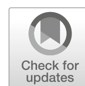

Lipid Intermediates in Bacterial Peptidoglycan Biosynthesis

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Hélène Barreteau, Didier Blanot, Dominique Mengin-Lecreulx, and Thierry Touzé

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Abstract

Peptidoglycan constitutes one of the major "Achilles heels" of bacteria because it is an essential component for cell integrity, and its metabolism is the target for a great number of antibacterials of different natures, e.g., antibiotics such as β-lactams and vancomycin, host immune system antimicrobial peptides, and bacteriocins. Peptidoglycan synthesis requires the translocation, across the plasma membrane, of the polymer building block, a disaccharide-pentapeptide. This event is performed via the attachment of the subunit to a lipid carrier, undecaprenyl-phosphate. Lipid intermediates called lipids I and II are generated through the sequential transfer of *N*-acetylmuramoyl-pentapeptide and *N*-acetylglucosamine moieties from nucleotide precursors to the lipid carrier by MraY and MurG transglycosylases, respectively. The last membrane

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intermediate, lipid II (undecaprenyl-pyrophosphate-*N*-acetylmuramoyl(-pentapeptide)-*N*-acetylglucosamine), can be further enzymatically modified through the addition of functional groups, amino acids, or peptides, before being flipped towards the outer leaflet of the plasma membrane where the final transfer of the peptidoglycan subunits to the growing polymer is catalyzed by penicillin-binding proteins. The integral membrane proteins FtsW, MurJ, and AmJ are thought to play a major role in the translocation process; however, the exact mechanism and the role of these molecular determinants is yet to be established. The lipid carrier is generated via a pathway involving two steps, first a polymerization reaction of isopentenyl-pyrophosphate catalyzed by the essential cytosoluble UppS enzyme, yielding undecaprenyl-pyrophosphate, followed by a dephosphorylation step ensured by a yet unknown enzyme. At each final transfer of a subunit to the elongating peptidoglycan, the lipid carrier is released in the pyrophosphate form, which is recycled to guarantee the high rate of polymer synthesis. Several integral membrane undecaprenyl-pyrophosphate phosphatases, from two distinct protein families and having their active site facing the extracytoplasmic side, have been identified, BacA and PAP2 enzymes. These enzymes can readily dephosphorylate the released lipid carrier precursor. Thereafter, the lipid is flipped back to the inner side of the membrane, by a yet unknown mechanism, in order to be reused as a glycan acceptor for a new round of peptidoglycan polymerization.

1 Introduction

Peptidoglycan (PG) is an essential component of the cell envelope of almost all bacteria. It is a complex heteropolymer composed of long glycan chains made up of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues linked by β1→4 bonds. Moreover, the glycan chains are cross-linked by short peptides attached to the D-lactoyl group of each MurNAc residue (Vollmer et al. 2008). Its main function is to preserve cell integrity by withstanding the inner osmotic pressure. It also contributes to the maintenance of a defined cell shape and is involved in the processes of cell elongation and division (den Blaauwen et al. 2008). Since the inhibition of its biosynthesis or its degradation generally causes cell lysis, PG constitutes an attractive target for the design of new antibacterial agents (Bugg et al. 2011). The biosynthesis of this polymer (Fig. 1a) is a three-stage process, which takes place in the cytoplasm (synthesis of nucleotide precursors), at the plasma membrane (synthesis of lipid intermediates and their translocation), and in the extracytoplasmic space (PG polymerization) (Lovering et al. 2012). The second stage can be summarized as follows: first, the 1-phospho-MurNAc-pentapeptide moiety from UDP-MurNAc-pentapeptide is transferred to the membrane polyprenol carrier undecaprenyl-phosphate (C_{55}-P), yielding lipid I. Thereafter, the addition of a GlcNAc residue from UDP-GlcNAc to lipid I leads to the formation of lipid II which in many bacteria, especially Gram-positive species, is enzymatically

