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Penicillin-Binding Proteins in *Bacillus subtilis* Mutants

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The penicillin-binding proteins (PBPs) from mutants of *Bacillus subtilis* were studied and related to morphology. In a previously described cloxacillin-resistant mutant of *B. subtilis* strain Porton, PBP 2a had an altered mobility by sodium dodecyl sulfate gel electrophoresis and was present in increased amounts. In addition, PBPs 1a and 1b were missing in this mutant. The only morphological change seen was a decrease in size of about 15%. Studies of two Triton-resistant morphological mutants of *B. subtilis* 168, Tr49 (small diameter) and Tr61 (helical form), revealed no change in the number of PBPs compared with that of the parent strain. However, PBPs 1a and 1b had an altered mobility in the mutant Tr49.

The discovery of multiple penicillin-binding proteins (PBPs) in all of the bacteria studied so far indicates that the mechanism of action of penicillin is complex and that there are multiple killing targets (1, 2, 8). By using improved methods, it was found recently that *Bacillus subtilis* has seven or eight PBPs (PBPs 1a, 1b, 2a, 2b, [2c], 3, 4, and 5) (5), rather than the five originally described (1). Consequently, the PBP pattern and the morphology of the cloxacillin-resistant *B. subtilis* mutant (3) have been reexamined. In addition, the PBPs of two Triton-resistant *B. subtilis* mutants with known morphological changes were studied. One of them has a reduced diameter, and the other grows in helices (7).

MATERIALS AND METHODS

B. subtilis strain Porton and its cloxacillin-resistant mutant 5 (selected by a stepwise procedure [3]) and *B. subtilis* 168 and its Triton-resistant mutants Tr49 and Tr61 (provided by M. J. Tilby, Max Planck Institut für Immunbiologie, Tübingen, West Germany [7]) were employed.

Binding of [^{14}C]penicillin to membranes, solubilization and subsequent purification of PBPs by affinity chromatography employing 7-aminocephalosporanic acid (7-ACA) or 6-aminopenicillanic acid as ligands, and competition studies of binding of cloxacillin or cephalexin to PBPs in mutant 5 were performed in essence as described (3, 5). For scanning electron microscopy, bacteria were grown to mid-log phase in antibiotic M3 medium (Difco Laboratories) and harvested by gentle centrifugation (2,000 rpm in a Sorvall GSA rotor). The cells were washed once in 0.1 M sodium phosphate buffer (pH 7.5) and resuspended in the same buffer containing 0.1% glutaraldehyde. The final suspension contained 5×10^9 cells per ml and was kept at 4°C for at least 4 days. The fixed cells were collected by filtration on a Nuclepore filter and dehydrated by stepwise washing with 0, 25, 50, 75, and 100% acetone, respectively. The dehydrated samples were dried and coated with coal-gold (4) before being analyzed with a Hitachi HHS-2R scanning electron microscope.

RESULTS

Studies of PBPs in the cloxacillin-resistant mutant 5. In an earlier report from this laboratory (3) a cloxacillin-resistant strain, mutant 5 of *B. subtilis* strain Porton, was described in which PBP 2 had an altered mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an increase in resistance to cloxacillin, which parallelled the increased resistance of this mutant to cloxacillin. In addition, PBPs 1a and 1b were missing in this mutant. Improved techniques have led to an increase in the number of detectable PBPs in *B. subtilis*, and in particular, PBP 2 is now known to consist of two and possibly three protein components (PBPs 2a, 2b, and [2c]) (5). These findings required a reexamination of the change in PBP 2 in mutant 5. The result of [^{14}C]penicillin G binding to membrane showed clearly that the protein with altered mobility in mutant 5 was PBP 2a; in addition, this protein was markedly increased in amount (Fig. 1B and C). The absence of detectable amounts of PBPs 1a and 1b was confirmed. Analysis of the purified PBPs from mutant 5 by SDS-PAGE, using Coomassie blue staining for protein, supports the results obtained in studies of [^{14}C]penicillin binding to the membrane-associated PBPs detectable by fluorography. By using 7-ACA as ligand in covalent affinity chromatography of solubilized membranes from mutant 5, Kleppe and Strominger (5) were able to elute with hydroxylamine a mixture of only PBPs 2b, 2c, and 4 from the

