Characterization of an *Enterococcus hirae* Penicillin-Binding Protein 3 with Low Penicillin Affinity

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Enterococcus hirae S185, a clinical isolate from swine intestine, exhibits a relatively high resistance to penicillin and contains two 77-kDa penicillin-binding proteins 3 of high (PBP 3^s) and low (PBP 3^r) affinity to penicillin, respectively. A laboratory mutant S185^r has been obtained which overproduces PBP 3^r and has a highly increased resistance to penicillin. Peptide fragments specifically produced by trypsin and SV8 protease digestions of PBP 3^r were isolated, and the amino acid sequences of their amino terminal regions were determined. On the basis of these sequences, oligonucleotides were synthesized and used as primers to generate, by polymerization chain reaction, a 233-bp DNA fragment the sequence of which translated into a 73-amino-acid peptide segment of PBP 3^r. These structural data led to the conclusion that the *E. hirae* PBP 3^r and the methicillin-resistant staphylococcal PBP 2' are members of the same class of high-M_r PBPs. As shown by immunological tests, PBP 3^r is not related to PBP 3^s but, in contrast, is related to the 71-kDa PBP 5 of low penicillin affinity which is responsible for penicillin resistance in *E. hirae* ATCC 9790 and R40.

The emergence among important bacterial pathogens of high-M_r penicillin-binding proteins (PBPs) having low affinity for the drug is a serious threat for the future of chemotherapy (19, 24). Resistance may arise by the remodeling of some targetted PBPs into altered forms which exhibit low intrinsic sensitivity, by de novo synthesis of a PBP of low affinity, or by overproduction of a preexisting highly resistant PBP (9, 22, 23).

The relatively low sensitivity of *Enterococcus hirae* ATCC 9790 to penicillin has been attributed to the occurrence, in small amounts, of a high-M_r PBP of low affinity, the 71-kDa PBP 5. Laboratory mutants such as *E. hirae* R40 have been obtained which overproduce PBP 5 and, as a corollary, are highly penicillin resistant (9).

In contrast to *E. hirae* R40, *E. hirae* S185, another penicillin-resistant strain isolated from swine intestine, contained a small amount of PBP 5 but a large amount of the 77-kDa PBP 3. Given that PBP 3 in strains ATCC 9790 and R40 is very sensitive to penicillin, experiments were undertaken to unravel the underlying mechanism of this new type of penicillin resistance.

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MATERIALS AND METHODS

Bacterial strains and MIC determination. *E. hirae* S185 was a gift from L. Devriese, University of Ghent, Ghent, Belgium. *E. hirae* R40 (9), NT1/20 (2), and Rev14 (10) were gifts from R. Fontana and P. Canepari, University of Verona, Verona, Italy. MAX Efficiency *Escherichia coli* DH5αF'IQ competent strain was from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). *E. coli* HB101 was also used.

MIC values were determined in liquid medium as described previously (4).

Membranes. *E. hirae* cells grown unshaken at 37°C in 500 ml of SB medium (5) and collected at the late exponential phase ($A_{550} = 6.0$) were suspended in 100 ml of 5 mM sodium phosphate (pH 7.0) containing 1 mM $MgCl_2$ and lysed with a mixture of lysozyme (10 mg), DNase (200 µg), RNase (100 µg), and muramidase (1 mg) as described previously (8). Membranes were purified by several washings and centrifugations. They were stored in the frozen state (10 mg of total proteins ml^{-1}) in 40 mM sodium phosphate (pH 7.0) containing 5% (vol/vol) glycerol. The proteins were measured by using the Lowry method as modified by Coyette et al. (3).

Labeling with benzyl[^{14}C]penicillin, SDS-PAGE, and fluorography. Samples were labeled with benzyl[^{14}C]penicillin (54 Ci mol^{-1}; Amersham International, Buckinghamshire, United Kingdom) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography of the gels was performed as described previously (8). The PBPs were estimated by densitometry of the fluorograms by using a model 620 densitometer (Bio-Rad) and *Streptomyces* R61 PBP as the standard (11). The values of the second-order rate constant of protein acylation and the antibiotic concentrations and incubation times necessary to achieve a certain extent of saturation of the PBPs were calculated as described previously (12, 15).

Amino acid sequencing. Depending on the molecular mass, the peptides were subjected to SDS-PAGE (8.5 or 15% acrylamide) and electroblotted on poly(vinylidene difluoride) Immobilon membrane filters (Millipore Corp.) by using a Bio-Rad Mini Trans-Blot cell (17). Automated microsequences were performed on a 477-A pulsed liquid sequenator with on-line analysis of the amino acid phenylthiohydantoin derivatives by using a 120-A analyser (Applied Biosystems, Foster City, Calif.).

