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MINI REVIEW

Formation of the glycan chains in the synthesis of bacterial peptidoglycan[1]

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The main structural features of bacterial peptidoglycan are linear glycan chains interlinked by short peptides. The glycan chains are composed of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), all linkages between sugars being β,1→4. On the outside of the cytoplasmic membrane, two types of activities are involved in the polymerization of the peptidoglycan monomer unit: glycosyltransferases that catalyze the formation of the linear glycan chains and transpeptidases that catalyze the formation of the peptide cross-bridges. Contrary to the transpeptidation step, for which there is an abundant literature that has been regularly reviewed, the transglycosylation step has been studied to a far lesser extent. The aim of the present review is to summarize and evaluate the molecular and cellullar data concerning the formation of the glycan chains in the synthesis of peptidoglycan. Early work concerned the use of various *in vivo* and *in vitro* systems for the study of the polymerization steps, the attachment of newly made material to preexisting peptidoglycan, and the mechanism of action of antibiotics. The synthesis of the glycan chains is catalyzed by the N-terminal glycosyltransferase module of class A high-molecular-mass penicillin-binding proteins and by nonpenicillin-binding monofunctional glycosyltransferases. The multiplicity of these activities in a given organism presumably reflects a variety of *in vivo* functions. The topological localization of the incorporation of nascent peptidoglycan into the cell wall has revealed that bacteria have at least two peptidoglycan-synthesizing systems: one for septation, the other one for elongation or cell wall thickening. Owing to its location on the outside of the cytoplasmic membrane and its specificity, the transglycosylation step is an interesting target for antibacterials. Glycopeptides and moenomycins are the best studied antibiotics known to interfere with this step. Their mode of action and structure–activity relationships have been extensively studied. Attempts to synthesize other specific transglycosylation inhibitors have recently been made.

Key words: peptidoglycan/transglycosylation/penicillin-binding proteins/monofunctional glycosyl-transferases/ lycopeptide and moenomycin antibiotics.

Introduction

Peptidoglycan (or murein) is a continuous covalent macromolecular structure located on the outside of the cytoplasmic membrane of almost all eubacteria, and it is found exclusively in these organisms (Schleifer and Kandler, 1972; Rogers *et al.*, 1980). Its main function is to preserve cell integrity by withstanding the internal osmotic pressure. It is also responsible for the maintenance of a defined cell shape and is intimately involved in the cell division process (Nanninga, 1998). Its absence from cells will in a hypotonic medium lead to swelling and to the rupture of the cytoplasmic membrane. Under certain conditions, cells lacking peptidoglycan can be maintained as protoplasts, or spheroplasts, but they lose their shape and cell division is impeded or greatly perturbed. The main structural features of this giant macromolecule are linear glycan chains interlinked by short peptides (Figure 1). The glycan chains are composed of alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), all linkages between sugars being β,1→4. The carboxyl group of each *N*-acetylmuramic acid residue is substituted by a peptide subunit, which is most often L-alanyl-γ-D-glutamyl-diaminopimelyl(or L-lysyl)-D-alanyl-D-alanine in nascent peptidoglycan and which subsequently loses one or both D-alanine residues in mature peptidoglycan. Neighboring glycan chains are interlinked either by a direct peptide linkage between a peptide subunit of a chain with one of another chain or by a short peptide bridge between two peptide subunits.

The biosynthesis of peptidoglycan has been investigated in various organisms, and an overall view valid for both Gram-positive and Gram-negative bacteria has emerged (Rogers *et al.*, 1980; Ward, 1984; Bugg and Walsh, 1992; Matsuhashi, 1994; van Heijenoort, 1994, 1998). The first stage concerns the assembly of the disaccharide-peptide monomer unit via a series of UDP precursors and lipid intermediates (Figure 2). Six cytoplasmic steps (mediated by MurA to MurF) lead to the formation of the UDP-MurNAc-pentapeptide precursor from UDP-GlcNAc. Thereafter, the transfer of the phospho-MurNAc-pentapeptide moiety of UDP-MurNAc-pentapeptide to the membrane acceptor, undecaprenyl phosphate, is catalyzed by transferase MraY and yields lipid I. The addition of GlcNAc to lipid I by transferase MurG leads to lipid II, which carries the complete disaccharide peptide monomer unit: GlcNAc-MurNAc-L-Ala-γ-D-Glu-A_2pm(or L-Lys)-D-Ala-D-Ala. In the course

[1]In the peptidoglycan literature the term transglycosylase has been used not only for the activities catalyzing the formation of the peptidoglycan chains but also for activites degrading the macromolecule. To avoid confusion with these extensively studied autolytic activities the term glycosyltransferase was preferred here.

