**Main Text for**

# Polar localization of putative phospholipid transporters in *Escherichia coli*

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**Abstract**

The Gram-negative bacterial cell envelope comprises an outer membrane (OM) with an asymmetric arrangement of lipopolysaccharides (LPS) and phospholipids (PLs), protecting them from both physical and chemical threats. To build the OM, PLs must be transported across the cell envelope; this process has remained elusive until recently, where three collectively essential AsmA-superfamily proteins – YhdP, TamB, and YdbH – are proposed to function as anterograde PL transporters in *Escherichia coli*. Here, we discover that all three proteins are strongly enriched at the cell poles. We also identify a novel interacting partner of YhdP, which provides possible insights into its localization mechanism. Our observation raises the possibility that anterograde PL transport could be spatially restricted to the cell poles, and highlights the importance of understanding the spatial-temporal regulation of OM biogenesis in coordination with cell growth and division.

## Main text

The outer membrane (OM) is an essential barrier that protects Gram-negative bacteria from antibiotics and detergents (1). Critical to this function is the asymmetric arrangement of lipids in the OM, which is presumably established via the direct placement of lipopolysaccharides (LPS) and phospholipids (PLs) into the outer and inner leaflets, respectively (2). LPS is transported from the inner membrane (IM) to the OM along the continuous hydrophobic groove of the seven-component Lpt bridge, eventually inserted into the outer leaflet all around the cells except the poles (3, 4). By comparison, how PLs are moved bidirectionally across the cell envelope has been less clear (2). Recently, it was proposed that three AsmA-superfamily proteins, YhdP, TamB, and YdbH, are involved in this essential process of anterograde PL transport in *Escherichia coli* (5, 6). These trans-envelope proteins are structural homologs of eukaryotic bridge-like lipid transport proteins, featuring repeating β-groove motifs along their predicted/solved structures compatible with lipid binding and transport (7, 8). Indeed, experimental and *in silico* evidences that support PL binding in these hydrophobic grooves are emerging (9-11), though mechanistic understanding of transport is lacking. For example, it is not clear what other factor(s) might be required as part of the functional complexes of these AsmA-superfamily proteins, nor is it known where these complexes may assemble in the cell. YdbH interacts with the OM lipoprotein YnbE (12), and TamB interacts with the OM β-barrel protein (OMP) TamA, the latter also in fact has an established function in OMP assembly (13, 14). In this regard, much less is known about YhdP.

## Results

**YhdP interacts with the cell division protein DedD.** To identify potential interacting partner(s) of YhdP, we expressed a functional C-terminally 8xHis-tagged version of YhdP at low levels in an *E. coli* wild-type strain (Figure S1), and performed affinity purification after isolating and solubilizing the total membrane fraction. YhdP-His migrated as a ~150 kDa band on SDS-PAGE (Figure 1A). An additional band between 35-40 kDa was readily observed only in the sample containing YhdP-His (Figure 1A), suggesting a possible interacting partner. Interestingly, tandem mass spectrometry identified this co-purified band as the cell division protein DedD. To further validate this interaction, we performed reciprocal purification, and demonstrated that N-terminally 6xHis-tagged DedD pulled down C-terminally 3xFLAG-tagged YhdP (Figure 1B, S1). We conclude that YhdP stably and specifically interacts with DedD in cells.

**YhdP localizes to the cell poles.** DedD is a single-pass IM protein containing a periplasmic sporulation-related repeat (SPOR) domain (15), which is known to bind “denuded” peptidoglycan at the cell septum; it localizes to mid-cell and influences the division process via an unclear mechanism (15). We therefore wondered if the observed interaction with DedD is important for the intracellular distribution of YhdP. To

study YhdP localization, we constructed and expressed a fully functional N-terminal sfGFP fusion of YhdP in cells (Figure S2). Strikingly, sfGFP-YhdP was found predominantly at the cell poles, and this polar localization did not change in the absence of DedD (Figure 2A). We sometimes observed slight fluorescence signal at mid-cell, which however did not correlate with the cell division cycle.

