# Lipid Flippases for Bacterial Peptidoglycan Biosynthesis

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**Supplementary Issue: Cellular Anatomy of Lipid Traffic**

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**ABSTRACT:** The biosynthesis of cellular polysaccharides and glycoconjugates often involves lipid-linked intermediates that need to be translocated across membranes. Essential pathways such as *N*-glycosylation in eukaryotes and biogenesis of the peptidoglycan (PG) cell wall in bacteria share a common strategy where nucleotide-sugars are used to build a membrane-bound oligosaccharide precursor that is linked to a phosphorylated isoprenoid lipid. Once made, these lipid-linked intermediates must be translocated across a membrane so that they can serve as substrates in a different cellular compartment. How translocation occurs is poorly understood, although it clearly requires a transporter or flippase. Identification of these transporters is notoriously difficult, and, in particular, the identity of the flippase of lipid II, an intermediate required for PG biogenesis, has been the subject of much debate. Here, I will review the body of work that has recently fueled this controversy, centered on proposed flippase candidates FtsW, MurJ, and AmJ.

**KEYWORDS:** MOP exporter, MATE transporter, murein, MviN, YdaH

**SUPPLEMENT:** Cellular Anatomy of Lipid Traffic

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## Introduction

Bacteria are unicellular organisms that often live in environments where the external osmolarity is lower than that in their cytoplasm. To protect themselves from the osmotic lysis that this difference in pressure would cause, most bacteria surround their cytoplasmic membrane with a rigid cell wall. This cell wall, composed of peptidoglycan (PG), is a polymeric macromolecule built with glycan chains that are interconnected through peptide bridges.[1,2] The resulting structure, or sacculus, is incredibly stable and serves as a scaffold for other envelope structures.

Building the PG matrix is a complex, highly controlled process.[1,3] When a bacterium grows, it must add new material into the preexisting PG structure in order to accommodate the increase in cell size. Then, when the bacterium enters the division program, it must synthesize a septum containing a PG cell wall that will separate both daughter cells. The final three-dimensional structure of the PG sacculus is genetically programed and provides the characteristic cell shape (eg, rod, sphere, spiral) to each bacterial species.[4]

Bacteria use a highly conserved pathway to build their PG sacculus by polymerizing a disaccharide-pentapeptide (Figs. 1 and 2) subunit into long glycan chains that are cross-linked by peptide bonds. Although there are variations mostly in the stem peptide and mode of cross-linking, the chemical composition of the PG cell wall is highly conserved among bacteria.[2] Nonetheless, there is a distinct difference between the PG cell wall from the so-called Gram-negative bacteria and that from their Gram-positive counterparts.[5] Gram-negative bacteria possess two membranes (i.e. the inner or cytoplasmic and outer membranes (OMs)) that are separated by an aqueous compartment called the periplasm, where a single layer of PG resides. In contrast, Gram-positive bacteria only contain one (cytoplasmic) membrane that is surrounded by a thick, multi-layered PG cell wall. For simplicity, I will refer next to the PG biogenesis of *Escherichia coli*, the Gram-negative bacterium used in the studies most relevant to this review.

The steps in PG biogenesis proceed in a linear pathway that spans several cellular compartments (Fig. 2). A similar pathway strategy is used across nature for the synthesis of many glycopolymers and glycoconjugates, including the pathway involved in *N*-glycosylation.[6] In PG biogenesis, nucleotide precursors UDP-N-acetylmuramic acid pentapeptide and UDP-N-acetylglucosamine (UDP-GlcNAc) are made in the cytoplasm,[7–9] lipid intermediates lipid I and lipid II are synthesized in the inner leaflet of the cytoplasmic membrane,[10–13] and the glycan chains are polymerized and cross-linked in the periplasm using GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala disaccharide-pentapeptide as the building block (Fig. 2).[14–16] Thus, while synthesis of the

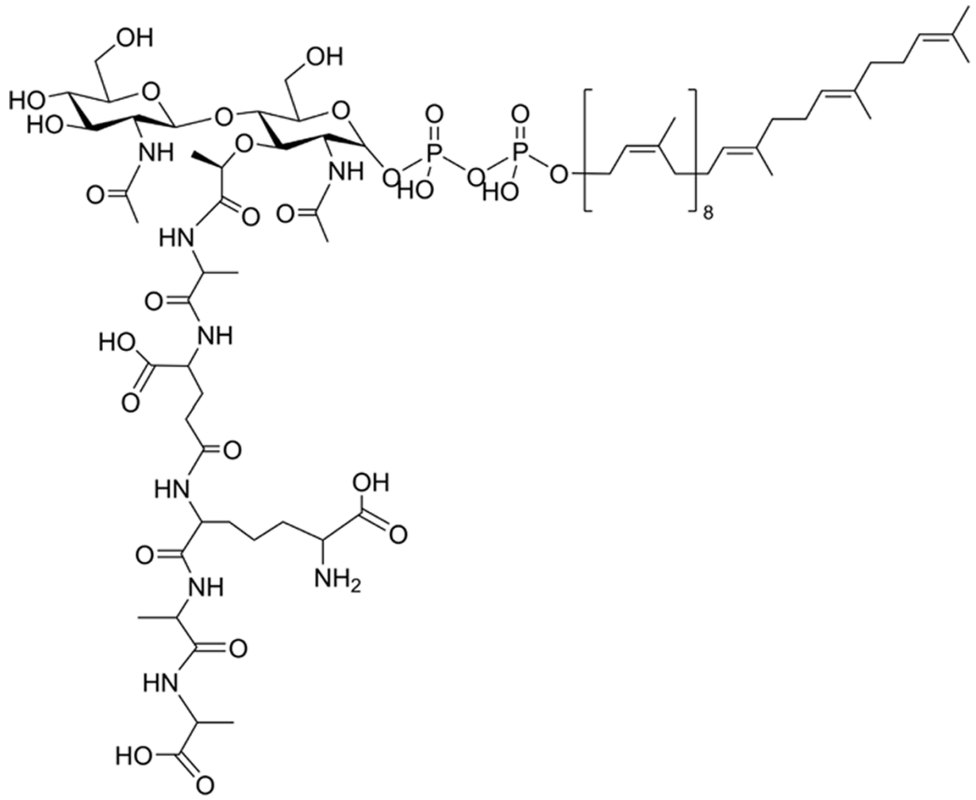

**Figure 1.** Structure of lipid II from *E. coli*. Undecaprenol is linked by a pyrophosphate to the PG building block composed of a GlcNAc-MurNAc disaccharide and an L-Ala-γ-D-Glu-A2pm-D-Ala-D-Ala stem pentapeptide.
**Abbreviations:** GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; A2pm, *meso*-diaminopimelic acid.

