JOURNAL OF BACTERIOLOGY, Aug. 1996, p. 4948–4957 Vol. 178, No. 16
0021-9193/96/$04.00+0

Structure of the Low-Affinity Penicillin-Binding Protein 5 PBP5fm in Wild-Type and Highly Penicillin-Resistant Strains of *Enterococcus faecium*

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Received 12 February 1996/Accepted 17 June 1996

Among its penicillin-binding proteins (PBPs), *Enterococcus faecium* possesses a low-affinity PBP5, PBP5fm, which is the main target involved in β-lactam resistance. A 7.7-kb *Eco*RI chromosomal fragment of *E. faecium* D63r containing the *pbp5fm* gene was cloned and sequenced. Two open reading frames (ORFs) were found. A 2,037-bp ORF encoded the deduced 73.8-kDa PBP5fm, the amino acid sequences of which were, respectively, 99.8, 78.5, and 62% homologous to those of the low-affinity plasmid-encoded PBP3r of *Enterococcus hirae* S185r and the chromosome-encoded PBP5 of *E. hirae* R40 and *Enterococcus faecalis* 56R. A second 597-bp ORF, designated *psrfm*, was found 2.3 kb upstream of *pbp5fm*. It appeared to be 285 bp shorter than and 74% homologous with the regulatory gene *psr* of *E. hirae* ATCC 9790. Different clinical isolates of *E. faecium*, for which a wide range of benzylpenicillin MICs were observed, showed that the increases in MICs were related to two mechanisms. For some strains of intermediate resistance (MICs of 16 to 64 μg/ml), the increased level of resistance could be explained by the presence of larger quantities of PBP5fm which had an affinity for benzylpenicillin (second-order rate constant of protein acylation [k_{+2}/K] values of 17 to 25 M^{-1} s^{-1}) that remained unchanged. For the two most highly resistant strains, EFM-1 (MIC, 90 μg/ml) and H80721 (MIC, 512 μg/ml), the resistance was related to different amino acid substitutions yielding very-low-affinity PBP5fm variants ($k_{+2}/K \leq 1.5$ M^{-1} s^{-1}) which were synthesized in small quantities. More specifically, it appeared, with a three-dimensional model of the C-terminal domain of PBP5fm, that the substitutions of Met-485, located in the third position after the conserved SDN triad, by Thr in EFM-1 and by Ala in H80721 were the most likely cause of the decreasing affinity of PBP5fm observed in these strains.

The genus *Enterococcus*, closely related to the genus *Streptococcus*, is involved in different clinical infections (37). Apart from their physiological properties, enterococci also differ from streptococci in that they generally are naturally 10- to 1,000-fold less susceptible to penicillins than streptococci (37). It was demonstrated that the natural low susceptibility of enterococci to penicillin is linked to the presence of at least one high-molecular-mass penicillin-binding protein (PBP) which has a low affinity for β-lactams (1, 17, 50). *Enterococcus faecium* appears to be the enterococcal species most resistant to β-lactam antibiotics, for which there are a wide range of benzylpenicillin MICs (0.5 to ≥ 64 μg/ml) for clinical isolates (22, 30, 50). Recently it became obvious that a new population of clinical *E. faecium* isolates for which the MICs of benzylpenicillin were very high (256 to 512 μg/ml) had emerged in different countries (15, 22, 23, 30).

Among laboratory mutants and clinical isolates of *E. faecium*, two mechanisms have been shown to be involved in the high-level resistance to benzylpenicillin. These strains produced either an increased quantity of the essential low-affinity PBP5fm (15, 17) or, as described more recently, a PBP5fm with an apparent decreased affinity for benzylpenicillin (15, 30). The low-affinity-PBP-encoding genes of different enterococci, such as the chromosomal *pbp5* genes of *Enterococcus hirae* ATCC 9790 (11) and *Enterococcus faecalis* 56R (43) and the plasmid-borne gene *pbp3r* of *E. hirae* S185r (40, 41), have been cloned and sequenced. The present study was undertaken to clone and sequence the PBP5fm-encoding genes of clinical *E. faecium* isolates which presented different levels of resistance to benzylpenicillin with the aim of determining possible structural differences between the corresponding proteins, which could explain the increases in penicillin resistance.

(Part of this work was conducted by W. Zorzi in partial fulfillment of the requirements for a Ph.D. degree from the University of Liège, Belgium.)

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids pBR322, pGEM-3Zf(+), and pTA12 were used for gene cloning experiments.

Media and MIC determination. *Enterococcus* and *Escherichia coli* strains were grown in brain-heart infusion (BHI) broth (Difco) and Luria-Bertani medium or 2XYT broth (16 g of Bacto Tryptone, 10 g of yeast extract, 5 g of NaCl, 1 liter of water, pH 7.0) (42), respectively. The MICs were determined in BHI broth as described previously (8).

Isolation of cytoplasmic membranes. *E. faecium* cells were grown in 500 ml of BHI broth at 37°C with gentle shaking and collected at the late exponential phase (corrected A_{550}, 5). Once resuspended in 100 ml of 5 mM sodium phosphate (pH 7.0) containing 1 mM $MgCl_2$, the cells were lysed with a mixture of lysozyme (20 mg), DNase (200 μg), and RNase (200 μg) as described previously (39). Membranes were purified by several washings and centrifugations (100,000 × *g*) in the same buffer. They were stored frozen (−20°C) in 40 mM sodium phosphate (pH 7.0) containing 5% (vol/vol) glycerol. The protein concentrations were measured according to the method of Lowry et al. as modified by Coyette et al. (7).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. faecium		
D26	Clinical isolate	Reference Center for Streptococci and Staphylococci (Institut Pasteur, Paris)
D63, D344, D359	Pen^r clinical isolates	50
D366	Pen^r clinical isolate	49
EFM-1	Pen^r clinical isolate	24
D63r	Pen^r mutant derived from D63	This work
H80721	Pen^r clinical isolate	This work
LMG11423	Type strain	Culture collection of the Laboratorium voor Microbiologie, Faculteit Wetenschaffen, Universiteit Gent-LMG
22166	Clinical isolate	Liège University Hospital
E. hirae		
ATCC 9790	Type strain	8
R40	Pen^r mutant derived from ATCC 9790	17
S185r	Pen^r mutant derived from the clinical isolate S185	39, 40
E. coli		
RR1	Δ(*gpt-protA*)*62 leuB6 thi-1 lacY1 hsdS$_B$20 rpsL20* (Str^r) *ara-14 galK2 xyl-5 mtl-1 supE44 mcrB$_B$*	42
Plasmids		
pBR322	Low-copy vector, Ap^r Tet^r	Promega (Leiden, The Netherlands)
pGEM-3Zf(+)	High-copy vector, Ap^r	Promega (Leiden, The Netherlands)
pTA12	High-copy vector, Ap^r	36
pDML517	pBR322 carrying a ca. 7.7-kb *Eco*RI fragment of the D63r genome	This work