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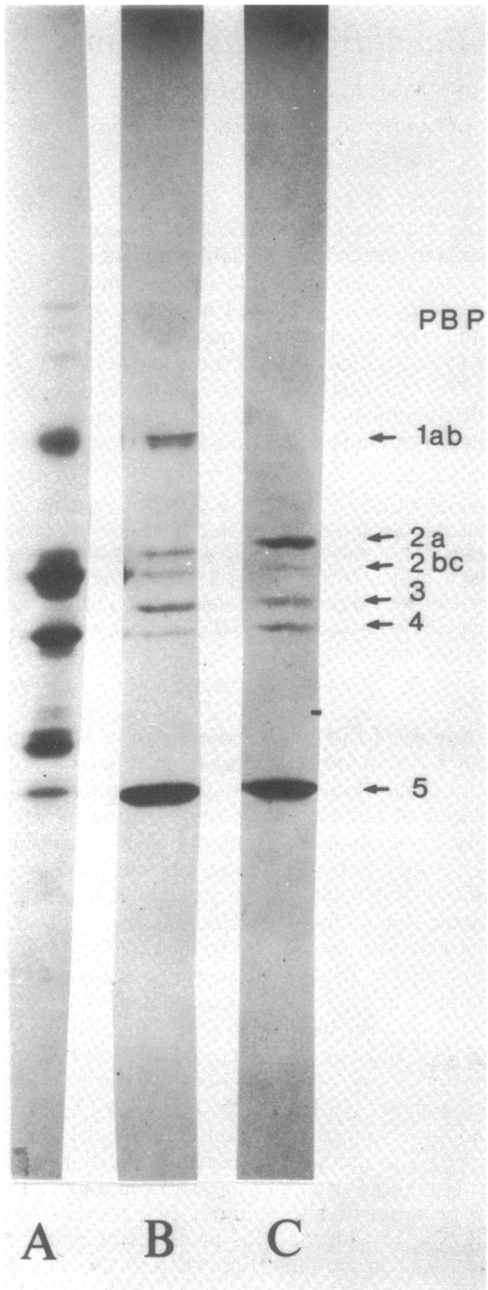

FIG. 1. Analysis of PBPs in particulate membranes from *B. subtilis* strain Porton and cloxacillin-resistant mutant 5. Particulate membranes were incubated with saturating concentrations of [^{14}C]penicillin G before proteins were separated on a 7.5 to 15% polyacrylamide gradient gel. [^{14}C]Penicillin G-labeled proteins were detected by fluorography. (A) Standard PBPs purified with a 7-ACA resin (5), (B) particulate membranes from strain Porton, and (C) particulate membranes from mutant 5.

affinity resin. The "flow through" of the 7-ACA column was subsequently applied to a 6-aminopenicillanic acid affinity column, from which a mixture of the altered PBP 2a and the major PBP 5 (D-alanine carboxypeptidase) was eluted, together with minor amounts of PBP 3 or 4 or both. No PBP 1a or 1b could be isolated from the mutant.

Analysis of PBPs in Triton-resistant *B. subtilis* strains. No major differences in protein pattern were found when particulate membranes from *B. subtilis* mutants Tr49 and Tr61 and the parent strain 168 were analyzed by SDS-PAGE and Coomassie blue staining. However, a slight but reproducible increase in mobility of PBPs 1a and 1b was observed on fluorograms of [^{14}C]penicillin G-labeled proteins from mutant Tr49 (Fig. 2b). This change in mobility was also observed when purified PBPs from this small-diameter mutant were analyzed by SDS-PAGE and detected by Coomassie blue staining (data not shown). An interesting observation is the difference in mobility of the major PBP 5 (D-alanine carboxypeptidase) between *B. subtilis* strain Porton and strain 168 and the conservation of the other PBPs (Fig. 2).

Competition of [^{14}C]penicillin G binding to membranes by cloxacillin and cephalosporin C. Was the previously observed resistance of PBP 2 to cloxacillin due to a change in PBP 2a, 2b, or in both? Fluorograms of separated membrane proteins, which had been preincubated with various amounts of cloxacillin before the addition of saturating amounts of [^{14}C]penicillin G, demonstrated clearly that the change in resistance to cloxacillin (3) was in PBP 2a only. An increased resistance of PBP 2a to cephalosporin C was also observed to the same extent as PBP 2a was resistant to cloxacillin. No change in sensitivity to penicillins or to cephalosporins was observed among any of the Triton-resistant mutants.

Morphology studies. Scanning electron microscopy was used to study any morphological changes between the cloxacillin-resistant mutant 5 and its parent, *B. subtilis* strain Porton. The cells of mutant 5 had a decrease in diameter and length of approximately 16%, but no apparent change in general shape (Fig. 3a and b). This technique was also used to confirm the altered morphology described for the Triton-resistant mutants Tr49 and Tr61. These micrographs demonstrate clearly the helical shape of mutant Tr61 and the reduced diameter of mutant Tr49 when compared with the parent, *B. subtilis* 168 (Fig. 3c, d, and e) (7).

