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Review

Current understanding of *de novo* synthesis of bacterial lipid carrier (undecaprenyl phosphate): More enzymes to be discovered

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An isoprenoid named undecaprenyl phosphate (Und-p) is the only known lipid carrier in bacteria. It is involved in the translocation of hydrophilic intermediates of cell wall components across the hydrophobic phospholipid bilayer of the cell membrane for subsequent polymerization in cell wall synthesis. Und-p is made available by both *de novo* synthesis and recycling. Evidences are emerging that in addition to dephosphorylation of undecaprenyl pyrophosphate (Und-pp), the phosphorylation of undecaprenol (Und-OH) into Und-p exists as an alternative pathway in Gram-positive bacteria but not in Gram-negative bacteria. This review gives an overview of the current knowledge in the synthesis of Und-p. It also hypothesizes the presence of yet to be identified Und-pp phosphatases at the inner cytoplasmic membrane that function in addition to a known phosphatase, named undecaprenyl pyrophosphate phosphatase (Upp-P) in *de novo* synthesis of Und-p. As the processes involved in cell wall synthesis remains the most promising antimicrobial therapeutic means, a more thorough understanding of the synthesis of the bacterial lipid carrier will not only improve the knowledge of cell wall synthesis but may also lead to the identification of potential drug targets and vaccine candidates.

Key words: Lipid carrier, undecaprenyl phosphate, undecaprenol, undecaprenol kinase, cell wall synthesis.

INTRODUCTION

Polyisoprenoids are polymers of five carbon isoprene units used as lipid carriers across the three domains of life (Jones et al., 2009). At the centre of the synthesis of bacterial cell wall is an isoprenoid lipid carrier named undecaprenyl phosphate (Und-p). It is involved in the translocation of glycan biosynthetic intermediates of carbohydrate polymers across the hydrophobic phospholipid bilayer of bacteria cell membrane to the externally located site of polymerization during cell growth, and division (Lennarz and Scher, 1972; Bouhss et al., 2008). The intermediates of peptidoglycan lipopolysaccharide, teichoic acid and enterobacterial common antigen are all translocated by the Und-p, reflecting its importance to the life of the cell. As such, the availability of Und-p has been shown to be a limiting factor and a site of control in the biosynthesis of cell wall components (Higashi et al., 1970; Anderson et al., 1972; Tatar et al., 2007; Valvano, 2008). Und-p has long been known to be derived from dephosphorylation of its precursor, undecaprenyl pyrophosphate (Und-pp) by the action of Und-pp phosphatases. These phosphatases have drawn much attention lately because of the essential role they play in the availability of the lipid carrier. The first Und-pp phosphatase gene to be characterized was identified in *Escherichia coli* and named *uppP* (formerly *bacA*) (Cain et al., 1993; El Ghachi et al., 2004). Biochemical characterization showed its importance as it accounts for about 75% of Und-pp phosphatases activity in *E. coli* while bioinformatics reveals that *uppP* is largely distributed as single copy in most bacteria

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Abbreviations: DMAPP, Dimethylallyl pyrophosphate; **FPP,** farnesyl pyrophosphate; **G3P,** glyeraldehyde -3-phosphate; **GPP,** geranyl pyrophosphate; **IPP,** isopentenyl pyrophosphate; **MEP,** 2-C-methyl-erythritol-4-phosphate; **Und-pp,** undecaprenyl pyrophosphate; **Und-p,** undecaprenyl phosphate.

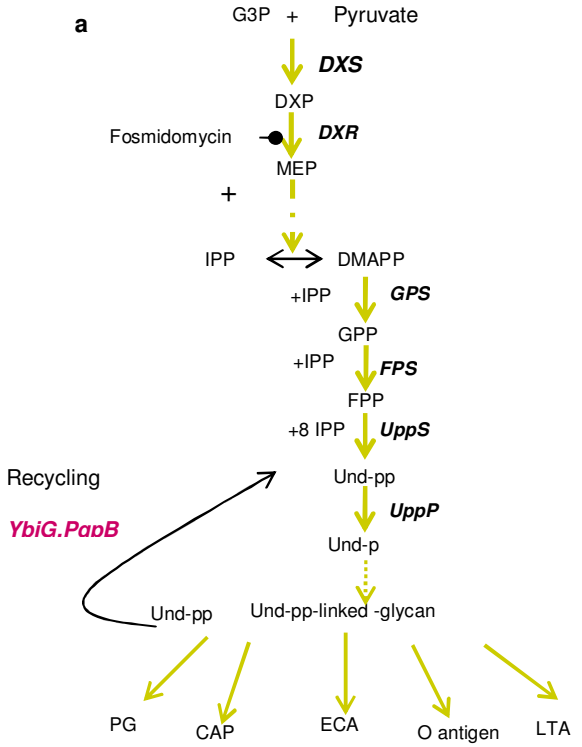

Figure 1. Synthesis of Und-p as a lipid carrier in *E. coli*. *E. coli* uses the mevalonate pathway in generating IPP, Isopentenyl pyrophosphate and DMAPP, Dimethylallyl pyrophosphate. The key enzymes involved in the pathway are in bold italics. G3P: Glyeraldehyde -3-phosphate, GPP: Geranyl pyrophosphate, FPP: Farnesyl pyrophosphate, MEP: 2-C-methyl-erythritol-4-phosphate, Und-pp: Undecaprenyl pyrophosphate, Und-p: Undecaprenyl phosphate, GPS: Geranyl pyrophosphate synthase, FPS: Farnesyl pyrophosphate synthase, DXS: deoxy-xylulose-5-phosphate synthase, DXR: deoxy-xylulose-5-phosphate reductoisomerase, UppS: Und-pp synthase, UppP: Und-pp phosphatases. PG: Peptidoglycan, CAP: Capsule, ECA: Enterobacteria common antigen, LTA: Lipoteichoic acid translocated by Und-p. Fosmidomycin is indicated to inhibit DXR. Figure adapted with modification from (Tatar et al., 2007).