Amplification by PCR, cloning, and nucleotide sequencing. The DNA recombinant techniques used were described previously (20). The *E. hirae* S185^r DNA was prepared as described previously (16), which includes treatments with lysozyme in the presence of sucrose, SDS, and protease K. The two nucleotide primers (see Results and Fig. 8) were

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synthesized by Eurogentec, Liège, Belgium. Polymerase chain reaction (PCR) amplification was performed on 100-μl samples containing the E. hirae S185^r DNA (2 μg), the primers (1 μM each), deoxynucleoside triphosphates (200 μM), the Taq DNA polymerase (2.5 U; Perkin Elmer-Cetus, Norwalk, Conn.), and 0.2% (wt/vol) gelatin. The buffer was 10 mM Tris hydrochloride (pH 8.4) containing 50 mM KCl and 2 mM MgCl$_2$. Samples were covered with paraffin and submitted to 30 amplification cycles in a programmable heater as follows: 1 min of denaturation at 94°C, 1.5 min of annealing at 55°C, and 1.5 min of polymerization at 72°C. The 233-bp DNA product was treated as follows: (i) subjected to PAGE (7% acrylamide) in TBE buffer (1 mM EDTA–40 mM Tris-borate buffer [pH 8]) by using a Bio-Rad Mini-Protean apparatus; (ii) eluted from the gel by shaking the relevant strip for 15 h at 37°C in 1 ml of 100 mM Tris hydrochloride (pH 8.0) containing 500 mM NaCl and 5 mM EDTA; (iii) filtered on a 0.22-μm-pore-size membrane filter (Millipore); (iv) precipitated with ethanol; (v) digested with BamHI and EcoRI restriction enzymes (Bethesda Research Laboratories); and (vi) cloned in M13tg130 and M13tg131 (Amersham International, Buckinghamshire, United Kingdom). MAX Efficiency E. coli DH5αF'IQ competent cells served for transformation experiments. Nucleotide sequencing was performed with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) by using the dideoxynucleotide chain termination method (21).

Antibodies. Two antisera were used. One of them was raised against the largest water-soluble tryptic fragments derived from PBP 5 of strain R40 (7). The other was raised against a large tryptic fragment prepared from PBP 3^r (the 64-kDa t-PBP 3^r; see Results). Adult rabbits were injected three times at 15-day intervals with 100 μg of the peptide emulsified in complete Freund adjuvant. Anti-PBP 3^r antibodies were purified by immunoadsorption on E. coli HB101 and E. hirae Rev14 cell lysates (13). Immunodetection was performed with the antiserum at a final 5,000-fold dilution on proteins or peptides transferred from polyacrylamide slab gels onto 0.45-μm-pore-size HA type nitrocellulose sheets (Millipore). Transfer was made by using a Bio-Rad Trans-Blot SD cell and 20 mM Tris–192 mM glycine buffer (pH 8.3) containing 20% (vol/vol) methanol. The antibody-antigen complexes were detected by using an alkaline phosphatase-coupled anti-rabbit goat antiserum (Bio-Rad instruction manual, Immun-blot assay kit, catalog no. 170-6509 and 170-6511).

RESULTS

PBP profiles in E. hirae ATCC 9790, R40, S185, and S185^r: occurrence in strains S185 and S185^r of two 77-kDa PBP 3^s and PBP 3^r of high and low penicillin sensitivity, respectively. E. hirae S185^r was derived from strain S185 by four serial cultures on agar plates containing 32, 64, 128, and 256 μg of benzylpenicillin ml^{-1}. E. hirae ATCC 9790, R40, S185, and S185^r contain the six species-specific membrane-bound PBPs, namely, PBP 1 (119 kDa), PBP 2 (84 kDa), PBP 3 (77 kDa), PBP 4 (75 kDa), PBP 5 (71 kDa), and PBP 6 (43 kDa) (Fig. 1, lanes 1 through 4). The 69-kDa PBP 4* is a spontaneous breakdown product of PBP 4 formed during preparation and storage of the membranes (4).

Although they have similar PBP profiles, the four enterococcal strains differ from each other by the amounts of PBPs 3 and 5 that they contain. In comparison with strain ATCC 9790 (Fig. 1, lane 1), membranes of strain R40 contained relatively less PBP 3 and more PBP 5 (lane 2). In contrast,
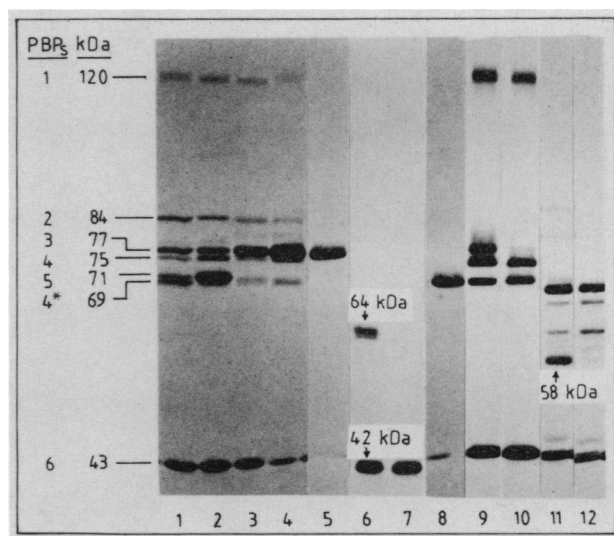

FIG. 1. SDS-PAGE and fluorography of benzyl[^{14}C]penicillin-labeled membranes of E. hirae strains and water-soluble tryptic peptide fragments 64-kDa t-PBP 3^r, 42-kDa t-PBP 3^r, and 58-kDa t-PBP 3^s. Lanes: 1 through 4, membranes (200 μg of protein) of E. hirae ATCC 9790, R40, S185, and S185^r, respectively; 5, membrane (200 μg) of E. hirae S185^r in which PBP 3^r was selectively labeled with 100 μM benzyl[^{14}C]penicillin for 60 min; 6 and 7, supernatant fractions of the S185^r membranes (300 μg) after treatment with trypsin at pH 7.0 (lane 6) or at pH 7.8 and in the presence of 10 mM MgCl$_2$ (lane 7); 8, membranes (200 μg) of E. hirae R40 in which PBP 5 was selectively labeled with 100 μM benzyl[^{14}C]penicillin for 60 min; 9 and 10, membranes (150 μg) of E. hirae NT1/20 as such (lane 9) or in which PBP 3^s was selectively protected against labeling with 100 μM benzyl[^{14}C]penicillin for 60 min (lane 10); 11 and 12, supernatant fractions of the membranes shown in lanes 9 and 10, respectively, after treatment with trypsin. The acrylamide concentration used for the SDS-PAGE was 7.2% (wt/vol).