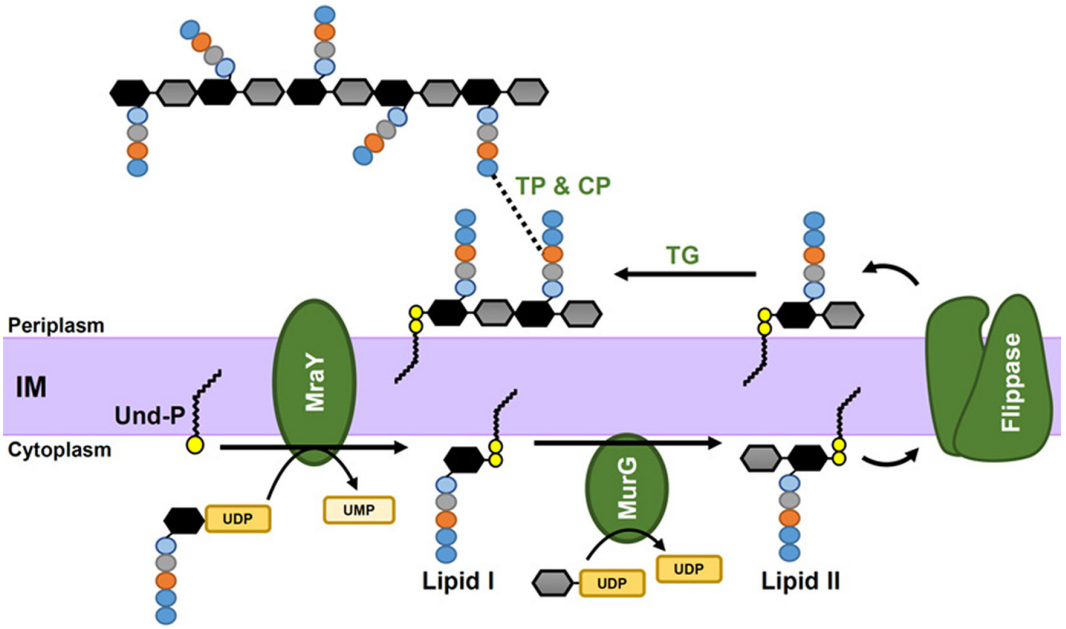


**Figure 2.** Schematic of the PG biogenesis pathway in *E. coli*. Synthesis of PG precursors begins in the cytoplasm where nucleotide-linked precursors UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramic acid-L-Ala-γ-D-Glu-A2pm-D-Ala-D-Ala are made. The latter precursor is linked to Und-P by MraY to generate lipid I. Then, MurG utilizes lipid I and UDP-*N*-acetylglucosamine to synthesize lipid II. A lipid II flippase translocates lipid II across the inner membrane (IM) so that transglycosylases (TG) can polymerize the disaccharide-pentapeptide into glycan chains. In addition, TPs catalyze peptide bonds between stem peptides that are properly oriented in adjacent glycan chains, while CPs remove the terminal D-Ala residue of stem peptides. For more detailed description, refer to relevant reviews.[1,2,12]

disaccharide-pentapeptide takes place in the cytoplasm, its polymerization occurs in the periplasm. Consequently, this building block must be flipped across the membrane, and it does so in the form of lipid II, a disaccharide-pentapeptide conjugate of undecaprenyl pyrophosphate (Und-PP), which is a 55-carbon polyisoprenyl lipid (Fig. 1).[10,17–21] It is estimated that at steady state, *E. coli* contains only 1,000–2,000 molecules of lipid II per cell.[22] Even in Gram-positive organisms, which build a thicker PG saccule than *E. coli*, lipid II is only estimated to be <1 mol percent of the amount of phospholipids that constitute their cytoplasmic membrane.[23] Therefore, lipid II translocation must be fast and efficient to keep up with the growth rate of the PG matrix, which is coupled to cell growth.

How bacterial cells translocate lipid II has been the center of great controversy. Given the structure and chemical composition of lipid II (Fig. 1), the PG community agrees that translocation of this large amphipathic peptidyl-glycolipid across the cytoplasmic membrane requires a flippase(s). Following the credit card swiping model proposed for polar lipids,[6,24] it is thought that this flippase provides a conduit for the hydrophilic moiety of lipid II so that it can traverse the hydrophobic core of the membrane, while the lipid portion of the molecule likely remains in the membrane during transport. What has been debated is the identity of the flippase(s) that translocates lipid II.

It is important to note that bacteria synthesize other Und-PP-linked oligo- and polysaccharides that are also flipped across the cytoplasmic membrane. Several transporters belonging to the Wzx and ATP-binding cassette (ABC) families have been assigned to perform this function.[25–29] Briefly, Wzx proteins transport Und-PP-linked oligosaccharides that bacteria utilize to build polysaccharides that eventually are displayed on the cell surface. The best characterized member of the Wzx family flips Und-PP-linked O-antigen subunits to the periplasmic leaflet of the cytoplasmic membrane, where they are polymerized and subsequently ligated to lipopolysaccharides (LPS) before they are transported to the cell surface of the Gram-negative bacteria.[25,26] In contrast to those relevant to Wzx-dependent transport, some Und-PP-linked oligosaccharides are polymerized into Und-PP-linked polysaccharides in the cytoplasmic leaflet of the bilayer prior to membrane translocation. To transport this type of substrate, ABC transporters use the energy derived from the binding and hydrolysis of ATP in the cytoplasm.[27–29]

As mentioned above, the identity of the bacterial lipid II flippase has been highly controversial, with the debate mainly focused on two proteins, FtsW and MurJ.[30] Recently, a third protein, AmJ, has also been identified as a transporter of lipid II.[31] Next, I will present the arguments and counterarguments for each of these cases. I should disclose here my own research-based bias to MurJ,[32–36] which I hope I kept neutralized during the exposition of each case. Ultimately, you, the reader, should decide which of these proteins, if any, flips lipid II during PG biogenesis.