[a] Pen^r, penicillin resistant; Ap^r, ampicillin resistant; Tet^r, tetracycline resistant.

Labeling of PBPs with benzyl-[^{14}C]penicillin and immunodetection of PBP5fm. Samples of membranes were generally labeled for 30 min at 37°C with 1 to 100 μM benzyl-[^{14}C]penicillin (54 Ci/mol; Amersham International, Buckinghamshire, United Kingdom). Specific labeling of PBP5fm with 100 μM benzyl-[^{14}C]penicillin was obtained after the other PBPs were saturated by a 30-min pretreatment with nonradioactive benzylpenicillin (1 μM). The PBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [7.2% acrylamide; wt/vol]). Fluorography of the gels was performed as described previously (12). The PBP intensities were estimated by densitometry of the fluorograms with a model 620 densitometer (Bio-Rad) or a video camera (with the CAM software from Cybertech-Dalton, Berlin, Germany), and *Streptomyces* sp. strain R61 DD-carboxypeptidase was used as the standard (18). The values of the second-order rate constant of protein acylation (k_{+2}/K) were calculated as described previously (21, 32).

PBP5fm was also detected in membrane preparations after SDS-PAGE (8.5% acrylamide [wt/vol]) and Western blotting (immunoblotting) as described previously (39). Three antisera were used in this study: one raised against PBP5 of *E. hirae* R40 (11) and two others raised against PBP3r of *E. hirae* S185r and PBP5fm of *E. faecium* EFM-1, as described previously (39).

Molecular biology techniques. The techniques for extraction of total *E. faecium* DNA and DNA recombination were essentially those described previously (11, 42). The oligonucleotides used in this work were synthesized by Eurogentec (Liège, Belgium). The different PCR-generated fragments were amplified with the *Taq* DNA polymerase from Boehringer (Mannheim, Germany) during 30 cycles as follows: 1 min of denaturation of 94°C, 1 min of annealing at 55°C, and 20 to 120 s of polymerization at 72°C. Oligonucleotides ON1* (5′-G$_{1241}$GGAC AAAGTAATCGGGTTGTACCC-3′) and ON2 (5′-T$_{1019}$GACCACGCAAGA AGCAACAAGAGGG-3′) were used to amplify the PCR1 fragment (see Fig. 2). Oligonucleotides ON2 and ON3* (5′-G$_{1752}$AATGGTTGTTCAGGATTTTCTT C-3′) were used to amplify the PCR2 fragment (see Fig. 2).

Southern blots and slot blots were prepared on nylon sheets (Hybond-N; Amersham International) with the LKB VacuGene XL kit from Pharmacia Biotech (Uppsala, Sweden) and the Bio-Dot SF blotting microfiltration unit from Bio-Rad (Richmond, Calif.), respectively, according to the manufacturers' instructions. The blotted DNAs were hybridized with digoxigenin-labeled probes: either at the 3′ end for the oligonucleotide probe ON1* or during amplification for the PCR probe, as specified by the manufacturer (Boehringer). Hybridizations were detected by chemiluminescence with the Lumigen-PPD as described by Boehringer.

Nucleotide sequencing. Nucleotide sequencing was carried out with the T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and [^{35}S]dATP labeling or the Autoread sequencing kit (Pharmacia Biotech) with fluorescent primers or incorporation of fluorescent dUTP. In the latter case, electrophoresis was performed with an Automated Laser Fluorescent A.L.F. DNA Sequencer apparatus (EMBL, Heidelberg, Germany) (2).

Construction of the 3-D model of the C-terminal domain of PBP5fm. The three-dimensional (3-D) model was constructed from the alignment of the amino acid sequence of the PBP5fm C-terminal domain on the known 3-D structure of the β-lactamase of *Staphylococcus aureus* PC1 (25, 26) by homology modeling with the COMPOSER programs (4, 5, 46, 47). The model structure was then refined by energy minimization with AMBER (48).

Nucleotide sequence accession number. The EMBL accession numbers for the nucleotide sequences of *pbp5fm* of D366, D63r, EFM-1 and H80721 and of *psrfm* of D63r are X84859, X84860, S84861, X84862, and X84858, respectively.

