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Molecular Cloning of the Gene of a Penicillin-Binding Protein Supposed to Cause High Resistance to β-Lactam Antibiotics in *Staphylococcus aureus*

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A novel penicillin-binding protein, PBP-2′ (M_r about 75,000), is known to be induced in excessively large amount by most β-lactam compounds in cells of a clinically isolated strain of *Staphylococcus aureus*, TK784, that is highly resistant to β-lactams and also most other antibiotics. This protein has very low affinities to most β-lactam compounds and has been supposed to be the cause of the resistance of the cells to β-lactams. A 14-kilobase DNA fragment was isolated from the cells that carried the gene encoding this penicillin-binding protein and also a genetically linked marker that is responsible for the resistance to tobramycin. This DNA was cloned on plasmid pACYC184 and was shown to cause both production of PBP-2′ and resistance to tobramycin in *Escherichia coli* cells. However, the formation of PBP-2′ in *E. coli* was only moderate and was independent of normal inducer β-lactams. The PBP-2′ formed in the *E. coli* cells showed slow kinetics of binding to β-lactams similar to that of PBP-2′ formed in the original *S. aureus* cells and gave a similar pattern of peptides to the latter when digested with the proteolytic V8 enzyme of *S. aureus*.

The development of strains of staphylococci that are highly resistant to β-lactams (methicillin-resistant *Staphylococcus aureus*) is becoming a serious problem in antibacterial chemotherapy (3, 12, 17, 18). Most resistant strains isolated in some hospitals contain a new penicillin-binding protein (PBP) (M_r about 75,000) that has low affinities to most β-lactam antibiotics. Formation of this protein is greatly enhanced by contact of the cell with β-lactam antibiotics (12, 17). Therefore, this new penicillin-binding protein has been thought to cause the very high resistance of the staphylococcal cells to β-lactams. For development of appropriate chemotherapeutic methods, cloning of the gene coding for this protein and studies on the mechanism of induction of the protein by β-lactams and on the structure and functions of the protein are urgently required. Such studies should also be of great interest from the standpoint of general microbiology, as they should reveal the mechanism of evolution of this inducible PBP and the physiological and biochemical functions of PBPs, which have been studied extensively in gram-negative *Escherichia coli* (8, 10) but scarcely at all in gram-positive bacteria.

MATERIALS AND METHODS

Strains used and culture conditions. A β-lactam-resistant, multiple-drug-resistant strain of *Staphylococcus aureus*, TK784, was isolated in Teikyo University Hospital (17). TK784E is a β-lactam-susceptible, tobramycin-susceptible revertant of TK784. *Escherichia coli* K-12 strains K802 (*hsdR hsdM*$^+$ *gal met supE*) and JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ$^-$ Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15) (19) were obtained from Eiichi Ohtsubo. *E. coli* plasmid pACYC184 was obtained from Hiroshi Matsuzawa, and plasmid pUC19 (19), used for preparation of a hybridization probe, was from Eiichi Ohtsubo.

Cells were grown at 30°C in a modified Lennox broth (7) called L′ broth containing 10 g of polypeptone (Daigo Eiyo Kagaku Co., Osaka), 5 g of yeast extract (Daigo Eiyo Kagaku), 5 g of NaCl, 1 g of glucose, and 20 mg of thymine per liter and adjusted to pH 7.0 with NaOH. Solid medium was prepared by the addition of 1.5% agar (Wako Pure Chemical Co., Osaka).

