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 3h) and low (PBP 3l) affinity to penicillin, respectively. A laboratory mutant S1855 has been obtained which overproduces PBP 3l and has a highly increased resistance to penicillin. Peptide fragments specifically produced by trypsin and SV8 protease digestions of PBP 3h 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 3h. These structural data led to the conclusion that the E. hirae PBP 3h and the methicillin-resistant staphylococcal PBP 2' are members of the same class of high-M, PBPs. As shown by immunological tests, PBP 3l is not related to PBP 3h 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, 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 targeted 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, 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 3h. 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 (A550 = 6.0) were suspended in 100 ml of 5 mM sodium phosphate (pH 7.0) containing 1 mM MgCl2 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[14C]penicillin, SDS-PAGE, and fluorography. Samples were labeled with benzyl[14C]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 electrophoreted 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 phenylthiodyantoin 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′ 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
synthesized by Eurogentec, Liège, Belgium. Polymerase
chain reaction (PCR) amplification was performed on 100-μl
samples containing the E. hirae S185\textsuperscript{a} 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\textsubscript{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) sub-
pected 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 King-
dom). MAX Efficiency E. coli DH5α F\textsuperscript{+}IQ competent cells
served for transformation experiments. Nucleotide sequenc-
ing was performed with the Sequenase kit (U.S. Biochemical
Corp., Cleveland, Ohio) by using the deoxyribonucleotide
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\textsuperscript{a} (the
64-kDa t-PBP 3; 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\textsuperscript{a} anti-
bodies 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 goat anti-rabbit 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\textsuperscript{a}:
ocurrence in strains S185 and S185\textsuperscript{a} of two 77-kDa PBP 3\textsuperscript{a}
and PBP 3\textsuperscript{b} of high and low penicillin sensitivity, respectively.

*E. hirae* S185\textsuperscript{a} was derived from strain S185 by four serial
cultures on agar plates containing 32, 64, 128, and 256 μg of
benzylpenicillin ml\textsuperscript{-1}. *E. hirae* ATCC 9790, R40, S185,
and S185\textsuperscript{a} 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\textsuperscript{a} is a sponta-
eneous breakdown product of PBP 4 formed during prepara-
tion and storage of the membranes (4).

Although they have similar PBP profiles, the four enter-
occoccal 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,
strains ATCC 9790 and 20 M⁻¹ s⁻¹ for PBP 3' (as observed with PBP 5 of strain R40).

To discriminate PBP 3' from PBP 3', membranes of strain S185' (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' (as well as PBPs 1 and 2) and 20-fold lower than that necessary to saturate PBP 3' 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[14C]penicillin for 60 min at 37°C, thus causing complete derivatization of both PBP 3' 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' in the membranes of strain S185' (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' were estimated on the basis of these and other data (Table 1). E. hirae ATCC 9790 and R40 lack PBP 3'. PBP 3' and PBP 5 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'.

Trypsin digestion of the 77-kDa PBP 3': the 64-kDa t-PBP 3'

and 42-kDa t-PBP 3'. Isolation of the membrane-bound 77-kDa PBP 3' was not attempted. Instead, membranes of strain S185' in which PBP 3' 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 X 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 MgCl₂ 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' from membranes of strain S185'.

Trypsin digestion of the 77-kDa PBP 3': the 58-kDa t-PBP 3' peptide fragment. Identification of the trypsin degradation product(s) of the 77-kDa PBP 3' rested upon the use of E. hirae NT1/20. Strain NT1/20 has a PBP profile similar to that

![FIG. 2. Effects of increasing concentrations of benzylpenicillin on growth in SBA broth of E. hirae ATCC 9790, R40, S185, and S185'. Absorbances were 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.](image)

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

| TABLE 1. PBPs as percentage of total proteins of the membranes of E. hirae strains |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| PBP                            | ATCC 9790       | R40             | S185            | S185'           |
| 1                              | 0.34            | 0.18            | 0.25            | 0.11            |
| 2                              | 0.22            | 0.17            | 0.14            | 0.09            |
| 3'                             | 0.4             | 0.2             | 0.32            | 0.4             |
| 3r                             | 0.3             | 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            |
of strain ATCC 9790 and thus lacks PBP 3', 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' (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 MgCl₂ 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[¹⁴C]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' 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' and therefore was called 58-kDa t-PBP 3'. 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', 42-kDa t-PBP 3', and 58-kDa t-PBP 3'. As derived from saturation curves, the 64-kDa t-PBP 3' and the 42-kDa t-PBP 3' 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' (=20 M⁻¹ s⁻¹), and the 58-kDa t-PBP 3' 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'. Accordingly, at each step of the purification procedure, the tryptic fragments were identified on the basis of their molecular mass and affinity for benzyl[¹⁴C]penicillin.