membranes of strain S185 contained more PBP 3 and less PBP 5 (lane 3). In membranes of strain S185^r, these differences were more pronounced. PBP 3 and PBP 5 represented 23 and 12%, respectively, of the total PBPs in membranes of strain ATCC 9790, 10 and 47% in strain R40, 41 and 6% in strain S185, and 76 and 2% in strain S185^r.

The four enterococcal strains also have widely different benzylpenicillin MIC values: 1 μg ml^{-1} for strain ATCC 9790, 16 μg ml^{-1} for strain S185, 80 μg ml^{-1} for strain R40, and 175 μg ml^{-1} for strain S185^r. Figure 2 shows the effect of increasing concentrations of benzylpenicillin on bacterial growth in SBa broth (5).

Penicillin resistance of strain R40 was proposed to be attributable to the presence of a high level of PBP 5 which has a low affinity for penicillin (9). Given that PBP 3 in strain ATCC 9790 (and R40) is very susceptible to derivatization by benzylpenicillin (4, 9), the high level of penicillin resistance of strains S185 and S185^r (which contain large amounts of PBP 3 and small amounts of PBP 5) was unexpected. Further study revealed that saturation of PBP 3 by benzylpenicillin was monophasic in strains ATCC 9790 and R40, biphasic in strains S185 and S185^r (Fig. 3), and strongly suggested the concomitant presence of a highly penicillin-sensitive 77-kDa PBP 3^s and a much-less-penicillin-sensitive 77-kDa PBP 3^r. As derived from benzylpenicillin concentrations required to achieve 50% saturation after 5 min of incubation at 37°C, the values of the second-order rate constant of protein acylation were 5,000 M^{-1} s^{-1} for PBP 3^s (as observed with PBP 3 of
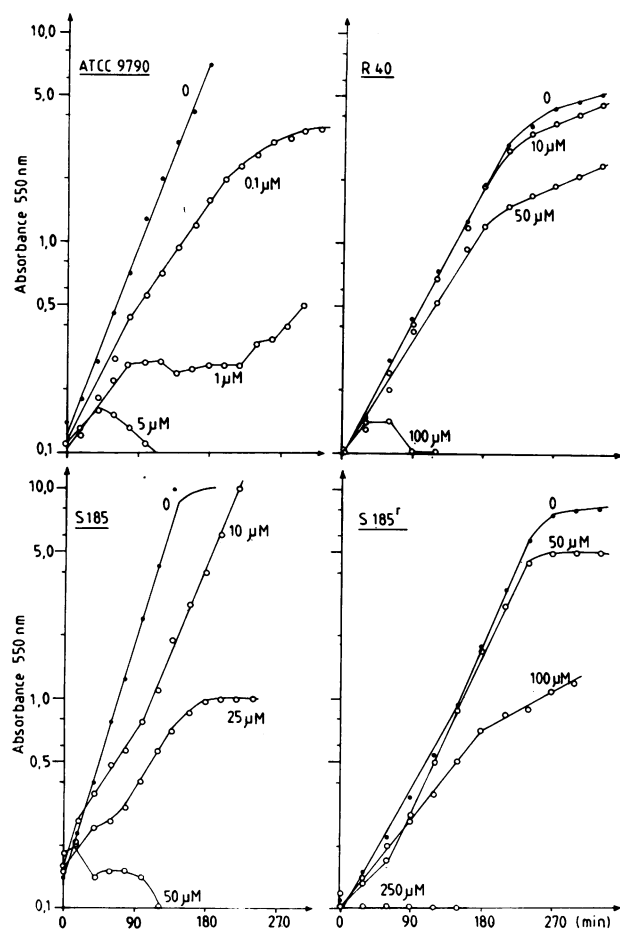
FIG. 2. Effects of increasing concentrations of benzylpenicillin on growth in SBa broth of *E. hirae* ATCC 9790, R40, S185, and S185^r. Absorbances are corrected to agree with Beer Lambert's law. Benzylpenicillin, at the indicated final concentrations (1 μM = 0.356 μg ml^{-1}; 5 μM = 1.78 μg ml^{-1}; 25 μM = 8.9 μg ml^{-1}) was added at time zero.

strain ATCC 9790) and 20 M^{-1} s^{-1} for PBP 3^r (as observed with PBP 5 of strain R40).