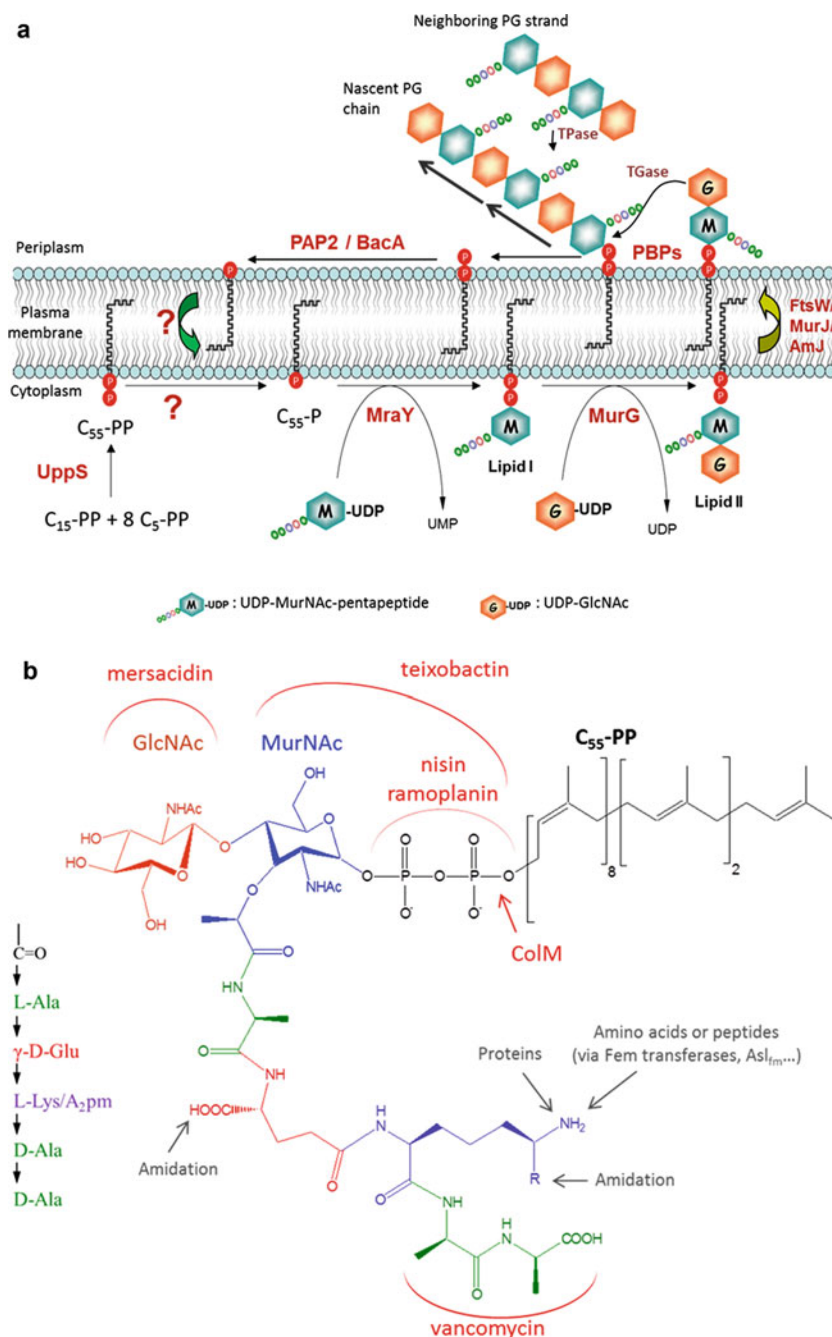

Fig. 1 (**a**) Schematic representation of PG biosynthesis and C_{55}-P metabolism. (**b**) Chemical structure of lipid II. Possible enzymatic modifications and antibacterial acting sites are indicated. TPase and TGase: transpeptidase and transglycosylase reactions, respectively. *A_2pm* diaminopimelic acid, *ColM* colicin M, *R* H (L-Lys) or COOH (A_2pm)

modified (Fig. 1b). The disaccharide-pentapeptide moiety is then translocated through the membrane to reach the outer face, where the transglycosylation (TGase) and transpeptidation (TPase) reactions (stage 3), catalyzed by penicillin-binding proteins (PBPs), take place. The TGase reaction releases the lipid carrier in the undecaprenyl pyrophosphate form (C_{55}-PP) that is actively recycled. The whole biosynthesis of PG has been the subject of several reviews (Barreteau et al. 2008; Bouhss et al. 2008; Sauvage et al. 2008; van Heijenoort 2010; Teo and Roper 2015). Here, we essentially focus on the metabolisms of the lipid I, lipid II, and C_{55}-P.

2 Biosynthesis of Lipid I by MraY

The formation of lipid I from UDP-MurNAc-pentapeptide and C_{55}-P is catalyzed by MraY, which belongs to the superfamily of polyprenyl phosphate *N*-acetylhexosamine 1-phosphate transferases (PNPT) (Price and Momany 2005), including enzymes involved in the biosynthesis of other cell surface polymers (WecA, TagO, etc.). MraY is an integral membrane enzyme with ten transmembrane segments and periplasmic N- and C-terminal ends (Bouhss et al. 1999). Cytoplasmic loops (especially the so-called loop E) are involved in substrate recognition and catalysis. Owing to its integral membrane nature, MraY proved to be difficult to purify. Nevertheless, orthologs from *Bacillus subtilis*, *Escherichia coli*, and some other species were purified using either suitable detergents or nanodisc-containing cell-free systems (Bouhss et al. 2004; Henrich et al. 2016). Crystal structures of MraY from *Aquifex aeolicus* have been solved as the apoenzyme (Fig. 2a) (Chung et al. 2013) or in complex with muraymycin D2 (MD2; Fig. 2b) (Chung et al. 2016), a naturally occurring ribosamino-uridine inhibitor which is competitive toward the nucleotide substrate (Tanino et al. 2011). Both the apoenzyme and the enzyme-inhibitor complex crystallized as a dimer with a hydrophobic tunnel at the dimer interface large enough to accommodate phospholipids. After binding MD2, MraY undergoes large conformational rearrangements near the active site, especially at the level of loop E (Fig. 2b). A two-step catalytic mechanism with an enzyme-phospho-MurNAc-pentapeptide covalent intermediate was first proposed from experiments carried out with crude membrane extracts (van Heijenoort 2010). However, recent data obtained with the pure enzyme are rather in favor of a one-step mechanism without covalent intermediate (Fig. 2c), consistent with a random bi-bi model (Al-Dabbagh et al. 2016; Liu et al. 2016). Site-directed mutagenesis and examination of the crystal structure helped identifying certain active site residues. Three aspartyl and one histidyl residues are particularly important for catalysis: D98, D99, D231, and H289 in *B. subtilis* and D117, D118, D265, and H324 in *A. aeolicus*. One of the aspartyl residues (D231/265) interacts with an essential Mg^{2+} ion which binds the pyrophosphate bridge of the nucleotide substrate (Chung et al. 2013). Another residue (D98/117) deprotonates a hydroxyl group of the lipid substrate prior to the nucleophilic attack of UDP-MurNAc-pentapeptide by C_{55}-P (Al-Dabbagh et al.