(El Ghachi et al., 2004). Unsurprisingly, no copy of this gene was detected in *Mycoplasma* which lacks cell wall. Despite the essentiality of the function of this gene, many studies have shown that its deletion from the genome is not lethal in both Gram-positive and Gram-negative bacteria (Chalker et al., 2000; El Ghachi et al., 2004; Rose et al., 2004; Bernard et al., 2005).

To explain the non-essentiality of UppP, some members of type 2 phosphatidic acid phosphatase family (PAP2) (Stukey and Carman, 1997) were later demonstrated to be involved in dephosphorylating Und-pp in *E. coli*, *Bacillus subtilis* and *Cupriavidus metallidurans* (El Ghachi et al., 2005; Tatar et al., 2007; Touze et al., 2008; Hynninen et al., 2009). Since the availability of Und-p is essential for the survival of bacteria, a thorough understanding of all the enzymes involved in both its *de novo* synthesis and recycling will give a more complete overview of the bacterial cell wall biosynthesis in addition to the possible discovery of new drug targets. Moreover, elucidating the potential of additional enzymes known to possess Und-pp phosphatase activities may be beneficial in proffering solution to the challenging cases of bacterial drug resistance or unavailability of efficient vaccines.

This review emphasizes the contribution of undecaprenol kinase to the *de novo* synthesis of the essential Und-p in Gram-positive bacteria. It also hypothesizes the presence of unknown Und-pp phosphatases at the inner cytoplasmic membrane that functions together with UppP in *de novo* synthesis of Und-p in *E. coli*. Although Und-p is involved in various processes of cell envelope biosynthesis, peptidoglycan synthesis is used as a typical example of the *de novo* synthesis and recycling of Und-p in this review. Due to the implication of UppP in virulence and antibiotic resistance in some bacteria, a focus is also made on its potential as a drug target as well as in vaccine development against pathogenic bacteria including *Mycobacterium tuberculosis*.

Biosynthesis of undecaprenyl phosphate

All isoprenoids including Und-p originate from two five-carbon building blocks which are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Swiezewska and Danikiewicz, 2005; Valvano, 2008). These two molecules are products of two distinct biosynthetic pathways: the 2-C-methyl-erythritol 4-phosphate pathway (MEP) which forms IPP and DMAPP from glyceraldehyde-3-phosphate and pyruvate (Rohmer et al., 1993; Kuzuyama, 2002; Eisenreich et al., 2004) and the mevalonate pathway (MVA) which involves the formation of IPP and DMAPP from acetyl coA with mevalonate as an intermediate (Schroepfer, 1981; Hunter, 2007).

A major intermediate in the synthesis of polyisoprenoids is farnesyl pyrophosphate (FPP). It is made of fifteen carbon atoms resulting from the head to tail condensation of IPP and DMAPP by geranyl pyrophosphate phosphatase, followed by a further addition of IPP by farnesyl pyrophosphate synthase (Ogura and Koyama, 1998; Liang et al., 2002). The first committed step in the synthesis of bacterial Und-p is catalyzed by undecaprenyl pyrophosphate synthase (UppS), an essential prenytransferase that catalyses the addition of eight C5 IPP units in cis configuration unto all *trans* FPP to produce Und-pp (Shimizu et al., 1998; Liang et al., 2002). In order to generate the functional Und-p, Und-pp is dephosphorylated by Und-pp phosphatases (Figure 1).

The process of identifying the gene(s) encoding the essential Und-pp phosphatase activity started with the finding that the overexpression of two genes namely *bcrC* and *bacA* (now referred to as *uppP*) in *E. coli* resulted in bacitracin resistance (Cain et al., 1993). Cells overexpressing *uppP* were able to resist up to 200 µg ml^{-1} bacitracin that completely lysed control cells ((El Ghachi et al., 2004). Bacitracin is a mixture of related polycyclic peptides (Johnson et al., 1945) that binds tightly to the pyrophosphate motif of Und-pp in the presence of divalent cations thereby preventing its access to phosphatases. In the presence of bacitracin, the supply of Und-p is affected, consequently leading to lysis of the cell due to impaired cell wall formation (Kanof, 1970; Stone and Strominger, 1971; Storm, 1974).

280-fold increase in Und-pp phosphatase activity was observed in membrane extracts of *E. coli* overexpressing *uppP* compared with control cells, supporting its role as an Und-pp phosphatases (Cain et al., 1993; El Ghachi et al., 2004). It was thus explained that overexpression of UppP enhanced its competition with bacitracin for the pyrophosphate motif of Und-pp and accounts for the observed bacitracin resistance (El Ghachi et al., 2004). Amino acid sequence analysis shows that UppP possess a large cytoplasmic loop that is conserved in all bacteria (El Ghachi et al., 2005). Due to the cytoplasmic location of UppS (the enzyme that synthesizes Und-pp), and the conserved cytosolic loop of UppP it has been suggested to be involved in *de novo* synthesis rather than in the recycling of Und-p (El Ghachi et al., 2004; Tatar et al., 2007).

E. coli Δ*uppP* null mutant did not show any significant growth or morphological defect, neither was its sensitivity to bacitracin affected (El Ghachi et al., 2004). However, the Und-pp phosphatase activity in the mutant was reduced by 75%. These observations showed its importance, but non-essentiality as well as non exclusiveness in generating Und-p. The non-essentiality of *uppP* homologues was also observed in Gram-positive bacteria namely *Staphylococcus aureus*,

Streptococcus pneumoniae and *B. subtilis* and acid fast bacteria; *M. tuberculosis*, *M. bovis* BCG, and *M. smegmatis* (Cain et al., 1993; Chalker et al., 2000; Rose et al., 2004; Bernard et al., 2005). Computational analysis shows that paralogues of *uppP* do not exist in any of these bacteria (El Ghachi et al., 2005). It was thus later demonstrated that some integral membrane enzymes from type 2 phosphatidic acid phosphatase family (PAP2) possess Und-pp phosphatase activities sufficient to sustain the growth of bacterial cells and are responsible for the viability of *uppP* mutants (El Ghachi et al., 2005). In *E. coli*, these include LpxT and YbjG, which had been previously uncharacterized, and PgpB, a phosphatidyl glycerol phosphate phosphatases (Tatar et al., 2007; Touze et al., 2008). PbrB in *Cupriavidus metallidurans*, and BcrC in *B. subtilis* have also been reported to possess Und-pp phosphatase activities (Bernard et al., 2005; Hynninen et al., 2009).