To discriminate PBP 3^r from PBP 3^s, membranes of strain S185^r (and strain R40 used as control) were first incubated with 15 μM nonradioactive benzylpenicillin for 10 min at 37°C. This antibiotic concentration was 10-fold higher than that necessary to saturate PBP 3^s (as well as PBPs 1 and 2) and 20-fold lower than that necessary to saturate PBP 3^r or PBP 5. In a second step, the PBPs left in a free form in the membranes were labeled by reaction with 100 μM benzyl[^{14}C]penicillin for 60 min at 37°C, thus causing complete derivatization of both PBP 3^r and PBP 5. As a result of this competition experiment involving nonradioactive and radioactive penicillin, the only labeled PBP seen in vast amounts was the PBP 3^r in the membranes of strain S185^r (Fig. 1, lane 5) and the PBP 5 in the membranes of strain R40 (Fig. 1, lane 8). The PBP contents of *E. hirae* ATCC 9790, R40, S185, and S185^r were estimated on the basis of these and other data (Table 1). *E. hirae* ATCC 9790 and R40 lack PBP 3^r. PBP 3^s and PBP 3^r occur in approximately equivalent amounts (0.3% of total membrane protein) in strain S185 and in a ratio of approximately 1 to 4 (0.4% and 1.6%, respectively) in strain S185^r.

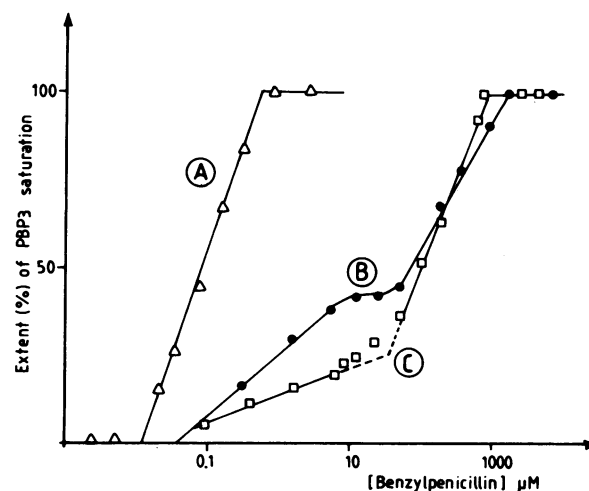**Trypsin digestion of the 77-kDa PBP 3^r: the 64-kDa t-PBP 3^r**

FIG. 3. Saturation by benzyl[^{14}C]penicillin of 77-kDa PBPs 3 of membranes of *E. hirae* ATCC 9790 (A), S185 (B), and S185^r (C). Membranes (100 μg in 10 μl) were incubated for 5 min at 37°C in the presence of increasing concentrations of benzyl[^{14}C]penicillin. Microdensity measurements of the 77-kDa PBPs 3 were made on the fluorograms.

and 42-kDa t-PBP 3^r. Isolation of the membrane-bound 77-kDa PBP 3^r was not attempted. Instead, membranes of strain S185^r in which PBP 3^r was selectively radioactively labeled as described above (Fig. 1, lane 5) were digested with trypsin (type XI; Sigma Chemical Co.) under the following conditions: 300 μg of total membrane protein plus 12 μg of trypsin in 30 μl of 25 mM sodium phosphate (pH 7.0); 30 min at 37°C. This trypsin treatment resulted in the release to the 100,000 × g supernatant of three water-soluble radioactive peptides of 64, 42, and 29 kDa (Fig. 1, lane 6). The relative amounts of each of these peptide fragments could be modified to some extent by adjusting the conditions of proteolytic treatment. In particular, the addition of 10 mM MgCl$_2$ and an elevated pH of 7.8 favored the production of the 42- and 29-kDa fragments (Fig. 1, lane 7). (Note that the 29-kDa fragment is not shown in Fig. 1.)

Membranes of strain R40 in which PBP 5 was specifically radioactively labeled (Fig. 1, lane 8) were also submitted to trypsin digestion. As shown in detail elsewhere (7), the tryptic digest profile of PBP 5 from membranes of strain R40 differed from that of PBP 3^r from membranes of strain S185^r.

Trypsin digestion of the 77-kDa PBP 3^s: the 58-kDa t-PBP 3^s peptide fragment. Identification of the trypsin degradation product(s) of the 77-kDa PBP 3^s rested upon the use of *E. hirae* NT1/20. Strain NT1/20 has a PBP profile similar to that

TABLE 1. PBPs as percentage of total proteins of the membranes of *E. hirae* strains

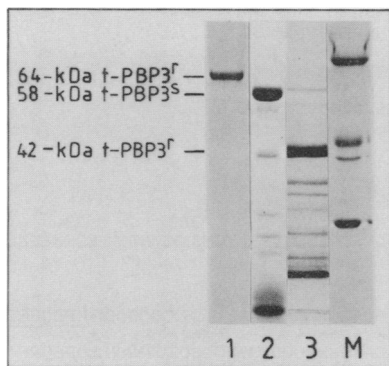| PBP	% of total proteins in strain:			
	ATCC 9790	R40	S185	S185^r
1	0.34	0.18	0.25	0.11
2	0.22	0.17	0.14	0.09
3^s	0.4	0.2	0.32	0.4
3^r	0.0	0.0	0.32	1.6
4	0.31	0.26	0.19	0.25
5	0.22	0.94	0.09	0.05
6	0.26	0.24	0.26	0.14

FIG. 4. SDS-PAGE and Coomassie blue staining of t-PBP 3^r and t-PBP 3^s peptide fragments. Lanes: 1, 64-kDa t-PBP 3^r (3 μg of protein); 2, 58-kDa t-PBP 3^s (≈8.5 μg); 3, 42-kDa t-PBP 3^r (≈7.5 μg); M, protein markers (66.3-kDa bovine serum albumin; 42.7-kDa ovalbumin; 38-kDa *Streptomyces* R61 PBP; 29-kDa carbonic anhydrase).

of strain ATCC 9790 and thus lacks PBP 3^r, but it also lacks PBP 2 (Fig. 1, lane 9) (2). As a corollary, the PBP which has the highest affinity for cefotaxime is PBP 3^s (second-order rate constant of protein acylation, 4,400 $M^{-1} s^{-1}$) (6).