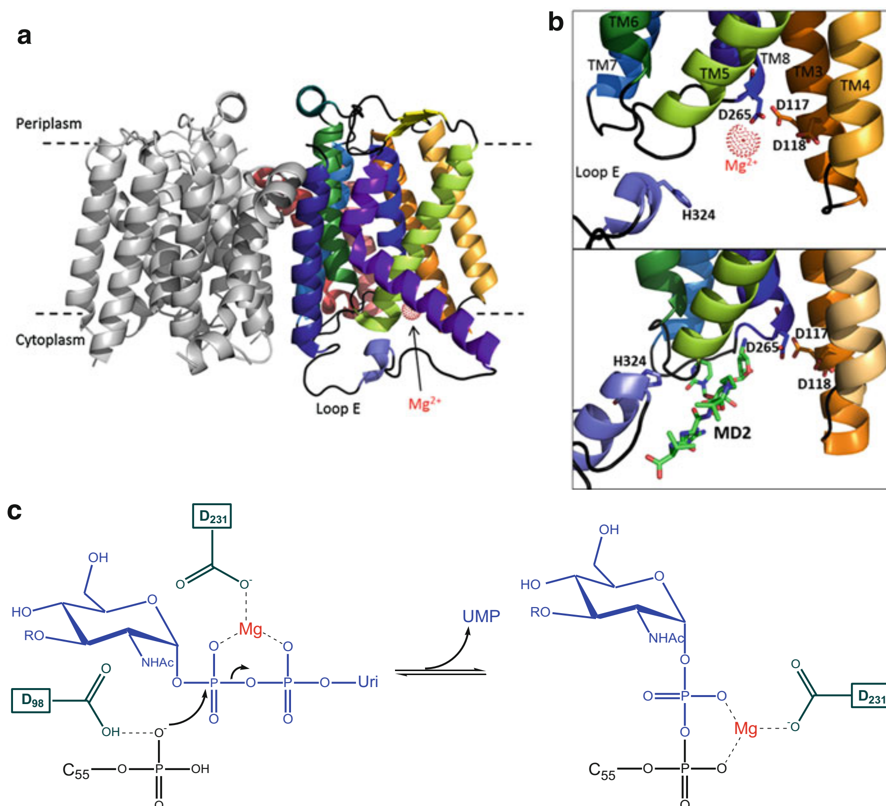

Fig. 2 (**a**) Structure of MraY from *A. aeolicus* (PDB entry, 4J72). Only one protomer is *colored*; the other one is shown in *grey*. Each transmembrane segment is colored from the *red* (N-terminus) to the *blue* (C-terminus). (**b**) Zoom at MraY active site in the apoenzyme (*up*) and in the complex with the MD2 inhibitor (*down*) (PDB entry, 5CKR). (**c**) Proposed one-step MraY reaction mechanism. The amino acid numbering is that of the *B. subtilis* sequence. *Uri* uridine, C_{55} undecaprenyl chain, *R* D-lactoyl-pentapeptide

2008). Surprisingly, MD2 interacts neither with the three aspartyl residues nor with the Mg^{2+} ion, suggesting that the mode of binding of this nucleoside antibiotic differs from that of UDP-MurNAc-pentapeptide (Chung et al. 2016). MraY is the target of many natural or synthetic nucleoside inhibitors belonging to the tunicamycin, ribosamino-uridine, uridylpeptide, and capuramycin classes (Dini 2005). However, owing to toxicity problems, none of them is of clinical use. MraY is also inhibited by the lysis protein E from bacteriophage ΦX174 (Zheng et al. 2009), as well as by cationic antimicrobial peptides containing the RWxxW motif (Bugg et al. 2016).

3 Biosynthesis of Lipid II by MurG

The formation of lipid II from lipid I and UDP-GlcNAc is catalyzed by MurG, which belongs to the glycosyltransferase B superfamily (Ünligil and Rini 2000). MurG is associated to the inner face of the plasma membrane (Bupp and van Heijenoort 1993). Its purification (Crouvoisier et al. 1999; Ha et al. 1999) and site-directed mutagenesis of invariant amino acids (Crouvoisier et al. 2007) have been reported for the *E. coli* enzyme, and the structures of the orthologs from *E. coli* and *Pseudomonas aeruginosa* have been solved (Brown et al. 2013; Ha et al. 2000; Hu et al. 2003). Crystal structures reveal that MurG contains two domains separated by a deep cleft (Fig. 3a). The C-terminal domain harbors the UDP-GlcNAc binding site, while lipid I is presumably bound by the N-terminal domain (Ha et al. 2000). The width of the cleft is reduced upon UDP-GlcNAc binding, the enzyme adopting a more closed conformation (Hu et al. 2003). MurG obeys an ordered bi-bi mechanism in which UDP-GlcNAc binds first (Chen et al. 2002). An as yet unidentified residue is believed to deprotonate the C4 hydroxyl group of the MurNAc moiety of lipid I, thereby generating an oxyanion which attacks the C1 of GlcNAc of the nucleotide substrate to form an oxycarbenium-ion-like transition state. Finally, the reaction results in the inversion of the anomeric configuration of GlcNAc in lipid II (Fig. 3b). MurG is inhibited by uridine-linked transition-state mimics (Trunkfield et al. 2010) as well as by compounds derived from the screening of chemical libraries (Hu et al. 2004). Recently, steroid-like compound murgocil was found to selectively hinder PG synthesis in *Staphylococci* through MurG inhibition (Mann et al. 2013).