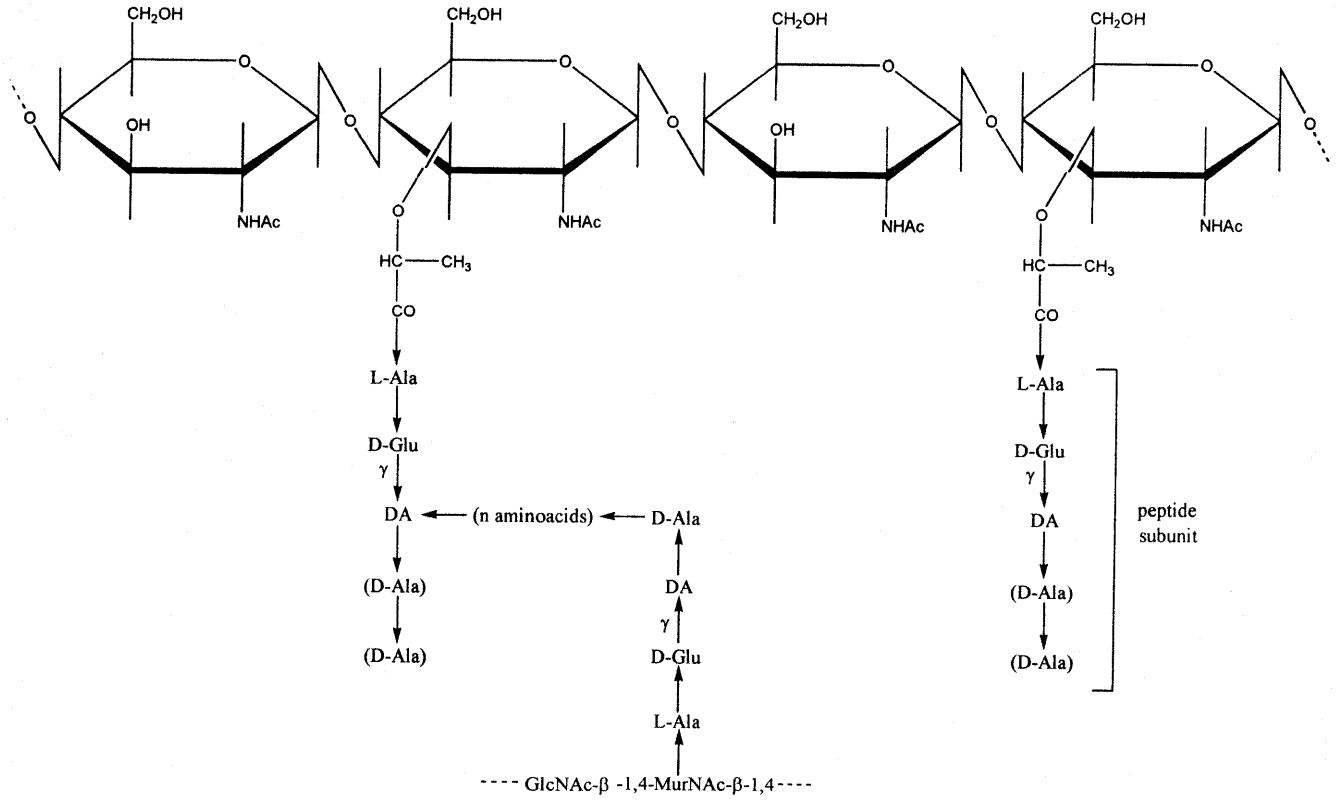


Fig. 1. Scheme of the primary structure of bacterial peptidoglycan. Abbreviations: GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid; DA: diamino acid (generally diaminopimelic acid or L-lysine); n: number of amino acids in the cross-bridge (n = 0 to 5 depending on the organism). D-Ala: often missing in the peptidoglycan of many organisms.

of the formation of the lipid intermediates the peptide subunit can undergo various modifications (amidation, addition of extra aminoacids, etc.). The final lipid intermediate is transfered by an unknown mechanism through the hydrophobic environment of the membrane to the externally located sites of incorporation of the monomer unit into growing peptidoglycan.

The second stage of peptidoglycan synthesis concerns the polymerization of the monomer unit on the outside surface of the cytoplasmic membrane and the binding of newly made material to the preexisting cell wall (Figure 2). The comparison of the structure of peptidoglycan with that of its precursors was the key in understanding the main aspects of the biochemical mechanisms underlying peptidoglycan polymerization. Two major types of membrane-bound activities are involved: glycosyltransferases[1] that catalyze the formation of the linear glycan chains and transpeptidases that catalyze the formation of the peptide cross-bridges. In growing cells, polymerization reactions are accompanied by concomitant or subsequent structural modifications of peptidoglycan. Its closed covalent structure must continuously adjust to the requirements of surface growth and cell division. Each organism possesses a variety of specific peptidoglycan hydrolases and certain of them are responsible for the structural adjustments (Shockman and Höltje, 1994; Smith *et al.*, 2000).

As targets of the β-lactam antibiotics and owing to their implication in certain mechanisms of resistance to these drugs, the transpeptidases have been extensively investigated. In particular, their active site has been well characterized (Ghuysen, 1991; Ghuysen and Dive, 1994). On the contrary, the transglycosylation step has been studied to a limited extent, although it is the initial key step in the assembly of the macromolecule from its monomer unit. This review is an attempt to bring together the essential molecular and cellular data with respect to the transglycosylation step. Early work dealing with the study of the polymerization reactions in various *in vivo* and *in vitro* systems is briefly summarized. Such approaches have been widely employed for studying the correlations between transglycosylation and transpeptidation, the attachment of newly synthesized material to preexisting peptidoglycan and the mechanisms of action of cell wall–targeted antibiotics. The main other topics of the review will concern: (1) the glycosyltransferases catalyzing the formation of the glycan chains; (2) the mechanisms of the transglycosylation reaction; (3) the *in vivo* functioning of the peptidoglycan glycosyltransferases; and (4) the inhibitors of the transglycosylation step.

Peptidoglycan polymerization in intact cells, membranes, crude cell walls, permeabilized cells, or protoplasts

In intact cells the synthesis of peptidoglycan has been followed by the incorporation of radiolabeled peptidoglycan constituents. Diaminopimelic acid has been used mostly in *Escherichia coli* (see references in Höltje and Schwarz, 1985 and Höltje, 1998; Wientjes *et al.*, 1985; Mengin-Lecreulx *et al.*, 1989; Prats and

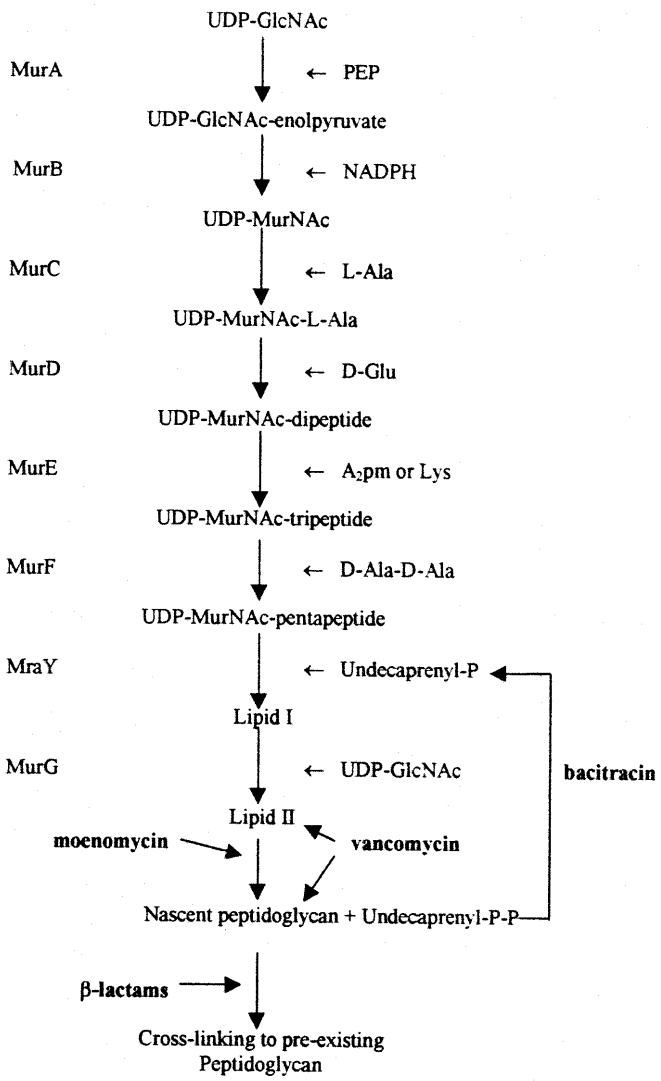


Fig. 2. Peptidoglycan synthesis and antibiotics interfering with polymerization.

