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Purification of Penicillin-Binding Protein 2 of *Escherichia coli*

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Penicillin-binding protein 2 (PBP-2) of *Escherichia coli* K-12 was purified by covalent affinity chromatography using 6-aminopenicillanic acid covalently coupled to carboxymethyl-Sepharose (6-APA-CM-Sepharose). Purification of PBP-2 was accomplished by prebinding the methoxy cephalosporin, cefoxitin, to the Triton X-100-solubilized PBPs of *E. coli* and then incubating the PBPs with 6-APA-CM-Sepharose. Cefoxitin readily binds to all the *E. coli* PBPs except PBP-2 and, thus, in the presence of cefoxitin, only PBP-2 could bind to the 6-APA-CM-Sepharose. The purification of a mixture of all of the PBPs of *E. coli* by affinity chromatography is also described.

Penicillin and other β-lactam antibiotics kill bacteria by inhibiting synthesis of the bacterial cell wall (1, 4, 29). Different approaches have been used to determine which steps of cell wall synthesis are inhibited. For example, in *Escherichia coli* and *Bacillus subtilis* penicillin-sensitive enzymes that may be involved in the synthesis of the peptidoglycan layer of the cell wall have been studied (3-5, 12, 13, 34). These enzymes have been identified as peptidoglycan transpeptidases and D-alanine carboxypeptidases, both of which are penicillin sensitive in vitro. Two penicillin-sensitive carboxypeptidase activities purified from *E. coli* (carboxypeptidases IA and IB, C) (34) are probably not required for normal cell growth (11, 19, 20). The major carboxypeptidase of *B. subtilis* is only inhibited by concentrations of cephalothin much greater than those required to kill the cell, and it is completely inhibited by concentrations of 6-aminopenicillanic acid (6-APA) which have no effect on the growth of *B. subtilis* (2).

Membrane-bound proteins of *B. subtilis* and *E. coli* which bind penicillin covalently have also been investigated as possible targets of penicillin action (25, 27, 30, 31). In the case of *E. coli*, these penicillin-binding proteins (PBPs) were solubilized with the ionic detergent Sarkosyl NL-97 and then separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) (25, 27). Seven *E. coli* PBPs have been identified by this method. Genetic and biochemical studies of the PBPs from *E. coli* have indicated that: (i) PBP-1 is involved in the elongation of the cell wall during the cell division cycle: (ii) PBP-2 has some function in the maintenance of production of the rod morphology of *E. coli* cells; and (iii) PBP-3 is involved in the formation of the septum during cell division (25, 32, 33). PBP-1 includes two protein components termed PBP-1A and PBP-1Bs, and PBP-1Bs may be a transpeptidase (26, 33). PBP-4, -5, and -6 have transpeptidase and carboxypeptidase activity in vitro (11, 20, 28, 34). PBP-4 is identical to carboxypeptidase IB of *E. coli*, and PBP-5 and PBP-6 are the polypeptides composing carboxypeptidase IA (11, 19, 20, 28, 34). Mutants lacking PBP-4 (*dacB*) grow normally (11, 20), as do those lacking the major activity of D-alanine carboxypeptidase IA (*dacA*) (19). The double mutant *dacA dacB* is also apparently normal (19). However, the *dacA* mutation has been shown to be a mutation of PBP-5 and, thus, this double mutant still has about 10% of the wild-type carboxypeptidase activity, due to the presence of an unaltered PBP-6 (21). Thus, the question of whether some D-alanine carboxypeptidase activity is essential for normal growth is still unanswered. PBP-4 has already been purified (34). A mixture of PBP-5 and -6 was obtained previously (34), and these two PBPs were recently separated (H. Amanuma, manuscript in preparation). Studies of the functions of *E. coli* PBP-1, -2, and -3 in cell wall synthesis would be greatly facilitated by purification of these proteins as well.