Recycling of undecaprenyl phosphate

De novo biosynthesis of Und-p is known to occur in small quantities and cells have been shown to rely on the recycling of previously used carrier (Higashi et al., 1967; van Heijenoort 2001). Some *E. coli* proteins have been implicated in Und-p recycling and are described later.

One of the enzymes reported to be involved in Und-p recycling is an *E. coli* enzyme named LpxT. It uses Und-pp as a phosphate donor in the formation of lipid A 1-diphosphate species releasing Und-p as a product (Stukey and Carman, 1997; Touze et al., 2008). Using a thermo-sensitive variant of MsbA, a lipid A flippase that is impaired at temperatures above 37°C (Doerrler et al., 2001) and temperature shift assay, it was reported that LpxT is functional only when MsbA is active (Touze et al., 2008). This confirms that the involvement of LpxT in the dephosphorylation of Und-pp actually takes place at the periplasm, an action that corresponds to the recycling rather than *de novo* synthesis of Und-p (Touze et al., 2008).

Another enzyme YbjG, was also shown to be involved in Und-p recycling in *E. coli*. A sandwich fusion was constructed in which PhoA, an alkaline phosphatase that folds correctly only at the reducing condition of the periplasm was inserted between Arg139 and Val140 close to the location of catalytic His145 of YbjG. With this construct, the alkaline phosphatases activity of PhoA was observed. In addition, topological model prediction of YbjG orientates its active site in the periplasmic compartment. This thus implicates YbjG in recycling rather than in de novo synthesis of Und-p (Tatar et al., 2007).

With similar experiments, the catalytic site of PgpB, an *E. coli* phosphatidyl glycerophosphate phosphatase which could also dephosphorylate Und-pp was found to be located in the periplasm (Touze et al., 2008). *pgpB* truncated at the 3' end to various lengths was fused with β-lactamase gene. Fusions with the amino acids located in the periplasm resulted in ampicillin resistance and the corresponding amino acid residues were shown to be located at the catalytic site of PgpB (Touze et al., 2008).

Also, PbrB, biochemically characterized to dephosphorylate Und-pp, was also reported to depend on PbrA, a P-1B type ATPase for its effect on lead resistance in *C. metallidurans* (Hynninen et al., 2009). PbrA acts as an efflux pump extruding divalent metal ions including lead ions from the cytosol (Borremans et al., 2001; Hynninen et al., 2009). The dependence of PbrB on PbrA suggests that

it relies on PbrA to extrude lead ions from the cytosol before it transfers phosphate group from Und-pp to the extruded lead ions thereby precipitating the ions in the phosphate form, a known mechanism of lead precipitation in bacteria (Levinson et al., 1996; Levinson and Mahler, 1998; Mire et al., 2004; Hynninen et al., 2009). It can therefore be implied that the active site of PbrB is located in the periplasm thus contributing to recycling of Und-p in this bacterium (Hynninen et al., 2009).

The various enzymes outlined above functions in recycling of Und-p in *E. coli*. However, no enzyme has been reported in its recycling in Gram-positive bacteria. Notably, BcrC of *B. subtillis* uses its Und-pp phosphatase activity to confer resistance to bacitracin (Bernard et al., 2005). The introduction of a copy of *bcrC* into a strain carrying double deletion of *bceA*, (an efflux pump) and *bcrC* (Δ*bceA*Δ*bcrC*) resulted in 8 fold increase in IC_{50} to bacitracin (0.9 to 7.4 µM) while the introduction of a copy of *bcrC* into *bcrC* single mutant (Δ*bcrC*) (with intact *bceA*) resulted into a 13 fold increase (31 to 420 µM) (Bernard et al., 2005). It therefore appears that the function of BcrC in conferring bacitracin resistance depends somewhat on the presence of BceA in this bacterium and thus may play role in Und-p recycling. Further experiments are needed verify this.

More enzymes to be discovered

In *E. coli*, the simultaneous inactivation of three genes, *uppP*, *ybjG* and *pgpB* was reportedly lethal (El Ghachi et al., 2005). It was thus suggested that the lethality of the triple mutation is an indication that UppP, PgpB and YbjG contributed to the total Und-pp phosphatase activity in E. coli (Touze et al., 2008). In further investigations, an *E. coli* mutant with conditional expression of *uppP* was constructed. In the mutant, *uppP* was supplied to double deletion mutant with genotype, *ΔuppPΔybjG* by plasmid *p*MAK705*uppP* whose expression is impaired at 42°C. A third mutation was introduced by deleting the chromosomal copy of *pgpB* resulting in a triple mutant with genotype *ΔuppPΔybjGΔpgpBpMAK705uppP.* As such, the triple mutant was viable at 30°C, but not at 42°C (El Ghachi et al., 2005). This further suggested that the three genes *uppP*, *ybjG* and *pgpB* contribute to the total Und-pp phosphatases activities in *E. coli.*

E. coli mutants with genotypes *ΔuppPΔybjGΔpgpBpMAK705uppP, ΔuppPΔybjG* and *ΔuppPΔpgpB* were reported to be viable without change in growth rate (El Ghachi et al., 2005; Touze et al., 2008). This suggests that a single chromosomal copy of *uppP*, pgpB or ybjG respectively in the mutants was enough for survival and sustenance of growth in E. coli (El Ghachi et al., 2005).

