

Deciphering the Metabolism of Undecaprenyl-Phosphate: The Bacterial Cell-Wall Unit Carrier at the Membrane Frontier

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During the biogenesis of bacterial cell-wall polysaccharides, such as peptidoglycan, cytoplasmic synthesized precursors should be trafficked across the plasma membrane. This essential process requires a dedicated lipid, undecaprenyl-phosphate that is used as a glycan lipid carrier. The sugar is linked to the lipid carrier at the inner face of the membrane and is translocated toward the periplasm, where the glycan moiety is transferred to the growing polymer. Undecaprenyl-phosphate originates from the dephosphorylation of its precursor undecaprenyl-diphosphate, with itself generated by *de novo* synthesis or by recycling after the final glycan transfer. Undecaprenyl-diphosphate is *de novo* synthesized by the cytosolic *cis*-prenyltransferase undecaprenyl-diphosphate synthase, which has been structurally and mechanistically characterized in great detail highlighting the condensation process. In contrast, the next step toward the formation of the lipid carrier, the dephosphorylation step, which has been overlooked for many years, has only started revealing surprising features. In contrast to the previous step, two unrelated families of integral membrane proteins exhibit undecaprenyl-diphosphate phosphatase activity: BacA and members of the phosphatidic acid phosphatase type 2 super-family, raising the question of the significance of this multiplicity. Moreover, these enzymes establish an unexpected link between the synthesis of bacterial cell-wall polymers and other biological processes. In the present review, the current knowledge in the field of the bacterial lipid carrier, its mechanism of action, biogenesis, recycling, regulation, and future perspective works are presented.

The Universal Glycan Lipid Carrier

THE USE OF LONG and linear-chain polyprenyl-phosphate lipids that facilitate the translocation of a sugar or glycan strand across a biological membrane is an extraordinary conserved process encountered in all kingdoms of life.^{39,101} They are involved in protein glycosylation in eukaryotic,¹⁰ archeal, and bacterial cells⁵² as well as for the biogenesis of bacterial cell-wall polysaccharides such as peptidoglycan,⁷ lipopolysaccharide (LPS) O-antigen,^{93,110} wall teichoic acids,⁸ capsular polysaccharides,¹²⁰ common enterobacterial antigen,⁸⁸ membrane-derived oligosaccharides,¹¹⁸ and exopolysaccharides. In mammals, defects in protein glycosylation referred to as congenital disorders of glycosylation¹¹⁹ engender severe malformations and developmental retards with outcomes ranging from death in infancy to late-onset diseases. In bacteria, the inhibition of the biosynthesis or recycling of the undecaprenyl-phosphate carrier lipid provokes the arrest of peptidoglycan synthesis and, consequently, cell lysis.⁷ This essential chemical carrier takes part in critical cellular processes, and, therefore, its metabolism represents a potential target for therapeutics such as new antibacterial agents.

These long carbon chain lipids are generated by sequential condensations of several isoprene units (the 5-carbon building block, isopentenyl-diphosphate).^{39,101} Among the living organisms, the structure of the lipid carrier varies, to some extent, in both the carbon chain length (number of polymerized isoprene units) and stereochemistry. In bacteria, the 55-carbon chain length generated by condensation of 11 isoprene units yielding undecaprenyl-phosphate (C₅₅-P) (Fig. 1) is the common rule; however, lower-sized decaprenyl-phosphate (C₅₀-P)⁵⁶ and nonaprenyl-phosphate (C₄₅-P)⁴⁸ homologues were found to assist glycan translocation in some mycobacterial species and *Paracoccus denitrificans*, respectively. The *cis* or *trans* configuration of the double bonds in the polyisoprenoid chain is another source of diversity. Typically, the isoprene units are added in a *cis* configuration on a short all-*trans* oligoprene precursor comprising one to three subunits. Finally, but not the least, the status of hydrogenation of the terminal isoprene unit proximal to the phosphate group (the α -position) appears either unsaturated, as found in bacteria, or saturated, as found in eukaryotes and archaea, with the latter form thus requiring an additional reduction reaction, leading to the so-called dolichyl-phosphate (Fig. 1). In all organisms, these

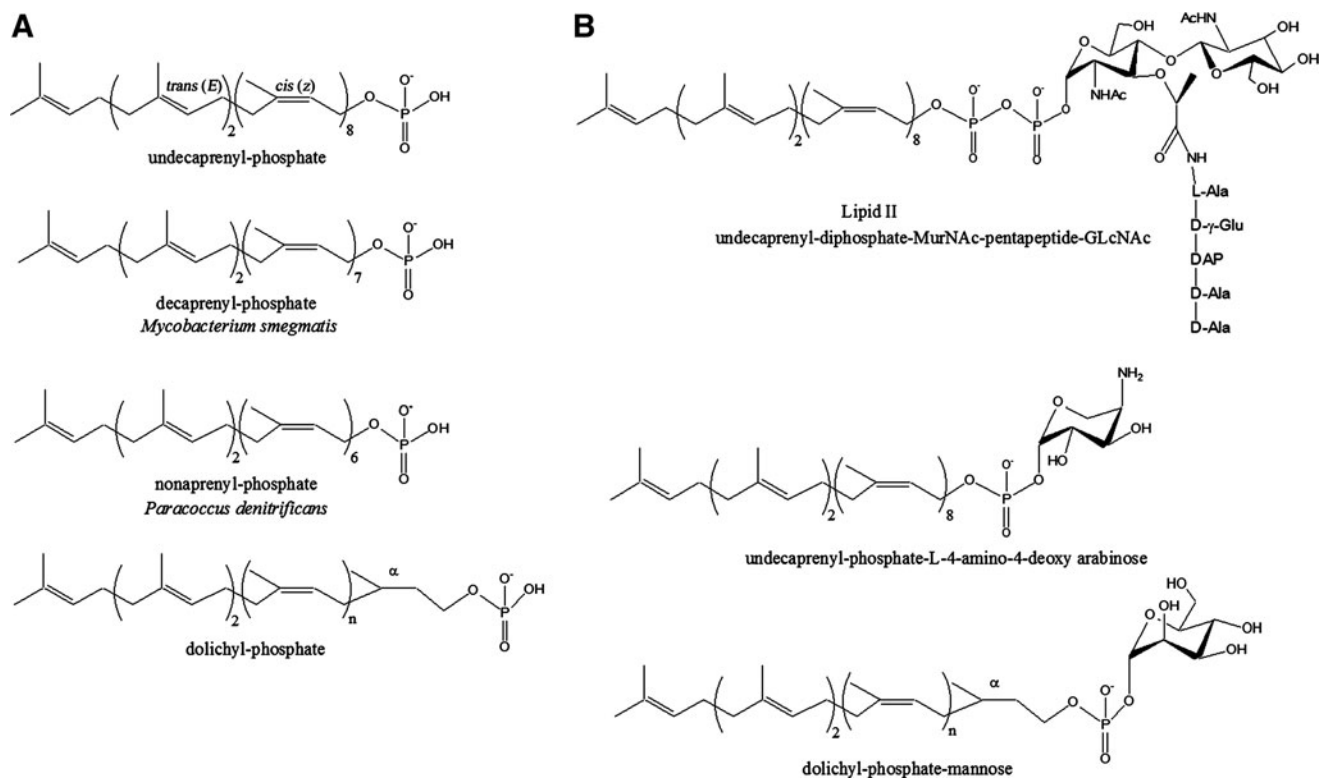


FIG. 1. Structures of different polyprenyl-phosphate carrier lipids (A) and lipid intermediates from various glycoconjugate biosynthetic pathways (B).

lipids fulfill a similar function, which is to facilitate translocation of a polar head group (mono- or oligosaccharides) across a highly hydrophobic environment; therefore, the driving force that presides over this observed structural diversity still remains unexplained.

The way in which these lipids carry out their function is apparently very well conserved.³⁹ The sugar/glycan is first transferred from a nucleotide-diphosphate activated donor to the lipid carrier, leading to the formation of a membrane-bound intermediate with a polyprenyl-diphosphate-glycan structure (Figs. 1 and 2). In some cases, the membrane intermediate displays a polyprenyl-monophosphate-glycan structure, as, for instance, in undecaprenyl-phosphate-L-4-amino-4-deoxy-arabinose that is involved in lipid A modification in Gram-negative bacteria,¹⁰⁷ or in dolichyl-phosphate-mannose which is responsible for protein mannosylation⁹⁶ (Fig. 1). The reason that there is such a divergence in the linkage nature is not understood. The initial step of glycan transfer occurs on the cytoplasmic side of the membrane, the endoplasmic reticulum membrane in eukaryotes, or the cytoplasmic membrane in prokaryotes. The resulting lipid intermediate is then translocated across the membrane so that the glycan moiety becomes accessible to enzymes catalyzing subsequent steps of glycan transfer or polymerization on the outer side of the membrane (Fig. 2). The fact that the sugar is linked to the lipid carrier is not sufficient *per se* to overcome the thermodynamic barrier which represents the translocation of a polar group across a lipid bilayer, and this event requires a dedicated flippase.⁹⁴ Nevertheless, given that polyprenyl-phosphate lipids are the preferred membrane-bound glycan carriers found in nature, the polyprenol-based structure, its length, geometry,

and membrane orientation, may play an active role in the flippase-catalyzed translocation process. Several biophysical studies have revealed that the presence of polyprenyl-phosphate or its derivatives (polyprenol, polyprenyl-diphosphate, or polyprenyl-sugar) in model membrane vesicles significantly increases their fluidity and ion permeability.^{109,112,117} It is reasonable to figure out that local destabilization of the membrane may, thus, be a determinant to overcome the translocation energetic barrier.