Cells expressing sfGFP-YhdP were slightly elongated (Figure 2B), indicative of division defects. This phenotype was similarly observed in strains expressing YhdP-His (Figure S3). We reasoned that expressing YhdP *in trans* might sequester DedD in these cells. Consistent with this idea, introducing additional copies of DedD partially reversed the elongated cell phenotype (Figure 2D). In this strain, sfGFP-YhdP still localized to the cell poles (Figure 2C), and slightly more consistently detected at mid-cell. While DedD does not influence the strong polar localization of YhdP, it appears to stabilize the appearance of YhdP at the mid-cell, when expressed at sufficient levels.

**TamB and YdbH also localizes predominantly to cell poles.** The revelation that YhdP is found at the cell poles led us to investigate the subcellular localization of the other two putative PL transport proteins. We constructed N-terminally sfGFP-tagged versions of TamB and YdbH, and demonstrated their functionalities (Figure S2). Interestingly, both sfGFP-TamB and sfGFP-YdbH appeared strongly enriched at the cell poles (Figure 2E). We also observed localization of TamB at the mid-cell in longer cells, though it was difficult to differentiate whether the protein appeared there before or after complete cell separation. (Figure 2E). Notably, expressing sfGFP-TamB or sfGFP-YdbH did not result in cell division defects (Figure 2F). Taken together, these observations established that the three collectively essential AsmA superfamily proteins in *E. coli* are polarly localized, implying that their possible role(s) in PL transport is likely spatially confined.

## Discussion

How YhdP, TamB, and YdbH are spatially enriched at the cell poles is intriguing. One possibility is that all these three AsmA superfamily proteins are recruited to the mid-cell at the late stage of division, and then stays at the new pole (eventually the “older” pole) to support cell growth. These proteins contain a large periplasmic domain that spans the cell envelope; the presence of the peptidoglycan mesh likely impedes their lateral diffusion once the protein bridges are formed. In the case of YhdP, its specific interaction with DedD may contribute to the initial appearance of YhdP at mid-cell, but is insufficient on its own. Additional undiscovered factor(s) are likely important for YhdP distribution, since removing DedD does not abolish polar localization, nor does it cause OM defects similar to a strain lacking YhdP (Figure S4). In the same vein, TamB and YdbH may be enriched at mid-cell, and then the cell poles. Of course, an equally possible scenario is that these AsmA-superfamily proteins could be “recruited” directly to the

cell poles, albeit via unknown mechanism(s). The exact molecular bases for polar localization of the putative PL transporters remain to be investigated.

The observed polar localization of YhdP, TamB, and YdbH imply that (anterograde) PL transport to the OM may be spatially confined to the cell poles. Consistent with this idea, removing YhdP was shown to partially rescue the unique IM shrinkage phenotype that specifically occurs at the cell pole, presumably due to high PL flux, in a strain with OM lipid dyshomeostasis (16). Polarly-confined PL transport contrasts strikingly with the assembly of new LPS and OMPs, which occurs all around the cell except the poles (3, 4, 17). This spatial segregation of OM biogenesis pathways is intriguing. Unlike LPS and OMPs, PLs are much more diffusive, and can be effectively re-distributed in the inner leaflet of the OM from the poles to support OM expansion elsewhere during growth. Furthermore, having the major constituents of the two leaflets of the OM be assembled at distinct localities may be optimal for the establishment of OM lipid asymmetry; inserting PLs into the inner leaflet where the outer leaflet is already filled (with LPS) could minimize scrambling and prevent mislocalization of PLs. While the field continues to gather experimental evidence of AsmA-superfamily proteins moving PLs directly, it will also be critical to test and understand the evolutionary advantage(s) to localize PL transport to the poles.

## Methods

**Strains, plasmids, and growth conditions.** Bacterial strains and plasmids are summarized in Tables S1 and S2. *E. coli* MG1655 is used as the wild-type for all experiment. Strains were grown in Luria-Bertani (LB) broth (1% tryptone and 0.5% yeast extract, 1% NaCl) at 37°C.

