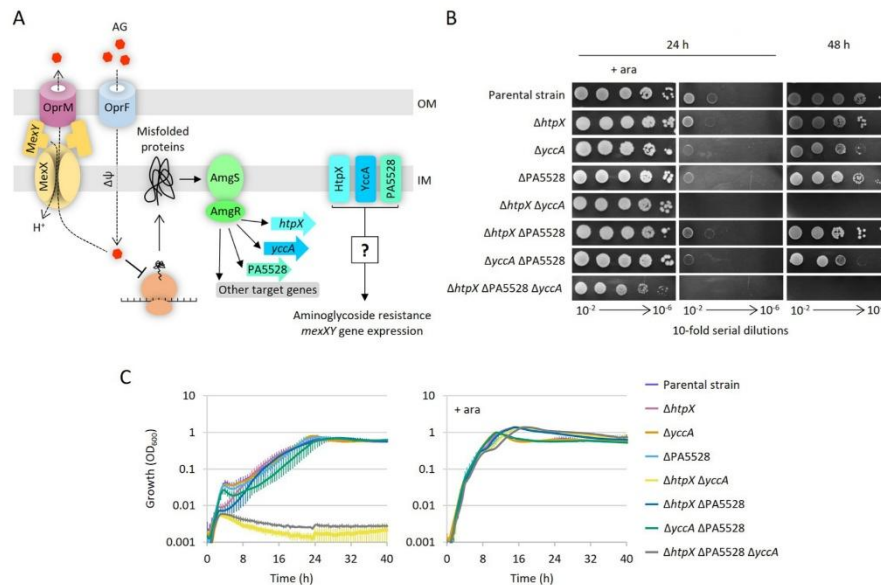


Fig. 5. (A) Schematic representation of the AmgRS signaling pathway in *P. aeruginosa* and its role in membrane stress adaptation and aminoglycoside resistance. (B) Plating efficiency of the conditional mutant PAO1 *araC* *P_{ara::lspA}* Δ *lspA* (parental strain) and its derivatives carrying single or multiple deletions in the *htpX*, *yccA*, and/or *PA5528* genes on MH agar plates supplemented or not with 0.5 % arabinose (+ ara). Images in panel B are representative of three experiments. (C) Growth curves of the same strains described in panel B cultured in MHI with or without 0.5 % arabinose (+ ara). Values are the mean ($\pm$ standard deviation) of three independent experiments.

the double mutants $\Delta htpX \Delta PA5528$ and $\Delta yccA \Delta PA5528$ did not show relevant growth differences with respect to the parental strain *lspA* *amgS*^{P381H} both in solid and liquid medium (Fig. 5B-C). In contrast, the growth of the double mutant $\Delta htpX \Delta yccA$ and of the triple mutant was abrogated in the absence of arabinose. Arabinose restored the growth of these mutants to levels comparable to the parental strain (Fig. 5B-C), indicating that the growth defects associated with the loss of both *HtpX* and *YccA* are specific to *LspA*-depleted cells. This result highlights *HtpX* and *YccA* as essential players of AmgRS-mediated rescue of *LspA*-depleted cells, leading us to speculate that these IM proteins somehow counteract the damage caused by impaired lipoprotein maturation in *P. aeruginosa*. However, ectopic overexpression of *HtpX* or *YccA* in the *lspA* conditional mutant was not able to restore growth under non-inducing conditions (Figure S6). This finding indicates that AmgRS-mediated induction of *htpX* and *yccA* gene expression is not sufficient to sustain the growth of *LspA*-depleted *P. aeruginosa* cells, implying that additional AmgRS-regulated factors are likely to contribute to this adaptive response.

4. Discussion

The experimental validation of gene essentiality and the investigation of the effects of the depletion or inactivation of essential proteins are critical for the selection of suitable targets for the rational design of antibacterial drugs (Hogan and Cardona, 2022). In this work, we focused on the lipoprotein-specific signal peptidase *LspA*, which was predicted to be essential in *P. aeruginosa* based on transposon random mutagenesis studies (Lee et al., 2015; Turner et al., 2015; Poulsen et al., 2019) and represents the target of both natural and synthetic antibacterials, which, however, are basically ineffective against *P. aeruginosa* (Gerth et al., 1982; Onishi et al., 1984; Kiho et al., 2003; Garland et al., 2020).

Here, we demonstrated that *LspA* depletion in *P. aeruginosa* causes

growth arrest, loss of cell viability, and severe defects in cell envelope integrity (Figs. 1 and 2), confirming the essentiality of this protein and its suitability as a target for the development of anti-*P. aeruginosa* compounds. A genetic screen for suppressor mutations able to restore the growth of *LspA*-depleted *P. aeruginosa* cells showed that the frequency at which these mutations occur is lower as compared to mutations supporting the growth of isogenic cells depleted of other essential proteins involved in either lipoprotein or LPS transport to the OM (Fig. 3). This suggests that off-target mutations that can compensate for growth-limiting levels (or activity) of *LspA* are rare in *P. aeruginosa*, further pointing out *LspA* as a promising antibacterial drug target.

The characterization of suppressor mutants revealed that activation of the TCS AmgRS can partly restore growth and cell envelope functionality in *LspA*-depleted cells (Fig. 4), and that this rescue effect is apparently related to increased expression of two membrane proteins (*HtpX* and *YccA*) likely involved in proteolytic control of membrane proteins (Fig. 5). It is important to emphasize, however, that this finding does not imply that *LspA* is dispensable for *P. aeruginosa* growth. Indeed, all our attempts to delete *lspA* from a strain with a constitutively active AmgRS system (PAO1 *amgS*^{P381H}) failed, indicating that AmgRS activation can support the growth of *LspA*-depleted cells but cannot overcome the essentiality of this protein. This is indirectly corroborated by membrane fractionation experiments showing that a representative OM protein is still partially localized in the OM in *LspA*-depleted cells (Fig. 4), suggesting that *LspA* depletion in the conditional mutant does not completely abrogate *LspA* activity.

AmgRS was originally identified for its role in intrinsic aminoglycoside resistance in *P. aeruginosa* (Lee et al., 2009; Lau et al., 2013). AmgRS controls the expression of dozens of genes, primarily involved in proteolysis and membrane transport, and has been proposed to play a role in the adaptive response to membrane stress, such as that caused by aminoglycoside-induced translational misreading (Lee et al., 2009). So, it is tempting to speculate that its activation in *LspA*-depleted cells

alleviates the damage caused by impaired lipoprotein processing and transport, rather than rescuing the defect in lipoprotein maturation itself. This hypothesis aligns with the observation that *P. aeruginosa* does not tolerate complete loss of LspA, even when AmgRS is induced. Interestingly, AmgRS-mediated aminoglycoside resistance requires three AmgRS-regulated IM proteins, a protease (HtpX), a modulator of FtsH activity (YccA), and a protein with unknown function (PA5528), as AmgRS was found to no longer confer aminoglycoside resistance in a *P. aeruginosa* triple mutant lacking these proteins (Hinz et al., 2011). In addition, HtpX and PA5528 are also necessary for AmgRS-mediated induction of the MexXY efflux pump, even if the underlying mechanism has not been elucidated (Lau et al., 2015). Overall, this body of evidence aligns with our finding that the concomitant loss of HtpX and YccA abolishes the growth-rescue effect of AmgRS activation in LspA-depleted cells (Fig. 5). Therefore, it can be argued that these membrane proteins contribute significantly to the stress response mediated by AmgRS. However, it remains unclear whether they directly mitigate the negative effects caused by LspA depletion or are somehow required to support the impact of AmgRS hyperactivation in LspA-depleted cells. The protease HtpX has been extensively characterized in *E. coli* (Sakoh et al., 2005; Yoshitani et al., 2019), but its physiological role and substrate specificity have not been investigated in *P. aeruginosa*. The possible function of YccA is even more elusive. Based on the observation that YccA is a substrate of the IM protease FtsH in *E. coli*, and that the expression of an FtsH-insensitive YccA variant can hamper FtsH proteolytic activity (Kihara et al., 1998), this protein has been proposed to function as an FtsH inhibitor (van Stelten et al., 2009). However, how YccA actually modulates FtsH in *E. coli* has not been clarified, and whether this putative function is conserved in *P. aeruginosa* is yet to be determined. Given this lack of information, any hypothesis about the mechanisms by which HtpX and YccA could make LspA depletion more tolerable for *P. aeruginosa* cells would be purely tentative without a thorough investigation of their biochemical activity and physiological role in this bacterium. Nevertheless, it is tempting to speculate that their activity might mitigate the detrimental effects of pro-/apolipoprotein accumulation in the IM resulting from impaired lipoprotein maturation (Hussain et al., 1980; Mychack and Janakiraman, 2021; Diao et al., 2021; Legood et al., 2022). It is also noteworthy that, while HtpX or YccA are required for AmgRS-mediated rescue of LspA-depleted cells, we observed that their overexpression alone is not sufficient to support the growth of these cells, strongly suggesting that other AmgRS-regulated genes are involved. Further studies will be needed to clarify the mechanism underlying the rescue effect mediated by HtpX and YccA in LspA-depleted *P. aeruginosa* cells and to identify the additional contributing factors.

5. Conclusion

Overall, this work experimentally confirms that LspA represents a promising target for novel anti-*P. aeruginosa* agents, as LspA is strictly essential for growth, and off-target mutations capable of compensating for growth-limiting levels of LspA appear to be comparatively rare. Moreover, LspA depletion causes severe defects in cell envelope integrity and increases antibiotic susceptibility, making LspA inhibition a valuable strategy also for the development of antibiotic adjuvants. However, activation of a TCS involved in membrane stress response (AmgRS) can alleviate the damage caused by LspA depletion in *P. aeruginosa*. This observation is consistent with previous studies showing that the TCSs CpxAR and BfmRS protect *E. coli* and *Acinetobacter baumannii*, respectively, from defects in OM lipoprotein biogenesis and/or transport (Grabowicz and Silhavy, 2017; May et al., 2019; Marotta et al., 2025). Even if these three TCSs are not orthologs, they have partially overlapping regulons and control envelope stress-related functions (Lee et al., 2009; Marotta et al., 2025). Thus, our finding further strengthens the link between envelope stress-responsive TCSs and lipoprotein maturation defects in Gram-negative bacteria, and

further highlights the importance of considering this aspect when evaluating the efficacy of antibacterials targeting lipoprotein maturation or transport.

CRedit authorship contribution statement

Davide Sposato: Conceptualization, Investigation, Validation, Visualization, Writing – original draft, Formal analysis. **Giorgia Molesini:** Conceptualization, Investigation, Validation, Formal analysis. **Christopher Riccardi:** Investigation, Data curation, Formal analysis. **Jessica Mercolino:** Investigation. **Luisa Torrini:** Investigation. **Ilaria Varone:** Investigation, Writing – original draft. **Manuela Cipolletti:** Investigation. **Filippo Acconcia:** Resources, Supervision, Writing – review & editing. **Giordano Rampioni:** Resources, Writing – review & editing. **Livia Leoni:** Resources, Writing – review & editing. **Paolo Visca:** Resources, Writing – review & editing, Funding acquisition. **Marco Fondi:** Data curation, Software, Supervision, Funding acquisition, Writing – review & editing. **Francesco Imperi:** Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Francesco Imperi reports financial support was provided by Italian Ministry of University and Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2026.100565.

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CHAPTER 8. Activation of the envelope stress-responsive two-component system AmgRS compensates for depletion of the essential lipoprotein signal peptidase LspA in *Pseudomonas aeruginosa*

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CHAPTER 8. Activation of the envelope stress-responsive two-component system AmgRS compensates for depletion of the essential lipoprotein signal peptidase LspA in *Pseudomonas aeruginosa*

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CHAPTER 9. CONCLUDING REMARKS

In this thesis novel insights into the molecular mechanisms involved in the assembly and the maintenance of the Gram-negative cell envelope were offered, thereby contributing to a better understanding of its structure and function.

First, a systematic investigation of AsmA-like proteins in the Gram-negative bacterium *P. aeruginosa* was provided, which revealed the presence of four essential members of this protein family. Three of these are homologous to the previously characterized *E. coli* proteins (*i.e.* TamB, YhdP and YdbH), and are functionally conserved in *P. aeruginosa*, as well as their relative contribution to GPL anterograde transport and OM homeostasis, with YhdP playing a more critical role than TamB and YdbH. Notably, we also identified an additional AsmA-like protein absent in *E. coli*, which we named EpaF, that is involved in the same functional pathway as the other essential AsmA-like proteins, thereby expanding the AsmA-like protein network involved in OM homeostasis. Through the generation of YhdP structural variants and YdbH-YhdP hybrid proteins, we showed that the C-terminal hydrophobic α -helices of YhdP are essential for its function in maintaining OM integrity, likely by mediating membrane interaction and/or facilitating GPL insertion into the OM. We demonstrated that the function of *P. aeruginosa* YdbH depends on the OM lipoprotein YnbE, but this requirement can be bypassed by the fusion of YdbH with the C-terminal hydrophobic region of YhdP, provided the fusion protein is long enough to span the periplasmic space. Notably, both *P. aeruginosa* YhdP and the long YdbH-YhdP fusion remain active in the heterologous host *E. coli*, supporting the idea that the C-terminal hydrophobic region of YhdP carries intrinsic, transferable properties crucial for AsmA-like protein function in Gram-negative bacteria. The combination of this hydrophobic region and the overall protein length compatible with a trans-periplasmic bridge likely underlies the dominant role of YhdP in maintaining OM homeostasis relative to other AsmA-like proteins. Overall, our findings are consistent with a model in which the hydrophobic C-terminus of YhdP promotes interaction with the OM, potentially enabling GPL delivery in the absence of additional protein partners. Despite the progress achieved in this thesis in defining the role of AsmA-like proteins in OM homeostasis, key mechanistic aspects of their function remain unresolved, including the biological significance of AsmA-like protein redundancy. Multiple members of this family coexist within the same organism and contribute to a shared functional pathway, yet with unequal importance. This redundancy may provide robustness to OM biogenesis, ensuring continuity of phospholipid transport under dynamic environmental conditions, although different AsmA-like proteins may be specialized for distinct physiological contexts or substrates. One intriguing possibility is that this diversification reflects lipid species-specificity. Gram-negative bacteria produce heterogeneous phospholipid populations that vary in headgroup composition and acyl chain properties, and it is

conceivable that individual AsmA-like proteins preferentially bind or transport specific GPL species, thereby fine-tuning OM composition and asymmetry. If so, what appears as redundancy may in fact represent functional specialization embedded within a shared transport framework. Future studies addressing lipid-binding specificity and structural determinants of substrate selection will be crucial to distinguish between true redundancy and species-specific specialization within the AsmA-like protein family. In addition to the essential AsmA-like proteins, the *P. aeruginosa* genome encodes other AsmA-like proteins whose roles remain unclear. Based on the assays performed in our studies, these proteins do not appear to play a major role in OM biogenesis or homeostasis under the conditions tested, although their divergence and conservation suggest they may fulfill context-dependent or yet unidentified functions. Finally, it is still unclear how GPLs are extracted from IM and loaded onto these proteins prior to transport toward the OM. Whether AsmA-like proteins directly extract phospholipids from the OM bilayer, receive them from yet unidentified accessory factors, or interact with dedicated lipid-donating complexes remains to be determined. Clarifying how GPLs are initially captured and subsequently delivered across the periplasm represents a central challenge for understanding this transport pathway. It has been proposed that proteins belonging to the DedA family proteins may act as functional partners of AsmA-like proteins, assisting in the extraction of GPLs from the IM. This hypothesis largely stems from evidence that eukaryotic homologs of DedA and AsmA-like proteins physically interact and cooperate in inter-organelle lipid trafficking, particularly during lipid transfer from the endoplasmic reticulum to the autophagosomal membrane. However, our observation that deletion of individual DedA proteins did not affect the growth of *P. aeruginosa* mutant strains whose viability depends on a single essential AsmA-like protein challenges this hypothesis, suggesting that DedA-mediated GPL transport is unlikely to represent the mechanism by which bacterial AsmA-like proteins extract GPLs from the IM. While the involvement of other IM proteins interacting with AsmA-like proteins and assisting in GPL uptake cannot be excluded, it is worth noting that molecular dynamics simulations predict YhdP to be capable of extracting GPLs from model membranes. This observation supports the idea that GPLs may enter the periplasmic groove of AsmA-like proteins independently of protein partners, potentially driven by GPL concentration gradients across the cell envelope. Future biochemical and biophysical studies will be required to clarify this aspect.

Our further studies on DedA family proteins led to the identification of two key players in Und-P recycling in *P. aeruginosa*, namely PA4029 and PA4011. On one hand, we observed that deletion of PA4029 increased sensitivity to the Und-P *de novo* synthesis-targeting antibiotic fosmidomycin and reduced *P. aeruginosa* ability to develop colistin resistance. In *P. aeruginosa* colistin resistance is usually linked to the addition of L-Ara4N to lipid A, which requires Und-P as lipid carrier for the

transport of the precursor. In this context, absence of PA4029 may reduce precursor flux, limiting the supply of lipid-linked intermediates, thus preventing L-Ara4N lipid A modification in favor of essential processes that require Und-P, such as peptidoglycan biosynthesis, thereby explaining the observed reduced capacity of evolve colistin resistance of Δ PA4029 cells. In addition, biochemical analyses confirmed that PA4029 binds Und-P in a concentration-dependent manner, and that this interaction can be disrupted by the Und-P-targeting antibiotic amphomycin. Moreover, binding to Und-P was clearly favored in mixed-lipid assays with other phospholipids, supporting the hypothesis that PA4029 is not a general lipid transporter but has high specificity for Und-P. Taken together, these findings place PA4029 within the DedA-mediated Und-P recycling pathway and underscore its importance for cell envelope homeostasis. Future work should focus on the direct *in vitro* reconstitution of flipping activity, as well as on high-resolution structural studies of PA4029 in complex with C55-P. On the other hand, PA4011 appears to play a key role in Und-P recycling by functioning as an Und-PP phosphatase. In addition to a DedA domain, PA4011 contains a PAP2-like domain homologous to those of the *E. coli* phosphatases YbjG, PgpB, and LpxT, which are known to participate in Und-PP dephosphorylation, together with UppP. Consistent with this functional relationship, deletion of the *P. aeruginosa* *uppP* ortholog in the Δ PA4011 background increased sensitivity to EDTA and fosmidomycin and caused growth arrest at 25°C, a condition under which *lpxT* expression is strongly reduced. Moreover, depletion of LpxT by conditional mutagenesis completely abolished growth of the Δ PA4011 Δ *uppP* strain. These genetic interactions indicate that PA4011, UppP, and LpxT contribute to a shared essential function, most likely the dephosphorylation of Und-PP, and that loss of multiple components of this pathway critically compromises Und-P availability. Finally, structure-function analyses based on PA4011 variants carrying amino acid substitutions in putative catalytic residues, as well as constructs expressing either the DedA or the PAP2-like domain alone, confirmed that PA4011 activity primarily relies on its phosphatase domain and suggested that it is independent of the DedA domain.

