Screen for Inhibitors of the Coupled Transglycosylase-Transpeptidase of Peptidoglycan Biosynthesis in *Escherichia coli*

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Class A high-molecular-weight penicillin-binding protein 1a (PBP1a) and PBP1b of *Escherichia coli* have both transglycosylase (TG) and transpeptidase (TP) activity. These enzymes are difficult to assay, since their substrates are difficult to prepare. We show the activity of PBP1a or PBP1b can be measured in membranes by cloning the PBP into an *E. coli* ponB::Spc' strain. Using this assay, we show that PBP1a is ~10-fold more sensitive to penicillin than PBP1b and that the 50% inhibitory concentration (IC50) of moenomycin, a TG inhibitor, is ~10-fold higher in the PBP transformants than in wild-type membranes; this increase in IC50 in transformants can be used to test the specificity of test compounds for inhibition of the TG. Alternatively, the coupled TG-TP activity of PBP1b can be directly measured in a two-step microplate assay. In the first step, radiolabeled lipid II, the TG substrate, was made in membranes of the *E. coli* ponB::Spc' strain by incubation with the peptidoglycan sugar precursors. In the second step, the TG-TP activity was assayed by adding a source of PBP1b to the membranes. The coupled TG-TP activity converts lipid II to cross-linked peptidoglycan, which was specifically captured by wheat germ agglutinin-coated scintillation proximity beads in the presence of 0.2% Sarkosyl (B. Chandrakala et al., Antimicrob. Agents Chemother. 48:30–40, 2004). The TG-TP assay was inhibited by penicillin and moenomycin as expected. Surprisingly, tunicamycin and nisin also inhibited the assay, and paper chromatography analysis revealed that both inhibited the transglycosylase. The assay can be used to screen for novel antibacterial agents.

Peptidoglycan, the major structural component of the bacterial cell wall, is essential for bacterial growth. Since the polymer is absent in humans, disruption of peptidoglycan synthesis is an attractive method for eliminating bacteria in a search for new antibacterial agents.

Peptidoglycan is a macromolecular polymer of a repeating disaccharide-peptide unit, where the peptide chains attached to adjacent sugar molecules are cross-linked. In the terminal stages of its synthesis, at the periplasm, the disaccharide units are polymerized by the transglycosylase (TG) enzyme, and the peptide chains are cross-linked by the transpeptidase (TP) (3, 29) (Fig. 1). The disaccharide unit N-acetylmuramyl(L-pentapeptide)-N-acetylglucosamine is assembled on a lipid carrier, undecaprenol phosphate, from the sugar precursors UDP-N-acetylglucosamine (UDP-GlcNAc) and UDP-N-acetylmuramyl(L-pentapeptide) [UDP-MurNAc(pp)], forming lipid II. The TG catalyzes the transfer of the disaccharide from lipid II to nascent peptidoglycan, releasing lipid pyrophosphate.

Class A high-molecular-weight penicillin-binding proteins (PBPs), such as PBP1a and -1b of *Escherichia coli*, are bifunctional molecules having both transglycosylase and transpeptidase activity on the same polypeptide (20, 29, 31). Either PBP1a or PBP1b needs to be functional for the cell to survive; the double mutant is not viable (38). Since the PBPs are located on the periplasmic surface, they are more accessible to drug molecules; this location also precludes problems associated with the permeability of the cell wall to the drug and resistance due to drug efflux. Both transglycosylase and transpeptidase are attractive targets for drug discovery (14). However, both of these enzymes are difficult to assay in a format amenable to high-throughput screening; this is true for the transpeptidase in particular.

The transglycosylase substrate, lipid II, is present in bacterial cells in small quantities and is tedious to isolate (21, 32). An alternative to isolating the substrate is allowing lipid II to accumulate in situ in membranes by using detergent to inhibit transglycosylation and subsequently removing the detergent to assay transglycosylation (5, 18). However, the product peptidoglycan is typically separated from lipid II by paper chromatography (5, 10). Using this method, the TG activity of PBP1b has been demonstrated previously (10, 15, 21, 29a, 30, 31). However, the activity of PBP1a (15) has been more difficult to measure, and the reaction may have to be performed on filter paper. More recently, lipid II and analogues have been chemically synthesized; these can be used to measure the TG activity, although the water solubility of the substrate is an issue and shorter-chain analogues are better substrates than the natural lipid (26, 33, 37). TG activity is inhibited by moenomycin (14, 31), which does not affect TP activity, and competition of its binding to the PBP has been proposed as an assay to isolate TG inhibitors (34).

