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Filling holes in peptidoglycan biogenesis of *Escherichia coli*

Natividad Ruiz

Department of Microbiology, The Ohio State University, Columbus, OH 43210, USA

Abstract

The peptidoglycan cell wall is an essential mesh-like structure in most bacteria. It is built outside the cytoplasmic membrane by polymerizing a disaccharide-pentapeptide into glycan chains that are crosslinked by peptides. The disaccharide-pentapeptide is synthesized as a lipid-linked precursor called lipid II, which is exported across the cytoplasmic membrane so that synthases can make new glycan chains. Growth of the peptidoglycan wall requires careful balancing of synthesis of glycan chains and hydrolysis of the preexisting structure to allow incorporation of new material. Recent studies in *Escherichia coli* have advanced our understanding of lipid II translocation across the membrane and how synthases are regulated to ensure proper envelope growth.

INTRODUCTION

The bacterial cell envelope provides structural integrity and shape to the cell, serves as a selective permeability barrier, and mediates interactions with the outside world. One of the best studied bacterial envelopes is that of *Escherichia coli*, which is considered the archetype of the Gram-negative envelope. The *E. coli* envelope is delimited by the inner and outer membranes (IM and OM, respectively), which are separated by the periplasm. The periplasm is a protein-rich aqueous compartment that contains a 3-6 nm-thick layer of cell wall composed of peptidoglycan (PG) [1,2].

The PG cell wall is a polymeric mesh-like structure that provides the cell its shape and protects it from osmotic lysis in hypotonic environments [3]. It is made by polymerizing a β -1,4-linked *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) disaccharide into long glycan strands that are crosslinked with peptides [4]. Although the exact 3-dimensional structure is hard to determine, electron cryotomography data support a model where the glycan chains are parallel to the IM [5]. Underscoring the importance of this structure is the fact that almost all bacteria possess a PG sacculus and that the overall composition and biogenesis of the PG wall is conserved, although the complexity and thickness of the structure varies among different types of bacterial envelopes [1,2]. Some variation in composition also exists among different bacterial species and, some, if not all, bacteria can modify their PG cell wall in response to environmental conditions [2,6,7]. The

Corresponding author: ruiz.82@osu.edu.

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essentiality of this macromolecule to bacterial physiology and the fact that its biogenesis is the target of many antibiotics has driven efforts to understand PG biogenesis. In this review, I will highlight studies that have recently filled gaps in two areas of PG research in *E. coli*: translocation of PG precursors across the IM and regulation of the activity of PG synthases.

An abridged version of PG biogenesis in *E. coli*

PG biogenesis starts in the cytoplasm with the synthesis of its building block, the disaccharide-pentapeptide GlcNAc-MurNAc-L-Ala- γ -D-Glu-A₂pm-D-Ala-D-Ala, and ends in the periplasm with its polymerization and crosslinking (Fig. 1) [4,8]. Cytoplasmic nucleotide precursors UDP-MurNAc-pentapeptide, UDP-GlcNAc, and the lipid carrier undecaprenyl-phosphate (Und-P) are utilized to synthesize the membrane-bound intermediate lipid II [9]. Lipid II is then translocated across the IM by a flippase and used by glycosyltransferases (GTs) to synthesize glycan chains that are crosslinked by transpeptidases (TPs) primarily by a peptide bond between an amino group on the third position of one stem peptide and a carbonyl group on the fourth position of the stem peptide on an adjacent glycan strand [3,10]. In *E. coli*, most PG synthesis is thought to be accomplished by PBP1a and PBP1b, two partially redundant, bifunctional enzymes with both GT and TP activities that belong to the class A penicillin-binding protein (PBP) family [3,11,12]. Additional factors catalyze modifications and proper assembly of the PG structure [3,13,14]. Of the aforementioned essential steps, translocation of lipid II across the IM is the most poorly understood.

Who flips lipid II?