RESULTS

Presence of a low-affinity PBP5fm with a size of apparently 77 kDa in the *E. faecium* strains. The different strains of *E. faecium* studied were all clinical isolates, except for the resistant strain *E. faecium* D63r, which was derived from clinical isolate D63 by five serial passages on agar plates containing benzylpenicillin concentrations from 2.5 to 25 μg/ml. The MICs are presented in Table 2. As illustrated in Fig. 1A for strains D63 and D63r, all harbored the six species-specific membrane-bound PBPs, namely PBP1 (120 kDa), PBP2 (88 kDa), PBP3 (86.5 kDa), PBP4 (84 kDa), PBP5 (PBP5fm) (77 kDa), and PBP6 (45 kDa) (17, 50). When the MICs of penicillin for the different *E. faecium* strains were compared with the second-order rate constant of PBP5fm acylation by penicillin (k_{+2}/K) and the quantity of PBP5fm (Table 2), it was observed that in all strains except EFM-1 and H80721, PBP5fm had very similar and low k_{+2}/K values, ranging from 15 to 24 M^{-1} s^{-1}. They were comparable to those of PBP5 of *E. hirae* ATCC 9790 and PBP3r of *E. hirae* S185r (k_{+2}/K = 9 and 20 M^{-1} s^{-1}, respectively) (11, 39). In these *E. faecium* strains, increasing MICs were apparently linked to increasing production of PBP5fm. This was illustrated by strain D63r, which, compared with its parental strain, D63, showed a 14-fold increase in the MIC, no significant change in the k_{+2}/K

TABLE 2. Relationship between MICs, the affinity of PBP5fm variants for benzylpenicillin, and their relative abundance in the membranes of different *E. faecium* strains[a]

E. faecium strain	Benzylpenicillin MIC (μg/ml)	k_{+2}/K (M^{-1} s^{-1})	% PBP5fm vs total membrane proteins
LMG11423	2.5	15	0.04
D63	5	24	0.08
D366	16	17	0.13
D359	16	20	0.13
D26	20	19	0.15
22166	40	20	0.32
D344	64	17	0.42
D63r	70	20	0.45
EFM-1	90	1.5	0.08
H80721	512	<1.3	0.08

[a] The MICs of benzylpenicillin were measured in BHI broth, the second-order rate constant (k_{+2}/K) values of PBP5fm acylation by benzyl-[^{14}C]penicillin were estimated after 30-min incubations, and the percentages of PBP5fm in the total membrane proteins were calculated by measuring the amount of PBP5fm on fluorograms and immunoblots and the amount of total proteins.

value, and a 6-fold increase in the quantity of PBP5fm. On the other hand, strains EFM-1 and H80721 seemed to belong to a quite distinct category, with much higher MICs, at least 10 times lower k_{+2}/K values, and no increase in PBP5fm production compared with the most sensitive strains. Therefore, one would expect the high MICs in the latter category to be related to the very low affinity of the membrane-bound PBP5fm. From these results, a relationship between the MICs of penicillin for *E. faecium* strains and the quantity of PBP5fm or its affinity for benzylpenicillin could be inferred. The latter observation is reminiscent of that made with some strains of *E. faecium* for which MICs of penicillin were above 64 μg/ml (15, 30).

Immunological properties of PBP5fm. In order to detect structural relationships between PBP5fm of *E. faecium* and other low-affinity PBPs present in different enterococci, a serum raised against PBP3r of *E. hirae* S185r (39) was tested by immunoblotting of cytoplasmic membrane proteins from different *E. faecium* strains. As shown in Fig. 1B, the anti-PBP3r serum reacted with the PBP5fm variants of all of the *E. faecium* strains studied and the PBP5 and PBP3r of *E. hirae* ATCC 9790 and S185r, respectively. Similar results were obtained with an antiserum raised against PBP5fm of EFM-1 and PBP5 of *E. hirae* R40 (data not shown). Note that if strain S185r produces a very low quantity of PBP5 usually detectable on immunoblots with anti-PBP5 antibodies (39), no PBP5 could be seen in Fig. 1B when anti-PBP3r antibodies were used. Interestingly, these antibodies reacted only weakly with PBP5 of *E. faecalis* (data not shown). This suggested that PBP3r of *E. hirae* S185r, PBP5 of *E. hirae* ATCC 9790, and PBP5fm of *E. faecium* shared more determinants among each other than with PBP5 of *E. faecalis*. In addition, these results confirmed that the different quantities of PBP5fm measured in the different strains described above were not biased by the affinity of the protein, especially in the case of the two very-low-affinity PBP5fms.

Identification of *pbp5fm* of *E. faecium* D63r. Since PBP5fm and PBP3r were immunologically and kinetically related, oligonucleotides ON1*, ON2, and ON3*, synthesized on the basis of the nucleotide sequence of *pbp3r*, were used as primers during PCR with total DNA of D63r (see Materials and Methods and Fig. 2). Two PCR fragments, the 222-bp PCR1 and the 733-bp PCR2, originating from the region coding for the N-terminal domain of PBP5fm were amplified (Fig. 2 and 3) and cloned into pTA12 and pGEM3-Zf(+), respectively. Their sequences were identical to those of the corresponding regions of *pbp3r*, except that C1107 in *pbp3r* (40) was replaced by T in *pbp5fm* (Fig. 4), a change which did not lead to an amino acid substitution.

The digoxigenin-labeled PCR1 fragment was used to probe the total DNA of *E. faecium* D63r digested separately with three restriction enzymes, *Eco*RI, *Nco*I, and *Hin*dIII. In each of the digests, only one fragment with a size of 7.7, 12, or 1 kb, respectively, was shown to hybridize with the probe. Similarly, only one *Eco*RI fragment from *E. faecium* D366, EFM-1, and H80721 hybridized with the PCR1 probe with similar intensities (data not shown). These results suggested that only a single copy of the gene was present and that the variations in amount of PBP5fm in these strains were not due to a gene dosage effect.

In contrast to what was observed with *E. hirae* S185r, in which the *pbp3r* gene was shown to be plasmid borne (41), plasmids prepared from *E. faecium* D63r never hybridized with the 222-bp probe.

Cloning and expression of *pbp5fm* in *E. coli* RR1. Fragments with sizes of 6 to 9 kb produced after *Eco*RI digestion of total DNA of D63r, separated by electrophoresis on 0.8% agarose gels and isolated by electroelution, were inserted into pBR322 to prepare a library which was used to transform *E. coli* RR1 cells. The digoxigenin-labeled probe ON1* strongly hybridized with 4 clones out of 500. They each harbored a recombinant plasmid containing the same 7.7-kb *Eco*RI insert. This insert, present in pDML517 (Fig. 2), contained a region, including *pbp5fm*, which had the same restriction sites as those found in *pbp3r* (40).