There are few reports of transpeptidase enzyme activity in membrane fractions (21, 31), since cross-linked peptidoglycan has very similar properties and cannot be separated from uncross-linked precursor. The only means of measuring transpeptidation is by analysis of the degree of cross-linking of the...
Peptidoglycan. This is a laborious procedure and needs large quantities of material and hence has more frequently been reported for whole cells (21, 31). The other method of measuring transpeptidase is by measuring the release of the terminal D-Ala residue from muramyl(pentapeptide) resulting from the cross-linking, which can be distinguished from carboxypeptidase by its dependence on peptidoglycan synthesis, i.e., inclusion of UDP-N-acetylgalcosamine in the reaction (16). This method requires labeling of the terminal D-Ala residue, which is laborious, and measurement of D-Ala is not easily performed in a microplate. The hallmark of peptidoglycan transpeptidases is their inhibition by penicillin and related β-lactams, giving rise to the term “PBPs”: penicillin binds covalently to the PBPs and inhibits the TP, while having no effect on transglycosylation (12, 28). This has given rise to indirect assays as indicators of the TP activity of the PBPs, most of which are binding assays and are not truly reflective of the enzyme activity. The most commonly used assays involve the binding of a β-lactam to the PBPs to screen for agents that compete with this binding, the conversion of nitrocefin to a colored product upon binding to the PBP, or the use of a synthetic peptide that gives a colored product on hydrolysis (1, 2, 17, 25). These indirect assays can be easily adapted to microplates.

An earlier report from our unit has shown that cross-linked peptidoglycan can be specifically detected in a microplate by capture with wheat germ agglutinin-coated scintillation proximity assay beads with detergent (8). In addition, this activity was shown to be a true measure of the enzyme activity of the TP (7). This makes it possible, in principle, to assay the coupled TG-TP activity, since cross-linked peptidoglycan can be distinguished from lipid II in a microplate format. Here we show that the plasmid-borne PBP1a gene can be transformed into an E. coli strain that lacks PBP1b, and its product can then be used to assay TG and TP activity. In vitro peptidoglycan synthesis, which is insignificant in the host strain, is reconstituted by overexpression of PBP1a. Further, the coupled peptidoglycan transglycosylase-transpeptidase of the PBPs can be directly measured in a two-step reaction. The substrate, lipid II, was made by incubating membranes of an E. coli strain lacking PBP1b with the peptidoglycan sugar precursors. In a second step, a source of PBP1b was added to these membranes to produce cross-linked peptidoglycan, which was captured using the scintillation proximity assay (SPA) beads. The assay can be used to screen in high throughput mode for inhibitors of both TG and TP.

 FIG. 1. Schematic of stages 2 and 3 of peptidoglycan biosynthesis pathway indicating reference inhibitors of the enzymes. Cross-linked peptidoglycan is formed in wild-type membranes (A), but lipid II is formed in membranes of the E. coli AMA1004 ponB::Sp′ strain (strain deficient in PBP1b) (B), However, in membranes of the same strain transformed with a plasmid expressing PBP1a or PBP1b, the pathway should be reconstituted and cross-linked peptidoglycan formed (C). The enzymes are referred to by their E. coli gene product names, except for transglycosylase, transpeptidase, and lipid pyrophosphatase, which are denoted by TG, TP, and LPase, respectively. In panel C, reference inhibitors of the enzymes are represented in italics. Open circles denote N-acetylmuramidase, and filled circles denote radiolabeled N-acetylgalactosamine. Squares symbolize N-acetylmuramyl(pentapeptide), with the vertical line attached to the square representing the pentapeptide chain attached to muramic acid; diagonal lines connecting the N-acetylmuramyl(pentapeptide) residues denote cross-links formed by the transpeptidase.

(38) Materials. Wheat germ agglutinin-coated (WGA)-SPA beads were purchased from Amersham International, Piscataway, N.J. Chloramphenicol was added to a concentration of 100 μg/ml. The cells were harvested 60 min after the addition of vancomycin. A hot-water extract of the culture cells was purified by gel and ion exchange chromatography. Fractions were monitored by sugar estimation, and the concentration was estimated from its absorbance at 262 nm using a molar extinction coefficient of 10,000 (19).

Enzyme preparation. Membranes from Escherichia coli AMA1004 or other strains were prepared as described earlier (7). Briefly, the cells were grown to an A600 of ~1.8, resuspended in 50 mM Tris-HCl (pH 7.5), 0.1 mM MgCl2, and lysed using a French pressure cell. The lysate was spun at low speed, and the supernatant obtained from this procedure was ultracentrifuged. The pellet was washed once, resuspended in the same buffer, and stored in aliquots at –70°C. The concentration of protein was estimated by use of Coomassie blue stain (Pierce Chemical Co.).

