# Glycosyl Transferase Activity of the *Escherichia coli* Penicillin-Binding Protein 1b: Specificity Profile for the Substrate†

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ABSTRACT: The glycosyl transferase of the *Escherichia coli* bifunctional penicillin-binding protein (PBP) 1b catalyzes the assembly of lipid-transported *N*-acetylglucosaminyl-$\beta$-1,4-*N*-acetylmuramoyl-L-Ala-$\gamma$-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala units (lipid II) into linear peptidoglycan chains. These units are linked, at C1 of *N*-acetylmuramic acid (MurNAc), to a $C_{55}$ undecaprenyl pyrophosphate. In an in vitro assay, lipid II functions both as a glycosyl donor and as a glycosyl acceptor substrate. Using substrate analogues, it is suggested that the specificity of the enzyme for the glycosyl donor substrate differs from that for the acceptor. The donor substrate requires the presence of both *N*-acetylglucosamine (GlcNAc) and MurNAc and a reactive group on C1 of the MurNAc and does not absolutely require the lipid chain which can be replaced by uridine. The enzyme appears to prefer an acceptor substrate containing a polyprenyl pyrophosphate on C1 of the MurNAc sugar. The problem of glycan chain elongation that presumably proceeds by the repetitive addition of disaccharide peptide units at their reducing end is discussed.

To fight antibiotic resistance, the discovery of new targets and new antibacterial agents is a challenging problem. Because it is exclusively present in bacteria and essential for their survival, the bacterial cell wall peptidoglycan remains a privileged target for inhibition by antibacterials. Peptidoglycan is a polymer formed by linear glycan chains composed of alternating *N*-acetylglucosamine (GlcNAc)[1] and *N*-acetylmuramic acid (MurNAc) residues, cross-linked by short peptides appended to MurNAc, all linkages between sugars being $\beta$-1,4. The precursors of peptidoglycan, UDP-GlcNAc and UDP-MurNAc-pentapeptide, are synthesized in the cytoplasm (*1*) and transferred onto a phosphorylated $C_{55}$-isoprenoid, a membrane soluble carrier, at the inner face of the cytoplasmic membrane with the assistance of the MraY and MurG transferases (*2*, *3*). The carrier-linked disaccharide-peptide (lipid II) is then transferred to the external face of the cytoplasmic membrane by an unknown process. Finally, polymerization of the peptidoglycan monomer unit is carried out by class A penicillin-binding proteins (PBPs) via their glycosyl transferase (GT) activity catalyzing glycan chain elongation and acyl-transferase (or transpeptidase) activity catalyzing peptide bond formation between adjacent glycan chains (*4*). These PBPs combine both activities in a single polypeptide chain.

*Escherichia coli* PBP1b has been the most investigated class A PBP in terms of membrane topology, purification, and catalytic activities. It catalyzes the conversion of lipid II into peptidoglycan in in vitro assays (*5*). Moenomycin, a glycolipid, and vancomycin derivatives inhibit the GT activity of PBP1b (*1*, *6*). Because of its location outside of the cytoplasmic membrane and its specificity, the glycosyl transferase is an interesting drug target that has not yet been fully explored.

The exact mechanism of GT reaction is still not well-known. The linear glycan chain assembly presumably proceeds by the repetitive addition of disaccharide peptide units either at their reducing end or at their nonreducing end (Figure 1). In the first case, which has been established in *Bacillus licheniformis* and *Micrococcus luteus*, the growing glycan chain linked to the undecaprenol pyrophosphate acts as the glycosyl donor substrate and is transferred to the 4-OH of the GlcNAc unit of lipid II which is the glycosyl acceptor substrate (*7*). In the second case, lipid II functions as a donor and its disaccharide peptide is transferred to the 4-OH of the GlcNAc of the growing glycan chain which is the acceptor substrate.

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[1] Abbreviations: PBPs, penicillin-binding proteins; GT, glycosyl transferase; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; $A_2$pm, diaminopimelic acid; DMSO, dimethyl sulfoxide.