Membranes (300 μg of total proteins in 15 μl of 40 mM sodium phosphate [pH 7.0] containing 1 mM $MgCl_2$ and 5% [vol/vol] glycerol) as such or pretreated with 5 μM cefotaxime for 10 min at 37°C were labeled with 100 μM benzyl[^{14}C]penicillin for 60 min at 37°C and then digested with 3 μg of trypsin for 10 min at 37°C. Note that the 5 μM cefotaxime concentration was fivefold higher than that necessary to saturate PBP 3^s almost completely (Fig. 1, lane 10). The membranes that were not pretreated with cefotaxime yielded three major radioactively labeled peptides of 69, 58, and 44 kDa, respectively (Fig. 1, lane 11). The pretreatment with cefotaxime selectively prevented the 58-kDa peptide from reacting with radioactive penicillin (Fig. 1, lane 12). Thus, this peptide was considered to originate from PBP 3^s and therefore was called 58-kDa t-PBP 3^s. The 69- and 44-kDa peptides were considered to be the t-PBP 4* and one of the t-PBP 5 fragments studied previously (7), respectively.

Properties and purification of the 64-kDa t-PBP 3^r, 42-kDa t-PBP 3^r, and 58-kDa t-PBP 3^s. As derived from saturation curves, the 64-kDa t-PBP 3^r and the 42-kDa t-PBP 3^r peptide fragments reacted with benzylpenicillin with a low second-order rate constant (10 $M^{-1} s^{-1}$) very similar to that observed with the membrane-bound PBP 3^r (≈20 $M^{-1} s^{-1}$), and the 58-kDa t-PBP 3^s peptide fragment reacted with a high second-order rate constant (5,000 $M^{-1} s^{-1}$) very similar to that observed with the membrane-bound PBP 3^s. Accordingly, at each step of the purification procedure, the tryptic fragments were identified on the basis of their molecular mass and affinity for benzyl[^{14}C]penicillin.

The membranes from strain S185 or S185^r and the conditions of trypsin digestion varied depending on the particular tryptic fragment to be isolated in sufficient quantity. Whatever the case, the peptide fragment(s) of interest could be isolated by using the two-step procedure described below with a trypsin digest of membranes of strain S185^r containing 1.5 g of total proteins (60 mg of trypsin and 200 ml of 25 mM sodium phosphate [pH 7]; 30 min at 37°C).

In step 1, the supernatant was filtered through a 0.22-μm-pore-size membrane filter (Millipore), supplemented with 25 ml of 1 M Tris hydrochloride (pH 7), and loaded onto a
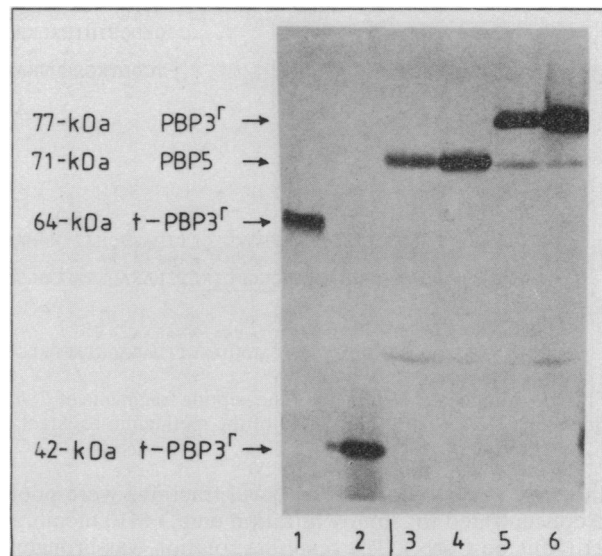

FIG. 5. Reaction of anti-64-kDa t-PBP 3^r antibodies with 64-kDa t-PBP 3^r (lane 1), 42-kDa t-PBP 3^r (lane 2), PBP 5 of membranes of *E. hirae* ATCC 9790 and R40 (lanes 3 and 4), and PBP 3^r of membranes of *E. hirae* S185 and S185^r (lanes 5 and 6). The amounts of proteins used were 2 μg (lanes 1 and 2) and 100 μg (lanes 3 through 6). The same pattern was obtained with the anti-t-PBP 5 antiserum.