The PBP content of the cytoplasmic membranes isolated from each of the four *E. coli* clones bearing a hybridization-positive recombinant plasmid was analyzed by immunoblotting and fluorography. PBP5fm was specifically labeled with benzyl-[^{14}C]penicillin as described in Materials and Methods, except

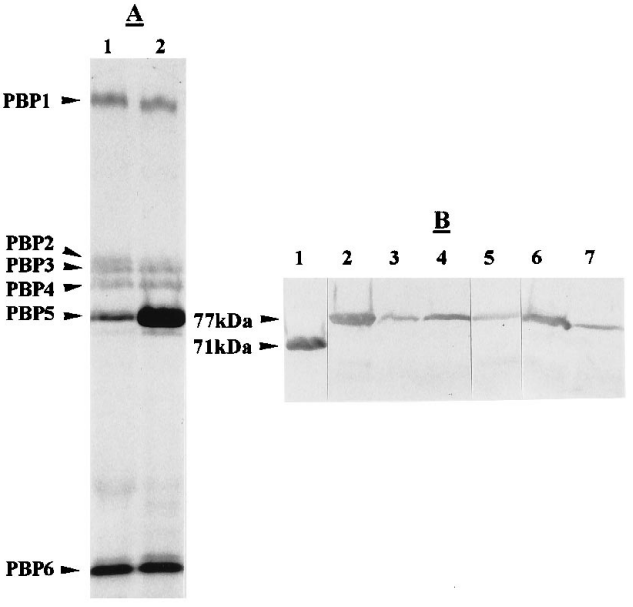


FIG. 1. Fluorogram and Western blot of *E. hirae* and *E. faecium* membranes after SDS-PAGE. (A) Fluorogram of benzyl-[^{14}C]penicillin-labeled membranes (300 μg of protein) of *E. faecium* D63 (lane 1) and D63r (lane 2). (B) Western blot of *E. hirae* and *E. faecium* membranes treated with polyclonal anti-PBP3r antibodies. *E. hirae* ATCC 9790 (lane 1) and S185r (lane 2); *E. faecium* EFM-1 (lane 3), D366 (lane 4), LMG11423 (lane 5), D63r (lane 6), and D63 (lane 7).

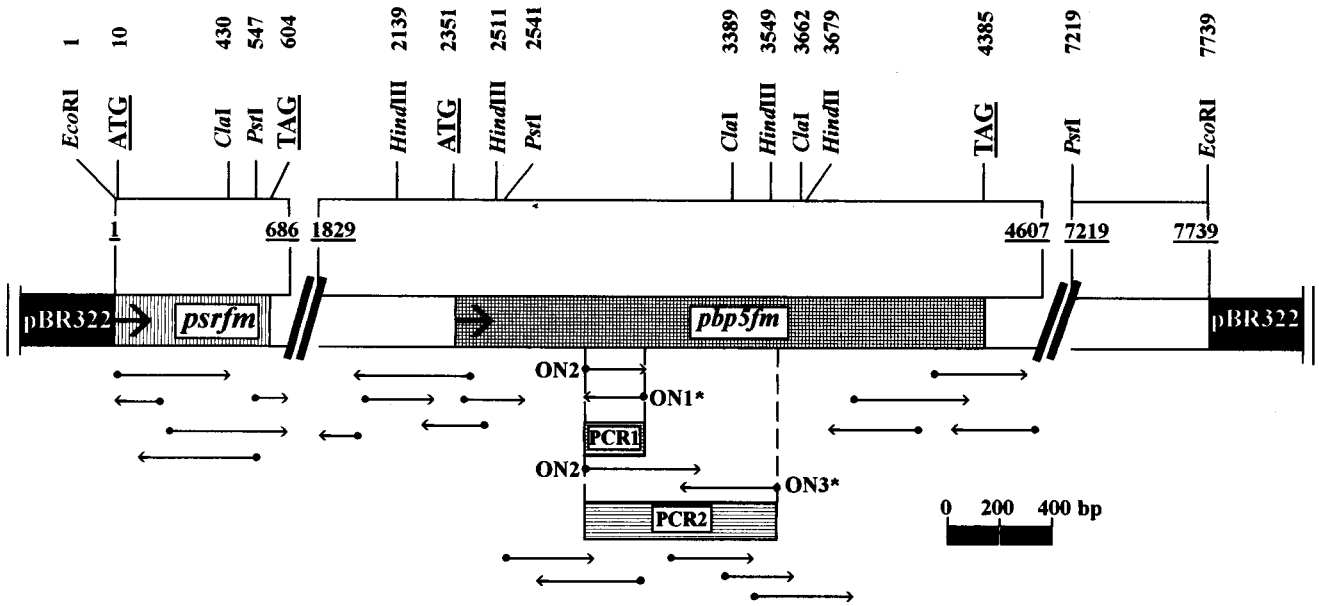


FIG. 2. Restriction map of the 7.73-kb insert of pDML517. The positions of the PCR1 and PCR2 fragments and the strategy for sequencing *psrfm* and *pbp5fm* from positions 1 to 686 and 1829 to 4607 are indicated.

that the membrane-associated β-lactamase was inactivated for 30 min at 37°C in the presence of 30 μM β-iodopenicillinate. The results indicated that the four clones produced a low-affinity PBP of a size similar to that of the membrane-bound PBP5fm of strains *E. faecium* D63r and D63 and which also reacted with the antibodies raised against PBP3r (data not shown).

Sequencing of *pbp5fm*. With the strategy shown in Fig. 2, sequencing of the pDML517 insert on both strands yielded a 2,037-bp open reading frame (ORF) (Fig. 3) which started with an ATG codon at position 523 and terminated with a TAA stop codon at position 2557. A perfect consensus ribosome binding site, AGGAGG (45), occurred 5 bp upstream from the ATG start codon. Computer analysis of the secondary structure of the corresponding RNA showed that the AGGAGG sequence was on a single-stranded region optimal for the translation of prokaryotic messengers (45). The TAA stop codon was followed by palindromic regions able to form a hairpin (stem-loop) structure in the corresponding RNA, typical of many prokaryotic terminators (31). The 39.8% GC content of the sequence was similar to that of other streptococcal genes (14). The *pbp5fm* gene could be translated into a 678-amino-acid protein (Fig. 3) which had a calculated mass of 73.8 kDa, a value slightly smaller than the 77 kDa estimated from SDS-PAGE analysis.