**Affinity purification, SDS-PAGE, and immunoblot.** For each strain, 1.5 L of cell culture was lysed via sonication and the membrane pellet collected via ultracentrifugation. Solubilized membrane fraction was loaded into a column packed with TALON cobalt resin (Clontech) and incubated for 1 h at 4 °C with rocking. The mixture was allowed to drain by gravity before washing with 10 × 10 mL and eluted with 4 mL of elution buffer containing 200 mM imidazole. Concentrated eluate was subjected to SDS-PAGE analyses and immunoblotting. The resulting gel was stained with silver stain kit (SilverQuest™ Silver Staining Kit, ThermoFisher) and subsequently Coomassie Blue, or transferred onto PVDF membrane for immunoblotting with α-His or α-FLAG antibodies. Detailed protocol is available in the supporting information.

**Microscopy**. Cell morphology and fluorescence localization of proteins were captured using an Olympus FV3000 confocal microscope at 100x magnification. Mid-log cultures were spotted on 1% agarose pad for image acquisition. Images were analyzed using ImageJ (FIJI) and the MicrobeJ plugin.

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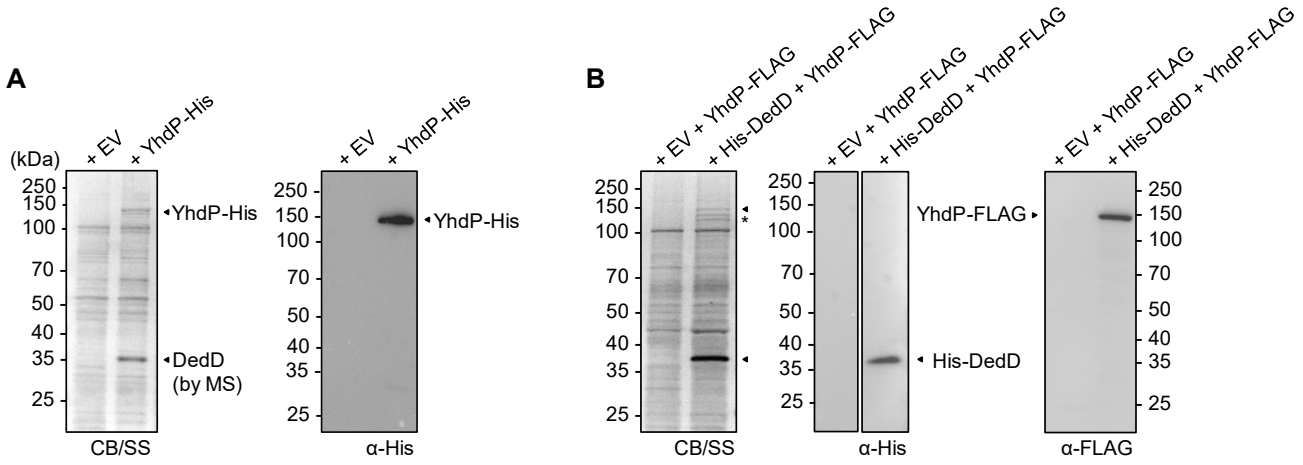
113 **Figure Legend**

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116 **Figure 1 YhdP interacts specifically with DedD.** (A) Affinity purification of MG1655 WT strain harboring
117 empty vector or pET23/42-*yhdP-His*. Samples were subjected to SDS-PAGE and immunoblot analyses
118 using α-His antibody. Tandem MS/MS sequencing of the indicated protein band revealed DedD as the
119 interacting partner. (B) Reciprocal affinity purification using WT strain harboring pBAD33-*His-dedD* and
120 pET23/42-*yhdP-FLAG*. Samples were subjected to SDS-PAGE stained with coomassie blue (CB) and
121 silver stain (SS), and immunoblot analyses using α-His and α-FLAG antibodies. (*) Two additional protein
122 bands co-purified may represent native YhdP and/or C-terminal degradation resulting in loss of FLAG
123 tag.