Several flippases have been identified, emphasizing the existence of a sharp specificity between the flippase and the nature of the glycan to be translocated.^{20,41,42,51,89,123} This underlies the fact that the flippase should recognize the sugar moiety at the membrane interface in the first place. In bacteria, the identification of flippases was facilitated, as their genes were generally clustered with genes involved in a particular polysaccharide biosynthesis pathway (FtsW for peptidoglycan,⁷⁵ Wzx for O-antigen,⁵⁰ etc.). Then, most flippases have been identified by genetic approaches and through the observation that their inactivation typically provoked the accumulation of a specific membrane-bound intermediate and the concomitant arrest of the biosynthesis of the corresponding polysaccharide. However, the function of these membrane proteins still requires biochemical validation via the reconstitution of the purified proteins in model membrane vesicles and the development of specific flippase assays. The flippase activity of FtsW toward the peptidoglycan lipid II intermediate [undecaprenyl-diphosphate-MurNAc-(pentapeptide)-GlcNAc] was recently addressed by using such an appropriate biochemical approach.⁷⁵ The lipid II translocation mechanism is considered ATP

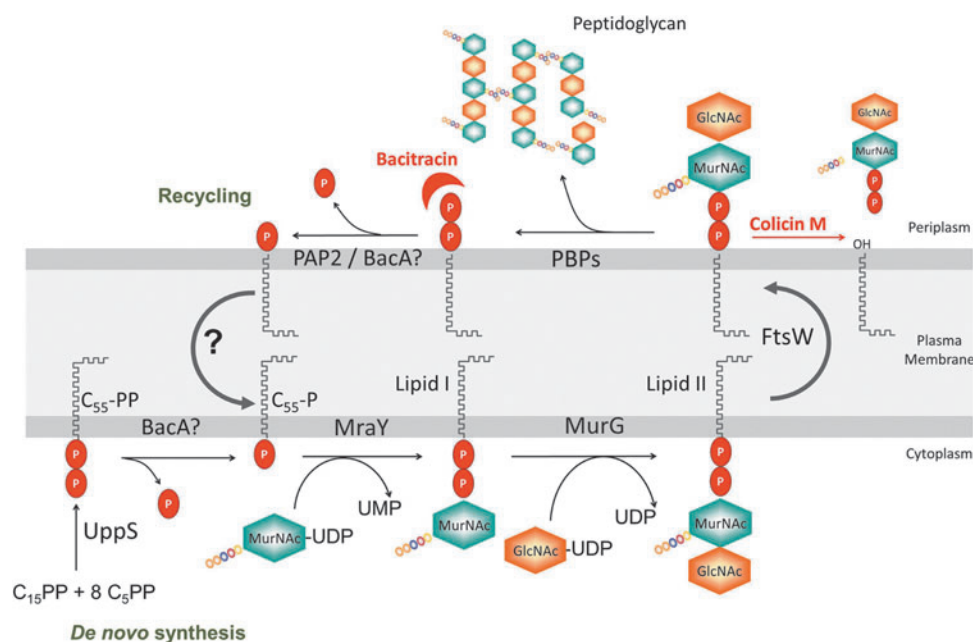


FIG. 2. C_{55} -P metabolism and membrane steps of peptidoglycan biosynthesis. C_{55} -PP is *de novo* synthesized by undecaprenyl-diphosphate synthase (UppS) enzyme in the cytoplasm, from which it partitions in the inner side of the plasma membrane. Subsequently, C_{55} -PP should be dephosphorylated to function as a lipid carrier in various polysaccharide biosynthetic pathways, such as the peptidoglycan synthesis. The enzymes MraY and MurG catalyze the successive transfers of the phospho-MurNAc-pentapeptide and GlcNAc motifs from the nucleotide precursors to the lipid carrier C_{55} -P, generating the lipid I and lipid II intermediates, respectively. Lipid II is then translocated by the flippase FtsW to the periplasmic side of the membrane, where polymerization reactions of peptidoglycan that are catalyzed by the penicillin-binding proteins (PBPs) occur. The lipid carrier is released in its pyrophosphate form (C_{55} -PP), which should be dephosphorylated and shuttled back to the inner face of the membrane to be reused. C_{55} -P metabolism is the target of bacitracin that sequesters C_{55} -PP, thereby inhibiting its dephosphorylation, and of colicin M which cleaves lipid II in dead-end products, C_{55} -OH and 1-pyrophospho-MurNAc(-peptide)-GlcNAc.

independent, with the subsequent polymerization step being considered the driving force for unidirectional translocation; however, the mechanism *per se* is absolutely not established and highlights will now arise from structural studies of these polyprenol-based flippases. Whether the flippase binds the polyprenol chain is not clear. It is possible that the C_{55} aliphatic chain remains largely embedded in the phospholipid bilayer, providing a part of the driving force by local destabilization in order to elicit the transmembrane movement. Biophysical studies led to the hypothesis that alcohol derivatives (polyprenol) may be oriented parallel to the membrane plan near the center of the bilayer, whereas polyprenyl-phosphate and other derivatives should be oriented perpendicular to the membrane plan with the charged head groups protruding at the hydrophilic surface.^{125,126} It is then possible that on binding of the diphosphate-sugar moiety by the flippase, reorientation of the aliphatic chain parallel to the membrane plan is induced, thereby favoring translocation, while the head group would diffuse alongside a hydrophilic flippase groove. Once translocated, the membrane-bound intermediate will act as an activated sugar donor, playing the same role as the typical nucleotide-diphosphate donor in the cytoplasmic space. The sugar is finally transferred from this membrane-bound donor to a specific acceptor molecule such as the peptidoglycan polymer growing chains, the lipid A-core for O-antigen LPS synthesis, or other nascent polymers.

As a by-product of the final extracytoplasmic transfer reaction, the lipid carrier is released in a diphosphate form (Fig. 2), except when the membrane-bound intermediate possesses a monophosphate linkage, in which case the lipid carrier is directly released in its active form. The released lipid will then be recycled, providing what is considered an important supply of the lipid carrier.⁹² It should be dephosphorylated and flipped back to the cytoplasmic side of the membrane to initiate another cycle of glycan translocation, a sequence of events that will be considered further next. The mechanism by which the flipping of the free-lipid carrier occurs is unknown; nevertheless, it was shown that the rate of polyprenyl-phosphate translocation across a protein-free phospholipid bilayer was too slow to satisfy the high rate of polysaccharide synthesis.^{71,72} About 5,000 lipid II molecules should be flipped per second to match the high rate at which the peptidoglycan is polymerized in *Escherichia coli* growing cells, thus implying a high rate of lipid recycling.^{73,113} It was then assumed that polyprenyl-phosphate flipping occurs via a protein-assisted mechanism, even though no specific flippase has yet been identified. It was recently hypothesized that the glycan-derivative flippase itself may carry out the flip of the polyprenyl-phosphate back to the inner side of the membrane once the sugar moiety has been transferred to the final extracytoplasmic acceptor molecule.⁹⁵ This hypothesis is based on the identification of a dolichyl-phosphate-mannosyl flippase activity in endoplasmic reticulum membranes that was found to be

inhibited by dolichyl-phosphate. These data provided evidence that the flippase binds to the isoprenoid chain itself, at least in part, raising the possibility that recycling of the free lipid may also be facilitated by the same flippase in a bi-directional process. In the proposed model, the flippase would display two binding sites located on opposite sides of the membrane and the glycan-charged polyprenol and free isoprenoid would be flipped in opposite directions. We can even hypothesize that the polyprenyl-phosphate would not be released after its translocation, thereby enabling a new sugar unit to be directly charged on the flippase-bound lipid carrier to initiate another cycle of transport. This model implies the existence of multienzyme complexes involving glycosyltransferases on both sides of the membrane, with the polyprenyl-diphosphate phosphatase and the flippase acting in a concerted manner. Biosynthetic complexes have been underlined for the biogenesis of specific polymers, as, for instance, in the peptidoglycan biosynthesis pathway,²² thus supporting such a model. This model of lipid carrier regeneration is highly speculative and other possible scenarios exist, which will be considered further next, but it offers a framework for future studies.