Comparison between *pbp5fm* and *pbp3r*. Comparison of the nucleotide sequences of *pbp3r* of *E. hirae* S185r and *pbp5fm* of *E. faecium* D63r yielded a very high identity index (99.8%). Only five nucleotide substitutions were observed in the ORFs. Four of them (C-975→T, C-1107→T, C-1785→A, and A-1980→G) were silent. Both genes thus encoded the same protein, except that Thr-25 (ACT) in PBP3r was replaced by Ala (GCT) in PBP5fm (Fig. 3). Analysis of the hydrophobic profiles indicated that the substitution occurred in the anchoring peptide.

Comparison of the regions adjacent to *pbp5fm* and *pbp3r* showed that upstream they were homologous only up to position 437 (i.e., position −87 with respect to the ATG indicated by an arrow in Fig. 3), while downstream they were very similar up to position 2778, with only two substitutions ([*pbp5fm*]C-2618→*pbp3r*]A and [*pbp5fm*]G-2698→[*pbp3r*]A) and a deletion of (*pbp3r* A-2577).

Presence of a gene homologous to *psr* in *E. faecium*. Since it was shown that a *psr* gene was involved in *pbp5* expression in *E. hirae* ATCC 9790 (33), we searched for a similar gene in *E. faecium* D63r. Sequencing of the 5′ region of the 7.7-kb insert of pDML517 according to the strategy shown in Fig. 2 led to the detection of a 597-bp ORF (Fig. 4) located about 2.3 kb upstream from the *pbp5fm* gene. Its ATG start codon was positioned 10 bp immediately downstream from the 5′ end of the *Eco*RI-cloning site (Fig. 2 and 4). This ORF had 74% homology to *psr* of *E. hirae* and was designated *psrfm*. However, *psrfm* was 285 bp shorter than the completed *psr* sequence (35) (Fig. 4). The deduced amino acid sequence of Psrfm showed 80% identity for the part shared with Psr of *E. hirae*.

Relationship between the sequences of different PBP5fm variants and their affinities for benzylpenicillin. Since there appeared to be a relationship between increasing benzylpenicillin MICs for *E. faecium* D366, EFM-1, and H80721 and decreasing second-order rate constant values of PBP5fm acylation by the same antibiotic (Table 2), it was of interest to compare the sequences of these proteins. The entire *pbp5fm* of each strain was amplified by PCR and sequenced. When the amino acid sequence of PBP3r of *E. hirae* S185r was used as a reference and compared with that of PBP5fm of strains D63r, D366, EFM-1, and H80721, high-homology indexes of 99.8, 97.8, 96.9, and 96.7%, respectively, were found. The differences found in the primary structure of these PBPs and their location are shown in Table 3 and in Fig. 3.

All of these proteins had 678 residues, except that of H80721, which had an additional serine (AGT codon), designated Ser-466′. Six substitutions in the PBP5fm sequences of EFM-1 and H80721 could be suspected to decrease the second-order rate of acylation (k_{+2}/K), which roughly reflects the affinity for benzylpenicillin (21). Two of them, at positions 485