Peptidoglycan synthesis assay. Peptidoglycan synthesis was performed in membranes of E. coli ponB::Sp′ AMA1004 or the same strain transformed with a plasmid bearing the PBP1b gene [E. coli ponB::Sp′ AMA1004(pBS98)] or the PBP1a gene [E. coli ponB::Sp′ AMA1004(pBS89)] (7). E. coli membranes (4 μg protein) were incubated for 90 min at 37°C with 15 μM UDP-MurNAc(Ac) and 2.5 μM UDP-[3H]GlcNAc (0.2 μCi) in a buffer of 50 mM HEPES-ammonia (pH 7.5), 10 mM MgCl2, 8% dimethyl sulfoxide in a total volume of 25 μl. The reaction was stopped by the addition of 5 μl of 90 mM EDTA; this was followed by capture of the product on 500 μg WGA-SPA beads in 170 μl of 50 mM HEPES-NaOH, pH 7.5, containing Sarkosyl at a final concentration of 0.2% (to 200 μl). Under these conditions, cross-linked peptidoglycan is specifically monitored (8, 24). In the case of reactions using membranes from E. coli ponB::Sp′ AMA1004, a parallel reaction to monitor the quantity of lipid II formed was carried out by the addition of beads without Sarkosyl (8). The blank used was a reaction without UDP-MurNAc(Ac) captured under similar conditions.
**Coupled transglycosylase-transpeptidase assay.** Membranes (4 μg protein) from a PBP1b-deficient E. coli strain (ponB::Spc° AMA1004) were preincubated for 120 min at 37°C with 15 μM UDP-MurNAc(pp) and 4.16 μM UDP-[3H]GlcNAc (0.6 μCi) in a buffer of 50 mM HEPES-ammonia (pH 7.5), 10 mM MgCl2 in a total volume of 15 μL. Formation of lipid II in this step was monitored by the addition of WGA-SPA beads in the absence of detergent (see below) (8). To ensure that lipid II was being monitored, a parallel reaction without UDP-MurNAc(pp) was incubated under identical conditions and captured with the same beads; the difference between the level of the product in this reaction and that in the complete reaction is a measure of the lipid II formed.

Subsequently, the transglycosylase-transpeptidase reaction was carried out by adding 10 μL of a solution containing UDP-GlcNAc (to dilute out the radioactive activity and prevent monitoring of lipid II synthesis during the second step) and a source of PBP1b in detergent such that the final concentration was 250 μM UDP-GlcNAc, 8% dimethyl sulfoxide, 0.04% N-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS), and 40 mM NaCl in a final volume of 25 μL. Test compounds, i.e., potential inhibitors of the transglycosylase or transpeptidase, were added at this step. The reaction mixture was incubated for 20 min (unless specified otherwise) at 37°C. The reaction was stopped by the addition of 5 μL of 90 mM EDTA. The product of the reaction was analyzed by addition of WGA-SPA beads or by use of paper chromatography. All reactions were carried out in triplicate. For peptidoglycan synthesis reactions, the omission of the first sugar nucleotide, UDP-MurNAc(pp), typically is used as an enzyme blank. However, in this assay the blank used consisted of a solution that had all components except PBP1b; this solution was added at the second step. For both types of analysis, the counts per minute (cpm) obtained in the blank reaction were subtracted from those from reactions containing PBP1b as a measure of the cross-linked peptidoglycan formed by the coupled TG-TP activity of the PBP added in the second step. For the SPA, the enzyme reaction was performed in flexible plates (1450-401) from Wallace, Finland. The product was captured by the addition of 170 μL of a suspension of WGA-SPA beads (500 μg/well) in N-laurylsarcosine–HEPES buffer such that the final concentration (in 200 μL) was 0.2% N-laurylsarcosine (Sarkosyl) and 50 mM HEPES-ammonia (pH 7.5). Under these conditions, the beads selectively measure radioactive cross-linked peptidoglycan (7, 8). Radioactivity was measured with a Microbeta Trilux instrument 3 h after the addition of the beads. The signal was quite stable, and samples could be counted from 3 to 48 h after bead addition.

For the paper chromatography, 20 μL of the reaction mixture was spotted on Whatman 3MM paper, which was then dried and chromatographed overnight in isobutyric acid:1 M ammonia (5:3 vol/vol). Peptidoglycan stays at the origin, whereas lipid I and lipid II have an Rf of ~0.9 (5, 10, 24, 29). These regions were cut out, and after the paper had dried, the radioactivity was measured with a liquid scintillation counter (using Optiphase HiSafe 2 scintillation fluid; Wallac, Turku, Finland).