Pharmacia Q-Sepharose fast-flow column (2.6 by 40 cm) (gel volume, 58 ml; total capacity, 1 g). A gradient of NaCl (0 to 1 M) in 25 mM Tris hydrochloride-25 mM sodium phosphate (pH 7) eluted the 64-, 42-, and 58-kDa peptide fragments at 0.17 to 0.22 M NaCl.
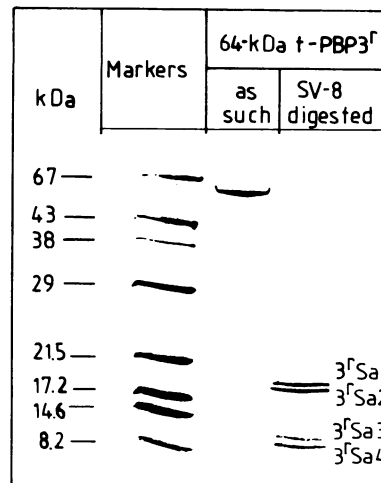

FIG. 6. Degradation of 64-kDa t-PBP 3^r into subfragments 3^r Sa1, 3^r Sa2, 3^r Sa3, and 3^r Sa4 by treatment with *S. aureus* SV8 protease. The figure was obtained by SDS-PAGE (15% acrylamide–2 M urea) and Coomassie blue staining of the electroblot made on an Immobilon membrane filter (Millipore). The 64-kDa t-PBP 3^r reacted with benzyl[^{14}C]penicillin. Subfragments 3^r Sa1, 3^r Sa2, and 3^r Sa4, but not 3^r Sa3, were radioactive (not shown). Protein markers are as follows: 66.3-kDa bovine serum albumin, 42.7-kDa ovalbumin, 38-kDa *Streptomyces* R61 PBP, 29-kDa carbonic anhydrase, 21.5-kDa trypsin soybean inhibitor, and 17.2-, 14.6-, and 8.2-kDa myoglobin CNBr cleavage products.
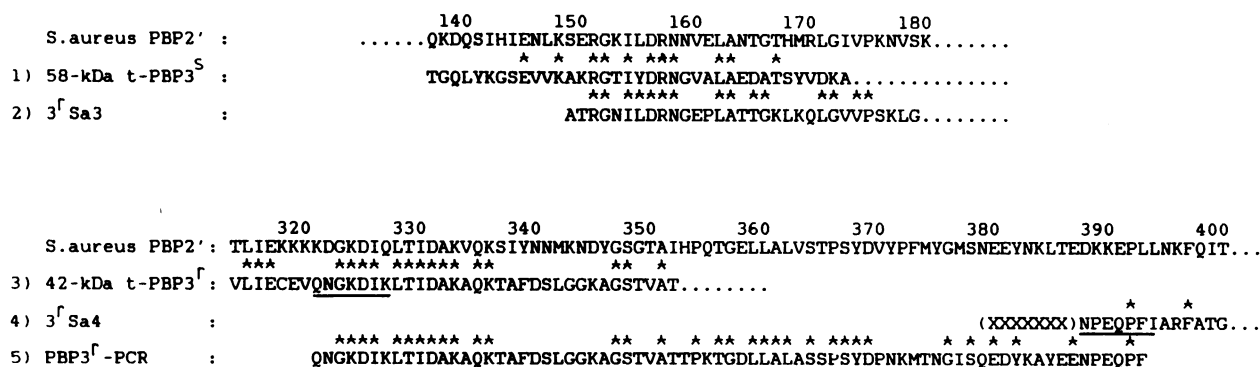
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                                         140        150       160       170       180
        S.aureus PBP2' :              ......QKDQSIHIENLKSERGKILDRNNVELANTGTHMRLGIVPKNVSK.......
                                            *  *  ** * *** ** *
   1) 58-kDa t-PBP3^S :                TGQLYKGSEVVKAKRGTIYDRNGVALAEDATSYVDKA..............
                                         ** ***** ** **  ** **
   2) 3^r Sa3        :                  ATRGNILDRNGEPLATTGKLKQLGVVPSKLG........
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                       320        330       340       350       360       370       380       390       400
     S.aureus PBP2': TLIEKKKKDGKDIQLTIDAKVQKSIYNNMKNDYGSGTAIHPQTGELLALVSTPSYDVYPFMYGMSNEEYNKLTEDKKEPLLNKFQIT...
                       ***    **** ****** **             ** *
  3) 42-kDa t-PBP3^r: VLIECEVQNGKDIKLTIDAKAQKTAFDSLGGKAGSTVAT.......
                                                                                                     *    *
  4) 3^r Sa4        :                                                                        (XXXXXXX)NPEQPFIARFATG...
                       **** ****** **          ** *  * ** **** * ****     * * *  *   *
  5) PBP3^r-PCR      : QNGKDIKLTIDAKAQKTAFDSLGGKAGSTVATTPKTGDLLALASSPSYDPNKMTNGISQEDYKAYEENPEQPF
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FIG. 7. Amino acid alignment of the peptide fragments of E. hirae PBP 3^s (sequence 1) and PBP 3^r (sequences 2 through 5) with the peptide segments Q137-K180 and T314-T400 of the methicillin-resistant PBP 2' of S. aureus. For underlined sequences, see Fig. 8.

In step 2, the 0.17 to 0.22 M NaCl fractions were pooled and concentrated to 5 ml by filtration on a YM10 membrane filter (Amicon Corp.). The resulting solution was brought to 1.7 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate (pH 7), and samples containing at most 10 mg of total protein were filtered on a 1-ml phenyl-Superose HR5/5 column (Pharmacia). Upon treatment with a decreasing gradient of $(NH_4)_2SO_4$ concentration in the same buffer, the 58-kDa t-PBP 3^s and the 42-kDa t-PBP 3^r fragments eluted at about 0.75 M $(NH_4)_2SO_4$ (overall yield, ≈20%) and the 64-kDa t-PBP 3^r eluted at 0.22 M $(NH_4)_2SO_4$ (overall yield, 60%). The 64-kDa t-PBP 3^r thus obtained was 95 to 100% pure (Fig. 4, lane 1). In turn, it was estimated (Fig. 4, lane 2) that about 50% of the total proteins were accounted for by the 58-kDa t-PBP 3^s and that the 42-kDa t-PBP 3^r was a minor component of this fraction. Improved yield (about 40%) in the 42-kDa t-PBP 3^r required trypsin treatment of the membranes at pH 7.8 in the presence of 10 mM $MgCl_2$ (Fig. 4, lane 3).