Preparation of DNA. Total cellular DNA was prepared from *S. aureus* TK784 cells in the late logarithmic phase in L′ broth by the protocol of Silhavy et al. (13), with some modifications for application to staphylococcal cells. The cells from 200 ml of culture were washed with and suspended in 10 ml of 50 mM Tris hydrochloride buffer–5 mM EDTA–50 mM NaCl, adjusted to pH 8.0. Lysostaphin (Calbiochem-Behring, La Jolla, Calif.) and RNase A (Sigma Chemical Co., St. Louis, Mo.) were added to final concentrations of 20 and 200 μg/ml, respectively, and the suspension was incubated with mild shaking at 37°C for 30 min. Then 2 ml of 0.5% sodium dodecyl sulfate (SDS)–50 mM Tris–0.4 M EDTA–1 mg of proteinase K (Sigma) per ml, adjusted to pH 7.5, was added with thorough mixing, and the preparation was heated at 50°C for 60 min. The lysed suspension was mixed with an equal volume of phenol (saturated with 100 mM Tris hydrochloride buffer, pH 8.0) and emulsified in a capped tube by repeatedly inverting the tube for 5 min. The resulting emulsion was centrifuged at 1,000 × *g* for 15 min, the aqueous layer was collected and then treated twice with phenol, and the final aqueous layer was extracted three times each with an equal volume of phenol-chloroform (1:1) and chloroform-isoamyl alcohol (24:1). DNA was precipitated from the aqueous phase by inverting the solution very gently after the addition of 0.1 volume of 3 M sodium acetate (pH 8.0) and 2 volumes of ethanol at −80°C. The threadlike

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precipitate was spooled out with a glass rod and dissolved in 4 ml of 50 mM Tris hydrochloride buffer (pH 7.5) containing 1 mM EDTA.

Plasmid DNA preparation. Large-scale preparation of plasmid DNA and rapid electrophoretic analysis of recombinant plasmids were carried out by the alkaline lysis procedure of Birnboim and Doly (1). Transformation was carried out by the method of Norgard et al. (11).

Southern blotting. Chromosomal and plasmid DNA fragments were separated on 0.7% agarose gels containing 0.5 μg of ethidium bromide per ml by electrophoresis as described previously (14). For preparation of a radioactive probe, plasmid DNA was labeled with [α-^{32}P]dCTP (410 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) by the strand-specific labeling method of Hu and Messing (5). Further procedures for Southern transfer and hybridization were as described previously (14).

Detection of PBPs. The procedure for detection of PBPs was essentially as described previously (15), except that [^{3}H] benzylpenicillin was used. Membranes of *S. aureus* or *E. coli* were prepared by differential centrifugation of sonically disrupted cells (with 50 μg of lysostaphin per ml for *S. aureus* cells) in 10 mM potassium phosphate buffer (pH 7.0). The binding reaction was carried out for 10 min at 30°C with 60 μM [^{3}H]benzylpenicillin. With staphylococcal membranes, termination of the binding reaction by adding unlabeled benzylpenicillin (final concentration, 9 mM) was immediately followed by the addition of SDS (final concentration, 1%) and 2-mercaptoethanol (final concentration, 10%) and boiling the mixture for 2 min, omitting the step of removal of the outer membrane fraction by centrifugation. The ^{3}H-labeled proteins were separated by SDS-polyacrylamide gel electrophoresis (6), and the radioactive area was detected by fluorography after preillumination of the X-ray film.

Antibiotics. The following antibiotics were gifts from the indicated companies: cephalosporin 6315S (Shionogi Pharmaceutical Co., Osaka); ampicillin, cloxacillin, and cefminox (MT-141) (Meiji Seika Co., Tokyo); mecillinam (Takeda Chemical Industries Co., Osaka); ceftizoxime (Fujisawa Pharmaceutical Co., Osaka); cefmetazole (Sankyo Co., Tokyo); and clavulanic acid (Beecham Laboratories, Bristol, Tenn.). Benzylpenicillin potassium salt, tobramycin, and chloramphenicol were commercial products. [^{3}H]benzylpenicillin (N-ethylpiperidinium salt, 113.8 mCi/mg) was a generous gift from P. Cassidy, Merck Sharp & Dohme, Rahway, N.J. [^{14}C]cefminox (20 Ci/mol) was from Meiji Seika Co.