Mecillinam (6-β-amidinopenicillanic acid) (see Fig. 1) has a unique effect on *E. coli* cells. At concentrations of the antibiotic of 0.1 μg per ml or less, *E. coli* cells become spherical, and then they eventually lyse (17, 22). A round morphology occurs with a high frequency among mecillinam-resistant mutants (10, 18). PBP-2 has a very high specificity for binding mecillinam (24, 25). The concentration of mecillinam at which PBP-2 is 50% saturated is 0.02 μg per ml per 10 min at 30°C. The other PBPs of *E. coli* do not bind mecillinam at this concentration (25). A mutant lacking PBP-2 has been isolated

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(24-26). The mutant is a temperature-sensitive mutant of *E. coli* which is resistant to mecillinam at 30°C. Cells of this mutant, SP6, have a round morphology instead of a rod morphology at 30°C and do not grow at 42°C. Genetic analysis of SP6 and of a second temperature-sensitive mutant which is rod-shaped at 30°C but grows as a sphere at 42°C indicates that the *rodA* gene is either the structural gene for PBP-2 or is very closely linked to that gene (9). The *rodA* gene, a gene maintaining the rod shape of the cell, and the genes for PBP-2 and mecillinam resistance are all cotransducible with the *lip* gene at the same frequency (9). These results led to the conclusion that PBP-2 has a function in maintaining or producing the normal rod morphology of *E. coli*. Purification of PBP-2 is a prerequisite to further biochemical studies of the functions which it may perform in cell wall biosynthesis. This paper describes the purification of *E. coli* PBP-2, as well as that of a mixture of all of the PBPs of *E. coli* by covalent penicillin affinity chromatography (3).

MATERIALS AND METHODS

Preparations of membranes. Cells of *E. coli* K-12 strain H2143, grown to log phase in a medium described previously (34), were used to prepare *E. coli* membranes. Cells (300 g) were suspended in 300 ml of 0.02 M potassium phosphate buffer (pH 7.0), and the cells were then ground with 300 ml of glass beads (Potters Industries) in a Gifford Wood maxi-mill for 30 min at 5°C. The ground cells were then centrifuged for 20 min at 5,000 rpm in a Sorvall centrifuge to remove whole cells and debris. The supernatant was then centrifuged at 38,000 rpm in a model B60 ultracentrifuge (International Equipment Co., Needham Heights, Mass.), using an A170 rotor for 40 min. The membrane pellet obtained was then suspended in 500 ml of 0.02 M potassium phosphate buffer (pH 7.0) and centrifuged again at 38,000 rpm for 40 min. The pellet was washed again in this manner with 500 ml of the same buffer. The washed membranes were suspended in 0.02 M potassium phosphate buffer (pH 7.0) to a protein concentration of 20 mg per ml.

Preparation of 6-APA-CM-Sepharose or aminopropyl mecillinam-Sepharose. Using the method of Cuatrecasas and Parikh (7), carbo methyl (CM)-Sepharose-CL-6B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was reacted with *N*-hydroxysuccinimide to form the *N*-hydroxysuccinimide ester of the carboxymethyl groups of the CM-Sepharose. 6-APA or aminopropyl mecillinam (Fig. 1) could be coupled to the CM-Sepharose by the reaction of their primary amino groups with the *N*-hydroxysuccinimide ester.

Affinity chromatography of the PBPs. The *E. coli* PBPs were solubilized from membranes of strain H2143 (500 mg of protein) by adding to them an equal volume of buffer containing 0.1 M potassium phosphate (pH 7.0), 2% Triton X-100, 1 M NaCl, 0.06 M $MgCl_2$ and incubating at room temperature for 30 min.

