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Lipid Intermediates in the Biosynthesis of Bacterial Peptidoglycan

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INTRODUCTION

Over the past 50 years, both the structure and the biosynthesis of bacterial peptidoglycan (murein) have been extensively investigated, owing to its importance as an essential structural cell wall component (131, 145, 152), to its involvement in cellular morphogenesis (61, 71, 124, 147, 150, 186), and to the fact that steps of its biosynthesis are specific targets for well-known antibiotics and potential ones for novel antibacterials (66, 76, 145). An overall view of its biosynthesis valid for both gram-positive and gram-negative bacteria has emerged as a multistep process (references 34, 72, 73, 115, 145, 179, 180, and 190 and references therein). Briefly, the assembly of the disaccharide-peptide monomer unit is achieved via a linear pathway with a series of UDP nucleotide precursors and lipid intermediates (Fig. 1). The cytoplasmic steps lead to the formation of the UDP-MurNAc-pentapeptide precursor from UDP-GlcNAc and are mediated by the MurA to MurF synthetases. Thereafter, the transfer of the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide to a membrane acceptor, undecaprenyl phosphate, is catalyzed by transferase MraY, yielding lipid I. Addition of *N*-acetylglucosamine to the *N*-acetylmuramic acid residue of lipid I by transferase MurG leads to lipid II, which carries the complete disaccharide-peptide monomer unit: GlcNAc-β-(1→4)-MurNAc-L-Ala-γ-D-Glu-A_2pm (or L-Lys)-D-Ala-D-Ala.

Lipid II is transferred by an as-yet-unknown mechanism through the hydrophobic environment of the cytoplasmic membrane to externally located sites where polymerization of the disaccharide-peptide monomer involves two major types of membrane-bound activities: glycosyltransferases (GTs), which catalyze the formation of the linear glycan chains, and transpeptidases, which catalyze the formation of the peptide cross-bridges and the binding of nascent peptidoglycan to the preexisting cell wall (references 72, 73, and 179 and references therein). Prior to the polymerization reactions, the lipid intermediates can undergo secondary modifications mostly in their peptide moiety (amidation, addition of extra amino acids, and esterification, etc.). Since these reactions are not necessarily complete, lipid intermediates must be considered in many cases as complex mixtures of modified forms.

The discovery in the mid-1960s of the lipid intermediates of peptidoglycan synthesis (37, 118) and the determination of their structures (4, 87) were important for understanding their role as the specific link between the intracellular synthesis of the disaccharide-peptide monomer unit and the extracytoplasmic polymerization reactions. However, for several decades they were further studied to a limited extent owing to the tedious work required for their preparation as well as to poor knowledge of the membrane enzymes involved in their metabolism. Renewed interest was brought about by the now-ready availability of these enzymes, the resolution of the crystal structure of several ones, and their possible use as targets for the search of novel antibacterials. In turn, this led to the preparation of the lipid intermediates and analogues by new methods and to the development of new specific assays. It has also appeared that they play an important role in the covalent attachment of proteins to the cell wall of gram-positive bacteria (113), in the modes of action of antibiotics with which they form specific complexes (references 30 and 79 and references therein), and in mechanisms of resistance to antibiotics. Although their role in peptidoglycan biosynthesis has been reported in many reviews, no systematic survey of their properties and functions has been made. This review is an attempt to bring together and critically evaluate the dispersed data concerning these lipid intermediates.

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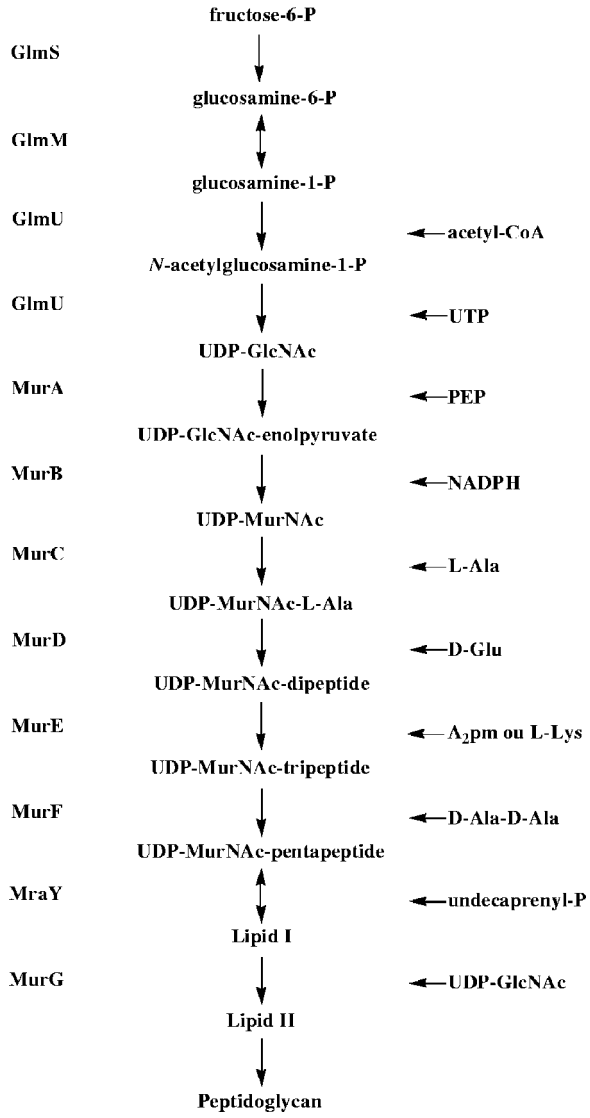

FIG. 1. Stepwise assembly of the peptidoglycan monomer unit. A_2pm, diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid.

DETECTION OF THE LIPID INTERMEDIATES IN CELL-FREE SYSTEMS

The existence of lipids I and II and their respective roles in the pathway were established by the study of cell-free peptidoglycan-synthesizing systems using UDP-GlcNAc, radiolabeled UDP-MurNAc-pentapeptide, and particulate preparations from *Staphylococcus aureus* (37, 118). The participation in this process of a perchloric acid-precipitable membrane intermediate was proposed (163). In assays with membranes from *S. aureus* or *Micrococcus luteus*, it was identified as a lipid intermediate (lipid I) and shown to be used as a substrate for the formation of a second intermediate (lipid II) by the addition of an *N*-acetylglucosamine residue (3). Both lipids I and II were detected as radiolabeled material migrating near the front of paper chromatograms run in isobutyric acid-1 M ammonia (5:3). It is noteworthy that at the same time a lipid intermediate was discovered in the biosynthesis of the *Salmonella* O-antigen (195). Thereafter, in vitro peptidoglycan-synthesizing activity was demonstrated with membrane or crude cell wall preparations from various organisms, and the lipid intermediates were detected in a similar way (references 145, 179, and 190 and references therein). As closely related compounds, lipids I and II were not separable by paper chromatography (4). Only recently was their clear separation by thin-layer chromatography described (29, 153, 176).

DETECTION OF THE LIPID INTERMEDIATES IN INTACT CELLS AND THEIR POOL LEVELS

The cellular pools of the lipid intermediates remain a poorly addressed question. Knowledge of their levels and variations is however essential for understanding many aspects of the membrane steps of peptidoglycan synthesis. In numerous experiments, the in vivo synthesis of peptidoglycan was followed by specific radiolabeling (reference 179 and references therein), but in only a few cases were the pools of the lipid precursors considered (28, 65, 81, 97, 121, 139). They were recovered from labeled cells in different ways (organic solvents, detergents, lysozyme), purified by paper or/and column chromatography, and quantitated. Their relative amounts were determined by analysis of the muropeptides obtained after mild acid hydrolysis (81, 97, 121, 139). In *Escherichia coli*, the lipid I pool was estimated at most at 700 molecules per cell and that of lipid II at 1,000 to 2,000 (182) by comparing the UDP-MurNAc-pentapeptide pool (120) with the relative pools of UDP-MurNAc-pentapeptide, lipid I, and lipid II (97). Rough estimates can also be made from other published data (139) and lead to 2,000 molecules per cell for lipid I and 3,000 for lipid II. It seems that the pool levels are higher in gram-positive organisms, in agreement with these organisms' known higher peptidoglycan content. In *Bacillus megaterium*, the lipid II pool was estimated at 34,000 molecules per cell (65). The binding of ramoplanin to *S. aureus* cells showed that the lipid intermediates amounted at most to 50,000 molecules per cell (160). Similarly, 70,000 and 200,000 molecules per cell were found with mersacidin in *M. luteus* and *Staphylococcus simulans*, respectively (31). Although limited, these studies revealed several interesting features. In *E. coli*, there is always an excess of lipid II over lipid I (81, 97, 121, 139), and the relative pools of UDP-MurNAc-pentapeptide, lipid I, and lipid II were found to remain constant during the cell cycle (97). A two- to threefold accumulation of lipid II was observed when cells were treated with moenomycin, an inhibitor of peptidoglycan polymerization (98).