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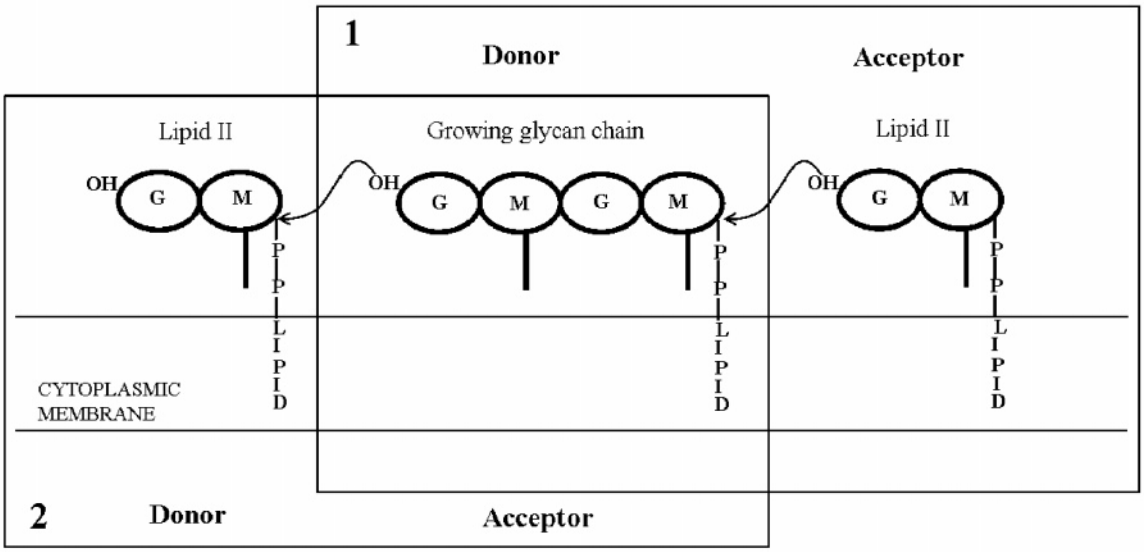



FIGURE 1: Polymerization of a glycan chain: (1) addition of disaccharide peptide units at the reducing end of the glycan chain and (2) addition of disaccharide peptide units at the nonreducing end of the glycan chain. M, MurNAc-pentapeptide; G, GlcNAc; lipid-P-P, undecaprenyl-pyrophosphate.

The biochemical analysis of glycan polymerization remains a difficult task. This is mainly due to the lipidic nature of the substrate which is difficult both to obtain and to handle. Some progress has been made in the past few years. Chemical synthesis of lipid II has been developed to overcome the substrate limitations (*8*−*10*). A biological method for producing lipid II from membranes has been developed by Breukink (*11*). Schwartz et al. have developed a HPLC analytical system coupled with a postreaction tagging of the lipid II with fluorescamine and a continuous fluorescence assay by using dansyl-lipid II (*8*, *12*).

The objective of this work is to learn more about the *E. coli* PBP1b specificity profile for its substrates by modifying the lipidic part and or the peptide moiety. These studies should help us to understand the catalytic mechanism of the glycosyl transferase and design a simple in vitro assay.

## EXPERIMENTAL PROCEDURES

*Materials.* Di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, tetra-*N*-acetylchitotetraose, penta-*N*-acetylchitopentaose, hexa-*N*-acetylchitohexaose, 4-methylumbelliferyl-$\beta$-D-*N*,*N*′-diacetylchitobiose, and MurNAc-(L-Ala-D-*iso*-Gln)-GlcNAc were from Sigma-Aldrich. Fluorescein-labeled ampicillin was synthesized as described previously (*13*).

*Bacterial Strains, Plasmids, and Growth Media*. *E. coli* BL21(DE3) was supplied by Novagen Inc. (Madison, WI). The pDML 924 plasmid carrying the *ponB* gene encoding His-tagged (M46−N844) PBP1b was described previously (*5*). The bacteria were grown at 37 °C in Luria-Bertani (LB) rich medium containing 50 $\mu$g/mL kanamycin.

*Production and Purification of the His-Tagged (M46*−*N844) PBP1b from E. coli.* His-tagged (M46−N844) PBP1b was produced in *E. coli* BL21(DE3)/pDML924 and purified according to the procedure described by Terrak et al. (*5*). The purification yield was ∼1 mg/L of culture. The 95% pure PBP1b was stored at −20 °C in buffer A [25 mM Tris-HCl (pH 7.5), 0.7% CHAPS, and 0.5 M NaCl] at a concentration of 1 mg/mL.