Synthesis of Bacterial Undecaprenyl-Diphosphate

The *de novo* synthesis of bacterial undecaprenyl-phosphate (C₅₅-P) proceeds in two sequential steps: (1) the biosynthesis of its precursor undecaprenyl-diphosphate (C₅₅-PP) via eight consecutive condensation reactions of the building block, isopentenyl-diphosphate (C₅-PP), onto farnesyl-diphosphate (C₁₅-PP) and (2) the dephosphorylation of the latter precursor, yielding the monophosphate “active” form. All isoprenoids (linear isoprenoids, carotenoids, retinoids, ubiquinones, etc.) are synthesized by sequential head-to-tail 1'-4 condensations of the five-carbon building block, the homoallylic substrate, to an allylic diphosphate substrate of varying carbon chain length.¹⁶ These reactions are catalyzed by prenyltransferases, which belong to two families according to the stereochemical outcome, *cis* (Z-prenyltransferases) or *trans* (E-prenyltransferases), of the double bond in the newly added isoprene.^{80,116} Each prenyltransferase is specific to the allylic substrate it elongates as well as to the length of the final product (*i.e.*, the number of consecutive condensation reactions). *All-trans*-farnesyl-diphosphate (C₁₅-PP) results from the condensation of one isoprene unit on dimethylallyl-diphosphate, yielding geranyl-diphosphate (C₁₀-PP), followed by a second condensation reaction catalyzed by the farnesyl-diphosphate synthase, which is the prototype of the *trans*-prenyltransferase family.^{64,79} The synthesis of C₁₅-PP is not specific to the metabolism of C₅₅-P, as this compound is also used as a precursor for the formation of a great variety of isoprenoids.

The *cis*-prenyltransferase undecaprenyl-diphosphate synthase (UppS) catalyzes the elongation of C₁₅-PP with eight isoprene units, yielding di-*trans*, octa-*cis*-undecaprenyl-diphosphate.¹⁰⁴ The UppS-encoding gene was identified in 1998 in *Micrococcus luteus*, thereby identifying the first *cis*-prenyltransferase enzyme.⁹⁸ This was achieved by screening *E. coli* clones transformed by a genomic DNA library of *M. luteus* for increased expression of prenyltransferase activity. UppS enzymes from other bacteria were then identified by sequence homology, and this gene was shown to be essential

in *E. coli* and *Streptococcus pneumoniae*.^{2,55} UppS is a soluble cytoplasmic protein that synthesizes a membrane-embedded lipid. The dissociation rate of C₅₅-PP from UppS was shown *in vitro* to be much faster in the presence of detergent Triton X-100,⁶⁸ suggesting that *in vivo* the enzyme should be in close proximity to the cytosolic face of the membrane to enable the rapid release of the product and its concomitant partition in the phospholipid bilayer. UppS activity was also shown to be dependent on the presence of the divalent cation Mg²⁺ for C₅-PP binding and for catalysis, but not for initial C₁₅-PP binding.^{13,83} Several UppS three-dimensional (3D) structures, with^{13,14,37} or without^{29,59} bound substrates, were solved, which along with intensive mutagenesis^{30,31,58} and kinetic studies^{68,103} provided great knowledge on the functioning of this biosynthetic step.

The condensation reaction catalyzed by prenyltransferases occurs via a nucleophilic attack of the C1-atom from the allylic substrate bearing the leaving phosphate group by the C4-atom of C₅-PP (Fig. 3).⁷⁹ The attack is followed by the stereospecific removal of a proton from the C₅-PP C2-atom to generate the new double bond. This can take place through a concerted or a sequential mechanism, depending on whether the release of the pyrophosphate group and the nucleophilic attack happen simultaneously or not, with a carbocation intermediate being formed only in the sequential mechanism. Data from Liang and collaborators strongly suggested that UppS may display a concerted mechanism; these authors, indeed, showed that it was impossible to trap a carbocation intermediate during UppS catalysis by using an electron-withdrawing approach to slow down the reaction.^{67,68} In contrast, *trans*-prenyltransferases such as the farnesyl-diphosphate synthase obviously catalyze the condensation reaction through a sequential mechanism and formation of an isolable allylic carbocation intermediate species. In the latter process, the allylic substrate first undergoes an ionization, leading to the release of the pyrophosphate group, which is followed by the condensation event and finally, by proton elimination (Fig. 3).

UppS forms a homodimer in which the two 29-kDa subunits are tightly associated via a coiled-coil structure between two long α -helices ($\alpha 5$) arising from each monomer and the edges of a central β -strand ($\beta 6$) and small α -helices ($\alpha 6$) (Fig. 4A).²⁹ The overall structure shows a central β -sheet core surrounded by five out of seven α -helices. Most of the conserved residues among UppS enzymes are located within two long α -helices ($\alpha 2$ and $\alpha 3$) and four β -strands ($\beta 1$ – $\beta 4$), all together delineating a large 30Å-deep hydrophobic cleft that was shown to accommodate the elongating isoprenoid carbon tail (Fig. 4B).¹³ The entrance of the active-site tunnel, in which the binding of negatively charged pyrophosphate groups from the substrates and the catalysis occur, displays several positively charged residues along with an acidic aspartate residue (Asp26) that was shown to be critical for catalysis, and an important flexible loop connecting $\alpha 3$ to $\beta 2$, the so-called entrance loop.

UppS structures revealed conformational changes occurring on C₁₅-PP binding,¹³ providing better insights into the molecular mechanism of the enzyme. These changes occurred on the tunnel $\alpha 3$ helix that was straight in the apo-enzyme (the open form), and appeared kinked in the C₁₅-PP-bound structure (the closed form) with tighter space

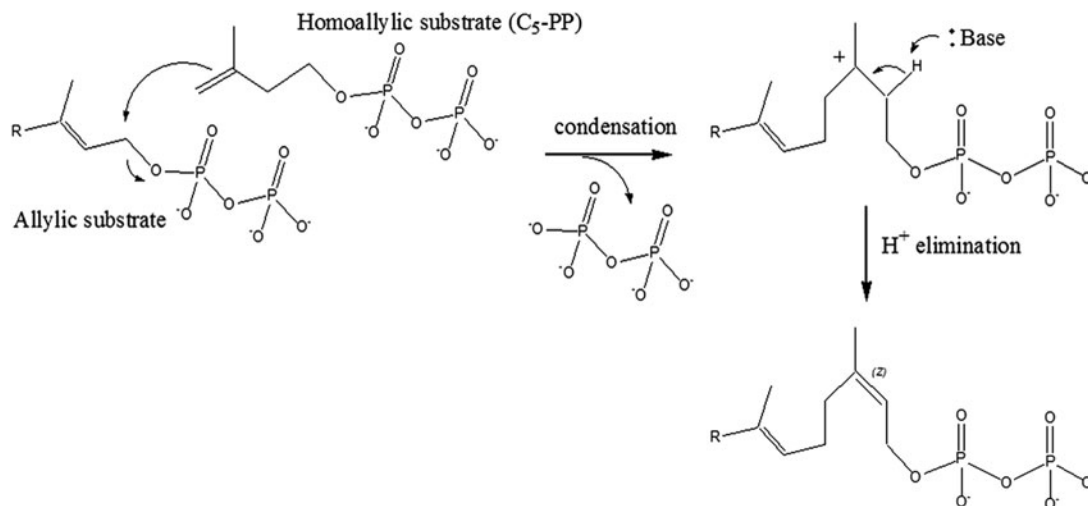
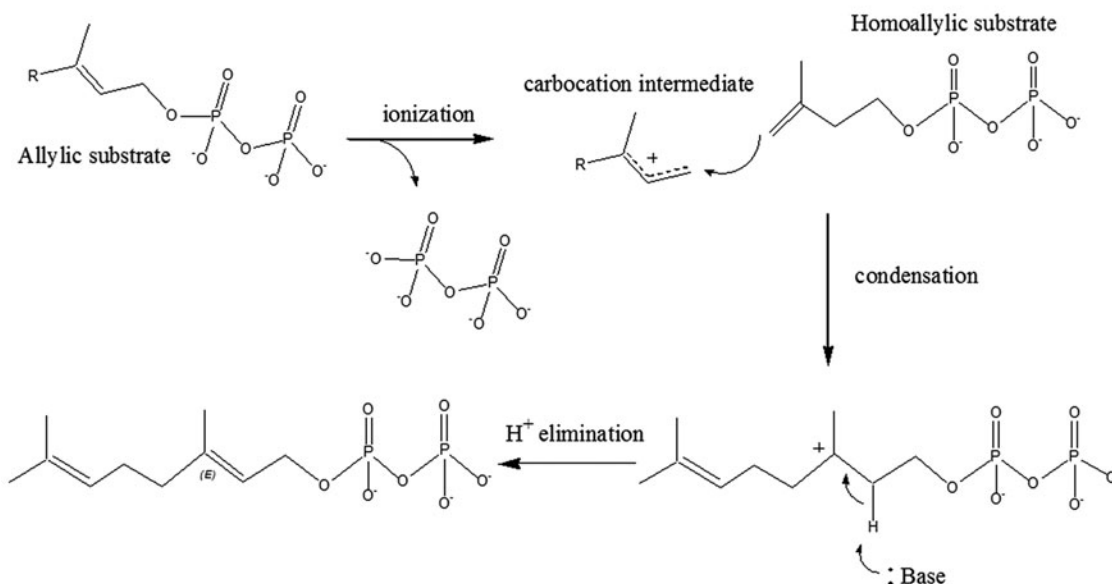
A Concerted mechanism (*cis*-prenyltransferases)**B Sequential mechanism (*trans*-prenyltransferases)**

