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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 diacylglyceryl 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 (Vogeley 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 μL 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 (OD_{600}) 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 $OD_{600} = 1$. 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 0.5 % 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 OD_{600} 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 $OD_{600} = 3$. Equal volumes (150 μL) of bacterial suspensions and HEPES solution containing or not propidium iodide (PI; 40 μg/mL) or 1-N-phenylnaphthylamine (NPN; 20 μM) were mixed, and 100 μL of each sample were aliquoted on a black flat-bottom 96-well microtiter plate. OD_{600} 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 OD_{600} 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 $OD_{600} = 1$. One hundred-μL 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 (OD_{600}) 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 $OD_{600} = 0.08$ in sterile saline solution, and swabbed onto MH agar plates, supplemented with 0.075 % arabinose for the conditional mutants. Disks containing rifampicin (5 μg), streptomycin (10 μg), gentamycin (10 μg), imipenem (10 μg), novobiocin (30 μg), tobramycin (10 μg), or 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 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 $OD_{600} = 1$. 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 μL aliquots of the $OD_{600} = 1$ 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 × 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 × *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 × *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 × *g* for 20 min. Cell membranes were collected by centrifuging the supernatant for 1 h at 210,000 × *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 × *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 × 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_{ara}::*lspA* Δ*lspA*, 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

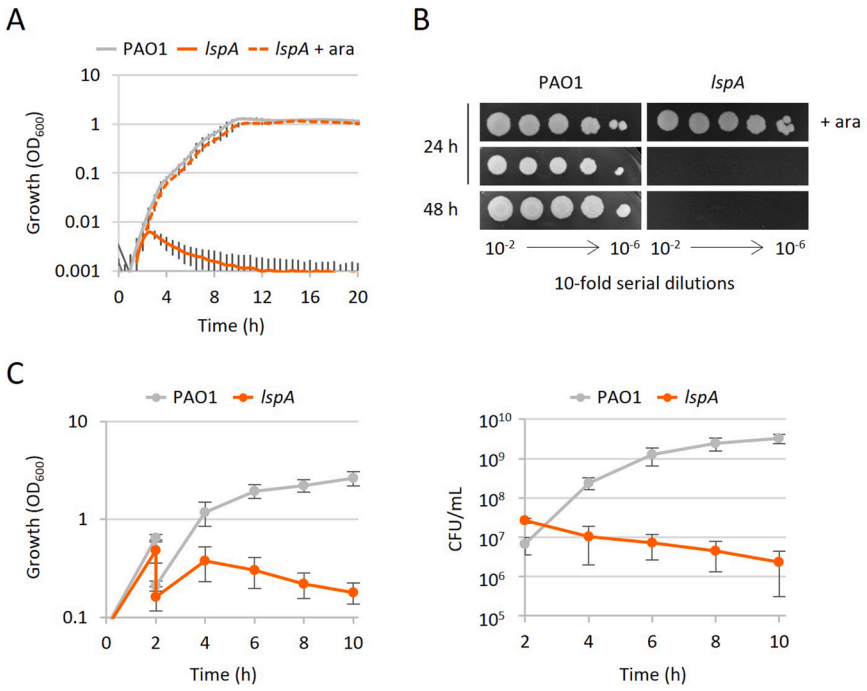

Fig. 1. **(A)** Growth curves of *P. aeruginosa* PAO1 and the PAO1 *araC* P_{ara}::*lspA* Δ*lspA* conditional mutant (*lspA*) cultured in microtiter plates in MH, supplemented or not with 0.5 % arabinose (+ ara). **(B)** Plating efficiency of the same strains described in panel A on MH agar plates supplemented 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. OD_{600} and cell viability (CFU/mL) were monitored every 2 h. Values in panel A and C are the mean (± 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.