ISOLATION AND PURIFICATION OF THE LIPID INTERMEDIATES

The isolation and purification of the lipid intermediates presented several challenges with respect to their presence at low pool levels in overwhelming amounts of phospholipids. The radiolabeled lipid intermediates that accumulated in the *M. luteus* and *S. aureus* cell-free systems were totally solubilized by *n*-butanol in the presence of isobutyric acid or, preferably, 6 M pyridium acetate, pH 4.2, and purified by successive column chromatographies on DEAE-cellulose, silicic acid, and Sephadex LH-20 (4, 87). The lipid intermediates were to some

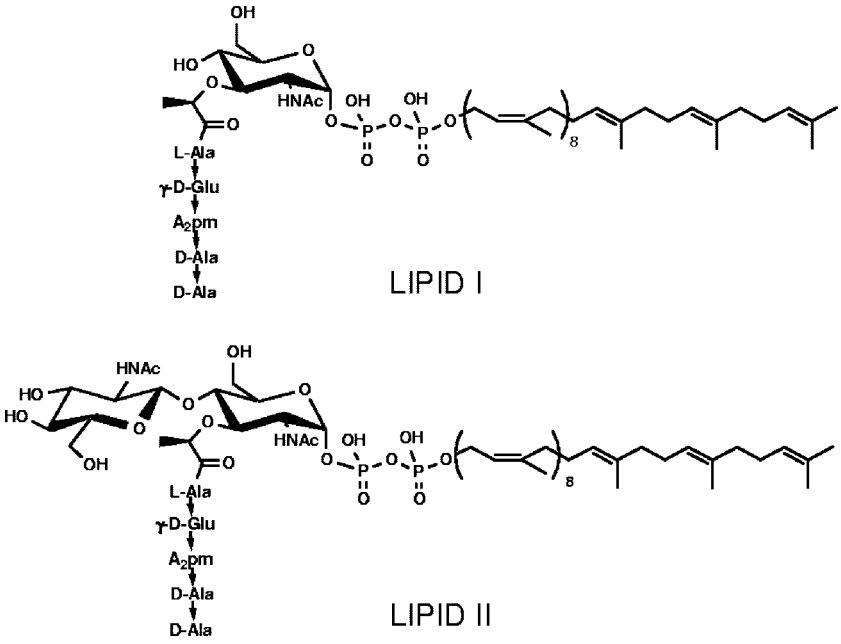

FIG. 2. Structures of lipids I and II.

extent extractible with petroleum ether, diethyl ether, chloroform, or chloroform-methanol. Depending on the initial absence or presence of UDP-GlcNAc and glycine in the cell-free peptidoglycan-synthesizing system, either lipid I or lipid II or a modified form was secured (4). Blocking polymerization by ristocetin or vancomycin in peptidoglycan-synthesizing systems promoted accumulation of the lipid intermediates (2, 3, 134, 141, 160; references 31 and 66 and references therein).

Subsequently, radiolabeled lipids I and II were prepared in a similar way from cell-free systems of several organisms: lipid I from staphylococci (136), *B. megaterium* (166), *Micrococcus flavus* (153), *E. coli* (107), and *Mycobacterium smegmatis* (107), and lipid II from *E. coli* (80, 107, 123, 168, 173, 181, 182), *Selenomonas ruminantium* (94), *B. megaterium* (167), *M. luteus* (31), *M. flavus* (153), and *M. smegmatis* (107). The absence or presence of UDP-GlcNAc in these systems determined the accumulation of lipid I only or of both intermediates. In the latter case, lipids I and II could be distinguished by analysis of their muropeptide content after removal of their lipid moiety by mild acid hydrolysis (62, 95, 107, 157). When determined, yields based on the initial amount of radiolabeled UDP-MurNAc-pentapeptide used ranged from less than 1% to up to 18%, and preparations amounted at most to a few μmols. Addition of purified MurG to the *M. flavus* system led to a high lipid II accumulation with depletion of the endogenous undecaprenyl phosphate pool (99).

STRUCTURE OF THE LIPID INTERMEDIATES

The comparison of the structure of peptidoglycan with that of its precursors was the key in understanding the biochemical reactions underlying peptidoglycan polymerization. The structure of lipid II was first established with purified preparations from *S. aueus* and *M. luteus* (4, 87) and characterized as undecaprenyl pyrophosphoryl disaccharide-pentapeptide (Fig. 2). This work implied the determination of the amino acid, hexosamine, and phosphate compositions. The status of the two phosphate residues found per disaccharide-pentapeptide was determined by mild acid hydrolysis (20 min at pH 4 and 100°C), which released pyrophosphate disaccharide-pentapeptide and a lipid moiety conclusively identified by mass spectrometry as undecaprenol. Stronger acid hydrolysis (0.1 N HCl or 1 N acetic acid for 15 min at 100°C) led to disaccharide-pentapeptide, whereas pyrophosphatase led to the corresponding phospho derivative. A pyrophosphate thus joins the reducing end of the *N*-acetylmuramic acid residue to undecaprenol. The structure of lipid I was defined in a similar way and by its relationship as the immediate precursor to lipid II (Fig. 2). The structures of the lipid intermediates from various organisms and those of analogues were studied by mass spectrometry without prior modification (29, 153), after conversion to reduced muropeptides (107), or after specific enzymatic degradation by colicin M into pyrophospho-muropeptides (60). It is noteworthy that the α-anomeric configuration of the *N*-acetylmuramic acid residue in UDP-MurNAc-pentapeptide is conserved in lipids I and II (Fig. 2), whereas the addition of *N*-acetylglucosamine to lipid I by MurG is accompanied by an inversion of the α-anomeric configuration it has in UDP-GlcNAc (see Fig. 4).

The stereochemistry of bacterial undecaprenol (Fig. 2) isolated as the free alcohol or secured after mild acid hydrolysis of the phosphorylated derivative was characterized by mass, infrared, and nuclear magnetic resonance (NMR) spectrometries (75, 151, 196; reference 174 and references therein). NMR spectrometry indicated the presence of two internal *E*, one α-*Z*, seven internal *Z*, and one methyl-terminal isoprene residues per molecule. This stereochemistry was further confirmed by the elucidation of the biosynthesis of undecaprenol (reference 96 and references therein). In bacteria, the un-

decaprenyl diphosphate synthase catalyzes the *Z*-prenyl chain elongation onto (all-*E*)-farnesyl diphosphate as a primer to yield undecaprenyl diphosphate with *E*,*Z*-mixed stereochemistry. Subsequent dephosphorylation yields undecaprenyl phosphate, used as the substrate by the MraY transferase for the formation of lipid I (reference 59 and references therein). The bacterial undecaprenol isolated directly or from phosphorylated derivatives contained small amounts of nonaprenol, decaprenol, and dodecaprenol (75, 87, 151, 173). Recently, structural analysis of compounds identified as lipids I and II from *M. smegmatis* demonstrated that their lipid moiety was mainly decaprenol and not undecaprenol as in all previously studied cases (107).

SYNTHESIS OF THE LIPID INTERMEDIATES AND ANALOGUES

The low pool levels of the lipid intermediates, their limited accumulation in cell-free systems, and the tedious work involved in their isolation and purification have restricted their availability for the study of the membrane steps of the peptidoglycan pathway. Their synthesis by chemical and enzymatic methods offers an alternative which has been successfully followed over the past few years. Efforts have concerned both lipids I and II and analogues. They have enabled the development of the study of the MraY and MurG transferases and of the peptidoglycan GTs as well as the search for specific inhibitors.