FIG. 3. Mechanisms of C₅-PP condensation on an allylic substrate by *cis*-prenyltransferases (A) and *trans*-prenyltransferases (B).

in the tunnel for better binding of the allylic substrate. The closed conformation likely ensures the correct positioning of the allylic substrate, enabling its nucleophilic attack by C₅-PP later on. In addition, in contrast to the apo form in which the structure of the entrance loop could not be determined, likely due to its high degree of flexibility, the electron density of the loop was clearly visualized in the C₁₅-PP-bound structure. The structure of a ternary complex composed of UppS, C₅-PP, and a C₁₅-PP analog (C₁₅-sPP, in which the oxygen atom connecting the farnesyl and pyrophosphate groups was replaced by a sulfur atom to compromise catalysis) has revealed the central role played by the Asp26 residue and the metal ion in the catalytic process (Fig. 4C).³⁷ In the latter complex, the Mg²⁺ was not bound to C₅-PP to which it is primarily bound (in fact, UppS binds C₅-PP in complex with Mg²⁺), but was, instead, coordi-

nated by the C₁₅-sPP pyrophosphate group and Asp26 side chain. In contrast, in an inactive UppS mutant in which the aspartate residue was replaced by an alanine, the Mg²⁺ ion remained associated to C₅-PP and no Mg²⁺ was associated to C₁₅-sPP. It was then hypothesized that the Asp26 residue plays a key role in catalysis by controlling the migration of the metal ion from C₅-PP to C₁₅-PP, thereby facilitating the allylic pyrophosphate group dissociation which was concomitant to the nucleophilic attack. This structure-based hypothesis also strongly supported the concerted mechanism that had already been suggested by chemical and biochemical approaches.

It was hypothesized that once it has reached its final length after the successive condensation reactions, the lipid product pushes away the entrance loop, a movement which then drives the relaxation of the binding site tunnel to adopt

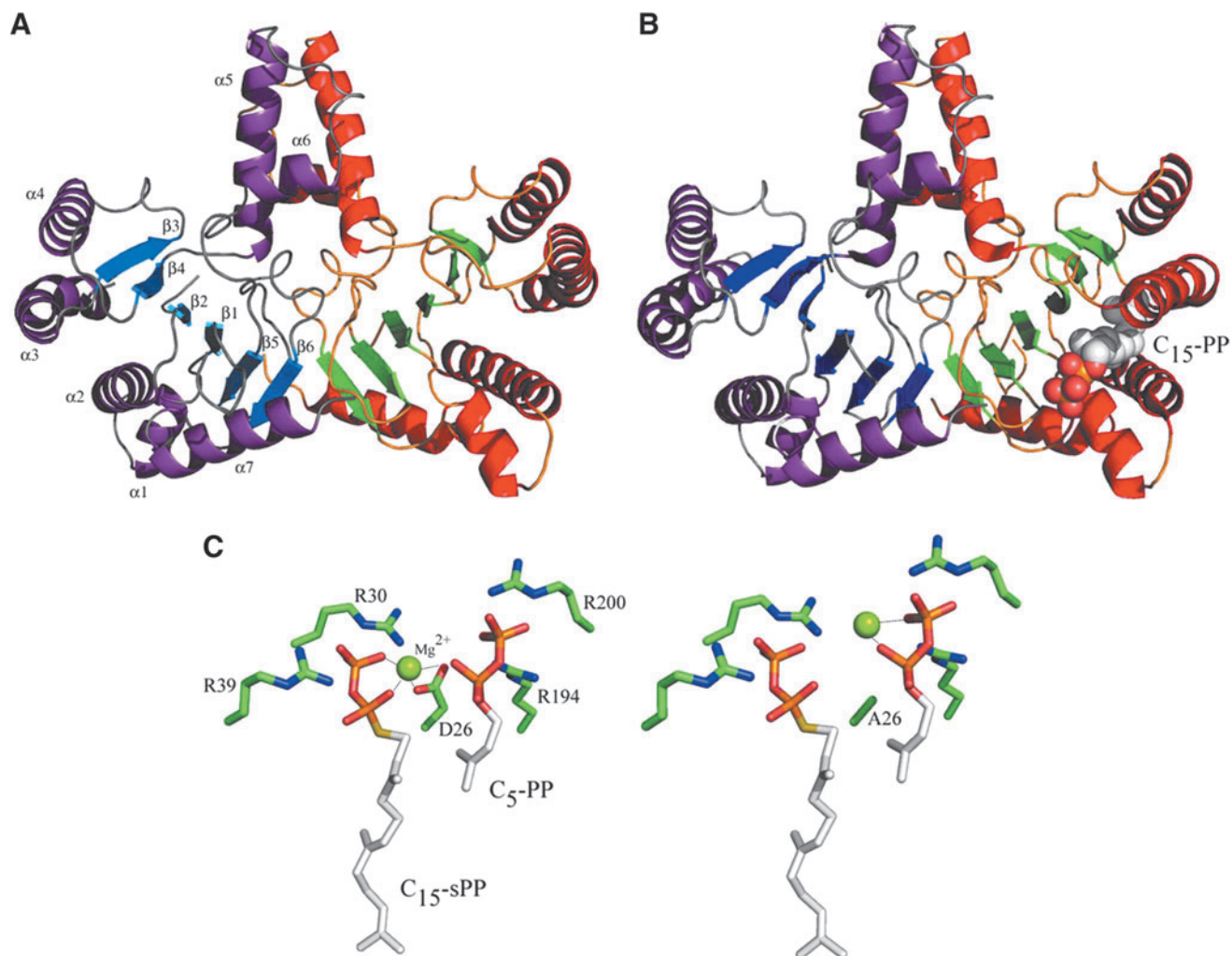


FIG. 4. Structures of UppS enzyme from *Escherichia coli*. Overall structure of UppS (1UEH atomic coordinate): one dimer shown with purple α -helices and blue β -strands and the other monomer shown with red α -helices and green β -strands (A); structure of UppS in complex with C_{15} -PP homoallylic substrate (represented with spheres) (1V7U atomic coordinate), highlighting conformational changes occurring in the $\alpha 3$ -helix on substrate binding (B). Details of the ternary complex of UppS with Mg^{2+} , C_{15} -sPP, and C_5 -PP of wild-type enzyme (left) and a D26A mutant (right) (1X06, 1X07, 1X08, and 1X09 atomic coordinates) emphasizing the condensation mechanism and the role played by the aspartate 26 residue in this process (C).

its open conformation, thus releasing C_{55} -PP and enabling a new cycle of synthesis.⁵⁹ The length of the final isoprenoid tail was shown to be controlled via a molecular ruler mechanism. The bottom of the tunnel is covered by bulky residues that should block a further elongation reaction via steric hindrance. The leucine residue Leu137 in *E. coli* UppS appeared particularly critical in such a process, as its replacement by an alanine residue resulted in the synthesis of a longer-chain product.⁵⁹

As an essential bacterial enzyme, UppS represents a potential target for the search of new antibacterial agents. Several reports have already described the identification of UppS inhibitors by using either a rational design or high-throughput screening, validating the use of this enzyme as a drug target. For instance, various bisphosphonate compounds mimicking diphosphate substrates have been found to inhibit UppS via a competition with C_{15} -PP for enzyme binding.^{36,127} Three dimensional structures of UppS-inhibitor

complexes showed that several of these compounds occupied the enzyme active-site tunnel. High-throughput screening also provided several leads that were found to inhibit the growth of *S. pneumoniae* with satisfactory minimum inhibitory concentrations as a result of UppS activity inhibition.^{21,61,85}