While our genetic and physiological data strongly support a role for PA4011 in Und-PP dephosphorylation, definitive confirmation of this function will require *in vitro* biochemical assays directly demonstrating its catalytic activity toward Und-PP. Moreover, the specific contribution of the DedA domain to PA4011 function remains unclear. Our results show that the PAP2-like domain retains its function even in the absence of the DedA domain, indicating that the latter is not essential for enzymatic activity or for proper folding of the PAP2-like domain in the inner membrane. Notably, overexpression of the wild-type PA4011 is highly toxic, whereas the PAP2-like domain is not. Although this result should be interpreted with caution, it may indicate that the DedA domain of PA4011 is unlikely to be a mere evolutionary remnant but may instead have a biological function in

P. aeruginosa cells. Similarly, the physiological roles of other DedA family proteins remain unresolved, as their deletion did not result in detectable phenotypes under the conditions tested, suggesting that these proteins may have context-dependent or distinct functions unrelated to Und-P(P) recycling.

Finally, we identified the lipoprotein signal peptidase LspA as an essential and promising antibacterial target in *P. aeruginosa*. Depletion of LspA caused growth arrest, loss of viability, and severe defects in cell envelope integrity, confirming its suitability for anti-*P. aeruginosa* drug development. We observed that mutations compensating for growth-limiting levels of LspA arise at a lower frequency than for other essential envelope-related proteins, indicating limited potential for resistance through off-target mutations. However, we uncovered that activation of the envelope stress-responsive two-component system AmgRS partially alleviates the defects caused by LspA depletion, yet it remains insufficient to bypass LspA essentiality, as complete deletion of *lspA* remained lethal. These findings suggest that AmgRS mitigates envelope stress resulting from impaired lipoprotein maturation rather than restoring lipoprotein processing itself.

This finding aligns with previous studies indicating that the TCSs CpxAR and BfmRS help protect *E. coli* and *A. baumannii*, respectively, from defects in OM lipoprotein biogenesis or transport. Although these three TCSs are not direct orthologs, they share partially overlapping regulons and govern functions related to envelope stress. Consequently, our results reinforce the connection between envelope stress-responsive TCSs and lipoprotein maturation defects in Gram-negative bacteria, emphasizing the critical need to account for this factor when assessing the effectiveness of antibacterials targeting lipoprotein maturation or transport.

APPENDIX. Manuscripts I co-authored that are not directly related to the main topic of my PhD thesis

During my PhD, I also worked on a side project aimed at characterizing the *P. aeruginosa* *apaH* gene, which resulted in a publication in which I am a co-first author (“The diadenosine tetraphosphate hydrolase ApaH contributes to *Pseudomonas aeruginosa* pathogenicity”, published in PLoS Pathogens in 2024). ApaH encodes a hydrolase of diadenosine tetraphosphate (Ap₄A), a compound with a still poorly understood role in bacteria. While Ap₄A production generally increases under stress conditions, its classification as either a second messenger or a damage metabolite remains debated. In this study, we demonstrated that the absence of ApaH, which leads to intracellular accumulation of Ap₄A, impaired *P. aeruginosa* growth under iron-depleted conditions and caused a decrease in the production of various virulence factors, both in laboratory and clinical strains. Most importantly, *apaH* deletion reduced the bacterium’s ability to cause infection in different models, including a murine pulmonary infection model. Taken together, these results highlighted ApaH as a promising target for the development of anti-virulence compounds.

Finally, during the last months of my PhD, I also participated in a collaboration with the group of Prof. Federica Briani (Department of Biosciences, University of Milan) for a study aimed at investigating the adsorption mechanisms of the *P. aeruginosa* bacteriophage DEV, leading to the identification of the essential OMP LptD as the phage secondary receptor (“*Pseudomonas aeruginosa* DEV phage exploits the essential LptD outer membrane protein as receptor for adsorption”, accepted for publication in mBio on December 15th, 2025). My contribution to this work consisted in the generation of *P. aeruginosa* mutants expressing LptD variants lacking specific extracellular loops. These mutants were instrumental for confirming the interaction interface between LptD and the phage receptor-binding proteins and assessing the effects of LptD alterations on phage adsorption efficiency.

Both these works are provided in this Appendix.

PLOS PATHOGENS

RESEARCH ARTICLE

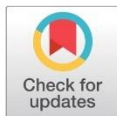
The diadenosine tetraphosphate hydrolase ApaH contributes to *Pseudomonas aeruginosa* pathogenicity

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Abstract

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* causes a wide range of infections that are difficult to treat, largely because of the spread of antibiotic-resistant isolates. Antivirulence therapy, *i.e.* the use of drugs that inhibit the expression or activity of virulence factors, is currently considered an attractive strategy to reduce *P. aeruginosa* pathogenicity and complement antibiotic treatments. Because of the multifactorial nature of *P. aeruginosa* virulence and the broad arsenal of virulence factors this bacterium can produce, the regulatory networks that control the expression of multiple virulence traits have been extensively explored as potential targets for antivirulence drug development. The intracellular signaling molecule diadenosine tetraphosphate (Ap4A) has been reported to control stress resistance and virulence-related traits in some bacteria, but its role has not been investigated in *P. aeruginosa* so far. To fill this gap, we generated a mutant of the reference strain *P. aeruginosa* PAO1 that lacks the Ap4A-hydrolysing enzyme ApaH and, consequently, accumulates high intracellular levels of Ap4A. Phenotypic and transcriptomic analyses revealed that the lack of ApaH causes a drastic reduction in the expression of several virulence factors, including extracellular proteases, elastases, siderophores, and quorum sensing signal molecules. Accordingly, infection assays in plant and animal models demonstrated that ApaH-deficient cells are significantly impaired in infectivity and persistence in different hosts, including mice. Finally, deletion of *apaH* in *P. aeruginosa* clinical isolates demonstrated that the positive effect of ApaH on the production of virulence-related traits and on infectivity is conserved in *P. aeruginosa*. This study provides the first evidence that the Ap4A-hydrolysing enzyme ApaH is important for *P. aeruginosa* virulence, highlighting this protein as a novel potential target for antivirulence therapies against *P. aeruginosa*.

OPEN ACCESS

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files. RNA-seq raw data have been deposited in the NCBI Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE255863>). These data will be made public when the manuscript is accepted for publication.

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Author summary

Novel therapeutic interventions are urgently needed to address the threat of antimicrobial resistance. Inhibition of the virulence potential of bacterial pathogens, the so called anti-virulence approach, is an alternative or complementary strategy to standard antibiotic-based therapies. The identification of suitable targets is essential to promote and advance antivirulence drug discovery programs. By combining *in vitro* analyses and *in vivo* infection assays, we demonstrated that the enzyme ApaH, which is responsible for the degradation of the intracellular signaling molecule diadenosine tetraphosphate, contributes to the virulence of the important human pathogen *Pseudomonas aeruginosa*. Indeed, loss of ApaH drastically reduces the production of several virulence factors in both laboratory and clinical isolates, and impairs *P. aeruginosa* ability to cause infection in plant, insect and rodent models. These results, together with the absence of ApaH homologues in mammals, make ApaH a promising target for the development of anti-*P. aeruginosa* anti-virulence drugs.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium widely studied for its pathogenic nature, particularly in the context of opportunistic infections in hospitalized, immunocompromised and cystic fibrosis (CF) individuals [1]. A recent study associated *P. aeruginosa* with more than 500,000 deaths in 2019 globally [2]. The bacterium is well known for its metabolic plasticity, capability to thrive in many different environments, large arsenal of virulence factors, and resistance to multiple antibiotics [3–5]. The latter is due to inherent low membrane permeability and expression of several efflux pumps, as well as to the ability to readily acquire new resistance determinants via mutations or horizontal gene transfer [6,7].

Successfully thriving in diverse environments requires complex regulatory networks that help bacterial cells perceive the environment and adapt their behavior. *P. aeruginosa* has a large genome (6.3 Mb for the reference strain PAO1), 10% of which is dedicated to regulatory networks, reflecting its high versatility and the wide range of environments and hosts it can inhabit [8]. The contribution of quorum sensing (QS) systems, two-component systems, and nucleotide-based second messengers to *P. aeruginosa* stress response, metabolic versatility, switch from planktonic to biofilm lifestyle, and virulence gene regulation has been extensively investigated [3,9–11]. Concerning the roles of nucleotide-based second messengers in *P. aeruginosa*, the stringent response alarmone guanosine tetraphosphate (ppGpp) promotes survival under nutrient starvation and antibiotic tolerance [12], cyclic di-GMP (c-di-GMP) is the primary intracellular signal that controls biofilm development [13], while cyclic AMP triggers virulence gene expression in response to surface sensing [14]. Notably, to date the role of another widely distributed nucleotide-based second messenger, *i.e.* diadenosine tetraphosphate (Ap4A), has not been explored in this bacterium.

Ap4A (or Ap3A) has been found in all kingdoms of life and consists of two adenosines linked by a polyphosphate chain containing four (or three) phosphates [15,16]. Ap4A is primarily generated as a side product of aminoacyl-tRNA synthetase activity [17] and its levels increase in response to several types of stress [18]. Ap4A intracellular homeostasis relies on the activity of Ap4A-hydrolyzing enzymes, such as the diadenosine tetraphosphatase ApaH or members of the versatile family of Nudix hydrolases [18–20]. ApaH appears to play a prominent role in Ap4A homeostasis in Gram-negative bacteria [18,21]. Since its discovery decades

ago, there has been debate about the function of Ap4A, leading to the alternative hypotheses that Ap4A may be either a damage metabolite or an intracellular signaling molecule [18,22].

Several studies have demonstrated that Ap4A has pleiotropic effects in bacteria. In the model organism *Escherichia coli*, Ap4A has been found to affect the timing of cell division [23], repress motility [24], increase sensitivity to heat and oxidative stress [25], and reduce tolerance to aminoglycoside antibiotics [26,27]. Regarding other bacteria, Ap4A has a negative impact on sporulation in *Myxococcus xanthus* [28], reduces oxidative stress resistance in *Helicobacter pylori* [29], and limits invasion of mammalian cells by *Salmonella enterica* [21], while it promotes biofilm formation in *Pseudomonas fluorescens* [30].

By combining genetic, transcriptomic, and phenotypic *in vitro* analyses with *in vivo* infection assays, in this study we demonstrate that ApaH inactivation in *P. aeruginosa* leads to intracellular accumulation of Ap4A and drastically reduces virulence gene expression and pathogenicity. This work paves the way for the investigation of regulatory mechanisms underlying Ap4A-mediated control of *P. aeruginosa* virulence and for the exploitation of ApaH as a novel potential target for antivirulence drug discovery.

Results and discussion

Effect of Ap4A accumulation on *P. aeruginosa* growth and biofilm formation

In order to verify the influence of Ap4A in *P. aeruginosa* physiology, we generated a clean *apaH* deletion mutant in the reference strain PAO1. Growth assays in the rich medium Lysogeny broth (LB) showed that the *apaH* mutant has slightly reduced growth rates (doubling time of 37 and 40 minutes for the wild type and mutant, respectively) and anticipates the entry into the stationary phase, reaching cell densities 2.3-fold lower than the wild type strain (Fig 1A). Mass spectrometry analysis showed that Ap4A levels in cell extracts are 25-fold higher in the *apaH* mutant with respect to the wild type strain (Fig 1B). The ectopic expression of *apaH* from the pMEapaH plasmid in the *apaH* mutant restored both growth and Ap4A intracellular concentration at wild type levels (Figs 1A, 1B and S1). It was reported that Ap4A promotes biofilm formation in *P. fluorescens*, in line with the increase in c-di-GMP intracellular levels observed in ApaH-deficient *P. fluorescens* cells [30]. We therefore investigated the impact of Ap4A on biofilm formation in *P. aeruginosa*. Surprisingly, we did not observe any difference

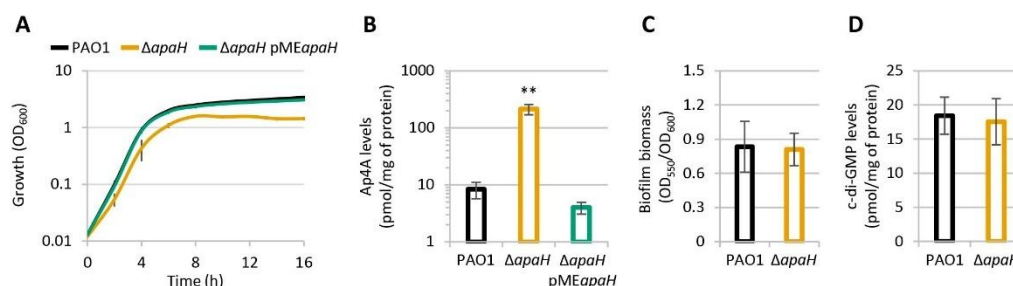


Fig 1. (A) Growth curves and (B) intracellular Ap4A levels of the wild type strain *P. aeruginosa* PAO1 and the isogenic deletion mutant *ΔapaH*, carrying or not the pMEapaH plasmid, cultured in flasks at 37°C in LB, supplemented with 100 μM IPTG in the case of *ΔapaH* pMEapaH. The control strains with the empty plasmid pME6032 are shown in S1 Fig. (C) Biofilm formation of PAO1 and *ΔapaH* in LB in 96-well polystyrene microtiter plates after 24-h incubation at 37°C. (D) Intracellular c-di-GMP levels of PAO1 and *ΔapaH* cultured in LB at 37°C. Cells for Ap4A and c-di-GMP quantification were collected after 12 h of growth. Values are the mean (± standard deviation) of at least three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.001$) with respect to PAO1 (ANOVA).

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in either biofilm formation or c-di-GMP intracellular levels between wild type and $\Delta apaH$ cells (Fig 1C and 1D). Overall, these experiments demonstrate that the lack of ApaH causes a marked increase in the Ap4A intracellular levels also in *P. aeruginosa* and that Ap4A accumulation has a negative impact on planktonic growth in this bacterium. In contrast, increased Ap4A levels do not appear to affect biofilm formation or c-di-GMP homeostasis in *P. aeruginosa*, suggesting that the cellular pathways affected by Ap4A may differ significantly also between closely related species. Notably, a slight 20% decrease in the ADP levels was observed in the $\Delta apaH$ mutant with respect to the wild type, while ATP levels were comparable between the two strains (S2 Fig). The effect of ApaH depletion on ADP levels seems to be specific, as no differences were observed in GDP and GTP levels (S2 Fig). This result is in line with the activity of ApaH as a symmetric diadenosine tetraphosphatase which cleaves Ap4A in two ADP molecules [19,20], and also suggests that Ap4A accumulation has a minor impact on the energy status of the cells.

Ap4A does not affect *P. aeruginosa* susceptibility to antibiotics

Previous works showed that *apaH* inactivation in *P. aeruginosa* (i) slightly induces the expression of the colistin resistance gene *eptA*, without however affecting colistin resistance [31], and (ii) makes *P. aeruginosa* cells harboring an aminoglycoside resistance plasmid more susceptible to killing by millimolar concentrations of kanamycin [26]. To verify whether Ap4A has a general effect on antibiotic resistance in *P. aeruginosa*, the resistance profile of PAO1 and $\Delta apaH$ cells was compared through the Kirby-Bauer disc diffusion assay. As shown in Fig 2A, *apaH* inactivation and, thus, Ap4A accumulation does not increase sensitivity to any of the antibiotics tested, including clinically relevant antibiotics used to treat *P. aeruginosa* infections (i.e., ciprofloxacin, imipenem, tobramycin, gentamycin) or antibiotics that are only active against *P. aeruginosa* mutants with defects in cell envelope integrity (i.e., novobiocin, erythromycin, rifampicin) [32]. It was previously reported that Ap4A can enhance the bactericidal activity of aminoglycoside antibiotics in *E. coli* and, likely, other bacteria [26]. Therefore, we performed time-kill assays for two aminoglycosides, namely kanamycin and gentamicin. ApaH-deficient cells showed a slightly delayed killing by kanamycin with respect to wild type cells, while the susceptibility of the wild type and $\Delta apaH$ strains to gentamicin killing was comparable (Fig 2B). Overall, these results argue against a relevant contribution of Ap4A in intrinsic antibiotic resistance in *P. aeruginosa*. The discrepancy between our and previous results [26] regarding kanamycin sensitivity is possibly due to the different genetic backgrounds and experimental conditions, as we used the wild type strain PAO1, while Ji and coauthors used PAO1 carrying an aminoglycoside resistance plasmid challenged with a very high kanamycin concentration (1,000 $\mu\text{g/mL}$) [26].

