The DedA superfamily member PetA is required for the transbilayer distribution of phosphatidylethanolamine in bacterial membranes

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The sorting of phospholipids between the inner and outer leaflets of the membrane bilayer is a fundamental problem in all organisms. Despite years of investigation, most of the enzymes that catalyze phospholipid reorientation in bacteria remain unknown. Studies from almost half a century ago in Bacillus subtilis and Bacillus megaterium revealed that newly synthesized phosphatidylethanolamine (PE) is rapidly translocated to the outer leaflet of the bilayer [Rothman & Kennedy, Proc. Natl. Acad. Sci. U.S.A. 74, 1821–1825 (1977)] but the identity of the putative PE flippase has eluded discovery. Recently, members of the DedA superfamily have been implicated in flipping the bacterial lipid carrier undecaprenyl phosphate and in scrambling eukaryotic phospholipids in vitro. Here, using the antimicrobial peptide duramycin that targets outward-facing PE, we show that Bacillus subtilis cells lacking the DedA paralog PetA (formerly YbfM) have increased resistance to duramycin. Sensitivity to duramycin is restored by expression of B. subtilis PetA or homologs from other bacteria. Analysis of duramycin-mediated killing upon induction of PE synthesis indicates that PetA is required for efficient PE transport. Finally, using fluorescently labeled duramycin we demonstrate that cells lacking PetA have reduced PE in their outer leaflet compared to wildtype. We conclude that PetA is a long-sought PE transporter. These data combined with bioinformatic analysis of other DedA paralogs argue that the primary role of DedA superfamily members is transporting distinct lipids across the membrane bilayer.

Significance
How phospholipids get distributed between the inner and outer leaflets of biological membranes is poorly understood. While progress has been made in identifying transporters in eukaryotic cells, the identity of flippases in bacteria has remained elusive. Here, we provide evidence that the DedA superfamily member PetA is a lipid transporter that distributes phosphatidylethanolamine between the inner and outer leaflets of Bacillus subtilis membranes. Our work suggests that the primary role of DedA family members is in transporting distinct lipids across the membrane.

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The authors declare no competing interest.

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and VMP1, were found to redistribute phospholipids with diverse headgroups across the lipid bilayer in proteoliposomes (16, 17).

Here, we provide evidence that *B. subtilis* PetA, a DedA superfamily member, is the long-sought PE transporter proposed by Rothman and Kennedy almost a half century ago. We show that cells lacking PetA are more resistant to the antimicrobial peptide (AMP) duramycin that targets outward-facing PE and show that homologs from other bacteria can restore sensitivity to the ∆petA mutant. Using fluorescently labeled duramycin we demonstrate that cells lacking PetA have reduced PE in their outerleaflet compared to wildtype, but their total pools of PE are indistinguishable. Finally, we present bioinformatic analysis of bacterial proteins with domain fusions suggesting that DedA domains are capable of transporting diverse phospholipids. These findings combined with studies on the UndP transporter UptA and eukaryotic TMEM41B and VMP1 argue that the primary function of proteins in the DedA superfamily is transporting distinct lipids across the bilayer.

**Results**

*ybfM* Is in an Operon with Genes Involved in PE Biosynthesis. *B. subtilis* encodes six DedA paralogs. In recent work, we showed that one of them (UptA, formerly YngC) is an undecaprenyl phosphate (UndP) flippase that recycles the lipid carrier from the outer to the inner leaflet of the cytoplasmic membrane (14). We speculated that other members of this large protein family are transporters that act on distinct lipids. The *B. subtilis* *ybfM* gene encodes a DedA paralog and is co-expressed in an operon with *pssA* and *psd* that encode phosphatidylserine (PS) synthase and phosphatidylserine decarboxylase (Fig. 1B). These two enzymes are required for the synthesis of phosphatidylethanolamine (PE) (Fig. 1A). Genome neighborhood analysis revealed that *ybfM* is frequently present in an operon or adjacent to the PE biosynthetic genes (Fig. 1B). These findings led us to hypothesize that YbfM facilitates the transbilayer movement of PE. Based on the results described below, we have renamed *ybfM*, petA for PE transporter Δ.