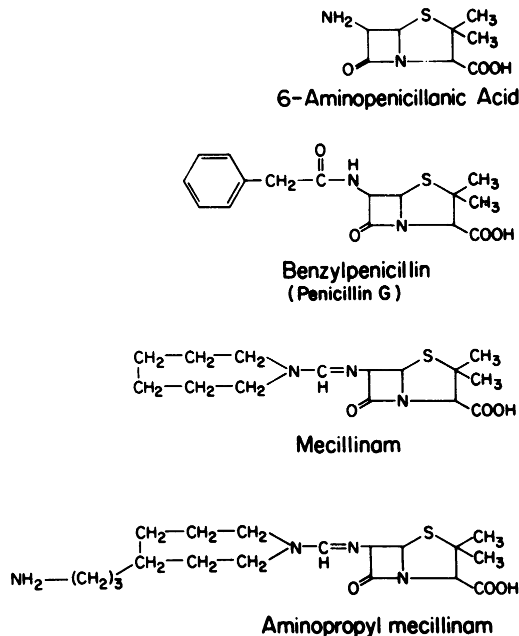

FIG. 1. *Chemical structures of β-lactam antibiotics used.*

The membranes were stirred during this incubation period. The membrane mixture was then centrifuged at 38,000 rpm for 30 min with an IEC model B60 ultracentrifuge. The supernatant contained the crude solubilized PBPs. In the case where PBP-2 alone was to be isolated, cefoxitin or cephradine was added to the solubilized PBPs, and the mixture was incubated at room temperature for 20 min. If all the PBPs were to be isolated, this step was eliminated. An equal volume of 0.02 M potassium phosphate buffer (pH 7.0) was added to the PBPs, and then the PBPs were incubated with 10 ml of 6-APA-CM-Sepharose for 30 min at room temperature. The 6-APA-CM-Sepharose was then filtered in a sintered glass filter and washed successively with 300 ml of buffer composed of 0.05 M potassium phosphate buffer (pH 7.0), 0.1% Triton X-100, 1 mM 2-mercaptoethanol, 300 ml of the same buffer containing 0.1 M NaCl, and 300 ml of the buffer containing 2 M NaCl. This extensive washing was necessary to remove proteins which had bound to the 6-APA-CM-Sepharose non-specifically through, for example, hydrophobic interactions (23).

The washed 6-APA-CM-Sepharose was then poured into a 20-ml glass column. To elute the PBPs from the 6-APA-CM-Sepharose, at room temperature 10 ml of a buffer containing 0.05 M potassium phosphate (pH 7.0), 1% Triton X-100, and 0.4 M NH_2OH was passed through the column and collected. After 30 min had elapsed, an additional 10 ml of the buffer was passed through the column and collected, and after the following 30-min period, a final 10-ml fraction of the buffer was collected from the column.

The three fractions of buffer containing the eluted PBPs were combined and dialyzed overnight against 10 liters of 0.01 M Tris-hydrochloride buffer (pH 7.5)

containing 0.1% Triton X-100 and 1 mM 2-mercaptoethanol at 5°C. This dialysis removed the NH_2OH from the PBP preparation. The PBPs were then concentrated on a 0.2-ml column of DEAE-cellulose (DE-52, Whatman) and eluted from the DE-52 with three column volumes of a buffer containing 0.05 M Tris-hydrochloride (pH 7.5), 1% Triton X-100, and 0.5 M NaCl.

PB assays and SDS-PAGE of the PBPs. To 100 μl of the PBP preparation isolated, 10 μl of [^{14}C]penicillin G (54 μCi/μmol) or 2 μl of [^{14}C]mecillinam (64 μCi/μmol) was added. The final concentration of [^{14}C]penicillin G was 68 μg per ml, and the final concentration of mecillinam was 3 μg per ml. The PBP samples assayed usually contained 2 to 5 μg of protein. The assays were incubated for 10 min at 25°C, and then 400 μl of cold acetone was added to terminate the assay and precipitate the PBPs. The mixture was centrifuged at 7,000 rpm in a Sorvall centrifuge. Then the protein pellet obtained was suspended in 50 μl of buffer containing 0.625 M Tris-hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 3% SDS, and the samples were boiled for 5 min.

The PBPs were then subjected to SDS-PAGE by the method of Laemmli and Favre (15). SDS was present at a concentration of 0.1% in the gel. The upper stacking gel contained 6% acrylamide, and the lower separating gel contained 10% acrylamide. Radioactive protein bands in the gel were detected by fluorography by the method of Bonner and Laskey (6). Kodak XR-5 X-ray film was used in the fluorography procedure. The film was exposed to the gel for 2 weeks for the detection of all PBPs isolated.

Protein concentrations were determined by the method of Lowry et al. (16).

Chemicals. [^{14}C]penicillin G (54 μCi/μmol) was obtained from Amersham Corp., Arlington Heights, Ill. [^{14}C]mecillinam (64 μCi/μmol) was a gift from Leo Pharmaceutical Co., as were nonradioactive mecillinam and aminopropyl mecillinam. Cephradine and cefoxitin were kindly provided by the Merck Company.