ApaH inactivation widely affects *P. aeruginosa* gene expression

To investigate the possible role of Ap4A in *P. aeruginosa* physiology, we compared the transcriptional profiles of wild type and $\Delta apaH$ cells cultured to mid-exponential phase in LB by means of RNA sequencing (RNA-seq). Following statistical validation of the dataset, 1,280 differentially regulated genes (DEGs) with a fold change (FC) $\geq \pm 2.0$ and an adjusted *P* value < 0.05 were identified (S1 Table), corresponding to 22.3% of the PAO1 genome (<https://pseudomonas.com>) [33]. Of these, 799 were downregulated in ApaH-deficient cells (S1 Table). This indicates that the rise in the Ap4A intracellular level caused by *apaH* deletion has a huge impact on the *P. aeruginosa* transcriptome, with most of the DEGs being repressed by Ap4A (62.4%). By applying more stringent thresholds (i.e., FC $\geq \pm 3.0$ or $\geq \pm 4.0$) the number of DEGs drops to 477 and 267, respectively (S1 Table), highlighting that Ap4A has only a

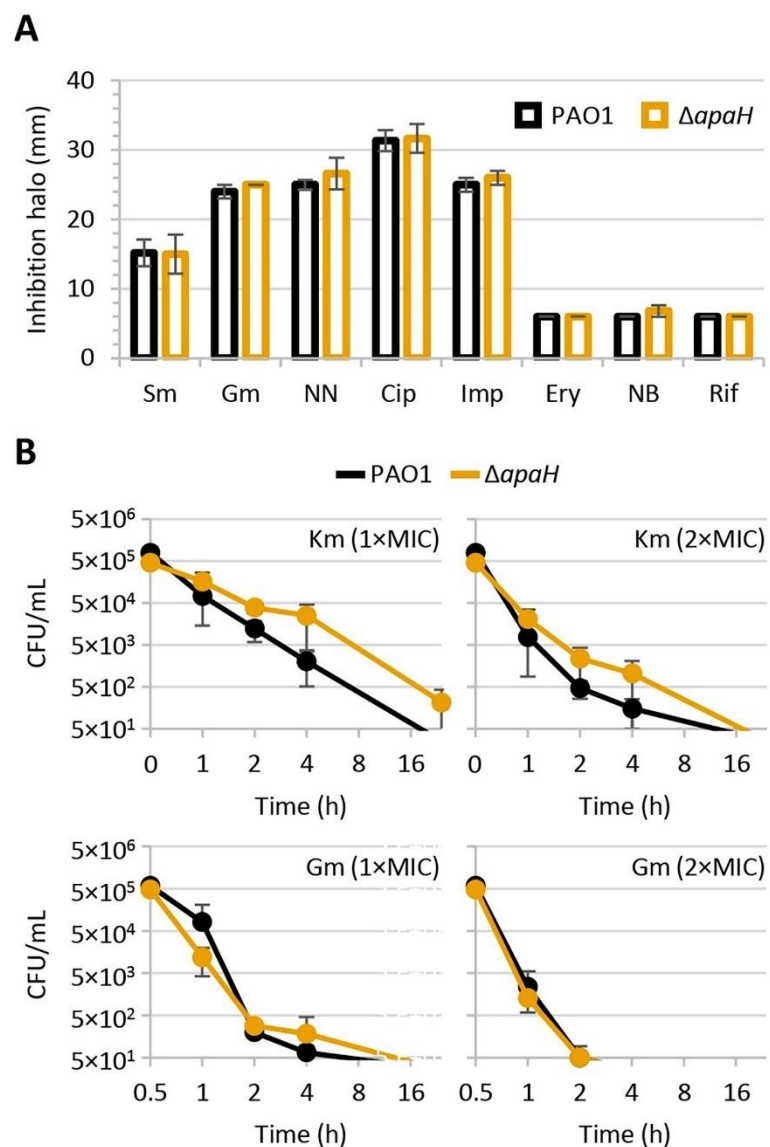


Fig 2. (A) Inhibition halos of streptomycin (Sm), gentamicin (Gm), tobramycin (NN), ciprofloxacin (Cip), imipenem (Imp), erythromycin (Ery), novobiocin (NB), or rifampicin (Rif) for *P. aeruginosa* PAO1 and the $\Delta apaH$ mutant in the Kirby-Bauer disc diffusion assay. (B) Time-kill curves of PAO1 and $\Delta apaH$ in the presence of kanamycin (Km) at 32 and 64 $\mu\text{g/mL}$ or Gm at 0.5 and 1 $\mu\text{g/mL}$, corresponding to 1× and 2×MIC for both strains. Values are the mean ($\pm$ standard deviation) of at least three independent assays.

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moderate effect on the transcription of most DEGs. Considering that *apaH* deletion slightly affects growth (Fig 1A), it is likely that some of these DEGs are affected indirectly due to changes in the physiological and/or metabolic status of the cells upon AP4A intracellular accumulation.

According to PseudoCAP annotations (<https://pseudomonas.com>), most of the highly affected DEGs ($FC \geq \pm 4.0$) are involved in virulence factors production, stress response, and translation (Fig 3). In particular, many genes important for *P. aeruginosa* pathogenicity were downregulated in ApaH-deficient cells, including those encoding extracellular proteases (*i.e.*, AprA, LasA, LasB, and protease IV), pyocyanin biosynthetic enzymes, type IV pili, the adhesin LecA, and enzymes responsible for the synthesis of the *pqs* QS signal molecules (PQS and HHQ) (S1 Table). Moreover, genes involved in iron uptake (*e.g.*, those responsible for the synthesis and transport of the two siderophores pyoverdine and pyochelin) were downregulated, while those involved in iron storage (*e.g.*, bacterioferritin genes) were upregulated in the *apaH* mutant relative to PAO1 (S1 Table), suggesting that ApaH deficiency and/or Ap4A accumulation dysregulate intracellular iron homeostasis. Among DEGs involved in translation, it is worth noting that almost all genes encoding ribosomal proteins were downregulated in the *apaH* mutant (S1 Table), suggesting that Ap4A might have a negative impact on protein translation. In this view, it is surprising that ApaH-proficient and -deficient cells showed comparable susceptibility to inhibitors of protein synthesis, such as the aminoglycosides gentamicin, kanamycin, streptomycin, and tobramycin (Fig 2). While most DEGs were downregulated in the *apaH* mutant (53 upregulated vs 214 downregulated genes at the $FC \geq \pm 4.0$ threshold), energy metabolism was the only functional category for which the number of up- and downregulated DEGs was comparable (Fig 3 and S1 Table), suggesting that intracellular Ap4A accumulation may induce some metabolic shifts in *P. aeruginosa*.

ApaH inactivation suppresses *P. aeruginosa* virulence traits and reduces heat tolerance

The RNA-seq analysis highlighted a relevant downregulation of many virulence genes in the *apaH* deletion mutant. We attempted to validate the transcriptomic data by comparing the

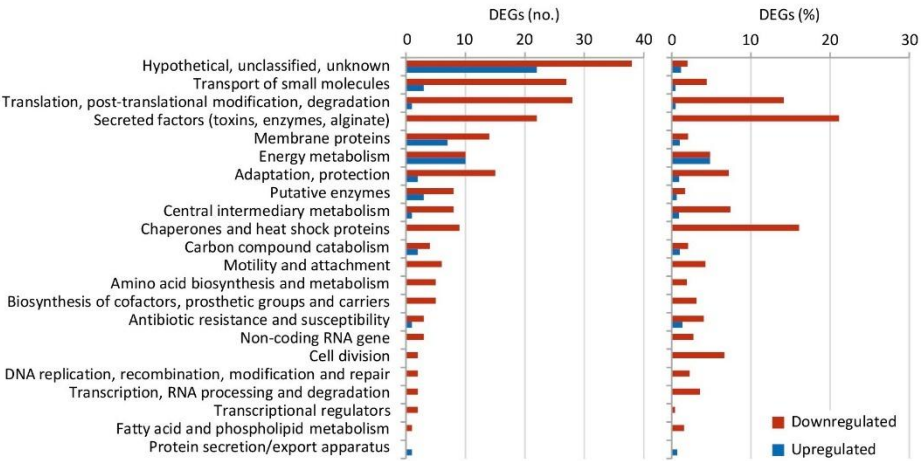


Fig 3. Functional analysis of differentially expressed genes (DEGs). Number (left panel) and relative percentage (right panel) of downregulated and upregulated genes (red and green bars, respectively) in the *apaH* mutant compared to PAO1 in the PseudoCAP functional categories listed on the left. Only DEGs with a fold change $\geq \pm 4$ were considered (S1 Table).

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production of selected virulence factors between the wild type and $\Delta apaH$ strains. The production of proteases, elastase and *pqs* signal molecules was almost completely abrogated in ApaH-deficient compared to wild type cells (Fig 4A–4C). To confirm that the effect of *apaH* deletion on the *pqs* QS system was specific, we also quantified the QS signal molecules of the *las* and *rhl* QS systems, whose synthase genes (*i.e.*, *lasI* and *rhlI*) were not identified among DEGs by RNA-seq (S1 Tables). No significant differences were observed in *las* and *rhl* signal production between wild type and $\Delta apaH$ cells (S3 Fig).

As discussed above, many genes involved in iron metabolism were dysregulated in ApaH-deficient cells. Accordingly, the *apaH* mutant was strongly impaired in growth in an iron-poor minimal medium (CAA), unless an excess of iron was exogenously provided (Fig 4D). This result suggests that the growth defect of the *apaH* mutant in CAA is primarily due to its inability to efficiently acquire and/or use iron. The production of the siderophore pyoverdine was indeed significantly reduced in cells lacking ApaH (Fig 4E), providing further phenotypic validation of RNA-seq data. Interestingly, increasing concentrations of iron promoted growth in a similar way in the $\Delta apaH$ and parental strains (S4 Fig). This implies that, although probably defective in iron acquisition, ApaH-deficient cells are still able to sense and utilize iron for cell metabolism. Notably, since the QS signal molecule PQS can bind iron and influence iron uptake (reviewed in 34), it cannot be excluded that the reduced production of PQS could at least in part account for the dysregulated iron metabolism observed in *P. aeruginosa* cells lacking ApaH.

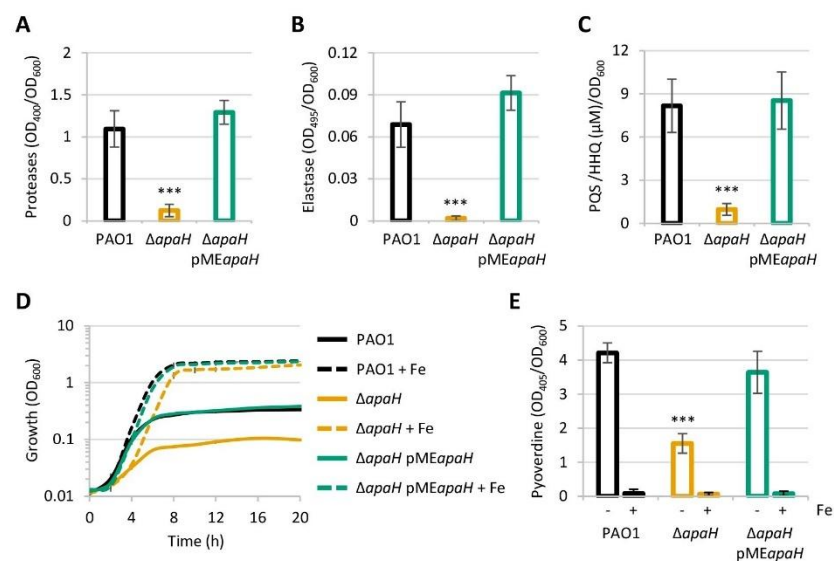


Fig 4. (A) Protease activity, (B) elastase activity, and (C) PQS/HHQ levels, normalized to cell density (OD₆₀₀), in the supernatants of *P. aeruginosa* PAO1 and the *apaH* mutant, carrying or not the pME_{apaH} plasmid, cultured at 37°C in LB, supplemented with 100 μ M IPTG in the case of $\Delta apaH$ pME_{apaH}. (D) Growth curves and (E) pyoverdine levels, normalized to cell density (OD₆₀₀), of the same strains cultured at 37°C in the iron-poor medium CAA, supplemented with 50 μ M FeCl₃ when indicated (+ Fe), and 100 μ M IPTG in the case of $\Delta apaH$ pME_{apaH}. Values are the mean ($\pm$ standard deviation) of at least three independent assays. Asterisks indicate a statistically significant difference ($P < 0.001$) with respect to PAO1 (ANOVA). The control strains with the empty plasmid pME6032 are shown in S6 Fig.

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RNA-seq analysis also revealed that many genes encoding chaperones and other heat shock proteins are downregulated in ApaH-deficient *P. aeruginosa* cells (S1 Table and Fig 3). Accordingly, we found that these cells are more sensitive to heat shock than wild type cells (S5 Fig), as previously observed in *E. coli* [25]. In contrast, while Ap4A accumulation was reported to increase susceptibility to oxidative stress in some bacteria [25,29], we did not observe dysregulation of the main oxidative stress response genes (S1 Table) or decrease in resistance to hydrogen peroxide or the superoxide-generating agent paraquat in the *P. aeruginosa* *apaH* mutant (S2 Table), further confirming that the effects of Ap4A may vary among different bacterial species.

As expected, the production of virulence factors and *pqs* signal molecules, as well as the ability to thrive under iron-depleted conditions and to tolerate heat shock, was restored to wild type levels by ectopic expression of *apaH* in Δ *apaH* cells (Figs 4, S5 and S6), confirming that the defective phenotypes were due to ApaH deficiency and/or Ap4A intracellular accumulation.

ApaH inactivation reduces *P. aeruginosa* pathogenicity in different infection models

Since ApaH is required for full expression of several *P. aeruginosa* virulence genes *in vitro*, this enzyme could represent a novel target to reduce *P. aeruginosa* pathogenicity. To test this hypothesis, we assessed the pathogenicity of ApaH-deficient cells in well-established plant and animal models, including lettuce leaves [35], *Galleria mellonella* insect larvae [36], and a mouse model of pulmonary infection [37].

In the lettuce leaf infection assay, the wild type strain caused severe rot (black coloration) of the entire midrib, while the Δ *apaH* strain induced some signs of tissue damage only at the inoculation site. Moreover, determination of colony forming units (CFUs) in infected midribs showed a 2-log reduction of bacterial load for the *apaH* mutant compared to the wild type strain (Fig 5A), indicative of impaired growth and/or persistence of ApaH-deficient cells *in planta*.

The *apaH* mutant also showed reduced infectivity in *G. mellonella* larvae, with a lethal dose 90% (LD₉₀) almost 5-fold higher than that of the wild type strain (Fig 5B). Accordingly, when the same (lethal) dose was injected, lethality was significantly delayed in larvae infected with ApaH-deficient cells with respect to those infected with wild type cells (S7 Fig).

Finally, the Δ *apaH* mutant was much less virulent than the wild type in a mouse model of acute lung infection, as 80% of mice infected with the mutant survived and fully recovered, while 100% of those infected with the wild type strain died (Fig 5C). This result prompted us to investigate the capability of Δ *apaH* mutant cells to persist in and spread from mice lungs. To this aim, mice were intra-tracheally infected and sacrificed 24 h post infection. Wild type infected mice showed high bacterial loads in the lungs and also in the spleen, indicative of efficient bacterial dissemination in the bloodstream. In contrast, mice infected with the *apaH* mutant showed lower persistence in the lungs and poor systemic dissemination (Fig 5D). No differences were observed in the number of leukocytes in bronchoalveolar lavage fluids between wild type- and Δ *apaH*-infected mice, including neutrophils (that are first responder immune cells following acute infections), macrophages (that have key roles in pathogen recognition), and total lymphocytes (that are mediators of cellular adaptive immunity during infections) (Fig 5E). This suggests that the immune system efficiently recognizes ApaH-deficient cells and that wild type cells better escape the host inflammatory response, reaching higher cell densities in the lungs and better disseminating in secondary organs like the spleen.

In conclusion, these *in vivo* assays demonstrate that the lack of ApaH severely impairs *P. aeruginosa* PAO1 persistence and infectivity in various infection models.

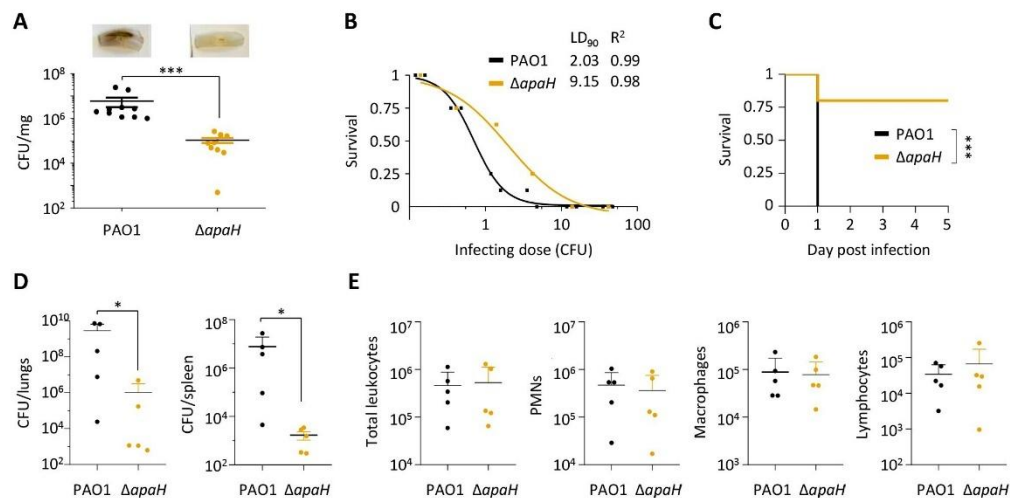


Fig 5. (A) Pathogenicity of *P. aeruginosa* PAO1 and the *apaH* mutant in the lettuce leaf virulence assay. Bacterial viable cells (CFUs) per mg of lettuce midribs at three days post injection are shown. Two independent experiments were performed, each with five biological replicates. A representative picture of the infected midribs is shown for each strain. (B) Dose-dependent survival curves of *G. mellonella* larvae infected with different doses of PAO1 or $\Delta apaH$ cells. Lethal dose 50% (LD₅₀) and R² values are shown in the figure. (C) Survival of mice (n = 5 per group) challenged with intratracheal injection of 10⁷ CFUs of PAO1 or $\Delta apaH$. (D) Total bacterial CFU in lungs or spleen from mice (n = 5 per group) intra-tracheally infected with 10⁷ CFU of PAO1 or $\Delta apaH$ and sacrificed within 24 h of infection (see Materials and methods for details). (E) Count of total leukocytes, polymorphonuclear neutrophils (PMN), macrophages, and lymphocytes in BALF from the mice described in panel D. Asterisks indicate statistically significant differences (**P* < 0.05, ****P* < 0.001) with respect to PAO1 (Mann-Whitney test for data in panels A, D, and E; Mantel-Cox test for data in panel C).