Dephosphorylation of Undecaprenyl-Diphosphate

The dephosphorylation of C_{55} -PP constitutes the ultimate step for the formation of the lipid carrier. This essential reaction is required not only for *de novo* synthesis but also for the recycling of the lipid carrier when it is released in a diphosphate form after each round of polymerization of a cell wall component (Fig. 2). For several decades, the C_{55} -PP dephosphorylation step was known to be the target of bacitracin, an antibiotic composed of a mixture of small cyclic peptides produced by *Bacillus licheniformis*.⁹⁹ The

most potent of these compounds, bacitracin A, tightly binds to C₅₅-PP. Though the sequestration of the lipid carrier precursor, bacitracin blocks the formation of C₅₅-P, thereby interrupting the synthesis of peptidoglycan and provoking cell death. In 1993, Cain *et al.* reported the identification of a gene from *E. coli* whose overexpression conferred increased bacitracin resistance; for this reason, the gene was named *bacA*.⁹ Due to its high degree of hydrophobicity, the 30-kDa BacA protein was predicted to be an integral membrane protein, which was further confirmed when BacA was purified from membrane extracts.²⁵ Using appropriate enzymatic assays, it was then unambiguously established that BacA catalyzed the dephosphorylation of C₅₅-PP to C₅₅-P. This was the first time that such an activity was identified to date. The resistance toward bacitracin observed on *bacA* overexpression then likely arose from a competition between the enzyme and the antibiotic for C₅₅-PP. Homologues of the *bacA* gene were then found in all sequenced bacterial genomes, except in the small-sized genome of *Helicobacter pylori*, and they were rarely found in eukaryotes. Surprisingly, although C₅₅-PP dephosphorylation constitutes an essential step for the biogenesis of the lipid carrier, the inactivation of the chromosomal *bacA* gene could be readily obtained in several bacteria such as *Staphylococcus aureus*, *S. pneumoniae*,¹² *Mycobacterium smegmatis*,⁹⁰ and *E. coli*,²⁵ without loss of cell viability. However, inactivation of this gene increased the susceptibility to bacitracin of all these species, and it was also found to attenuate the virulence of *S. aureus* and *S. pneumoniae* in the mouse model of infection and to reduce the ability of *M. smegmatis* to form biofilms. The way in which *bacA* is involved in these processes was not established. A common pool of C₅₅-P likely participates in the synthesis of various cell wall carbohydrates other than the essential peptidoglycan, such as the teichoic acids and the LPS O-antigen in Gram-positive and Gram-negative bacteria, respectively. Although these structures are not essential for growth, they are known to modulate the virulence of pathogenic bacteria by increasing their host colonization capacities or resistance toward host defenses.^{8,19} Given that BacA contributes to the formation of the lipid carrier, the availability of C₅₅-P pool might be altered in a *bacA* null mutant, thus likely compromising the synthesis of nonessential cell wall components; however, this assumption needs further investigations to be validated. To explain the nonessentiality of *bacA*, the C₅₅-PP phosphatase activity was measured in membrane extracts from an *E. coli* K12 *bacA* null mutant, revealing a residual activity of about 25% compared with that in the wild-type strain extracts.²⁵ This finding indicated that BacA accounted for 75% of the total wild-type activity and that another enzyme(s) was(were) responsible for the remaining activity. It should be noted that this residual activity was sufficient to sustain normal growth of *E. coli* in laboratory conditions.

The mechanisms by which certain bacteria are naturally resistant to bacitracin provided valuable insights into the identity of the other C₅₅-PP phosphatases. In bacitracin-resistant bacteria, such as the antibiotic producer itself (*B. licheniformis*)⁸⁶ or certain strains of *Bacillus subtilis*,⁸² and *Enterococcus faecalis*,⁶⁹ the resistance is conferred by the expression of three genes, namely *bcrA*, *bcrB*, and *bcrC*, which could be organized in operon (*B. licheniformis* and *E. faecalis*) or not (*B. subtilis*). In *B. subtilis* and *E. faecalis*,

the expression of these genes is induced by a transcriptional regulator that senses the presence of bacitracin in the milieu.^{69,81} Together, *bcrA* and *bcrB* encode an ABC transporter that pumps out bacitracin and *bcrC* was initially thought to encode a subunit of the bacitracin efflux pump itself. However, *bcrC* was found to confer bacitracin resistance in a way that was not dependent on the pump activity,⁵ raising the question of the BcrC mode of action. In *E. faecalis*, *bcrC* codes for a homologue of BacA, revealing that the expression of a C₅₅-PP phosphatase is specifically used as a means to resist bacitracin. In contrast, BcrC from *B. licheniformis* and *B. subtilis* was not related to the BacA family, but to another large superfamily of phosphatases named PAP2 (phosphatidic acid phosphatases of type 2).^{78,100} In 1999, Harel and collaborators revealed that *E. coli* cells' resistance to bacitracin was modulated by the expression of the YbjG protein,³⁸ which is a homologue of the BcrC protein from *Bacillus* species. The disruption of the chromosomal *ybjG* gene resulted in bacitracin susceptibility, whereas its overexpression conferred antibiotic resistance. Altogether, these findings suggested that these PAP2 proteins might display C₅₅-PP phosphatase activity, similar to BacA. The BcrC membrane protein from *B. subtilis* was purified and its C₅₅-PP phosphatase activity was clearly demonstrated.⁴ PAP2 enzymes from *Bacillus* species are likely devoted to bacitracin resistance via the depletion of the C₅₅-PP pool under the pressure of the antibiotic. Whether other PAP2 proteins encoded by housekeeping genes were also involved in the basic formation of C₅₅-P required further investigations.

A search for PAP2 encoding genes in *E. coli* underlined the existence of two proteins in addition to YbjG: the phosphatidylglycerol-phosphate phosphatase PgpB and a protein of unknown function, YeiU.²⁶ All three proteins were predicted to be integral membrane proteins with four to six transmembrane segments. The three corresponding chromosomal genes could be disrupted individually without an effect on cell viability and growth.²⁶ In contrast, the co-inactivation of *ybjG* and *pgpB* along with *bacA* was lethal, as demonstrated by the construction of a conditional triple mutant ($\Delta ybjG$, $\Delta pgpB$, and $\Delta bacA$) expressing a copy of *bacA* gene on a temperature-sensitive plasmid. After a shift at the restrictive temperature, the latter mutant was shown to accumulate soluble peptidoglycan nucleotide precursors (UDP-GlcNAc and UDP-MurNAc-pentapeptide) and then to lyse as a result of the arrest of peptidoglycan synthesis. This lethal phenotype was endorsed to a depletion of the C₅₅-P pool due to the absence of the three phosphatases. The data indicated that BacA and the PAP2 enzymes, except YeiU, were able to complement each other in supplying C₅₅-P for normal cell growth, raising the question of the relevance of such a redundancy. Noteworthy, Waechter, and collaborators identified a gene from *Saccharomyces cerevisiae*, called *CWH8*, which encodes an endoplasmic reticulum transmembrane protein exhibiting a dolichyl-diphosphate phosphatase activity that yields the active form of the eukaryotic lipid carrier, dolichyl-phosphate.²⁷ Mutations in *CWH8* resulted in the accumulation of dolichyl-diphosphate and defects in protein glycosylation. These authors further identified a homologue of the yeast *CWH8* gene in mammalian cells that was able to complement the *CWH8* mutation.⁹¹ Therefore, the two eukaryotic proteins

CWH8 and DOLPP1 belonging to the PAP2 superfamily likely carried out a similar function as the prokaryotic BacA, YbjG, and PgpB proteins in carrier lipid (re)generation.

The PAP2 phosphatases are characterized by a signature sequence displaying three distinct motifs designed C1, C2, and C3: K(X₆)RP-(X₁₂₋₅₄)-PSGH-(X₃₁₋₅₄)-SR(X₅)H(X₃)D, and members of this family are widespread among the three domains of life.¹⁰⁰ The homology between PAP2 members is relatively low and generally restricted to the signature sequence. The PAP2 superfamily comprises different types of enzymes: mammalian glucose-6-phosphatases, nonspecific acid phosphatases, various lipid phosphatases, vanadium peroxidases, and several uncharacterized enzymes. Interestingly, this family contains cytoplasmic soluble proteins as well as integral membrane proteins⁷⁸ such as the identified bacterial C₅₅-PP phosphatases. The 3D structure of a soluble PAP2 enzyme, the nonspecific acid phosphatase from *Shimwellia blattae* (formerly known as *Escherichia blattae*), was reported in complex with molybdate, giving insights into the architecture of the active site and the catalytic mechanism of this class of enzyme.⁴⁹ The active site of these histidine phosphatases is shaped by the three signature motifs. The catalysis occurs via a nucleophilic attack of the phosphate ester bound by a catalytic histidine residue, leading to the covalent binding of a phosphate group to a nitrogen atom of the imidazole ring.^{33,34,49} From the phosphoenzyme intermediate structure, it was deduced that this catalytic histidine residue belonged to the C3 motif. The formation of this phosphoenzyme intermediate is believed to be established via a charge-relay system involving the aspartate residue from motif C3 and the protonation of the substrate leaving group (in our case C₅₅-P) by the histidine residue from motif C2. In a second step, the phosphoenzyme intermediate should be hydrolyzed to release the enzyme and the phosphate group. By acting this time as a base, the C2-motif histidine may facilitate a nucleophilic attack of the phosphate group by water (releasing inorganic phosphate) or another acceptor molecule (in a phosphotransfer reaction). These catalytic triad residues His_{C2}-Asp_{C3}-His_{C3} are conserved in *E. coli* PAP2 proteins, whereas the other signature residues are mostly variant, suggesting that the basic catalytic mechanism is conserved in all PAP2 members, in particular in membrane PAP2 hydrolases.