Initially, analogues of lipid I were synthesized for use in the study of transferase MurG. Structural variations were introduced in any one of the lipid, diphosphoryl, sugar, or peptide moieties of lipid I. The general strategy was to chemically couple an appropriate protected derivative of the phospho-*N*-acetylmuramoyl-pentapeptide to a lipid phosphate. The first functional substrate analogue described was (*R*,*S*)-α-dihydroheptaprenyl-pyrophosphoryl-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-*meso*-A_2pm (N^{ε}-dansyl)-D-Ala-D-Ala, obtained by a semisynthetic route (10). UDP-MurNAc-pentapeptide was enzymatically degraded to phospho-MurNAc-pentapeptide, which was dansylated and coupled to the lipid phosphate activated by the phosphoroimidazolidate method to form the pyrophosphate linkage. The next important step was the chemical synthesis of a water-soluble substrate analogue with a 10-carbon citronellyl lipid chain and the same one with an N^{ε}-biotinylated lysine residue (77, 119). The diphenyl chlorophosphate activation method was used for the assembly of the pyrophosphate bond. In following work, numerous lipid I analogues with modified lipid moieties were prepared in a similar way by use of diphenyl chlorophosphate or 1,1′-carbonyldiimidazole (11, 39, 103, 197). Lipid I analogues were also synthesized in which the anomeric diphosphoryl lipid moiety was replaced by a phosphate group or noncharged groups such as methoxy or thiophenoxy (158). Furthermore, citronellyl-lipid I analogues containing α-D-*N*-acetylglucosaminyl, α-D-glucosyl, and α-D-*N*-acetylmuramyl carbohydrates were made (47). At the same time, two chemical syntheses of natural lysine-containing lipid I were reported (183, 197). They involved the coupling of protected phospho-*N*-acetylmuramoyl-pentapeptide to undecaprenyl monophosphate by application of the 1,1′-carbonyldiimidazole (197) or the phosphoroimidazolidate (183) method. Finally, the availability of purified MraY transferase has enabled the enzymatic synthesis of natural A_2pm-containing lipid I from undecaprenyl phosphate and UDP-MurNAc-pentapeptide (25, 60).

The synthesis of lipid II and its analogues was recently reviewed in detail (191). Both chemoenzymatic and chemical approaches were followed. A first synthesis concerned a soluble analogue of lipid II, citronellyl-pyrophosphoryl-*N*-acetylmuramoyl-[L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala]-β-(1→4)-GlcNAc, which was obtained by the MurG-catalyzed transfer of *N*-acetylglucosamine from UDP-GlcNAc onto the corresponding chemically synthesized lipid I analogue (105). In a similar way, numerous lipid II analogues with modified lipid moieties were prepared (39, 197). In particular, the natural lysine-containing lipid II was prepared in this way (197). Thereafter, its total chemical synthesis was reported by two groups (154, 184). As with lipid I, the strategy involved first the synthesis of a protected *N*-acetylglucosaminyl-β-1,4-(phospho)*N*-acetylmuramoyl pentapeptide and then its coupling to undecaprenyl phosphate by the 1,1′-carbonyldiimidazole method. Lipid II analogues with an ε-amino-dansylated Lys (154, 184) or A_2pm (11) residue were also synthesized. In another approach, lipid II and analogues with different lipid moieties were secured by incubating membranes from *M. flavus* with polyprenyl, geranyl, or farnesyl phosphate in the presence of UDP-GlcNAc and UDP-MurNAc-pentapeptide (29). Presumably, the efficiency of this system was due to the low specificities of membrane-associated MraY and MurG for the different lipid moieties considered. More recently, purified MurG was used to synthesize natural A_2pm-containing lipid II from the corresponding lipid I (60). The chemical synthesis of heptaprenyl-lipid IV comprising two disaccharide-peptide units has also been described (201).

BIOSYNTHESIS OF LIPID I

The literature dealing with transferase MraY has been reviewed in detail elsewhere (57, 138, 180). Recent data have concerned its purification, the study of its catalytic mechanism, the development of enzymatic assays, and the search for specific inhibitors. Owing presumably to its complex transmembrane structure (22), the overproduction and purification of MraY were long-pending problems. They have now been successfully overcome with MraY from *Bacillus subtilis*, which enabled the study of kinetic parameters and various properties (25). MraY belongs to the UDP-D-*N*-acetylhexosamine: polyprenol phosphate D-*N*-acetylhexosamine 1-P transferase family, which includes other cell wall transferases such as WecA and WbpL (138). Comparative analysis of these transferases and mutations in the cytoplasmic loops led to the proposal of a catalytic mechanism (Fig. 3) implying different groups of MraY and the conservation of the α-anomeric configuration of UDP-MurNAc-pentapeptide in lipid I (104, 138).

The specificity of MraY for its nucleotide substrate was previously reviewed (138, 180). Recently, it was shown to have a broad specificity for its lipid phosphate substrate accepting dolichol-type isoprenyl phosphates, phytyl phosphate, and water-soluble prenyl phosphates (29). Over the past few years, new enzymatic assays mostly designed for high-throughput screening and the study of specific inhibitors were developed

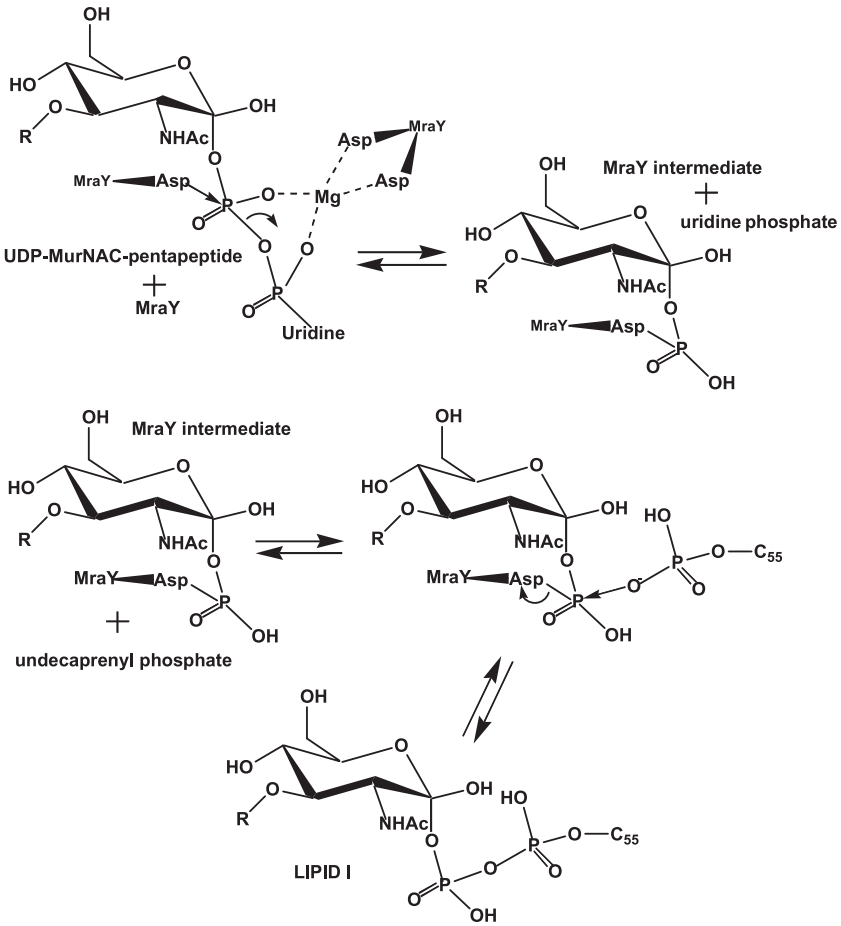

FIG. 3. Multistep MraY-catalyzed formation of lipid I. Scheme established with data from references 104, 125, and 138. R, D-lactoyl-peptide.