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To further evaluate the impact of Ap4A levels and ApaH activity on virulence, we wondered whether ApaH overexpression and the consequent reduction in Ap4A levels (S1 Fig) could affect the virulence of the wild type strain PAO1. In line with the results of the phenotypic assays (S6 Fig), ApaH overexpression resulted in a minor increase in the mRNA levels of some virulence genes previously identified as DEGs by RNA-seq, and did not affect infectivity in lettuce leaves (S8 Fig). In contrast, ApaH-overexpressing PAO1 cells appeared more virulent than the corresponding empty-plasmid controls in the *G. mellonella* infection model (S8 Fig). While these results show an inverse correlation between Ap4A intracellular levels and *P. aeruginosa* virulence, they also demonstrate that the negative effect caused by Ap4A accumulation on virulence is much more pronounced than the positive effect caused by Ap4A reduction. This highlights the importance of *P. aeruginosa* ApaH in maintaining the intracellular concentration of Ap4A below levels that would be detrimental to virulence gene expression, thus corroborating ApaH as a potential target for antivirulence therapy against *P. aeruginosa*.

The impact of ApaH on virulence is conserved in *P. aeruginosa* clinical isolates

P. aeruginosa strains are extremely diverse at the genetic level [38], and versatility across strains was also observed in the regulatory networks that control metabolism, stress response, and virulence [39–41]. Therefore, we decided to verify whether the ApaH-mediated control of virulence traits is conserved in *P. aeruginosa* strains other than PAO1.

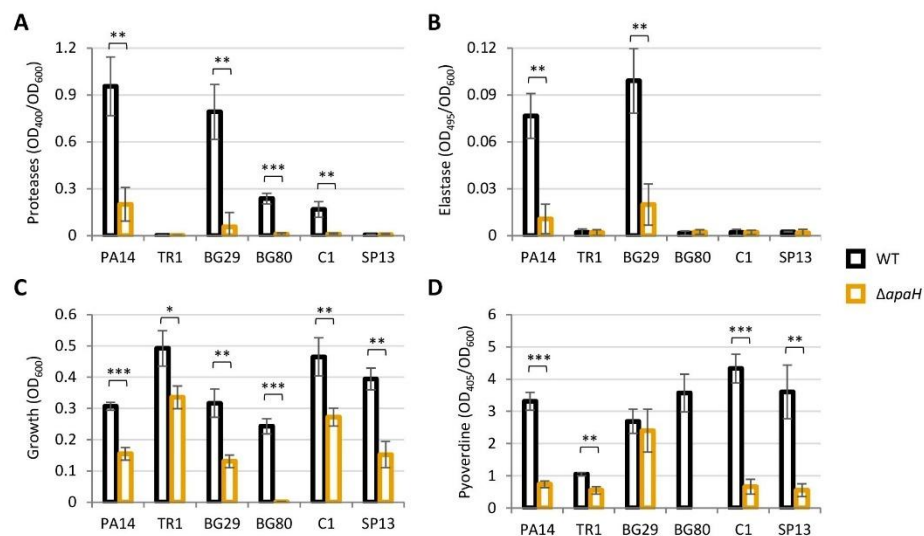


Fig 6. (A) Protease and (B) elastase activity, normalized to cell density (OD_{600}), in the supernatants of *P. aeruginosa* PA14 and the indicated *P. aeruginosa* clinical isolates (WT) or the cognate *apaH* deletion mutants ($\Delta apaH$) cultured at 37°C in LB. (C) Maximum growth yields and (D) pyoverdine levels normalized to cell density (OD_{600}), of the same strains cultured at 37°C in the iron-poor medium CAA. Pyoverdine levels were not measured for BG80 $\Delta apaH$ as this mutant did not grow in CAA (see panel C). Values are the mean ($\pm$ standard deviation) of three independent assays. Asterisks indicate statistically significant differences between each $\Delta apaH$ mutant and the corresponding parental strain (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; unpaired *t* test).

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To this aim, we first generated an *apaH* deletion mutant in *P. aeruginosa* PA14, another widely used reference strain that is distantly related to PAO1 [42,43]. Phenotypic assays confirmed that ApaH is important for virulence gene expression and adaptation to iron deficiency also in PA14. Indeed, the PA14 *apaH* mutant mirrored the behavior of the PAO1 *apaH* mutant, being significantly impaired in protease, elastase and siderophore production, as well as in the ability to grow under iron-depleted conditions (Fig 6).

Then, we attempted to generate a small collection of *apaH* deletion mutants from *P. aeruginosa* clinical isolates. Although we started with ten isolates, we obtained *apaH* mutants only for five of them (S3 Table), including strains isolated from CF lungs (i.e., TR1, BG29, and BG80) [44,45] and strains from bloodstream infections (i.e., C1 and SP13) [46,47]. Notably, the remaining isolates were not genetically intractable, as transconjugants with the suicide plasmid integrated into the chromosome were obtained for all clinical strains. Thus, the difficulty in deleting *apaH* suggests that, in some *P. aeruginosa* strains, the intracellular accumulation of Ap4A might be highly detrimental to cell growth and/or viability. However, this hypothesis has not been experimentally investigated in this study. Expression of virulence factors was highly variable among clinical isolates, as two and four of them produced almost undetectable levels of proteases and elastase, respectively, under the conditions tested (Fig 6A and 6B). Nonetheless, *apaH* inactivation significantly reduced protease and elastase production in the isolates that released quantifiable amounts of these virulence factors (Fig 6A and 6B). Moreover, growth in an iron-depleted medium was significantly impaired for all *apaH* mutants, although at different extents (Fig 6C). This growth defect could be almost completely abolished by exogenous iron supplementation (S9 Fig). Finally, all *apaH* mutants but BG29 $\Delta apaH$ showed lower pyoverdine production with respect to the corresponding parental strain (Fig 6D).

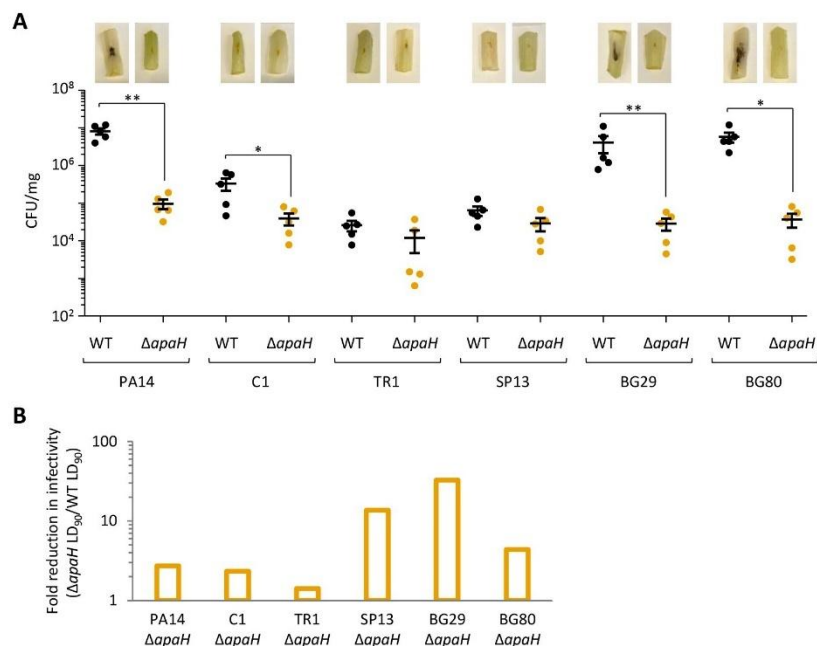


Fig 7. (A) Pathogenicity of *P. aeruginosa* PA14 and the indicated *P. aeruginosa* clinical isolates (WT) or the cognate *apaH* deletion mutants ($\Delta apaH$) in the lettuce leaf virulence assay. Bacterial viable cells (CFUs) per mg of lettuce midribs at three days post injection are shown. Five biological replicates for each strain were analyzed. A representative picture of the infected midribs is shown for each strain. **(B)** Reduction in infectivity, calculated as the ratio between the lethal dose 90% (LD₉₀) of the $\Delta apaH$ mutant and the LD₉₀ of the corresponding wild type strain, observed for *apaH* mutants of *P. aeruginosa* PA14 and the indicated *P. aeruginosa* clinical isolates in *G. mellonella* larvae. The dose-dependent survival curves used to calculate the LD₉₀ values and the corresponding R² values are shown in S10 Fig.

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Finally, we used the lettuce and *G. mellonella* infection models to confirm that ApaH is important for pathogenicity also in PA14 and clinical isolates. As shown in Fig 7A, *apaH* deletion significantly impaired infectivity in lettuce leaves in all but two isolates. Interestingly, these two isolates (TR1 and SP13) showed poor ability to proliferate and cause damage in lettuce leaves, at levels comparable to the $\Delta apaH$ mutants of the other strains (Fig 7A), suggesting that the modest effect of ApaH may be due to poor virulence of these two strains in the lettuce model of infection. All the $\Delta apaH$ mutants also resulted less virulent than the parental strains in *G. mellonella* larvae, although great variability was observed among isolates, as the increase in the LD₉₀ values with respect to the corresponding parental strains ranged between 1.4 fold for TR1 $\Delta apaH$ and 30 fold for BG29 $\Delta apaH$ (Figs 7B and S10).

Although the number of clinical isolates analyzed in this study is small, overall these analyses indicate that the positive effect of ApaH on *P. aeruginosa* virulence is not restricted to laboratory-adapted strains.

Conclusions

Although first discovered more than 50 years ago, the physiological role of Ap4A remains elusive [20]. The role of Ap4A in bacteria has been studied mainly by generating mutant strains

defective in Ap4A-degrading enzyme(s), which accumulate Ap4A at high intracellular levels. This approach highlighted pleiotropic and variable effects caused by Ap4A accumulation in different bacteria, including loss of motility, higher heat and oxidative stress sensitivity, lower antibiotic tolerance, reduced invasiveness in mammalian cells, and/or enhanced biofilm-forming capability [24–30]. Here, we showed that the lack of the diadenosine tetraphosphatase ApaH strongly reduces the infectivity of the difficult-to-treat pathogen *P. aeruginosa* in different infection models, in line with the downregulation of many virulence genes observed *in vitro*. In contrast, no relevant effect of ApaH was observed on biofilm formation, oxidative stress and antibiotic resistance in *P. aeruginosa*. We also provided evidence that the importance of ApaH for virulence gene expression may be conserved in clinical isolates, although our analysis was limited to a small number of strains.

The mechanisms by which Ap4A exert its effects in bacterial cells are still elusive. Proteomic studies with Ap4A-derived probes identified several putative Ap4A targets in *E. coli*, including chaperones, stress response proteins, and core metabolic enzymes [25,48,49]. Recently, the interaction of Ap4A with inosine-5'-monophosphate dehydrogenase (IMPDH), a key enzyme for nucleotide biosynthesis, has been thoroughly characterized in *Bacillus subtilis* [50]. Thus, it is plausible that Ap4A can regulate at least some phenotypes by binding to specific protein partners. On the other hand, it has been recently demonstrated that Ap4A can be incorporated by *E. coli* RNA polymerase into nascent transcripts as a protective 5'-cap that increases RNA stability [51,52], and that specific promoter features can affect the efficiency of RNA capping [53]. This finding raises the possibility that the phenotypes observed for mutants lacking the ApaH enzyme might not only result from Ap4A-mediated control of protein effectors, but also from altered RNA lifetimes and consequent modulation of gene expression. The evidence that ApaH plays an important role in *P. aeruginosa* virulence gene regulation will hopefully drive future biochemical and genetic studies to investigate the molecular mechanisms underlying ApaH-mediated control of gene expression in this bacterium.

The spread of drug resistance in bacterial pathogens has pushed the researchers toward searching for antibiotic alternatives, including compounds with antivirulence activity [54,55]. This work provides the first direct evidence that ApaH inactivation could be a suitable strategy to inhibit bacterial pathogenicity *in vivo*. Notably, phosphatases distantly related to bacterial ApaH enzymes, termed ApaH-like phosphatases (ALPHs), have been identified in some eukaryotes but not in Vertebrata [56,57], implying that specific ApaH inhibitors should not affect Ap4A homeostasis in human cells. Early studies developed and tested substrate analogue inhibitors of *E. coli* ApaH, mainly to characterize the biochemical properties of the enzyme [58]. These studies, combined with the structural-functional characterization of *P. aeruginosa* ApaH, could pave the way for the design of ApaH inhibitors to be tested and developed as antivirulence drugs to treat *P. aeruginosa* infections.

Materials and methods

Ethics statement

Animal protocol was approved by the Italian Ministry of Health (Approval # 855) according to the Italian legislative decree 26/2014.

Bacterial strains, plasmids, and growth conditions

The strains and plasmids used in this study are listed in S3 Table. Bacteria were routinely cultured in LB (Lennox formulation) for genetic manipulation. For specific assays, LB, Mueller-Hinton (MH), Tryptic Soy Broth (TSB), or Casamino acids (CAA) medium (0.5% Casamino acids, 0.4 mM MgCl₂) [59] were used, as indicated in the text. When required, antibiotics were

added at the following concentration for *E. coli* (the concentrations used for *P. aeruginosa* are shown in brackets): ampicillin, 100 µg/mL; tetracycline, 12.5 µg/mL (50–100 µg/mL); carbenicillin (500 µg/mL); nalidixic acid, 15 µg/mL; chloramphenicol, 30 µg/mL (375–650 µg/mL). When specified, growth media were supplemented with isopropyl β-D-1-thiogalactopyranoside (IPTG) or FeCl₃ at the indicated concentrations.

Generation of mutants

The *apaH* deletion mutants were generated using the previously described suicide plasmid pDM4Δ*apaH*, which contains the upstream and downstream regions of the *apaH* coding sequence cloned adjacent to each other [31]. The plasmid pDM4Δ*apaH* was transferred into *P. aeruginosa* strains by conjugation, and transconjugants were selected on LB agar plates containing 15 µg/mL nalidixic acid and 375 µg/mL chloramphenicol. For some clinical strains, chloramphenicol concentration was increased up to 650 µg/mL. Deletion mutants were obtained by homologous recombination and sucrose-based selection as previously described [60], screened by colony PCR using the primer pair *apaH*↑_FW/*apaH*↓_RV [31], and confirmed by DNA sequencing.

Growth assays

Planktonic growth assays were performed either in flasks or in microtiter plates, as indicated. Bacterial strains were precultured in LB and then refreshed 1:1,000 in LB or CAA. Bacterial cultures were incubated in flasks at 37°C and 200 rpm or aliquoted in the wells of 96-well microtiter plates (200 µL in each well) and incubated at 37°C in a Spark 10M microtiter plate reader (Tecan). Growth was measured over time as the optical density at 600 nm (OD₆₀₀) of the bacterial cultures.

Biofilm assay

Biofilm formation was assessed through the microtiter dish biofilm assay [61], with minor modifications. Briefly, bacterial cells cultured overnight in LB were inoculated in the same medium at OD₆₀₀ = 0.002 and aliquoted in 96-well polystyrene microtiter plates (200 µL per well). Five replicate wells for each strain were used in each experiment. After 24-h incubation at 37°C under static conditions, the OD₆₀₀ of the bacterial cultures was measured in a Spark 10M microtiter plate reader (Tecan). Then, planktonic cells were removed, wells were washed four times with distilled water, and attached cells were stained with 0.1% crystal violet for 15 min. After four washes with water, biofilm-bound dye was eluted with 30% acetic acid for 15 min and the OD₅₅₀ was measured in a Spark 10M microtiter plate reader (Tecan). For each well, the OD₅₅₀ value was divided by the OD₆₀₀ of the bacterial culture to normalize biofilm formation to planktonic growth.

MIC assays

Antibiotic, H₂O₂ and paraquat MICs were determined through the broth microdilution method. Strains were precultured in MH and then refreshed at ca. 5×10⁵ cells/mL in the same medium supplemented with increasing concentrations of each antibiotic/compound in 96-well microtiter plates. MIC was visually recorded after 24-h incubation at 37°C. At least three independent experiments were performed for each strain/antibiotic.

Kirby-Bauer disc diffusion assay

Bacterial cell suspensions in saline were normalized at 0.5 McFarland Standard and swabbed onto MH agar plates. Discs containing gentamicin (10 µg), streptomycin (10 µg), tobramycin (10 µg), ciprofloxacin (5 µg), erythromycin (15 µg), novobiocin (30 µg), rifampicin (5 µg), or

imipenem (10 µg) (Becton Dickinson) were placed on the surface of the inoculated plates, and growth inhibition halo diameters were measured after 24-h incubation at 37°C.

Time-kill assays

Late-exponential phase bacterial cultures in MH were diluted in the same medium at ca. 5×10^5 CFU/mL in the presence of gentamicin or kanamycin concentrations corresponding to 1× or 2×MIC. Bacterial cultures were incubated at 37°C with vigorous shaking (200 rpm) and, at different time points, serial dilutions were prepared in saline and plated on MH agar plates for CFU counting.

Quantification of intracellular nucleotide levels

Cells for Ap4A, c-di-GMP, ATP, ADP, GTP, and GDP quantification were collected after 12 h of growth in LB at 37°C. Both extraction and quantification of Ap4A and c-di-GMP by liquid chromatography coupled with tandem mass spectrometry were performed as previously described [62,63]. ATP, ADP, GTP and GDP were extracted with the same protocol used for Ap4A and quantified under the same chromatographic conditions [62]. Mass spectrometric detection was performed in positive ionization mode, with mass transitions as follows: ATP m/z : 508 → 136; ADP m/z : 428 → 136; GTP m/z : 524 → 152; GDP 444 → 152. The amounts of Ap4A, c-di-GMP, ATP, ADP, GTP, and GDP were normalized to the cellular protein content of the corresponding cell extracts determined using the DC protein assay kit (Bio-Rad) and bovine serum albumin as the standard.

RNA extraction, RNA-seq and RT-qPCR

Total RNA was extracted from three independent biological replicates for each sample. Bacteria were cultured in LB at 37°C until mid-exponential growth phase. One-mL aliquots of bacterial cultures were mixed with 2 mL of RNA Protect Bacteria Reagent (Qiagen), and RNA was purified using RNeasy Mini Kit (Qiagen). Eluted RNA samples were treated with TURBO DNase (Thermo Fisher Scientific) and SUPERase-In (Ambion) for 1 h at 37°C. DNase I was removed upon RNA purification with the RNeasy Column Purification Kit (Qiagen). RNA-seq was performed at GENEWIZ (Azenta Life Sciences, Leipzig, Germany). RNA quantification and quality assessment, rRNA depletion, library preparation, sequencing, and statistical analysis of the data were performed as previously described [64]. The Wald test was used to generate *P* values and log2 fold changes that were converted to FCs. Only DEGs with an adjusted *P* value < 0.05 were considered statistically significant (S1 Table). RNA-seq data have been deposited in the NCBI Gene Expression Omnibus database [65] and are accessible through GEO Series accession number GSE255863 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE255863>).