The fact that the dephosphorylation of C₅₅-PP is an essential step for the (re)generation of the lipid carrier makes this reaction an attractive target for new antibacterial agents. However, now that we established that this activity can be ascribed to three distinct proteins, any one of which being sufficient to sustain normal cell growth, this implies that a potent inhibitor would have to inactivate all three proteins to display an antibacterial activity. The fact that BacA and PAP2 enzymes belong to two unrelated protein families suggests they have different structures and catalytic mechanisms, which may render the discovery of an effective inhibitor of the C₅₅-PP dephosphorylation step even trickier.

Recycling Versus De Novo Synthesis

As already mentioned, the dephosphorylation of C₅₅-PP is performed in the course of *de novo* synthesis after the UppS reaction, as well as for the recycling of the lipid carrier after the final glycosyltransferase reaction (Fig. 2). While *de novo*

synthesized C₅₅-PP likely partitions in the inner leaflet of the plasma membrane, the periplasmic oriented glycosyltransferases release C₅₅-PP in the outer leaflet. Given that multiple C₅₅-PP phosphatases have been identified, this raised the question of their respective sites of reaction and involvement in either *de novo* synthesis or recycling (or both). The membrane topology of *E. coli* PAP2 enzymes was addressed by the construction of hybrid proteins in which they were fused to reporter proteins being active at only one side of the membrane (β -lactamase, alkaline phosphatase, or Green Fluorescent Protein). PgpB was shown to contain six transmembrane segments and to have its active site residues facing the periplasm.¹⁰⁵ Similarly, YbjG and YeiU signature residues were found to be oriented toward the periplasmic space.¹⁰² It should be noted that the eukaryotic PAP2 homologue CWH8 was also found to be active on the extracytoplasmic side of the membrane, that is, the endoplasmic reticulum lumen, similar to the prokaryotic PAP2 enzymes.²⁷ In contrast, the membrane topology of the BacA protein, which lacks any feature of a typical phosphatase, remains to be explored. It has been speculated that, according to their topology, the PAP2 enzymes may be exclusively involved in recycling, whereas BacA would participate in *de novo* synthesis by displaying a cytoplasmic-oriented active site. Nevertheless, this simple model presents a paradox in the fact that any one of the BacA, YbjG, or PgpB proteins can sustain normal cell growth, which suggests that each of them can perform C₅₅-PP dephosphorylation in the course of *de novo* synthesis as well as recycling, as both processes are supposed to be essential. One way to explain this paradox is that C₅₅-PP and C₅₅-P could be translocated indifferently from one side of the membrane to the other in order to be dephosphorylated and glycosylated at the respective appropriate sites. Another possibility is that the three phosphatases BacA, PgpB, and YbjG are oriented likewise toward the periplasmic space, which then easily explains why they complement each other. In this case, another nonidentified cytoplasmic-oriented C₅₅-PP phosphatase should exist; if not, dephosphorylation of the *de novo* synthesized C₅₅-PP should also occur at the periplasmic side, which implies that C₅₅-PP has first to be flipped toward the periplasm to be dephosphorylated and then flipped back to the cytoplasm to be glycosylated. The fact that synthesis and dephosphorylation of C₅₅-PP would occur on opposite sides of the membrane would have the benefit to confer a driving force for unidirectional transbilayer movement of the lipid; that is, an accumulation of C₅₅-PP in the inner leaflet would favor its translocation toward the outer leaflet, with the opposite way for C₅₅-P. In any case, the identity of a C₅₅-PP and/or C₅₅-P flippase remains a central question. Not only it was hypothesized that the polyprenol-sugar flippase could play this role but it is also possible that the integral membrane C₅₅-PP phosphatases participate in this process with or without the aid of a flippase.

Links Between C₅₅-PP Metabolism and Other Cellular Functions

The *E. coli* PgpB protein was first identified in 1983 by Raetz and collaborators as an enzyme participating in the biosynthesis of phosphatidylglycerol, an essential lipid

representing about 25% of total *E. coli* cell membrane phospholipids.⁴⁷ It is synthesized by dephosphorylation of phosphatidylglycerol-phosphate, a precursor present at extremely low levels in *E. coli* cell membranes (~0.1%).¹⁵ Three enzymes called PgpA, PgpB, and PgpC that do not exhibit significant sequence similarity were shown to catalyze this dephosphorylation step.^{32,66} While single and double *pgp* mutants grew quite normally and contained wild-type levels of phosphatidylglycerol in their membranes, the co-inactivation of all three genes was found to be lethal. Given the apparent role of PgpB in two distinct metabolisms, the substrate specificity of the purified PgpB enzyme was assessed *in vitro*. The activity of this enzyme toward C₅₅-PP was low (~100-fold lower) compared with that measured for other substrates such as the diacylglycerol-diphosphate phospholipid or the shorter C₁₅-PP isoprenoid, raising the question of the physiological function of this protein.¹⁰⁵ Nevertheless, we showed that the dephosphorylation rate of C₅₅-PP in detergent micelles was increased by the addition of phospholipids, which was not the case for the other substrates. This suggested that the long-chain isoprenoid lipid may adopt in detergent micelles a conformation that prevents maximal binding and/or hydrolysis by PgpB. Therefore, the apparent kinetic constants might be largely underestimated relative to physiological constants. Moreover, the fact that chromosomally expressed PgpB was able to sustain normal cell growth when the other enzymes involved in either C₅₅-PP dephosphorylation²⁶ or phosphatidylglycerol⁶⁶ synthesis were absent clearly indicated that PgpB displays a dual physiological function and that this promiscuous protein establishes a link between two distinct essential pathways.

Among *E. coli* PAP2 enzymes, YeiU was initially considered as being unable to catalyze the formation of C₅₅-P *in vivo*, as the expression of its gene from either the chromosome or a high-copy number plasmid did not restore the viability of the conditional triple mutant.²⁶ In fact, not only *E. coli* YeiU but also its homologues from *Salmonella typhimurium* and *Salmonella enterica* were found to catalyze the specific transfer of the distal phosphate group from C₅₅-PP to the lipid A moiety of LPS, yielding C₅₅-P and lipid A 1-diphosphate (Fig. 5).^{54,106,111} The identification of C₅₅-PP as the physiological phosphate donor was clearly established by demonstrating that the antibiotic bacitracin, which specifically binds C₅₅-PP, inhibited the formation of lipid A 1-diphosphate *in vivo*.¹⁰⁶ Thus, YeiU also displayed a C₅₅-PP phosphatase activity that was coupled to a phosphotransfer reaction, creating an unexpected link between C₅₅-P metabolism and the lipid A remodeling pathway. YeiU was renamed LpxT according to the nomenclature of proteins involved in lipid A synthesis/modification processes. Interestingly, the C₅₅-PP phosphatase activity of LpxT was not dependent on the presence of lipid A when assayed *in vitro*. Thus, the reason that LpxT was unable to complement the conditional triple mutant remains to be elucidated.

Lipid A is the essential hydrophobic moiety of the LPS, constituting the outer leaflet of the outer membrane of all Gram-negative bacteria.⁸⁷ Its predominant structure consists of a hexa-acylated disaccharide of glucosamine that is phosphorylated at both the 1 and 4' positions. This basic structure undergoes multiple modifications that are regu-

lated by environmental conditions. Although not essential for growth, these modifications are critical for resistance to certain bactericidal agents and for evasion of host immune defenses.⁷⁷ In rich medium, about one third of *E. coli* lipid A is modified by the addition of another phosphate group at the 1-position, yielding a lipid A 1-diphosphate species, and this modification is totally abolished in a *lpxT* null mutant.¹⁰⁶ Given that *E. coli* cells contain about 10⁶ lipid A molecules, LpxT should, therefore, contribute notably to the pool of C₅₅-P. The physiological relevance of this lipid A modification, which increases the negative charge of the bacterial surface, is still unknown, as *lpxT* mutants from *E. coli*, *S. typhimurium*, and *S. enterica* did not display any apparent phenotype as compared with parental strains. Under certain circumstances, such as the presence of a high concentration of Fe³⁺ in the extracellular medium, the two-component system PmrA/PmrB induces the expression of ArnT and EptA, which in contrast to LpxT neutralize lipid A phosphate negative charges through the addition of positively charged amino groups.^{17,77} Indeed, ArnT¹⁰⁷ and EptA⁶⁰ specifically catalyze the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) to the monophosphate groups at the 4' and 1 positions of lipid A, respectively. These modifications confer resistance to Fe³⁺ and cationic antibacterial peptides (CAMPs) such as polymyxin B by limiting their binding to the cell surface. They were also shown to decrease the recognition of lipid A by the host immune TLR4 signaling system, thereby enabling bacteria to escape host defenses.⁸⁴ Thus, EptA and LpxT accomplish two different modifications, that is, the addition of a pEtN or a phosphate group, at the same (1-phosphate) position of the lipid A. While EptA transcription is increased under PmrA/PmrB-induction conditions, LpxT activity was recently demonstrated to be post-translationally repressed via the binding of a small membrane peptide called PmrR,⁵⁴ whose expression is induced by phosphorylated PmrA, thus favoring the addition of the pEtN group instead of a phosphate group in these conditions. It was hypothesized that this mode of regulation of LpxT by PmrR was more efficient than a transcriptional repression to enable a rapid fine-tuning of cell surface properties (charges content). Interestingly, a search for PmrA/PmrB-regulated genes revealed the *ybjG* gene, whose transcription appeared induced by phosphorylated PmrA.⁷⁰ This led to the hypothesis that YbjG expressed in these conditions may compensate for the lack of LpxT in the (re)generation of the C₅₅-P carrier lipid.¹⁷