4 Modifications of Lipid Intermediates

PG is subjected to many types of modifications leading to an important variability of its chemical structure within the bacterial world, which can be involved in resistance towards various antibacterial agents or modulate bacterial recognition by host immune systems. These modifications occur at various steps of its biosynthesis, but those undergone at the level of lipids I and II are especially amidation and extra amino acids or protein attachment (Fig. 1b). For example, the LtsA protein from *Corynebacterium glutamicum* is responsible for the amidation of PG *meso*-A_2pm residues (Levefaudes et al. 2015). LtsA catalyzes the transfer of an amino group from L-glutamine onto the carboxyl group of lipid II *meso*-A_2pm. Amidation can also occur on the glutamate residue of lipid II, as in *Staphylococcus aureus* where the glutamine amidotransferase-like protein GatD and the Mur ligase homolog MurT in concert catalyze the formation of D-isoglutamine at position 2 of the peptide stem (Figueiredo et al. 2012; Münch et al. 2012). PG is the point of covalent attachment of cell envelope proteins (Dramsi et al. 2008). In Gram-negative bacteria, Braun's lipoprotein seems to be the unique PG-linked protein (Braun and Rehn 1969). This 58 amino acid-long triacylated protein is anchored in the outer membrane. In *E. coli*, the side chain amine of the C-terminal lysine residue of Braun's lipoprotein is linked to the α-carboxyl group of the *meso*-A_2pm residue of lipid II, and the

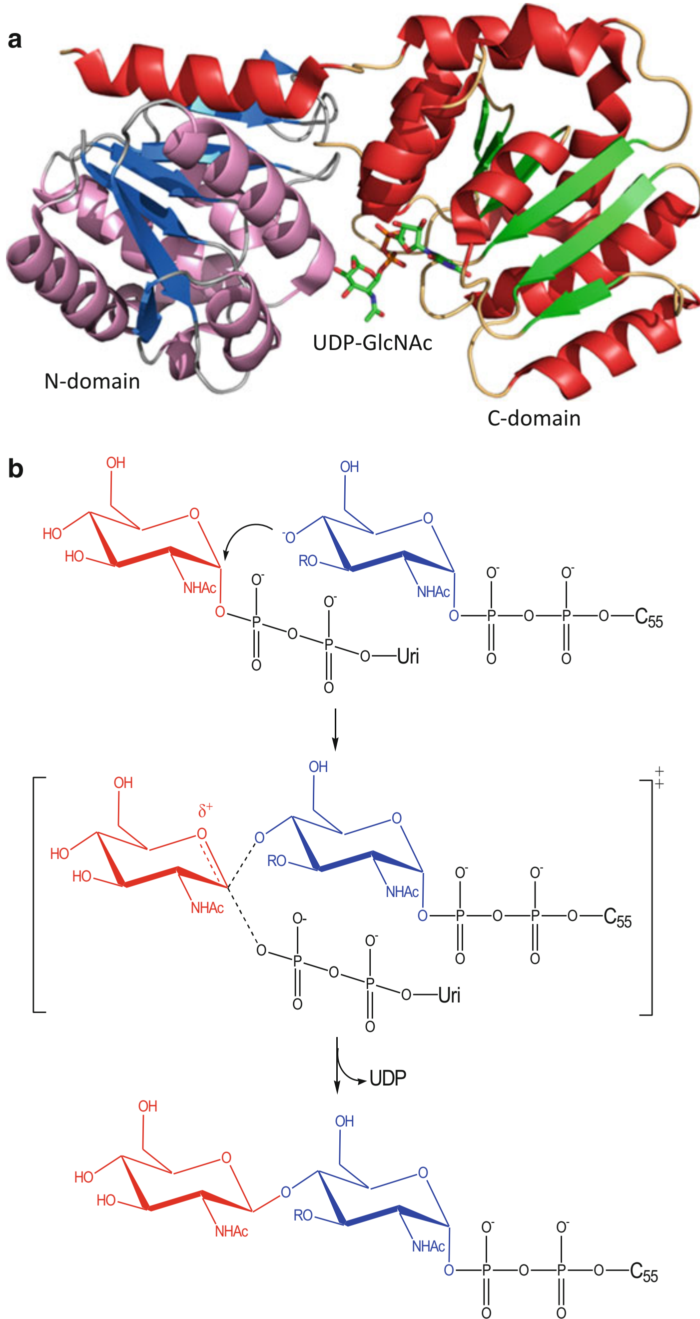

Fig. 3 (**a**) Structure of the *E. coli* MurG:UDP-GlcNAc complex (PDB entry, 1NLM). (**b**) Reaction mechanism of MurG. *C_{55}* undecaprenyl chain, *Uri* uridine, *R* D-lactoyl-pentapeptide

formation of this bond is catalyzed by one of the four L,D-transpeptidase activities identified in this species (Magnet et al. 2007). The major modifications of lipid II are performed in Gram-positive bacteria by different enzymes responsible for the addition of interpeptide bridges required for PG polymerization (transpeptidation) in these species. The Fem transferases synthesize interpeptide bridges from activated glycine or L-amino acids as aminoacyl-tRNAs (Mainardi et al. 2008). For instance, FemX, FemA, and FemB peptidyltransferases from *S. aureus* synthesize a pentaglycine interpeptide bridge by successive incorporations of a first glycine residue (FemX), the next two glycines (FemA), and finally the last two glycines (FemB) to the ε-amino group of L-Lys of lipid II peptide stem (Schneider et al. 2004). In *Enterococcus faecium*, the interpeptide bridge consists of a single D-amino acid (D-aspartic acid), the addition of which is performed by Asl_{fm}, an enzyme of the ATP-grasp superfamily; activation occurs through the formation of β-aspartyl phosphate in an ATP-dependent reaction (Bellais et al. 2006). The amino group of the interpeptide bridge of lipid II from Gram-positive bacteria is also the site of attachment of specific proteins. This covalent attachment is ensured by the sortase family of transpeptidases, which have been shown to be essential in pathogenesis (Bradshaw et al. 2015).