RESULTS

Isolation of *E. coli* PBPs by affinity chromatography. The PBPs of *B. subtilis* and *Staphylococcus aureus* have been purified with Sepharose 4B-200 (Sigma Chemical Co., St. Louis, Mo.) containing the spacer arm 6-aminohexanoic acid to which 6-APA had been coupled (6-APA-AH-Sepharose 4B-200). These purifications involve solubilization of bacterial membranes with Triton X-100 and incubation of the detergent extract with Sepharose covalently substituted with a penicillin or cephalosporin, followed by elution of the PBPs with hydroxylamine (3, 14). A similar technique was used to purify the *E. coli* PBPs. Initial attempts to purify the *E. coli* PBPs yielded preparations of PBPs which were badly contaminated with other proteins which bound to the 6-APA-AH-Sepharose 4B-200 non-specifically. It is likely that the nonspecific binding resulted from hydrophobic interactions between the membrane proteins and the hexamethylene spacer arm of the 6-APA-AH-Sepharose 4B-200 (23).

The extent of nonspecific binding to the affinity chromatography resin was significantly reduced by the use of a resin with a shorter spacer (6-APA-CM-Sepharose), which was formed by coupling 6-APA to CM-Sepharose (Pharmacia) using the method of Cuatrecasas and Parikh (7). It had previously been shown that this penicillin affinity chromatography resin could be used to purify the D-alanine carboxypeptidase of *Bacillus stearothermophilus* (35).

In addition to the *E. coli* PBPs previously identified by Spratt and Pardee (27), four other proteins which bind [^{14}C]penicillin are present in the PBP preparation obtained by affinity chromatography using 6-APA-CM-Sepharose (Fig. 2). These are the two bands present between PBP-3 and PBP-4, called PBP-3A and PBP-3B, and the two bands below PBP-6, called PBP-7 and -8. Very little PBP-5 is present in the PBP preparation compared to the amount of

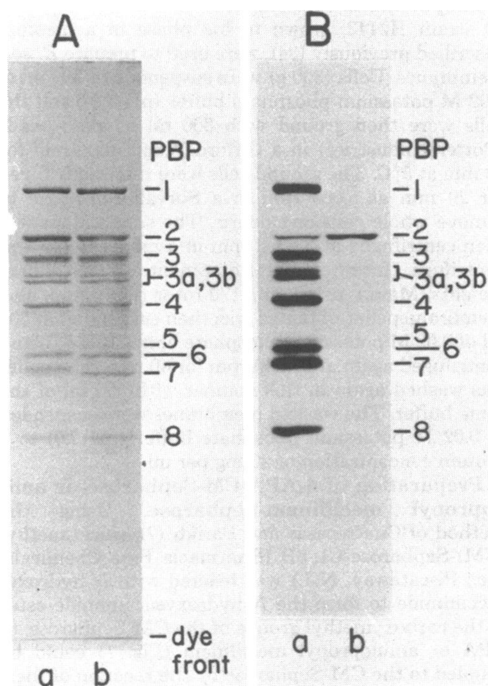

FIG. 2. *PAGE of E. coli PBPs purified by affinity chromatography. (A) Coomassie blue-stained polyacrylamide gel: lanes a and b are duplicate samples of the PBP preparation. (B) Fluorogram of the polyacrylamide slab gel: lane a, binding of [^{14}C]penicillin G to the PBPs; lane b, binding of [^{14}C]mecillinam to the PBPs. See the text for details.*

PBP-6. However, when these proteins are purified by the method of Tamura et al. (34), they are present in approximately equal amounts. The small amount of PBP-5 in the PBP preparation is due to the fact that PBP-5 does not bind to 6-APA-CM-Sepharose, although it will bind to similar affinity columns with longer spacer arms (H. Amanuma, manuscript in preparation).

In Fig. 2B, lane b, the binding of [^{14}C]mecillinam to the PBPs is shown. At the concentration of mecillinam used in the assays, only PBP-2 should bind mecillinam (25). Thus, the major protein band binding mecillinam is PBP-2. The results indicate also that a very small amount of [^{14}C]mecillinam binds to PBP-3A and PBP-4. All the protein bands in the PBP preparation correspond to PBPs, with the exception of three very minor protein bands. These proteins may be present due to residual nonspecific binding to the 6-APA-CM-Sepharose. No attempt has been made thus far to further characterize PBP-3A, -3B, -7, and -8. However, these PBPs appear consistently in the PBP preparations prepared by the affinity chromatography procedure described here.