de Pedro, 1989), in a few other Gram-negative bacteria (Hebeler and Young, 1976; Rosenthal, 1979; Greenway and Perkins, 1985), and more rarely in Gram-positive bacteria (Mauck *et al.*, 1971; Fuchs-Cleveland and Gilvarg, 1976). Lysine has been restricted to Gram-positive bacteria (Boothby *et al.*, 1971; Mirelman *et al.*, 1974), whereas *N*-acetyl-glucosamine has been used both in Gram-positive (Tynecka and Ward, 1975; Cheung *et al.*, 1983; Sinha and Neuhaus, 1991; Pooley and Karamata, 2000) and Gram-negative (Martin and Gmeiner, 1979; Greenway and Perkins, 1985; Rosenthal, 1979; Rosenthal and Dziarski, 1994; Payie and Clarke, 1997) organisms. Less frequently have glutamate (Mauck *et al.*, 1971; Hoffmann *et al.*, 1974; Keglevic *et al.*, 1974), alanine (Wise and Park, 1965; Waxman *et al.*, 1980; Rosenthal, 1994 and references therein), glycine (Wise and Park, 1965), and acetate (Dezélée and Shockman, 1975) been used. Peptidoglycan contents can also be followed by chemical quantification of a specific constituent, such as A_2pm (Mengin-Lecreulx and van Heijenoort, 1985). All these methods imply a separation of the polymerized material from its precursors.

In vitro peptidoglycan-synthesizing activity has been elicited with membrane preparations from various organisms (Chatterjee and Park, 1964; Meadow *et al.*, 1964; Anderson *et al.*, 1966; Izaki *et al.*, 1966; Araki *et al.*, 1966; Plapp and Strominger, 1970; Reynolds, 1971; Wickus and Strominger, 1972; Hammes and Neuhaus, 1974; Pellon *et al.*, 1976; van Heijenoort *et al.*, 1978; Kamio *et al.*, 1982; Kraus *et al.*, 1985). Crude cell walls from Gram-positive organisms retain to some extent the functional spatial interrelationships between the membrane and the cell wall and were found to be particularly appropriate for the *in vitro* study of the polymerization reactions (Mirelman and Sharon, 1972; Mirelman and Sharon , 1972; Ward, 1974; Ward and Perkins, 1974; Hammes and Kandler, 1976; Weston *et al.*, 1977; Kalomiris *et al.*, 1982). Ether- or toluene-permeabilized cells (Schrader and Fan, 1974; Mirelman *et al.*, 1977; Brown and Perkins, 1979; Mirelman and Nuchamowitz, 1979; Giles and Reynolds, 1979; Maass and Pelzer, 1981; Metz *et al.*, 1983; Kraus *et al.*, 1985; Pisabarro *et al.*, 1986) as well as protoplasts (Elliott *et al.*, 1975; Rosenthal and Shockman, 1975) have also been used.

In these various systems nucleotide precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide, one or the other being radiolabeled, have generally been employed as substrates, thereby implying a synthesis of the lipid intermediates prior to polymerization. In a few cases, the pentapeptide nucleotide was replaced by UDP-MurNAc-tri-, tetra-, or hexa-peptide (Izaki *et al.*, 1968; Plapp and Strominger, 1970; Hammes and Kandler, 1976; Pisabarro *et al.*, 1986). Isolated lipid II has also been used as substrate with membrane preparations (Strominger *et al.*, 1966; Izaki *et al.*, 1968; Taku and Fan, 1976; Kamio *et al.*, 1982). An assay specific for transglycosylation was developed by using purified lipid II, *E. coli* membranes, and penicillin as transpeptidation inhibitor (van Heijenoort *et al.*, 1978).

Incorporation of radioactivity has been taken as a measure of peptidoglycan polymerization and the resulting polymerized material has been characterized in different ways: mobility on paper chromatography, solubility or insolubility in SDS or TCA, structural analysis, glycan chain length, and extent of cross-linking. It should be stressed that the efficiency of these systems is generally very low when compared to peptidoglycan synthesis in growing cells: For instance, the *in vitro* specific polymerizing activity accounted for 0.1 to 1% of the *in vivo* level in *Staphylococcus aureus* (Anderson *et al.*, 1966) and for 2% in *E. coli* (Kraus *et al.*, 1985). However, a high specific activity was reported with membranes from *Gaffkya homari* (Hammes and Neuhaus, 1974). Furthermore, the structure of the *in vitro* polymerized material can differ in many ways from that of native peptidoglycan (Kraus *et al.*, 1985). It must thus be cautioned that results obtained with such systems could reflect imperfectly the true *in vivo* processes.

Correlation between transglycosylation and transpeptidation

In normally growing bacteria, transglycosylation catalyzing the formation of the glycan chains and transpeptidation catalyzing the cross-linking between peptide subunits are continuous, tightly coupled reactions. The problems of their correlation and of the attachment of newly synthesized

material to preexisting peptidoglycan have been investigated with numerous *in vivo* and *in vitro* systems. The conclusions drawn from these studies (Rogers *et al.*, 1980; Ward, 1984) can be briefly summarized as follows.