*Lipid II and GT Activity*. The *meso*-[$^{14}$C]diaminopimelic acid-labeled lipid II (specific activity of 0.126 $\mu$Ci/nmol) was prepared as described previously (*5*). The fluorescent analogue of lipid II, the α-dihydroheptaprenyl-pyrophosphoryl-MurNAc-[L-Ala-$\gamma$-D-Glu-*meso*-A$_2$pm($N^{\epsilon}$-dansyl)-D-Ala-D-Ala]-GlcNAc, had a α-dihydroheptaprenyl ($C_{35}$) instead of the undecaprenyl ($C_{55}$) group and bore a dansyl group on the $\epsilon$-amino function of the *meso*-diaminopimelyl residue (*14*). A typical GT assay was performed as follows. $^{14}$C-labeled lipid II (1.5 $\mu$M) and purified PBP1b (18 nM) were incubated in 50 mM Tris-HCl (pH 7.5), 0.046% CHAPS, 33 mM NaCl, 10 mM $MgCl_2$, 0.5% decyl PEG, 12.5% 1-octanol, and 25% DMSO (buffer A+) for 15 min at 30 °C, in a total volume of 30 $\mu$L. In some cases, PBP1b (1.12−10 $\mu$M) was first incubated with benzylpenicillin (100 $\mu$M) for 10 min at 30 °C in buffer A to inactivate its transpeptidase activity (*5*). The GT reaction was stopped by addition of 10 $\mu$L of isobutyric acid. Then, the products were separated by overnight chromatography (15−16 h) on Whatman No. 1 paper with an isobutyric acid/1 M ammonia mixture (5:3) as the mobile phase. The polymerized peptidoglycan remained immobile on the chromatograms. After the chromatograms had dried for ∼6 h, the radioactive compounds were detected with a Phosphor Imager Scanner and analyzed with Quantity One (*5*). The components tested as potential substrates were added to the reaction mixture at concentrations indicated in Figure 2.

*Enzymatic Synthesis of UDP-Disaccharide Derivatives.* It has been shown that *E. coli* MurG catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to UDP-MurNAc-pentapeptide to give rise to UDP-disaccharide-pentapeptide (*15*). The synthesis of the UDP-MurNAc-(L-Ala-$\gamma$-D-Glu-

*meso*-$A_2$pm-D-Ala-D-Ala)-GlcNAc was performed as follows. UDP-GlcNAc (1 mM, Sigma-Aldrich) and UDP-MurNAc-L-Ala-$\gamma$-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala (4 mM) were incubated in the presence of 200 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 35% (v/v) DMSO, and MurG (90 $\mu$g) (prepared as described in ref *16*) in a total volume of 250 $\mu$L, for 24 h at 24 °C. The reaction was stopped by heating the mixture at 100 °C for 3 min and the mixture lyophilized. The mixture was solubilized in 50 mM ammonium formate buffer (pH 4.8) and loaded onto a Nucleosil $5C_{18}$ column (250 mm × 4.6 mm; Alltech Templemars) using ammonium formate buffer at 0.6 mL/min as the mobile phase. Detection was monitored at 262 nm. The peak corresponding to UDP-disaccharide-pentapeptide, which had a retention time of 24.5 min, was collected. (The retention times for UDP-GlcNAc and UDP-MurNAc-pentapeptide were 4.4 and 11.4 min, respectively.) The *m*/*z* ratio of the $[M - H]^-$ ion was 1396.0 Da, in agreement with the theoretical mean value of 1397.16 Da. The yield (determined by quantitative amino acid analysis) was 40 nmol (16%). Upon overnight chromatography on Whatman No. 1 paper developed in an isobutyric acid/1 M ammonia mixture (5:3), the compound has an $R_f$ value of ~0.1.

The same compound was also synthesized from UDP-[$^{14}$C]-GlcNAc (258 nmol, 2.5 $\mu$Ci; Amersham-Pharmacia Biotech) and UDP-MurNAc-pentapeptide (1 mmol): yield of 54 nmol, 0.52 $\mu$Ci (21%); radiochemical purity (determined by HPLC with on-line scintillation counting) of 98%.

*Chemical Synthesis of UDP-Disaccharide Derivatives.* UDP-MurNAc-GlcNAc, UDP-MurNAc-(L-Ala-L-Lys-$NH_2$)-GlcNAc, UDP-MurNAc-[L-Ala-L-Lys-(dansyl-$NH_2$)]-GlcNAc, and UDP-MurNAc-[L-Ala-D-*iso*-Gln-($NH_2$)]-GlcNAc were synthesized as described by Lioux et al. (*17*).