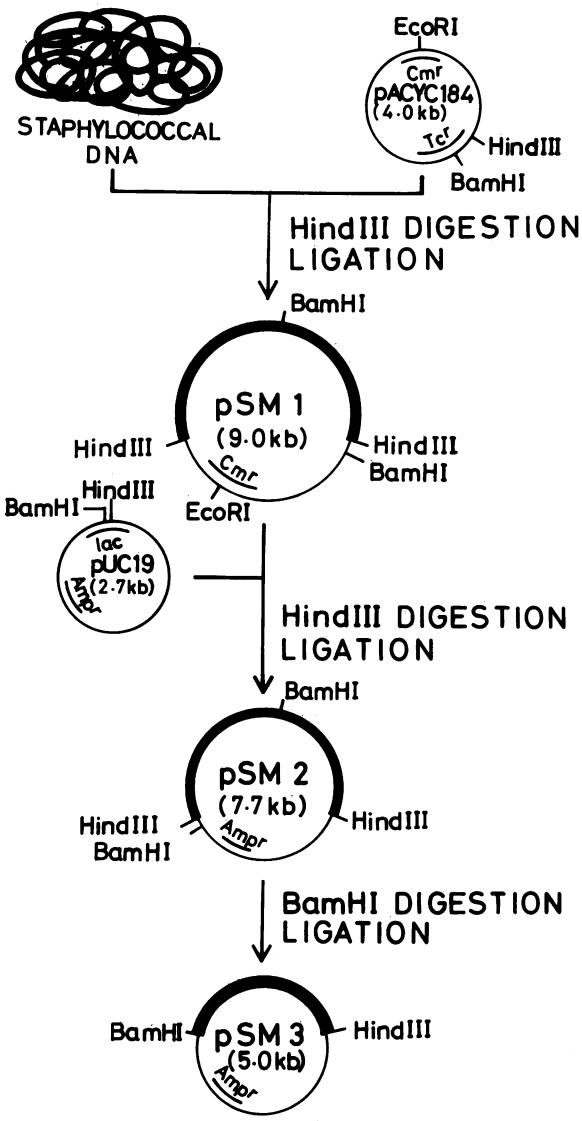

FIG. 1. Cloning of staphylococcal tobramycin resistance for preparation of a probe. The thick line represents staphylococcal chromosomal DNA; the thin lines represent plasmid DNA. Selection in each of the three transformation steps was for tobramycin resistance.

RESULTS

Cloning of PBP-2′ of *S. aureus*. The strategy used for cloning the β-lactam-inducible staphylococcal PBP, PBP-2′, was based upon a previous observation of Ubukata et al. (17) that the resistances of *S. aureus* TK784 cells to two kinds of antibiotics, β-lactams and aminoglycosides such as tobramycin, were closely genetically linked to each other; maintenance of the resistant staphylococcal cells overnight at a high temperature (43.5°C) caused simultaneous loss of the resistances to both antibiotics (sensitive strain TK784E). We hoped that when the cloned gene encoding PBP-2′ from *S. aureus* was introduced into *E. coli* cells, the cell would show resistance to tobramycin, if not to β-lactams, because staphylococcal PBP may not function in *E. coli* in forming the *E. coli* peptidoglycan, even if the protein is formed. The strain used, *S. aureus* TK784, contained a 20-kilobase (kb) plasmid, but this plasmid was found not to carry genes for tobramycin or penicillin resistance. For shotgun cloning, the total staphylococcal DNA was digested by restriction enzymes *Eco*RI or *Hin*dIII, and the DNA fragments formed by each enzyme were ligated to *E. coli* plasmid pACYC184 that had also been treated with the same restriction enzyme (Fig. 1) and used to transform cells of *E. coli* strain K802. Colonies that grew on L′ agar plates in the presence of 25 μg of tobramycin per ml and 25 μg of chloramphenicol per ml were obtained when plasmids were prepared with restriction enzyme *Hin*dIII. The tobramycin-resistant *E. coli* cells contained a plasmid (pSM1) that carried a 5.0-kb staphylococcal DNA fragment (Fig. 1). These resistant cells, however, did not show altered sensitivity to ampicillin or produce PBP-2′, as judged by the SDS-polyacrylamide gel electrophoretic examination. To locate the flanking nucleotide sequences that were expected to contain the gene of PBP-2′, the 5.0-kb