In *Cupriavidus metallidurans* CH34, a PAP2 phosphatase-encoding gene, named *pbrB*, was detected within a gene cluster known to participate in a lead resistance mechanism.⁴⁶ This cluster, *pbrTRABCD*, was carried on a megaplasmid and consisted of two divergently transcribed operons: *pbrTR* and *pbrABCD*.⁶ The expression of the latter operon was regulated by the zinc/cadmium/lead-responsive element PbrR.⁷⁶ *PbrB* and *pbrC* formed a single gene encoding a putative membrane PAP2 enzyme that was fused to a putative signal peptidase. The precise function of the *pbrABCD* encoded proteins in the heavy metal resistance mechanism of this species was investigated.⁴⁶ It was demonstrated that lead resistance was achieved through the cooperation of a zinc/cadmium/lead-efflux pump P-type ATPase, PbrA, and a C₅₅-PP phosphatase, PbrB, the activity

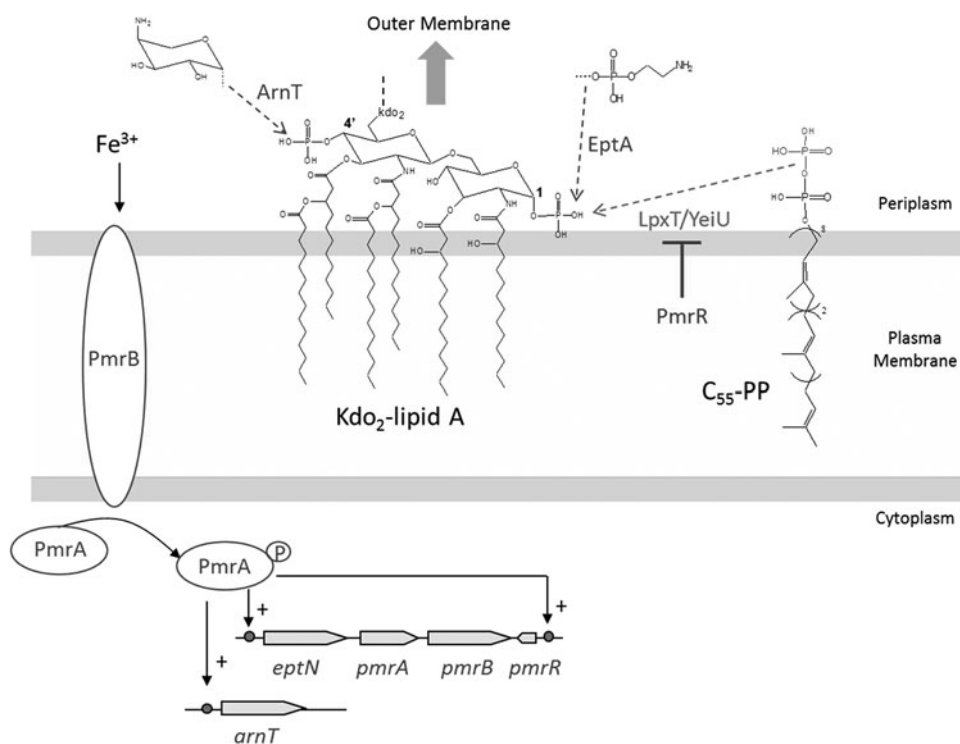


FIG. 5. Phosphorylation of lipid A by LpxT in *E. coli*, *Salmonella enterica*, and *Salmonella typhimurium*. LpxT transfers the distal phosphate group from C₅₅-PP to the 1-phosphate group of nascent lipid A at the periplasmic side of the plasma membrane, releasing lipid A-1-diphosphate and C₅₅-P. The 6' position of lipid A is linked to two Kdo (3-deoxy-D-manno-octulosonic acid) residues from the core region. Subsequently, the core-lipid A (followed or not by the outermost O-antigen depending on the bacterial species) is exported toward the outer membrane and forms the outer leaflet. In rich media, the modification, whose role is yet to be discovered, occurs in about one third of total lipid A molecules. The activation of the PmrA/PmrB two-component system, by high extracellular concentrations of Fe³⁺, induces the expression of a small-membrane peptide, PmrR, which inhibits LpxT; along with ArnT and EptA, which catalyze the modification of lipid A with 4-amino-4-deoxy-L-arabinose (to the 4'-phosphate group) and phosphoethanolamine (to the 1-phosphate group), respectively. The latter lipid A decorations confer increased resistance to Fe³⁺ and cationic antibacterial peptides (CAMPs) and attenuate the recognition of lipid A by the host immune TLR4 system.

of which was not dependent on the presence of the PbrC moiety. The PbrA protein exports the Pb²⁺ ions to the periplasm and, subsequently, inorganic phosphate groups released by PbrB-dependent dephosphorylation of C₅₅-PP precipitate lead, thus preventing its re-entry into the cell.⁴⁶ The precipitation of lead phosphate as a means of resistance to this heavy metal had been previously observed in different bacterial species^{62,74}; however, the molecular determinants had not been determined. It should be noted that the *pbrB* gene was able to complement the conditional *E. coli* mutant strain in which all identified C₅₅-PP phosphatase encoding genes (*ybjG*, *pgpB*, *bacA*, and *lpxT*) had been inactivated, and the C₅₅-PP phosphatase activity of the PbrB protein was clearly established *in vitro*.⁴⁶ Here, the metabolism of C₅₅-P is linked to a mechanism of lead resistance based on the overexpression of a specific C₅₅-PP phosphatase, which is reminiscent of the process by which certain bacteria are resistant to bacitracin. These different mechanisms rely on the existence of an available pool of C₅₅-PP that can be mobilized in specific situations. As is the case for the phosphorylation of lipid A, C₅₅-PP could be used for purposes that are beyond the basic formation of the lipid carrier. We highlighted the unexpected fact that C₅₅-PP constitutes a donor of the phosphate group in the periplasm, a compartment which does not contain nucleotide-phosphate

compounds that usually play this role in the cytoplasm. We can, therefore, envisage the existence of a periplasmic network of phosphorylations involving C₅₅-PP as a phosphate donor and diverse acceptor molecules. The different C₅₅-PP phosphatases could then be implied in specific phospho-transfer reactions, explaining their multiplicity. Periplasmic C₅₅-PP could also represent a supply of energy. We can hypothesize that the C₅₅-PP phosphatases could use the energy released through C₅₅-PP hydrolysis to catalyze, by themselves or with a dedicated flippase, the translocation of the carrier lipid back to the cytoplasmic side of the membrane. This hypothesis is supported by the fact that all the C₅₅-PP phosphatases are integral membrane proteins, which is an essential pattern for a flippase.

Formation and Role of C₅₅-P Derivatives

In eukaryotes and archaea, the *de novo* biosynthesis pathway leading to dolichyl-phosphate includes the formation of dolichol, the alcohol derivative of the lipid carrier.^{11,23} Such an alcohol intermediate is not found in the C₅₅-PP pathway in prokaryotes. However, a significant pool of undecaprenol (C₅₅-OH) has been observed in many Gram-positive bacteria,^{3,35,44,108} whose origin and function are not clearly established. In eukaryotes, dolichol is

generated by dephosphorylation of the *de novo* synthesized polyprenyl-diphosphate followed by a reduction of its α -isoprene unit. While the enzymes responsible for the removal of the pyrophosphate group of polyprenyl-diphosphate have not yet been identified, the second step was recently shown to be catalyzed by the steroid 5α -reductase type 3 (SDR5A3) in humans.¹¹ Interestingly, the dolichol derivative in mammalian cells can account for as much as 90% of the total polyprenol pool.⁵⁷ The role of this derivative is obviously to generate the lipid carrier, but it possibly plays additional cellular functions. In order to generate the active form of the lipid carrier, a dolichol kinase subsequently catalyzes its phosphorylation. The *SEC59* gene from yeast was the first gene shown to encode a CTP-dependent dolichol kinase,⁴³ and orthologues with a similar function were identified in mammals.²⁸