For quantitative reverse transcription PCR (RT-qPCR), cDNA was reverse transcribed from 0.5 µg of total RNA with Prime Script RT Reagent Kit (Takara). The cDNA was used as the template for RT-qPCR in a AriaMx Real-Time PCR System (Agilent) using TB Green Premier EX Taq master mix (Takara). The primers used for RT-qPCR are listed in S4 Table. Relative expression of each gene with respect to the housekeeping gene *rpoD* was calculated using the $2^{-\Delta\Delta Ct}$ method [66].

Virulence factor assays and quantification of QS signal molecules

Total protease and LasB activities were measured in cell-free supernatants, obtained from bacterial cultures grown for 8 h in LB, using the azocasein and elastin-Congo red assays, respectively, as previously described [67].

Pyoverdine was measured as the optical density at 405 nm (OD_{405}) of cell-free supernatants obtained from bacterial cultures grown for 20 h in CAA, appropriately diluted in 100 mM Tris-HCl (pH 8.0), and then normalized to the OD_{600} of the corresponding bacterial culture [45].

Levels of 3OC₁₂-HSL, C₄-HSL, and PQS/HHQ signal molecules were determined in cell-free supernatants, obtained from bacterial cultures grown for 8 h in LB, using the biosensor strains PA14-R3 [68], C4-HSL-Rep [69], and AQ-Rep [70] (S3 Table), as previously described [64], and then normalized to the OD_{600} of the corresponding bacterial culture.

Heat shock sensitivity assay

Heat sensitivity of *P. aeruginosa* cells was assessed as previously described [71]. Briefly, cells were cultured in LB at 20°C until late-exponential growth phase, harvested by centrifugation, resuspended in saline, and then incubated at 50°C. After 30, 60, 120 and 240 min of incubation, aliquots of the cell suspensions were collected, serially diluted in saline and plated on LB agar plates for CFU counting.

Infection assays

The lettuce leaf infection assay was performed as described [34], with few modifications. Briefly, 10 μ L of bacterial cells resuspended at an OD_{600} of 1 in 10 mM MgSO₄ were inoculated into the midribs of fresh Romaine lettuce leaves. The midribs were incubated for three days at 30°C, photographed, and ground in saline with a plastic pestle until a nearly homogenous mixture was obtained. Serial ten-fold dilutions were prepared in saline and plated onto LB agar plates for viable cell counting.

The *G. mellonella* infection assay was performed by injecting serial dilutions of bacterial cell suspensions in saline into the hemolymph of *G. mellonella* larvae as described [35]. Eight larvae were infected with each infecting dose and two independent experiments were performed. Infected larvae were incubated at 30°C for up to three days to monitor mortality. Kaplan-Meier curves, dose-response curves, LD₅₀ and R² values were determined using GraphPad Prism as previously described [72].

For mouse infection assays, 10⁷ mid-exponential cells of PAO1 or Δ apaH, cultured in TSB and resuspended in Dulbecco's phosphate buffered saline (DBPS), were intra-tracheally injected into C57Bl6/N mice (8–12 week-old, 20–22 g, Charles River), previously anesthetized with an intraperitoneal injection of 500 mg/kg of 2,2,2-tribromoethanol. For assessing lethality, mice were monitored daily and sacrificed with CO₂ inhalation when they reached a humane endpoint with weight loss > 20% and evidence of severe clinical disease (e.g., scruffy coat, loss of appetite, severe inactivity, or painful posture). Determination of bacterial load in lungs and spleen and analysis of leukocytes in bronchoalveolar lavage fluids (BALF) were carried out as previously reported [36] for infected mice sacrificed with CO₂ inhalation at 24 h post infection.

Statistical analysis

Statistical analyses were performed with the software GraphPad InStat, using the unpaired *t* test, ANOVA test, Mann-Whitney test or Mantel-Cox test, as indicated in the figure legends.

Supporting information

S1 Table. DEGs between PAO1 and Δ apaH cells identified by RNA-seq.
(XLSX)

S2 Table. MIC of hydrogen peroxide (H₂O₂) and paraquat (PQ) for *P. aeruginosa* PAO1 and the isogenic Δ *apaH* mutant.

(PDF)

S3 Table. Bacterial strains and plasmids used in this study.

(PDF)

S4 Table. Primers used for RT-qPCR.

(PDF)

S1 Fig. (A) Growth curves of the wild type strain *P. aeruginosa* PAO1 and the *apaH* mutant carrying the pME*apaH* plasmid or the empty plasmid pME6032, cultured at 37°C in LB supplemented with 100 μ M IPTG. (B) Intracellular Ap4A levels of the same strains cultured for 12 h under the conditions described in panel A. Values are the mean ($\pm$ standard deviation) of three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.01$) with respect to PAO1 pME6032 (ANOVA).

(PDF)

S2 Fig. (A) Intracellular levels of ATP, ADP, GDP and GTP in *P. aeruginosa* PAO1 and the *apaH* mutant cultured at 37°C in LB. (B) Intracellular levels of ATP, ADP, GDP and GTP in *P. aeruginosa* PAO1 and the *apaH* mutant carrying the empty plasmid pME6032 or the plasmid pME*apaH*, cultured at 37°C in LB supplemented with 100 μ M IPTG. Values are the mean ($\pm$ standard deviation) of three biological replicates. Asterisks indicate a statistically significant difference ($P < 0.05$) with respect to PAO1 (panel A; unpaired *t* test) or PAO1 pME6032 (panel B; ANOVA).

(PDF)

S3 Fig. Levels of the *rhl* QS signal molecule C₄-HSL (A) and the *las* QS signal molecule 3OC₁₂-HSL (B), normalized to cell density (OD₆₀₀), in the supernatants of *P. aeruginosa* PAO1 and the *apaH* mutant, carrying or not the empty plasmid pME6032 or the plasmid pME*apaH*, cultured at 37°C in LB, supplemented with 100 μ M IPTG in the case of strains carrying the plasmids. Values are the mean ($\pm$ standard deviation) of three independent assays. No statistically significant differences ($P > 0.05$) were observed with respect to PAO1 unpaired *t* test) or PAO1 pME6032 (ANOVA).

(PDF)

S4 Fig. Growth curves of *P. aeruginosa* PAO1 and the *apaH* mutant in the iron-poor medium CAA supplemented or not with the indicated concentrations of FeCl₃ in microtiter plates at 37°C in an automatic microtiter plate reader. Values are the mean of three technical replicates and the curves are representative of three biological replicates.

(PDF)

S5 Fig. Heat tolerance of (A) *P. aeruginosa* PAO1 and the *apaH* mutant or (B) the same strains carrying the empty plasmid pME6032 or the plasmid pME*apaH*. Cells were cultured at 20°C in LB, supplemented with 100 μ M IPTG for plasmid-harboring strains, and subjected to a heat shock at 50°C. Cell viability was monitored as CFU/mL at different time points. Values are the mean ($\pm$ standard deviation) of at least three independent assays. Asterisks indicate a statistically significant difference ($P < 0.05$) with respect to PAO1 (panel A; unpaired *t* test) or PAO1 pME6032 (panel B; ANOVA).

(PDF)

S6 Fig. (A) Protease activity, (B) elastase activity, and (C) PQS/HHQ levels, normalized to cell density (OD₆₀₀), in the supernatants of *P. aeruginosa* PAO1 and the *apaH* mutant, carrying

the empty plasmid pME6032 or the plasmid pME*apaH*, cultured at 37°C in LB supplemented with 100 μ M IPTG. (D) Growth curves and (E) pyoverdine levels, normalized to cell density (OD_{600}), of the same strains cultured at 37°C in the iron-poor medium CAA, supplemented with 100 μ M IPTG and 50 μ M $FeCl_3$ when indicated (+ Fe). Values are the mean ($\pm$ standard deviation) of three independent assays. Asterisks indicate a statistically significant difference ($P < 0.001$) with respect to PAO1 pME6032 (ANOVA).

(PDF)

S7 Fig. Kaplan-Meier survival curves of *G. mellonella* larvae infected with 13.8 ($\pm$ 2.8) PAO1 cells or 13.9 ($\pm$ 0.2) Δ *apaH* cells. Sixteen larvae were infected with each strain in two independent experiments. Asterisks indicate a statistically significant difference ($P < 0.001$) with respect to PAO1 (Mantel-Cox test).

(PDF)

S8 Fig. (A) Relative mRNA levels of selected virulence genes (*prpL*, *pvdD*, *aprA*, *lasA*, *lasB*), determined by RT-qPCR, in *P. aeruginosa* PAO1 carrying the empty plasmid pME6032 or the plasmid pME*apaH*, cultured in LB supplemented with 100 μ M IPTG until mid-exponential phase. Values are the mean ($\pm$ standard deviation) of three biological replicates. **(B)** Pathogenicity in the lettuce leaf virulence assay of PAO1 pME6032 and PAO1 pME*apaH* precultured in LB supplemented with 100 μ M IPTG. Bacterial viable cells (CFUs) per mg of lettuce midribs at three days post injection are shown. Five biological replicates for each strain were analyzed. Representative pictures of the infected midribs are shown. **(C)** Dose-dependent survival curves of *G. mellonella* larvae infected with different doses of PAO1 pME6032 or PAO1 pME*apaH* precultured in LB supplemented with 100 μ M IPTG. Lethal dose 90% (LD_{90}) and R^2 values are shown in the figure.

(PDF)

S9 Fig. Maximum growth yields of *P. aeruginosa* PA14 and the indicated *P. aeruginosa* clinical isolates (WT) or the cognate *apaH* mutants (Δ *apaH*) in CAA medium supplemented with 50 μ M $FeCl_3$ over 24 h of growth at 37°C. Values are the mean ($\pm$ standard deviation) of three independent assays. The asterisk indicates a statistically significant difference ($P < 0.05$) between the Δ *apaH* mutant and its parental strain C1 (unpaired *t* test).

(PDF)

S10 Fig. Dose-dependent survival curves of *G. mellonella* larvae infected with different doses of the indicated wild type strains (black lines and symbols) or the corresponding Δ *apaH* mutants (orange lines and symbols). Lethal dose 90% (LD_{90}) and R^2 values are shown in the figure.

(PDF)

S1 Data. Raw data of all experiments.

(PDF)

Author Contributions

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| Bacteriophages | Research Article

Pseudomonas aeruginosa DEV phage exploits the essential LptD outer membrane protein as receptor for adsorption

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ABSTRACT *Pseudomonas aeruginosa* bacteriophage (phage) DEV is a podovirus of the *Schitoviridae* family, related to the prototypical *Escherichia coli* phage N4. N4 uses the novel glycan receptor (NGR) surface glycan, presumably bound by the gp66 appendages, and the NGR transporter NfrA, recognized by the phage gp65 tail sheath, as receptors for adsorption. In contrast, DEV relies on the O-antigen moiety of lipopolysaccharide (LPS) as the primary receptor recognized by the gp53 long tail fibers. However, DEV can infect deep-rough strains that lack the O-antigen moiety by using another, still unknown receptor. Here, we provide evidence that the essential LPS transporter LptD serves as the DEV secondary receptor and that DEV gp54 is its cognate receptor-binding protein. gp54 is encoded within the essential *gp56-gp55-gp54* operon, which also includes *gp56*, the short tail fiber gene. Using cryogenic electron microscopy, AlphaFold modeling, and genetic analysis, we show that DEV gp56, gp55, and gp54 assemble into a receptor-binding fiber (RBF) positioned laterally to a previously uncharacterized tail plug protein, gp74. The DEV RBF is functionally equivalent to the N4 sheath protein gp65, which associates with the tail plug gp53. Thus, DEV and N4 both use a glycan and its surface-exposing transporter as receptors for adsorption. To our knowledge, this is the first example of a *P. aeruginosa* phage using an essential outer membrane protein as a receptor, with implications for phage therapy.

IMPORTANCE *Pseudomonas aeruginosa* phage DEV uses the O-antigen of lipopolysaccharide as its primary receptor. In this study, we found that LptD, an essential and highly conserved outer membrane protein, serves as the secondary receptor for DEV. This interaction is mediated by a specialized receptor-binding fiber composed of the DEV proteins gp54, gp55, and gp56. We posit that the *gp56-gp55-gp54* genes form a functional module, possibly disseminated via horizontal gene transfer among distantly related phages, involved in tail sealing and the regulated unplugging of the tail upon interaction with the bacterial receptor. Given the high conservation of receptor-binding proteins among phages in the DEV *Litunavirus* genus, we anticipate that other members of this genus may also use LptD as their receptor. Since *Litunaviruses* are actively explored for phage therapy, insights into the interaction between DEV and its receptors could help develop more effective and targeted phage-based treatments.

KEYWORDS bacteriophage genetics, bacteriophage evolution, bacteriophage therapy, bacteriophage receptor, bacteriophage structure

Bacteriophage (phage) infection begins with the recognition of a surface-exposed bacterial molecule, the primary receptor, by a phage receptor-binding protein (RBP). In Gram-negative bacteria, this receptor is often the lipopolysaccharide (LPS), a complex glycolipid located in the outer leaflet of the outer membrane (OM) (1–4). The LPS consists of three parts: the lipid A, the innermost and highly conserved portion; the

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core oligosaccharide, with low intraspecies variability; and the O-antigen, a polymer of repeated oligosaccharide units with high intraspecies variability in composition and repeat number (5). This configuration, where the core is capped with the O-antigen, is termed “smooth” LPS in juxtaposition with “rough” and “deep-rough” LPS species lacking either the O-antigen only or the O-antigen and the outermost portion of the core oligosaccharide, respectively. Both the O-antigen and core moieties can act as receptors for phages infecting bacterial strains with smooth or rough LPS, respectively. Additionally, some LPS-binding phages possess a secondary receptor, typically an OM protein (OMP) (2, 4). In smooth strains, the OMPs may be concealed by the LPS polysaccharide portion extending above the OM layer; thus, the infecting phage must first interact with the LPS to reach the secondary receptor. In contrast, in rough strains with short LPS, secondary receptors may be exposed and accessible. The ability to recognize a secondary receptor broadens the host spectrum of phages exploiting the O-antigen as the primary receptor to include rough strains (6).

The utilization of two bacterial receptors belonging to different chemical classes does not necessarily imply that the phage employs different structures to bind them. For example, the prototypical *Escherichia coli* myovirus (i.e., a phage with a long contractile tail) T4 binds both the LPS and the OmpC porin with different portions of the long tail fibers (7–9). On the contrary, the *E. coli* siphovirus (i.e., a phage with a long non-contractile tail) T5 exploits the long tail fibers to bind the O-antigen and the tailspike at the tail tip to interact with the ferrichrome receptor FhuA (10–15).

In this work, we studied the adsorption of phage DEV to its *Pseudomonas aeruginosa* host. DEV is a podovirus, that is, a phage with a short, non-contractile tail, belonging to the *Schitoviridae* family like the *E. coli* phage N4 (16–18). DEV can propagate both in the smooth PAO1 *P. aeruginosa* strain and in deep-rough *galU*, *algC*, or *wapH* PAO1 mutants, but not in a *wzy* mutant producing an LPS species in which the core is capped with a single O-antigen repeat (19). A DEV $\Delta 53$ deletion mutant lacking the *gp53* tail fiber gene does not grow in wild-type (wt) PAO1 or *wzy* mutant, whereas it is still able to infect the *galU*, *algC*, or *wapH* deep-rough mutants (20). Based on these data, we postulated that (i) when infecting the smooth strain PAO1, DEV attaches to the O-antigen with the *gp53* tail fibers, (ii) DEV has another receptor besides the O-antigen that is recognized by a phage RBP other than *gp53*, and (iii) the second phage receptor is accessible in deep-rough mutants but not in PAO1 or in the *wzy* mutant (20).

In this paper, we present evidence supporting LPS transporter LptD as the DEV secondary receptor and DEV *gp54* as the phage's second RBP. The *gp54* gene is part of an essential operon that includes the *gp56* gene, which encodes a short tail fiber we previously localized at the DEV tail tip by structural analysis (20). We provide structural evidence that DEV *gp54*, *gp55*, and *gp56* form a specialized receptor-binding fiber (RBF) complex. Surprisingly, this complex attaches laterally to a newly identified plug, *gp74*, which is conserved in N4-like phages. The DEV RBF complex interacts with both the LPS transporter LptD through *gp54* and with the plug *gp74* via *gp56*, explaining why deletion of *gp56* abolishes infectivity and causes loss of DNA from DEV virions.

RESULTS

LPS depletion stimulates DEV $\Delta 53$ adsorption

We explored the hypothesis that DEV, like other podoviruses, may first attach to the O-antigen and then exploit the LPS core oligosaccharide as a secondary receptor (4, 21). In this case, a strain that does not produce any LPS form should be non-permissive for DEV adsorption. To test this hypothesis, we used a PAO1 derivative with the *lpxA* gene under the control of the *E. coli* *araBp* promoter (22). In this mutant, transcription of *lpxA* is inducible with arabinose. *lpxA* encodes an essential enzyme of the lipid A biosynthetic pathway, and its depletion blocks the biosynthesis of all forms of LPS (i.e., uncapped and capped with either the common polysaccharide antigen or the O-antigen) (23–25).

We found that in the absence of arabinose, *araBp-lpxA* PAO1 cultures stopped growing after five to six divisions, and the arrested cultures were highly depleted of LPS.

However, DEV adsorption was only slightly affected (Fig. 1A through C). Interestingly, the gp53 tail fiber-defective mutant DEV $\Delta 53$, which does not adsorb to PAO1, could adsorb to LPS-depleted PAO1 with efficiency similar to that of the wt DEV (Fig. 1B), suggesting that the LPS is not required for DEV $\Delta 53$ infection.

We also tested whether DEV loses infectivity upon incubation with purified LPS, as it was observed for E217, a phage targeting the LPS O-antigen for adsorption (26, 27). Notably, incubation of DEV with the LPS extracted from PAO1 or from the *galU* mutant did not reduce phage infectivity (Fig. 1D).

