Main Text for

Polar localization of putative phospholipid transporters in *Escherichia coli*

Wee Boon Tan[1,2,#], Zhi-Soon Chong[1,#], Jacob Wye Meng Cheong[1,#], Jiang Yeow[1], and Shu-Sin Chng[1,2,*]

Affiliations:

[1]Department of Chemistry, National University of Singapore, Singapore 117543.

[2]Singapore Center for Environmental Life Sciences Engineering, National University of Singapore (SCELSE-NUS), Singapore 117456.

[#] These authors contributed equally

* To whom correspondence should be addressed.

E-mail: chmchngs@nus.edu.sg

ORCID ID:

Wee Boon Tan: 0000-0002-5055-4517

Shu-Sin Chng: 0000-0001-5466-7183

Jiang Yeow: 0000-0001-5384-7211

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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 identify a novel interacting partner of YhdP, and discover that all three AsmA-superfamily proteins are recruited to, and strongly enriched, at the cell poles. 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.

Importance

The outer membrane of Gram-negative bacteria serves as an effective permeability barrier, and confers intrinsic antibiotic resistance. This barrier function requires distinct distribution of lipids across the bilayer, yet how phospholipids, the most basic building block, get transported and assembled into the outer membrane is not well understood. In this study, we describe the observation revealing that three putative phospholipid transporters are mostly present at the cell poles in *Escherichia coli*, highlighting possible polar localization of lipid transport to ultimately support outer membrane expansion and structure during growth and division. Our work provides new insights into outer membrane biogenesis, and informs future strategies for antibiotics development against lipid transport processes.

Observation

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.

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. We could no longer detect this unique band in the Δ*dedD* strain (Figure 1A). 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 were also able to predict a structural model of a YhdP-DedD heterodimer using AlphaFold3 (15), revealing topologically sound interactions at both the transmembrane and periplasmic regions (Figure S2). We conclude that YhdP 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 (16), 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 (16). We therefore

wondered if the observed interaction with DedD is important for the intracellular distribution of YhdP. To study localization, we constructed N-terminal sfGFP fusions of both DedD and YhdP. We showed that sfGFP-YhdP is fully functional in maintaining the OM barrier (Figure S3). As expected, sfGFP-DedD localized to the cell septa during division (Figure 2A). In striking contrast, however, sfGFP-YhdP was found predominantly at the cell poles. We further demonstrated that this polar localization is not dependent on DedD (Figure 2A).

Cells expressing sfGFP-YhdP were slightly elongated (Figure S4), indicative of division defects. This phenotype was similarly observed in strains expressing YhdP-His (Figure S5). 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 S4). While DedD and YhdP do not appear to co-localize, there are functional implications for their observed interaction.

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 stable expression and functionalities (Figure S2). Interestingly, both sfGFP-TamB and sfGFP-YdbH appeared strongly enriched at the cell poles (Figure 2B). 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 2B). Notably, expressing sfGFP-TamB or sfGFP-YdbH did not result in cell division defects (Figure S4). 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.

The putative PL transporters are targeted directly to the poles. How YhdP, TamB, and YdbH are spatially enriched at the cell poles is unclear. These AsmA-superfamily proteins could be recruited directly to the poles. Alternatively, they may be first recruited to the mid-cell at the late stage of division, and then stay at the new pole. To ascertain if the polar signal(s) appears before or after the completion of cell division, we performed time-lapse fluorescence microscopy with our strains. Interestingly, all three sfGFP-YhdP, sfGFP-TamB, and sfGFP-YdbH only accumulated strongly at the new poles after two daughter cells had clearly separated (Figure 2C-E, Supplemental Movies 1-3). Notably, these putative PL transporters contain a large periplasmic domain that spans the cell envelope, therefore are likely immobile once the protein bridges are formed, due to the presence of the peptidoglycan mesh in the periplasm. Consistent with minimal lateral diffusion, the fluorescence signals for all three proteins remained largely static once formed (Supplemental Movies 1-3). While the exact molecular bases for

polar localization of the putative PL transporters remain to be investigated, we conclude that YhdP, TamB, and YdbH are directly targeted to the cell poles (instead of division sites) and remain immobile thereafter.