## Case for FtsW

In order to generate new-born cells, rod-shaped *E. coli* cells double their length by growing their cell envelope, including the PG cell wall, along the long cell axis. Then, in a highly concerted constriction program, they build a PG septum at mid-cell and split it while invaginating the inner and OMs so that the two daughter cells can separate.[37,38] The *ftsW* gene is part of an operon required for PG biosynthesis[39,40] and it owes its name to a mutant allele that confers the filamentous temperature-sensitive phenotype characteristic of *fts* mutations that cause defects in cell division.[40] Cells carrying *fts* alleles can typically grow and divide normally at low temperatures; however, at high temperatures, these mutants continue to grow laterally but are unable to undergo cell division, resulting in long filamentous cells that ultimately die. Their phenotype is caused by defects in the assembly or function of the divisome, the dynamic multiprotein complex that controls and executes cell division.[37,38] FtsW is required for cell division[41] and localizes to the septum[42] where it recruits the essential PG transpeptidase (TP) FtsI (or PBP3).[43–45]

FtsW is a polytopic membrane composed of 10 transmembrane domains (TMDs).[46] Analysis of its amino acid sequence reveals high similarity to two other membrane proteins, RodA and SpoVE.[47] RodA is a conserved protein required for maintaining the rod shape of *E. coli* cells,[48] while SpoVE is required for sporulation in the Gram-positive bacterium *Bacillus subtilis*.[47,49] Together, FtsW, RodA, and SpoVE are the founding members of the shape, elongation, division, and sporulation (SEDS) family of proteins.[50] Notably, *E. coli* has both RodA and FtsW, and these proteins are thought to perform the same function during elongation and division, respectively, at each cell cycle.

The suggestion that FtsW and RodA might be lipid II flippases was made decades ago. Once it was recognized that lipid II translocation across the cytoplasmic membrane is a step required for PG biogenesis, the hunt for the lipid II flippase(s) began. It was reasoned that the flippase would be a membrane protein required for PG synthesis. The first candidates to be suggested as lipid II flippases were FtsW and RodA based on the fact that they are polytopic membrane proteins required for septal and lateral growth, respectively, and PG synthesis.[51,52] At present, there are no data in the literature supporting that RodA is involved in lipid II translocation. In contrast, in recent years, the role of FtsW in lipid II translocation has been tested using in vitro biochemical experiments.[53,54] Nevertheless, we still await experimental evidence confirming this function in vivo. In fact, as described below, recent in vivo studies on MurJ dispute FtsW functioning as a lipid II flippase in *E. coli*.[36] On the flip side, pun intended, in vivo experiments support that the opposing candidate, MurJ, is a lipid II flippase, and attempts to reconstitute its activity in vitro have failed.[36,54]

An important obstacle faced by those studying lipid transport is the lack of a biochemical assay to probe transport. The first advance in studying the mechanism of lipid II

translocation was accomplished when the Breukink group developed a method to monitor the translocation of fluorescently 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labeled lipid II (NBD-lipid II; NBD group at the third position in the stem peptide)[55] in liposomes and cell-derived vesicles.[56] This assay was modeled after one developed to examine the asymmetric localization of NBD-labeled phospholipids across lipid bilayers.[57] Briefly, NBD-lipid II is first incorporated into a lipid bilayer (artificial liposome or cell-derived) where it should be equally distributed (stage I; Fig. 3A). Subsequent addition of dithionite preferentially eliminates fluorescence of NBD-lipid II localized in the outer leaflet because of its slow permeability across the bilayer and its ability to reduce fluorescent NBD-lipid II to nonfluorescent 7-amino-2,1,3-benzodioxol-4-yl (ABD)-lipid II (stage II; Fig. 3A). Further addition of a detergent that disrupts the bilayer totally eliminates fluorescence in the sample (stage III; Fig. 3A). Utilizing this assay, the authors could not detect spontaneous translocation of lipid II in protein-free, artificial unilamellar bilayers in a three-hour period, even if NBD-lipid II was synthesized