Purification of PBP-2. Since PBP-2 selectively binds mecillinam, it was thought that it would be possible to purify PBP-2 by affinity chromatography using Sepharose to which a derivative of mecillinam, aminopropyl mecillinam, had been coupled (Fig. 1). However, this procedure was unsuccessful because it was not possible to couple the aminopropyl mecillinam to the Sepharose so that the concentration of mecillinam per milliliter of Sepharose was low enough to prevent binding of PBPs other than PBP-2.

Another possible method of isolating PBP-2 would be to add to the solubilized PBP mixture a penicillin or cephalosporin which bound to all the PBPs except PBP-2 and then incubate the PBPs with 6-APA-CM-Sepharose. Under these conditions only PBP-2 should bind to the 6-APA-CM-Sepharose. Cephradine and cefoxitin were used since these cephalosporins bind to PBP-2 only at very high concentrations compared to the concentrations at which they can bind to the other PBPs (25).

The *E. coli* PBPs were solubilized with a buffer containing Triton X-100 and 0.5 M NaCl as described. Then, either 600 μg of cefoxitin per ml or 400 μg of cephradine per ml was added to the solubilized proteins, and the mixture was incubated for 20 min at 25°C. The PBPs were then incubated with 6-APA-CM-Sepharose, the Sepharose was washed, and the PBPs were eluted with a buffer containing 0.4 M NH_2OH.

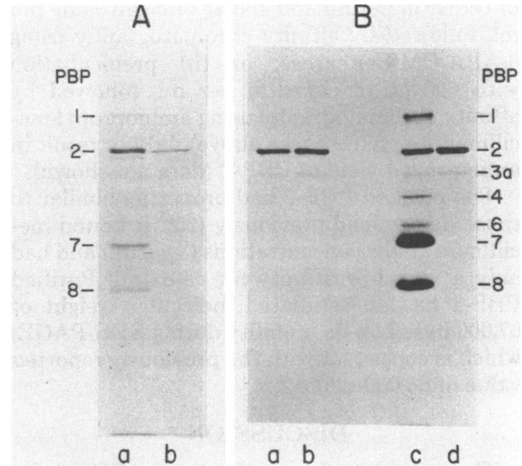

FIG. 3. *PAGE of PBPs purified when cephradine or cefoxitin was prebound to the PBPs before affinity chromatography. (A) Coomassie blue-stained polyacrylamide slab gel: lane a, cephradine (400 μg/ml) was prebound to the PBPs; lane b, cefoxitin (600 μg/ml) was prebound to the PBPs. (B) Fluorogram of the gel showing binding of [^{14}C]penicillin G and [^{14}C]mecillinam to the PBP preparations: lane a, binding of penicillin G to the cefoxitin-pretreated PBP preparation; lane b, binding of mecillinam to the cefoxitin-pretreated PBP preparation; lane c, binding of penicillin G to the cephradine-pretreated PBP preparation; lane d, binding of mecillinam to the cephradine-pretreated PBP preparation.*

The results are shown in Fig. 3.

Figure 3A shows the Coomassie blue-stained gel containing samples of the preparation obtained, and Fig. 3B shows a fluorogram indicating the results of assays for the binding of [^{14}C]-mecillinam and [^{14}C]penicillin G to the PBP preparations. The preparation obtained by binding cefoxitin to the solubilized PBPs before affinity chromatography has only one major protein band in it (Fig. 3A, lane b). This band corresponds to the major protein band binding both penicillin G and mecillinam in the preparation and is at a position in the gel corresponding to PBP-2 (Fig. 3B, lanes a and b). There is also a minor protein band in this preparation which binds penicillin G and mecillinam. Its position in the gel indicates that it is PBP-3A. The PBP preparation obtained by incubating the solubilized PBPs with cephradine before affinity chromatography contains significant amounts of the other PBPs in addition to PBP-2 (Fig. 3A, lane a and Fig. 3B, lane c). However, more PBP-2 was present in this prepartion than in the preparation treated with cefoxitin.

Modification of the procedure for purification of PBP-2 by (i) preincubation of the Triton-solubilized membrane proteins with both 200 μg

of cefoxitin per ml and 400 μg of cephradine per ml, followed by affinity chromatography using 6-APA-CM-Sepharose; or (ii) preincubation with 600 μg of cefoxitin per ml, followed by affinity chromatography using aminopropyl mecillinam-Sepharose (see above) did not result in an improved yield of PBP-2 (data not shown).