Discussion

Despite the strong interaction, majority of YhdP and DedD do not appear to occupy the same physical subcellular space. DedD is also not required for YhdP function in maintaining the OM barrier (Figure S6). While DedD diffuses away from the division site as daughter cells split, a low, residual level of DedD is also present everywhere, including the poles (Figure S7, Supplemental Movie 4). We speculate that the polar subpopulation of DedD could interact stably with, and to support, YhdP, e.g. facilitating assembly through the cell wall mesh. The exact functional implication for YhdP-DedD interaction requires further investigation.

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 (17). 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, 18). 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. Besides, 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

Strain and plasmid information and experimental procedures for affinity purification and confocal microscopy are detailed in the supporting information.

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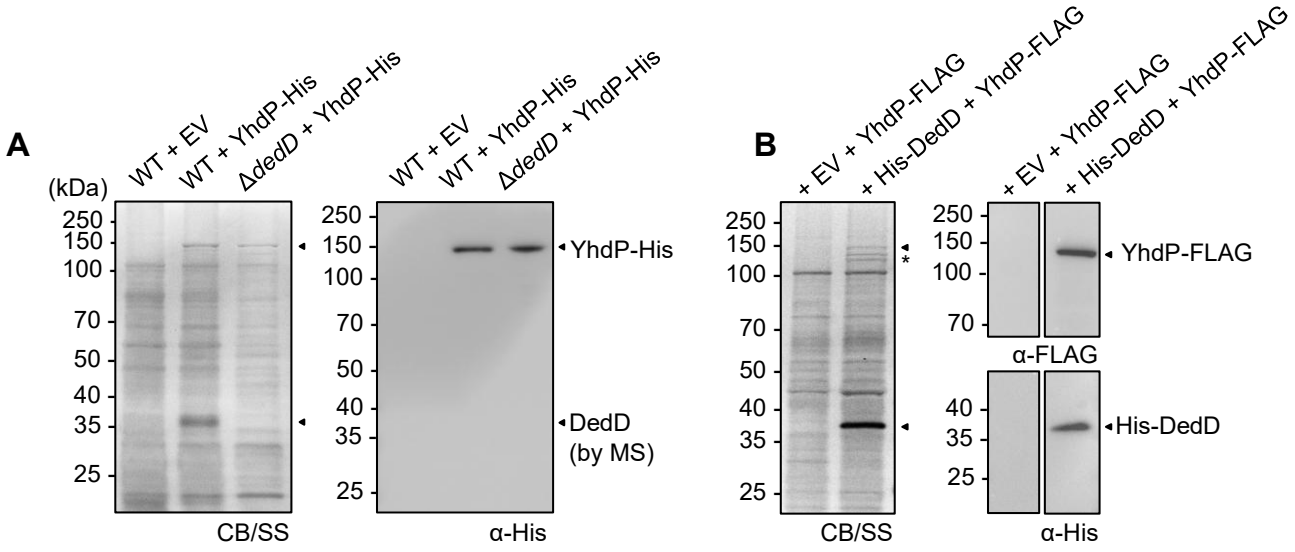
116 **Figure Legend**