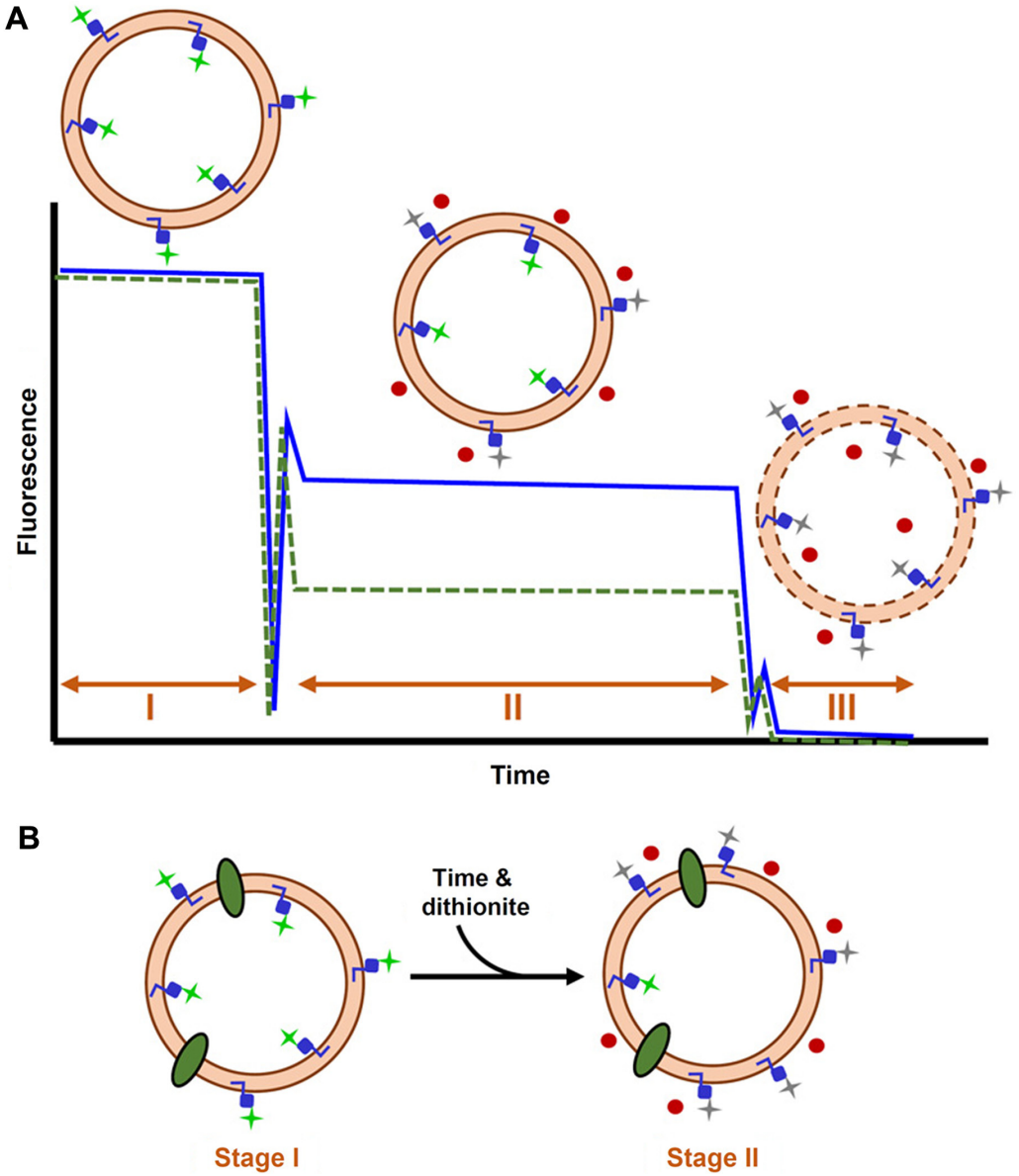


**Figure 3.** Assay to measure in vitro the translocation of lipid II in liposomes. (**A**) As previously described,[53,54] liposomes (unilamellar vesicles) are first loaded with NBD-lipid II, which distributes symmetrically in both leaflets (stage I). At this stage, all NBD-lipid II can fluoresce, so fluorescence signal (solid blue line in graph) from NBD (green star) is maximal. Then, addition of dithionite (red circle, stage II) reduces the NBD in lipid II molecules localized in the outer leaflet of the vesicle to nonfluorescent ABD (gray star), causing a reduction in fluorescence. Residual fluorescence is completely eliminated upon treatment of vesicles with detergents in the presence of dithionite (stage III). The solid blue line in the graph represents the fluorescence signal obtained in liposomes lacking flippase activity, while the dotted green line corresponds to the fluorescence signal obtained in liposomes containing a flippase (as shown in **B**). (**B**) An illustration of how addition of a lipid II flippase (green oval) to liposomes preloaded with NBD-lipid II can drive translocation of the lipid across the bilayer (stage II, **A**), causing a decrease in the fluorescence signal (dotted green line, **A**) in the presence of dithionite.

by MurG in situ.[56] In contrast, they detected lipid II translocation in a modified assay using membrane vesicles derived from *E. coli* cells without the addition of an energy source.[56] Together, these data suggested that translocation of lipid II across cell-derived vesicles was mediated by a flippase(s) through an ATP- and pmf-independent mechanism.

This dithionite-based assay was subsequently used to test whether purified FtsW translocates NBD-lipid II across proteoliposomes (Fig. 3).[54] Specifically, detergent-purified FtsW was incorporated into unilamellar vesicles loaded with NBD-lipid II. While adding dithionite to protein-free liposomes eliminated ~50% of the fluorescence corresponding to the NBD-lipid II molecules randomly incorporated into the outer leaflet of the bilayer, there was a reduction of up to 70% in fluorescence in liposomes containing FtsW. This additional FtsW-dependent decrease in fluorescence was not detected when liposomes contained other control proteins, even the lipid II flippase candidate MurJ. In addition, this in vitro reconstitution assay was complemented by a novel Förster resonance energy transfer (FRET)-based assay to examine translocation of NBD-lipid II across right-side-out membrane vesicles derived from cells where FtsW was either overproduced or depleted.[54] The Breukink group took advantage of the fact that the membrane-impermeable antibiotic vancomycin binds to the D-Ala-D-Ala terminal portion of the stem peptide of lipid II.[54,58] In their assay, they monitored the energy transfer between NBD-lipid II (donor) and tetramethylrhodamine cadaverine (TMR)-vancomycin (acceptor). If the two fluorophores were in close proximity in the same leaflet, energy transfer between NBD and TMR would occur, resulting in a decrease in the NBD signal and a concomitant increase in the TMR fluorescence. This study showed that there was an increase in FRET signal in right-side-out vesicles derived from cells overproducing FtsW and a decrease in those derived from cells depleted of FstW. In contrast, altering the levels of MurJ did not have an effect. From these data, the authors concluded that FtsW is a lipid II flippase.[54]

Mohammadi et al recently addressed two important tests missing in their earlier study: (a) substrate specificity of the reported FtsW-dependent translocation and (b) dependence of transport on FtsW function (ie, testing inactive FtsW proteins).[53] With respect to the substrate specificity issue, they first tested whether purified FtsW could flip NBD-phospholipids across artificial unilamellar vesicles using the dithionite-based assay (Fig. 3).[53,54] Their results showed that the addition of wild-type FtsW to liposomes promoted the translocation of the three phospholipids tested, phosphatidylethanolamine and phosphatidylglycerol, which are the major phospholipids in *E. coli*, and phosphatidylcholine, which *E. coli* does not produce. Furthermore, they showed that FtsW also promoted the translocation of several NBD-lipid II analogs larger than NBD-lipid II. From this set of experiments, the authors concluded that FtsW flips both phospholipids and lipid II, and that there is a size and shape limit for substrates.[53]

The requirement for FtsW function was tested by utilizing a collection of FtsW variants that either lacked some of its 10 TMDs or carried specific residue substitutions.[53] Surprisingly, truncated FtsW proteins lacking TMD 10, TMDs 7–10, or TMDs 5–10 were still able to induce translocation of NBD-lipid II across proteoliposomes. Thus, addition of a truncated FtsW variant containing only TMDs 1–4 was sufficient to flip lipid II. This study also showed that full-length FtsW proteins containing single residue substitutions (R145A and K153N) in TMD 4 exhibited dominant-negative effects in *E. coli* cells and could not promote translocation of NBD-lipid II in their in vitro assay. However, addition of these mutant proteins to liposomes still caused the translocation of NBD-phospholipids. Based on these data, the authors concluded that FtsW transports lipid II and phospholipids through different mechanisms of facilitated diffusion and hypothesized that transport occurs through a pore-like structure.[53]