1. Transglycosylation can proceed independently from transpeptidation as exemplified by the formation of uncross-linked or low cross-linked soluble peptidoglycan in various cell-free systems and in protoplasts. Furthermore, the treatment of growing cells or cell-free peptidoglycan-synthesizing systems with β-lactam antibiotics, which are specific inhibitors of the transpeptidation reactions, results in the formation of soluble uncross-linked peptidoglycan material. In a few cases it has been shown that the soluble material can function as an intermediate in the synthesis of cell wall–linked peptidoglycan.
2. The question remains as to whether nascent peptidoglycan is present or not as soluble material in normally growing cells. It has been proposed that perhaps small amounts of nascent uncross-linked peptidoglycan are transiently synthesized prior to cross-linking (Ward, 1984). It should be stressed that when considering low levels of soluble peptidoglycan material it is difficult to distinguish between true nascent material and possible autolytic degradation products.
3. Conversely, a transpeptidation reaction without prior or concomitant formation of glycan chains has been reported only in very special cases (Kraus *et al.*, 1985) or with model peptides (Lepage *et al.*, 1997). Moreover, the specific inhibition of transglycosylation by moenomycin-type antibiotics does not lead to any polymerization by transpeptidation (Huber, 1979 and references therein; Suzuki *et al.*, 1980). In peptidoglycan the glycan chains are generally quite longer than the peptide chains, and only some of the monomer units are involved in cross-bridges. This makes the addition of monomer units to preexisting peptidoglycan by transpeptidation and their subsequent interlinking by transglycosylation very unlikely.

Taken together, these facts suggest that in growing cells the polymerization of the monomer unit proceeds essentially by tranglycosylation, which precedes cross-linking to the cell wall. Transpeptidation is responsible not only for the formation of cross-bridges in the new material but also for the transfer of newly made material to preexisting peptidoglycan. It follows glycan chain formation or is at best concomitant with it. The possibility of some attachment of newly made material to old cell wall by transglycosylation has not been entirely ruled out (Mirelman and Sharon, 1972; Weston *et al.*, 1977). The average length of the glycan chains synthesized in intact cells or in cell-free systems can vary from 10 to over 100 disaccharide units. The length distribution of the glycan chains was determined in *E. coli* (Harz *et al.*, 1990).

Peptidoglycan multimodular bifunctional polymerases

Virtually all eubacteria possess a set of minor membrane proteins designated as penicillin-binding proteins (PBPs) that are the specific targets of the β-lactam antibiotics and that are involved in the late steps of peptidoglycan synthesis (references in Ghuysen, 1991; Ghuysen and Dive, 1994). They are detected by their ability to covalently bind radiolabeled penicillin and the stability of the penicillin-protein complexes has greatly facilitated their study. PBPs vary from species to species in number, size, amount, and affinity for β-lactam antibiotics. High-molecular-mass PBPs (HMM-PBPs) are essentially two-domain proteins that belong either to class A or class B, depending on the structure and the catalytic activity of their N-terminal domain (Goffin and Ghuysen, 1998). The C-terminal domain of both classes is responsible for transpeptidation activity and β-lactam antibiotics covalently bind to its catalytic center. In class A HMM-PBPs, the N-terminal domain is responsible for their glycosyltransferase activity, whereas in class B the N-terminal is presumably involved in interactions with other membrane proteins (Marrec-Fairley *et al.*, 2000). Therefore, class A HMM-PBPs are bifunctional enzymes capable of catalyzing both transglycosylation and transpeptidation. About 30 class A HMM-PBPs from both Gram-positive and Gram-negative bacteria have now been identified on the basis of genome sequence data (Goffin and Ghuysen, 1998). Their non-penicillin-binding domains are a continuum of diverging sequences. The alignments of their amino acid sequences revealed five conserved motifs.

PBP1b from *E. coli* has been by far the most investigated class A HMM-PBP in terms of location, membrane topology, purification, and glycosyltransferase activity. The organization of this membrane-associated polymerase was analyzed by studying β-lactamase fusion proteins (Edelman *et al.*, 1987) and by immunoelectron microscopy (den Blaauwen and Nanninga, 1990). PBP1b contains a 63-amino acid N-terminal cytoplasmic tail, followed by a 24-amino acid transmembrane domain and a 757-amino acid periplasmic region with the transglycosylation and transpeptidation domains clearly separated by an inert linker (Nakagawa *et al.*, 1984; Lefèvre *et al.*, 1997). The existence of a well-defined transglycosylation domain was futher substantiated by the production of truncated forms retaining glycosyltransferase activity (Nakagawa *et al.*, 1984; Terrak *et al.*, 1999). An additional membrane association site not apparent by hydropathy analysis was localized in the first 163 aminoacids of the periplasmic N-terminal glycosyltransferase domain of a form lacking the cytoplasmic and transmembrane domains (Wang *et al.*, 1996). A similar situation was also more recently observed in the glycosyltransferase domain of PBP2a from *Streptococcus pneumoniae* (Di Guilmi *et al.*, 1999). In the cell envelope of *E. coli,* PBP1b is present as a monomer and as dimers, one of which is closely associated with peptidoglycan (Zijderveld *et al.*, 1995). Evidence has been brought by immunoelectron microscopy for its localization at the adhesion sites of the envelope (Bayer *et al.*, 1990). Such a clustered distribution of peptidoglycan-polymerizing activity was further substantiated by fractionation procedures (Ishidate *et al.*, 1986). The periplasmic localization of the transglycosylation domain is in agreement with a polymerization of the disaccharide-peptide monomer unit taking place on the outside of the cytoplasmic membrane.

To date, the overproduction and purification of class A polymerases with a biochemically characterized glycosyltransferase activity have been restricted essentially to the three present in *E. coli*: PBP1a (Tamura *et al.*, 1980; Ishino *et al.*, 1980), PBP1b (Tamura *et al.*, 1980; Suzuki *et al.*, 1980; Nakagawa *et al.*, 1984; van Heijenoort *et al.*, 1992; Terrak *et al.*, 1999), and PBP1c (Schiffer and Höltje, 1999). The alignment of their sequences covering both the transglycosylation

and transpeptidation domains revealed a clear homology between all three PBPs (Schiffer and Höltje, 1999). When assayed with lipid II as substrate and penicillin as transpeptidation inhibitor, their specific glycosyltransferase activity was greatly dependent on the presence of a solvent (CH_3OH, DMSO, 1-octanol) and/or a detergent (decyl-PEG, sodium deoxycholate, etc.) required for the solubilization of both the substrate and the enzyme. Various properties of *E. coli* PBP1b, in particular kinetic parameters, were studied (Nakagawa *et al.*, 1984; Terrak *et al.*, 1999). In the best assay yet described (Terrak *et al.*, 1999), the reaction proceeds with an efficiency of 39,000 M^{-1} s^{-1}. The accessibility to reasonable amounts of lipid II remains a critical point for the development of enzymatic studies. Its preparation is a tedious affair that has been carried out only in a few cases (see references in: Umbreit and Strominger, 1972; Hara and Suzuki, 1984; van Heijenoort *et al.*, 1992). Other class A polymerases have been purified: PBP1a and PBP2a from *S. pneumoniae* (Di Guilmi *et al.*, 1998, 1999). However, appropriate glycosyltransferase assays remain to be developed for them. A polymerase purified from *Bacillus megaterium* that catalyzed the *in vitro* synthesis of uncross-linked peptidoglycan was tentatively identified as PBP4, but it is not yet known if it belongs to class A polymerases (Taku *et al.*, 1982).