However, as described previously, the active sites of YbjG and PgpB face the periplasm and thus are implicated in recycling. The growth and survival of the *E. coli* cells in the absence of UppP (presumably functioning in *de novo* synthesis) and only a single chromosomal copy of either YbjG or PgpB implicated only in recycling requires an explanation. It may be that it is not only UppP that functions as Und-pp phosphatase in *de novo* synthesis of Und-p. This is because the cell needs the production of Und-p *de novo* before recycling takes place. Figure 2 depicts the scenario.

In addition, the cell wall of *E. coli ΔuppP* mutant appeared intact as implied from the unchanged growth rate and sensitivity to antibiotics observed (El Ghachi et al., 2005). The cell walls of *S. aureus* and *S. pneumoniae uppP* mutants also appeared to remain intact as judged by flow cytometry, microscopy, unchanged growth rate and sensitivity to osmotic stress (Chalker et al., 2000). However, while the sensitivity of *E. coli ΔuppP* mutant to bacitracin and other antibiotics remained unchanged, there were 16 and 1,000 fold increase in sensitivities of *S. aureus* and *S. pneumoniae uppP* mutants respectively to bacitracin alone but not to other antibiotics including those targeting the cell wall or cell membrane such as vancomycin and polymyxin B (Leutgeb, 1969; Chalker et al., 2000).

For the hypersensitivities to bacitracin observed with *S. aureus* and *S. pneumoniae ΔuppP* mutants, the authors explained that Und-p is produced solely by dephosphorylation of Und-pp. Therefore, treatment with bacitracin sequestered the available Und-pp, accounting for the hypersensitivity to bacitracin (Chalkern et al., 2000). If Und-p is produced solely by Und-pp phosphatase, it is expected that the deletion of *uppP* would have been lethal due to the absence of Und-p produced *de novo*, since *uppP* is present as a single copy in these bacteria. And if other phosphatases perform the function of UppP, thus rescuing the cell from its deletion and ensuring the observed intact cell wall, there should be no change in sensitivity to any antibiotic (bacitracin inclusive) in these bacteria as observed in *E. coli ΔuppP* mutants. An explanation for the hypersensitivity to only bacitracin among other antibiotics by Gram-positive *S. aureus* and *S. pneumoniae ΔuppP* mutants will fill a knowledge gap that may be existing in bacterial cell wall biosynthesis.

It is reasonable to hypothesize that there should be a bypass pathway that does not require dephosphorylation of Und-pp that functions to provide the essential lipid carrier (Und-p), in Gram-positive bacteria. The pathway may account for the intact cell walls of *S. aureus* and *S. pneumoniae ΔuppP* mutants and the observed hypersensitivity to only bacitracin among others. It can also be hypothesized that if the active site of UppP faces the cytoplasm as widely speculated, other unknown

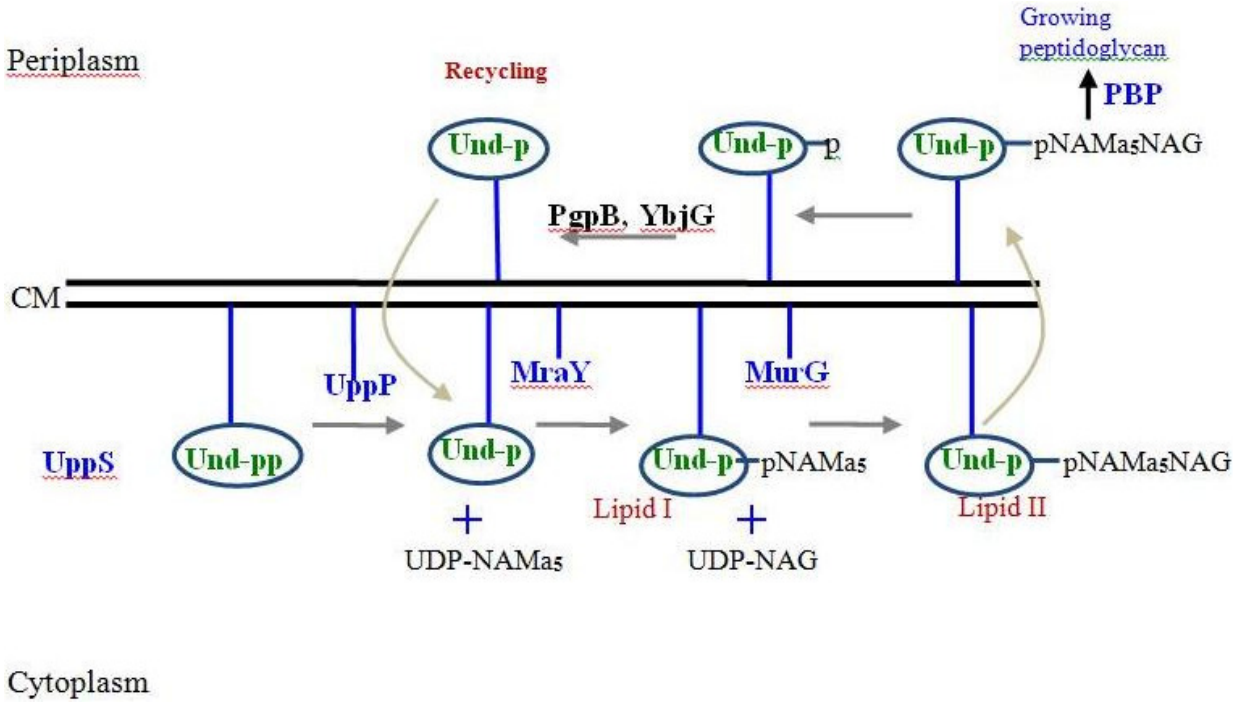

Figure 2. This diagram illustrates *de novo* synthesis and recycling of Und-p typified by peptidoglycan synthesis.

phosphatases with active sites facing the cytoplasm functions in addition to UppP in *de novo* synthesis of Und-p.