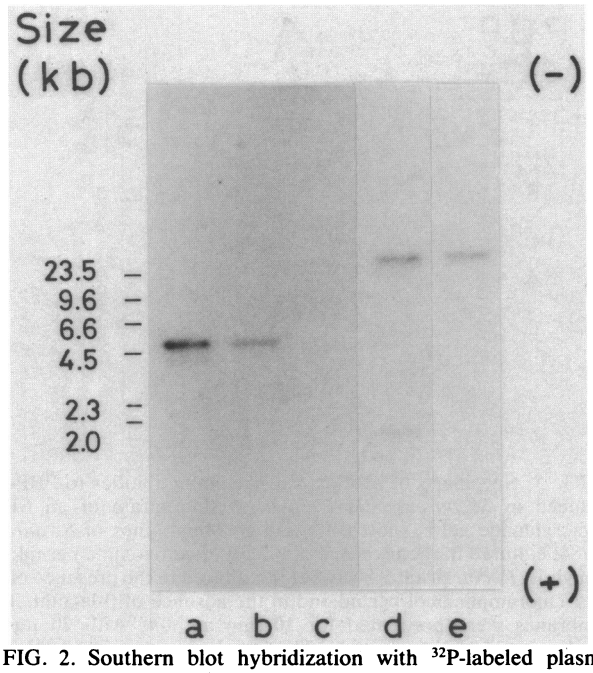

FIG. 2. Southern blot hybridization with ^{32}P-labeled plasmid pSM3 carrying the tobramycin resistance gene as a probe. An autoradiogram is shown. Lanes: a, *Hin*dIII-digested plasmid pSM1; b, *Hin*dIII-digested *S. aureus* TK784 total cell DNA; c, *Hin*dIII-digested *S. aureus* TK784E total cell DNA; d, *Eco*RI-digested *S. aureus* TK784 total cell DNA; e, *Pst*I-digested *S. aureus* TK784 total cell DNA.

fragment was transferred into another plasmid, pUC19, to form pSM2 (host, JM109; white colonies on L′ plates containing 50 mg of 5-bromo-4-chloro-3-indolyl-β-galactoside, 20 mg of isopropyl-β-thio-galactopyranoside, and 50 mg of ampicillin per liter) and was shortened to 2.3 kb by treatment with *Bam*HI. After self-ligation of the plasmid DNAs and transformation, tobramycin-resistant cells were selected (pSM3, Fig. 1). The 2.3-kb fragment containing the tobramycin resistance gene was then used to locate larger restriction fragments of total staphylococcal DNA by Southern blot hybridization (Fig. 2). Cutting with *Hin*dIII, *Eco*RI, or *Pst*I provided fragments of 5.0 kb (*Hin*dIII, track b), 23.5 and 2 kb (*Eco*RI, track d), and 23.5 kb (*Pst*I, track e) that bound to the probe. No radioactive area was observed when DNA from the susceptible strain TK784E was used (track c). The area containing the 23.5-kb *Eco*RI fragment on the gel was cut out, and the DNA fragments were extracted, ligated with plasmid pACYC184 previously cut with *Eco*RI, and used to transform *E. coli* K802 cells. Tobramycin (25 μg/ml)-resistant cells were isolated and were tested for the presence of plasmids and PBPs. *E. coli* cells that contained a 23.5-kb staphylococcal fragment (*Eco*RI sites at both ends) in the plasmid (pSM4, Fig. 3) also contained a new PBP that had mobility very similar to that of staphylococcal PBP-2′ on SDS-polyacrylamide gel electrophoresis (Fig. 4). When the plasmid pSM4 was extracted from strain K802(pSM4) and introduced into another strain (*E. coli* JM109) by selection with 25 μg of tetracycline per ml, this other strain again showed resistance to tobramycin and production of PBP-2′.