Thus, the DEV secondary receptor is not an LPS species.

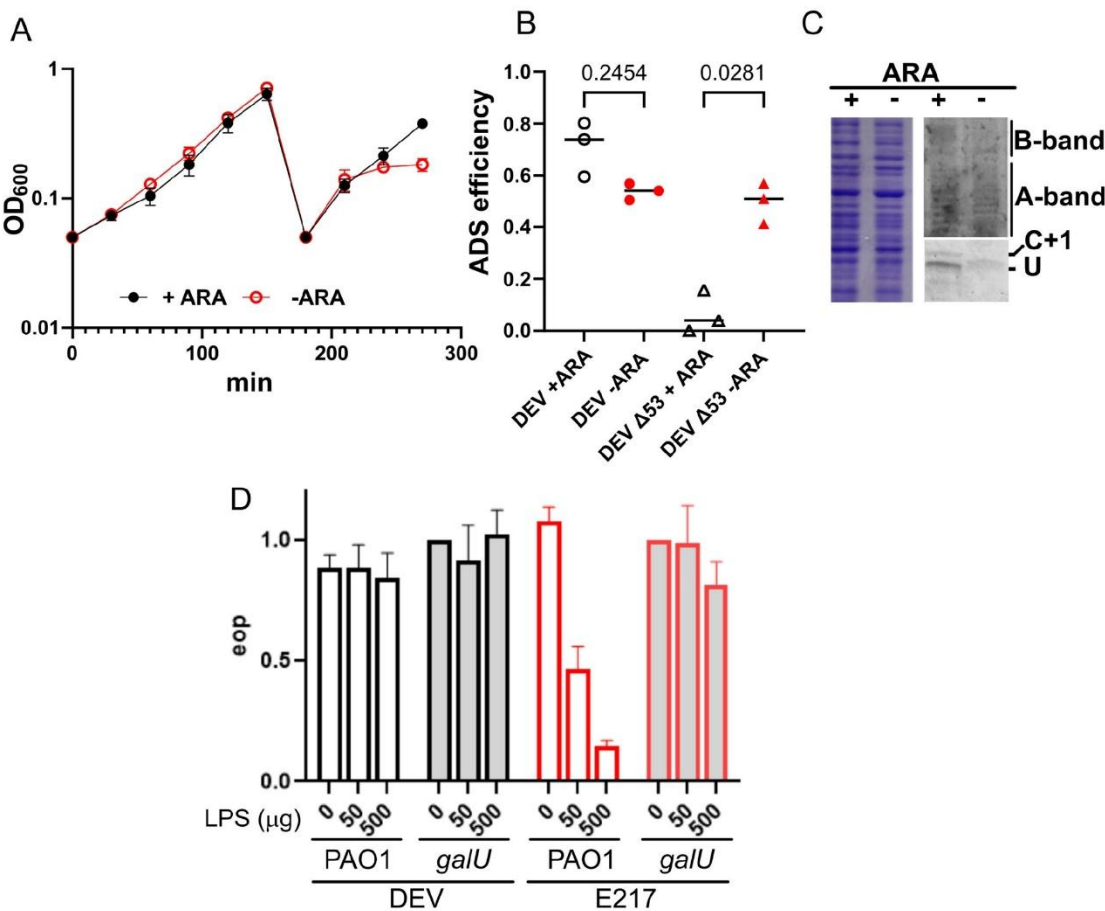


FIG 1 DEV adsorption upon LPS depletion. (A) Growth of the *araBp-lpxA* strain in presence or absence of 0.2% arabinose (ARA). The adsorption assay (B) and LPS analysis (C) were executed on bacteria collected at the last time point (270 min). (B) Adsorption (ADS) efficiency of DEV (circles) and DEV $\Delta 53$ (triangles) in the presence or absence of arabinose. The significance of the difference (P) estimated with one-way ANOVA and Šidák's multiple comparisons test is reported. (C) LPS (right panels) and proteins (left panel) extracted from cultures grown in the presence or absence of arabinose. Proteins were analyzed by SDS-PAGE and Coomassie staining and LPS by 18% Tricine gel electrophoresis (lower panel) and 12% SDS-PAGE (upper panel) and emerald-green staining. LPS species were recognized based on their electrophoretic profile (19). B-band, serotype-specific LPS; A-band, common antigen LPS; C+1, LPS capped with a single O-antigen repeat; u, uncapped LPS. (D) Pre-incubation with LPS extracted from PAO1 (empty bars) inactivated E217 but not DEV. LPS extracted from the *galU* mutant (filled bars) did not impair infection by either phage. Bars indicate average efficiency of plating (EOP, $N = 2$) with range.

Mutations in *lptD* and *rpoH* confer DEV resistance to the PAO1 *galU* mutant

To identify the secondary receptor of DEV, we searched for mutations that confer resistance to DEV in a *galU* defective strain, which lacks the DEV primary receptor, the O-antigen-capped LPS (19, 25). PAO1 *galU* cultures were plated on plates spread with DEV and the colonies tested for resistance to both wt DEV and *gp53*-defective DEV Δ53. The genomes of two independent (i.e., selected from independent bacterial cultures) *galU* derivatives resistant to both phages were sequenced to identify the mutation(s) responsible for phage resistance. Both mutants retained the *galU* frameshift mutation present in the parental strain (19) and had an additional mutation in either the *lptD* or *rpoH* gene, encoding the LPS transporter LptD or the alternative sigma factor σ^H , respectively. Specifically, we found a 33 bp in-frame deletion (mutation *lptD*^{Δ33}) that eliminates residues 880–890 in the 924 amino acids of LptD and a T>G substitution in *rpoH*, resulting in the replacement of the conserved valine at position 164 with glycine (V164G) in σ^H . No other mutations were detected, implying that the mutations identified in *lptD* or *rpoH* are responsible for DEV resistance in deep-rough *galU* strains (Table 1; Fig. 2A and B).

Since LptD is located in the OM, whereas σ^H is a cytoplasmic protein, we focused on LptD as a potential secondary receptor for DEV. To confirm that the mutation in *lptD* was responsible for the DEV-resistant phenotype of the *galU lptD*^{Δ33} mutant, we checked DEV plating efficiency on *galU lptD*^{Δ33} carrying the pLptD plasmid, which expresses the wt LptD protein. Both wt DEV and DEV Δ53 were able to grow in the complemented *galU lptD*^{Δ33}/pLptD strain (Fig. 2C), confirming that the *lptD*^{Δ33} mutation is responsible for DEV resistance. We then tested DEV growth in the *galU lptD*^{Δ33} strain expressing wt GalU from the pGalU plasmid to assess whether the *lptD*^{Δ33} mutation caused DEV resistance only in the absence of GalU or also in its presence. DEV was able to form plaques on the complemented host, demonstrating that the *lptD*^{Δ33} mutation does not block phage growth as long as the smooth LPS can be produced. Notably, E217 and E10 phages, which depend on the O-antigen for adsorption and do not infect the *galU* mutant (19, 26), were able to grow in the *galU lptD*^{Δ33}/pGalU strain (Fig. 2C), suggesting that the *lptD*^{Δ33} mutation does not strongly impact LPS transport. Accordingly, no significant difference in the LPS level was observed between *galU* and *galU lptD*^{Δ33} mutants (Fig. 2B).

DEV adsorption efficiency is low in the absence of the O-antigen and responds to LptD levels

We measured the adsorption efficiency of DEV and DEV Δ53 to the *galU* and *galU lptD*^{Δ33} mutants compared with PAO1. Despite DEV's high efficiency of plating (EOP) on the *galU* mutant, its adsorption to this strain was significantly reduced (from over 80% for PAO1 to 10%–15% for the *galU* mutant), similar to the adsorption to the DEV-resistant *galU lptD*^{Δ33} mutant. Accordingly, DEV Δ53 showed low adsorption to all tested strains (Fig. 3A). Complementation of the *galU* mutation with pGalU increased DEV adsorption to the *galU lptD*^{Δ33} mutant, but it did not improve Δ53 adsorption, as expected. In contrast, complementation of the *lptD*^{Δ33} mutation with pLptD similarly increased DEV and Δ53 adsorption to around 40%, with some fluctuations in the adsorption efficiency (Fig. 3B).

TABLE 1 Efficiency of plating of DEV and DEV Δ53 on PAO1 double mutants

Mutant	DEV		DEV Δ53	
	Average ^a	SD ^b	Average ^a	SD ^b
PAO1	1.0	0.03	<0.003	n.a.
<i>galU</i>	0.9	0.07	1.0	0.11
<i>galU lptD</i> ^{Δ33}	<0.0003	n.a.	<0.003	n.a.
<i>galU rpoH</i>	0.006	0.0005	<0.003	n.a.

^aEfficiency of plating (EOP) average (*N* = 3), determined as the ratio between the titer on each replicate culture and the average titer calculated on the reference strains (wt PAO1 for DEV, and PAO1 *galU* for DEV Δ53).

^bn.a., not applicable.

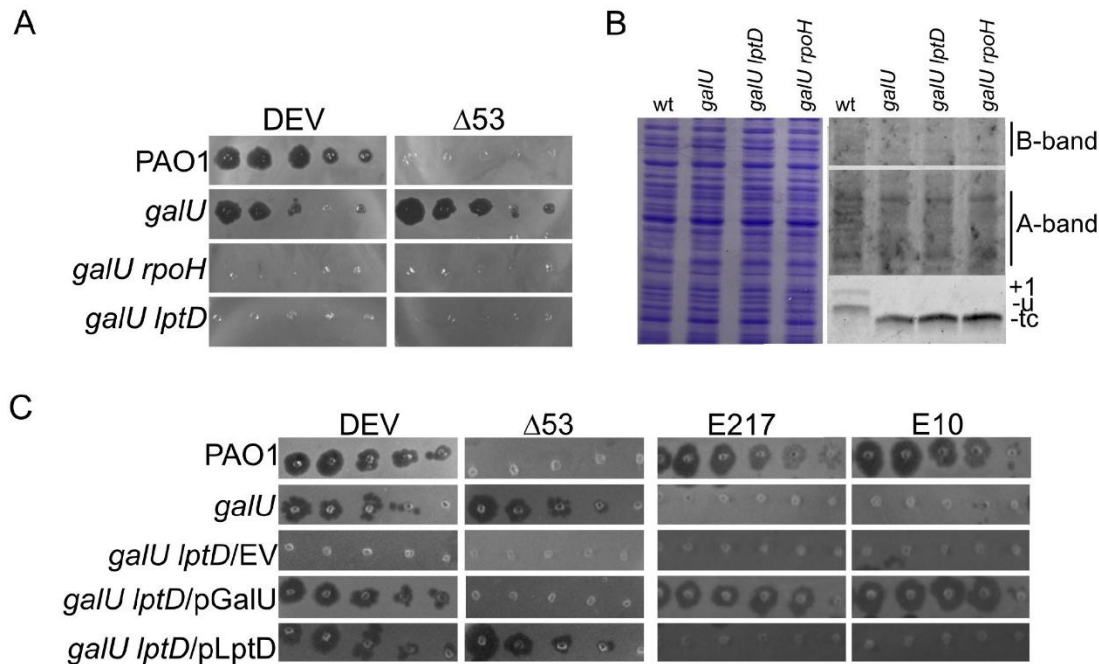


FIG 2 Effect of *lptD* and *rpoH* mutations on the LPS profile and phage growth. (A and C) Tenfold serial dilutions (from left to right) of the indicated phages were replicated on PAO1 or the PAO1 mutants indicated on the left of the panels. The plates were incubated for 16 h at 37°C. EV, empty vector. (B) LPS extracted from cultures of PAO1 (wt) or the PAO1 mutants indicated on top of the lanes, grown at 37°C. Samples corresponding to the same total OD₆₀₀ were split and used to extract proteins as a sampling control (left panel) and LPS (right panels). Proteins and LPS were analyzed as described in the legend of Fig. 1. B-band, serotype-specific LPS; A-band, common antigen LPS; +1, LPS capped with a single O-antigen repeat; u, uncapped LPS; tc, LPS with truncated core oligosaccharide. The *galU lptD*^{Δ33} mutant is indicated as *galU lptD* in all panels.

Therefore, without the O-antigen or the O-antigen binding protein gp53, DEV adsorption becomes inefficient and/or unstable and depends on LptD levels.

We validated this finding by testing whether low LptD levels decreased DEV adsorption. Since LptE depletion prevents LptD insertion into the OM, we used a PAO1 derivative with arabinose-dependent *lptE* expression (strain *araBp-lptE*) (28). The mutant did not stop growing in the absence of arabinose, suggesting that LptE depletion was only partially achieved (Fig. 3C). In spite of this, DEV adsorption was reduced in *araBp-lptE* bacteria grown without arabinose, whereas, as expected, $\Delta 53$ adsorption to this smooth strain remained low in the presence or absence of arabinose (Fig. 3C). We then assessed whether DEV adsorbed to an *E. coli* strain exposing *P. aeruginosa* LptD on the cell surface. We used a $\Delta waaG::kan$ deep-rough *E. coli* strain (29) expressing LptD and LptE of *P. aeruginosa* from compatible plasmids. We observed that DEV adsorption increased slightly but consistently in the presence of plasmids expressing *P. aeruginosa* proteins compared to the empty vectors. In contrast, E217 adsorption, which requires *P. aeruginosa* LPS (19, 26), remained similarly low in both strains (Fig. 3D). Overall, these results support the hypothesis that LptD is the DEV secondary receptor.

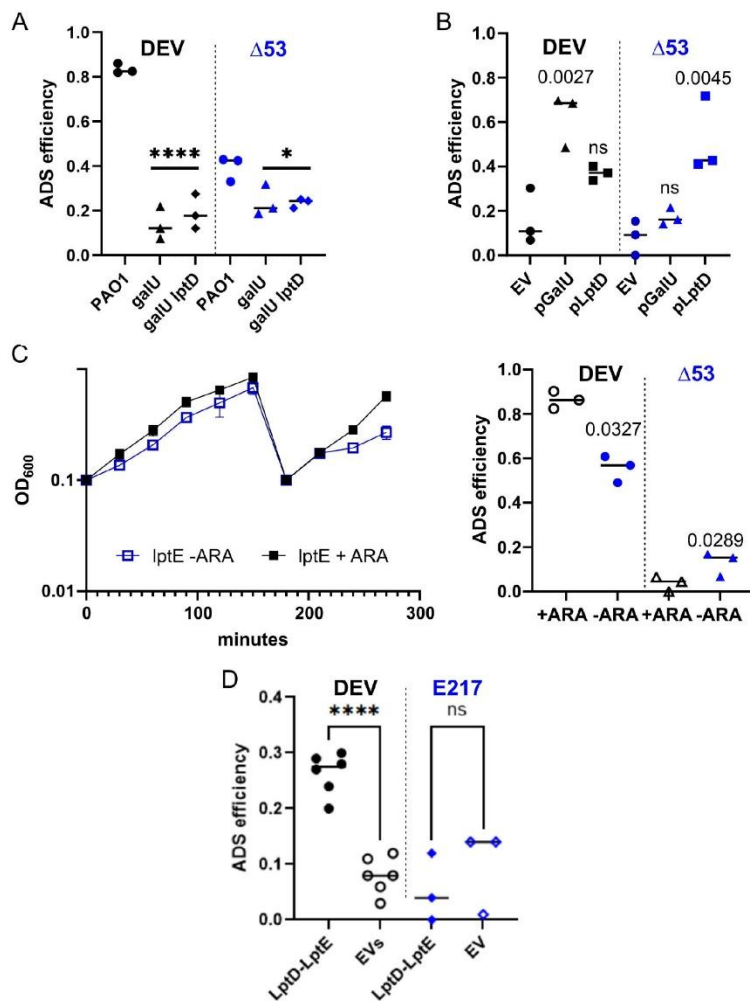


FIG 3 Efficiency of LptD-mediated DEV adsorption. Efficiency of adsorption to the indicated *P. aeruginosa* strains (A) or to the *galU lptD*^{Δ53} double mutant carrying the indicated plasmids (B) In panel A, the *galU lptD*^{Δ53} mutant is indicated as *galU lptD*. EV, empty vector, pGM931. The symbols represent replicate samples, and the lines represent the average. *P* was calculated with one-way ANOVA and Šidák's multiple comparisons test. Only *P* values relative to the comparisons between PAO1 and the mutant strains (A, ****, <0.0001; *, <0.05) or between the control *galU lptD*^{Δ53}/EV and the strains carrying the plasmids pGalU or pLptD (B) are reported for clarity. ns, not significant. (C) Growth of the *araBp-lptE* strain (left panel) and adsorption efficiency of DEV and DEV Δ53 (right panel) in the presence or absence of 0.2% arabinose (ARA). The adsorption assay was performed on bacteria collected at the last time point. The significance of the difference (*P*) estimated with a two-tailed *t*-test is reported. (D) Adsorption efficiency of DEV (circles) and E217 (diamonds) to *E. coli* ΔwaaG expressing *P. aeruginosa* LptD and LptE from pLptD and pGM-lptE compatible plasmids (LptD-LptE). EVs, empty vectors. *P* was calculated with one-way ANOVA and Tukey *post hoc* test. ****, <0.0001; ns, not significant.

The gp56-55-54 operon is essential

It has been proposed that several *E. coli* siphoviruses of the *Drexlerviridae* family may utilize LptD as their secondary receptor recognized by a RBP encoded by one of two genes flanking a gene homologous to phage λ gpJ, which encodes the distal tail fiber located at the tail tip (21). Interestingly, the final 70% of DEV gp56 short fiber has around 30% identity to gpJ-like proteins of *Drexlerviridae* (Table S1). According to genomic sequence analysis, the gp56 gene is part of an operon with gp55 and gp54, with the start codons of gp55 and gp54 overlapping the stop codons of gp56 and gp55, respectively. Reverse transcription-PCR (RT-PCR) analysis confirmed that the gp56, gp55, and gp54 genes are co-transcribed into a single mRNA (Fig. 4A). gp54 was identified as a structural protein according to mass-spectrometry analysis of DEV particles, whereas the identification of gp55 as a structural protein was uncertain (20). Both gp54 and gp55 are highly conserved among *Litunaviruses* (Tables S2 and S3), and gp54 and gp55 orthologs were reported as structural proteins of LIT1, a *Litunavirus* highly similar to DEV (20, 30).