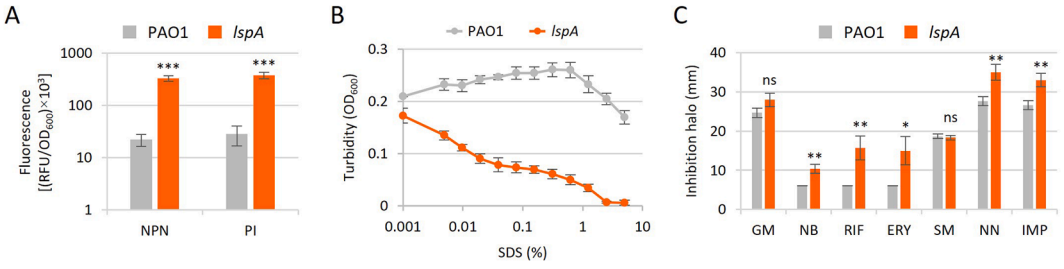

Fig. 2. **(A)** NPN- and PI-mediated fluorescence, measured as relative fluorescence units (RFU) normalized to the OD_{600} of the bacterial suspension, of *P. aeruginosa* PAO1 and the PAO1 *araC* P_{ara}::*lspA* Δ*lspA* conditional mutant (*lspA*) cultured in MH using the dual-refresh culturing strategy shown in Fig. 1C. **(B)** Sensitivity to the lytic effect of SDS, measured as a decrease in cell suspension turbidity (OD_{600}), 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 (± 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).

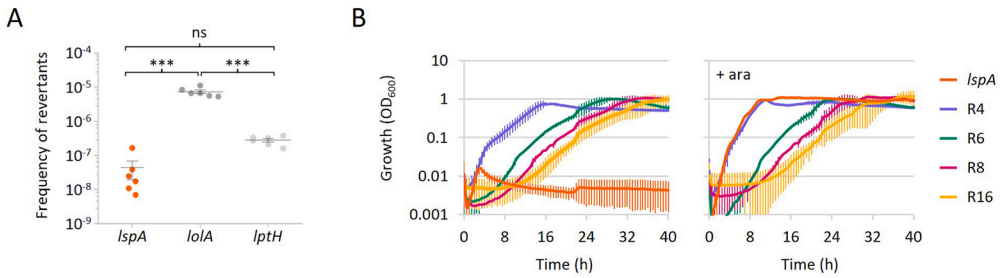

Fig. 3. (A) Frequency of revertant mutants of the PAO1 *araC* P_{ara}::*lspA ΔlspA* (*lspA*), PAO1 *araC* P_{ara}::*lolA ΔlolA* (*lolA*), and PAO1 *araC* P_{ara}::*lptH ΔlptH* (*lptH*) conditional mutants. Six independent experiments were performed for each strain. The asterisks indicate statistically 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 (± 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.