The purified PBP-2 had properties similar to those determined previously (25). It bound mecillinam at low concentrations (3 μg/ml) and had only a very low affinity for cefoxitin. Purified PBP-2 has an estimated molecular weight of 67,000 based on its mobility during SDS-PAGE, which is consistent with the previously reported value of 66,000 (25).

DISCUSSION

The procedure for purification of PBP-2 described here successfully separates PBP-2 from *E. coli* membranes and the rest of the *E. coli* PBPs (except for a very small amount of PBP-3A). A major problem of the purification of PBP-2 is the small amount of the protein present in the *E. coli* cell membrane. Spratt estimated that there are only 20 molecules of PBP-2 present per cell, and the protein binds only 0.7% of the total [^{14}C]penicillin G which can bind to membranes of *E. coli* (25). The results of our purification procedure indicated that from 300 g of membrane protein, possibly as much as 50 to 100 μg of PBP-2 could be isolated. Purification of PBP-2 from a mutant containing elevated levels of PBP-2 would provide better yields of PBP-2. Cloning of the PBP-2 gene is another approach to obtaining larger amounts of this protein.

The preparation of all the *E. coli* PBPs purified by affinity chromatography consistently contained four PBPs not previously identified, PBP-3A, -3B, -7, and -8. These PBPs do not correspond to any of the seven Sarkosyl-solubilized PBPs which have been consistently detected by the method of Spratt and Pardee (27). However, PBP-7 may be similar to an eighth PBP which appears occasionally in the Sarkosyl-solubilized preparations. The reason PBP-3A, -3B, -7, and -8 appear only in the purified *E. coli* PBP preparation may be that: (i) these PBPs do not bind [^{14}C]penicillin G when the penicillin is incubated with the intact membrane as in the procedure of Spratt and Pardee (27) (they may be inaccessible to penicillin in the membrane); (ii) separation of the crude Sarkosyl-solubilized PBPs by slab gel electrophoresis is not sensitive enough to detect PBP-3A, -3B, -7, and -8 under the conditions of electrophoresis employed; (iii) PBP-3A, -3B, -7, and -8 might be proteolytic digestion products of the other *E. coli* PBPs which are produced during the purification procedure. The latter possibility may be unlikely, however, since PBP-3A, -3B, -7, and -8 appear consistently and in the same relative amounts in the purified PBP preparations.

Further characterization of purified PBP-2 should contribute to the determination of the exact role of the protein in the maintenance or production of the rod morphology of *E. coli* cells. Thus far, studies of PBP-2 have encompassed only the determination of the specificity of the protein for different penicillins and cephalosporins (25). These studies were performed by binding the antibiotics to *E. coli* membranes, solubilizing the PBPs with Sarkosyl, and then separating them by SDS-PAGE. The effects of NH_2OH and 2-mercaptoethanol on the release of penicillin from PBP-2 were also investigated. It is important to determine whether PBP-2 has any D-alanine carboxypeptidase or transpeptidase activity and whether there is any other enzyme activity involved in cell wall synthesis. However, no peptidoglycan transpeptidase or D-alanine carboxypeptidase activity has been detected in the purified PBP-2 preparations under the assay conditions employed by Tamura et al. (34) for these activities (data not shown). It is possible that under different conditions enzyme activity might be detected. For example, Triton X-100 was present in the assays performed. Some other detergent, such as a Span or Pegosperse detergent (8), may stimulate the enzyme activity of the purified PBP-2 to a greater extent than Triton X-100. Alternatively, it may be necessary to incorporate PBP-2 into lipid vesicles made from *E. coli* lipids to reconstitute its enzymatic activity. This has been possible with the *B. stearothermophilus* D-alanine carboxypeptidase (34). It is likely that the actual substrate of PBP-2 is a macromolecular precursor of the completed peptidoglycan structure rather than the lower-molecular-weight substrates used in the assays for PBP-2 (34). Alternatively, the substrate may be linked to the lipid carrier, C_{55}-isoprenol. A wide variety of such precursors must be isolated and tested to determine which of them is the actual substrate of PBP-2.

ACKNOWLEDGMENTS

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