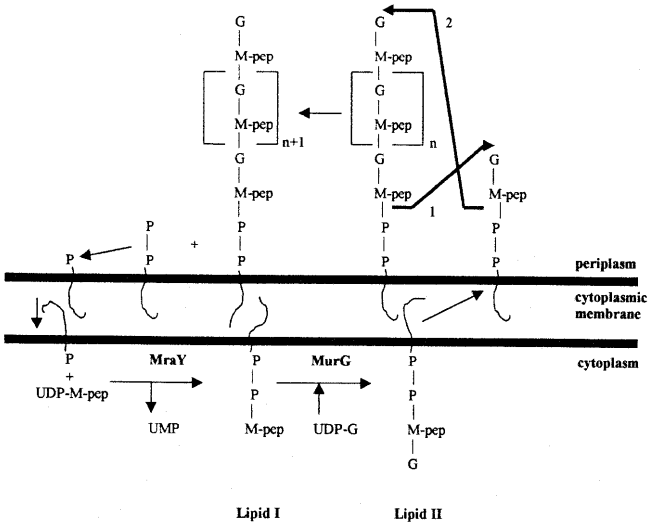


Fig. 3. Extension of a glycan chain by transglycosylation. 1: elongation at the reducing end of the glycan chain; 2: elongation at the nonreducing end of the glycan chain; G: GlcNAc; M: MurNAC; P : undecaprenyl phosphate; P-P : undecaprenyl pyrophosphate; pep: pentapeptide.

Monofunctional glycosyltransferases

A number of membrane-bound, non-penicillin-binding, monofunctional glycosyltransferases (Mgt) capable of catalyzing only the formation of uncross-linked peptidoglycan were found in *E. coli* (Hara and Suzuki, 1984), *Micrococcus luteus* (Park and Matsuhashi, 1984), *S. aureus* (Park and Matsuhashi, 1984), and *S. pneumoniae* (Park and Burman , 1985). They were solubilized, partially purified and characterized. They accounted for a large part of the *in vitro* measurable glycosyltransferase activity determined with lipid II as substrate. More recently, in a variety of bacterial strains (*E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Ralstonia eutropha*, *S. aureus*) genes encoding monofunctional glycosyltransferases were detected on the basis of sequence homology with genes encoding class A HMM-PBPs (Spratt *et al.*, 1996; Di Berardino *et al.*, 1996; Paik *et al.*, 1997). In *E. coli* overexpression of the the *mgt* gene led to a significant increased *in vitro* peptidoglycan-synthesizing activity (Di Berardino *et al.*, 1996). A soluble form of the *R. eutropha mgt* gene product was obtained, thereby opening the way to a more easy approach to a structural analysis of a peptidoglycan glycosyltransferase domain (Paik *et al.*, 1997).

Mechanism of the transglycosylation reaction

The exact mechanism of the transglycosylation reaction is still not known. In the periplasm the linear assembly of the glycan chains presumably proceeds by the repetitive addition of dissaccharide peptide units either at their reducing end (1 in Figure 3) or at their nonreducing end (2 in Figure 3). In the first case, the growing glycan chain attached to undecaprenyl pyrophosphate is the glycosyl donor substrate and is transferred to the 4-OH group of the GlcNAc unit of lipid II, which is the glycosyl acceptor substrate. This mechanism has in fact been established for transglycosylation in a few Gram-positive organisms (Ward and Perkins, 1973; Fuchs-Cleveland and Gilvarg, 1976; Weston *et al.*, 1977). It is noteworthy that lipopolysaccharide O-antigene polymerization proceeds in a similar manner (Raetz, 1996). The other possibility is that lipid II is functioning as the donor and its disaccharide-peptide is transferred to the 4-OH group of the GlcNAc of the growing chain, which is the acceptor and is not necessarily bound to undecaprenyl pyrophosphate. In the *in vitro* polymerization reaction with lipid II and purified PBP1b, chain elongation is initiated in the absence of any growing chain (Suzuki *et al.*, 1980; Nakagawa *et al.*, 1984), suggesting that lipid II can be recognized by both the donor and acceptor sites of the enzyme and that even with an elongation at the nonreducing end the growing chain must be initially bound to undecaprenyl pyrophosphate. However, both isolated mature peptidoglycan and various forms of nascent peptidoglycan are generally devoid of any linkage to undecaprenyl pyrophosphate. Only in a few cases was such a linkage found (Ward and Perkins, 1973; Fuchs-Cleveland and Gilvarg, 1976; Thorpe and Perkins, 1979). Therefore, some enzymatic process leading to the release of the glycan chain from undecaprenyl pyrophosphate must take place.

Very little is known about the structure of the catalytic center of peptidoglycan glycosyltransferases. Unfortunately, crystallization of a purified form of *E. coli* PBP1b has not led to the elucidation of its three-dimensional structure (Ishino *et al.*, 1988). However, the 3D structures of four *N*-acetylmuramidases (three lysozymes and transglycosylase Slt70) involved in the breakdown of the peptidoglycan glycan chains are now known (Thunnissen *et al.*, 1995). They have the same catalytic machinery and the overall packing of the secondary structure elements of their catalytic centers is similar. By the cleavage of β-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine bonds and the release of disaccharide peptide units, they each catalyze a reaction that is the reverse of the polymerization reaction.