Radioactive N-acetylglycosamine was incorporated into peptidoglycan, and the quantity of peptidoglycan formed in the reaction was measured as cpm of N-acetylglycosamine incorporated into the product. For the SPA, it is difficult to determine the counting efficiency, so all results are represented as cpm. For the paper chromatography analysis, the counting efficiency was low and, for the data shown here, resulted in 350 to 600 cpm per pmol of N-acetylgalcosamine.

Graphs were plotted using the Graphpad Prism software; error bars are used to indicate the standard error of the mean, but in some figures these may not be obvious, since they are smaller than the symbols. Where the purity of the starting material is not defined, compound concentrations are expressed in units other than μM.

**Source of PBPs.** PBP1b was purified by affinity chromatography from E. coli ΔponB::Spc° AMA1004(pBS96). Membranes made from this strain (made as described above) were treated with 1% octyl-glucoside, 1 M NaCl, 100 mM Tris-HCl (pH 7.5) for 60 min at room temperature. Following centrifugation at 150,000 x g for 45 min, the supernatant was loaded onto ampicillin-Sepharose. The column was washed with 0.5% octyl-glucoside, 2 M NaCl in the same buffer, and the PBPs were eluted with 1 M hydroxylamine, 0.5% octyl-glucoside, 2 M NaCl, 100 mM Tris-HCl (pH 8.0) at room temperature over 4 h. The eluate was dialyzed against 0.5% octyl-glucoside, 2 M NaCl, Tris-HCl (pH 7.5) and stored in aliquots at -70°C. The major PBP purified was PBP1b. Ampicillin was coupled to Affigel 10 by incubation with the gel for 2 h followed by washing with 100 mM Tris-HCl, pH 8.0.

An alternative source of PBP1b was a detergent extract of membranes from the same strain. The membranes were incubated for 60 min on ice with 1% CHAPS, 1 M NaCl, 100 mM Tris-HCl (pH 7.5) at a concentration of 5 mg/ml protein and subsequently centrifuged at 150,000 x g for 15 min. The supernatant was used as a crude source of PBP1b. This was made fresh each time, although it can also be stored in aliquots at -70°C.

### RESULTS

**Assay of PBP1b or PBP1a activity in transformants.** Since the enzymes involved in the late stages of peptidoglycan synthesis are membrane associated (Fig. 1), cross-linked peptidoglycan synthesis can be monitored in E. coli membranes incubated with the sugar precursors UDP-MurNAc(pp) and UDP-[3H]GlcNAc (7). In membranes from a strain of E. coli deficient in PBP1b under the same conditions, lipid II is formed instead of peptidoglycan; lipid II can be captured by the WGA-SPA beads in the absence of the detergent (Table 1) (8, 24). However, in membranes of the PBP1b-deficient strain transformed with a plasmid bearing a copy of the PBP1a gene, cross-linked peptidoglycan was formed by the regenerated pathway (Fig. 1 and Table 1).

Cross-linked peptidoglycan was also formed in a transformant with a copy of the PBP1a gene on a plasmid (Table 1). This was surprising, since the parent strain, ponB::Spc° AMA1004, failed to produce measurable peptidoglycan despite the presence of an intact PBP1a gene (8). PBP1a enzyme is difficult to assay, and specific conditions may be required (10, 15). This result indicates that although wild-type levels of PBP1a cannot be assayed, levels of enzyme that are increased by cloning the gene on a plasmid may permit it to be detected under conditions similar to those for PBP1b.

The cross-linked peptidoglycan formed in the transformant membranes is a result of the sequential activities of five enzymes: those of MraY, MurG, and lipid pyrophosphorylase and the TG and TP activities of PBP1a or PBP2b. However, a comparison of the effect of inhibitors on lipid II synthesis in the parent PBP1b-deficient strain to that of synthesis of cross-linked peptidoglycan in the PBP transformants can be used to specifically measure the sensitivity of the cloned PBP1b or PBP1a activity to inhibitors. Thus, inhibitors of the MraY, MurG, or lipid pyrophosphorylase should show inhibition of the parental PBP1b-deficient strain as well as of the transformants.