*Digestion of the Synthesized Peptidoglycan.* The peptidoglycan spots were cut off from the paper and incubated in 200 $\mu$L of 25 mM Tris-HCl (pH 7.5) containing 2 mg/mL lysozyme for 17 h at 37 °C. The pieces of paper were then washed with 100 $\mu$L of water, and the different fractions were pooled and lyophilized. The lyophilized material was dissolved in 30 $\mu$L of water and centrifuged to eliminate the paper debris before chromatography.

*Glycan Chain Synthesis.* Glycan chains were synthesized by incubating dansyl-lipid II (7.5 $\mu$M) for 22 h at 30 °C with PBP1b (1.12 $\mu$M), the transpeptidase activity of which had been inactivated by benzylpenicillin. The resulting mixture was treated with 0.05 M HCl for 5 min at 100 °C to remove the dihydroheptaprenyl pyrophosphate moiety and was neutralized with 0.05 M NaOH (*18*).

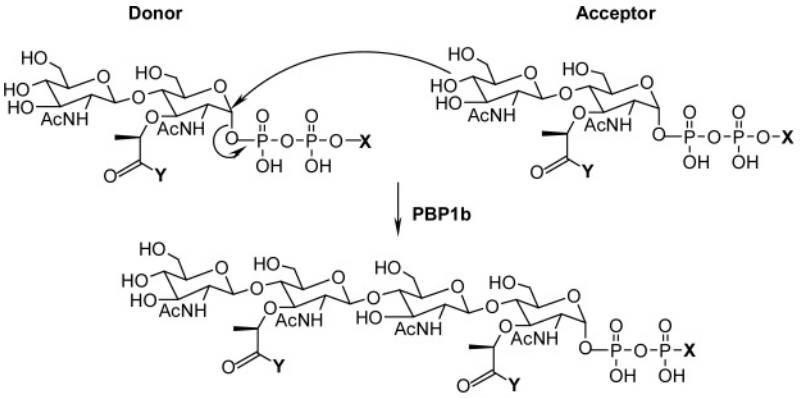
*Mass Spectral Analyses.* Mass spectral analyses were performed using nano-electrospray ionization mass spectrometry. Typically, approximately 1 pmol/$\mu$L samples were prepared in a 50% acetonitrile/0.1% formic acid mixture and loaded into gold-coated borosilicate needles (Protana, Odense, Denmark). The needle was placed into the source of a Micromass (Manchester, U.K.) Q-TOF I system, and a voltage of 1.1 kV was applied. Spectra were collected during 3−5 min, and data were processed using the Masslynx software delivered with the instrument.

## RESULTS

*E. coli*-purified PBP1b catalyzes the in vitro glycan chain polymerization with the natural lipid II precursor, undecaprenyl-pyrophosphoryl-MurNAc-(L-Ala-$\gamma$-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala)-GlcNAc, compound 1 (Figure 2), as the substrate with a $k_{cat}$ value of $70 \times 10^{-3}$ $s^{-1}$ and a $k_{cat}/K_m$ efficiency of ~39000 $M^{-1}$ $s^{-1}$ (*5*). The purified protein shows an affinity for $\beta$-lactams very similar to that of the membrane-bound enzyme (*5*). It bound fluorescein-labeled ampicillin in a 1:1 ratio (data not shown) (*19*). In in vitro assays with the lipid II precursor, the rate of glycan chain synthesis is not modified by the presence of penicillin which inhibits the transpeptidase activity (*5*). It was shown that the replacement of undecaprenyl with betulaheptaprenyl ($C_{35}$) and that of *meso*-$A_2$pm with Lys in lipid II did not modify the $k_{cat}$ and $k_{cat}/K_m$ values measured with an extract of PBP1b (*6*). The α-dihydroheptaprenyl-pyrophosphoryl-MurNAc-[L-Ala-$\gamma$-D-Glu-*meso*-$A_2$pm($N^\epsilon$-dansyl)-D-Ala-D-Ala]-GlcNAc, compound 2 (Figure 2) (*14*), was a substrate for PBP1b. When the enzyme (0.9 $\mu$M) was incubated in the presence of dansyl-lipid II (7.5 $\mu$M), a decrease in the fluorescence was observed, and after incubation for 17 h, lipid II was consumed (data not shown). In addition, incubation of the enzyme (18 nM) for 15 min at 30 °C in the presence of [$^{14}$C]lipid II (1.5 $\mu$M) and dansyl-lipid II (3 $\mu$M) decreased the percentage of synthesized radioactive peptidoglycan by 80% (Figure 2). Quantification of the fluorescence required more material than that of radioactivity which explains why radioactive lipid II was used in most of the experiments described below.