5 Flipping of Lipid II

Once synthesized and possibly modified, lipid II is translocated toward the outer leaflet of the plasma membrane to allow the transfer of the PG building block (disaccharide-peptide) to the growing polymer by the PBPs (TGase and TPase reactions) (Fig. 1a). The knowledge of the molecular determinant(s) and the mechanism of this flipping event represent the “Holy Grail” in this research field, and recent progresses have opened intense debates as several protein “flippase” candidates have arisen. The integral membrane FtsW protein and its orthologs (RodA, SpoVE) from the SEDS (shape, elongation, division, and sporulation) superfamily have long been considered as playing a central role in this process because of their presence in virtually all PG-containing bacteria, their essentiality, and their interaction with other PG biosynthesis enzymes in the so-called elongasome and divisome complexes (Boyle et al. 1997; Fraipont et al. 2011). Moreover, Mohammadi et al. have reported biochemical evidence for a flippase activity of FtsW after its reconstitution into lipid II-containing liposomes (Mohammadi et al. 2011). In their in vitro assays, a fluorescent 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD) analogue of lipid II was used, which emphasized that the presence of FtsW in liposomes enhances the translocation of NBD-lipid II from one leaflet of the bilayer to the other, as shown by a sharp increase in fluorescence extinction with a fluorescence quencher. These data, supporting a direct role of FtsW in flipping lipid II, did not put an end to the story as MurJ (or MviN), another contender for this activity, was strengthened. The integral membrane protein MurJ, which is essential for PG biosynthesis in *E. coli*, belongs to the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) exporter superfamily (Hvorup et al. 2003; Inoue et al. 2008; Ruiz 2008). Wzx, another member of this

superfamily, mediates, by an as yet unknown mechanism, the translocation of C_{55}-PP-linked intermediates in the biosynthesis pathways of enterobacterial common antigen (ECA), O-antigen from lipopolysaccharides (LPS), capsule, and other cell surface polymers (Islam and Lam 2013). Sham et al. recently designed an elegant in vivo assay to demonstrate the involvement of MurJ in lipid II translocation (Sham et al. 2014). They expressed in Δ*murJ E. coli* cells a functional cysteine-containing variant of MurJ which can be inhibited by a sulfhydryl-reactive reagent. Then, they showed that upon MurJ inhibition, the newly synthesized lipid II was no more accessible to the colicin M toxin, a lipid II-degrading enzyme acting exclusively at the periplasmic side of the membrane (see below), strongly suggesting an arrest of lipid II translocation in these conditions. The authors concluded that MurJ was the essential lipid II flippase and that other factors catalyzing this event in *E. coli* were therefore unlikely to exist. Nevertheless, the support of MurJ as a general lipid II flippase was hindered by the fact that MurJ was not essential in *B. subtilis* and that no flippase activity was observed in Mohammadi et al.'s in vitro assay in MurJ-containing liposomes (Fay and Dworkin 2009; Mohammadi et al. 2011). The nonessentiality of MurJ in *B. subtilis* let the MurJ's supporters to argue that an alternate lipid II flippase could exist in this bacterium. A search for such an ancillary translocase highlighted a synthetic lethal phenotype caused by the inactivation of MurJ together with AmJ (alternate to MurJ), another membrane protein with no sequence similarity with MurJ, FtsW, or other transporters (Meeske et al. 2015). Furthermore, AmJ from *B. subtilis* was able to support the growth of MurJ-inactivated *E. coli* cells, undoubtedly showing that MurJ and AmJ display a redundant function. As to whether FtsW and/or MurJ/AmJ are themselves part of the translocase or function as essential partners or regulators of the latter and the mechanism by which this event occurs remain largely to be deciphered, and this exciting debate seems to be far from being closed (Ruiz 2016).

6 Synthesis and Recycling of the Lipid Carrier

C_{55}-P is a general lipid carrier for cell surface polymers subunits, which are synthesized in the cytoplasm and must be transported throughout the plasma membrane (e.g., PG, teichoic acids, LPS O-antigen) (Manat et al. 2014). Therefore, a unique C_{55}-P pool must be shared by several metabolic pathways in a single cell, implying a fine-tuned synchronization in the synthesis of these various polymers. The (re)generation and delivery of this essential lipid should thus be tightly controlled. However, to date, little is known about these regulation processes. C_{55}-P originates from the dephosphorylation of its precursor, C_{55}-PP, itself being generated by de novo synthesis or released after each TGase reaction at the periplasmic side of the membrane (Fig. 1a). The de novo synthesis of C_{55}-PP is catalyzed by the essential cytosoluble UppS enzyme belonging to the *cis* prenyltransferase family (Teng and Liang 2012). UppS performs eight sequential condensations of the five-carbon building block, the homoallylic isopentenyl pyrophosphate substrate (C_5-PP), with the allylic 15-carbon-chain *trans*, *trans*-farnesyl pyrophosphate