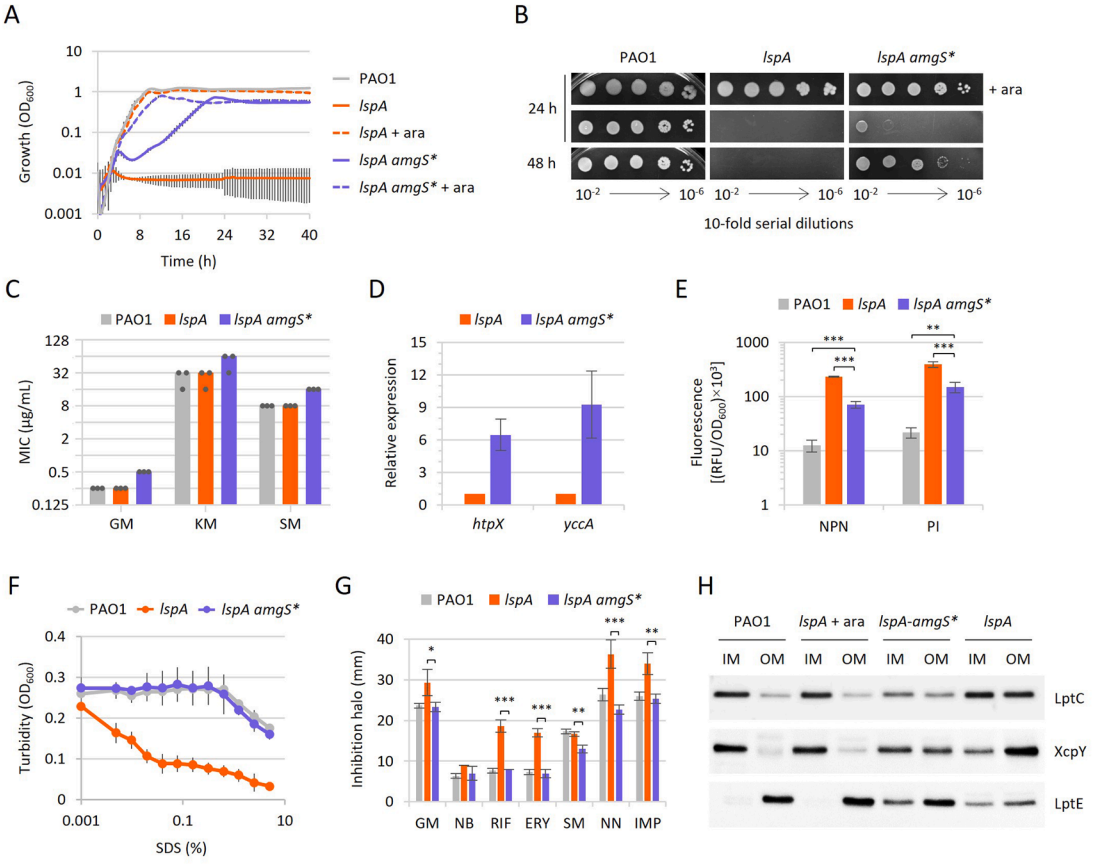

Fig. 4. (A) Growth curves of *P. aeruginosa* PAO1 and the PAO1 *araC* P_{ara}::*lspA ΔlspA* (*lspA*) and PAO1 *araC* P_{ara}::*lspA ΔlspA amgS*^{P381H} (*lspA amgS**) conditional mutants in MH, supplemented or not with 0.5 % arabinose (+ ara). **(B)** Plating efficiency of the same strains described 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 OD_{600} 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 (OD_{600}), 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 (± 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 (*htpX* 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 htpXand 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 *htpX* 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

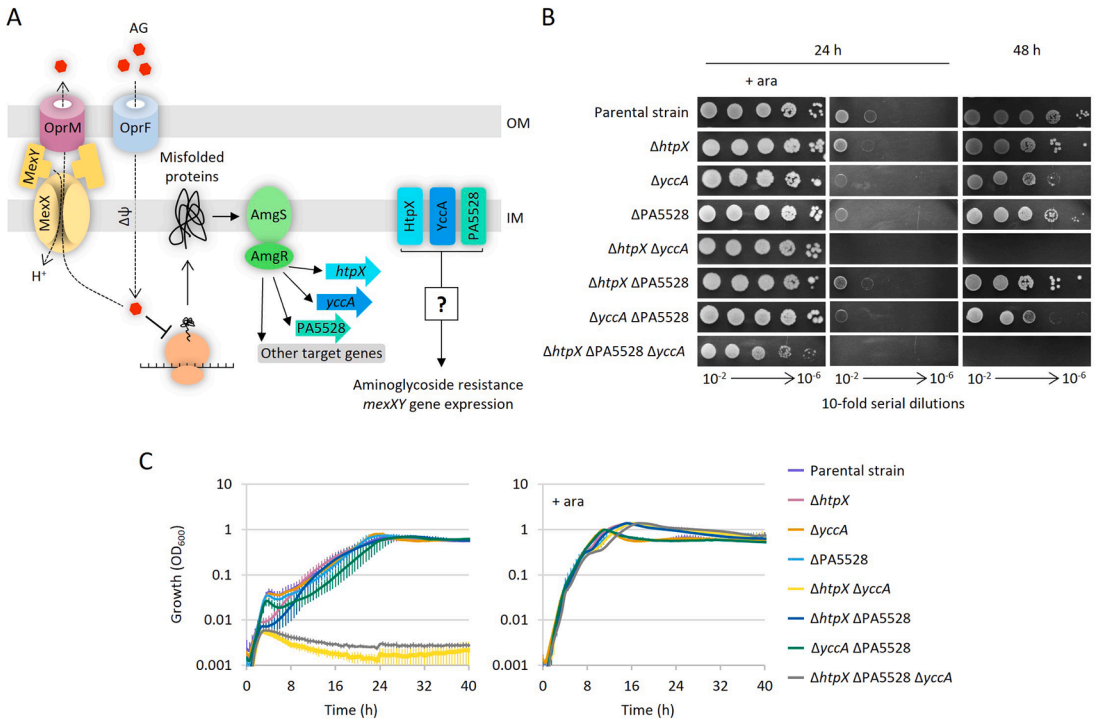

Fig. 5. **(A)** Schematic representation of the AmgRS signaling pathway in *P. aeruginosa* and its role in membrane stress adaptation and aminoglycoside resistance. **(B)** Plating efficiency of the conditional mutant PAO1 *araC* P_{ara}::*lspA* Δ*lspA* (parental strain) and its derivatives carrying 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 MH with or without 0.5 % arabinose (+ ara). Values are the mean (± standard deviation) of three independent experiments.

the double mutants Δ*htpX* ΔPA5528 and Δ*yccA* ΔPA5528 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 Δ*htpX* Δ*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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