But inhibitors of the PBPs, e.g., penicillin, should show

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**TABLE 1. Comparison of cross-linked peptidoglycan synthesis in wild-type E. coli, PBP1b-deficient mutant, and transformants**

<table>
<thead>
<tr>
<th>Membrane of E. coli strain (protein encoded by plasmid)</th>
<th>WGA-SPA + Sarkosyl</th>
<th>WGA-SPA (no detergent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ponB::Spc° AMA1004 (pBS89) (PBP1b)</td>
<td>3,902 ± 99</td>
<td>ND</td>
</tr>
<tr>
<td>ponB::Spc° AMA1004 (pBS96) (PBP1b)</td>
<td>121 ± 34</td>
<td>2,398 ± 121</td>
</tr>
<tr>
<td>ponB::Spc° AMA1004 (pBS98) (PBP1a)</td>
<td>5,700 ± 172</td>
<td>ND</td>
</tr>
<tr>
<td>ponB::Spc° AMA1004 (PBP1b)</td>
<td>4,784 ± 386</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Membranes of E. coli AMA1004, the PBP1b-deficient AMA1004 ponB::Spc° strain, and the latter strain transformed with a plasmid encoding either PBP1a (pBS89) or PBP1b (pBS96) under the control of their respective cognate promoters were incubated under conditions for peptidoglycan synthesis. Reaction products were captured with WGA-SPA beads plus N-laurylsarcosine or with the same beads in the absence of detergent (where lipid II is the final product). Cross-linked peptidoglycan was formed in the transformant strains and in wild-type E. coli but not in the PBP1b-deficient strain. ND, not determined.
inhibition of the transformant strain alone. This could be used to test if an unknown compound is a PBP inhibitor rather than an inhibitor of the earlier enzymes in the pathway, i.e., MraY, MurG, or lipid pyrophosphorylase.

Accordingly, we tested the effect of inhibitors on all three membranes (Fig. 2). As expected, tunicamycin, an inhibitor of MraY, inhibited both the PBP1b-deficient strain and the strain transformed with PBP1a or PBP1b. On the other hand, moenomycin and penicillin, inhibitors of transglycosylase and transpeptidase, respectively, inhibited the transformants only and not the PBP1b-deficient strain. Interestingly, penicillin inhibited peptidoglycan synthesis in the PBP1b transformant with a 50% inhibitory concentration (IC50) of 4 ± 1.5 μM but that of the PBP1a transformant with an IC50 approximately 10 times lower (<0.3 μM). This must reflect the different sensitivities of PBP1a and PBP1b to penicillin. Supporting this is the fact that the IC50 of the PBP1b transformant is similar to that of wild-type E. coli, where the major activity is contributed by PBP1b (8, 10).

This result prompted us to test the effect of cephalaxin and cephradine on peptidoglycan synthesis in membranes of the transformants. As expected, these two antibiotics, which bind with higher affinity to PBP1a and PBP3 than to PBP1b (9), showed IC50 values of ~2.7 ± 0.5 and 7.2 ± 1.2 μM, respectively, on peptidoglycan synthesis in the PBP1a transformant membranes, while having no effect on the PBP1b transformant or on wild-type membranes (8).

The IC50s for moenomycin were similar with the PBP1b and PBP1a transformants (~150 nM), but these values were more than 10 times higher than the moenomycin IC50 with wild-type E. coli (Table 2). This difference must be a reflection of the increased copy number of the PBP in membranes of the transformants. For unknown test compounds, this difference could be used as an indicator of specificity of interaction with the PBPs. A summary of IC50s is shown in Table 2.

**Transglycosylase-transpeptidase assay feasibility.** In the above-described transformant strains, the plasmid-encoded PBPs presumably integrate into the membrane in a fashion similar to that of the native enzyme. If the radioactive lipid II that accumulates in membranes of a PBP1b-deficient strain can be converted to cross-linked peptidoglycan by the addition of an exogenous source of PBP1b, this could be used to develop a direct assay for the transglycosylase-transpeptidase activities of the PBP.

The feasibility of a two-step assay was tested (Fig. 3). In the first step, lipid II is formed by incubation of the PBP1b-deficient membranes (E. coli ponB::Spc) with sugar precursors; formation of lipid II can be monitored by capture with WGA-SPA beads (24). Following this step, the radiolabel was diluted out and the coupled transglycosylase-transpeptidase assayed by the addition of purified E. coli PBP1b (0.6 μg) to the membranes and incubation for 10 min at 37°C. At the end of this incubation, any cross-linked peptidoglycan formed was captured by the addition of WGA-SPA beads and Sarkosyl.

Cross-linked peptidoglycan was indeed formed under these conditions, indicating that the exogenously added PBP1b is able to access the lipid II formed in the membranes (Table 3).