(26, 92, 159, 161, 200). As a target in the search for novel antibacterials, MraY presents many advantages (essentiality, ubiquity to eubacteria, specificity). The known inhibitors of MraY can be classified into three different groups (57): (i) the nucleosides (comprising the tunicamycins, ribosamine-uridines, uridylpeptides, and capuramycins), (ii) lipopeptide amphomycin, and (iii) protein E. Despite the number of described inhibitors, not one has yet entered clinical use as an antibiotic.

BIOSYNTHESIS OF LIPID II

The MurG transferase is responsible for the synthesis of lipid II by catalyzing the transfer of *N*-acetyl-D-glucosamine from the UDP-GlcNAc donor onto the C-4 hydroxyl of the *N*-acetylmuramic acid residue of the lipid I acceptor (Fig. 4). The formation of the β-(1→4) linkage is accompanied by an inversion of the anomeric configuration of the *N*-acetylglucosamine residue. The study of MurG has mostly concerned the *E. coli* enzyme, and elsewhere the literature has been reviewed in detail up to 2000 (180). Since then, considerable progress has been made. MurG was shown to belong to the GT-B superfamily (175). Its X-ray structure and that of its complex with UDP-GlcNAc were resolved (78, 90). Compared to the free protein, there is a change in the relative organization of the N- and C-terminal domains of the complex, which adopts a more closed conformation. The binding site for UDP-GlcNAc appears to be in the C-terminal domain, whereas the primary acceptor binding site is located in the N-terminal domain. The location of the UDP-GlcNAc donor was confirmed by mutational analysis (46, 90).

A direct assay for MurG activity was initially described with radiolabeled lipid I (166). Owing to its difficult availability in large amounts (136, 166), assays based on a reaction coupled with MraY were developed (45, 121, 160). Various coupled assays have since been used for high-throughput screening purposes (26, 50, 92, 140, 200). Direct assays with synthetic lipid I analogues were first carried out with compounds containing C_{10} and C_{35} chains (10, 11, 119). Their efficiency was greatly enhanced by the use of a soluble lipid substrate and a chaotropic agent such as dimethyl sulfoxide. More recently, fluorescence assays were devised for conveniently monitoring the MurG activity (39, 86, 102, 103). Activity measurements with vesicles containing lipid I showed that MurG activity was increased by the presence of cardiolipin (177).

The availability of adequate substrates and convenient assays enabled the determination of the kinetic parameters of *E. coli* MurG (11, 77) and the study of its specificity profile (39, 77, 90, 103). By use of a radioactive C_{35} lipid II analogue, it was

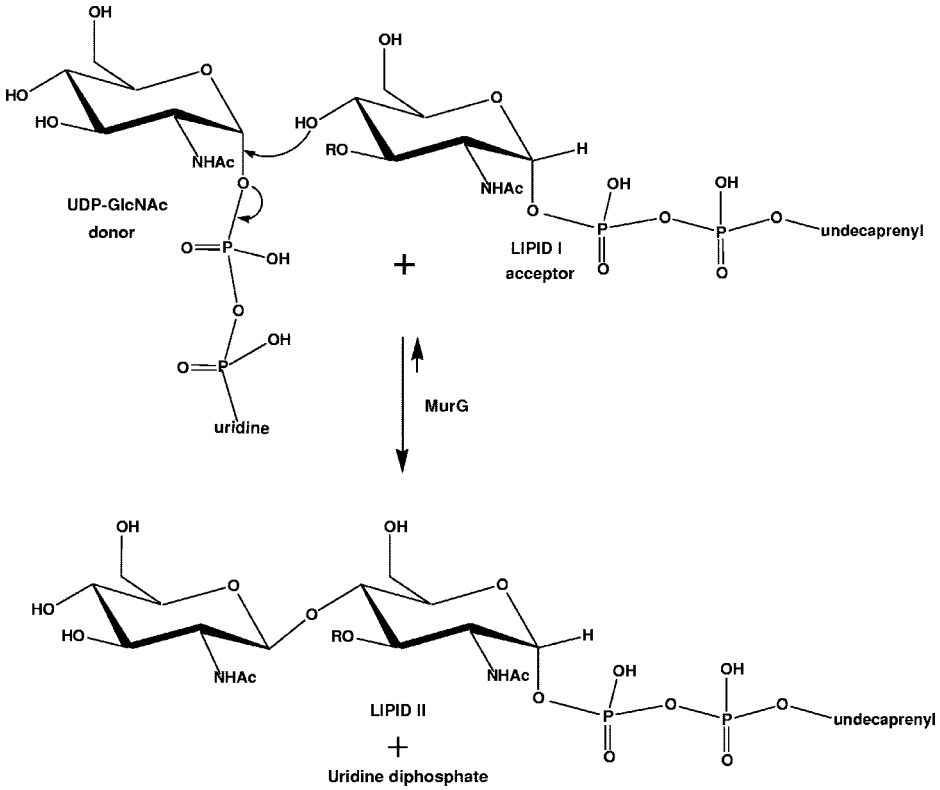

FIG. 4. MurG-catalyzed formation of lipid II.

established that MurG can catalyze to a certain extent the reverse reaction, namely, the production of radioactive UDP-GlcNAc (11). Interestingly, biotinylated UDP-MurNAc-pentapeptide functions as an acceptor substrate, albeit much less efficiently than lipid I (77). The specificity of the acceptor site was investigated with analogues of lipid I modified in its lipid moiety (39, 103). From comparison of kinetic parameters, it was concluded that the lipid chain interacts with the enzyme (39). Those with a saturated alkyl chain were better substrates than natural lipid I but not as good as one with a C_{20} tetraprenol (39, 103). This indicated that double bonds were not crucial for recognition and that the interaction of the lipid chain with MurG was simply hydrophobic. There is a length requirement for the lipid chain, which should be long enough to interact hydrophobically (103). The UDP-GlcNAc donor site of MurG had a high specificity for UDP and also showed good selectivity for GlcNAc, indicating that the equatorial hydroxyl at C-4 was critical (77). UDP was found to be a competitive inhibitor of the UDP-GlcNAc donor and a noncompetitive inhibitor of the lipid I acceptor. Product inhibition and dead-end inhibition analyses demonstrated an ordered Bi Bi mechanism in which the UDP-GlcNAc donor binds first (39). This means that the two substrates bind to MurG before the first product is formed and that the sequence of the addition of the two substrates and of the release of the two products follows an obligatory order (Fig. 4).

Like MraY, transferase MurG is an interesting potential antibiotic target due to its essentiality, its ubiquity in eubacteria, and the specificity of the reaction it catalyzes. A number of acceptor and donor substrate analogues were found to have an inhibitory effect (77). A fluorescence-based substrate displacement assay was used for the high-throughput screening of large compound collections against MurG (86, 91). In this way, competitive inhibitors with a neutral core mimicking the diphosphate moiety of the UDP-GlcNAc donor substrate were identified. Ramoplanin (160, 188) and glycopeptides (103, 140) were shown to inhibit the MurG activity in vitro. Initially, ramoplanin was proposed to act by binding to lipid I and sequestering it from the reaction (160), but more-recent experiments showed that it interacts directly with the enzyme (85). The inhibitory effect of glycopeptides is presumably due to their interaction with the C-terminal D-Ala-D-Ala sequence of lipid I, as previously established with C-terminal D-Ala-D-Ala-containing precursors and many peptides (66, 93, 126, 141) but to date not with lipid I. Since these drugs do not penetrate the cell membrane, their in vitro effect on MurG activity is of no significance for their in vivo mode of action, which involves their interaction with extracytoplasmically located lipid II (see last section).