PetA Expression Sensitizes Cells to Duramycin. To investigate whether PetA affects the distribution of PE between the inner and outer leaflets, we used the antimicrobial peptide duramycin. Duramycin is a 19 amino acid AMP that specifically binds PE and disrupts the membrane causing rapid cell death (18, 19). Importantly, duramycin and its derivatives have been shown to specifically bind surface-exposed PE and have been used to screen for phospholipid transporters (20) and detect PE asymmetries in yeast (21, 22). To test whether PetA affected surface-exposed PE, we used minimal inhibitory concentration (MIC) assays to compare the sensitivity of wildtype *B. subtilis* with strains lacking petA or the PE biosynthetic genes. As reported previously, *pssA* and *psd* deletion strains were completely resistant to duramycin (19, 23) and the absence of LPG or cardiolipin did not affect the sensitivity of *B. subtilis* to duramycin (SI Appendix, Fig. S1), consistent with the specificity of duramycin for PE. Cells lacking PetA were twofold to fourfold more resistant to duramycin than wild-type (Fig. 2A), suggesting the mutant had less surface-exposed PE. Expression of *petA* in trans restored duramycin sensitivity to the ∆petA mutant, indicating that the deletion was not affecting expression of *pssA* or *psd*. Furthermore, expression of PetA homologs from diverse *Bacillus* species that displayed synteny with PE biosynthetic genes restored duramycin sensitivity to the *B. subtilis* ∆petA mutant (Fig. 2C). By contrast, overexpression of the DedA paralog UptA that transports UndP did not restore duramycin sensitivity to the ∆petA mutant (Fig. 2C).

To rule out the possibility that the total PE pool is lower in the ∆petA mutant compared to wildtype, we analyzed the abundance of all major phospholipids using thin layer chromatography (TLC) (SI Appendix, Fig. S1). As previously reported (24), the ∆pssA and ∆psd mutants completely lacked PE, and the cells lacking Psd accumulated PS since PS could not be converted to PE (Figs. 1A and 2B). However, and importantly, the ∆petA mutant had PE levels that were comparable to wildtype and the petA complementation strain (Fig. 2B and SI Appendix, Fig. S1).

To further characterize the contribution of PetA to duramycin sensitivity, we deleted the entire *pssA-petA-psd* operon and reconstructed it at two neutral genomic loci. The *pssA* and *psd* genes were placed under the control of an IPTG (isopropyl-β-d-thiogalactopyranoside) -regulated promoter in strains with or without petA fused to a xylose-regulated...
promoter (SI Appendix, Fig. S2A). These strains were grown with different concentrations of IPTG to titrate the levels of PE and analyzed for their sensitivity to duramycin. As expression of the PE synthesis enzymes increased, the sensitivity to duramycin of both the petA+ and petA− strains increased (SI Appendix, Fig. S2B) and at each IPTG concentration, the two strains had comparable levels of PE (SI Appendix, Fig. S2C). At every IPTG concentration tested, the strain expressing PetA was more sensitive to duramycin (SI Appendix, Fig. S2B). The increased sensitivity ranged from 2- to 16-fold depending on the level of PE. As part of this analysis, we identified IPTG concentrations in which the strain lacking PetA produced more PE than the strain expressing PetA, yet the petA− strain was more resistant to duramycin (SI Appendix, Fig. S2B). Since PE is synthesized on the inner leaflet of the membrane, these results argue that PetA functions in the transport of PE to outer leaflet of the membrane bilayer (Fig. 1A).

PetA Enhances the Rate of Appearance of Outward-Facing PE. In the seminal study of Rothman and Kennedy (8), newly synthesized PE in both B. subtilis and B. megaterium was rapidly flipped to the outer leaflet of the cell surface. Using the IPTG-regulated promoter fusion to pssA and pss described above, we induced de novo PE synthesis in cultures containing duramycin and monitored the rate at which the petA+ and petA− strains stopped growing and lysed (Fig. 2D). As anticipated, prior to IPTG addition and therefore in the absence of PE, the two strains grew identically and were completely resistant to duramycin (Fig. 2D). Upon addition of IPTG, both strains ceased growing and began to lyse. However, as can be seen in Fig. 2D, at all duramycin concentrations tested the petA+ strain was more rapidly killed than the petA− strain. We conclude that PetA increases the rate at which newly synthesized PE is distributed to the outer leaflet of the bilayer and is likely to be the long-sought transporter described by Rothman and Kennedy.