**TABLE 2. Effect of reference inhibitors (IC50s) on peptidoglycan synthesis in wild-type, mutant, and transformant membranes**

| Inhibitor (IC50 unit) | Wild type ponB::Spc (PBP1b) ponB::Spc (PBP1a) ponB::Spc |
|----------------------|----------------------|----------------------|----------------------|
| Tunicamycin (μg/ml) | 0.4 ± 0.1 | 0.17 | 0.08 ± 0.06 | 0.4 ± 0.1 |
| Penicillin (μM)     | 3 ± 0.08 | 4 ± 1.5 | -0.25 | >1,000 |
| Cephalaxin (μM)     | ~1,000 | >1,000 | 2.7 ± 0.5 | >1,000 |
| Cephradine (μM)     | ~590 | >1,000 | 7.2 ± 1.2 | >1,000 |
| Moenomycin (nM)     | 13 ± 4 | 148 ± 2 | 128 ± 3 | >1,000 |

a The genotypic characteristic of the strain is indicated; the plasmid-encoded proteins produced by the transformants are indicated in parentheses. Reactions using membranes of the PBP1b-deficient strain (E. coli AMA1004 ponB::Spc strain) were captured by use of WGA-SPA beads in the absence of detergent; all others were captured by use of WGA-SPA plus N-laurylsarcosine.
Inhibition of this reaction by moenomycin and penicillin, standard inhibitors of the transglycosylase and transpeptidase, respectively, confirmed that the reaction measured was indeed a product of these two enzymatic activities. Nisin, a MurG inhibitor, also inhibited the reaction; however, in the light of data showing that it binds lipid II (36), this is not surprising.

For an assay to be used in high-throughput screening, it is impractical to purify large quantities of PBP1b. Since PBP1b was shown to bind lipid II (36), this is not surprising. Tunicamycin typically inhibits MraY, but since it was added at step 2, after the specific activity of UDP-[3H] GlcNAc was reduced ~100-fold by the addition of excess UDP-GlcNAc, the inhibition observed cannot be due to the inhibition of MraY. To confirm this, the products were analyzed by paper chromatography. The inhibition of MraY should result in no radioactivity in lipid II (7, 27), but if tunicamycin was inhibited by nisin can be explained by its binding to lipid II, the substrate for the transglycosylase (36), the inhibition by tunicamycin was as expected; the IC50s are similar to those observed before for the inhibition of the peptidoglycan synthesis pathway (Table 2) (7). Nisin and tunicamycin also inhibited the coupled transglycosylase-transpeptidase reaction with IC50s of 4 ± 3 μg/ml and 3.2 ± 1 μg/ml, respectively, while vancomycin had an IC50 of 48 ± 14 μM.

Bacitracin (10 μM) did not inhibit the reaction, nor did other drugs such as sparfloxacin (300 μg/ml) or novobiocin (100 μM), indicating that the observed inhibition by tunicamycin and other antibiotics was specific.

**Validation by paper chromatography.** While the inhibition by nisin can be explained by its binding to lipid II, the substrate for the transglycosylase (36), the inhibition by tunicamycin was surprising. Tunicamycin typically inhibits MraY, but since it was added at step 2, after the specific activity of UDP-[3H]GlcNAc was reduced ~100-fold by the addition of excess UDP-GlcNAc, the inhibition observed cannot be due to the inhibition of MraY.

**TABLE 3. Feasibility of assaying the coupled transglycosylase-transpeptidase of PBP1b**

<table>
<thead>
<tr>
<th>Incubation type (time; additive)</th>
<th>cpm (mean ± SD) or % inhibition by shown compounds for:</th>
<th>Pure PBP1b</th>
<th>Detergent extract of membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank (0 min; EDTA)</td>
<td>1,600 ± 12</td>
<td>1,600 ± 12</td>
<td></td>
</tr>
<tr>
<td>Complete (20 min)</td>
<td>4,098 ± 500</td>
<td>3,344 ± 224</td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moenomycin (0.1 μM)</td>
<td>106</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Penicillin (1,000 μM)</td>
<td>96</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Vancomycin (300 μM)</td>
<td>91</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Nisin (1 mg/ml)</td>
<td>81</td>
<td>103</td>
<td></td>
</tr>
</tbody>
</table>

*Membranes from the E. coli AMA1004 ponB::Spc* strain were incubated with peptidoglycan sugar precursors as described in the text; in a second step, they were incubated for 10 min with 0.6 μg of purified PBP1b or with 0.45 μg of a detergent extract of membranes from an E. coli strain overexpressing PBP1b. The enzyme blank had EDTA added at zero time, i.e., before the start of step 2 of the incubation; the cpm of blank and complete reactions are shown. In the lower half of the table, the percentages of inhibition by reference compounds are shown.
camycin was inhibiting the transglycosylase, radioactive lipid II would be formed but not converted to peptidoglycan. Thus, paper chromatography should show radioactive lipid II in the tunicamycin-inhibited reaction if the TG were inhibited.