BIOSYNTHESIS OF MODIFIED LIPID INTERMEDIATES

The basic structure of bacterial peptidoglycan is that of an heteropolymer in which linear glycan chains are composed of alternating β-(1→4)-linked units of GlcNAc and MurNAc peptide and in which neighboring glycan chains are cross-linked by a direct peptide bond between peptide subunits. However, a variety of structural variations is encountered in the peptidoglycans of most if not all bacteria (references 145, 152, and 180 and references therein). This is clearly illustrated

TABLE 1. Specific peptidyltransferases catalyzing the assembly of the interpeptide bridge in *E. faecalis*, *E. faecium*, *L. lactis*, *S. aureus*, *S. pneumoniae*, and *W. viridescens*

Peptidyltransferase	Organism	Amino acid added	Reference(s)
AslA	*L. lactis*	D-Asp	185
Aslfm	*E. faecium*	D-Asp	15, 162
BppA1	*E. faecalis*	Ala	23/24
BppA2	*E. faecalis*	Ala	
FmhB	*S. aureus*	Gly	16, 114, 153
FemA	*S. aureus*	Gly	
FemB	*S. aureus*	Gly	
FemX	*W. viridenscens*	Ala	18, 84, 108, 135
MurM	*S. pneumoniae*	Ala/Ser	62
MurN	*S. pneumoniae*	Ala	

by the complexity of the muropeptide composition initially observed for *E. coli* (69, 70) and later in many other organisms (for examples, see references 9, 19, 21, 41, 51, 67, and 137). The importance of these modifications varies, and the physiological significance of many of them is still poorly understood.

Among the observed modifications, the presence of a peptide bridge between the peptide subunits is an important structural feature of many gram-positive organisms (reference 152 and references therein). It is assembled on the precursors by the stepwise addition of amino acids onto the ε-amino group of the L-lysine residue of the pentapeptide subunit and accepted by the subsequent steps of the pathway. The actual cross-linking between the free N-terminal end of the peptide bridge of one subunit and the carboxyl of D-Ala^4 of another subunit takes place by transpeptidation during polymerization. Specific peptidyltransferases forming the FemABX protein family (references 146 and 185 and references therein) catalyze each step of the assembly of the stem peptide (Table 1), and two different mechanisms have been described. One entails the addition of L-amino acids or glycine from aminoacyl-tRNAs (23, 24, 62, 84, 108, 114, 135, 153), while the other one, which is ATP dependent and presumably specific for D-amino acids, proceeds without aminoacyl-tRNAs (15, 162, 185).

In *Streptococcus pneumoniae* and *S. aureus*, lipid II was identified as the main in vivo substrate for the assembly of the peptide bridge (62, 153). In contrast, in *Weissella viridescens* (formerly *Lactobacillus viridescens*), the in vivo addition of L-alanine onto UDP-MurNAc-pentapeptide was substantiated by the presence of a high UDP-MurNAc-hexapeptide pool and the absence of UDP-MurNAc-pentapeptide (83). The peptidyltransferases from *Enterococcus faecalis* (BppA1 and BppA2) and *Enterococcus faecium* (Aslfm) were overproduced and purified by using UDP-MurNAc-pentapeptide as an in vitro substrate (15, 23, 24). However, since the turnover observed with this substrate was very low and since no large UDP-MurNAc-hexapeptide pool level has been encountered in studies of enterococci (20), it can be assumed that in vivo the addition of the bridging amino acids occurs on the lipid intermediates. It remains to be determined whether this takes place on lipid I, on lipid II, or on both.

Therefore, the assembly of the peptide bridges seems to proceed in many cases on the lipid intermediates. However, UDP-MurNAc-hexapeptide and -heptapeptide precursors were detected, although at low pool levels, in staphylococci, enterococci, and streptococci after treatment with antibiotics inhibiting late steps of peptidoglycan synthesis or under conditions of glycine depletion (20, 112, 165). This could be due either to the addition of the bridging amino acid directly onto UDP-MurNAc-pentapeptide accumulating to a high pool level under these circumstances or to the reversibility of the MraY reaction driven by the accumulation of the lipid intermediates. In *E. faecium* and *Lactococcus lactis*, D-*iso*-Asn found in the peptide bridge is not added to the precursor but is formed by amidation of the already added D-*iso*-Asp residue (15, 185). It is not yet clear whether amidation takes place on the D-*iso*-Asp-containing lipid or on nascent peptidoglycan.

Other modifications of lipids I and II were described. A cell-free system from *Micrococcus luteus* was shown to catalyze the ATP-dependent addition of glycine onto the α-carboxyl of the D-glutamic acid residue of both lipid intermediates, although less efficiently on lipid I (95). Only a slight addition was observed for UDP-MurNAc-pentapeptide. Similarly, with a cell-free system from *S. aureus*, both lipids I and II acted as acceptors of ammonia in an ATP-dependent reaction in which the α-carboxyl of the D-glutamic acid residue was amidated (157). No direct amidation of UDP-MurNAc-pentapeptide was observed. The in vitro addition of cadaverine onto the D-glutamic residue of lipid II was described with a particulate fraction from *S. ruminantium* (94). For *M. smegmatis*, it was recently shown by mass spectrometry that the muropeptide originating from lipid II was a complex mixture in which the muramic acid residue and the pentapeptide were modified singly or in combination (107). The muramic acid residue was present as such or N acetylated, or N glycolylated, whereas the carboxylic functions of the pentapeptide were modified to various extents by amidation and methylation. The absence of any modification of the lipid I peptide subunit strongly suggested that in *M. smegmatis* those observed with lipid II occur after its formation from lipid I (107). This implies either a specific preference of the modifying enzymes for lipid II or their inaccessibility to lipid I owing to its possible direct channeling from MraY to MurG.

Yet another important modification of lipid II was observed in the cell wall sorting pathway of surface proteins in *S. aureus* (113). In this process, sortase A, a membrane-anchored transpeptidase, cleaves the threonine-glycine bond of the LPXTG motif of the surface protein, generating an acyl intermediate which is attacked by the N-terminal amino group of the peptide bridge of modified lipid II. In this way, C-terminal threonine of the surface protein is linked to the lipid precursor. This intermediate, undecaprenyl-pyrophosphoryl-MurNAc-[L-Ala-γ-D-Gln-N^{ε}(surface protein-LPXT-Gly_5)-L-Lys-D-Ala-D-Ala]-β-(1→4)-GlcNAc, functions as a substrate for the subsequent steps of peptidoglycan polymerization. It is believed that in this way the mature surface protein is incorporated as anchored to the cell wall and displayed on the cell surface (113).

The modifications undergone by the lipid intermediates are not necessarily all complete and often lead to complex pools, which can also be due in some cases to variations of the structure or pool levels of the cytoplasmic precursors. Direct analysis of the lipid intermediate pools, as carried out with *M. smegmatis* (107), is not yet a usual approach. Generally, it is assumed that structurally related precursors are recognized

similarly by the different steps of the pathway and that the heterogeneity of the lipid II pool is reflected to some extent by the muropeptide composition. This is valid for MraY, MurG, the lipid II translocation, and the GT steps, which have the same low-specificity profile for modifications in the peptide subunits, but not necessarily for the transpeptidation steps, which can depend more on the structure of the peptide subunit (see discussion in references 178 and 180). In the peptidoglycan literature, there are many examples of the simultaneous functioning of different sets of lipid intermediates. Briefly, attention will be focused here on three gram-positive pathogens, namely, *S. pneumoniae*, enterococci, and *S. aureus*, which have been extensively studied in connection with the mode of action of the β-lactam and glycopeptide antibiotics and with mechanisms of resistance. In these organisms, the incomplete formation of the peptide bridges was established by high-pressure liquid chromatography analysis of the muropeptide compositions (19, 51, 52, 62, 67, 109).

In *S. pneumoniae*, the MurM and MurN peptidyltransferases catalyze the addition of the first (Ala or Ser) and second (Ala) amino acid of the dipeptide bridge, respectively, onto lipid II (62). The proportions of unbranched, incomplete, and branched muropeptides vary with the *murM* allele of the strain considered (62). In particular, penicillin-resistant strains contain mostly dipeptide bridges, but inactivation of the *murM* gene leads to their disappearance, to loss of the resistance phenotype, and to increased susceptivity to antibiotic-induced lysis (63). The mechanisms connecting these phenotypes are not understood, but they make MurM a potential target for antiresistance agents, as recently illustrated by the synthesis of MurM inhibitors (44).