Thus, they were helpful for shedding light on the organization of the catalytic center and mechanism of *E. coli* PBP 1b (Terrak *et al.*, 1999). In the transglycosylation reaction it is likely that the sugar units of both lipid II and of a large piece of the growing chain are recognized by multiple sites of the polymerase, as already established in a number of glycosyltransferases that recognize both a glycosyl donor and a glycosyl acceptor (Kosmol *et al.*, 1997 and references therein). These sites could be related to the binding sites of the peptidoglycan N-acetylmuramidases, which have several subsites each for one sugar unit of peptidoglycan (Thunnissen *et al.*, 1995). The peptide moiety of the monomer unit plays a minor role in the recognition process as exemplified with shorter or modified peptide subunits (see references in van Heijenoort and Gutmann, 2000).

A speculative transition state involving the formation of an oxicarbenium cation was proposed for the peptidoglycan glycosyltransferases (Kosmol *et al.*, 1997; Ritzeler *et al.*, 1997; Terrak *et al.*, 1999). The transglycosylation reaction is an SN2 displacement leading to an inversion of configuration at C1, from the α-configuration in the precurseur to the β-configuration in the glycosylated nucleophile acceptor (Figure 4). From the analysis of residues that are central to catalysis in the N-acetylmuramidases, the role of possible homologous residues of PBP1b was investgated (Terrak *et al.*, 1999). Conserved dicarboxylic amino acid residues are probably elements of the glycosyltransferase catalytic center. It was proposed that in *E. coli* PBP1b the Glu-233 γCOOH donates its proton to the oxygen atom of the scissile phosphoester bond of the growing chain, leading to the formation of an oxicarbenium cation that undergoes attack by the 4-OH group of a nucleophile *N*-acetylglucosamine. Asp-234 and Glu-290 could be involved in the stabilization of the oxicarbenium cation and the activation of the 4-OH group.

In vivo functioning of the peptidoglycan glycosyltransferases

In a given organism there are several peptidoglycan glycosyltransferases (Goffin and Ghuysen, 1998). For instance, there are four in *E. coli* (PBP1a, PBP1b, PBP1c, and Mgt) and four in *S. pneumoniae* (PBP1a, PBP1b, PBP2a, and Mgt).

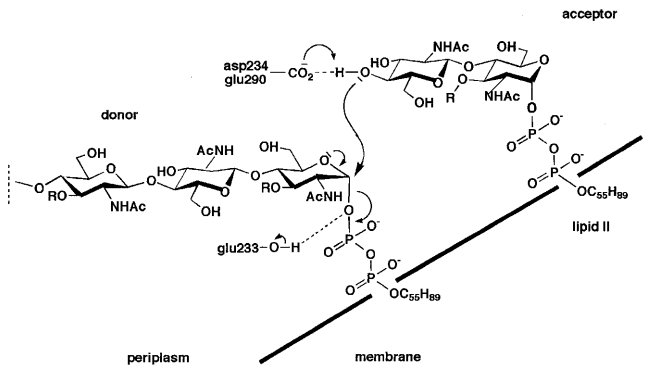


Fig. 4. Mechanism of transglycosylation with chain elongation at the reducing end. R = D-lactoyl-pentapeptide. The indicated dicarboxylic amino acid residues are those of the glycosyltransferase module of *E. coli* PBP1b presumably involved in the catalytic process.

Presumably, this multiplicity corresponds to a variety of functions. However, not all peptidoglycan glycosyltransferases are essential. In *E. coli* PBP1c and Mgt can be deleted, whereas either PBP1a or PBP1b must be present (Suzuki *et al.*, 1978; Spratt, 1983; Di Berardino *et al.*, 1996; Denome *et al.*, 1999). In *S. pneumoniae* class A PBPs 1a, 1b, and 2a are not individually essential but PBPs 1a and 2a together are (Hoskins *et al.*, 1999; Paik *et al.*, 1999). It has been proposed that nonessential peptidoglycan glycosyltransferases could functionally contribute to the permeability characteristics of the cell envelope (Dijkstra and Keck, 1996). They could be involved in localized peptidoglycan rearrangements accompanying the assembly of envelope-spanning complexes and various transfer processes through the envelope.

Considering that peptidoglycan is a continuous structure intricately associated with other macromolecules of the cell envelope, its assembly must proceed in coordination with other cell syntheses and also with the different steps of the cell cycle, such as elongation and septation. It is thus a highly complex process that is not readily accessible to biochemical analysis, and it involves both spatial and temporal controls. The topological localization of the incorporation of nascent peptidoglycan has been analyzed by various labeling experiments, mostly in *E. coli* (see leading references in Höltje, 1998) and by electron microscopy in cocci (Shockman *et al.*, 1974). Bacteria have at least two peptidoglycan-synthesizing systems. In rod-shaped organisms one is responsible for cell elongation, whereas the other one is involved in cell septation. In cocci most material is incorporated during the septation process and a second system is involved in cell wall thickening. It is noteworthy that there are a number of similarities between the polymerization of bacterial peptidoglycan and the formation of cell wall chitin in yeast (references in Osmond *et al.*, 1999; Valdivieso *et al.*, 1999). Chitin consists mainly of unbranched chains of β,1→4 linked N-acetylglucosamine residues and thus resembles the glycan chains of peptidoglycan. Although making up only 1–2% of the cell wall of *Saccharomyces cerevisiae*, like peptidoglycan, it is important for cell integrity. It is found in a ring at the base of the emerging bud, at the septum and in the lateral wall. Furthermore, *S. cerevisiae* has a multiplicity of functionally distinct chitin synthases.

In *E. coli* it was established that single glycan chains are inserted into the peptidoglycan during cell elongation (see references in Höltje, 1998). This process requires the accompanying hydrolytic cleavage of preexisting peptide cross-bridges or glycan chains and the cross-linking by transpeptidation to old material. In one model it was proposed that the bifunctional polymerase moves around the sacculus and inserts a single new strand between preexisting ones (Park and Burman, 1985). In another model it was postulated that new material is attached to the existing peptidoglycan before covalent bonds are cleaved to promote its insertion into the stress-bearing layer (Höltje, 1998). To take into account the complexity of the spatial and temporal constraints, as well as the multiple functional interactions, it was hypothesized that there are two peptidoglycan-synthesizing multienzyme complexes: one for elongation, the other one for septation (Höltje, 1998). Polymerases PBP1a and/or PBP1b would be present in both complexes, and their roles would be to provide a common peptidoglycan primer that could be processed for elongation or septation by proteins specific of each complex (Wientjes and Nanninga,

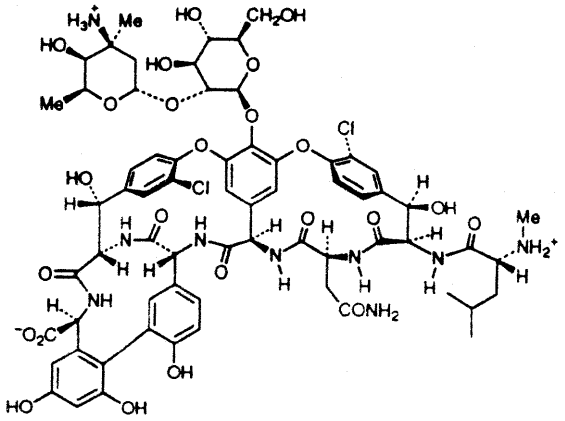
1991). Recently, possible *in vivo* protein–protein interactions between PBP1b and other putative proteins of these complexes were substantiated by *in vitro* binding experiments (Vollmer *et al.*, 1999).