The chromatography analysis was also used to confirm the substrate lipid II, the product of the reaction, and inhibition by other compounds. In the paper chromatogram, both cross-linked and un-cross-linked peptidoglycan remain at the origin, while lipid II runs at an $R_f$ of 0.9. Counts per minute in 1-cm segments are shown starting 1 cm below the origin. For ease of visualizing the data, the paper chromatography analysis of the blank, complete, and inhibited reactions is split into two graphs; the blank and complete reactions are plotted in both graphs for the sake of reference. Bottom right panel symbols: P, penicillin; V, vancomycin; M, moenomycin. The inhibitors used were as follows (concentrations shown in parentheses): tunicamycin (30 μg/ml), nisin (100 μg/ml), vancomycin (1 mM), penicillin (1 mM), and moenomycin (100 nM).

FIG. 4. Coupled transglycosylase-transpeptidase assay. (Top left) Effect of different incubation times at step 2 of the coupled TG-TP SPA. (Top right) Dependence of activity on the quantity of PBP1b-containing detergent extract of membrane in the TG-TP SPA; the quantity of detergent extract was varied while the detergent concentration was kept constant. (Bottom) Paper chromatographic analysis of products of the coupled TG-TP assay. Peptidoglycan, both cross-linked and un-cross-linked, remains at the origin of the chromatogram at an $R_f$ of 0, while lipid II runs at an $R_f$ of ~0.9. Counts per minute in 1-cm segments are shown starting 1 cm below the origin. For ease of visualizing the data, the paper chromatography analysis of the blank, complete, and inhibited reactions is split into two graphs; the blank and complete reactions are plotted in both graphs for the sake of reference. Bottom right panel symbols: P, penicillin; V, vancomycin; M, moenomycin. The inhibitors used were as follows (concentrations shown in parentheses): tunicamycin (30 μg/ml), nisin (100 μg/ml), vancomycin (1 mM), penicillin (1 mM), and moenomycin (100 nM).

FIG. 5. Effect of tunicamycin (A), nisin (B), moenomycin (C), vancomycin (D), and penicillin G (E) on the coupled TG-TP SPA. The coupled transglycosylase-transpeptidase SPA was performed as described in Materials and Methods in the presence of different concentrations of inhibitor, and the percent inhibition is plotted as a function of log inhibitor concentration. Where the starting material is not pure, the inhibitor concentrations are expressed in μg/ml.
whereas lipid II runs near the solvent front (16, 21, 30). Thus, in the complete reaction (the product is cross-linked peptidoglycan) or in a reaction where the transpeptidase is inhibited (the product is un-cross-linked peptidoglycan), radioactivity will be seen at the origin of the chromatogram. If the transglycosylase is inhibited, however, no conversion of lipid II will occur, and this will be reflected in the lack of radioactivity at the origin but counts at the lipid II region.

In the blank reaction (time zero addition of EDTA), the major product was lipid II ($R_f \sim 0.9$), part of which was converted to peptidoglycan upon incubation for 20 min with a source of PBP1b (the complete reaction is shown in Fig. 4, bottom left).

Both moenomycin and nisin prevented conversion of lipid II to peptidoglycan, giving the same quantity of peptidoglycan (cpm at the origin) as the blank reaction and indicating that both inhibit the transglycosylase. Interestingly, the radioactive lipid II peak observed with nisin is more diffuse than that of the blank reaction or that inhibited by moenomycin. We have observed this in many experiments with nisin; it is probably the result of the binding of nisin to lipid II, which alters its mobility. Tunicamycin showed inhibition of peptidoglycan synthesis, with a chromatographic profile similar to that of moenomycin and nisin and with no peptidoglycan at the origin. However, lipid II levels were similar to the zero time reaction, indicating that the transglycosylation was inhibited by tunicamycin and that the inhibition of MraY was not responsible for the inhibition seen in this assay.

As expected, penicillin, which inhibited the TG-TP SPA, shows no reduction in the radioactivity at the origin compared with that for the complete reaction, confirming that it inhibits the transpeptidase and not the transglycosylase. Vancomycin, which inhibited the SPA with an IC$_{50}$ of $\sim 48$ $\mu$M, showed only partial inhibition of peptidoglycan synthesis in the paper chromatographic analysis at 1 mM, suggesting that the inhibition observed with the SPA is a result of the cumulative inhibition of both the transglycosylase and the transpeptidase.