In enterococci, the mechanism of resistance to β-lactams was shown to involve the production of low-affinity penicillin-binding proteins (PBPs) (reference 43 and references therein). Inactivation of the BppA2 peptidyltransferase catalyzing the addition of the second L-alanine residue of the dipeptide bridge was associated with decreased β-lactam resistance, as seen for *S. pneumoniae* (24). More recently, a new mechanism of resistance was described for high-level β-lactam-resistant mutants isolated from *E. faecium* lacking low-affinity PBP5 (110, 111). It entailed the formation of a high UDP-MurNAc-tetrapeptide pool used by a β-lactam-insensitive L,D-transpeptidation reaction. The presence of both UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentapeptide allowed for the functioning of two peptidoglycan pathways with two sets of lipid intermediate pools. A similar situation was observed with the mechanism of resistance to glycopeptides which emerged in *E. faecium* by acquisition of transposon Tn*1546* (101), mediating the production of precursors with C-terminal D-lactate instead of D-alanine (7, 8). The substitution led to a 1,000-fold reduction in the affinity of vancomycin for its target (33). Upon induction by vancomycin, the two pathways functioned in parallel with presumably mixed lipid intermediate pools, as in the case of high-level β-lactam resistance. However, a novel mechanism of vancomycin resistance was recently discovered in an *E. faecium* high-level β-lactam-resistant mutant devoid of UDP-MurNAc-pentapeptide (43). The presence of an overwhelming UDP-MurNAc-tetrapeptide pool not only favored preferentially the L,D-transpeptidation pathway but also excluded the binding of glycopeptides to lipid II and nascent peptidoglycan, both lacking the C-terminal D-Ala-D-Ala target.

In *S. aureus*, heterogeneity of the lipid intermediate pools occurs under different circumstances. In a strain with an altered MurE lysine-adding enzyme, the UDP-MurNAc-Ala-D-Glu precursor accumulates and L-Ala-D-Glu peptide subunits are predominant in peptidoglycan (129). This implied the functioning of two pathways, one with UDP-MurNAc-dipeptide up to the transglycosylation step included and the other with enough UDP-MurNAc-pentapeptide to insure a certain extent of transpeptidation. The interpeptide bridge of *S. aureus* is assembled by the stepwise addition of glycine, serine, or alanine residues onto lipid II (153) catalyzed by peptidyltransferases FmhB, FemA, and FemB (146). The incompleteness of these reactions is reflected in the muropeptide composition (51). Interestingly, the presence of the *mecA* gene, which is the key component of broad-spectrum β-lactam resistance in *S. aureus*, had little effect on the heterogeneity of the muropeptide composition (51). A high-level *vanA*-type vancomycin resistance similar to that encountered in studies with enterococci was recently observed for *S. aureus* clinical strains (reference 156 and references therein). The impact of the presence of the *vanA* mechanism on the structure of peptidoglycan was investigated in an *S. aureus* β-lactam-resistant strain by analysis of the cytoplasmic precursor pools and the muropeptide composition after growth in the absence or presence of one or the other antibiotic (156). Clearly, different pathways and, to some extent, different enzymes were used by the *mecA* and *vanA* mechanisms.

CELLULAR LOCATION OF THE LIPID INTERMEDIATES AND TRANSLOCATION OF LIPID II

The presence of the lipid intermediates in particulate preparations, their structure, their properties, and their functions all substantiated a cellular location in the cytoplasmic membrane. For *E. coli*, the physical association with the cytoplasmic membrane was established by sucrose gradient centrifugation after specific radiolabeling (28). Lipid I is located on the inside surface of the cytoplasmic membrane, since it is formed there by MraY and used there as the substrate by MurG. Indeed, topological analysis of MraY from both *E. coli* and *S. aureus* suggested the involvement of its cytoplasmic loops in substrate recognition and in the catalytic process (22, 138). Furthermore, MurG was shown to be associated with the cytoplasmic side (35, 122). Thus, lipid II is entirely assembled on the inner surface of the cytoplasmic membrane. This is also true for its modified forms, since their formation implies the use of cytoplasmic constituents (amino acids, aminoacyl-tRNAs, and ATP, etc.). However, the protein-associated lipid II intermediate formed by sortase A appears to be exclusively located on the outside surface of the cytoplasmic membrane (113).

Lipid II or its modified forms are used as the substrate in the extracytoplasmic polymerization processes. Considering its low pool level, its translocation across the cytoplasmic membrane must be a fast and unidirectional process to sustain a steady peptidoglycan synthesis in growing cells. More than 4 decades after the discovery of this translocation, its mechanism remains unknown. This is a general problem regarding the translo-

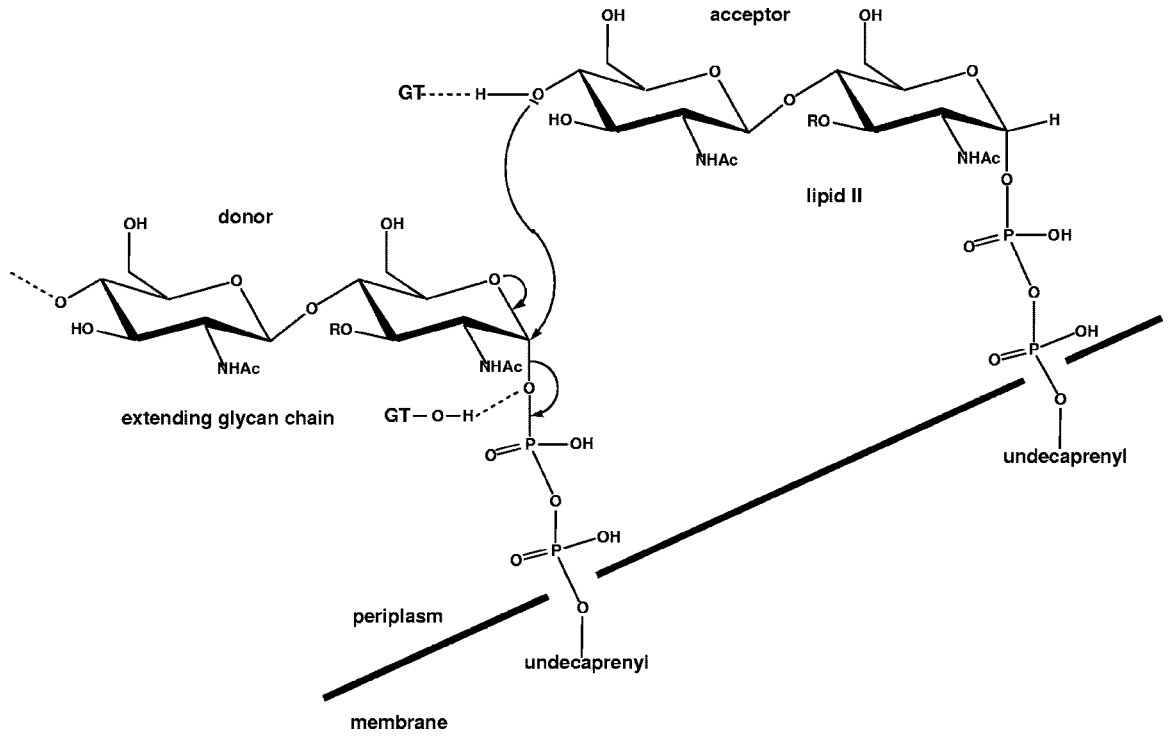

FIG. 5. Mechanism of transglycosylation with chain elongation at the reducing end. R, D-lactoyl-peptide.

cation of glycosyl carrier lipid-linked sugar chains through biological membranes (202). The biosyntheses of bacterial peptidoglycan, lipopolysaccharide O-antigen, and various exopolysaccharides share common properties with that of N-linked glycoproteins in the endoplasmic reticulum of eukaryotic cells (32). In these cases, an oligosaccharide intermediate is assembled on an isoprenoid lipid carrier at the cytoplasmic side of the membrane and subsequently translocated. It was proposed that the lipid polymorphism resulting from the nonlamellar lipid phase induced by polyisoprenols may provide a hydrophilic pore or channel to facilitate the translocation of lipid-linked oligosaccharides (references 202 and 203 and references therein). Such a mechanism could be responsible for the reversible translocation of lipid I postulated to explain the cleavage of the pentapeptide unit by the extracytoplasmic $VanY_D$ DD-carboxypeptidase and the formation of UDP-MurNAc-tetrapeptide in the cytoplasm of a vancomycin-resistant *E. faecium* strain (143).