Two other compounds are involved in the transglycosylation reaction, namely, its substrate lipid II and released undecaprenyl pyrophosphate. The rate of transport of lipid II through the membrane and that of its input in the reaction must match the rate of peptidoglycan synthesis, which is about 1000 units per second in *E. coli* (van Heijenoort *et al.*, 1992). Because the lipid II pool is low (1000–2000 copies per cell) in this organism, it has been discussed (van Heijenoort, 1994) whether it freely diffuses over the whole membrane or whether it is tunneled directly to the periplasmic peptidoglycan polymerases from transferases MraY and MurG, which catalyze its synthesis. This would imply a highly integrated coordinated process between the synthesis, transport, and use of the lipid substrate, as illustrated by the possible association of MraY and MurG in a peptidoglycan-synthesizing complex (Nanninga, 1998). In *S. aureus* there is evidence for lipid intermediate-protein interactions, which could indicate an association with MraY in a peptidoglycan-synthesizing complex (Weppner and Neuhaus, 1978). The undecaprenyl pyrophosphate released in the course of the transglycosylation reaction, both from the donor and the acceptor, is dephosphorylated to undecaprenyl phosphate, which is again available for the formation of lipid intermediates (Rogers *et al.*, 1980; Ward, 1984). The phosphatase catalyzing the dephosporylation was studied in one case (Goldman and Strominger, 1972).

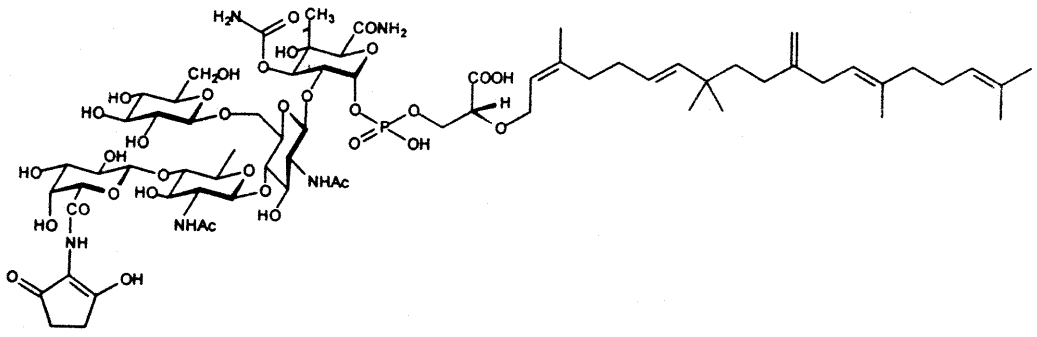
Inhibitors of the transglycosylation reaction

Glycopeptides and moenomycins are the two best studied groups of antibiotics known to interfere with the transglycosylation reaction (see references in Huber, 1979 and Reynolds, 1989). Although their modes of action differ, they possess certain common features: large molecular masses, no penetration into cells, preferential *in vivo* action on Gram-positive organisms, and an accumulation of peptidoglycan precursors in intact cells and in cell free systems. The nonpenetration of such inhibitors further substantiates the localization of the polymerization reactions on the outside of the cytoplasmic membrane.

Vancomycin (Figure 5) and teicoplanin are the main two members of the glycopeptide group clinically used against Gram-positive pathogens. The different aspects of the work on



VANCOMYCIN



MOENOMYCIN A

Fig. 5. Antibiotics interfering with the transglycosylation reaction.

these antibiotics has been extensively reviewed (see referecnes in Reynolds, 1989 and Williams and Bardsley, 1999). Moreover, the emergence of vancomycin resistance in enterococci has led to the characterization of a modified peptidoglycan metabolism and to an important literature on the subject (see references in Arthur *et al.*, 1996). The mode of action of glycopeptides involves the specific noncovalent binding to the C-terminal D-alanyl-D-alanine motif of the peptidoglycan monomer unit. The structures of various glycopeptides, including vancomycin, have been derived from nuclear magnetic resonance and X-ray studies and have led to a model of the binding involving specific hydrogen bonds and other noncovalent interactions (Williams, 1996). Because glycopeptides do not cross the membrane, complexes are formed only with the D-alanyl-D-alanine termini of exported lipid II or with those of nascent peptidoglycan. In the first case, the resulting segregation of the lipid substrate will lead to an arrest of glycan chain elongation; in the second case the binding to the peptide subunits of growing chains can by steric hindrance have a shielding effect on both transglycosylation and transpeptidation. Presently, it is difficult to assess whether both mechanisms are functioning *in vivo* or whether one is predominant. Recently, it has been established that other factors are involved in the antibacterial activity of glycopeptides (Williams and Bardsley, 1999 and references therein). In particular, it was found that glycopeptides (other than teicoplanin) form dimers in aqueous solution and that this dimerization is cooperative with ligand binding. It was proposed that dimerization facilitates the binding between the antibiotic dimer and the membrane-anchored peptidoglycan precursors, thereby leading to the formation of a complex. The structure–activity relationships of the vanomycin-type glycopeptide antibiotics have been evaluated in detail (Nagarajan, 1993).

