The peptidoglycan transglycosylase of *Bacillus megaterium* has been purified approximately 500-fold from a crude membrane fraction. This protein is likely to be the one previously called PG-II and was assayed by its ability to reconstitute with a crude phospho-N-acetylmuramyl-pentapeptide translocase preparation and partially purified N-acetylmuramylaminotransferase to give peptidoglycan synthesis from nucleotide precursors. The protein was identified as the peptidoglycan transglycosylase by its ability to synthesize lysozyme-sensitive peptidoglycan from undecaprenylphosphoryl-diaminopimelate of another. In *Escherichia coli*, the enzyme has transglycosylase activity, shows no detectable transpeptidase activity, and binds penicillin, and migrates as one main band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**MATERIALS AND METHODS**

**Purification of PG-II**—The first three steps in the purification of PG-II were essentially as described previously (9). Briefly, protoplast membranes of *B. megaterium* were first extracted with 1.5 M LiCl in TM buffer (20 mM MgCl₂, 0.05 M Tris-HCl, pH 7.4), for 15 min at 30 °C to recover the transglycosylase (7, 8). The extracted membranes were collected by centrifugation at 48,000 × g for 20 min at 4 °C and then were solubilized with 1 M LiCl and 2% cholate (9). The solubilized proteins were precipitated with 0.7 parts saturated ammonium sulfate in H₂O and the precipitate was dissolved in H buffer (2% cholate, 0.1 M LiCl, 5 mM ethylenediaminetetraacetate, 1 mM 2-mercaptoethanol, 0.05 M Tris-HCl, pH 7.4). The dissolved material was then purified through three chromatographic steps involving DEAE-cellulose, hydroxylapatite, and Bio-Gel A-0.5m matrices as previously reported (9). After these three purification steps, the active fractions were concentrated by addition of an equal volume of saturated ammonium sulfate. The precipitate (0.67 mg) was dissolved in 30 ml of H buffer containing 1 mM 2-mercaptoethanol and loaded onto a second hydroxylapatite column (1.5 × 2.6 cm) equilibrated with the same buffer. The column was washed extensively with this buffer, followed by washing with the same buffer without cholate. The adsorbed enzyme was eluted with 0.5 M ammonium sulfate in 0.2 M sodium phosphate buffer, pH 6.8. 2-ml fractions were collected at a flow rate of 16 ml/hr. PG-II and transglycosylase activities were determined in 50-μl and 20-μl portions, respectively, of each fraction. Active fractions were pooled and concentrated by precipitation with ammonium sulfate (0.56 g/ml). The concentrated material was dissolved in 0.1 sodium phosphate buffer (pH 6.8) containing 25% glycerol and was used for all experiments.

**Assay for PG-II**—PG-II was assayed as described previously (9). Briefly, PG-II was reconstituted together with translocase and transglycosylase and the reaction was assayed for the synthesis of peptidoglycan from the nucleotide precursors UDP-MurNAc-pentapeptide and UDP-[¹⁴C] MurNAc. The reaction mixture (11.6 cpm/pmol) was prepared basically by adding precursors to each assay tube and the solvent was evaporated under vacuum. The enzyme was added in a final volume of 35 μl in 0.01 M MgCl₂, 0.1 M KC1, 1 mM dithiothreitol, 100 μg/ml of penicillin G, and 0.07 M Tris-HCl, pH 8.5. After incubation for 90-120 min, with the same time being used in any one assay, the reaction tubes were boiled and the contents were analyzed by paper chromatography as described above.

**Protein**—Protein was determined by the Lowry procedure (12).

**SDS-PAGE**—SDS-PAGE was prepared using the method of Laemmli and Favre (13).

**Analysis of Assay Products**—All assay mixtures were analyzed by paper chromatography in isobutyric acid, 1 N NH₄OH solvent as previously described (9). Under these conditions, peptidoglycan remained at the origin, bactoprenyl intermediates had an Rₚ of approximately 0.4, and disaccharide-peptide monomers resulting from lysozyme digestion of peptidoglycan had an Rₚ of approximately 0.5.