The scenario differs greatly in bacteria where no C₅₅-OH is generated during *de novo* synthesis of the lipid carrier, although this derivative could account for approximately 90% of the total polyprenol pool in certain bacteria^{45,108} (*S. aureus*, *E. faecalis*, *Listeria plantarum*, and *Listeria monocytogenes*). C₅₅-OH was not detectable in other bacteria such as in *E. coli*,³ leading to the hypothesis that its presence was a characteristic of Gram-positive bacteria; however, further studies are required before drawing definitive conclusions. It was speculated that C₅₅-OH could not only constitute a reserve pool of lipid carrier which can be mobilized in emergency or specific conditions but it may also play unidentified functions, as in eukaryotes. C₅₅-OH can arise from the dephosphorylation of C₅₅-PP and/or C₅₅-P, or can be a by-product of a biosynthetic pathway that is yet to be discovered. None of the bacterial C₅₅-PP phosphatases identified to date catalyzed the formation of C₅₅-OH,^{26,105} that is, exhibited C₅₅-P phosphatase activity. The potential use of this derivative as a source of lipid carrier relies on the detection of a C₅₅-OH kinase activity in bacteria that produced C₅₅-OH. In *B. subtilis* and *Streptococcus mutant*, this kinase was shown to be the product of the *dgkA* gene.^{53,65} A DgkA enzyme was first described as an ATP-dependent diacylglycerol kinase in *E. coli*.¹¹⁵ Thus, *dgkA*-encoded enzymes likely evolved toward two divergent functions in bacteria, as the *E. coli* DgkA enzyme is specific for the diacylglycerol substrate, while the DgkA enzyme from *B. subtilis* and *S. pneumoniae* are specific for C₅₅-OH. The fact that *E. coli* DgkA does not phosphorylate C₅₅-OH is consistent with the absence of this derivative in this bacterium. The DgkA enzymes constitute a family of small-membrane proteins (~15-kDa) whose catalytic mechanism has not been yet determined. They do not share any common features with water-soluble kinases or eukaryotic dolichol kinases. Most studies relevant to this family of enzymes were performed on diacylglycerol kinases and yet little is known about the specific properties of C₅₅-OH kinases.¹¹⁵ The 3D structure of the *E. coli* DgkA protein was determined by solution NMR spectroscopy¹¹⁴ and more recently, by crystallography.⁶³ To what extent the structure and the catalytic mechanism of Gram-positive DgkA enzymes differ from their *E. coli* homologue remains to be established. Interestingly, Gram-negative DgkA enzymes recycle diacylglycerol into phosphatidic acid that will re-enter the glycerophospholipid biosynthesis pathway.¹¹⁵ The dia-

cylglycerol appears at the outer leaflet of the plasma membrane after the transfer of a phosphoglycerol group from phosphatidylglycerol to decorate nascent LPS molecules or membrane-derived oligosaccharides (periplasmic MDO are produced in low osmolarity conditions). Then, diacylglycerol should be flipped toward the inner leaflet of the plasma membrane to be subsequently phosphorylated by DgkA. *E. coli* DgkA displays a homotrimeric structure with each subunit forming a bundle of three transmembrane helices preceded by an N-terminal amphiphilic surface helix protruding at the inner face of the plasma membrane. DgkA presents three active site pockets that are likely shaped by the surface helix emerging from one subunit along with the edges of the three transmembrane segments from another subunit, as suggested by 3D structures.

As already mentioned, the precise role of C₅₅-OH in Gram-positive bacteria is unknown. Although the *dgkA* gene is not essential for growth, the conversion of C₅₅-OH into C₅₅-P was shown to be determinant under certain circumstances. For instance, a *dgkA*-null mutant of the oral pathogen *S. mutans* was more susceptible toward bacitracin than the parental strain,⁶⁵ suggesting that C₅₅-OH phosphorylation constitutes a bypass of the biosynthetic or recycling pathway when the latter is inhibited. For less evident reasons, the latter mutant was also affected for growth at low pH,¹²² the biosynthesis of certain lantibiotics,¹⁸ the formation of biofilms,¹²⁴ as well as for virulence.⁹⁷ Likewise, DgkA from *B. subtilis* was shown to be required for sporulation by a mechanism that is yet to be clearly established.¹ During sporulation, the bacterium synthesizes a peptidoglycan layer called the spore cortex which contributes greatly to the stability of the spore toward a wide range of harsh conditions. In the *dgkA* mutant, the cortex was found to degenerate after a short period of time after the entry in sporulation. Therefore, the C₅₅-OH pool may play a significant role in the formation of this specific peptidoglycan layer and transition from an actively growing cell to a dormant and stress-resistant spore. Interestingly, the chromosomal *dgkA* gene from the Gram-positive genus *Clostridium* was found to be fused to another gene encoding a phosphatase belonging to the PAP2 family. This intriguing finding suggests that *clostridia* DgkA are bifunctional enzymes which could catalyze the formation of C₅₅-P by either C₅₅-OH phosphorylation or C₅₅-PP dephosphorylation.

While *E. coli* cells do not usually present any detectable amount of C₅₅-OH within their membranes, the accumulation of this compound was found to occur when these cells were subjected to a bacteriocin called colicin M (ColM)²⁴ (Fig. 2). ColM was known for several decades to target the biosynthesis of peptidoglycan and the recycling of the lipid carrier, thus causing cell lysis. The mechanism of action of ColM was deciphered in 2006, highlighting a hydrolytic activity raised against the lipid II peptidoglycan membrane intermediate and causing the release of C₅₅-OH and 1-pyrophospho-MurNAc(-peptide)-GlcNAc as dead-end products. Interestingly, the overexpression of the *cbrA* gene (formerly *gidS*), whose function is unknown, was recently demonstrated to confer increased resistance of *E. coli* toward ColM.⁴⁰ Although the mechanism of action of CbrA in this process still remains to be established, it was interesting to find out that CbrA is an FAD-bound protein belonging to

the geranylgeranyl reductase family. Members of this family are widespread in bacteria and plants, where they are involved not only in the biosynthesis of pigments, but also in archaea for the biosynthesis of archaeal membrane lipids through the FAD-dependent reduction of isoprenoid-chain double bonds.¹²¹ Thus, the *E. coli* CbrA protein may likely catalyze a similar reaction. Given that CbrA may prevent the hydrolytic activity of ColM against lipid II, it was hypothesized that CbrA should catalyze the hydrogenation of the lipid II, most likely that of the double bond which is proximal to the pyrophosphate linkage. It is also conceivable that the lipid carrier itself or its precursors may be substrates. In any case, this reaction would generate a saturated form of lipid carrier but attempts to detect such derivatives in membranes of CbrA-overproducing cells have failed, raising the question of the specific role of CbrA. Whether the action of CbrA toward lipid II or the lipid carrier is physiological or fortuitous and only due to its overproduction remains unclear. If this function was to be confirmed in future studies, this would imply the existence of saturated derivatives of the lipid carrier in bacteria, which has never been emphasized. The fact that the expression of *cbrA* is induced under stress conditions would suggest a potential implication of these derivatives in adaptation to harsh conditions.

Conclusion

The ubiquitous polyprenyl-phosphate lipids play a critical function in the formation of extracytoplasmic glycoconjugates by enabling the portage of glycosyl units across the plasma membrane. The translocation process also requires a flippase that specifically recognizes a sugar-linked lipid carrier and terminates the traffic. While the identities of these molecular determinants are now known, at least for certain pathways, the mechanism of translocation is not established. This point will now constitute a central research issue, and the role played by these very peculiar and conserved lipids will have to be decrypted. Once the sugar moiety has been transferred to its final acceptor, the released lipid carrier is shuttled back to the inner side of the membrane to be recycled. This latter translocation process is also not known and, thus, represents another important issue.

In most bacteria, C₅₅-P accomplishes this essential task for the biosynthesis of the different cell wall elements. The first step in C₅₅-P metabolism, that is, the formation of C₅₅-PP, has been particularly well deciphered in the last two decades with the characterization of the essential UppS enzyme in great detail. More recently, the second step, that is, the dephosphorylation of C₅₅-PP, has revealed interesting features, such as the existence of several enzymes, from two different families, that catalyze this single reaction in one bacterium, questioning us about the significance of this redundancy. Perhaps, a clue to this question was given by the discovery that one of these enzymes transfers a phosphate group from C₅₅-PP to a specific extracytoplasmic acceptor molecule, that is, the lipid A. This finding now opens the way to the investigation of other potential phosphorylation reactions that could be important in fine-tuning extracytoplasmic activities and explain the enzyme multiplicity. No mechanistic or structural informations are yet known about the two kinds of C₅₅-PP phosphatases. Therefore, these integral membrane proteins should now be the object of intensive investigations.

Being central to the synthesis of the bacterial cell wall and since a part of it occurs in the extracytoplasmic space, the metabolism of C₅₅-P, its biosynthesis and recycling, represents an interesting target for the development of new antibacterials. A full knowledge of this pathway will likely open the way to new specific molecular targets.

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