Lipid II is a large amphipathic molecule that requires a transporter to traverse the IM [15]. In 2008, the essential IM protein MurJ was introduced as the lipid II flippase in *E. coli* [16,17]. Depletion of MurJ leads to the accumulation of nucleotide and lipid PG precursors, and the inhibition of PG biogenesis. These phenotypes, together with the fact that MurJ is related to flippases of polyisoprenoid-linked oligosaccharides [18], agreed with MurJ being the lipid II flippase in *E. coli* [16,17]. However, this model was disputed by *in vitro* experiments showing that the divisome protein FtsW, and not MurJ, could promote lipid II translocation across liposomes [19]. Consequently, FtsW was presented as the lipid II flippase for septal PG synthesis. By extension, it is implied that RodA, an FtsW's paralog required for PG synthesis during cell elongation, is the lipid II flippase for lateral growth. A debate about the identity of the lipid II flippase ensued [20,21].

The gold standard to ultimately demonstrate a protein's function is to reconstitute its activity *in vitro* using purified components. That said, any activity reconstituted *in vitro* should be corroborated with *in vivo* data to demonstrate its biological relevance. It is also true that reconstitution of an activity can fail for many technical reasons such as that the purified protein might not be properly folded. Therefore, the failure to demonstrate that MurJ can flip lipid II in an *in vitro* system should not be used as evidence that MurJ is not a lipid II flippase in cells [22]. In contrast, it is seemingly significant that FtsW promotes lipid II flipping in a liposome reconstitution system [19,22]. However, at present, we lack *in vivo* data demonstrating the relevance of these biochemical results. Furthermore, FtsW also translocates phospholipids across liposomes, calling into question its specificity for lipid II

flipping [22]. On the contrary, since the flippase controversy erupted, key evidence from *in vivo* experiments has emerged supporting that MurJ is the lipid II flippase in *E. coli*.

Extensive *in vivo* probing of MurJ structure using the substituted-cysteine accessibility method revealed that this protein has 14 transmembrane domains that fold into a structure similar to that of MATE exporters of amphipathic drugs [23,24]. The predicted structure includes a V-shaped solvent-exposed cavity that contains several charged residues required for function [25]. Furthermore, modification of some engineered cysteine substitutions in this cavity with sulfhydryl-reacting probes inactivates MurJ in *E. coli* [26]. This chemical genetic approach to specifically and rapidly inhibit MurJ was used in combination with the first *in vivo* assay to monitor translocation of lipid II across the membrane in living cells [26]. This assay is based on the ability of the toxin colicin M (ColM) to enter the periplasm and hydrolyze lipid II into undecaprenyl and pyrophosphate-disaccharide-pentapeptide, but not lipid II localized in the inner leaflet of the IM awaiting translocation. Thus, the amount of pyrophosphate-disaccharide-pentapeptide produced by ColM reflects the activity of lipid II flippases in cells. The combination of these tools showed that all of the lipid II flippase activity detectable in *E. coli* cells was inhibited upon rapid chemical inactivation of MurJ. In contrast, depletion of FtsW in a strain lacking *rodA* did not prevent ColM from hydrolyzing lipid II.

Family matters

These results demonstrate that MurJ, and not RodA or FtsW, is required for lipid II translocation but, on their own, they cannot distinguish whether MurJ is the flippase itself or a factor required to assist the lipid II flippase [26]. However, two additional crucial facts strongly argue for the former scenario. First, MurJ belongs to the MOP exporter superfamily, which includes members such as Wzx flippases of Und-PP-linked oligosaccharides similar to lipid II [18,27]. Second, like Wzx, MurJ adopts a structure with a central hydrophilic cavity located within the plane of the membrane that contains residues essential for function [23,25,28]. Thus, MurJ is evolutionarily related to flippases of Und-P-linked oligosaccharides and shares functionally relevant, structural features with them. It follows that the most reasonable and simplest conclusion from all these data is that MurJ is a lipid II flippase. As architect Louis H. Sullivan stated, “form ever follows function, and this is the law. Where function does not change, form does not change.” [29]