*Test of Potential Non-Lipid Acceptor Substrates for the Glycosyl Transferase Reaction.* Lysozyme catalyzes the cleavage of $\beta$-1,4-*N*-acetylmuramyl-*N*-acetylglucosamine bonds and the release of disaccharide-peptide units, a reaction that is the reverse of the polymerization reaction. Lysozyme also hydrolyzes chitin which consists mainly of $\beta$-1,4-linked *N*-acetylglucosamine residues and resembles the glycan chains of peptidoglycan. In the in vitro assay, lipid II functions both as a glycosyl donor and a glycosyl acceptor substrate (Figure 2). To characterize the specificity profile for the acceptor substrate, potential acceptors derived from chitin and peptidoglycan were tested in the presence of [$^{14}$C]-lipid II (1.5 $\mu$M) and PBP1b (18 nM) under conditions where the reaction rate was linear (*5*). Multimers (2−6 units) of GlcNAc (10 mM), compounds 8−12 (Figure 3), di-*N*-acetylchitobiose linked to 4-methylumbelliferyl (4.5 mM), compound 13 (Figure 3), and the disaccharide dipeptide, MurNAc-(L-Ala-D-*iso*-Gln)-GlcNAc (100 $\mu$M), did not show any competitive effect on the GT activity. Surprisingly, UDP-MurNAc-(L-Ala-$\gamma$-D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala)-GlcNAc (100 $\mu$M), compound 3 (Figure 2), which was enzymatically synthesized, inhibited 16% of the GT activity and 85% of the GT activity when it was preincubated with the enzyme. UDP (100 $\mu$M), UDP-MurNAc-pentapeptide (100 $\mu$M), or UDP-GlcNAc (100 $\mu$M) had no effect on the glycan chain formation, whereas moenomycin (100 nM), compound 14 (Figure 3), completely inhibited the reaction. These results showed that the UDP-disaccharide-pentapeptide was recognized by PBP1b.

To determine if simpler UDP-disaccharide-peptides were recognized by the enzyme, derivatives 4−7 (Figure 2) in which the peptide moiety was shortened, modified, or removed were chemically synthesized (*17*) and tested with *E. coli* PBP1b in the presence of [$^{14}$C]lipid II. As shown in Figure 2, none of these compounds inhibited the GT activity



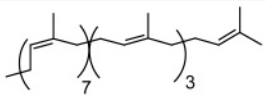
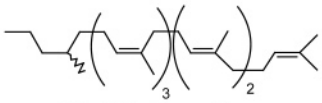
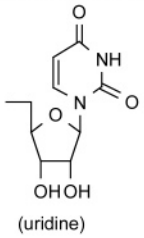

| Compound | X | Y | Inhibition [%] | |
|---|---|---|---|---|
| 1 |  (undecaprenyl) | L-Ala-γ–D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala | | |
| 2 |  (dihydroheptaprenyl) | L-Ala-γ–D-Glu-*meso*-$A_2$pm($N^{\varepsilon}$-dansyl)-D-Ala-D-Ala | 3 μM<br>1.5 μM | 80%<br>50% |
| 3 |  (uridine) | L-Ala-γ–D-Glu-*meso*-$A_2$pm-D-Ala-D-Ala | 100 μM | 16% |
| 4 | uridine | L-Ala-D-iso-Gln($NH_2$) | 100 μM | no |
| 5 | uridine | L-Ala-L-Lys-(dansyl-$NH_2$) | 100 μM | no |
| 6 | uridine | L-Ala-L-Lys-$NH_2$ | 100 μM | no |
| 7 | uridine | H | 100 μM | no |

FIGURE 2: Glycosyl transfer reaction and effect of the lipid II analogues on the GT activity of *E. coli* PBP1b. The glycosyl transferase activity was measured in the presence of [$^{14}$C]lipid II (1.5 $\mu$M) and PBP1b (18 nM) at 30 °C over the course of 15 min. Under these conditions, 20% of the substrate was consumed. The percent inhibition was calculated by comparing the reaction rates in the presence and absence of the compound. No means no inhibition occurred under the conditions that were used.

of PBP1b. The same results were obtained if the protein was preincubated with the compound before the incubation with [$^{14}$C]lipid II (data not shown). The peptide moiety of the UDP-disaccharide-peptide thus appears to play a role in the recognition by the enzyme.