A Hydrophilic Pocket in the Predicted PetA Structure Is Critical for Function. To gain mechanistic insight into PetA transport activity, we turned to structural modeling. Alphafold predicts that PetA resembles a transporter with re-entrant helices on both sides of the phospholipid bilayer (Fig. 3A). These helices are predicted to generate a hydrophilic pocket within the membrane that could bind the zwitterionic PE headgroup, while allowing the hydrophobic lipid tails to remain in the bilayer during transport. Several of the residues that line the pocket are conserved among the PetA homologs that restored duramycin sensitivity to the B. subtilis ΔpetA mutant (SI Appendix, Fig. S4A). To investigate whether these residues were important for function, we used a FLAG-tagged variant of the PetA homolog from Teribacillus halophilus (Th). The PetA(Th) protein was chosen because the epitope-tagged fusion was functional and readily detected by immunoblot (SI Appendix, Fig. S4 B and C). As can be seen in Fig. 3B, most of the conserved residues that line the hydrophilic pocket impaired the ability of the PetA(Th) to confer duramycin sensitivity. Importantly, all the mutant proteins were produced at levels similar to wildtype PetA(Th) (Fig. 3C). These data support

![Image](https://www.pnas.org)
the model that the hydrophilic pocket is important for PE transport and could bind the zwitterionic headgroup.

**PetA Promotes Surface-Exposed PE.** To more directly test whether PetA functions to increase surface-exposed PE, we turned to a fluorescent analog of duramycin (dura-FL) that has been used as a probe to quantify surface-exposed PE in both eukaryotic and bacterial cells (25, 26). First, we confirmed that dura-FL retains specificity for PE in *B. subtilis* membranes (*SI Appendix*, Fig. S5) and then used it to probe PE localization. For the latter experiments, we used the strains described above in which the *pssA-petA-psd* operon was reconstructed at two neutral loci under inducible control. We grew strains with and without petA in the presence of 7.5 µM IPTG and 10 mM xylose. Under these conditions, cells lacking petA were 8-16-fold more resistant to duramycin than cells producing PetA (*SI Appendix*, Fig. S2B). Cells from exponentially growing cultures were stained with dura-FL and analyzed by fluorescence microscopy. The petA+ cells had markedly higher dura-FL labeling than the petA- cells (Fig. 4).

![Fig. 3. A hydrophilic pocket in the predicted PetA structure is required for function. (A) Structural model of PetA predicted by AlphaFold2 colored based on surface charge distribution. Enlarged view of the hydrophilic pocket within the membrane is shown in the inset. Conserved residues, shown as sticks, are highlighted. (B) MIC assay of *B. subtilis* strains expressing the *T. halophilus* PetA ortholog with the indicated point mutants. (C) Representative immunoblot of the FLAG-PetA(Th) variants. SigA controls for loading.](image)

![Fig. 4. PetA promotes surface exposure of PE. (A) Representative micrographs of the indicated *B. subtilis* strains stained with fluorescent duramycin (dura-FL) in the presence or absence of the membrane permeabilizing AMP, melittin. PssA and Psd were expressed with 7.5 µM IPTG in the presence of PxylA-petA expressed with 10 mM xylose or in the absence of this inducible allele. Top, dura-FL staining. Bottom, overlays of phase-contrast and propidium iodide images. (Scale bar, 1 µm.) (B) Quantification of the dura-FL fluorescence from the strains shown in A. Bar represents median.](image)
as assayed by propidium iodide, indicating that the dura-FL probe had not permeabilized the lipid bilayer and was therefore reporting on outward-oriented PE (Fig. 4). To confirm that the cells grown in the presence and absence of PetA had equivalent amounts of PE, we performed the dura-FL labeling in the presence of melittin, a membrane permeabilizing AMP (27) that should allow dura-FL access to both the outer and inner leaflets of the membrane. As anticipated, virtually all cells stained with propidium iodide and had significantly higher dura-FL labeling. Importantly, the dura-FL labeling was similar in the presence and absence of PetA (Fig. 4).

**Discussion**

Altogether, our findings support the model that PetA catalyzes the transport of PE from the inner to the outer leaflet of the cytoplasmic membrane and likely represents the activity originally proposed by Rothman and Kennedy in 1977. The mechanism by which PetA flips PE is currently unknown, but our analysis of the residues predicted to line the hydrophilic pocket in PetA, suggests that this region binds the zwitterionic headgroup while the lipid tails remain in the bilayer. We speculate that PetA undergoes a conformational change upon PE binding that relocates this pocket toward the outer leaflet, reminiscent of the elevator-type mechanisms of small molecule transport across the bilayer (28). Future studies will be focused on investigating this and other models for PE flipping. It is noteworthy that PetA and UptA are predicted to have similar membrane topologies yet PetA functions in anterograde transport of PE and UptA in retrograde transport of UndP. The different directions of lipid transport of these related transporters suggest that DedA proteins are scramblases, serving as conduits that enable their lipid substrates to travel down their concentration gradients. Intriguingly, follow-up studies to Rothman and Kennedy’s 1977 paper established that PE transport in both *Bacillus* cells and membrane extracts was energy independent (10, 29, 30). Furthermore, the eukaryotic DedA superfamily members, TMEM41B and VMP1, have been found to catalyze energy-independent scrambling of phospholipids in proteoliposomes (16, 17).