**Assay for Transglycosylase**—The substrate bactoprenyl-phosphoryl-MurNAc-[¹³C]GlcNAc-pentapeptide (11.6 cpm/pmol) was prepared basically as described previously (8) and was purified by DEAE-cellulose (10) and Sephadex LH-20 gel filtration chromatography (11). For the transglycosylase assay, approximately 9000 cpm (520 pmol) of lipid substrate in chloroform/methanol (1:1) were first added to each assay tube and the solvent was evaporated under vacuum. The enzyme was added in a final volume of 35 μl in 0.01 M MgCl₂, 0.1 M KC1, 1 mM dithiothreitol, 100 μg/ml of penicillin G, and 0.07 M Tris-HCl, pH 8.5. After incubation for 90-120 min, with the same time being used in any one assay, the reaction tubes were boiled and the contents were analyzed by paper chromatography as described above.

**Chemicals**—Chemicals were as described previously (9) with tunicamycin, in addition, being purchased from Calbiochem.
Peptidoglycan Transglycosylase

Penicillin Binding—Penicillin binding was performed by previously reported procedures (14) using 

**TABLE I**

<table>
<thead>
<tr>
<th>Reconstituted components</th>
<th>With bacitracin</th>
<th>Without bacitracin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan</td>
<td>Lipid substrate</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>Translocase</td>
<td>9  26</td>
<td>5  45</td>
</tr>
<tr>
<td>Translocase plus transferase</td>
<td>14  345</td>
<td>14  509</td>
</tr>
<tr>
<td>Translocase plus transferase plus PG-II</td>
<td>232 233</td>
<td>235 254</td>
</tr>
<tr>
<td>Translocase plus transferase plus boiled PG-II</td>
<td>13 314</td>
<td>14 320</td>
</tr>
</tbody>
</table>

**RESULTS**

Purification of PG-II—In previous experiments, PG-II was prepared from membranes of *B. megaterium* solubilized with cholate and LiCl (9). This protein was assayed by combining it with crude translocase prepared by 0.1 M KOH extraction of membranes (9) and with partially purified transferase obtained by extracting toluene-treated *B. megaterium* cells with LiCl (7, 8). The combined enzymes in a cholate solution were dialyzed to remove the detergent, and the reconstituted preparation was tested for the ability to make chromatographically immobile peptidoglycan from UDP-MurNAc-pentapeptide and UDP-

**FIG. 1. Purity of isolated B. megaterium PG-II.** PG-II was purified as described under “Materials and Methods” and was analyzed by SDS-PAGE. A, original membranes (150 µg) from which PG-II was purified. B, purified PG-II (18 µg). The molecular weights of the reference standard molecules β-galactosidase (130,000), bovine serum albumin (68,000), and ovalbumin (43,000) are indicated.
Peptidoglycan Transglycosylase

The extent of PG-II purification is given in Table II. The quantity of PG-II could not be determined in the original membranes using the reconstitution assay because all the other peptidoglycan synthetic enzymes were also present, so the rate-limiting component in the synthesis may not have been PG-II. Furthermore, to be consistent, PG-II should have been assayed using the standard dissociation and reconstitution procedures, steps which obviously were not relevant when considering the original membranes. In subsequent steps where the yield of PG-II could be determined, the measurements in the early steps might well have been overestimates because there may have been contamination with pyrophosphatase. Consequently, part of the drop in the yield of PG-II during the purification may have been due to the loss of pyrophosphatase. Thus, the PG-II recovery of approximately one-quarter through the four chromatographic steps of Table II was at least as good as indicated in the table and may even have been better. This information suggested that PG-II was quite stable and remained active through a variety of treatments.

Purified PG-II was also assayed for peptidoglycan transglycosylase activity using the direct lipid substrate bactoprenyl-pyrophosphoryl-disaccharide-pentapeptide. This assay could be performed directly using the original membranes and the fractions from the second hydroxylapatite column because no cholate was present. In the intermediate steps, the detergent interfered with the assay, and differential recoveries from dialysis to remove the cholate led to inaccuracies in the transglycosylase determinations. From this direct assay, it seemed that more than 50% of the transglycosylase was recovered in the purified PG-II after the last hydroxylapatite step.

The apparent high recovery of both PG-II and transglycosylase through several purification steps strongly suggested that these two proteins are one and the same. Assuming the recovery of the transglycosylase to be accurately measured by the direct assay, the data in Table II indicated that this enzyme was purified approximately 500-fold.

Characterization of PG-II as the Peptidoglycan Transglycosylase—In the data in Table II, the transglycosylase was only identified as material able to make chromatographically immobile polymer from the substrate for peptidoglycan transglycosylase. Additional experiments were therefore performed to confirm that the assay did indeed measure peptidoglycan transglycosylase.