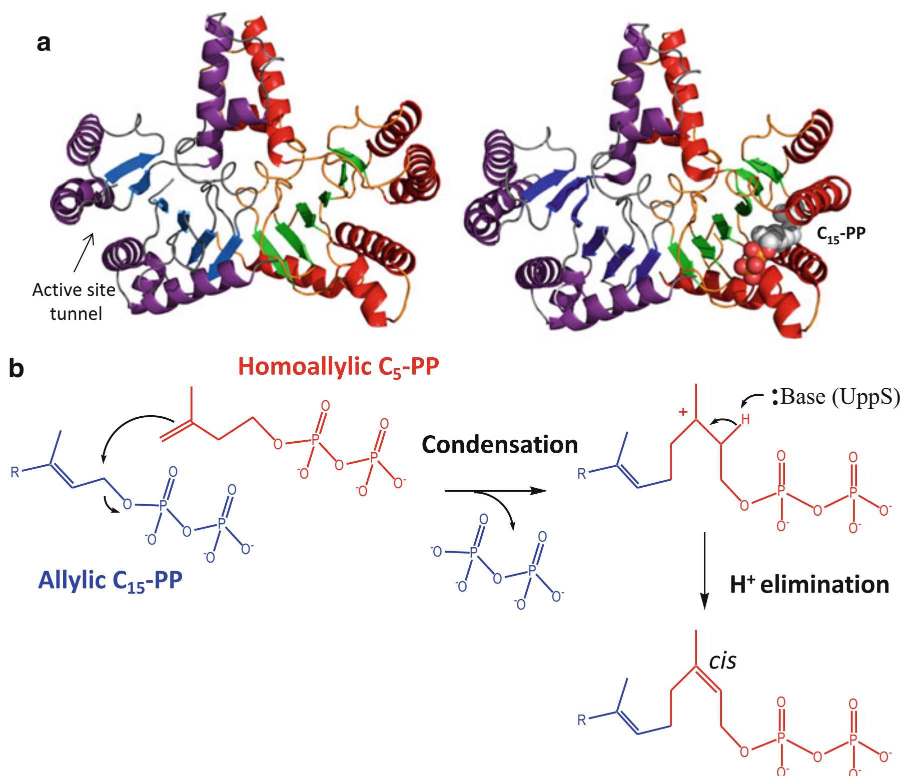

Fig. 4 (**a**) On the *left*, structure of UppS from *E. coli* in the apo-form (open conformation) (PDB entry, 1UEH). On the *right*, structure of UppS in complex with C_{15}-PP (closed conformation) (PDB entry, 1V7U). One monomer is represented with *purple* α-helices and *blue* β-strands and the other monomer is represented with *red* α-helices and *green* β-strands. (**b**) Mechanism of condensation by the *cis*-prenyltransferase UppS

(C_{15}-PP), yielding di-*trans*, octa-*cis*-C_{55}-PP. Several structures of UppS in the apo-form (Fujihashi et al. 2001) and in complex with C_{15}-PP, C_5-PP and the Mg^{2+} ion cofactor (Guo et al. 2005), together with several mutagenesis and kinetic studies (Teng and Liang 2012) have provided considerable knowledge of this catalytic process. As an essential enzyme, UppS is another potential target for novel antibacterials; therefore, UppS inhibitors are searched through different approaches (Jukič et al. 2016). UppS forms a dimer, with each protomer enclosing a deep hydrophobic cleft which accommodates the isoprenoid carbon tail during its elongation (Fig. 4a). UppS binds first the allylic C_{15}-PP substrate, triggering a conformational change that allows this binary complex to further bind C_5-PP, whose phosphate groups interact with an aspartyl residue from the active-site tunnel entrance (*E. coli* UppS D29 residue) via a magnesium bridge. A concerted mechanism was proposed, in which the release of the pyrophosphate group from the allylic substrate and the nucleophilic attack of C_{15}-PP C1-atom by C_5-PP C4-atom, ending in the formation of a new

double bond, occur simultaneously (i.e., no carbocation intermediate being formed) (Fig. 4b). The catalytic aspartyl D26 residue was then proposed to play a central role by controlling the migration of Mg^{2+} from C_5-PP to C_{15}-PP, where the metal ion may then facilitate the C_{15}-PP pyrophosphate group dissociation, concomitantly to the nucleophilic attack. The length of the final product is controlled by a molecular ruler mechanism, where bulky residues from the bottom of the tunnel block further condensation reaction via steric hindrance (Ko et al. 2001). Therefore, the newly synthesized C_{55}-PP pushes away a so-called entrance loop, which allows the relaxation of the active-site tunnel ready for a new cycle of synthesis.