PetA represents the fourth DedA family member implicated in lipid transport and suggests that the primary role of the proteins in this superfamily is in lipid redistribution. Further support for this idea comes from bioinformatic analysis of bacterial proteins with domain fusions. This analysis identified several fusions of DedA domains to enzymatic domains involved in lipid synthesis and processing (Fig. 5). One set of fusions replaces the MprF flippase domain with a DedA domain, suggesting lysophosphatidylglycerol is synthesized and transported by these DedA-MprF fusions (Fig. 5A). Other examples include fusions of phospholipid synthase and phospholipase domains with DedA domains (Fig. 5 B and C). The challenge for the future is to identify the substrates flipped by these and other DedA family members. Recent attempts to classify DedA domain-containing proteins based on sequence conservation defined three main clades (31). YbfM/PetA was found to cluster with UptA homologs involved in UndP recycling rather than the clade predicted to be phospholipid flippases. Our data showing that PetA functions in PE transport, highlights the challenges that lie ahead in assigning functions to this diverse family of proteins.

We note that cells lacking *petA* were not fully resistant to dura-mycin, indicating that PetA transport is not the only mechanism that distributes PE to the outer leaflet of the membrane. We cannot rule out the possibility that there is a second dedicated PE transporter, but it is also possible that in the absence of PetA, PE is non-specifically redistributed by other lipid transporters or spontaneously flops, albeit slower than PetA-catalyzed redistribution. Furthermore, while our data establishes a role for PetA in PE transport, it is possible that PetA can transport other phospholipids. However, the number of DedA paralogs encoded by *B. subtilis* and other bacteria suggests a degree of specialization...
and a more narrow substrate specificity. We note that individual deletions of the other five dedA genes had no impact on duramycin sensitivity and a strain lacking all six paralogs had duramycin sensitivity that was indistinguishable from the single petA deletion (SI Appendix, Fig. S6). Thus, we suspect that PE transport is specific to PetA among the DedA family members in B. subtilis. Whether PE flipping is mediated by a member of another family of transporters or potentially occurs spontaneously remains an important area of investigation for the future.

Most of the phenotypic characterization of bacterial DedA proteins comes from work in E. coli, which has 8 DedA paralogs. Individual mutants have mild or undetectable phenotypes but strains lacking two or more paralogs have pleiotropic phenotypes (32–35) that include impaired membrane potential (35) and changes in lipid composition (36). Gram-negative bacteria like E. coli, use phospholipids as substrates for the acylation of lipoproteins (3, 4), and the acylation and phosphoethanol modification of lipid A (2). These reactions occur in the outer leaflet of the cytoplasmic membrane. We hypothesize that defects in lipid sorting in the E. coli DedA mutants affect the rate of phospholipid consumption by these reactions and could explain the changes in membrane composition and defects in membrane potential observed.

Recent studies on the transport of phospholipids from the outer leaflet of the cytoplasmic membrane to the inner leaflet of the outer membrane in Gram-negative bacteria have revealed that AsmA family proteins act as bridges across the periplasmic space (SI Appendix, Fig. S7) (5–7). Interestingly, an AsmA-like protein in eukaryotes, Atg2, has been found to transport lipids from the endoplasmic reticulum (ER) to the developing autophagosome (SI Appendix, Fig. S7) (37). Furthermore, the DedA family members TMEM41b and VMP1 that scramble phospholipids in vitro (37) (SI Appendix, Fig. S7) (37). They are required for proper autophagosome formation (16, 17, 38–40).

General Methods. All B. subtilis strains were derived from the prototrophic strain PT79 (41). All B. subtilis experiments were performed at 37 °C with aeration in lysogeny broth (LB). Antibiotic concentrations used were: 100 µg/mL spectinomycin, 10 µg/mL kanamycin, 5 µg/mL chloramphenicol, 10 µg/mL tetracycline, 1 µg/mL erythromycin and 25 µg/mL lincomycin (MLS). All E. coli strains were derived from the prototrophic strain MC1061. E. coli growing cultures of were collected by centrifugation at 7000 RPM for 2 min. Cells were washed once with 1 × PBS (pH 7.4) and resuspended in 1/25th volume of 1 × PBS. Duramycin-FL-Labeling (Dura-FL) (Molecular Targeting Technologies) was added to a final concentration of 20 µg/mL during the dura-FL labeling step. Phase-contrast and fluorescence microscopy was performed with a Nikon Ti inverted microscope using a Plan Apo 100×/1.4 Oil Ph3 DM objective, a Lumencore SpectraX Light Emitting Diode illumination system and an Andor Zyla 4.2 Plus SCMOS camera. Chroma ET filter cubes (#49002 and 49008) were used for imaging, dura-FL and propidium idioxide, respectively. Exposure time of 1 s was used for dura-FL and 50 ms was used for propidium idioxide. Images were acquired with Nikon elements 4.3 software and analyzed using ImageJ (version2.3). Images were taken from multiple fields of view and images shown are representative of at least three independent experiments.