DISCUSSION

The relationship between the PBPs and enzymatic activities, penicillin-killing site, or mor-

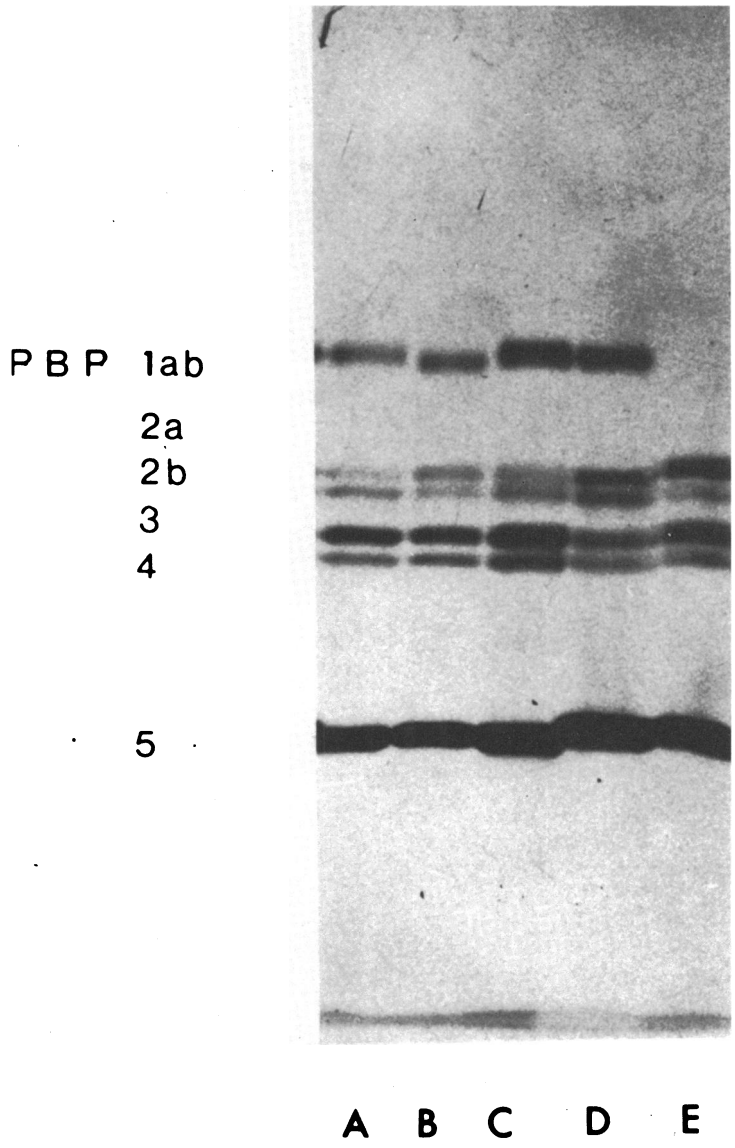

FIG. 2. Fluorogram showing PBPs in particulate membranes of *B. subtilis* strains. Particulate membranes were incubated with [^{14}C]penicillin G (50 μg/ml per min) as described in the text. Proteins were analyzed by SDS-PAGE (7.5%), and PBPs were detected by fluorography. (A) Strain 168, (B) mutant Tr49 (narrow diameter), (C) mutant Tr61 (helical), (D) strain Porton, and (E) cloxacillin-resistant mutant 5.

phology has been most extensively studied in *Escherichia coli*. In this organism, studies of differential sensitivity of PBPs to different β-lactam antibiotics, of mutants in which PBPs have been lost or have altered enzymatic activities, and of temperature-sensitive PBP mutants (reviewed in [6]) have established that apparently only PBPs 1b, 2, and 3 are essential for normal growth. By contrast, the loss of PBP 4 or of D-alanine carboxypeptidase activity of PBP 5 has no apparent consequence for the organism. The role of PBP 6 is not presently known.