Specificity profile of the anti-64-kDa t-PBP 3^r and anti-t-PBP 5 antibodies. The antibodies raised against the purified 64-kDa t-PBP 3^r and those raised against purified t-PBP 5 fragments of E. hirae R40 reacted with the 64- and 42-kDa t-PBPs 3^r (Fig. 5, lanes 1 and 2), the PBP 3^r of membranes of strains S185 (lane 5) and S185^r (lane 6), the PBP 5 of membranes of strains ATCC 9790 (lane 3) and R40 (lane 4),

and the purified t-PBP 5 fragments (not shown in Fig. 5). A very small amount of membrane-bound PBP 5 was found in strains S185 and S185^r (lanes 5 and 6). Treatment with the antisera failed to abolish penicillin binding. The antibodies did not react with the 77-kDa PBP 3^s in membranes of strains ATCC 9790 and R40 (lanes 3 and 4) nor with the 58-kDa t-PBP 3^s (lane 2).

SV8 protease hydrolysis of the 64-kDa t-PBP 3^r to the 3^r Sa1, 3^r Sa2, 3^r Sa3, and 3^r Sa4 peptide fragments. Edman degradation of the purified 64-kDa t-PBP 3^r failed. Consequently, the peptide, previously labeled by reaction with benzyl[^{14}C]penicillin, was carboxymethylated and then digested with the Staphylococcus aureus SV8 protease [60 μg of peptide, 1.2 μg of protease (Miles Scientific, Naperville, Ill.), and 250 μl of 100 mM $(NH_4)_2CO_3$ containing 1 mM $CaCl_2$; 8 h at 37°C]. SDS-PAGE (15% acrylamide) followed by Coomassie blue staining and fluorography revealed three radioactively labeled peptides of 19.7 kDa (3^r Sa1), 17.5 kDa (3^r Sa2), and 8.5 kDa (3^r Sa4), and one nonradioactively labeled peptide of 9.6 kDa (3^r Sa3) (Fig. 6).

Amino acid sequences. Samples containing the purified 42-kDa t-PBP 3^r and 58-kDa t-PBP 3^s and samples containing the four peptide fragments (3^r Sa1, 3^r Sa2, 3^r Sa3, and 3^r Sa4) were subjected to SDS-PAGE. After electroblotting, each purified peptide (about 500 pmol) was subjected to auto-

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Oligonucleotide  n° 1
                              G
   5'           G  T  G     A      3'
      G,GGATC C-C A -AA -GG -AA -GAT-ATT-AA
      BamHI        A  C  T   A        C
                                      C
               Q - N - G - K - D - I -(K)

Oligonucleotide  n° 2
              G           G
   3'  G  A      T   A              5'
      TT -GG -C TT-GT -GG -AAA-TAC TTAAGG
         A  T      C   T          |_____|
            C           C           EcoRI
      N  - P  - E - Q - P - F -(I)
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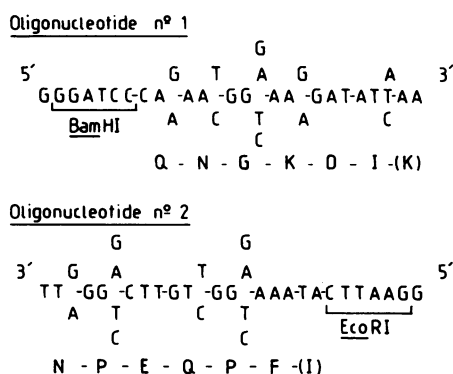
FIG. 8. Synthetic nucleotides used as primers for PCR amplification of a 233-bp DNA fragment encoding the peptide PBP 3^r-PCR shown in Fig. 7. Oligonucleotide 1 encodes the sequence Q8-K14 of the 42-kDa t-PBP 3^r. Oligonucleotide 2 is complementary of the nucleotide sequence encoding the sequence N8-I14 of 3^r Sa4. The amino acid sequences are underlined in Fig. 7.
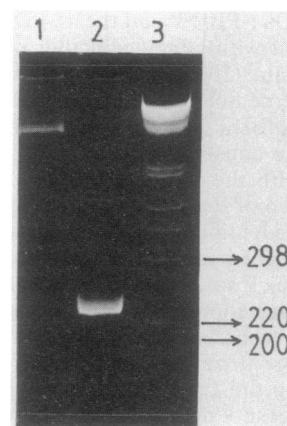

FIG. 9. PAGE analysis of the PCR products generated by using the two oligonucleotides of Fig. 8 as primers and the DNA from E. hirae R40 (lane 1) and E. hirae S185^r (lane 2) as template. Lane 3 shows DNA size markers (298, 220, and 200 bp of the 1-kb ladder) (BRL).