Mechanism of lipid II transport

By analogy to MATE exporters, MurJ could function using an alternating-access mechanism that relies on the gradient of a counter ion to drive transport. In this model, the essential charged residues in the cavity of MurJ could interact with either the hydrophilic moiety of lipid II or the counter ion [21,24,25]. Alternatively, since those essential residues are localized in the periplasmic half of the solvent-exposed cavity, MurJ could first engage with lipid II in an inward-facing conformation through a low-affinity site; this interaction could result in a conformational change that could open the periplasmic side of the cavity, allowing lipid II to interact with higher affinity with the aforementioned charged residues, thereby driving transport directionally to the periplasmic side of the membrane [21].

In addition, there might be more than one mechanism to translocate lipid II. Recently, AmJ, a 6-transmembrane domain protein unrelated to MurJ, was shown to be functionally redundant with MurJ in the Gram-positive bacterium *Bacillus subtilis* [30]. Moreover, AmJ can also substitute for native MurJ in *E. coli*. Transcription of *amJ* is activated by PG stress, suggesting that AmJ is directly involved in PG biogenesis under certain conditions. However, given that some flippases of Und-P-linked saccharides are promiscuous [31,32], it is also possible that AmJ could be a flippase of a different substrate(s) capable of also translocating lipid II.

Regulation of PG synthases across the cell envelope

Once lipid II is flipped across the IM, GTs use it to synthesize PG glycan chains [3]. Building of the PG sacculus requires coordination of glycan synthesis and hydrolysis of the preexisting mesh so that the new material can be incorporated through crosslinks without compromising cell integrity. Factors involved in this process are localized in the IM, OM, and periplasm, and recent work discussed below suggests that growth of the PG sacculus is coordinated with that of the OM, possibly to ensure that envelope layers grow in unison.

E. coli has two major bifunctional PBPs at the IM with GT and TP activities, PBP1a and PBP1b, which are encoded by *ponA* and *ponB*, respectively [33]. Individually, each of these PBPs is dispensable, but the loss of both results in rapid death [11,12]. Thus, *ponA* and *ponB* null alleles are synthetically lethal. Genetic approaches that relied on this synthetic lethality identified the OM lipoproteins LpoA and LpoB as factors that activate PBP1a and PBP1b, respectively [34,35]. Recent structural analyses have revealed that both Lpo proteins are long enough to span the periplasm and directly contact their cognate PBPs at the IM [36,37]. Upon contact, each Lpo factor activates its partner through different mechanisms (Fig. 2). Interaction of the C-terminal domain of LpoA with PBP1a stimulates its TP activity through an unknown mechanism, which somehow also results in an enhancement of its GT activity [35,38]. In contrast, LpoB directly increases the GT activity of PBP1b, which indirectly stimulates its TP activity [38]. Specifically, LpoB interacts with the non-catalytic UB2H domain of PBP1b [35,36], and suppressor mutations in *ponB* that bypass LpoB-mediated activation suggest that this interaction causes a conformational change in PBP1b that extends to the GT domain [39]. Possibly this change increases GT activity by removing a block where nascent glycan chains are predicted to exit the GT domain [39,40].

The fact that Lpo factors are OM lipoproteins suggested that they might coordinate OM and PG biogenesis [34,35]. In addition, *lpoB*⁻ and *ponB*⁻ cells exhibit synthetic phenotypes with deletions of genes encoding the Tol-Pal system, a trans-envelope protein complex that coordinates membrane constriction during cell division [35,41-43]. In dividing *E. coli* cells, cleavage of septal PG is coordinated with the invagination of the IM and OM. The FtsZ ring-like structure in the cytoplasm provides the IM contractile force [44], periplasmic amidases regulated by IM and OM proteins control septal cleavage [45,46], and the Tol-Pal pathway promotes OM constriction [41]. Recently, CpoB (formerly YbgF), which binds in a cleft between the UB2H and TPase domains of PBP1b, was identified as a factor that coordinates PG synthesis and OM constriction by connecting Tol and PBP1b-LpoB [47].