Moenomycins are phosphoglycolipid compounds produced as complexes of very similar components by various *Streptomyces* species and are used as growth promoter in animal nutrition (Huber, 1979). The structure (Figure 5) of the main constituent, moenomycin A, was determined by Welzel and co-workers (Welzel *et al.*, 1981, 1983). A very similar structure was reported for the related antibiotic, pholipomycin (Takahashi *et al.*, 1983; Scherkenbeck *et al.*, 1993). More recently, a 3-D structure of moenomycin A in aqueous solution was presented (Kurz *et al.*, 1998). Moenomycins interfere with the transglycosylation reaction as substantiated by their effect on the polymerizing activity of cell-free systems (Huber, 1979 and references therein; van Heijenoort and van Heijenoort, 1980) and that of *E. coli* PBP1b assayed with lipid II (Suzuki *et al.*, 1980; Nakagawa *et al.*, 1984). Moreover, moenomycin was shown to bind reversibly to *E. coli* PBP1b (van Heijenoort *et al.*, 1987). Owing to structural similarities between moenomycin and lipid II it has been proposed that the drug might compete with the substrate for a binding site on the enzyme (Linnett and Strominger, 1973; van Heijenoort *et al.*, 1987). However, from the study of simplified models of peptidoglycan and moenomycin A, it was found they could be superimposed on the basis of the sugar chains with a perfect overlapping of four sugar rings (Ritzeler *et al.*, 1997). This clearly suggests that moenomycin can compete with the growing chain for binding to the polymerase and is in agreement with the possible presence on the polymerase of binding sites for several sugar units. It is noteworthy that not all class A HMM-PBPs are sensitive to moenomycin (Paik *et al.*, 1999; Hoskins *et al.*, 1999). Similarly, the *S. aureus* and *S. pneumoniae* Mgts are sensitive to moenomycin (Park and Matsuhashi, 1984; Park and Burman , 1985) but not the *E. coli* and *M. luteus* Mgts (Hara and Suzuki, 1984; Park and Matsuhashi, 1984). Furthermore, moenomycin can interact with proteins devoid of peptidoglycan glycosyltransferase activity at concentrations 100- to 1000-fold higher than that inhibiting PBP1b (Graves-Woodward and Pratt, 1999, and references therein).

The structure–activity relationships of moenomycin A were evaluated by degradation studies and synthesis of analogues (El-Abadla *et al.*, 1999 and references therein). Minimum structural requirements for biological activity were determined with *in vitro* and *in vivo* assays and led to a core structure that retained glycosyltransferase inhibitory activity. The oligosaccharide part of moenomycin is recognized with high selectivity. There are two recognition sites for moenomycin-type transglycosylation inhibitors on the enzyme: one at the donor site and one at the acceptor site. Like moenomycin A itself, structural analogues with at least three sugars bind presumably to the donor site. They are active against Gram-positive bacteria as well as in the *in vitro* enzyme assay. Moenomycin analogues with two sugars are more or less inactive *in vivo* but do inhibit the enzyme *in vitro* provided they have the right substitution pattern. Their binding to the donor or acceptor site is not yet clearly established. The C_{25} lipid moiety (Figure 5) appears to be essential because its removal (Marzian *et al.*, 1994) or the introduction of one or several OH groups into the lipid chain (Kempin *et al.*, 1995) caused complete loss of inhibitory activity. However, its role in the anchoring of the antibiotic to the cytoplasmic membrane has remained elusive. An experimental system allowing the investigation of the interaction of moenomycins with artificial membranes has recently been developed (Anikin *et al.*, 1999).

Yet other compounds have been found to inhibit glycan chain formation. This is the case of mersacidin which is a lantibiotic (Brötz *et al.*, 1997). Recently, vancomycin derivatives that are active against vancomycin-resistant organisms and that inhibit peptidoglycan synthesis without binding to D-Ala-D-Ala were described (Allen *et al.*, 1996; Ge *et al.*, 1999; Goldman *et al.*, 2000). They interfere with transglycosylation by an as yet unknown mechanism. Based on the conserved inhibitory activity of certain moenomycin-derived saccharides and on structural comparisons between the lipid substrate and the peptidoglycan monomer unit, attempts have recently been made to synthesize specific inhibitors (Hecker *et al.*, 1990; Qiao and Vederas, 1993; Moss and Southgate, 1993; Brooks *et al.*, 1995). None of the compounds that were tested were active because they lacked important structural features of moenomycins. More recently, the solid phase synthesis of a library of moenomycin disaccharide analogues has led to the identification of novel compounds *in vitro* (Sofia *et al.*, 1999). Although far less potent than moenomycin A, they possessed antibacterial activity and were inhibitors of *in vitro* peptidoglycan synthesis. Assays for the identification of compounds that interfere with the binding of moenomycin to the glycosyltransferase site of *E. coli* PBP 1a and PBP 1b (Vollmer and Höltje, 2000) or that inhibit the transglycosylation step (Branstrom *et al.*, 2000) have recently been proposed.

Concluding remarks

Most of our current knowledge about peptidoglycan glycosyltransferases concerns *E. coli* PBP1b. With the development of genomics many other ones should now be accessible. However, the study of their enzymatic properties and mechanism of catalysis is dependent on the ready availability of their lipid substrate and the development of efficient assays. The determination of the crystal structure of soluble forms of a peptidoglycan glycosyltransferase will be a crucial step for understanding the mechanism of the transglycosylation reaction and it is essential that efforts be made on this topic. These different approaches will also undoubtedly be helpful for the design of new specific inhibitors. By its location on the outside of the cytoplasmic membrane and its specificity, the transglycosylation step remains an attractive potential target for the search for novel antibacterials. The variable inhibitory effect of moenomycin suggests a possible variability in the binding sites of the sugar units. Because the substrate (lipid II) and the product (uncrosslinked glycan chains) are very similar in all peptidoglycan transglycosylation reactions, the structure of the catalytic site could be less variable, in which case transition state analogs would be more suitable general inhibitors. Among the many unanswered questions with respect to the *in vivo* functioning of the transglycosylation step a few are of particular interest. The mechanism of translocation of the lipid intermediate substrate and that of the undecaprenyl pyrophosphate product are yet completely unexplored. A possible approach could be to study the effects of conditional mutations in putative translocases on peptidoglycan synthesis. As for the monofunctional glycosyltransferases, further studies need to focus on their location in the cytoplasmic membrane, their essentiality, and their exact *in vivo* function. Finally, it should be stressed that unraveling the precise organization of the hypothetical peptidoglycan-synthesizing complexes will undoubtedly remain a highly challenging problem.

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Abbreviations

A_2pm, diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; HMM, high-molecular mass; Mgt, monofunctional glycosyltransferase; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein.

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