Such studies have been comparatively more difficult with gram-positive bacteria because genetic studies of these organisms are less developed. In the present study, an earlier finding has

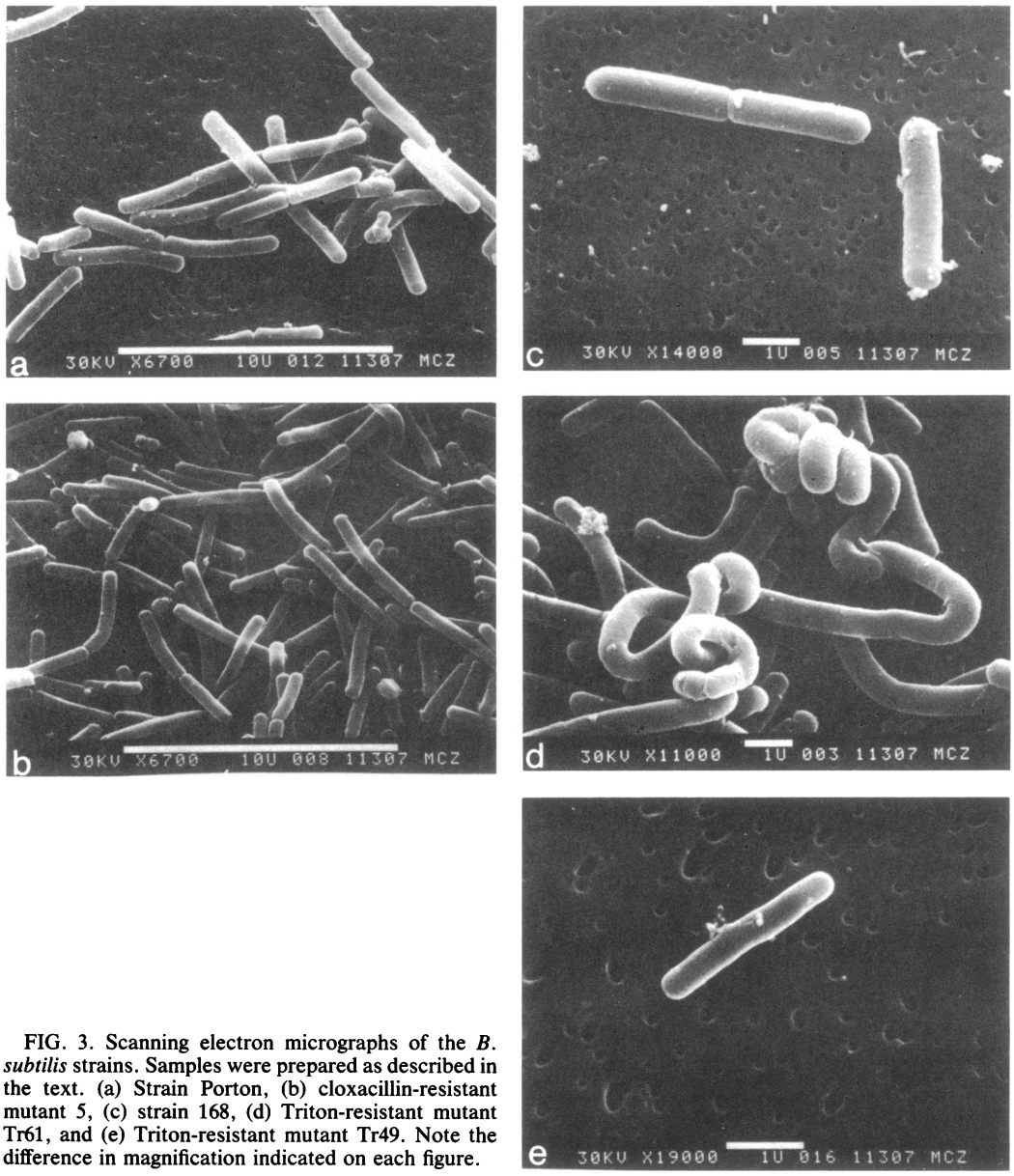

FIG. 3. Scanning electron micrographs of the *B. subtilis* strains. Samples were prepared as described in the text. (a) Strain Porton, (b) cloxacillin-resistant mutant 5, (c) strain 168, (d) Triton-resistant mutant Tr61, and (e) Triton-resistant mutant Tr49. Note the difference in magnification indicated on each figure.

been confirmed, and the reduced cloxacillin affinity of mutant 5 of *B. subtilis* strain Porton (3) can now be associated to an alteration of PBP 2a. In addition, overproduction of this PBP in mutant 5 may compensate in part for the loss of PBPs 1a and 1b. The length and diameter of this mutant were reduced. Similarly, an alteration of PBPs 1a and 1b was found in *B. subtilis* Tr49, a Triton-resistant mutant in which the diameter is reduced (7). Finally, no defect in the PBPs of the helical Triton-resistant mutant Tr61 was apparent by the techniques used here. The helical form could be the consequence of "unbalanced" peptidoglycan synthesis, perhaps resulting from an increase in activity of one of the enzymes (PBPs) involved in peptidoglycan synthesis.

ACKNOWLEDGMENTS

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