Regarding lipid II, fluorescence spectrometry experiments carried out with a dansylated derivative showed that the rate of unassisted movement across the membrane was not sufficient to sustain peptidoglycan synthesis (192). New experiments with a fluorescent lipid II analogue and lipid vesicles demonstrated that, in contrast to phospholipids, lipid II was unable to spontaneously move across the bilayer (176). However, it was translocated when *E. coli* inner membrane vesicles with a right-side-out orientation were used. This showed that an intact translocation machinery was present in such vesicles and likely composed of one or more membrane proteins. Additional experiments excluded the possibility that MurG could be responsible for lipid II translocation but suggested that the process is coupled to ongoing transglycosylation (176). Earlier results had suggested that the translocation of lipid II depended on ongoing phospholipid synthesis and was likely to be more than just a flip-flop mechanism (58). It is now essential to determine which membrane proteins are involved in the translocation process. In this respect, different reasons suggested that transmembrane protein FtsW might be a lipid II translocase (see references in reference 100). However, the absence of UDP-MurNAc-pentapeptide accumulation under conditions of FtsW depletion seemed to exclude this possibility (100). Interestingly, two distinct protein-mediated mechanisms were reported to be involved in the transfer of enterobacterial antigen (ECA) and O-antigen units across the membrane (1, 144). In particular, an assay of translocase activity was developed with sealed everted *E. coli* membrane vesicles to follow the transport of a radiolabeled analogue of the ECA glycolipid intermediate (144). Perhaps, this assay or the recently developed fluorescence spectrometry assay (176) could be appropriately used for the systematic search for translocases now that lipid II or analogues are more readily available. The choice of the membrane proteins to be overproduced, purified, and tested is not an easy matter, considering the complexity of the bacterial membrane proteome (48).

GLYCAN CHAIN FORMATION WITH LIPID II

Peptidoglycan glycan chains are assembled by the polymerization of the *N*-acetyl glucosaminyl-β-(1→4)-*N*-acetylmuramyl disaccharide unit of lipid II (or its modified forms) with the formation of β-(1→4) linkages (Fig. 5). The transglycosylation reaction is accompanied by the inversion of the α-anomeric configuration of the *N*-acetylmuramic acid residue, thus leading to linear chains containing exclusively β-(1→4) link-

TABLE 2. Peptidoglycan GTs and in vitro use of lipid II as the substrate

Type and name	Organism	Moenomycin	Reference(s)
Class A			
PBP 1b	*E. coli*		
Full length		+	123, 164, 169
Soluble forms		+	17, 155, 169
GT module		+	13
PBP 1a	*E. coli*		
Full length		ND	168, 172
PBP 4	*B. megaterium*		
Full length		ND	167
PBP 1a	*S. pneumoniae*		
Soluble form		+	54
PBP 2a	*S. pneumoniae*		
Soluble form		+	56
PBP 2	*S. aureus*		
Full length		+	106
Soluble form		+	12
PBP 4	*L. monocytogenes*		
Full length		+	199
PBP 1a	*T. maritima*		
GT module		+	127
MGT			
Full length	*E. coli*	−	53, 80
Full length	*M. luteus*	−	132
Full length	*S. pneumoniae*	+	133
Full length	*S. aureus*	+	132
Soluble forms		+	170, 189
Novel GT			
Full length	*B. subtilis*	+	117

ages. Elsewhere, literature on this process was reviewed in detail up to 2000 (179). In intact cells, transglycosylation is tightly coupled with transpeptidation, but it can proceed alone when transpeptidation is inhibited by penicillin, as observed in many studies with intact cells and free-cell systems. This uncoupling can even lead in some cases to a stimulation of the transglycosylation reaction (5, 94; references in reference 100). Specific periplasmic GTs are responsible for the formation of the glycan chains and come in two forms (references 72 and 179 and references therein): as N-terminal modules in bifunctional class A PBPs, which also contain C-terminal transpeptidase modules, and as monofunctional GTs (MGTs). They all show high sequence similarity, belong to the GT51 family in the sequence-based classification of GTs, and possess five conserved motifs (42, 72).

Initially, lipid II was used as the substrate with membrane preparations for studying in vitro peptidoglycan formation (reference 179 and references therein). A specific assay for transglycosylation was developed with lipid II, *E. coli* membranes, and penicillin as the transpeptidation inhibitor (181). Presently, more than 15 peptidoglycan-specific GTs have been overproduced and purified, and most of them have been assayed for their GT activity with lipid II as the substrate (Table 2). Analogues of lipid II have also been used as substrates (155, 197). Interestingly, heptaprenyl-lipid IV can also be used as the substrate in the presence or absence of lipid II (201). Inhibitors of the transglycosylation reaction either interact directly with the enzyme or form complexes with lipid II. Among the first type, moenomycin has the greatest potency and is the best-studied example (references 27, 130, 179, and 191 and references therein). Other inhibitors include derivatives of vancomycin, compounds partially mimicking moenomycin, and analogues of lipid II (references 39, 68, 74, 93, 179, and 191 and references therein). Complexes with lipid II will be discussed in the following section.

PBP1b from *E. coli* has been by far the most investigated class A PBP in terms of location, membrane topology, purification, and GT activity (reference 179 and references therein). Recent work has dealt with its kinetic characterization (155), the in vivo role of its dimeric form (36), the structural analysis of the in vitro-formed material (17), the successful preparation of a stable active form of its GT domain (13), and the study of its specificity profile (64). Efforts to study peptidoglycan GTs from gram-positive organisms were first undertaken with PBP1a and PBP2a from *S. pneumoniae* (54, 55). In particular, in vitro polymerization by a soluble form of PBP2a by use of dansylated lipid II as the substrate was observed with a catalytic efficiency of 10^{-3} M^{-1} s^{-1} (56). More recently, the overexpression and purification of PBP2 from *S. aureus* (12) and that of PBP4 from *Listeria monocytogenes* (199) were reported. They catalyzed polymerization using lipid II with catalytic efficiencies of 3.4×10^3 M^{-1} s^{-1} and 1.4×10^3 M^{-1} s^{-1}, respectively. These values are comparable to previous estimates for *E. coli* PBP1b (40, 155, 169). The GT domain of PBP1a from *Thermotoga maritima* was also functionally characterized (127). The recently reported resolution of the crystal structures of PBP2 from *S. aureus*, of its moenomycin-bound form, and of the GT domain of *Aquifex aeolicus* brings new insights to the mechanism of transglycosylation and its inhibition by moenomycin (106, 198).

Membrane-bound MGTs capable of catalyzing un-cross-linked glycan chain formation in various bacteria were identified (references 170 and 179 and references therein). MGTs from *E. coli* (80) and *S. aureus* (170, 189) were overproduced, purified, and partially characterized. In particular, a capacity to use lipid II with a catalytic efficiency of 5,800 M^{-1} s^{-1} was established for *S. aureus* MGT (170). Since some MGTs were shown to be nonessential, their exact physiological role remains poorly understood. It was proposed they could be involved in infection processes (170). Recently, mutants lacking all class A PBPs and possessing no MGT were described for *B. subtilis* (117) and *E. faecalis* (6). This showed that in some organisms they were not essential and that the transglycosylation reaction with lipid II was performed by a novel kind of GT activity displaying no similarity with known peptidoglycan GTs. It was demonstrated that the *B. subtilis* mutant was sensitive to moenomycin and that its membranes could use lipid II as the substrate for peptidoglycan synthesis (117). In the *E. faecalis* mutant, the deletion of the three class A PBP genes led to high-level resistance to moenomycin (6).