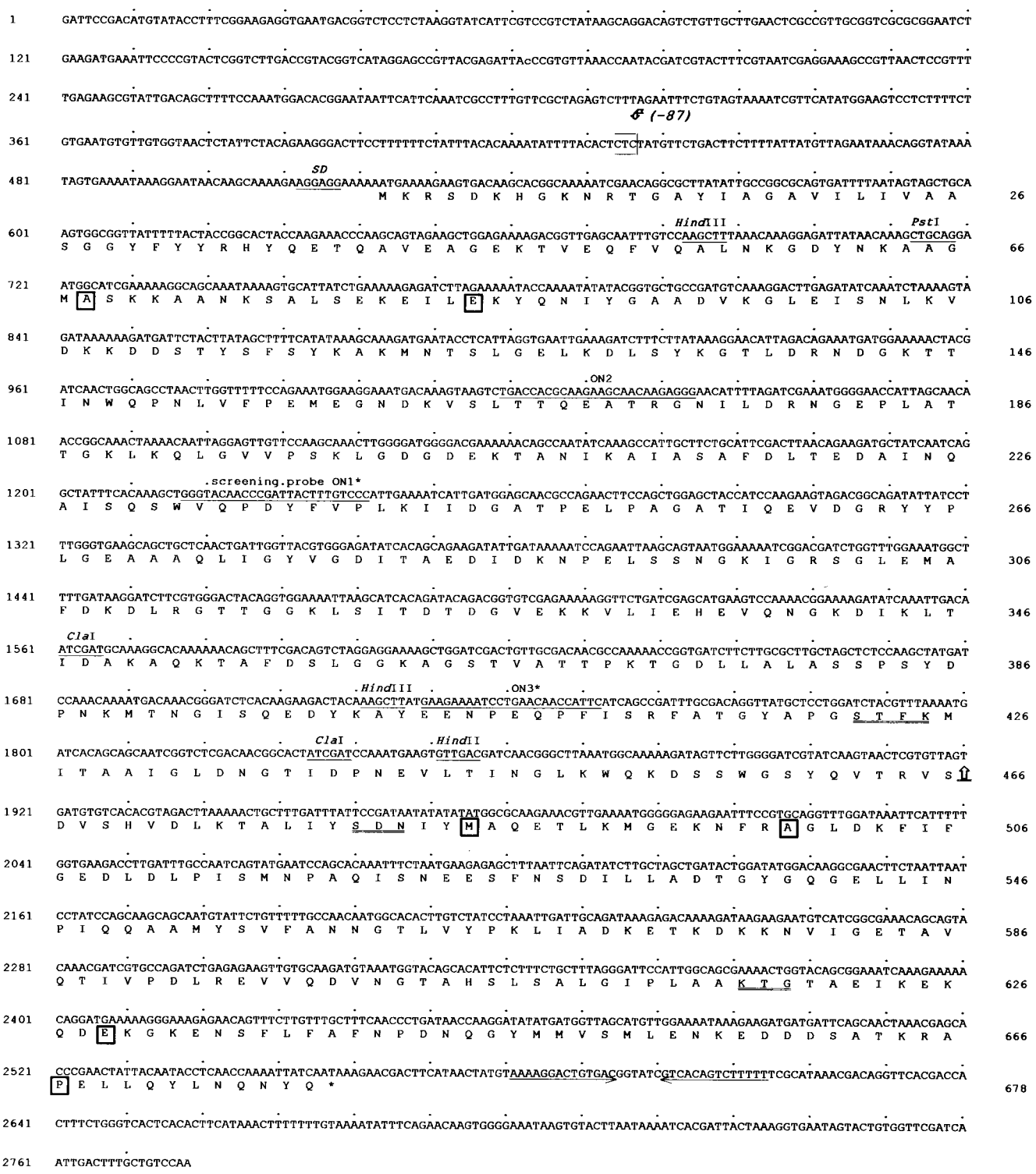


FIG. 3. Nucleotide sequence (corresponding to positions 1829 to 4607 of Fig. 2) of *pbp5fm* and amino acid sequence of PBP5fm of *E. faecium* D63r. The arrow ( ) at position −87 from ATG indicates the upper limit of homology between the sequences of *pbp5fm* and *pbp3r*. The putative Shine-Dalgarno sequence (*SD*), the restriction sites, and the positions of the oligonucleotides ON1*, ON2, and ON3*, which were used to amplify the PCR1 and PCR2 fragments, are underlined *, oligonucleotides chosen from the complementary strand. Conserved active-site motifs and residues which differ only in the very-low-affinity PBP5fm of EFM-1 and H80721 (see Table 3) are underlined (double line) and boxed frames, respectively. , position of the additional serine called Ser-466′ and found in the PBP5fm of H80721. Downstream from the stop codon, the two horizontal arrows correspond to inverted repeats forming a putative terminator.

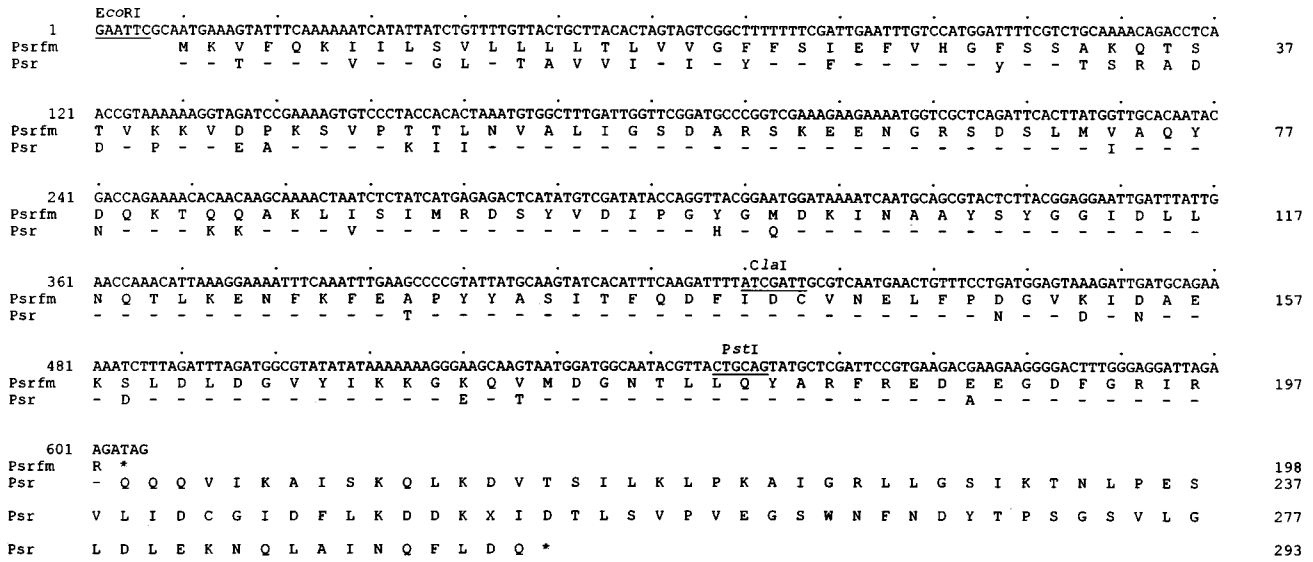


FIG. 4. Nucleotide sequence (corresponding to positions 1 to 606 of Fig. 2) of *psrfm* and amino acid sequence of Psrfm of *E. faecium* D63r. The amino acid sequence of Psr of *E. hirae* ATCC 9790 (35) is also given. The residues which are different from those of Psrfm are indicated; those which are identical are represented by a dash.

and 499, were located near the SDN-482 triad. More specifically, it should be emphasized that a decreasing affinity of PBP5fm for benzylpenicillin was observed when Met-485, present in the PBP5fm of D63r and D366, was substituted for by Thr in the PBP5fm of EFM-1 and by Ala in the PBP5fm of H80721 (Table 3). The variations observed in the N-terminal domain (positions 66 to 324) probably had no direct effect on the affinity of these PBPs. It was indeed demonstrated that, as

TABLE 3. Differences in the amino acid sequences of PBP5fm of *E. faecium* D63r, D366, EFM1, H80721, and PBP3r of *E. hirae* S185r[a]