The dephosphorylation of C_{55}-PP represents the ultimate step in the formation of the carrier lipid, which occurs in the course of de novo synthesis and also recycling (Fig. 1a). In contrast to the previous step that involves the single and essential UppS enzyme, the dephosphorylation of C_{55}-PP can be catalyzed by four different integral membrane enzymes in *E. coli* (El Ghachi et al. 2004, 2005). These enzymes belong to two distinct protein families: BacA, which constitutes a novel family of phosphatases present in a majority of bacteria, and three members of the ubiquitous PAP2 family (phosphatidic acid phosphatases of type 2) named PgpB, YbjG, and LpxT (formerly YeiU). Multiple genes inactivation is then required to elicit a lethal phenotype, raising the question of the significance of such a redundancy of enzymes for a single function. BacA requires a divalent metal ion for activity, with Ca^{2+} providing the highest activity as observed in vitro (Chang et al. 2014; Manat et al. 2015). BacA enzymes display two regions with a strong degree of conservation, which likely harbor active site residues. Mutagenesis studies have shown that BacA requires, especially, three amino acid residues belonging to these conserved regions (S24, E21, and R174) in order to display its activity both in vivo (functional complementation assay) and in vitro (kinetic analysis) (Manat et al. 2015). BacA membrane topology studies strongly suggested that these catalytic residues are facing the periplasm (Fig. 5). The PAP2 enzymes form a large family of integral membrane and soluble phosphatases with various substrates and physiological functions (Sigal et al. 2005). Interestingly, PgpB displays a dual function as being involved, in addition to C_{55}-P metabolism, in the synthesis of phosphatidylglycerol through phosphatidylglycerol phosphate dephosphorylation (Dillon et al. 1996).

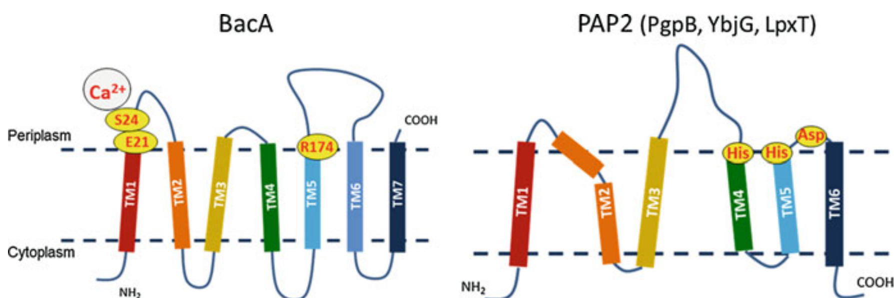

Fig. 5 Schematic representation of the C_{55}-PP phosphatases from *E. coli*

All PAP2 enzymes likely share a similar catalytic mechanism, which has been proposed to rely on a catalytic triad with one aspartyl and two histidyl residues. Based on biochemical and structural studies of different PAP2 enzymes, the catalysis should occur via a nucleophilic attack of the phosphate anhydride bond of C_{55}-PP by a catalytic histidine, leading to the formation of a phospho-histidine enzyme intermediate and the release of C_{55}-P (Ishikawa et al. 2000). The aspartyl residue may be involved in a charge relay system allowing this nucleophilic attack, while the additional histidine may protonate the leaving C_{55}-P product. In a second step, the phosphate group is released through its transfer to a water molecule, generating inorganic phosphate, or to another specific acceptor molecule. In such a way, LpxT enzyme catalyzes the transfer of a phosphate group from C_{55}-PP onto the lipid A moiety from LPS (Touzé et al. 2008a). Even though the physiological role of the resulting phosphorylated lipid A is yet unknown, LpxT takes part in a complex network of LPS modifications which are involved in bacterial adaptation to various conditions (Herrera et al. 2010; Kato et al. 2012). This unexpected phosphotransferase activity further suggests that other C_{55}-PP phosphatases may also exhibit similar activities with specific acceptor molecules (Manat et al. 2014). Interestingly, the active site residues of PAP2-type C_{55}-PP phosphatases are also oriented towards the periplasm, as demonstrated by several biochemical studies and recently confirmed by the 3D structure of PgpB (Fan et al. 2014; Tatar et al. 2007; Touzé et al. 2008b). The periplasmic orientation of the active sites of BacA and PAP2 suggests that these enzymes are rather involved in the recycling of C_{55}-PP. Indeed, the latter lipid is released at the outer leaflet of the plasma membrane as a product of TGase reactions, and it is therefore readily accessible to BacA and PAP2 enzymes. In contrast, it is unclear how the de novo synthesized C_{55}-PP, present at the inner leaflet after its synthesis by UppS, is dephosphorylated. As to whether a yet unidentified C_{55}-PP phosphatase with a cytoplasm-oriented active site exists or whether the C_{55}-PP flips toward the outer leaflet in order to be dephosphorylated needs further investigations. Another open issue concerns the mechanism by which the lipid carrier is translocated back to the inner leaflet, once dephosphorylated at the periplasmic side, in order to be reused as sugar acceptor for another cycle of PG (and other polymers) synthesis.

7 Antibacterials Targeting PG Lipid Intermediates and C_{55}-P (Re)generation

Lipid II is a well-validated drug target, especially in Gram-positive bacteria, where it is relatively well accessible from the outside due to the absence of an outer membrane. Many antibacterials bind lipid II in a noncovalent way. They are usually polar and of small size, and most of them are cyclic peptides, depsipeptides, or post-translationally modified peptides. Vancomycin is probably the most famous lipid II-targeting antibacterial, and it is also the only one of clinical use. This glycopeptide tightly binds to the D-Ala-D-Ala moiety of lipid II peptide stem, thereby inhibiting PG polymerization (TPase reactions) (Perkins