COMPLEXES BETWEEN THE LIPID INTERMEDIATES AND ANTIBIOTICS

It is now well established that antibiotics like ramoplanin, mersacidin, vancomycin, telavancin, nisin, epidermin, mannopeptides, and other glycopeptides and lantibiotics can bind noncovalently to the peptidoglycan lipid intermediates. Work on these complex-forming compounds has been extensively

reviewed (8, 14, 30, 34, 38, 79, 93, 116, 142, 148, 149, 188, 191, 194). In general, they are cyclic peptides, depsipeptides, or peptides with extra posttranslationally introduced ring rearrangements and amino acid modifications. Some contain sugar and/or lipid moieties. Owing to their size and polar structure, they penetrate with difficulty both the bacterial cytoplasmic membrane and the outer membrane of gram-negative bacteria. Therefore, in gram-positive bacteria they interact with extracytoplasmically located lipid II and by its sequestration they lead to the arrest of peptidoglycan polymerization. However, these antibiotics differ from one another in many respects, such as their affinity for lipid II, the interacting sites of lipid II, and the possession of additional and separate mechanisms of action affecting their antibacterial activity. This is illustrated by briefly outlining the properties and effects of some well-studied examples.

The affinity of ramoplanin for lipid II was determined with a fluorescent derivative, and the dissociation constant (10 to 100 nM) was in agreement with the value calculated (50 nM) from the inhibition kinetics of the transglycosylation reaction (188). Both approaches showed that ramoplanin binds in a 2:1 ratio to lipid II, and NMR results were consistent with a model in which lipid II binds in a dimer cleft. The direct correlation between the affinity for lipid II and the high bactericidal effect on various gram-positive organisms suggested that the sequestration of lipid II is its main mechanism of action. A tight specific association in an equimolar ratio was observed with mersacidin, which is a type B lantibiotic presumably acting similarly to ramoplanin (31). They both recognize the pyrophosphate and disaccharide moieties of lipid II, although they do not compete efficiently with one another.

The affinity of vancomycin for lipid II determined with a water-soluble form of the precursor was 10-fold lower than that of ramoplanin (93). Moreover, the 50% inhibitory concentration for inhibition of the transglycosylation reaction was ca. 10^{-5} M (123, 199). This could explain its lower bactericidal potency and the easy reversibility of its bacteriostatic effect. The complex involves the specific noncovalent binding of vancomycin to the C-terminal D-alanyl-D-alanine motif present in peptidoglycan precursors and nascent material (reference 93 and references therein). In intact cells, complex formation is limited to exported lipid II and nascent peptidoglycan. In the first case, the resulting sequestering of lipid II will lead to an arrest of glycan chain elongation by substrate depletion, whereas in the second case the binding to the D-alanyl-D-alanine motifs of growing chains inhibits transglycosylation and transpeptidation by steric hindrance. It is difficult to assess whether both mechanisms are functioning in vivo or whether one is predominant. Another aspect of the mechanism of action of vancomycin is the proposal that its dimerization in aqueous solution facilitates in a cooperative manner the binding to the membrane precursor (reference 194 and references therein), but this has been questioned (187).

Telavancin is a semisynthetic derivative of vancomycin with two extra chemical groups (reference 88 and references therein). Compared to vancomycin, it exhibits a superior antibacterial potency with a rapid bactericidal activity against a broad spectrum of gram-positive pathogens. By its preferential association with the cell membrane rather than with the cell wall, it leads to rapid membrane permeabilization and depolarization. This explains its greater potency for the inhibition of peptidoglycan synthesis in membrane systems despite its fivefold-reduced affinity for D-Ala-D-Ala termini compared to vancomycin. The direct correlation between membrane potential and viability suggests that its action on the membrane could be mostly responsible for its rapid bactericidal activity.

Nisin is the most prominent member of type A lantibiotics, which are elongated, amphiphilic, screw-shaped peptides with net positive charges (references 14, 30, 38, and 193 and references therein). It has antibacterial activity against various gram-positive bacteria and is widely used as a food preservative. Its primary mode of action is the formation in the cytoplasmic membrane of defined pores of 2 to 2.5 nm in diameter by use of lipid II as a docking molecule and as an integral part of the pore (30). In this way, nisin not only leads to the rapid permeabilization of the cell membrane but also sequesters lipid II no longer available for peptidoglycan biosynthesis. The affinity of nisin for lipid II was estimated to be 2×10^7 M^{-1} (30). Fluorescence and circular dichroism experiments with lipid vesicles demonstrated that nisin uses all available molecules of lipid II in the membrane to form pore complexes of remarkable stability, which have a uniform structure and consist of eight nisin and four lipid II molecules (82). NMR analysis of the in vitro-made stoichiometric 1:1 nisin-lipid II complex solubilized in dimethyl sulfoxide demonstrated a cage-like motif of nisin in which the pyrophosphoryl moiety of lipid II interacts with backbone amide groups of nisin (89). Besides the binding of pyrophosphate via hydrogen bonds, the structure of the nisin-lipid II complex revealed minor interactions with the first isoprene unit and the MurNAc residue (89).

CONCLUDING REMARKS

As illustrated here by many examples, chemically or enzymatically synthesized lipid intermediates and analogues are now essential tools for the study of the functioning of the peptidoglycan membrane pathway, of the catalytic mechanisms of its enzymes, of the mode of action of many antibiotics, and of mechanisms of antibiotic resistance. Their ready availability is thus of primary importance. Lipid I and analogues were synthesized mainly by chemical methods, but the successful synthesis of lipid I by use of purified MraY (25, 60) could be extended to that of analogues. A possible drawback of this approach is the easy reversibility of the MraY-catalyzed reaction requiring the use of high lipid phosphate concentrations or a specific way of removing formed UMP. Lipid II was prepared by both enzymatic (29, 60, 197) and chemical (154, 184) methods, but most analogues (11, 39, 197) were made by use of the MurG-catalyzed reaction, taking advantage of its limited reversibility (11). The enzymatic approach offers the possibility of flexibility for specific isotopic labeling, of easy scaling up, and of accessibility to a fairly wide range of scientists. Its success is based on the low specificities of MraY and MurG for the lipid and peptide moieties of their substrate. The chemical approach implies greater costs and a specific expertise in organic synthesis but allows access to a larger variety of structural modifications incompatible with the MraY and MurG substrate specificities. The choice of one approach or another therefore depends on the intended use of the lipid intermediate.

Various modifications of lipids I and II have been reviewed here. Undoubtedly, many more are yet to be identified, as recently exemplified with lipid II from *M. smegmatis* (107). As already pointed out, the physiological significance of the structural modifications observed for peptidoglycan is not always well understood, and it is necessary to clearly distinguish between modifications of the lipid intermediates from those taking place after polymerization on nascent peptidoglycan. Future studies will thus imply the systematic analysis of complex pools of lipid intermediates. Presently, the only approach is to analyze the fragments recovered after extraction and chemical processing. More convenient and efficient analytical methods must be developed. Among the cellular aspects still poorly addressed, the main one is the mechanism of translocation of lipid II. Perhaps lipid II-antibiotic complexes could be useful tools in these matters, as illustrated by their recent use in the study of the dynamic assembly of peptidoglycan in *B. subtilis* (49, 171).

The main enzymes of the metabolism of the lipid intermediates have now been purified, and the resolution of the crystal structures of MurG (78), FemA (16), FemX (18), *S. aureus* PBP2 (106), and the *Aquifex aeolicus* GT domain (198) have been achieved. Although MraY was successfully purified (25), its structural study is a difficult challenge owing to its 10-segment transmembrane topology but remains a necessity, considering the potency of its numerous known inhibitors (57). The question is why no inhibitor has yet come into clinical use after so many years of effort. Some of the reasons for this were recently discussed (57). The successful study of the catalytic mechanism of MurG illustrates quite well the advantages of combining biochemical and structural approaches. Due to their location on the outside of the cytoplasmic membrane, peptidoglycan GTs are potential targets in the search for novel antibacterials, and the recent structural study of two GT domains (106, 198) offers new perspectives for the study of these enzymes, which have not yet been explored extensively. The next expected progress is the resolution of the structure of a complex with lipid II or a substrate analogue. However, these structural approaches do not exclude the urgent necessity for more-advanced biochemical studies leading to a better understanding of their catalytic mechanism and of the modes of action of inhibitors as diverse as moenomycin (191) and new glycopeptide derivatives (79, 93). Finally, it should be stressed that a number of activities responsible for secondary modifications (amidation and esterification, etc.) of the lipid intermediates remain to be identified and studied.

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