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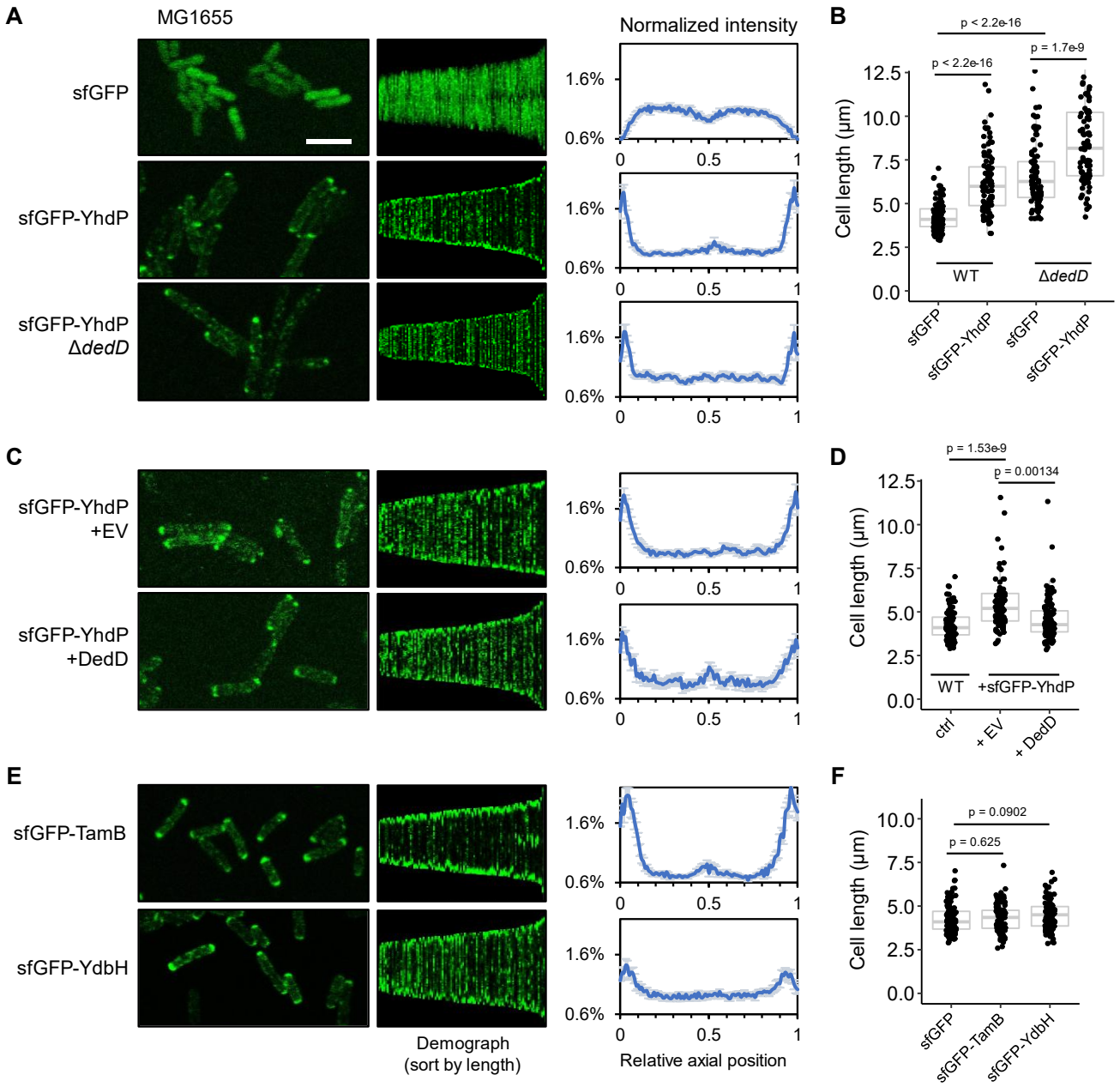


**Figure 2 YhdP, TamB, and YdbH are enriched at the cell poles.** (A), (C), (E) (*left*) Fluorescence microscopy images of MG1655 WT or Δ*dedD* cells harboring pET23/42-*sfgfp* (control), pET23/42-*sfgfp-yhdP*, pET23/42-*sfgfp-tamB*, or pET23/42-*sfgfp-ydbH*. The scale bar represents 5 µm. (*middle*) Demographic representations of the sfGFP fluorescence signals in the indicated strains, normalized along the long axis of individual cells and sorted according to cell length. (*right*) Average percentage distribution of the sfGFP signals along the long axis of cells (N=100). 95% confidence intervals are shown. (B), (D), (F) Quantification of cell length (N=100) of the indicated strains. Wilcoxon ranked sum test was used to test for statistical significance.

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