Initially, CpoB and PG biogenesis were linked because *cpoB*⁻ and *ponB*⁻ strains exhibit similar phenotypes [48]. The periplasmic protein CpoB associates with TolA and PBP1b at the IM and with LpoB at the OM [47,49]. *In vitro*, CpoB does not affect the GT activity of a PBP1B-LpoB complex but interferes with the LpoB-mediated activation of its TP activity. TolA neutralizes this inhibition possibly by preventing CpoB from associating with LpoB and, by itself, TolA stimulates the GT activity of PBP1b. The current model (Fig. 2B) poses that when CpoB binds to PBP1b, it interferes with the ability of LpoB to increase PBP1b's TP activity. When TolA is energized by the proton motive force through its partners TolQR during membrane constriction at the septum, it can reverse this inhibitory effect of CpoB on LpoB. Therefore, the ability of the Tol system to both promote OM constriction and tune PBP1b activity through CpoB allows it to regulate PG synthesis in response to the status of OM invagination during cell division [47]. Whether analogous factors link PBP1a-LpoA to OM growth remains unknown.

CONCLUSIONS

For decades, studies on PG biosynthesis primarily focused on the discovery and characterization of enzymes involved in PG biogenesis, leaving us with minimal knowledge of how cells translocate lipid II across the cytoplasmic membrane and coordinate PG synthesis to the growth of other envelope layers. The work highlighted here has made critical contributions to these two fundamental questions in PG biogenesis. The question of whether MurJ or FtsW/RodA flip lipid II in *E. coli* has received much attention in recent years. *In vivo* studies have provided evidence supporting MurJ and discounting FtsW/RodA, while support for FtsW remains limited to *in vitro* studies [19,22,23,25,26]. *In vitro* reconstitution of MurJ flippase activity will be needed to close the door on this controversy but, more importantly, to understand how MurJ functions. Specifically, we must understand how MurJ interacts with lipid II and determine the source of energy driving transport. The important question of what function FtsW and RodA perform in the cell must also be addressed.

The question of how *E. coli* coordinates the building and remodeling of the layers that constitute its envelope remains largely unknown. However, we recognize that this work of engineering must involve the proper construction of the PG structure to maintain the integrity and shape of the cell. As I have discussed, having discovered that Lpo proteins, CpoB, and the Tol system regulate PG synthases is an important step towards understanding PG biogenesis [34,35,47]. Looking ahead, we must elucidate the molecular details of this regulation and how it is coordinated with the biogenesis of other envelope components.

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HIGHLIGHTS

- Peptidoglycan (PG) precursor lipid II must be flipped across the membrane.
- Controversy about the identity of the lipid II flippase is discussed.
- *Escherichia coli* coordinates PG biogenesis with growth of other envelope layers.
- Recent studies about the regulation of PG synthases are reviewed.

Figure 1. IM-localized steps in PG biogenesis in *E. coli*

Lipid II is synthesized in the cytoplasmic leaflet of the IM and translocated by a flippase. Then, the GT activity of bifunctional PBPs (blue) use lipid II to synthesize glycan chains, while its TP activity crosslinks the new chains to the preexisting matrix.

Figure 2. Regulation of PBP1a and PBP1b

A. LpoA stimulates TP activity of PBP1a, resulting in an increase in GT activity. **B.** LpoB stimulates GT activity of PBP1b, resulting in an increase in TP activity (black arrows). CpoB negatively regulates this activation of TP activity by LpoB. TolA can stop the effect of CpoB on the TP activity of PBP1b and activate its GT activity. Stimulatory and inhibitory effects are denoted with pointed and blunt arrows, respectively.