The plasmid pSM4 was treated with *Bam*HI, which released a 14.5-kb *Bam*HI fragment of staphylococcal DNA. This fragment was inserted into pACYC184 at its *Bam*HI site (plasmid pSM5, Fig. 3). Cells containing this shortened plasmid, pSM5, also showed both tobramycin resistance and formation of PBP-2′.

The formation of PBP-2′ in *E. coli* cells that carried either one of the above plasmids was not, however, very great, being similar in amount to those of other *E. coli* PBPs, and was constitutive. None of β-lactam compounds so far tested enhanced the formation of PBP-2′ appreciably (compare Fig. 4 and 5). Production of the staphylococcal PBP in *E. coli* did not seem to exert an appreciable effect on the normal growth of the cells. Also insertion of the plasmids (pSM4 or pSM5) into *E. coli* mutant cells that are defective in PBP-1Bs, PBP-2, or PBP-3 did not rescue the phenotypical defect of the corresponding mutant cells (data not shown).

Identification of PBP-2′ formed in *E. coli*. The PBP-2′s formed in *S. aureus* and *E. coli* were identified first by comparing their affinities to some β-lactam antibiotics and then by comparing the peptide patterns of their proteolytic digests.

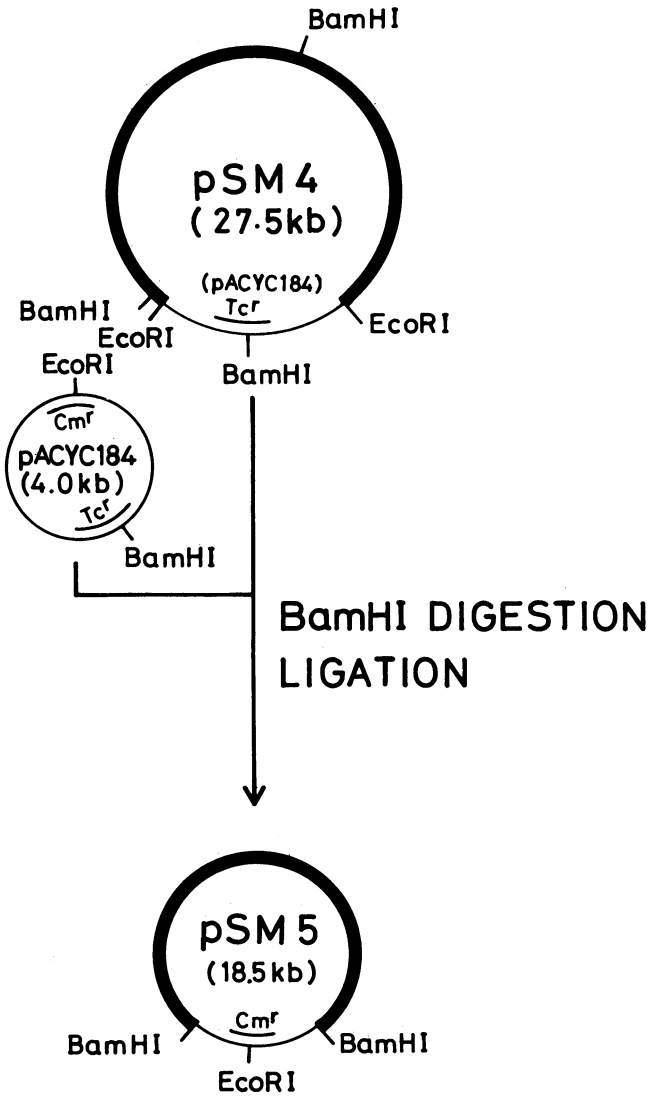

FIG. 3. Cloning of the staphylococcal PBP-2′ gene. A 23.5-kb *Eco*RI fragment of *S. aureus* TK784 DNA was ligated with pACYC184 at the *Eco*RI site, and a 14.5-kb *Bam*HI fragment was recloned by ligation with pACYC184 at the *Bam*HI site.