1969). The glycodepsipeptide ramoplanin, which is currently in the late stage of clinical development for the treatment of *Clostridium difficile* infections, has been known for a long time to inhibit TGase reactions. Recent studies have proposed ramoplanin to form membrane amphipathic symmetric dimers sequestrating lipid II, possibly at the level of its pyrophosphoryl moiety (Hamburger et al. 2009). Other antibacterials, such as a few lantibiotics and proteins from the colicin M family, target lipid II on various epitopes. Among the lantibiotics, which are gene-encoded peptides produced by some strains of bacilli (Willey and van der Donk 2007), the type-A nisin exhibits a dual mode of action by interfering with PG synthesis and also by disrupting the electric potential of the plasma membrane. Nisin uses the pyrophosphate moiety of lipid II as a docking site, which improves its efficiency to form pores in the membrane (Breukink and de Kruijff 2006). The type-B lantibiotic mersacidin is thought to bind to the GlcNAc moiety of lipid II, thereby inhibiting TGase reactions without pore formation (Brötz et al. 1998). Despite the fact that the molecular details of the latter interaction are yet to be resolved, an NMR study revealed conformational changes of mersacidin upon lipid II binding, suggesting a conformational adaptability of this class of antibiotic that might be central to their mode of action (Hsu et al. 2003). The colicin M protein and its orthologs display a unique phosphodiesterase enzymatic activity towards lipid II, releasing undecaprenol and pyrophospho-disaccharide-pentapeptide as dead end products, thus leading to cell death (El Ghachi et al. 2006). Unlike the other lipid II-targeting compounds, these proteins are produced by *Escherichia*, *Pseudomonas*, *Pectobacterium*, and *Burkholderia* genera and are active against a narrow range of related species (Barreteau et al. 2009; Grinter et al. 2012). This specificity is due to their mode of access to the periplasm of the target cell that relies on specific interactions with proteins used as receptors and translocation machineries (Cascales et al. 2007). In the search for new antibiotics, the exploitation of uncultured bacteria could be a fruitful approach. For example, the Gram-negative soil bacterium *Eleftheria terrae* produces a 1.2-kDa compound named teixobactin, which inhibits the growth of methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and *Mycobacterium tuberculosis*, but is not active against Gram-negative bacteria (Ling et al. 2015). Even though it also interacts in vitro with lipid I and C_{55}-PP, the formal mechanism of action of teixobactin was shown to rely on an interaction with the pyrophosphoryl and MurNAc moieties of lipid II.

The (re)generation of the lipid carrier is also the target of antibacterial compounds. Bacitracin, a mixture of related cyclic peptides produced by some strains of *B. subtilis*, tightly binds to the pyrophosphoryl moiety of C_{55}-PP. The sequestration of C_{55}-PP prevents an efficient production of the active form of lipid carrier, C_{55}-P, thus inhibiting PG biosynthesis (Siewert and Strominger 1967; Stone and Strominger 1971). In *E. coli*, the overexpression of C_{55}-PP phosphatases-encoding genes was proven to overcome bacitracin cytotoxicity (El Ghachi et al. 2004). C_{55}-P was also shown to be the target of friulimycin, produced by *Actinoplanes friuliensis* (Schneider et al. 2009), and of laspartomycin C, produced by *Streptomyces viridochromogenes* (Kleijn et al. 2016). Both lipopeptides are branched with C_{14}

and C_{15} fatty acids, respectively, and they act by C_{55}-P sequestration, preventing lipid II formation.

8 Research Needs

The biosynthesis of PG lipid intermediates by the essential MraY and MurG enzymes is now well decrypted, numerous kinetic and structural studies providing good knowledge of these enzymatic steps. Nevertheless, substrate recognition and mechanistic details are further required to get an in-depth comprehension of these reactions, which requires higher resolution, substrate-liganded protein structures. This knowledge offers a framework for the study of mechanisms of action of yet identified inhibitors and the design or the high-throughput screening of novel molecules for therapeutic applications. The enzymatic modifications of lipid II, which are bacterial strain-specific, represent an important field of investigation. These modifications are either essential, such as the addition of peptide bridges in Gram-positive bacteria, or required for adaptation to certain environmental conditions (e.g., resistance towards PG-targeting antibacterials and modulation of host immune response). The physiological functions of these modifications, the enzymatic processes underlying these structural changes, and their regulation need more research efforts. The design of antibacterials directed against these enzymatic steps can be an interesting approach to target specific pathogens without disturbing the whole microbiota. As already mentioned, the mechanism of lipid II translocation across the plasma membrane is the subject of a passionate controversy with several contenders as the true flippase. Whether these different candidates participate in that process and the way the latter is conducted remain largely to be deciphered through more genetic, biochemical, and structural studies. It will be of particular interest to highlight the role of the isoprenoid lipid, which is a universal glycan carrier. It is conceivable that the particular structure of this lipid may play a decisive role in the translocation event. This step is considered as an attractive drug target because it may be readily accessible from the extracytoplasmic side of the membrane. The (re) generation of the lipid carrier constitutes another interesting field of research especially with recent findings in the function and properties of the multiple C_{55}-PP phosphatases. The emphasized LpxT phosphotransferase reaction suggests that other PAP2 enzymes may perform comparable reactions with specific acceptor molecules. The physiological functions of such reactions, including the LpxT-dependent lipid A modification, will have to be further addressed. The role of the multiplicity of C_{55}-PP phosphatases in a single bacterium, their regulation, and their mechanism of action will also have to be examined. The fact that all C_{55}-PP phosphatases yet identified exhibit a periplasm-oriented active site suggests their involvement in lipid carrier recycling, raising the question of the de novo synthesis pathway which requires a similar enzymatic reaction. Finally, the mechanism of translocation of C_{55}-P back to the inner leaflet of the plasma membrane, to be reused, is another open question.

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