To determine if the disaccharide-pentapeptide from the UDP-disaccharide-peptide was incorporated into the glycan chains synthesized when the lipid II precursor was present, PBP1b (1.12 $\mu$M), the transpeptidase activity of which had first been inhibited with benzylpenicillin, was incubated at 30 °C for 21 h in buffer A+ in the presence of UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc (25 $\mu$M, 7.3 mCi/mmol) and nonradioactive dansylated lipid II (7.5 $\mu$M), and the reaction products were separated by paper chromatography. The results are shown in Figure 4 (track 1). A radioactive spot was present at the origin of the chromatogram and corresponded to the polymerized glycan chains. Under the conditions that were used, dansyl-lipid II was almost completely consumed, 5% of UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc was utilized, and approximately 15% of the disaccharide units in the synthesized peptidoglycan were [$^{14}$C]disaccharide-peptide. This result indicates that the UDP-disaccharide-pentapeptide is a substrate.

To ascertain whether this substrate analogue could yield a polymerized glycan chain in the absence of lipid II, UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc (25 $\mu$M) was incubated with PBP1b (1.13 $\mu$M) in buffer A+ for 21 h at 30 °C. Reactions were also carried out in buffer A+ without 1-octanol, decyl PEG, and DMSO, with more lipid II analogue (100 $\mu$M) and PBP1b (18 nM). Under all the tested conditions, no polymerization was detected. The reaction products were also analyzed by mass spectrometry. MS revealed the presence of only UDP-disaccharide-pentapeptide. These results indicate that the polyprenol moiety of the lipid II precursor, donor and/or acceptor, is required for GT activity.

*UDP-MurNAc-(L-Ala-γ-D-Glu-meso-$A_2$pm-D-Ala-D-Ala)-GlcNAc: A Glycosyl Donor and/or an Acceptor?* To test whether the UDP-disaccharide-pentapeptide could act as a glycosyl donor substrate and/or as a glycosyl acceptor

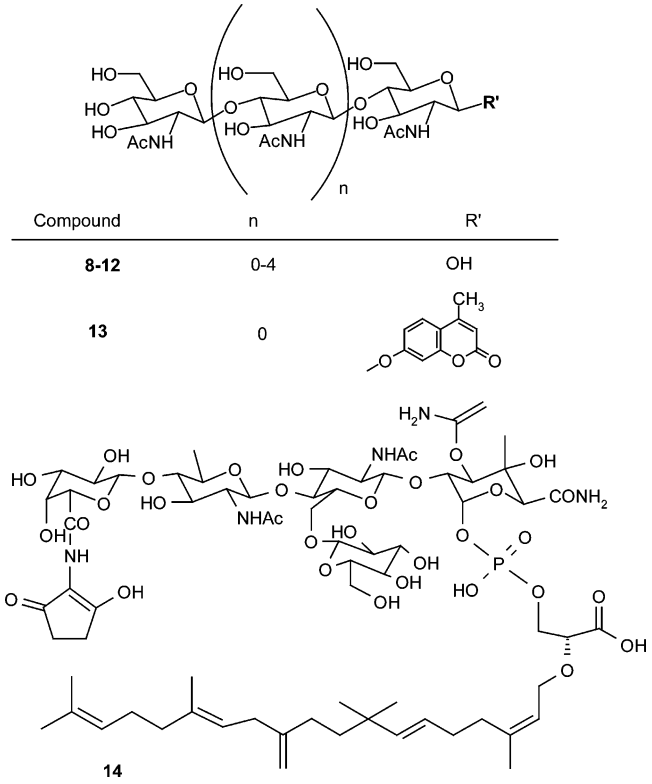



FIGURE 3: Structure of chitin derivatives and moenomycin.