Binding of [^{3}H]benzylpenicillin to PBP-2′ in *S. aureus* TK784 was scarcely inhibited by prior incubation (10 min at 30°C) with 6315S (50 μg/ml), a new cephalosporin antibiotic (T. Yoshida, T. Tsuji, S. Matsuura, S. Matsubara, and Y. Harada, Program Abstr. 23rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 265, 1983), or cloxacillin (data not shown), because the added β-lactams could not saturate the protein during this short preincubation time, whereas the binding of [^{3}H]benzylpenicillin to all other staphylococcal PBPs was strongly inhibited by similar preincubations (note that clavulanic acid was present at 20 μg/ml to inhibit the β-lactamase activity in the cells). No clavulanic acid was required when [^{14}C]cefminox (16), a 7α-methoxycephalosporin that is not destroyed by staphylococcal β-lactamases, was present (data not shown). Also the binding of PBP-2′ in *E. coli* to [^{3}H]benzylpenicillin was affected much less than those of other PBPs by prior incubation of the membrane fraction with 6315S (Fig. 5), cefminox, or mecillinam. These antibiotics bind strongly to other *E. coli* PBPs, and application of 6315S plus mecillinam (Fig. 6) almost completely inhibited the binding of [^{3}H]benzylpenicillin to all other *E. coli* PBPs, resulting in labeling of essentially only PBP-2′. Cloxacillin and cefminox plus mecillinam also showed similar effects (data not shown).

The ^{3}H-labeled PBP-2′s thus formed in the two bacteria were then treated with staphylococcal V8 protease, and the digests were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 6). During short-term incubation with V8 protease (10 min at 30°C at 2 μg/ml in the presence of 1% SDS) both PBP-2′s were degraded to a labeled penicillin-binding peptide with an apparent molecular weight of about 56,000, which was degraded further during longer incubation. Production of only one penicillin-binding peptide after short-term incubation with V8 protease of SDS-denatured PBP-2′ is quite unusual. Some PBPs treated with proteases before being denatured with SDS formed only one peptide (2, 9, 10), but not after SDS denaturation. Therefore we repeated the experiment of Fig. 6 by using a 15% polyacrylamide gel instead of 12% and again obtained a single band.

Binding kinetics. Like PBP-2′ in *S. aureus*, the protein produced in *E. coli* bound very slowly to [^{3}H]benzylpenicillin (binding of other staphylococcal PBPs is very rapid [4]). The binding increased almost linearly for 60 min upon incubation of the membranes with a sufficient amount of [^{3}H]benzylpenicillin at 30°C (Fig. 7).

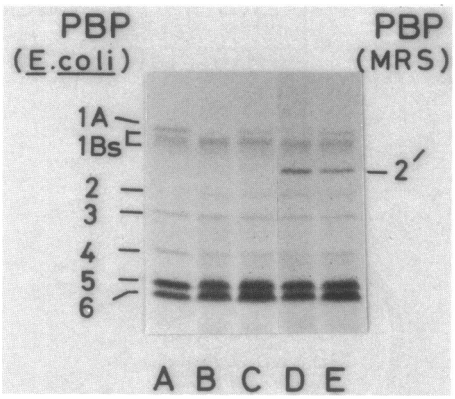

FIG. 4. Expression of the staphylococcal PBP-2′ gene in (A through C) *E. coli* K802 and (D and E) *E. coli* strain K802 containing plasmid pSM4. Cells were grown to 2 × 10^8 cells per ml (A), membranes were prepared, and PBP was assayed. For tracks B and D, 0.2 μg of cefmetazole per ml was present during the final 2 h of culture, and for tracks C and E, 0.2 μg of cefminox per ml was present. The figure shows a fluorogram of an SDS-polyacrylamide gel after electrophoresis. MRS, Methicillin-resistant *S. aureus*.