## Case for MurJ

MurJ (formerly MviN) is a 14-TMD membrane protein[34] required for PG biogenesis.[32,59–62] I identified MurJ as the only lipid II flippase candidate in *E. coli* using a reductionist bioinformatics search that took advantage of the small size of the genome of endosymbiotic bacteria that are closely related to the free-living bacterium *E. coli*.[32] Independently, the Kato group also found *murJ* as a gene required for PG biogenesis when analyzing a set of chromosomal deletion mutants of *E. coli*.[59] Both studies demonstrated that MurJ is required for cell viability and maintenance of cellular shape and integrity. Importantly, they also showed that depletion of MurJ caused a decrease in PG synthesis and an accumulation of PG nucleotide and lipid intermediates.[32,59] Although these data were in agreement with MurJ being an essential lipid II flippase, what led us to propose that MurJ itself was the long sought-after lipid II flippase[32,59] was the fact that MurJ belongs to the *m*ultidrug/*o*ligo-saccharidyl-lipid/*p*olysaccharide (MOP) exporter superfamily of proteins.[63]

Members of the MOP exporter superfamily include multidrug transporters[64–66] and the aforementioned Wzx proteins, which flip Und-PP-oligosaccharides across bacterial cytoplasmic membranes.[26,67–70] A notable side note is that a member of the MOP exporter superfamily is the eukaryotic Rft1 protein,[63] which has been the center of great controversy regarding its role in flipping polyisoprenoid-oligosaccharide precursors during *N*-glycosylation.[71–75] Briefly, the main arguments are the following. Helenius et al showed that in vivo depletion of the essential yeast protein Rft1 caused a decrease in *N*-glycosylation and an accumulation of the polyisoprenoid-oligosaccharide precursor that needs to be flipped across the membrane of the endoplasmic reticulum (ER) in this pathway.[72] Moreover, increasing production of Rft1 suppressed defects conferred by mutations that lead to the synthesis of an incomplete polyisoprenoid-oligosaccharide precursor of *N*-glycosylation that is poorly translocated across

the ER membrane. These results demonstrated that Rft1 is necessary for the membrane translocation of *N*-glycosylation precursors. However, Frank et al concluded that Rft1 is not the flippase itself because liposomes reconstituted with ER proteins extracted from cells depleted of Rft1 still translocated *N*-glycosylation polyisoprenoid-oligosaccharide precursors in an in vitro assay.[71]

The best studied members of the MOP exporter superfamily are multidrug exporters belonging to the *m*ultidrug *a*nd *t*oxic *e*xtrusion (MATE) family for which there is a large body of genetic, biochemical, and structural data.[63,76] Crystallography studies have revealed that their 12 TMDs are arranged into two six-helix bundles that form a V-shaped central cavity mainly lined by TMDs 1, 2, 7, and 8, which is essential for transport.[77–81] In general, MATE transporters extract amphipathic molecules from the bacterial cytoplasm by an antiport mechanism that takes advantage of the electrochemical gradient of protons or cations across the cytoplasmic membrane. It has been proposed that through an alternating-access model of transport, their central cavity opens to the cytoplasm in order to load the cargo and then undergoes a conformational change so that it opens to the other side of the membrane to deliver its cargo. During this process, a counter ion (proton or cation) is imported across the membrane. Interestingly, these structural studies have also revealed significant differences in how several members of the MATE family bind cations and substrates.[78–81]

Although there are still many unanswered questions regarding their mechanism of function, it is clear that a key structural and functional feature of the MATE transporters is their central cavity.[77–81] Also relevant to our discussion are recent studies on MOP exporter member Wzx suggesting that it might transport Und-PP-O-antigen molecules using a mechanism similar to that described for MATE transporters. A three-dimensional structural model has predicted that the 12-TMDs[82] of the *Pseudomonas aeruginosa* Wzx protein fold into a V-shaped structure with a central cationic cavity that might transport its anionic Und-PP-oligosaccharide substrate.[70] Although transport of the native substrate has not yet been reconstituted, in vitro studies have shown proton-dependent transport of anions in proteoliposomes containing purified Wzx.[69] Furthermore, a combination of in vivo and in vitro functional analyses of various Wzx mutant proteins has revealed the importance of charged and aromatic residues within the central cavity, suggesting that they interact with the substrate and/or protons.[69,70]

Given that structural and functional features might be conserved among members of the MOP exporter superfamily, my laboratory conducted structure–function analyses on MurJ. These studies generated a three-dimensional structural model that predicted that MurJ is structurally similar to other MOP exporters (Fig. 4).[34] Further, we probed this model in vivo by determining the accessibility of specific MurJ residues to the periplasm, the cytoplasm, and the hydrophobic

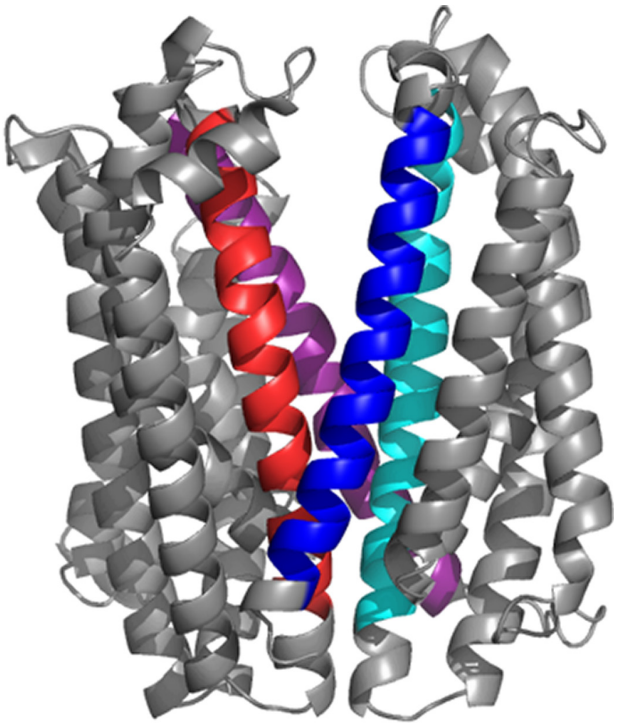

**Figure 4.** A structural model of MurJ. The front view of the model structure of MurJ of *E. coli* showing the central cavity opened toward the periplasm.[34] The cavity is mainly lined by TMDs 1 (blue), 2 (cyan), 7 (magenta), and 8 (red).

membrane environment. This detailed topological study validated the most salient features of the structural model.[34] Namely, it demonstrated that MurJ has 14 TMDs and TMDs 1–12 adopt a V-shaped structure with a solvent-exposed cavity mainly lined by TMDs 1, 2, 7, and 8 (Fig. 4). Moreover, functional studies revealed that the native charge of specific residues within this cavity is required for MurJ function in *E. coli*.[34,35]