Contribution of undecaprenol kinase to the pool of the lipid carrier

The hypersensitivity of the *S. pneumoniae* and *S. aureus uppP* mutants to only bacitracin (Chalker et al., 2000) despite intact cell walls can be explained if the Und-p needed for survival is generated by other enzymes apart from UppP in these bacteria. However, in the presence of bacitracin, these other enzymes could not compete effectively with bacitracin for Und-pp to generate Und-p and thus could not confer resistance. This other enzyme is likely involved in the conversion of undecaprenol (Und-OH) to Und-p by a kinase.

The presence of abundant Und-OH had long been detected in the membranes of some Gram-positive bacteria such as in *S. aureus* and *S. pyogenes* (Strominger et al., 1970), *Enterococcus faecalis*, *Lactobacillus plantarum* (Thorne and Kodicek, 1966; Gough et al., 1970; Thorne, 1973) and *Listeria monocytogenes* (Vilim et al., 1973). Recently, the amount of Und-OH in the cell wall of *S. aureus* was estimated to be 70 $nmolg^{-1}$ of cell dry weight. It was also reported to be the predominant form of undecaprenoids in this bacterium. In contrast, Und-OH was not detectable in the membrane extracts of *E. coli* (Barreteau et al., 2009).

The conversion of Und-OH to Und-p has been reported in membrane extracts of Gram-positive bacteria: *S. aureus* and *L. plantarum* (Higashi et al., 1970; Kalin and Allen, 1979). In addition, rather than phosphorylating diacylglycerol, homologue of *E. coli* diacylglycerol kinase, DgkA of *B. subtillis* functions as undecaprenol kinase while its sequence and conserved functional residues have a high degree of similarities with DgkA of *S. aureus*, *S. mutans* and *S. pneumoniae* (Jerga et al., 2007). A higher efficiency of phosphate group transfer to Und-OH compared to diacylglcerol was also observed with DgkA homologue of *S. mutans* (Lis and Kuramitsu, 2003; Shibata et al., 2009). These suggest that DgkA of Gram-positive bacteria converts Und-OH to Und-p. Phylogenetic tree presented in Figure 3 shows a clear distinction of DgkA homologues in representative Gram-positive and Gram-negative bacteria and agrees with sequence alignment reported by Jerga et al. (2007). It may therefore be possible that DgkA homologues in Gram-positive bacteria are the Und-OH kinases contributing to *de novo* synthesis of Und-p.

Supporting the involvement of undecaprenol kinase in the provision of Und-p is the finding that *S. mutans dgkA* mutant was found to exhibit increased sensitivity to bacitracin, reduced virulence and stress resistance than the parental strain (Shibata et al., 2009). The Und-p provided by DgkA might have ensured the translocation of cell wall intermediates thereby compensating the loss of UppP in the *S. aureus* and *S. pneumoniae* Δ*uppP* mutants. However the phosphatases activity needed for the conferment of bacitracin resistance might have been lost following the deletion of *uppP* (Kanof, 1970; Stone and Strominger, 1971).