Amino acid position	Difference in amino acid sequence				
	PBP3r of S185r	PBP5fm of:			
		D63r	D366	EFM-1	H80721
24	V	V	A	A	A
25	T	A	A	A	A
27	S	S	G	G	G
34	R	R	Q	Q	Q
66	G	G	E	E	E
68	A	A	A	T	T
85	E	E	E	D	D
100	E	E	Q	Q	Q
144	K	K	Q	Q	Q
172	T	T	A	A	A
177	L	L	I	I	I
204	D	D	G	D	G
216	A	A	S	S	S
324	T	T	A	A	A
401	A	A	S	A	A
466′[b]					S
485	M	M	M	T	A
496	N	N	K	K	K
499	A	A	I	T	T
525	E	E	D	D	D
586	V	V	V	L	V
598	Q	Q	R	Q	Q
629	E	E	E	V	V
667	P	P	P	S	S
k_{+2}/K (M^{-1} s^{-1})[c]	20	20	17	1.5	<1.3

[a] Modifications which concern only PBP5fm of strains EFM-1 and H80721 and whose locations are also presented in Fig. 3 are boxed. Positions 401 to 667 are in the C-terminal domain.

[b] 466′, position of the additional serine in the sequence of PBP5fm of strain H80721.

[c] See the legend to Table 2.

observed for PBP5 of *E. hirae* R40 (11), a 31-kDa tryptic digestion product of PBP5fm of *E. faecium* D63r corresponding to the C-terminal domain alone was still able to bind benzylpenicillin with the same kinetic properties ($k_{+2}/K = 17$ M^{-1} s^{-1}) as the native protein (52).

Comparison of PBP5fm with other low-affinity PBPs. The amino acid sequence of PBP5fm of strain D63r was compared with those of other known low-affinity PBPs, i.e., PBP3r and PBP5 of *E. hirae* (11, 40), PBP2′ of *S. aureus* (44), and PBP5 of *E. faecalis* 56R (43). From the alignment of the primary structures on the basis of the previous hydrophobic cluster analysis (40) emerged a consensus of strict identities (Fig. 5). The sequence of the *E. coli* PBP2 (3), a PBP of intermediate affinity for benzylpenicillin ($k_{+2}/K = 495$ M^{-1} s^{-1}), was aligned with the consensus to search for amino acid residues specific for low-affinity PBPs. Nine conserved boxes (labeled 1 to 9 in Fig. 5), analogous to those identified previously (40), were found in the N- and C-terminal domains of all of the PBPs. In the C-terminal domain, the three clusters of residues known to be part of the active-site cavity were detected: the tetrad S*TXK (where S* is the active-site serine), the triad SD(A)N, and the triad KT(S)G (19). Conserved amino acid residues which seemed to be specific for the low-affinity PBPs were identified all over these proteins, i.e., the N-terminal extension typical of the low-affinity PBPs (Fig. 5) (13, 40) and the N- and C-terminal domains. They appeared, however, to be much more numerous in the C-terminal domain, in which they even formed four conserved motifs. Three of them (designated A, B, and C in Fig. 5) were close to each other and were located between the SDN and KTG triads; the fourth (D) immediately followed the KTG triad.

DISCUSSION

Like other enterococci, *E. faecium* has a low-affinity PBP, PBP5fm, which is a major component in the development of resistance to β-lactams (this work and references 15, 16, 30, and 50).

Cloning of a 7.7-kb *Eco*RI fragment from *E. faecium* D63r DNA containing *pbp5fm* allowed sequencing of the gene and adjacent regions and their comparison with those of the plasmid-borne *pbp3r* of *E. hirae* S185r (41). The *pbp5fm* gene and its immediate environment (from position 437 to position 2778 [Fig. 3]) were identical to the corresponding *pbp3r* region. The strong homology found between PBP3r of *E. hirae* S185r and the enterococcal low-affinity PBPs (PBP5 of *E. faecalis* 56R, PBP5 of *E. hirae* ATCC 9790, and PBP5fm of all *E. faecium* strains studied, with 62, 78.5, and 96.7 to 99.8% identity, respectively) suggested that they had a common ancestor.

The high-level resistance to benzylpenicillin of different clinical isolates of *E. faecium* has been attributed either to an increased production of PBP5fm (references 1, 15, and 50 and this study) or its apparent lower affinity (references 15 and 30 and this study). No significant changes in affinity of PBP5fm were found in the different sensitive and intermediately resistant clinical isolates (Table 2); however, a 4- to 28-fold increase in the MICs of benzylpenicillin for the different strains was related to a 3- to 10-fold increase in the quantity of the 73.8-kDa PBP5fm. In this context, it was interesting to note the presence of a *psrfm* gene 285 bp shorter than *psr* about 2.3 kb upstream from *pbp5fm* in *E. faecium* D63r. Both *psr* and *psrfm* were 74% homologous, and their products showed 80% identity (35). Ligozzi et al. (33) reported that in a resistant mutant derived from *E. hirae* ATCC 9790, the increased expression of *pbp5* was linked to an 87-bp deletion overlapping the 5′ end of the *psr* repressor gene. Similarly, *E. faecium* D63r produced fivefold more PBP5fm than the parent strain, D63 (Table 2). However, in contrast to *E. hirae* R40 (33), the lack of repressor activity did not seem to be due to a modification of Psrfm, because preliminary experiments showed that the parent strain D63 harbored the same *psrfm* as strain D63r (9). Overproduction of PBP5fm could therefore result from either an alteration of the *psrfm* promoter, which was not sequenced here because it was not part of the 7.7-kb *Eco*RI insert of pDML517, or a modification of the target site of Psrfm, probably located between *psrfm* and *pbp5fm*.