Perhaps the most crucial test for peptidoglycan is its sensitivity to lysozyme. Therefore, chromatographically immobile polymer made by the transglycosylase from the lipid substrate was cleaved with this enzyme and the products were analyzed by paper chromatography (Fig. 2). The polymer was indeed degradable by lysozyme, largely to the disaccharide-peptide monomers generated by this enzyme from peptidoglycan.

In another test, catalysis by the transglycosylase was tested in the presence of several antibiotics known to affect cell wall biosynthesis (Table III). Of these antibiotics, only vancomycin, previously implicated (15) as inhibiting the polymerization step, had any negative effect on the synthesis. Although tunicamycin has been reported to inhibit the translocase (16, 17), there is no report that it blocks transglycosylation and, indeed, the peptidoglycan transglycosylase was not affected (Table III). Penicillin does not block the formation of uncross-linked peptidoglycan (9), and should not block transglycosylase. This result was also obtained (Table III). It has already been shown that bacitracin does not block the action of PG-II in a reconstituted peptidoglycan synthetic system, and it did not inhibit the transglycosylase (Table III). The reasons are not known for the small amount of stimulation of peptidoglycan synthesis by antibiotics other than vancomycin. These antibiotic studies both support previous reports of the mode of action of these inhibitors and provide additional evidence that PG-II is indeed the transglycosylase.

In further characterization experiments (Table IV), the transglycosylase was found to be neither a Mg2+-requiring nor peptide monomers generated by this enzyme from peptidoglycan.

TABLE II
Summary of purification of PG-II and peptidoglycan transglycosylase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>PG-II</th>
<th>Transglycosylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
<td>Yield</td>
<td>Purification</td>
</tr>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>Original membrane</td>
<td>229</td>
<td>400,000</td>
<td>100</td>
</tr>
<tr>
<td>Solubilized membrane</td>
<td>19</td>
<td>302,000</td>
<td></td>
</tr>
<tr>
<td>First hydroxylapatite</td>
<td>2.1</td>
<td>155,000</td>
<td></td>
</tr>
<tr>
<td>Bio-Gel A-0.5m</td>
<td>0.67</td>
<td>101,000</td>
<td></td>
</tr>
<tr>
<td>Second hydroxylapatite</td>
<td>0.28</td>
<td>75,000</td>
<td>59</td>
</tr>
</tbody>
</table>

Fig. 2. Lysozyme sensitivity of peptidoglycan synthesized by purified B. megaterium transglycosylase. Three identical assay mixtures using purified protein (1.5 ng of protein) were made with transglycosylase substrate for the synthesis of peptidoglycan. After incubation, one fraction was chromatographed immediately (top frame). Another (center frame) was further subjected to digestion with lysozyme at 10 μg/ml for 16 h at 30 °C before chromatography. In the third incubation (bottom frame), boiled enzyme was used. The positions of the immobile peptidoglycan, disaccharide monomers resulting from lysozyme digestion, and lipid substrate of the transglycosylase are indicated.
and could therefore have been dimers of disaccharide-peptidoglycan in the presence and absence of penicillin, and to examine more closely the transpeptidase activity of the purified transglycosylase, we used this enzyme to synthesize lysozyme (Fig. 2), a small amount of material was found which chromatographed more slowly than the peptidoglycan monomer and could therefore have been dimers of disaccharide-peptide cross-linked by transpeptidase in the presence of the antibiotic. However, in some experiments without active transglycosylase, material at this position was also observed. To examine more closely the transpeptidase activity of the purified transglycosylase, we used this enzyme to synthesize peptidoglycan in the presence and absence of penicillin, and a sulfhydryl enzyme, and the enzyme activity could be stimulated by low concentrations of deoxycholate.

In view of reports that E. coli transglycosylases both bind penicillin and have transpeptidase activities, the purified B. megaterium transglycosylase was also measured in these two regards.

The data in Fig. 3 show that the B. megaterium transglycosylase was indeed one of the major penicillin-binding proteins of the B. megaterium 899 membrane. The penicillin-binding pattern of this membrane is similar to that reported earlier for B. megaterium KM (14, 18).

The penicillin-binding proteins of B. megaterium KM were designated PBPs 1–5 in order of decreasing molecular weight (123,000; 94,000; 83,000; 70,000; and 45,000). The B. megaterium 899 penicillin-binding proteins have molecular weights of 126,000, 81,000, 71,000, 61,000, and 47,000. Since the B. megaterium 899 transglycosylase is the fourth from the largest, it may well correspond to PBP 4 of B. megaterium KM.