Structural Model Visualization. Alphafold2 predictions of PetA were downloaded from the AlphaFold Protein Structure Database (available at: https://alphafold.ebi.ac.uk/). ChimeraX1.3 was used to visualize the structural models and generate images. Residues critical for PetA function are shown as sticks.

Genomic Neighborhood and Domain Fusion Analysis. Gene neighborhood analysis was performed as previously described (14). Briefly, the Enzyme Similarity Tool (ETSI) (42) was used to generate sequence similarity networks (SSN) for the DedA protein family from the pfam entry SNARE_assoc (PF09335). Due to the large size of the SNARE_assoc family, the UniRef90 database was used. The SSN was then used as input for performing genomic neighborhood analysis using the Enzyme Function Initiative Genome Neighborhood Tool (EFI-GNI v1.0) available at: https://efi-gni.illinois.edu/efi-gni/index.php. Alignment score cutoff of 35% was used for the analysis. Gene neighborhood diagrams were generated to visualize the 10 nearest genes surrounding all dedA genes. Domain fusions were identified for the PF09335(SNARE_assoc) protein family on the InterPro website available at: https://www.ebi.ac.uk/interpro/entry/pfam/PF09335/.

MIC Assays. Exponentially growing cultures of B. subtilis were back-diluted 1:10,000 into 96-well microtiter plates containing the indicated concentrations of antibiotic and inducers. Plates were sealed with breathable membranes and grown with orbital shaking at 37 °C overnight. Plates were photographed after the overnight (~16 h) incubation. All MIC assays were performed in at least triplicate on the E. coli DedA mutants affected the rate of phospholipid consumption by these reactions and could explain the changes in membrane composition and defects in membrane potential observed.

Methods

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Growth Curve Assays. Exponentially growing cultures of B. subtilis were back-diluted to a starting OD of 0.05 in 25 mL LB containing 10 mM xylose and the indicated concentration of duramycin in 250-mL baffled flasks. Cultures were grown for 45 min with shaking with samples taken for OD measurements every 15 min. After 45 min of growth, IPTG was added to a final concentration of 20 µM and OD measurements were taken every 10 min thereafter. Growth curves were plotted with GraphPad Prism. Growth curves were performed at least three times and representative graphs are shown. All replicates gave comparable results.
Lipid Extractions. Three OD units of exponentially growing cultures of *B. subtilis* were collected by centrifugation at 3200g for 5 min. Cells were washed once with 1 mL of 1× PBS (pH 7.4) and resuspended in 1×PBS (pH 7.4) to a final volume of 300 μL. 750 μL of methanol and 375 μL of chloroform were added to cells (1:2:0.8) (CHCl3:MeOH:H2O). The mixture was incubated at room temperature for 1 h with occasional vortexing. The mixture was then centrifuged at 2000g for 10 min at 4°C to remove debris. The supernatant was added to a clean test tube with 375 μL chloroform and 375 μL of water (1:1:0.9) (CHCl3:MeOH:H2O) and incubated at room temperature for 10 min with occasional vortexing. Finally, the mixture was centrifuging at 2000×g for 10 min at 4°C. The organic phase was removed and dried under vacuum.

Thin-Layer Chromatography. The chromatography chamber was lined with Whatman paper and equilibrated for 2 h with chloroform–methanol–acetic acid (65:25:8, v/v). Silica 60 TLC plates (Supelco) were impregnated for 1 min in a 1.2% boric acid solution in 100% ethanol and activated at 100°C for 30 min. Dried lipid samples were resuspended in 5 μL of chloroform and 3 μL was immediately spotted onto the activated TLC plate and allowed to dry. The TLC plate was placed in the chromatography chamber until the solvent front was approximately 1 inch from top of the plate. The TLC plate was air dried and briefly submerged in primer-uline (5 mg/100 mL in 80:20 acetone–water) and then imaged under ultraviolet light. Images shown are representative of three biological replicates. All replicates gave comparable results.

Data, Materials, and Software Availability. UniproT accession codes for gene neighborhood analysis can be found in supplementary tables. Primers, synthetic DNA constructs and strains used can be found in supplementary tables. All study data are included in the article and/or SI Appendix.

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