To assess whether the proteins encoded by this operon, and in particular gp56, are essential, we tested the effect of $\Delta 56$ deletion on DEV propagation. This deletion removed the whole gp56 open reading frame (ORF), including a 45 bp long portion

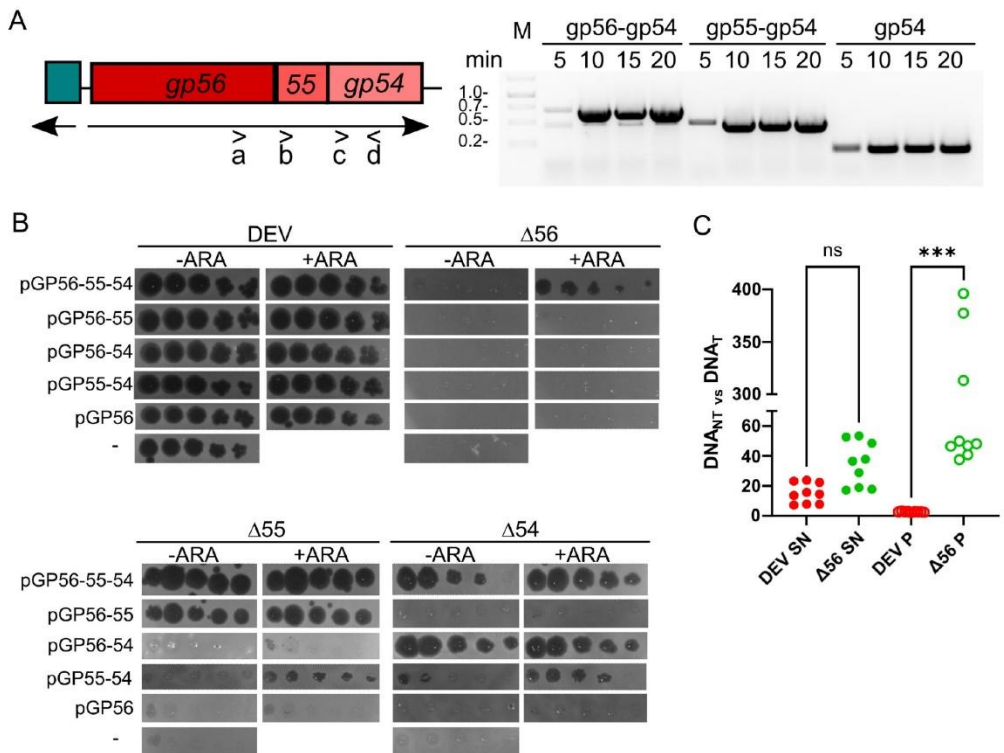


FIG 4 Expression and essential function of gp56-gp55-gp54 operon. (A) RT-PCR analysis of the gp56-gp55-gp54 operon transcription. The position of the a (4249), b (4250), c (4251), d (4252) oligonucleotides used for PCR amplification is shown on the left of the panel below the arrow indicating the operon transcription direction. gp56-gp54, gp55-gp54, and gp54 amplicons were obtained by RT-PCR with primers a-d, b-d, and c-d, respectively, on RNA extracted at different time points after DEV infection indicated in minutes on top of the lanes. M, MW marker. (B) EOP of mutant phages calculated by replica plating of 10-fold serial dilutions (from left to right) of phage lysates. The phages indicated above the panels were replica-plated onto PAO1 carrying the plasmids listed on the left. -, no plasmid. The plates were incubated for 16 h at 37°C. (C) DNA release from DEV particles. qPCR analysis of phage DNA content in pellets (P) and supernatants (SN) obtained after PEG- precipitation of phage lysates. DNA_{NT} vs DNA_T ratio between DNA amount in samples untreated (NT) vs treated (T) with DNase I. Significance of the difference was estimated with one-way analysis of variance and Šidák's multiple comparisons test. ns, not significant; ***, $P = 0.0009$.

of the upstream 5' untranslated region (UTR) and the first seven codons of *gp55*. The mutant phage did not form plaques on PAO1, whereas it grew in PAO1 carrying a plasmid with the entire *gp56-gp55-gp54* operon. Conversely, plasmids carrying *gp56* only or in combination with either *gp55* or *gp54* did not restore DEV $\Delta 56$ growth (Fig. 4B). This finding indicated that the *gp56*, *gp55*, and *gp54* genes are all essential and the $\Delta 56$ deletion, which also involves *gp55*, is polar on the downstream *gp54* gene. We did not further explore this aspect, which could be due to translation coupling between *gp55* and *gp54* and/or premature transcription termination of the poorly translated transcript.

To confirm the conclusion that *gp54* and *gp55* are essential genes, we constructed DEV $\Delta 54$ and $\Delta 55$ mutants, which carry deletions that eliminate only the *gp54* or the *gp55* ORF, respectively, and tested their growth. We found that the $\Delta 54$ and $\Delta 55$ mutants grew only if *gp54* or *gp55*, respectively, were expressed from complementing plasmids (Fig. 4B). This confirmed the essentiality of *gp54* and *gp55*.

The *gp56-55-54* operon controls tail closure

To assess whether the function of the proteins encoded in the *gp56-gp55-gp54* operon, and in particular of *gp56*, is to control tail plugging, we tested the effect of $\Delta 56$ deletion on DNA leakage by phage particles. DEV and DEV $\Delta 56$ virions were incubated for 3 h at 37°C and pelleted by PEG-NaCl precipitation. The supernatants and pellets were analyzed by real-time quantitative PCR (qPCR) using phage-specific oligonucleotides before and after DNase I treatment. The enzyme can degrade phage DNA unpackaged or even partially leaked from phage particles, but not the DNA fully packaged inside the phage head. As shown in Fig. 4C, DNase I treatment significantly reduced the DNA content in both supernatants (median DNA decrease of around 14- and 36.5-fold between treated (T) and untreated (NT) samples of DEV and DEV $\Delta 56$, respectively). In contrast, the impact of DNase I digestion on DEV DNA in the pellets was much lower (a median 2.6-fold decrease between treated and untreated samples). These results were anticipated, as supernatants are expected to contain unpackaged DNA, whereas pellets are enriched with DNA packaged in phage heads. On the other hand, DEV $\Delta 56$ DNA in the pellet was highly susceptible to DNase I digestion (a median 48-fold decrease between T and NT samples), suggesting that the phage genome leaked from the virions.

To strengthen this observation, we assessed the susceptibility of DEV $\Delta 56$ virion DNA to digestion with *Bam*HI, which recognizes 16 sites in the DEV genome. DEV $\Delta 56$ virions produced in PAO1 (defective virions) or PAO1 expressing *gp56-gp55-gp54* from a plasmid (complete virions) were incubated for 6 h at 37°C with the restriction enzyme, and the phage DNA was analyzed by Southern blotting using a DEV-specific probe (Fig. S1). We found that the DNA from complete virions remained mostly undigested, whereas the DNA from defective virions was completely digested. Overall, these results indicate that *gp56* controls the sealing of the phage tail.

gp54 mutations suppress DEV defective growth on the *galU lptD^{Δ33}* mutant

We then asked whether, like in *Drexlerviridae*, the *gp54* or *gp55* genes proximal to the *gpJ*-like *gp56* may encode the second RBP of phage DEV. To answer this question, we examined whether phage mutants that regained the ability to grow on the *galU lptD^{Δ33}* strain carried mutations in either of these genes. The rationale was that mutations restoring infectivity are likely to occur in the gene encoding the RBP, which physically interacts with the bacterial receptor. To increase the mutation frequency, we exposed a DEV lysate to UV rays before infecting a liquid culture of the PAO1 permissive host. After 20 min, the infected culture was plated on the *galU lptD^{Δ33}* double mutant. The *gp53-gp54-gp55-gp56* ORFs were amplified from a plaque formed by a mutant phage able to infect this strain (i.e., DEV g101; Fig. 5) and sequenced. Three base substitutions were identified: one resulting in the replacement of aspartate at position 799 with asparagine (D799N) in the *gp53* protein and the others resulting in the replacement of arginine at position 37 and threonine at position 181 with glycine and proline, respectively (R37G and T181P mutations), in *gp54*. No mutations were detected in *gp55* and *gp56*. This result

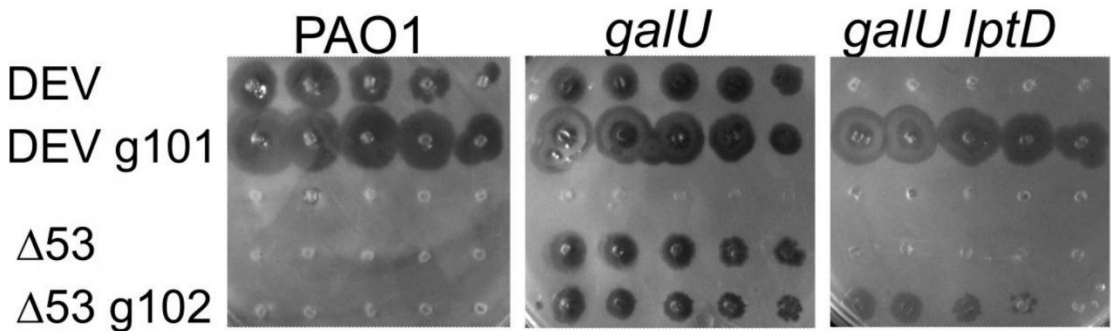


FIG 5 *gp54* mutations suppress DEV defective growth on *galU lptD*^{Δ53}. Replica-plating of 10-fold serial dilutions (from left to right) of the phages indicated on the left on PAO1 and PAO1 *galU* and *galU lptD*^{Δ53} (indicated as *galU lptD*) mutants. The plates were incubated for 16 h at 37°C.

was confirmed by whole genome sequencing of the mutant phage that showed that no other mutations were present in the phage genome.

To strengthen this observation, we looked for mutants of DEV Δ53 able to grow in *galU lptD*^{Δ53}. We exposed a DEV Δ53 lysate to UV radiation and infected a liquid culture of the *galU* permissive host. The infected culture was plated onto the *galU lptD*^{Δ53} mutant as described previously. One plaque was isolated and tested by replica-plating on the *galU* and the *galU lptD*^{Δ53} mutants, confirming the ability of this phage (i.e., DEV Δ53 g102) to grow in both strains (Fig. 5). The genome of the mutant phage was sequenced and compared with the DEV genome. Besides the *Δgp53* deletion, two missense mutations were identified in *gp54*, resulting in amino acid substitutions at positions 85 and 186, namely, valine to isoleucine (V85I) and asparagine to threonine (N186T). These data support the hypothesis that *gp54* is the RBP for the DEV secondary receptor.

Cryo-EM studies reveal DEV RBF complex

Our previous asymmetric reconstruction of the mature DEV virion (20) showed only weak density at the distal tip of the tail tube, insufficient to resolve individual protein components. Based on this observation, we initially hypothesized that the DEV short tail fiber *gp56* might function as a tail plug, analogous to the tail needles of other podophages such as P22 (31) and Sf6 (32). However, recent structural studies of phage N4 (33, 34) have identified the gene product 53 (*gp53*) as the *bona fide* tail plug. This protein, encoded by a gene located downstream of the structural genes for the capsid and tail tube (Fig. 6A), is conserved among N4-like phages, including DEV and LIT1, and has been detected in proteomic analyses of mature virions (30).

To test the hypothesis that *gp56* is not the DEV tail plug itself but may interact with the plug to stabilize the packaged genome, we vitrified at high titer DEV Δ53 mutant (20) lacking the long tail fibers (referred to as “appendages” in N4 [35]), and collected an extensive cryogenic electron microscopy (cryo-EM) data set to directly visualize the organization of *gp54*, *gp55*, and *gp56* in the DEV tail. Applying C12 rotational averaging yielded a 2.9 Å reconstruction of the entire tail, confirming the quality of this data set (Fig. S2A). As expected, asymmetric reconstructions of the tail apparatus from Δ53 virions exhibited substantially reduced noise relative to wt particles (Fig. S2B and S3). A 2D class average of the tail tip (Fig. 6B) revealed an elongated density positioned laterally to the tail tube. Consistently, a low-resolution focused reconstruction of the tail tip showed this elongated lateral density running approximately parallel to the tail tube, accompanied by a roughly triangular density at the distal end of the tail tube (Fig. 6C). These asymmetric density features are visible even at 14σ contour and appear to be equally occupied as the tail tube *gp75* (Fig. S4), suggesting they are a genuine structural component of DEV tail. We used AlphaFold3 to predict the structure of the *gp56*-*gp55*-*gp54* complex,

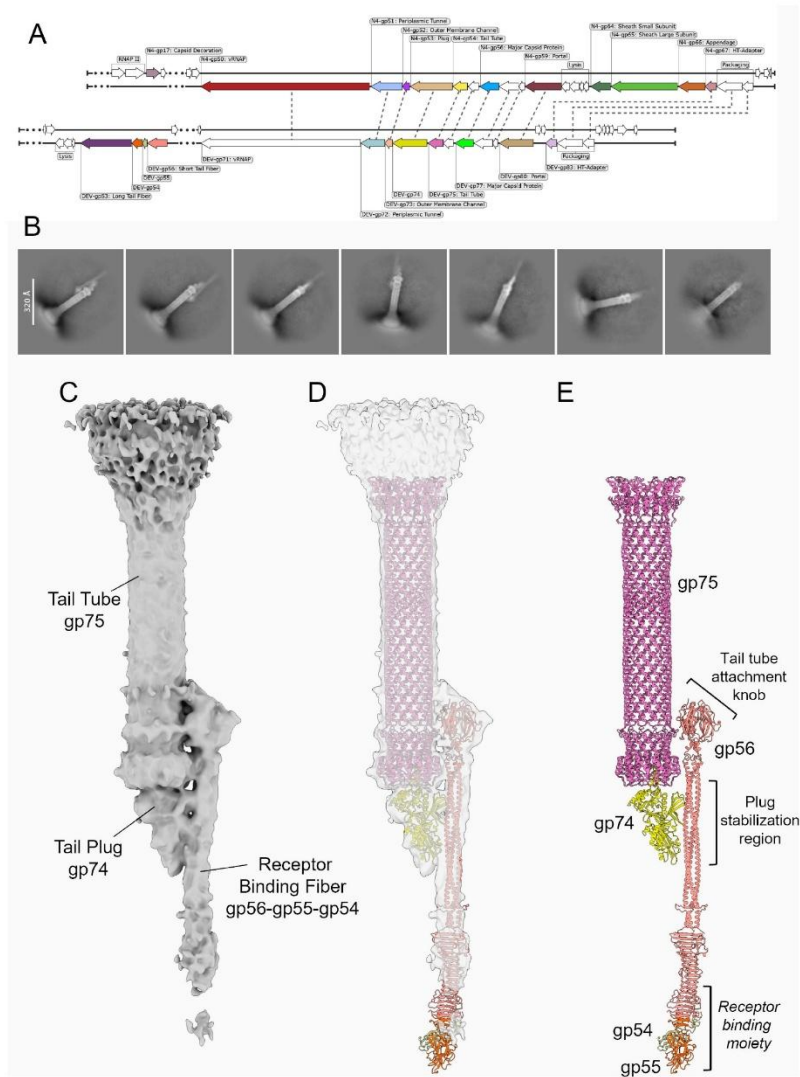


FIG 6 Cryo-EM analysis of DEV tails from DEV $\Delta 53$ virions lacking the long tail fiber. (A) Comparison of genomic organization between N4 (top) and DEV (bottom) highlights syntentic conservation of the plug gene: *gp53* in N4 and *gp74* in DEV. (B) Representative 2D class averages of the DEV tail tip obtained from low-noise micrographs of the $\Delta gp53$ mutant. (C) Focused reconstruction of the DEV tail showing the asymmetric interface formed by the tail tube (*gp75*), tail plug (*gp74*), and RBF complex (*gp56-gp55-gp54*). The density, shown in gray, is computed at 8.5 Å resolution (Fig. S2C) and contoured at 4 σ . A higher-contour view of this map (14 σ) is shown in Fig. S4. (D) Semitransparent representation of the density in panel C overlaid with the model of the tail tube *gp75* (magenta) and AlphaFold-predicted models of the tail plug *gp74* (light blue) and RBF complex (*gp56-gp55-gp54*). (E) Pseudo-atomic model of the DEV tail, comprising *gp75*, *gp74*, and the RBF complex (*gp56-gp55-gp54*), illustrating the overall asymmetry.

as well as DEV *gp74*, the putative DEV ortholog of the N4 tail plug *gp53*. Strikingly, the asymmetric elongated density observed in the focused reconstruction closely resembles an AlphaFold3 (36) model of the DEV *gp56* fiber in complex with *gp55* and *gp54* (Fig.

6D), which we docked into the density and refined using rigid-body fitting. Similarly, the density, occluding the dodecameric tail tube, aligns well with the AlphaFold model of DEV gp74, encoded immediately downstream of the tail tube gene gp75 (Fig. 6A). DEV gp74 exhibits a triangular architecture that caps the tail tube, forming a symmetry-mismatched 12:1 interface (Fig. 6D), as previously observed in phage N4 (33, 34). Visualization of the fitted atomic models suggests that gp74 establishes extensive lateral contacts with the gp56 fiber body (Fig. 6E), explaining the loss of DNA from DEV $\Delta 56$ virions (Fig. 4C). In contrast, gp54 and gp55, predicted to bind the C-terminal β -helix of gp56, form the receptor-binding moiety of RBF that remains largely disordered at the resolution of our reconstruction (Fig. 6E). The orientation of gp56 relative to the tail tube is nonetheless unambiguous, despite the limited resolution of the focused reconstruction. Gp56 N-terminal trimeric knob fits precisely into the density immediately above the tail tube tip (Fig. 6E), possibly functioning as an attachment knob. Together, these data provide structural evidence for a previously uncharacterized gp56-gp55-gp54 assembly, which we refer to as the RBF complex.

AlphaFold3 modeling of RBF-LptD interaction

The pseudo-atomic model of the DEV RBF heterotrimer predicts that one gp54 monomer and one gp55 monomer assemble at the tip of a gp56 homotrimer, distal to the capsid, to form a receptor-binding moiety that positions gp54 for recognition of LptD (Fig. 6E). In this structural model, gp56 acts as a structural adaptor between the tail tube and the receptor-binding subunit gp54. In contrast, gp55 stabilizes gp54's attachment to gp56, possibly contributing to receptor interaction (Fig. 7A). Therefore, AlphaFold3 modeling confirms the experimental observation that gp54 acts as DEV's RBP, which is exposed to the bacterium's OM through a structural fiber, gp56, and a potential adaptor, gp55.