The membranes from strain S185 or S185' 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' 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. 4. SDS-PAGE and Coomassie blue staining of t-PBP 3' and t-PBP 3' peptide fragments. Lanes: 1, 64-kDa t-PBP 3' (3 μg of protein); 2, 58-kDa t-PBP 3' (≈8.5 μg); 3, 42-kDa t-PBP 3' (=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).](image1)

![FIG. 5. Reaction of anti-64-kDa t-PBP 3' antibodies with 64-kDa t-PBP 3' (lane 1), 42-kDa t-PBP 3' (lane 2), PBP 5 of membranes of E. hirae ATCC 9790 and R40 (lanes 3 and 4), and PBP 3' of membranes of E. hirae S185 and S185' (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.](image2)

![FIG. 6. Degradation of 64-kDa t-PBP 3' into subfragments 3' Sa1, 3' Sa2, 3' Sa3, and 3' 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 electrophoretic pattern made on an Immunoblot membrane filter (Millipore). The 64-kDa t-PBP 3' reacted with benzyl[¹⁴C]penicillin. Subfragments 3' Sa1, 3' Sa2, and 3' Sa4, but not 3' 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.](image3)
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 (NH₄)₂SO₄ 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 (NH₄)₂SO₄ concentration in the same buffer, the 58-kDa t-PBP 3r and the 42-kDa t-PBP 3l fragments eluted at about 0.75 M (NH₄)₂SO₄ (overall yield, ~20%) and the 64-kDa t-PBP 3l eluted at 0.22 M (NH₄)₂SO₄ (overall yield, 60%). The 64-kDa t-PBP 3l 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 3l and that the 42-kDa t-PBP 3l was a minor component of this fraction. Improved yield (about 40%) in the 42-kDa t-PBP 3l required trypsin treatment of the membranes at pH 7.8 in the presence of 10 mM MgCl₂ (Fig. 4, lane 3).

Specificity profile of the anti-64-kDa t-PBP 3l and anti-t-PBP 5 antibodies. The antibodies raised against the purified 64-kDa t-PBP 3l and those raised against purified t-PBP 5 fragments of E. hirae R40 reacted with the 64- and 42-kDa t-PBPPS 3l (Fig. 5, lanes 1 and 2), the PBP 3l of membranes of strains S185 (lane 5) and S185* (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* (lanes 5 and 6). Treatment with the antisera failed to abolish penicillin binding. The antibodies did not react with the 77-kDa PBP 3l in membranes of strains ATCC 9790 and R40 (lanes 3 and 4) nor with the 58-kDa t-PBP 3l (lane 2). SV8 protease hydrolysis of the 64-kDa t-PBP 3l to the 3' Sa1, 3' Sa2, 3' Sa3, and 3' Sa4 peptide fragments. Edman degradation of the purified 64-kDa t-PBP 3l failed. Consequently, the peptide, previously labeled by reaction with benzyl[14C]penicillin, was carboxymethylated and then digested with the Staphylococcus aureus SV8 protease (60 µg of peptide, 1.2 µg of protease (Miles Scientific, Naperville, III.), and 250 µl of 100 mM (NH₄)₂CO₃ containing 1 mM CaCl₂; 8 h at 37°C). SDS-PAGE (15% acrylamide) followed by Coumassie blue staining and fluorography revealed three radioactively labeled peptides of 19.7 kDa (3' Sa1), 17.5 kDa (3' Sa2), and 8.5 kDa (3' Sa4), and one nonradioactively labeled peptide of 9.6 kDa (3' Sa3) (Fig. 6).

Amino acid sequences. Samples containing the purified 42-kDa t-PBP 3l and 58-kDa t-PBP 3l and samples containing the four peptide fragments (3' Sa1, 3' Sa2, 3' Sa3, and 3' Sa4) were subjected to SDS-PAGE. After electrophoretic, each purified peptide (about 500 pmol) was subjected to auto-
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' 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' Sa3 peptide (originating from the 64-kDa t-PBP 3') aligned with the peptide stretch S149-S179 of PBP 2', yielding 15 identities. Note that the 58-kDa t-PBP 3' and the 9.6-kDa 3' Sa3 aligned within the same region of PBP 2'.

(iii) The 39-amino-acid amino-terminal region of the 42-kDa t-PBP 3' aligned with the peptide stretch T314-1352 of PBP 2', yielding 18 identities.

(iv) The amino-terminal region of the 8.5-kDa 3' Sa4 [(αα),NPQPFDFARFATQ] 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' 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' 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' Sa4. Amplification by the PCR technique with these two oligonucleotides as primers and the E. hirae Sa185' 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'C-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' Sa1 and 17.5-kDa 3' 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'.

**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' which is much less susceptible to penicillin than the normal 77-kDa PBP 3'.

(ii) The 77-kDa PBP 3', like the E. hirae PBP 5 of low penicillin affinity, is another member of that class of physiologically important high-M, PBPs to which the methicillin-resistant staphylococcal PBP 2' belongs. Indeed, several peptide fragments of PBP 3' 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' 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, PBPs (Fig. 10) strongly suggests that they are markers of structural and functional significance.

(iii) The E. hirae 77-kDa PBP 3' and 71-kDa PBP 5 are immunologically related and are acetylated by benzylpenicillin with the same low second-order rate constant (≈10 to 20 M⁻¹ s⁻¹). PBP 3' 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' and PBP 5-encoding genes. Consequently, the PBP 3' gene is only present in E. hirae Sa185', while the PBP 5 gene is present in both E. hirae Sa185' and R40.

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

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**LITERATURE CITED**


5. Coyette, J., H. R. Perkins, J. Polacheck, G. D. Shockman,