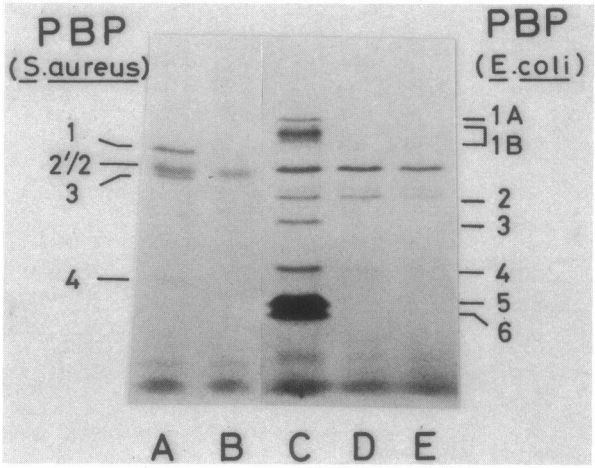

FIG. 5. Specificity of binding of β-lactam antibiotics to PBP-2′s produced in *S. aureus* and *E. coli*. A fluorogram of an SDS-polyacrylamide gel is shown. (A and B) Membranes of *S. aureus* TK784, cultured in the presence of 0.2 μg of ceftizoxime per ml; (C through E) *E. coli* strain K802(pSM5), cultured in the presence of 25 μg of chloramphenicol per ml and in the absence of β-lactam. The membranes were pretreated for 10 min at 30°C with 20 μg of clavulanic acid per ml (track A), 20 μg of clavulanic acid per ml plus 150 μg of 6315S per ml (track B), in the absence of β-lactam compound (track C), with 150 μg of 6315S per ml (track D), or 400 μg of 6315S per ml (track E) before labeling with [^{3}H]benzylpenicillin. The PBP-2′ formed in *E. coli* seemed to have slightly lower mobility than that formed in *S. aureus*.

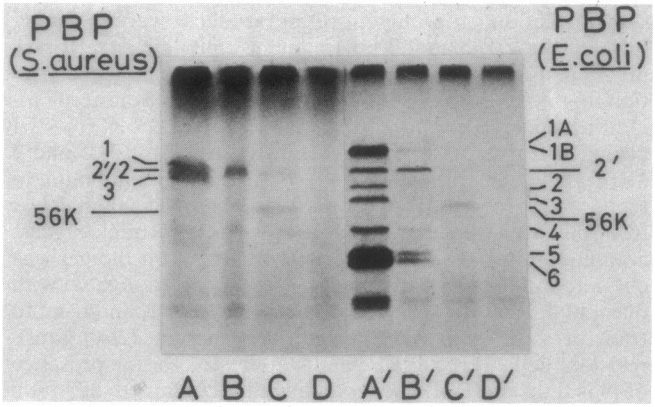

FIG. 6. Peptide pattern of PBP-2′s formed in *S. aureus* and *E. coli* after treatment with staphylococcal V8 protease. Membranes of *S. aureus* and *E. coli* (tracks A and A′ are essentially the same as tracks A and C, respectively, in Fig. 5) were pretreated with 20 μg of clavulanic acid per ml and 50 μg of 6315S per ml (tracks B through D) or 400 μg of 6315S per ml and 20 μg of mecillinam per ml for 10 min at 30°C (tracks B′ through D′), labeled with [^{3}H]benzylpenicillin, and boiled in the presence of 1% SDS and 10% 2-mercaptoethanol as described in Materials and Methods. Then the preparations were digested with staphylococcal V8 protease at a final concentration of 2 μg/ml at 30°C for 10 min (tracks C and C′) or 20 min (tracks D and D′). ^{3}H-labeled peptides were then separated by SDS-polyacrylamide (12%) gel electrophoresis. A fluorogram is shown.