The most logical and simplest explanation of the results on MurJ discussed so far is that MurJ is the lipid II flippase: MurJ is required for PG biogenesis, it belongs to the MOP exporter superfamily, it adopts a structure that resembles that of MATE transporters, it has a solvent-exposed hydrophilic cavity, and depleting MurJ from cells causes the accumulation of lipid-linked PG precursors.[32,34,35,59] Nevertheless, several studies have called this proposal into question. Purified FtsW, and not purified MurJ, had been shown to promote lipid II translocation in the in vitro reconstitution system described above.[54] In addition, although *ytgP*, the *murJ* ortholog in Gram-positive bacteria,[33] had been shown to be essential in *Staphylococcus aureus* and *Streptococcus pneumoniae*,[83,84] two independent studies disputed the essentiality of the function of YtgP in *B. subtilis*, a bacterium encoding multiple YtgP homologs.[33,85,86] Because *B. subtilis* cells lacking four of the *ytgP* paralogs are viable, it was disputed that these proteins could not perform the essential function of translocating lipid II.[85,87] As described below, the reason for this apparent discrepancy is that *B. subtilis* encodes AmJ, a protein that is redundant with YtgP (MurJ).[31]

Ultimately, the question that sparked studies on FtsW and MurJ is the following: which protein(s) flips lipid II in the cell? Focusing on the FtsW–MurJ controversy, a question that needed to be addressed was the following: do any of these two proteins flip or at least participate in the flipping of lipid II in cells? The Bernhardt, Kahne, and Ruiz groups joined forces to address this question in *E. coli*. First, we developed a method to monitor in vivo the translocation of lipid II across the cytoplasmic membrane in *E. coli* (Fig. 5).[36] This method takes advantage of the colicin M (ColM) toxin that some strains of *E. coli* produce to kill other members of its own species.[88,89] Specifically, after ColM is secreted into the environment, it can cross the OM of target cells to enter the periplasm. There, it inhibits PG biogenesis by hydrolyzing periplasmic lipid II into Und and PP-disaccharide-pentapeptide, which can be further cleaved by periplasmic carboxypeptidases (CPs) into a PP-disaccharide-tetrapeptide product.[36,88] Because ColM cannot cross the cytoplasmic membrane of target cells, it is specific for periplasmic (or flipped) lipid II. Therefore, if cells are treated with ColM for a given period, the amount of PP-disaccharide-tetrapeptide should reflect how much lipid II was flipped in that period. Second, we developed a method to specifically and rapidly inhibit MurJ function with a small molecule. To do this, we utilized a functional MurJ variant

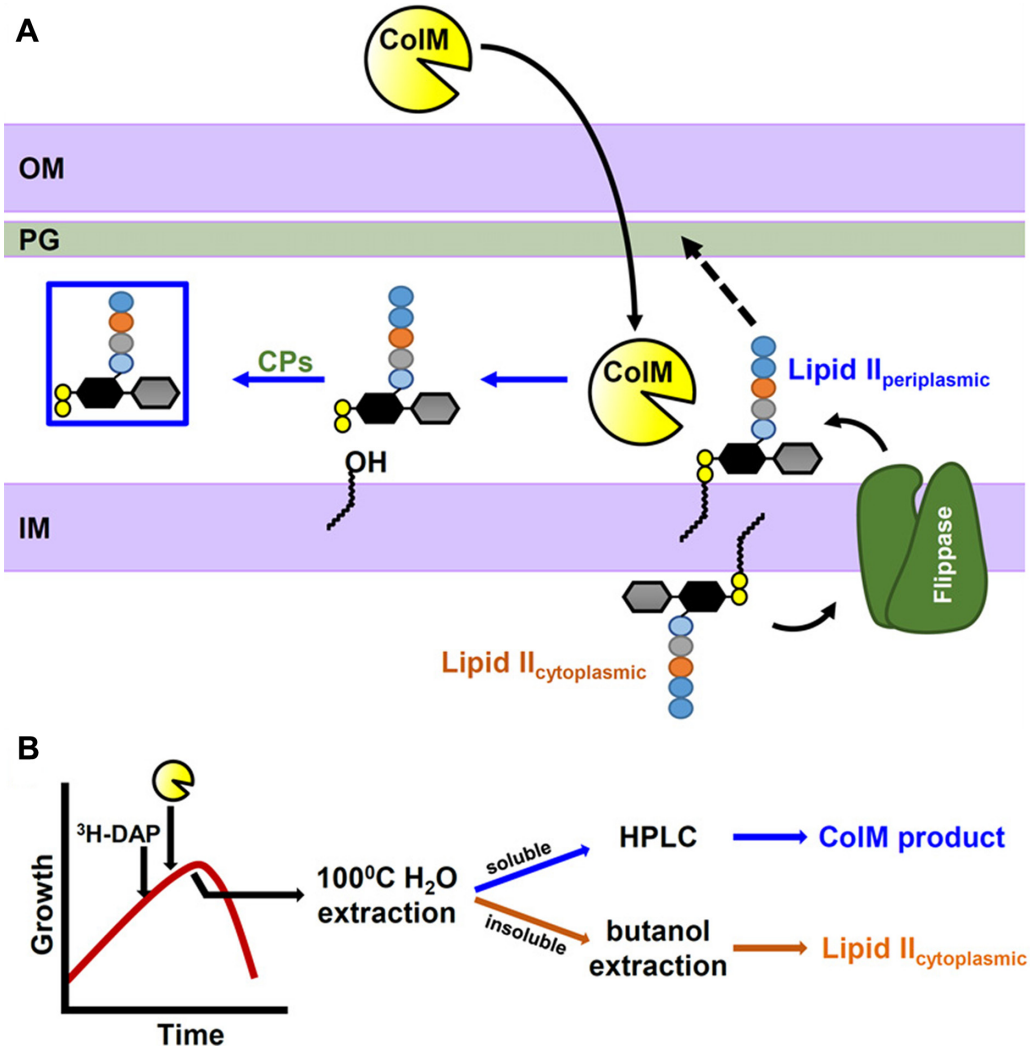


**Figure 5.** Assay to measure in vivo the translocation of lipid II in *E. coli* cells. (**A**) As previously described,[31,36] purified toxin ColM is added to actively growing *E. coli* cells. ColM crosses the OM to enter the periplasm, where it inhibits PG biogenesis by cleaving lipid II that has been flipped to the periplasm (lipid $II_{periplasmic}$). ColM cleaves lipid II into membrane-bound undecaprenol and soluble PP-disaccharide-pentapeptide, which is further converted by periplasmic CPs into PP-disaccharide-tetrapeptide (marked by blue box). (**B**) A schematic showing the experimental details of the assay.[31,36] PG precursors are specifically labeled with $^3$H-*meso*-diaminopimelic acid ($^3$H-DAP) and then treated or not with ColM. Before cell lysis occurs (drop in growth curve), cells are collected and extracted with boiling water. Species in the water-soluble fraction are separated using high-pressure liquid chromatography and radioactivity present in the ColM disaccharide-tetrapeptide product (blue box, **A**) is then measured. Radiolabeled lipid II that is not cleaved by ColM (lipid $II_{cytoplasmic}$) is measured after extracting the water-insoluble fraction with butanol. When flippase activity is not impaired, treatment with ColM leads to the appearance of signal in the fraction corresponding to the ColM product and the disappearance of signal from the lipid $II_{cytoplasmic}$ fraction. When flippase activity is inhibited, the amount of signal in the lipid $II_{cytoplasmic}$ fraction increases and the treatment of ColM does not lead to the appearance of signal corresponding to the ColM product.[36]