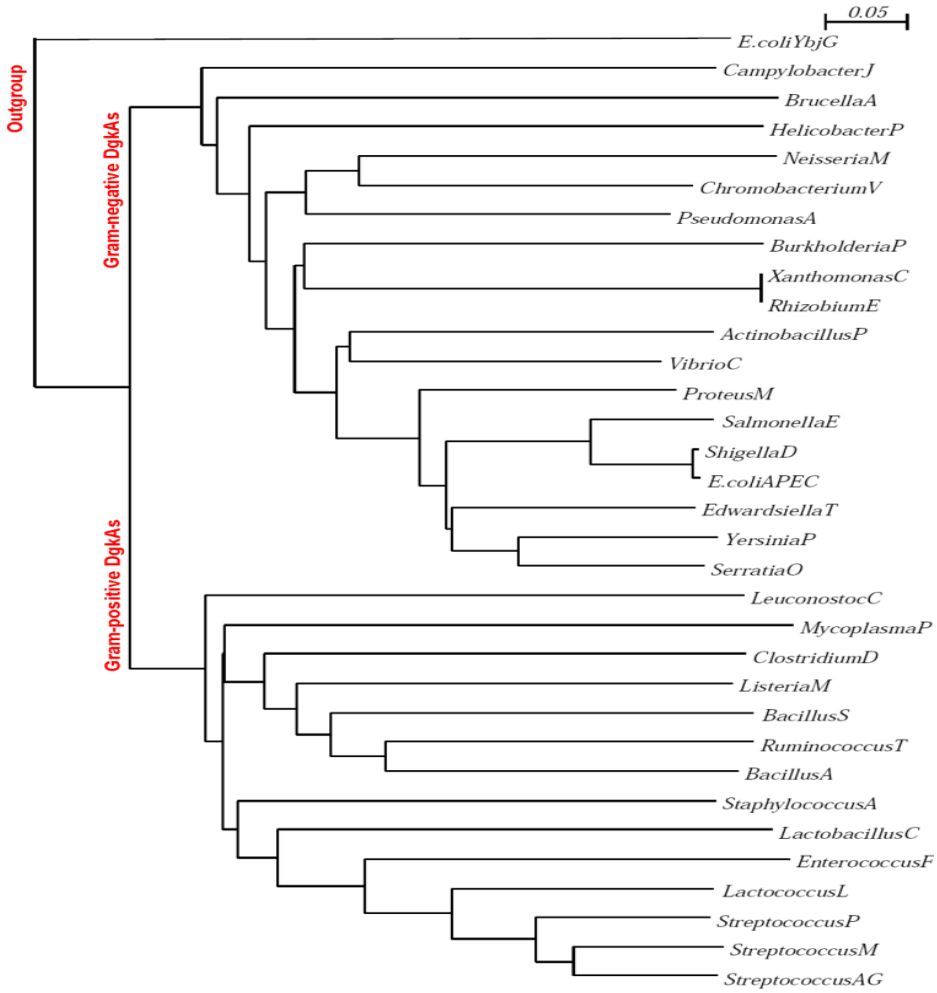

Figure 3. Phylogenetic tree (created using ClustalX 2.0.12 with default parameters) of DgkAs in Gram-positive and Gram-negative bacteria. *E.coli* YbjG (Und-pp phosphatases) is used as an outgroup. *LactococcusL* (*Lactococcus lactis*), *EnterococcusF* (*Enterococcus faecium), ClostridiumD (Clostridium difficile), StaphylococcusA (Staphylococcus aureus), BacillusA (Bacillus anthracis),StreptococcusAG (S agalactiae), ListeriaM (Listeria monocytogenes), StreptococcusP (S pneumonia), StreptococcusM (S. mutans),BacillusS (Bacillus subtillis), RuminococcusT (Ruminicoccus torques), LactobacillusC (Lactococcus casei), LeuconostocC (L. citreum), MycoplasmaP (Mycoplasma penetrans), SerratiaO (Serratia odorifera), E.coliAPEC (E.coli APEC), BrucellaA (Brucella abortus),YersiniaP (Yersinia pestis), SalmonellaE (Salmonella enterica), ShigellaD (Shigella dysenteriae), CampylobacterJ (Campylobacter jejuni), BurkholderiaP (Burkholderia pseudomallei), RhizobiumE (Rhizobium etli), HelicobacterP (Helicobacter pylori), VibrioC (Vibrio cholera), ProteusM (Proteus mirabilis),SynechococcusSP (Synechococcus sp.), EdwardsiellaT (Edwardsiella tarda), ActinobacillusP (Actinobacillus pleuropneumoniae), PseudomonasA (Pseudomonas aeruginosa), ChromobacteriumV (Chromobacterium violaceum), XanthomonasC (Xanthomonas campestris), NeisseriaM (Neisseria meningitides).* The tree reveals a clear distinction between the DgkAs of the two groups of bacteria . This supports that, a different role for DgkA in Gram-positive bacteria may not limited to *S. mutans* and *B. subtilis* in which it has been characterized

Figure 4 gives a pictorial view of possible involvement of an Und-OH (DgkA) when *uppP* is deleted in *S. aureus* and *S. pneumoniae*.

The origin of Und-OH in Gram-positive bacteria is yet unclear. The only known pathway in the generation of bacteria undecaprenoids is through the essential UppS enzyme whose product is Und-pp as shown in Figure 1. This suggests that Und-OH might have been generated from Und-pp or Und-p. Und-OH, originally absent, was detected in *E. coli* membrane extracts only after treatment

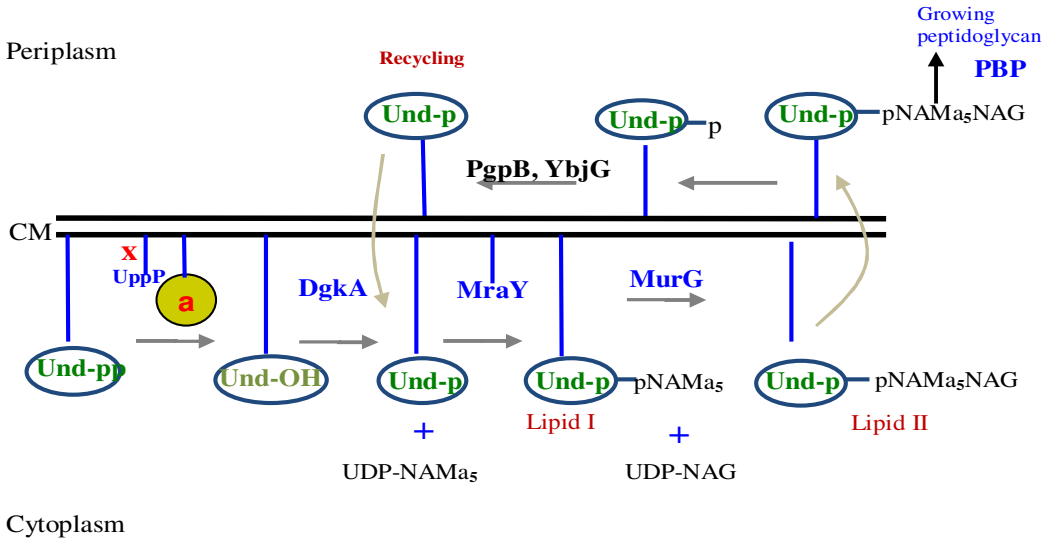

Figure 4. Scheme for a bypass pathway when *uppP* is deleted (depicted by red 'X') in *S. aureus* and *S. pneumoniae*. Contribution of undecaprenol kinase (DgkA) to *de novo* pool of Und-p in Gram-positive bacteria typified by peptidoglycan synthesis. a?: an unknown phosphatase functioning in the synthesis of Und-OH which will then be phosphorylated into functional Und-p by DgkA. CM: inner cell membrane, Und-OH: Undecaprenol , PgpB ,YbjG: recycling enzymes, UppS: Und-pp synthase ,UppP: Und-pp phosphatase in *de novo* synthesis, Und-p: undecaprenyl phosphate, Und-pp: undecaprenyl pyrophosphate, NAG: N-acetyl glucosamine, $NAMa_5$:N-acetyl muramic acid pentapeptide. MraY: phospho-N-acetylmuramoyl-pentapeptide transferase, MurG: Glycosyl transferase. Lipid I: Und-p-p- $NAMa_5$, Lipid II: Und-p-p- $NAMa_5$NAG.

with colicin M which degrades both lipid I (Und-p-$pNAMa_5$) and lipid II (Und-p-$pNAMa_5$NAG) (El Ghachi et al., 2006; Barreteau et al., 2009). This supports the notion that Und-OH is formed from either Und-pp or Und-p and that, the enzymes that catalyses this reaction may be present in Gram-positive bacteria but absent in Gram-negative bacteria. The discovery of enzymes playing this role designated at ***a*** in Figure 4 will thus be highly informative. Characterization of DgkA homologues of *S. aureus* and *S. pneumoniae* as possible Und-OH kinases and coinactivation of *dgkA* and *uppP* in Gram-positive bacteria will help in clarifying the contribution of DgkA to cell wall synthesis in these bacteria.