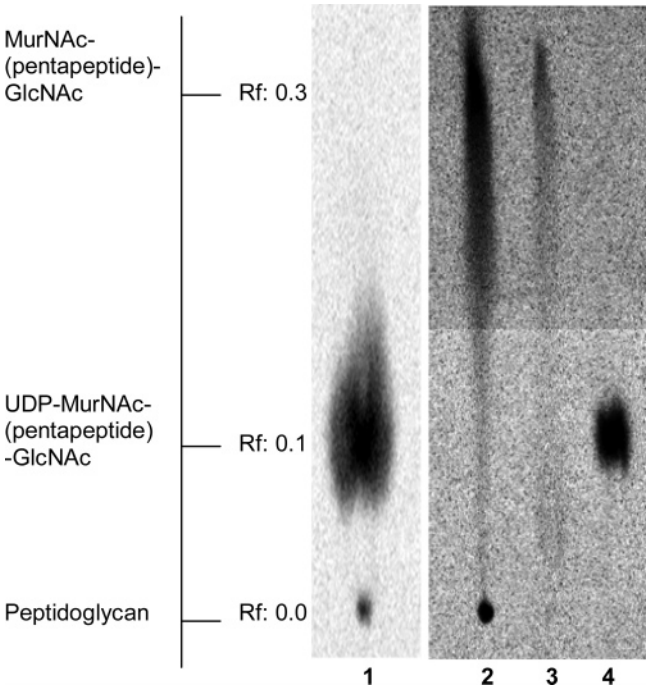



FIGURE 4: Paper chromatography analysis of the material polymerized by PBP1b in the presence of dansyl-lipid II and UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc (track 1) or the products obtained upon lysozyme digestion of the material polymerized by PBP1b in the presence of [$^{14}$C]lipid II (track 2) or in the presence of dansyl-lipid II and UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc (track 3). The UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc treated or not treated with lysozyme migrates with the same $R_f$ value of 0.1 (track 4). Lipid II migrates with an $R_f$ value of 0.8.

substrate, PBP1b was incubated with dansyl-lipid II and UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc. If the UDP-[$^{14}$C]disaccharide-peptide acts as an acceptor, UDP will be present at the reducing end of the glycan chain and the digestion of the peptidoglycan with lysozyme will liberate the UDP-[$^{14}$C]disaccharide-peptide. On the other hand, if the analogue acts as a donor, the digestion of the peptidoglycan with lysozyme will liberate the labeled [$^{14}$C]disaccharide-peptide and no UDP-[$^{14}$C]disaccharide-peptide should be detected. The polymerized materials obtained by incubating the benzylpenicilloylated PBP1b either with [$^{14}$C]lipid II or with dansyl-lipid II and UDP-MurNAc-(pentapeptide)-[$^{14}$C]-GlcNAc were isolated by chromatography, eluted from the chromatogram, and digested with lysozyme (see Experimental Procedures). The products of digestion were analyzed by paper chromatography. Figure 4 shows the results. Glauner and Höltje have shown that muramidase cleaves close to the terminus of a glycan chain linked to the undecaprenyl-pyrophosphate (*20*). We verified that lysozyme had no effect on UDP-disaccharide-peptide. Most of the digested products of the reaction containing the UDP-[$^{14}$C]disaccharide-pentapeptide (>80%) migrated with the same $R_f$ as the disaccharide-pentapeptide, and despite a smear of radioactivity, no spot of UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc was detected. However, a faint radioactive spot (<20%) below the level of the UDP-[$^{14}$C]disaccharide-pentapeptide was observed. Attempts to identify the nature of this spot by mass spectrometry analysis failed because of the very small amount of material. The digestion of the polymerized peptidoglycan after incubation of the penicilloylated PBP1b with [$^{14}$C]lipid II was also incomplete, as shown by the presence of radioactivity at the origin of the chromatogram (13%) and a smear of radioactivity.

These results suggest that the UDP-MurNAc-(pentapeptide)-GlcNAc acts mainly as a donor and agrees with the fact that there is no peptidoglycan synthesis without the addition of dansyl-lipid II, which could act as the acceptor. However, we could not rule out the possibility that the UDP-disaccharide-pentapeptide could act as an extremely poor acceptor in the presence of dansyl-lipid II.

In addition, to determine whether the glycan chains free of the polyprenyl pyrophosphate can act as an acceptor, dansyl-glycan chains were synthesized (see Experimental Procedures) and incubated overnight at 30 °C with PBP1b (1.12 $\mu$M) and UDP-MurNAc-(pentapeptide)-[$^{14}$C]GlcNAc (31.4 $\mu$M). No radioactivity was found at the level of the peptidoglycan spot after chromatography. Thus, glycan strands by themselves do not seem to act as acceptor substrates in the presence of UDP-MurNAc-(pentapeptide)-GlcNAc.