(MurJA29C) carrying a single Cys substitution localized in the central cavity of MurJ. Haploid cells producing only MurJA29C were viable and could synthesize PG. However, if these mutant cells were treated with MTSES (2-Sulfonatoethyl methanethiosulfonate), a small molecule that reacts with free Cys residues to form adducts, they rapidly lysed because they could not synthesize PG. This lethality was shown to be specific for the presence of the A29C substitution and was caused by the loss of function of the MurJA29C variant. Together, these methods allowed us to specifically inhibit MurJ in cells and rapidly detect the effect that this inhibition had in lipid II translocation using the ColM-based flippase assay. In essence, from these data, we concluded that inhibiting MurJ resulted in the loss of all the measurable translocation and the accumulation of lipid II that could only be cleaved by ColM if the cytoplasmic membrane was lysed. Furthermore, we also showed that depletion of FtsW in a strain-lacking RodA did not reduce lipid II translocation.[36]

From these results[36] and the fact that MurJ, a member of the MOP exporter superfamily, is essential[32,59,63] and structurally similar to MOP exporters, and possesses a solvent-exposed cavity that is essential for function,[34,35] we concluded that MurJ is the lipid II flippase in *E. coli*. Indeed, based on the credit card swipe model proposed for the transport of polar lipids[6,24] and the mechanism of function proposed for MATE exporters,[78–81] my group has also proposed that MurJ flips lipid II using an alternating-access mechanism: during transport, charged residues in the cavity of MurJ interact with the hydrophilic moiety of lipid II, while its lipid portion stays in the membrane, possibly sliding between TMDs 1 and 8 or 1 and 5.[35] Whether the negative charges located in the cavity that we showed to be essential interact with the substrate, promote intramolecular interactions, or interact with a counter ion remains unknown. Moreover, it is interesting that all of the charged residues that are critical for MurJ function are localized to the top half of the central cavity.[35] Therefore, it is possible that directional transport is not driven by an antiport mechanism but by an increase in the binding affinity of MurJ for lipid II as transport proceeds from the cytoplasmic bottom half of the cavity (lower affinity) to the external upper half (higher affinity). It is also possible that MurJ might not use an alternating-access mechanism at all, as has recently been proposed by the Locher group for PglK, the ABC transporter of the Und-PP-oligosaccharide used in *N*-glycosylation in bacteria.[90] In their structural study, the authors proposed a novel mechanism where the cavity does not open to the cytoplasmic side of the membrane. Instead, the polyisoprenoid end of the molecule engages with a helix in the periplasmic face of the membrane, inducing the interaction of the pyrophosphate-oligosaccharide portion of the substrate with positively charged residues within the outward-facing cavity and driving transport.

## Case for AmJ

As described above, an argument used against MurJ being a lipid II flippase was the fact that a mutant lacking *ytgP*, the *murJ* ortholog in Gram-positive bacteria,[33] and three other paralogs was viable.[85,87] Recently, the Bernhardt and Rudner laboratories have demonstrated that *ytgP* is not essential in *B. subtilis* because this organism encodes a protein, AmJ, that is functionally redundant with YtgP (MurJ).[31] After showing that a *B. subtilis* mutant strain lacking all 10 members of the MOP exporter superfamily is viable, the authors used a genetic screen for synthetic lethality with the Δ*ytgP* allele to identify proteins that might function as lipid II flippases. Specifically, they used a strain lacking the four MOP exporters most closely related to MurJ (including YtgP) and transposon mutagenesis in a screen designed to identify genes whose inactivation would cause lethality only in this mutant devoid of MurJ-like proteins. Assuming that YtgP (MurJ) is a lipid II flippase, the rationale of the screen was that if another protein functions as the lipid II flippase in the absence of YtgP, its genetic inactivation would cause lethality, since lipid II translocation is a process essential for cell viability. With this strategy, the authors identified AmJ (formerly YdaH) as an *a*lternate to *M*ur*J*. They further demonstrated that *amJ* and *ytgP* are indeed a synthetic lethal pair required for PG biogenesis in *B. subtilis*, and that in *E. coli,* production of *B. subtilis* AmJ can substitute for native MurJ in lipid II translocation.[31]

Analysis of the primary sequence of AmJ reveals that this polytopic membrane protein has no similarity to MurJ or any member of either the MOP exporter superfamily or ABC transporters, suggesting that AmJ is the founding member of a new type of protein involved in the translocation of Und-PP-linked sugars.[31] In addition, AmJ is not widely conserved in bacteria and its transcription is regulated by $\sigma^M$,[91,92] a sigma factor that is positively regulated by cell wall stress.[91,93] In fact, *amJ* transcription is upregulated in the absence of YtgP in *B. subtilis*.[31] These findings have led to the proposal that having AmJ, which is very different from MurJ, might benefit organisms that somehow might come into contact with environmental conditions that inhibit MurJ (YtgP).[31] Alternatively, AmJ could be a transporter of a yet-to-be-identified substrate and promiscuously transport lipid II since some, but not all, transporters of Und-PP-linked oligosaccharides from both the Wzx and ABC transporter types have relaxed substrate specificity.[94–100]

Finding AmJ raises the question of whether there could be additional types of transporters capable of translocating Und-PP-oligosaccharides. Indeed, the Raetz laboratory proposed that ArnE and ArnF (formerly, PmrM and PmrL) work, possibly in a complex, to translocate Und-PP-L-aminoarabinose across the cytoplasmic membrane of *E. coli* and *Salmonella*.[101] Each of these proteins is predicted to have four TMDs, and they are distantly related to the drug/metabolite transporter superfamily.[102] However, how these transporters function remains unknown.