We used AlphaFold3 (36) to model the interface between the DEV RBF (gp56-gp55-gp54) complex and its LptD receptor (Fig. 7B). This model positions gp54 directly at the LptD surface, interacting with surface-exposed loops extending from the β -barrel (Fig. S5) (37). Although the predicted interface between the gp54:gp55 complex and LptD has a low confidence score (Fig. 7B) and should therefore be interpreted with caution, the gp54 residues affected by mutations that restore DEV infectivity in the *lptD* ^{$\Delta 33$} mutant are predicted to lie at this interface, supporting a key role in receptor recognition (Fig. 7C). Based on AlphaFold3 predictions that LptD loop 6 (and possibly loop 3) interacts with gp54, while loops 9 and 11 may interact with gp55 (Fig. S6), we examined the plating efficiency of DEV and DEV $\Delta 53$ in *lptD* mutants lacking these loops. As shown in Fig. 7D, loops 3 and 11 were dispensable for phage growth. By contrast, both phages failed to grow in the Δ loop6 mutant and showed thermosensitive plating on the Δ loop9 mutant, consistent with impaired adsorption in the absence of loop 6 and unstable adsorption to the loop 9 mutant at high temperature.

DISCUSSION

The adsorption phase of phage infection begins with an initial interaction with a primary receptor, followed by a second step of irreversible binding to a secondary receptor that triggers genome delivery. For some bacteriophages, the receptors are different derivatives or spatially distinct domains of the same molecule, while for others, they are separate molecular entities. The evidence presented in this work places the *P. aeruginosa* DEV phage in the latter group, supporting LptD as the secondary receptor for DEV and gp54 as the RBP recognizing it.

Specifically, we found that the *lptD* ^{$\Delta 33$} mutation renders the deep-rough *galU* mutant resistant to DEV. This deletion removes 11 residues located in loop 13 and $\beta 26$ -strand of LptD, altering the extracellular-facing region of the protein and potentially affecting the lateral gate through which the LPS is transferred from LptD to the OM (37–39) (Fig. S5). *lptD* mutations are known to increase OM permeability by altering its composition, indirectly elevating phospholipid levels, and causing palmitoylation of LPS (40). This raises the possibility that the *lptD* ^{$\Delta 33$} mutation may indirectly inhibit DEV adsorption

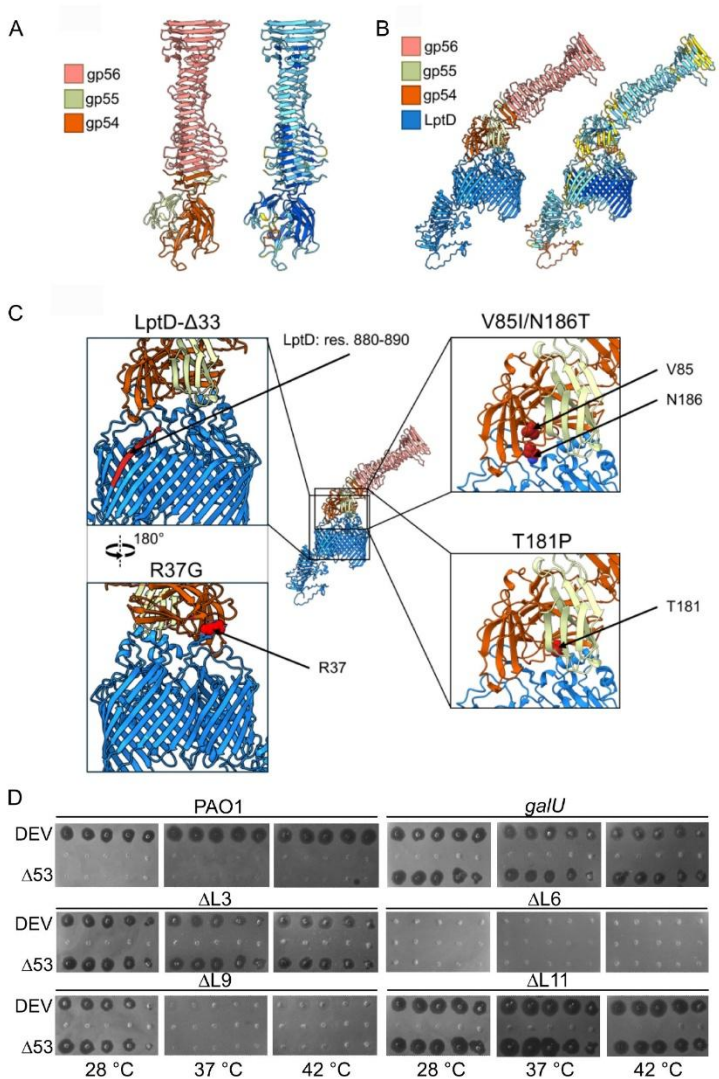


FIG 7 AlphaFold3 predictions of putative DEV receptor binding complex and phenotype of mutants devoid of LptD loops. (A) AlphaFold3 prediction of the trimeric gp56 C-terminus (res. 273–429), one copy of full-length gp55 and one copy of full-length gp54. Structure is colored by gene product (left) and according to the AlphaFold3 predicted local distance difference test (pLDDT) (right). The interface predicted template modeling (ipTM) and predicted template modeling (pTM) scores of the complex are 0.8 and 0.81, respectively. (B) AlphaFold3 prediction of receptor-binding complex with full-length LptD. Structure is colored by gene product (left) and pLDDT (right). The ipTM and pTM of the structure are 0.35 and 0.48, respectively. (C) Residues and regions identified in this study as relevant for adsorption are mapped in red onto the wt receptor and receptor-binding complex prediction. Residues 880–890 missing in LptD-Δ33 (top left); gp54 valine 85 and asparagine 186 (V85 and N186, top right); gp54 arginine 37 (R37, bottom left); gp54 proline 181 (P181, bottom right). (D) Replica plating of tenfold serial dilutions (from left to right) of the phages indicated on the left on PAO1 *galU* and *galU lptD* mutants lacking loop 3 (ΔL3), loop 6 (ΔL6), loop 9 (ΔL9), or loop 11 (ΔL11). The plates were incubated for 16 h at the indicated temperatures.

by altering the OM. Against this hypothesis, DEV resistance is also shown by a mutant lacking the LptD loop 6, which is far from LptD lateral gate and located at the interface with gp54 in the AlphaFold3 model. Moreover, the depletion of LpxA, which severely impairs both growth and LPS content, actually enhanced $\Delta 53$ phage attachment to PAO1, and ectopic expression of *P. aeruginosa* LptD and LptE in *E. coli* enabled DEV adsorption to *E. coli*, a species distantly related to *P. aeruginosa* and unlikely to produce surface molecules recognized by *P. aeruginosa* phages. However, the adsorption efficiency remained modest, possibly due to inefficient targeting of *P. aeruginosa* LptD to the *E. coli* membrane. Indeed, both wt DEV and the $\Delta 53$ mutant show high plating efficiency but low adsorption efficiency on deep-rough PAO1 mutants, suggesting that without the gp53-LPS interaction, phage attachment to the secondary receptor may be unstable and influenced by the experimental procedure used in adsorption assays.

To our knowledge, DEV is the first podovirus and the first *P. aeruginosa* phage proposed to use LptD as a receptor. Genetic evidence from *gp54* mutations suppressing the *galU lptD^{Δ53}* resistance to DEV supports a direct interaction between gp54 and LptD, identifying gp54 as the RBP. gp54 is encoded in an operon with the gpJ-like short tail fiber gp56 and gp55. In this study, we provide structural evidence that gp56 functions as a structural module that recruits gp55 and gp54, connecting them to the tail tube while stabilizing the tail plug, which we map to the conserved factor gp74. The asymmetry of the gp56-gp55-gp54 complex, which we designate here as the RBF, highlights the structural complexity of the DEV tail. The RBF represents a unique module that couples receptor recognition to tail unplugging, while permitting evolutionary diversification of the gp54 subunit to accommodate different hosts. The role of gp55, an essential DEV protein, appears to be that of a structural adaptor linking the tail fiber gp56 to the RBP gp54. Moreover, gp55 may help stabilize the interaction with the receptor. This is supported by the thermosensitive growth of DEV in the LptD mutant lacking loop 9, which in the AlphaFold3 model is close to gp55.

We propose that DEV and potentially other podoviruses of the *Schitoviridae* family employ an adsorption strategy analogous to that of bacteriophage T5 (and other siphoviruses). These phages sequentially recognize LPS and an OMP, which serve as primary and secondary receptors and are engaged by the lateral tail fibers and the tail tip complex, respectively (21, 41). Specifically, DEV may initiate host recognition through a reversible interaction between its gp53 tail fibers and the O-antigen. This step does not inactivate the phage, consistent with the observation that purified LPS fails to neutralize DEV. Instead, it likely stabilizes virion attachment and positions the phage near its secondary receptor, LptD, which is normally masked by the O-antigen layer in smooth strains (6, 42). Irreversible adsorption then occurs when the RBF complex engages LptD, similar to how T5 binds its final receptor FhuA (41). We propose that this interaction triggers tail-tip unplugging by destabilizing the interaction between the gp56 coiled coil domain and the plug gp74 (Fig. 6E), allowing the release of ejection proteins and the assembly of the DEV ejectosome (20).

Interestingly, although the *Schitoviridae* phages N4 and DEV utilize different host receptors, they appear to share a common strategy in which the primary receptor is transported by the secondary receptor. In fact, the *E. coli* phage N4 relies on the Novel Glycan Receptor (NGR) surface glycan, presumably bound by 12 appendages (gp66), and the NGR transporter NfrA, recognized by the phage gp65 tail sheath, as receptors for infection (43–45). Similarly, N4 employs the sheath protein gp65 to stabilize the tail plug gp53 and engage the NfrA receptor (33, 34), whereas DEV uses the RBF complex to stabilize gp74 and interact with LptD. In both phages, receptor binding via the RBP, gp65 in N4 and gp54 in DEV, triggers destabilization of the plug, leading to release of the ejection proteins (46). This mechanism may enhance adsorption efficiency by concentrating the phage close to the secondary receptor, given that both LPS and NGR are likely densely localized near their respective transporters, LptD and NfrA (47).

The identification of LptD as a phage receptor is potentially relevant for phage therapy, as LptD is an essential and conserved component of the OM, possibly limiting

the emergence of phage resistance via receptor modification or loss, or creating an evolutionary trade-off where mutations conferring phage resistance negatively impact cell envelope integrity and, consequently, antibiotic resistance (28). Given the high conservation of gp54 among *Litunaviruses*, we anticipate that other phages in this genus might use LptD as their receptor. However, the O-antigen and outer core effectively shield LptD, preventing phage access. Therefore, the host range of phages that use LptD as a terminal receptor is likely constrained by their ability to recognize and bind the primary receptor, typically the O-antigen. This initial interaction would be the principal determinant of host susceptibility, representing a significant bottleneck in the therapeutic application of LptD-targeting phages. Overcoming the barrier posed by the O-antigen will be essential for enabling these phages to infect a broad spectrum of clinically relevant strains.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids

Bacterial strains, bacteriophages, and plasmids are listed in Table S4, oligonucleotides in Table S5. Genome coordinates refer to PAO1 (GenBank accession number [NC_002516.2](#)) or DEV (GenBank accession no. [MF490238.1](#)). Details about the construction of plasmids, mutant bacterial strains, and phages are provided in Supplemental Methods. DEV deletion mutants were constructed by CRISPR-Cas mutagenesis (20). *lptD* mutants lacking specific extracellular loops were obtained by homologous recombination and sucrose-based selection (48). Bacterial cultures were grown in LB broth at 37°C unless otherwise stated. The cultures were supplemented with 300 µg/mL carbenicillin or 30 µg/mL chloramphenicol for plasmid maintenance. 0.05% or 0.2% (wt/vol) arabinose was added to induce expression from the *araBp* promoter when needed. Depletion of LpxA or LptE in *P. aeruginosa* strains with conditional expression was achieved as described in Supplemental Methods. *E. coli* cultures were maintained with 100 µg/mL ampicillin or 30 µg/mL chloramphenicol, as appropriate.

Basic techniques of phage handling

Phage lysates were generated as described (16). Methods applied to measure phage EOP, adsorption efficiency, and inactivation by LPS were previously described (19, 26) and are detailed in Supplemental Methods.

Isolation of DEV-resistant *galU* derivatives

To isolate DEV-resistant *galU* mutants, 0.1 mL of two independent cultures of PAER6b at OD₆₀₀ = 1.0 was plated onto two plates spread with 5×10^8 PFU of DEV. Two bacterial colonies were taken from different plates and streaked. Cultures of a sub-clone per each streak were tested as indicators for DEV replica plating to confirm resistance.

Bacteria and phage genome sequencing

Bacterial genomic DNA was extracted from two DEV-resistant PAER6b derivatives with the Genomic Purification Kit (Gentra System-Puregene). Phage DNA was extracted from 1 mL of high titer filtered lysate ($\sim 10^{10}$ PFU/mL) of DEV mutants using a modified phenol:chloroform:isoamyl alcohol method (Supplemental Methods). Bacteria and phage DNA were sequenced on NextSeq 2000 Illumina NGS platform at the SeqCenter (Pittsburgh, PA, USA) applying a sequencing package providing at minimum 400 (for bacteria) or 200 (for phages) Mb of 2×151 bp paired reads. Variant calling against PAO1 or DEV reference genomes was performed at the SeqCenter with the breseq software, version 0.36.1 (49). The average depth of sequencing coverage was >300 for bacterial genomes and >3,000 for phage genomes. Besides the new mutation in either *rpoH* or

lptD, the two bacterial genomes contained already known polymorphisms with respect to the reference genomic sequence (19).

LPS analysis

LPS was extracted from 1 OD₆₀₀ of exponential bacterial cultures using hot phenol and diethyl ether and analyzed by 18% tricine SDS-PAGE and 12% Bis-Tris SDS-PAGE as previously described (19). The gels were stained using the Pro-Q Emerald 300 staining kit (Thermo Fisher Scientific).

Phage UV mutagenesis

Fifty microliters of phage suspension at 10⁸ PFU/mL of DEV was irradiated with an UV dose of 10 mJ ($\lambda = 254$ nm). Ten microliters of the suspension was mixed with 140 μ L of a PAO1 culture at OD₆₀₀ = 0.5 in a 96-well plate. The plate was incubated at 37°C for 120 min and centrifuged 10 min at 4,500 rcf and 4°C. The supernatant was serially diluted and replica-plated onto the susceptible PAO1 and PAER6b hosts and the resistant PAER67 *galU lptD* ^{Δ 33} strain. Mutant phages forming turbid plaques were isolated on PAER67. DEV Δ 53 UV-mutagenesis was similarly performed (Supplemental Methods).

RT-PCR analysis of *gp56-55-54* operon transcription

RNA extraction from PAO1 cultures was performed at different time points after the infection with DEV and 2 μ g of RNA was reverse-transcribed as described (20). 0.5 μ L aliquots of the 10 μ L cDNA mixtures were PCR-amplified with forward primers 4249, 4250 or 4251, mapping within *gp56*, *gp55*, and *gp54*, respectively, and the reverse primer 4252 mapping within *gp54*. Mock reverse transcription reactions (i.e., without reverse transcriptase) were PCR-amplified to check DNA contamination, which was found to be undetectable.

DNA leakage assay from DEV Δ 56 virions

For the DNase I sensitivity assay, two PAO1 cultures in 10 mL of LB at OD₆₀₀ = 0.1 were infected with DEV or DEV Δ 56 with a MOI = 5 and incubated 50 min at 37°C. The infected cultures were pelleted to eliminate uninfected bacteria and cell debris, the supernatants filtered and the phages precipitated overnight at 4°C with 105 g/L of PEG₆₀₀₀ and 58 g/L of NaCl. After centrifugation for 30 min at 20,000 $\times$ g at 4°C, the pellets were resuspended in 1 mL of TN buffer and left 3 weeks at 4°C. Phage suspensions were then incubated 3 h at 37°C with slow shaking. The samples were PEG-precipitated as described before, the pellets resuspended in 1 mL of TN buffer, and the DNA was ethanol-precipitated from 0.5 mL of the supernatants and resuspended in 50 mL of water. Half of each sample was incubated with 10 mg/L DNase I for 30 min at 37°C and for 4 min at 93°C to inactivate the enzyme. The same volume of DNase I-treated (T) and not treated (NT) samples was analyzed by qPCR with primers specific for DEV *gp77* gene (4103–4104). The difference in DNA content was calculated as $2^{(Cq_{NT}-Cq_T)}$, where Cq_{NT} and Cq_T are the quantification cycles for NT and T samples, respectively. *Bam*HI sensitivity assay is described in Supplemental Methods.

Cryo-EM analysis of DEV Δ 53 virions and AlphaFold modeling

Vitrification and cryo-EM analysis of DEV Δ 53 virions is detailed in Supplemental Methods. All AlphaFold models of individual polypeptide chains and complexes were predicted using the AlphaFold3 server (36), as detailed in the Supplemental Methods. Mutation sites discussed in this study were then colored red to roughly localize putative binding interfaces using ChimeraX (50).

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J.N.N., C.A.A., N.F.B., S.A., F.F., F.A.F., and D.S. did the experiments. N.F.B. and G.C. performed cryo-EM structural analysis and AlphaFold3 structural predictions. D.S. and F.I. constructed *lptD* mutants and provided strains with conditional expression of essential genes. F.B. designed and supervised the project. All authors contributed to the writing of the manuscript.

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DATA AVAILABILITY

The cryo-EM asymmetric reconstruction of the DEV Δ53 virion tail and the focused reconstruction of the tail tip have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMD-74075 and EMD-74091. The corresponding atomic models have been deposited in the Protein Data Bank under accession numbers 9ZDW and 9ZE4. All other data supporting the findings of this study are available from the corresponding author upon request.

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental Material (mBio03561-25-S0001.pdf). Supplemental methods, Tables S1 to S5, and Fig. S1 to S6.

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