## DISCUSSION AND CONCLUSION

The glycosyl transferase of *E. coli* penicillin-binding protein (PBP) 1b catalyzes the assembly of lipid-transported *N*-acetylglucosaminyl-$\beta$-1,4-*N*-acetylmuramoyl-L-Ala-D-Glu-*meso*-A2pm-D-Ala-D-Ala units into linear peptidoglycan chains. Carbon 1 of the lipid-linked *N*-acetylmuramic acid undergoes attack by the acceptor nucleophile OH at C4 of *N*-acetylglucosamine. The reaction leads to an inversion of configuration at C1, from the $\alpha$-configuration in the precursor to the $\beta$-configuration in the glycosylated acceptor. Glycan chain elongation occurs either by transfer of the growing chain attached to the undecaprenyl-pyrophosphate, which is the donor, to C4 of *N*-acetylglucosamine of a lipid II precursor molecule (way 1, Figure 1) or by transfer of a lipid-

linked disaccharide functioning as a glycosyl donor to C4 of the growing chain, which is the acceptor substrate and is not necessarily linked to the undecaprenyl (way 2, Figure 1) (*1*, *21*). To gain insight into the catalytic mechanism of the glycosyl transferase, the specificity profile of the enzyme for the lipid II substrate was determined by using substrate analogues. The results of the experiments presented above suggest that the specificity of the enzyme for the glycosyl donor substrate differs from that for the acceptor.

Our data show that the UDP-disaccharide-pentapeptide (compound 3, Figure 2), in which the lipid moiety of the lipid II precursor is replaced with uridine, is recognized by *E. coli* PBP1b and functions mainly as a glycosyl donor, albeit less efficiently than the lipid II precursor. By contrast, UDP-MurNAc-pentapeptide, UDP-GlcNAc, and MurNAc-(dipeptide)-GlcNAc are not substrates. These results suggest that the donor substrate requires the presence of both GlcNAc and MurNAc and a reactive group on C1 of MurNAc but does not absolutely require the polyprenol chain which can be replaced with uridine. The UDP-disaccharide-dipeptide or -free of peptide has no effect on the reaction measured in the presence of lipid II, indicating that the GT is sensitive to the presence of the peptide chain which can be involved in the recognition of such a type of substrate. It is known that the specificity of the GT for the peptide moiety of lipid II is not very stringent, as glycan chain formation catalyzed by *E. coli* PBP1b accepts tripeptide subunits (*18*, *22*) or dansyl-*meso*-$A_2$pm (this study), lysine (*23*), or dansyl-lysine (*6*) in the pentapeptide subunit. Although the extrapolation of the results with the UDP-disaccharide-peptide to lipid II is dangerous, we are tempted to conclude that to be a substrate the precursor requires at least a tripeptide subunit.

Multimers of GlcNAc, chitobiose linked to 4-methylumbelliferyl or MurNAc-(dipeptide)-GlcNAc which contain a hydroxyl group at C4 of GlcNAc, are potential acceptors but are not recognized by the enzyme (Figure 3). If the UDP-disaccharide-peptide functions as an acceptor, it is an extremely poor one. Glycan chains containing the pentapeptide free of lipid did not act as acceptors in the presence of the UDP-disaccharide peptide acting as a donor. It thus appeared that the presence of a polyprenyl chain through a pyrophosphate bond on C1 of MurNAc is recommended for an acceptor substrate since both the absence of the reactive group or the presence of an UDP at this position does not permit the glycosyl transferase reaction or does so very poorly.

These experiments do not allow us to decide whether the growing saccharidic chain acts as a donor [as suggested in Gram-positive bacteria (*7*, *21*)] or as an acceptor in the transglycosylation reaction. However, it appears that PBP1b shows a strong preference for acceptor molecules containing a lipid moiety. It can be reasonably expected that, in the enzyme active site, the distance between the lipid anchor and the nucleophilic OH group of the acceptor remains constant. If the number of disaccharide units in the latter molecule progressively increases, it is difficult to imagine how this can be achieved. Therefore, the most direct conclusion is that the growing chain is probably the donor. However, additional experiments will be needed to completely rule out the possibility that it could be an acceptor.

At another level, the fact that glycan chains free of lipid do not appear to be acceptor substrates suggests that, in growing cells, the attachment of newly made material to the old cell wall does not occur by transglycosylation and occurs only by transpeptidation. These data reinforce the idea that class A PBP(s) could manufacture peptidoglycan primers that would be transferred by class B PBP(s) through a transpeptidation reaction to the preexisting peptidoglycan during elongation and cell division (*24*).

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