**DISCUSSION**

The coupled TG-TP scintillation proximity assay we describe can be performed in a microdilution plate, making possible its adaptation for high-throughput screening. Although we have used only PBP1b in this assay, in principle other bifunctional PBPs from *E. coli* or other bacteria could also be used.

An earlier report (8) described an indirect method to distinguish inhibitors of the PBPs from inhibitors of the other three membrane-associated enzymes of peptidoglycan synthesis in a pathway of reactions culminating in cross-linked peptidoglycan. However, in that system, inhibition of the TG or TP by compounds that also inhibit MraY or MurG, e.g., tunicamycin and nisin, cannot be detected. Similarly, while this assay has a distinct advantage over others (6) in that it is able to detect transpeptidase inhibitors, it cannot distinguish TG from TP inhibitors, and the effect of TG inhibitors on TP will be concealed. Other assays, e.g., paper chromatography, will have to be used to deconvolute inhibitors into those targeting the TG or TP.

While inhibition of the TG-TP assay by penicillin and moenomycin is expected, that by nisin and tunicamycin is not. To our knowledge, this is the first report of tunicamycin inhibition of transglycosylase activity. Tunicamycin is a UDP-MurNAc(pp)-competitive inhibitor of MraY (4), and inhibition in this assay cannot be explained easily. One possibility is that the PBPs form a complex with the other membrane proteins involved in peptidoglycan synthesis (22, 35) and that inhibition is via tunicamycin binding to MraY. On the other hand, the lack of a convenient assay for the transglycosylase would have prevented the testing of many compounds, and it is possible that tunicamycin also directly interacts with the transglycosylase. Nisin, like ramoplanin, binds lipid intermediates; ramoplanin has been shown to inhibit the transglycosylase (18), so our result is not unexpected.

There is much promiscuity in the inhibitors of peptidoglycan synthesis. Vancomycin inhibits MraY, MurG, and the transglycosylase and transpeptidase (13) by binding to the terminal $\alpha$-D-Ala-$\alpha$-D-Ala residues of peptidoglycan, lipid I, lipid II, and the sugar precursor UDP-MurNAc(pp). Ramoplanin binds lipid I and lipid II and inhibits MurG and the transglycosylase (18). Here, we show that both nisin and tunicamycin inhibit the transglycosylase. It thus appears that moenomycin and penicillin are the only two specific inhibitors in this pathway; this raises the question of how tunicamycin and nisin inhibit bacterial growth. It is quite likely that the primary killing mechanism is due to the inhibition of the TG-TP, since these are periplasmic enzymes and presumably encounter a higher concentration of inhibitors than do enzymes that are present on the inner surface of the cytoplasmic membrane, such as MraY and MurG.

The activity of PBP1a is difficult to assay, with most of the activity in wild-type *E. coli* membranes being contributed by PBP1b (8, 10). For the first time, we show that PBP1a can easily be monitored by expressing it on a plasmid in a host strain inactive for PBP1b. The same method can be used to study the activity of heterologous bifunctional PBPs by cloning them into an *E. coli* strain deficient in PBP1b. This method of testing the activity of a PBP is advantageous for PBPs that may be sensitive to purification and that cannot be tested by the direct TG-TP assay. It can also easily be used to study the activity of mutants of the PBP.

In addition, the increased IC$_{50}$ of a test compound on membranes overexpressing either PBP1a or -1b can be used as an indication of specific binding to the PBP. This effect was seen with moenomycin but, surprisingly, not with penicillin (Table 2). It is possible that there are so many PBPs in the cell that the overall sink of penicillin-binding proteins is not significantly increased by overexpression of either PBP1a or PBP1b; in wild-type cells, each of these represents only $\sim 10\%$ of the total PBP pool in cells (11). Moenomycin is an inhibitor of the TG, and since only PBP1b, PBP1a, and PBP3 among all PBPs have the TG domain, we expect the pool of moenomycin-binding proteins will significantly increase by overexpression of PBP1a or -1b. This increased IC$_{50}$ could result in a higher MIC for the growth of the bacteria containing higher levels of the PBP and could be used to confirm the mechanism of bacterial growth inhibition for unknown compounds. Unfortunately, since moenomycin does not inhibit growth of *E. coli*, this could not be tested.

For a long time, peptidoglycan synthesis has been a favorite target for the development of antibacterial drugs because of the success of antibiotics, e.g., the $\beta$-lactams and glycopeptides,
that act on these targets. An assay such as the one described here could be used to search for novel antibacterial agents.

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