117

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

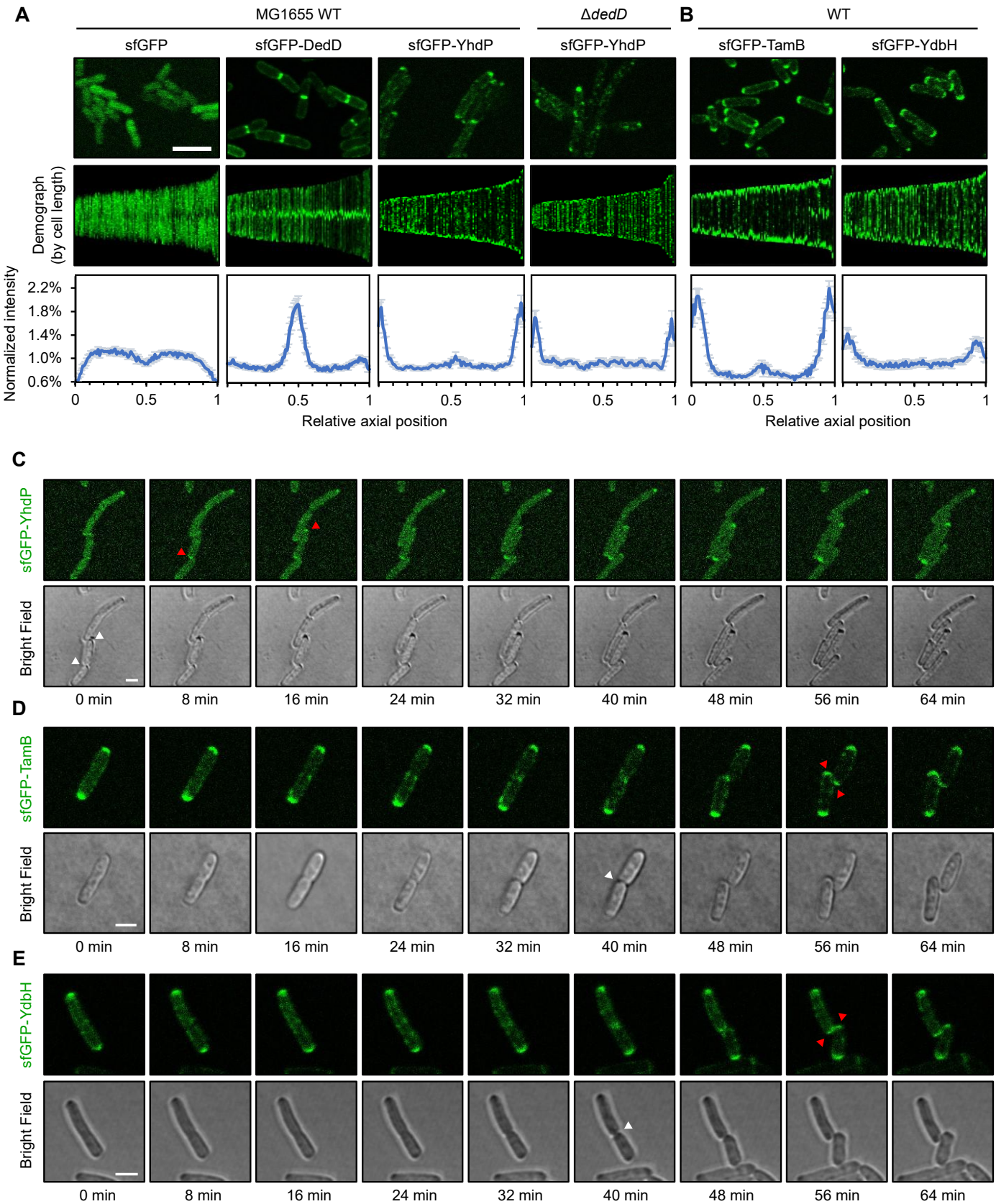

A
MG1655 WT
ΔdedD
B
WT
sfGFP
sfGFP-DedD
sfGFP-YhdP
sfGFP-YhdP
sfGFP-TamB
sfGFP-YdbH
Demograph (by cell length)
Normalized intensity
2.2%
1.8%
1.4%
1.0%
0.6%
0
0.5
1
Relative axial position
C
sfGFP-YhdP
Bright Field
D
sfGFP-TamB
Bright Field
E
sfGFP-YdbH
Bright Field
0 min
8 min
16 min
24 min
32 min
40 min
48 min
56 min
64 min

Figure 2 YhdP, TamB, and YdbH are enriched at the cell poles. (A),(B) (*top*) Fluorescence microscopy images of WT or Δ*dedD* cells harboring pET23/42-*sfgfp*, pET23/42-*sfgfp-dedD,* pET23/42-*sfgfp-yhdP*, pET23/42-*sfgfp-tamB*, or pET23/42-*sfgfp-ydbH*. Scale bar represents 5 μm. (*middle*) Demograph of the fluorescence sorted by cell length. (*bottom*) Distribution of the fluorescence along the long axis of cells (N=100). Error bars represent 95% confidence intervals. (C),(D),(E) Fluorescence time-lapse microscopy tracking the appearance of polar fluorescence signal in cell expressing sfGFP-YhdP, sfGFP-TamB, or sfGFP-YdbH. Images were originally taken at 2-minute intervals (see Supplemental Movies 1-3) and presented here at 8-minute intervals between frames. Scale bars represent 2 μm. White arrow(s) indicates completion of cell division and generation of new pole, only after which the fluorescence signals for all three putative PL transporters started to accumulate (red arrows).

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