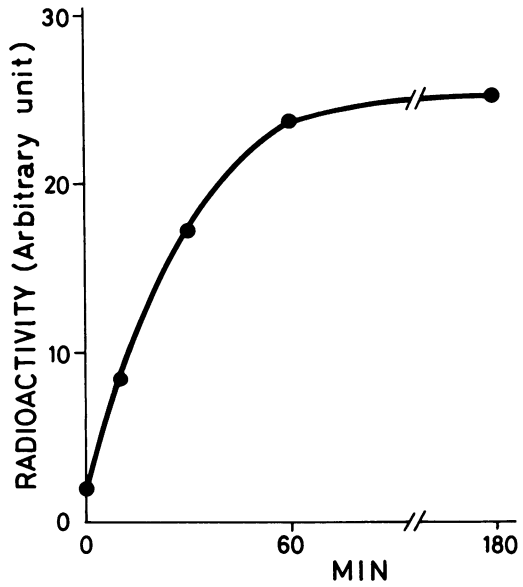

FIG. 7. Time course of [^{3}H]benzylpenicillin binding and chasing. Membranes of uninduced *E. coli* K802(pSM4) cells were labeled with 60 μm [^{3}H]benzylpenicillin at 30°C for the times indicated. The radioactivity at the position of PBP-2′ on the electrophoresis gel was estimated by densitometric tracing of the X-ray film.

DISCUSSION

The gene of a penicillin-binding protein of *S. aureus*, PBP-2′, was cloned in *E. coli* cells, leading to the expression of a protein very similar to the original PBP-2′ in *S. aureus*. Although its expression in *E. coli* was not inducible, the two proteins seemed to be very similar in molecular weight, as judged by the mobilities in SDS-polyacrylamide gel electrophoresis (although sometimes the PBP-2′ formed in *E. coli* appeared to have slightly lower mobility than that formed in *S. aureus*), their peptide patterns obtained after proteolytic digestion, and their very low binding affinities to several β-lactam antibiotics.

This cloning experiment incidentally provided the first case, to our knowledge, of cloning a PBP of one species in another species and the possibility of investigating whether a PBP of one species may function in another. The staphylococcal gene was expressed in *E. coli*, but the product protein did not seem to carry out a functional peptidoglycan-synthetic reaction in *E. coli*. This was expected, because the structure of peptidoglycans in these two organisms are very different and the peptidoglycan transpeptidase (crosslinking enzyme activity) of the former species may not cross-link the peptidoglycan in the latter species.

The source and evolution of this inducible, β-lactam-resistant PBP that gives staphylococci high resistance to β-lactams are unknown. One possibility is that it was formed by recombination between the β-lactamase gene (regulatory region and possibly a part of the reading frame) and some usual PBP gene to form a fused gene that codes for an inducible PBP with low affinity to β-lactams. This possibility must be examined by complete DNA sequencing of the PBP-2′ gene.

The loss of β-lactam inducibility of PBP-2′ when the gene is expressed in *E. coli* indicates either that the regulatory gene (or site) for the expression is separate from the structural gene and was not on the cloned DNA fragment, or that the regulatory mechanism does not function in *E. coli* cells. Further work is required on this problem.

In addition, the gene coding for PBP-2′ in the smallest possible DNA fragment must be returned to *S. aureus* cells to see whether this PBP alone is capable of inducing high β-lactam resistance in *S. aureus*. Now that at least the reading frame of the PBP-2′ gene has been obtained on a plasmid, this should not take too long.

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