Unknown undecaprenyl pyrophosphate phosphatase in *de novo* synthesis of undecaprenyl phosphate in bacteria

The essentiality of undecaprenyl pyrophosphate synthase (UppS) which is committed to the synthesis of Und-pp (Keenan and Allen, 1974; Shimizu et al., 1998; Apfel et al., 1999), coupled with absence of Und-OH in *E. coli* (Barreteau et al., 2009) brings to mind that the pathway of dephosphorylating Und-pp into Und-p is important in this bacterium. Essentially, the survival of *E. coli* cells with single copy of either YbjG or PgpB (recycling) in the absence of UppP (*de novo* synthesis) suggests the presence of yet to be identified phosphatases functioning *de novo* together with PgpB or YbjG to ensure survival.

Computational analysis shows that, while UppS is present in *Treponema pallidum* and *Rickettsia sp* as in other cell wall containing bacteria, UppP which performs the immediate downstream action is absent. Since UppP is absent, the dephosphorylation of Und-pp into functional Und-p may be performed by other enzymes in *T. pallidum* and *Rickettsia sp* if the active sites of YbjG or PgpB present in these bacteria are in the periplasmic space as demonstrated in *E. coli*.

Additionally, the subinhibitory concentration of fosmidomycin (Figure 1) partially impaired the activity of deoxy-xylulose-5-phosphate reductase (an enzyme functioning upstream of Und-pp synthesis), thereby reducing the amount of Und-pp formed de novo. *E. coli mutant* with simultaneous deletion of *uppP* and *pgpB* exhibited 54% survival as compared to 73% survival observed with wild type (Tatar et al., 2007). Owing to the absence of *uppP* in the mutant, there should be another enzyme that functions in *de novo* dephosphorylation of the residual Und-pp left after fosmidomycin treatment. This other phosphatase coupled with the recycling by enzymes like YbjG and LpxT may account for such a relatively high survival of the Δ*uppP*Δ*pgpB* mutant compared to wild type.

However, the lethality of *E. coli* triple mutant (*ΔuppPΔybjGΔpgpB*) indicates that the contribution of the proposed alternative Und-pp phosphatases (still present in the triple mutant) may not be enough to sustain cell growth without efficient recycling. This needed recycling might

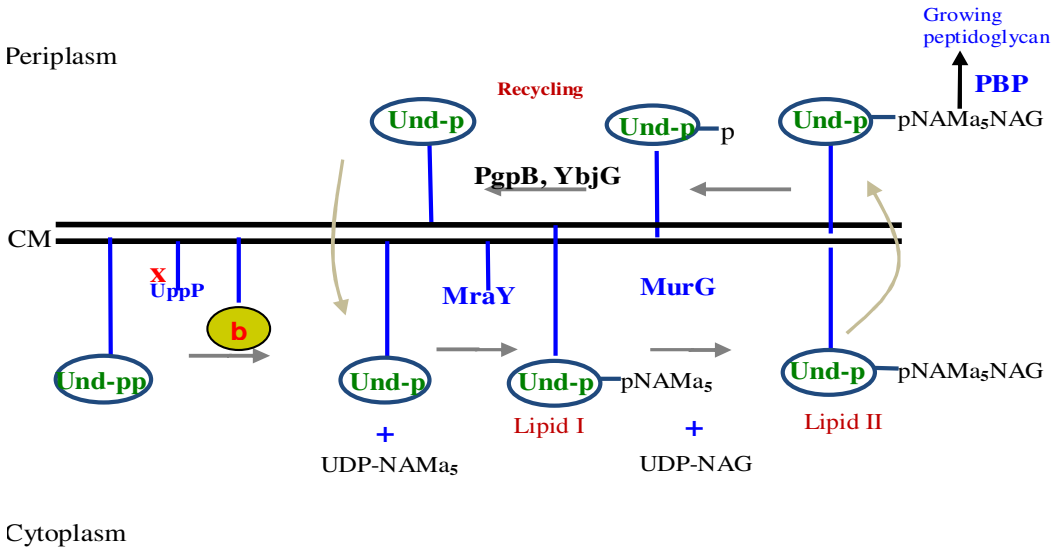

Figure 5. Probable contribution of other Und-pp phosphatases to the *de novo* pool of Und-p in *E. coli* when uppP is deleted (depicted by red'X') typified by peptidoglycan synthesis. **b**: Unknown Und-pp phosphatases in the generation of Und-p. CM: inner cell membrane, Und-OH: Undecaprenol , PgpB ,YbjG: recycling enzymes, UppS: Und-pp synthase, UppP: Und-pp phosphatase in *de novo* synthesis, Und-p: undecaprenyl phosphate, Und-pp: undecaprenyl pyrophosphate, NAG: N-acetyl glucosamine, $NAMa_5$:N-acetyl muramic acid pentapeptide. MraY: phospho-N-acetylmuramoyl-pentapeptide transferase, MurG: Glycosyl transferase. Lipid I: Und-p-p- $NAMa_5$, Lipid II: Und-p-p- $NAMa_5$,NAG.

have been provided by the PgpB or YbjG in the viable *E. coli ΔuppPΔybjG, ΔuppPΔpgpB* mutants respectively. It is therefore proposed that, in the absence of UppP, the unknown Und-pp phosphatases dephosphorylate the Und-pp produced by UppS *de novo* to provide the needed Und-p. The yet to be identified Und-pp phosphatases may account for the intact cell wall of *E. coli ΔuppP* mutants.

Figure 5 depicts the probable involvement of the unknown phosphatases in *de novo* synthesis of Und-p in the absence of UppP in *E.coli*.

Possible involvement of UppP in vaccine or drug target screening

In addition to its clear role in cell wall synthesis, homologues of UppP in different bacteria have been implicated in other functions such as virulence, antibiotic resistance and biofilm formation (Chalker et al., 2000; Rose et al., 2004; Touze et al., 2008; Hynninen et al., 2009; Vandal et al., 2009). As such its involvement in the development of vaccine and in drug screening should be considered.