When peptidoglycan made by isolated B. megaterium transglycosylase in the presence of penicillin was digested with lysozyme (Fig. 2), a small amount of material was found which chromatographed more slowly than the peptidoglycan monomer and could therefore have been dimers of disaccharide-peptide cross-linked by transpeptidase in the presence of the antibiotic. However, in some experiments without active transglycosylase, material at this position was also observed. To examine more closely the transpeptidase activity of the purified transglycosylase, we used this enzyme to synthesize peptidoglycan in the presence and absence of penicillin, and a sulfhydryl enzyme, and the enzyme activity could be stimulated by low concentrations of deoxycholate.

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then we digested the high molecular weight peptidoglycan at the origins of the chromatograms with lysozyme (Fig. 4). Chromatography of the digestion products clearly showed that the polymer could be completely digested by lysozyme to disaccharide-peptide monomers. There were no lysozyme-resistant dimers due to transpeptidase linking of two peptide side chains. Therefore, our isolated transglycosylase had no transpeptidase activity.

**DISCUSSION**

The data in this paper show that it is possible to purify a protein we have previously identified as PG-II through several purification steps with relatively little loss of activity. This protein is $M_r = 61,000$ both by measurements of enzyme activity of fractions separated by gel filtration column chromatography and by protein staining of SDS-PAGE gels. This agreement reinforces the conclusion that the same protein is being measured in both instances.

Additional tests showed that this highly purified preparation also retained 60% of the peptidoglycan transglycosidase activity. Thus, PG-II is very likely to be the transglycosylase itself. Positive identification of the enzyme as transglycosylase was based on the ability of the enzyme to use the lipid substrate for the transglycosylase to generate lysozyme-sensitive product and to have antibiotic sensitivities appropriate for the transglycosylase.

The transglycosylase activity is neither stimulated by Mg$^{2+}$ ions nor inhibited by sulfhydryl inhibitors. The lack of effect of several antibiotics further confirms the mode of action previously ascribed to the compounds tested. This confirmation gives added confidence in the use of these antibiotics in the specific dissection of biochemical events.

As for *E. coli* (1–6), the *B. megaterium* transglycosylase is a penicillin-binding protein. However, the purified enzyme did not have transpeptidase activity. It is entirely possible that the native protein in the cell has this activity, with the activity being unstable and lost on purification, while the transglycosylase activity and the penicillin-binding activity normally associated with the transpeptidase were more stable and were retained.

Our ability to purify a penicillin-binding protein in *B. megaterium* by assaying for transglycosylase activity is complementary to isolation of transglycosylases of *E. coli* by β-lactam binding (1–6). The experiments reinforce each other with respect to the conclusion that the same protein can have transglycosylase and penicillin-binding activities. Our experiments further show that proteins with both properties can be found for Gram-positive as well as Gram-negative bacteria.

As mentioned under “Results,” *B. megaterium* KM and 899 bacteria both have five PBPs, although the molecular weights differ somewhat between the two strains. However, the PBPs of the two strains are likely to correspond in function in order of decreasing molecular weight because the analogous molecules have approximately the same sizes, and at least one, PBP 5 of both strains, is known to have the same activity, that of a D-alanyl-D-carboxypeptidase (14).2 Enzyme activities for the other isolated *B. megaterium* PBPs have not been reported (14, 18).

If the PBPs of the two strains do correspond, PBPs 1 and 4 are likely to be of importance to cell physiology. PBP 4 has been shown in this communication to be a transglycosylase capable of synthesizing peptidoglycan, so it should be important to cell growth and morphogenesis.

PBP 1 is of interest in light of results with *E. coli*. In this bacterium, PBPs 1a, 1b, and 3 are the targets of lethal penicillin action, and all three are transglycosylases (1–6, 19–21). By analogy, the *B. megaterium* PBPs which are the sites of penicillin-binding would also be transglycosylases. Indeed, *B. megaterium* PBP 1 has been shown to be the site of lethal attack by penicillin (22, 23). Thus, in *B. megaterium*, PBP 1 as well as PBP 4 might be a physiologically important transglycosylase.

In view of the finding in *E. coli* that PBPs 1a and 1b are important for cell elongation, while PBP 3 is implicated in septum formation (19–21), it is possible that the *B. megaterium* PBPs 1 and 4 might also be differentially involved in these two cell processes.

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Purification of the peptidoglycan transglycosylase of Bacillus megaterium.
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