## Counterarguments

You have now read the arguments based on positive and negative results that support FtsW and discount MurJ (and AmJ),

respectively, or vice versa. The FtsW argument is fueled by results obtained in an in vitro reconstitution system but lacks support from in vivo evidence. On the contrary, the MurJ model is fueled by a collection of evidence obtained in vivo but lacks proof from a reconstitution system.

**Pro-MurJ (and AmJ) counterargument against FtsW.** It should first be noted that the lack of lipid II flippase activity of MurJ in the in vitro assay[54] could be the result of trivial explanations, such as the inactivation of the protein during purification. In addition, validity of the in vitro reconstitution system supporting FtsW as a lipid II flippase requires that the addition of purified FtsW does not cause disorder of the lipid membrane, which could result in a nonspecific increase in permeability to either dithionite or lipid II.[57,103] Although other proteins tested did not induce lipid II translocation, this issue is protein-specific and it has not been thoroughly ruled out for FtsW. In fact, this artifact could explain why in the dithionite-based assay, FtsW is reported to rapidly flip lipid II and several types of phospholipids.[53,54] More importantly, regardless of whether the in vitro flippase assay is flawed or not, ultimately, we want to know if FtsW flips lipid II in cells. At present, there is no in vivo experimental evidence supporting FtsW's function as a lipid II flippase in a cell. On the contrary, data from experiments designed to address this issue for FtsW, MurJ, and AmJ have indicated that MurJ and AmJ are required in lipid II translocation in *E. coli*, while FtsW and RodA are not.[31,36] Therefore, the simplest explanation of these results and those demonstrating that MurJ is essential in *E. coli* and structurally similar to related transporters of lipid II-like substrates is that MurJ is the lipid II flippase in this bacterium. Furthering this reasoning, given that AmJ can substitute for MurJ in *E. coli* and that *amJ* and *murJ* ortholog are a synthetic lethal pair in *B. subtilis*,[31] it follows that AmJ is a transporter that can flip lipid II in cells; whether AmJ is specific to lipid II or not awaits investigation.

**Pro-FtsW counterargument against MurJ.** In an in vitro reconstitution system, purified MurJ, unlike FtsW, has failed to induce lipid II translocation across liposomes.[54] Furthermore, levels of MurJ, unlike those of FtsW, do not correlate with lipid II translocation in an in vitro assay using cell-derived vesicles.[54] Therefore, FtsW, and not MurJ, is a lipid II flippase. The in vivo system supporting MurJ as a lipid II flippase does not allow one to discern whether the effect on lipid II translocation is direct or indirect. Only an in vitro reconstitution system can, and such a system has shown that FtsW, and not MurJ, can flip lipid II. Moreover, the in vivo studies by Sham et al[36] did not report values of radioactivity ($^3$H-DAP; Fig. 5) present in the fraction corresponding to the mature PG sacculus that might reflect undetected flippase activity even when MurJ is inhibited. Thus, it is possible that when MurJ was inhibited, there was still some level of lipid II translocation occurring (that could be mediated by FtsW) that could not be detected in the in vivo assay because of the notable background level (or low sensitivity of detection) of this assay.

## Could Both FtsW and MurJ be Lipid II Flippases?

Both FtsW and MurJ are essential in *E. coli*, implying that they perform nonredundant essential functions. Therefore, if both FtsW and MurJ were lipid II flippases, they either would have to be redundant but perform additional essential functions or should each serve as a lipid II flippase required for a unique purpose during growth and division. With respect to the first point, although FtsW is known to be essential for the proper assembly of the divisome,[43–45] there are no data hinting that MurJ could perform an additional function. With respect to both FtsW and MurJ being lipid II flippases required for a unique purpose, we should remember that FtsW's paralog RodA is likely to perform the same function during cell elongation that FtsW plays during cell division, calling into question in what other circumstance MurJ would be essential. In addition, the in vivo assay showed that all the detectable flippase activity was abolished upon MurJ inactivation.[31,36] Therefore, if all three proteins were lipid II flippases, these data would argue that MurJ would be the main flippase and that the activity of RodA and FtsW flippases is minimal (below detection) but essential. There are no current data or model in PG biogenesis that would explain this latter situation. Furthermore, the idea that the cell uses different flippases for specific essential functions seems only possible if lipid II is not free (diffusible) in the membrane. Some members of the PG community believe that lipid II is handed off from MurG to a flippase and then to a transglycosylase in a PG-synthesizing multiprotein complex. To my knowledge, there is no experimental evidence supporting this model; furthermore, a recent study by Grabowicz et al might argue against it.[104] In this work, the authors isolated a mutant of *E. coli* that produces a variant of WaaL (or RfaL), the ligase that normally adds the polysaccharide portion of Und-PP-O-antigen to the core of a glycolipid in order to synthesize LPS.[105] This ligation occurs in the periplasmic side of the cytoplasmic membrane. Interestingly, the mutation in *waaL* causes the altered WaaL protein to have relaxed substrate specificity so that it can utilize the disaccharide-pentapeptide moiety from lipid II as a substrate and ligate it to produce a novel form of LPS.[104] Although it is unclear whether the *waaL* mutant cells produce normal or elevated levels of lipid II, the fact that flipped, periplasmic lipid II is accessible to the mutant WaaL protein suggests that the lipid II flippase does not directly hand off lipid II after transport to the PG transglycosylases.

Based on these arguments, it seems unlikely that both FtsW and MurJ function as lipid II flippases in *E. coli*. What is clear is that more studies are needed to understand how FtsW (and RodA) and MurJ (and AmJ) function.

## Instructions to the Jury

This review was intended to serve as a guide that highlights the recent investigative developments in the essential process of translocation of lipid II across the bacterial cytoplasmic

membrane. In the past few years, studies have focused on the identification of the lipid II flippase and have resulted in two opposing camps in the PG community: those who defend FtsW based on an in vitro reconstitution system and those who defend MurJ and AmJ based on data generated in vivo. Using this review as a guide, I now encourage you to carefully examine the evidence, the primary literature, and decide whether or not the controversy about the identity of the lipid II flippase has been resolved beyond a reasonable doubt or, at least, based on the preponderance of the evidence.

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## Author Contributions

Wrote the first draft of the manuscript: NR. Agree with manuscript results and conclusions: NR. Jointly developed the structure and arguments for the paper: NR. Made critical revisions and approved final version: NR. Author reviewed and approved of the final manuscript.

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