Role in virulence

While there was no change in cell wall integrity, the *S. aureus* and *S. pneumoniae uppP* mutants were shown to be attenuated in mouse model of infection (Chalker et al., 2000). *S. pneumoniae uppP* mutant displayed 4.6 log10 attenuation in comparison to wild type, a value approaching the maximum attenuation of 6 logs earlier detected in *S. pneumoniae* respiratory tract infection model (Chalker et al., 2000). Similar observation was reported in H37Rv, the pathogenic strain of *M. tuberculosis* (Rose et al., 2004). *M. tuberculosis* (H37Rv) Rv2136c mutant exhibited reduced virulence in immunocompromised $Rag1^{-/-}$ mice. The median survival of mice infected with H37Rv wild type was 26 days while that of the mice infected with the mutant was as long as 70 days . Similarly, $IFN\gamma^{-/-}$ mice were less susceptible to the mutant strain (median survival of 80 days) than to the wild type (median survival of 30 days) (Rose et al., 2004).

In a related work, it was shown that H37Rv, with a single transposon disruptive insertion in Rv2136c did not exhibit any difference in morphology as depicted with scanning and transmission electron microscope (Vandal et al., 2009). However, the mutant was shown to be 11 fold and 19 fold more susceptible to heat and hydrogen peroxide respectively than the wild type. Severe attenuation was also observed *in vivo* as demonstrated by complete clearance of the bacteria after 56 days post infection (Vandal et al., 2009). Although *Mycobacterium bovis* (BCG) is an attenuated strain, faster clearance of the mutant than the wild type in the mouse model of infection suggests the contribution of the gene to survival in host cells (Rose et al., 2004).

As also stated by (Chalker et al., 2000), these findings thus suggest that, although other enzymes compensates

for the loss of *uppP* homologues in these bacteria in ensuring intact cell walls, they could not compensate for its probable involvement in virulence.

Role in antibiotic resistance

M. tuberculosis mutant with transposon insertion in Rv2136c shows 32 fold, 8 fold and 2 fold increased susceptibility erythromycin, rifampin and streptomycin respectively (Vandal et al., 2009). The association of UppP homologues and PAP2 enzymes with bacitracin resistance has also been reported largely in bacteria such as *E. coli*, *Bacillus sp*, *Streptococcus sp*., and *S. aureus* among others (Chalker et al., 2000; El Ghachi et al., 2004; Rose et al., 2004; El Ghachi et al., 2005). Although no increased susceptibility to other antibiotics investigated was observed with *uppP* null mutants of *S. aureus* and *S. pneumoniae* (Chalker et al., 2000), further studies are needed to elucidate the involvement of UppP in antibiotic resistance in other bacteria.

Coupled with its role in virulence and antibiotic resistance, the absence of UppP in eukaryotic cells makes it an attractive potential antimicrobial drug target. Drugs targeting UppP may perform synergistic functions.

A major mechanism of *M. tuberculosis* resistance to antibiotics is the complex cell wall (Zhang and Yew 2009). For example, fosmidomycin successfully inhibited the essential mycobacterial deoxy-xylulose-5-phosphate reductase (Figure 1) *in vitro* but was reportedly inactive against whole *M. tuberculosis* cells. The resistance observed was attributed to lack of antibiotic uptake (Dhiman et al., 2005; Henriksson et al., 2007; Brown and Parish, 2008). In essence, focusing on proteins involved in cell wall synthesis as drug target in *M. tuberculosis* is still an important area demanding attention. In support of possible synergistic role, it was earlier reported that sub-inhibitory concentration of bacitracin supposedly inhibiting the function of Rv2136c resulted in about tenfold increase in susceptibility of *M. tuberculosis* to clarithromycin, an antibiotic that had been classified as ineffective against *M. tuberculosis* (Bosne-David et al., 2000). All possible drug targets (Eoh et al., 2009) including Rv2136c should therefore be evaluated in this bacteria. Such drugs can synergize the currently used drugs by reducing the number of drugs required to treat the disease or shorten the required length of time of drug usage.

Similarly, increasing resistance of *S. pneumoniae* to commonly used antibiotics including macrolides and penicillins has been reported (Chalker et al., 2000; Ortqvist et al., 2005). Drugs that can target UppP homologue of *S. pneumoniae* thus weakening the cells as observed by reduced virulence of *S. pneumoniae* Δ*uppP* mutant may provide a synergistic effect thus improving the efficacy of compounds currently used to combat the bacteria.

CONCLUSION AND OPEN RESEARCH ISSUES

In addition to the lack of outer cell membrane, the hypersensitivity of *S. aureus* and *S. pneumoniae* Δ*uppP* mutants to only bacitracin among other antibiotics may be attributed to the lack of Und-pp phosphatase activity required to compete with bacitracin for resistance. The intact cell wall observed despite deletion of *uppP* homologue was also explained to be most probably due to the presence of Und-OH kinase that generates the essential Und-p alternatively from Und-OH. Investigation into the enzymes involved in the generation of Und-OH from either Und-p or Und-pp in Gram-positive bacteria will be informative as the conversion of Und-OH to the essential Und-p appears to play a significant role in the biosynthesis of cell wall components.

De novo synthesis of Und-p was also suggested to involve other yet to be identified enzymes with active sites located in the cytoplasm. Discovery of this proposed enzyme will give an improved picture of the enzymes playing role in cell wall biosynthesis as well as adaptation of the bacteria to varying environment such as exposure to antibiotics. Additionally, there is need for experimental evidence to ascertain the cytoplasmic location of the conserved loop of UppP in bacteria and thus, its involvement in *de novo* synthesis of Und-p.

The potential use of UppP homologues as well as DgkA homologues of Gram-positive bacteria as drug targets in synergy with other currently used but ineffective antibiotics in the treatment of resistant pathogenic bacteria also warrants further investigations. Importantly, the study of bacterial isoprenoids metabolism has only been done in few representative bacteria, studies in more Gram-positive and Gram-negative bacteria are needed for better understanding of cell wall synthesis.

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