E.hirae PBP3^r	:		RGNILDRNGEPLA		GKDIKLTIDAKAQ		TGDLLALASSPSYD
S.aureus PBP2'	:	151	RGKILDRNNVELA	323	GKDIQLTIDAKVQ	356	TGELLALVSTPSYD
E.coli PBP2	:	68	RGIIYDRNGIPLA	247	GHDIYLTLDLKLQ	280	TGGVLALVSTPSYD
E.coli PBP3	:	71	RGMITDRSGRPLA	229	AHNLALSIDERLQ	269	TGEVLAMANSPSYN
S.pneumoniae PBP2X	:	76	RGTIYDRNGVPIA	258	GKDVYTTISSPLQ	298	TGEILATTQRPTFD
N.gonorrhoeae PBP2	:	75	RGTVSDRNGAVLA	232	GKDIILSLDQRIQ	272	TGEILALANTPAYD

		I L		GKD TI		L YD
Consensus	:	RG- -DR---- A		--- ----Q		TG-VLA----P-
		V I		AHN SL		I FN

FIG. 10. Homologous boxes occurring in the amino-terminal regions of several high-M_r PBPs, i.e., S. aureus PBP 2' (22), E. coli PBP 2 (1), E. coli PBP 3 (18), Streptococcus pneumoniae PBP 2X (14), and Neisseria gonorrhoeae (23).

mated microsequence analysis. Comparison of the data thus obtained with PBP 2', of known primary structure, of methicillin-resistant S. aureus (22) led to the following observations (Fig. 7).

(i) The 37-amino-acid amino-terminal region of the 58-kDa t-PBP 3^s aligned with the peptide stretch Q137-I173 of PBP 2', yielding 11 identities from residue 145 to residue 167.

(ii) The 31-amino-acid amino-terminal region of the 9.6-kDa 3^r Sa3 peptide (originating from the 64-kDa t-PBP 3^r) aligned with the peptide stretch S149-S179 of PBP 2', yielding 15 identities. Note that the 58-kDa t-PBP 3^s and the 9.6-kDa 3^r Sa3 aligned within the same region of PBP 2'.

(iii) The 39-amino-acid amino-terminal region of the 42-kDa t-PBP 3^r aligned with the peptide stretch T314-I352 of PBP 2', yielding 18 identities.

(iv) The amino-terminal region of the 8.5-kDa 3^r Sa4 [($\alpha\alpha$)$_7$NPEQPFIARFATG] lacked similarity with any peptide segment of PBP 2', but it was necessarily located downstream of that region of the 42-kDa t-PBP 3^r which had been sequenced. Accordingly, the two oligonucleotides shown in Fig. 8 were synthesized. Oligonucleotide 1 had a BamHI site at the 5' OH end and coded for the sequence Q8-K14 of the 42-kDa t-PBP 3^r, and oligonucleotide 2 had an EcoRI site at the 5' OH end and was complementary of the nucleotide sequence coding for the sequence N8-I14 of 3^r Sa4. Amplification by the PCR technique with these two oligonucleotides as primers and the E. hirae S185^r DNA as template generated a 233-bp DNA fragment (Fig. 9, lane 2), the sequence of which translated into a 73-amino-acid peptide. This peptide, called PBP 3^r-PCR, aligned with the K321-L393 segment of PBP 2', yielding 33 identities (Fig. 7). Note that the 233-bp DNA fragment was not produced when the E. hirae R40 DNA was used as the control (Fig. 9, lane 1).

(v) The 19.7-kDa 3^r Sa1 and 17.5-kDa 3^r Sa2 (not shown in Fig. 7) arose by cleavage of the E-V bond, at positions 6 and 7 of the 42-kDa t-PBP 3^r.

DISCUSSION

From the work presented here, one can draw the following conclusions.

(i) Development of resistance to penicillin among enterococci can be the result of the emergence of a novel 77-kDa PBP 3^r which is much less susceptible to penicillin than the normal 77-kDa PBP 3^s.

(ii) The 77-kDa PBP 3^r, like the E. hirae PBP 5 of low penicillin affinity, is another member of that class of physiologically important high-M_r PBPs to which the methicillin-resistant staphylococcal PBP 2' belongs. Indeed, several peptide fragments of PBP 3^r align well with two peptide segments which in the staphylococcal PBP 2' extend from S149 to S179 (31 residues) and from T314 to T400 (87

residues), respectively. Alignment of the 118 amino acids of PBP 3^r and PBP 2' generates 52 strict identities (40%) and highlights a common signature consisting of three boxes of very high homology. The fact that these boxes are conserved in the amino-terminal domains of other high-M_r PBPs (Fig. 10) strongly suggests that they are markers of structural and functional significance.

(iii) The E. hirae 77-kDa PBP 3^r and 71-kDa PBP 5 are immunologically related and are acylated by benzylpenicillin with the same low second-order rate constant ($\approx$10 to 20 M^{-1} s^{-1}). PBP 3^r and PBP 5 are probably similar proteins, yet they have different tryptic digest profiles. In addition, the oligonucleotide primers used in this work discriminate the PBP 3^r- and PBP 5-encoding genes. Consequently, the PBP 3^r gene is only present in E. hirae S185^r, while the PBP 5 gene is present in both E. hirae S185^r and R40.

(iv) The E. hirae PBP 3^r (or the PBP 5) is not immunologically related to PBP 3^s, suggesting that PBP 3^r is not a protein mutant that would have emerged by limited remodeling of the penicillin-binding domain of PBP 3^s. PBP 3^s, however, possesses at least the same marker (Fig. 10, R151-A163) as that found in PBP 3^r and in the staphylococcal PBP 2'.

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