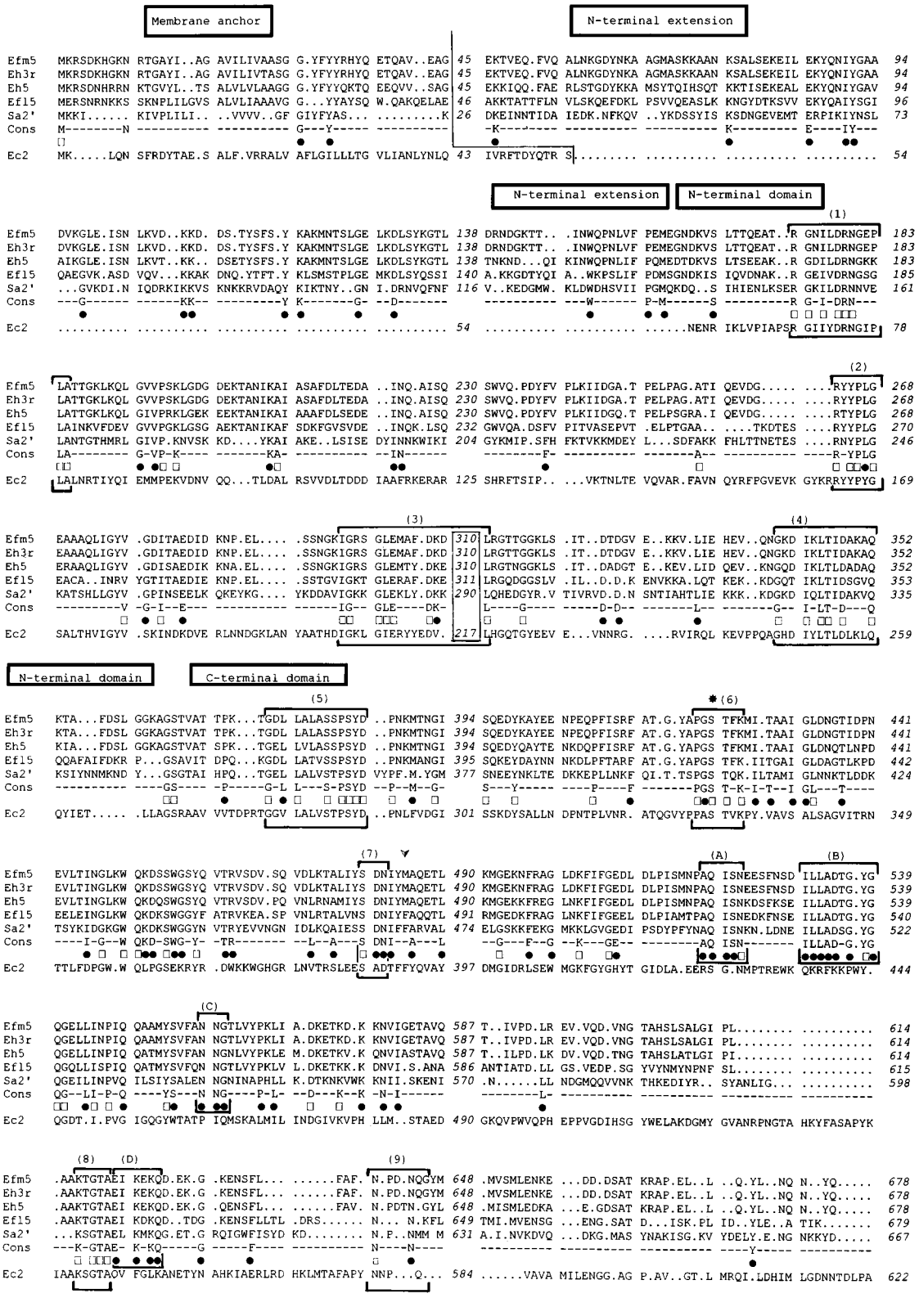
On the other hand, a decreased affinity (by at least 10-fold) of the quantitatively unaltered PBP5fm could explain the very high benzylpenicillin resistance of strains EFM-1 and H80721, for which the MICs were 90 and 512 μg/ml, respectively. Sequence comparison of the PBP5fm C-terminal domains revealed that the modification of four amino acids and the insertion between positions 466 and 467 of one serine residue (Ser-466′) in H80721 (Table 3), could be involved in lowering the affinity of PBP5fm.

A three-dimensional model of the C-terminal domain of PBP5fm (from residue 374 to the end in Fig. 3) was built in order to tentatively determine the positions of these residues with respect to the active site (Fig. 6 and 7). This model was constructed by analogy with the 3-D structure of the β-lactamase of *S. aureus* PC1 (25), despite the relatively low isology (23%) between these two sequences. To facilitate predictions, a three-step procedure was used: first, the sequence of the C-terminal domain of *S. aureus* PBP2′ was fitted on the *S. aureus* β-lactamase model (40% isology), next the PBP5fm sequence was aligned on that of the PBP2′ C-terminal domain (56% isology), and finally, a model of the C-terminal domain of PBP5fm was built, on the basis of these alignments, from the X-ray structure of the β-lactamase. The configuration of three peptide fragments of PBP5fm (Ile-411 to Pro-420; Phe-504 to Gln-540, and Glu-629 to Lys-632), represented by dotted lines (I, II, and III, respectively) in Fig. 6, could not be predicted, because they had no equivalent in the 3-D structure of the β-lactamase.

Although the 3-D structure of the PBP5fm C-terminal domain was hypothetical, it was quite close to the tertiary structure established recently for the transpeptidase domain of *Streptococcus pneumoniae* PBP2x (38), which was quite similar to that of class A β-lactamases and consequently of the *S. aureus* PC1 enzyme. In addition, that model could be an excellent working tool to plan directed mutagenesis experiments.

On the basis of that model, residues Glu-629 and Pro-667 appeared to be located far from the active site (Fig. 6). Residue Ser-466′ was positioned on the external face of the C-terminal domain, which made its ability to alter the affinity of PBP5fm difficult to predict. However, in penicillin-resistant

FIG. 5. Amino acid alignments of different low-affinity PBPs with PBP2 of *E. coli*. Efm5, PBP5fm of *E. faecium* D63r; Eh3r, PBP3r of *E. hirae* S185^r; Eh5, PBP5 of *E. hirae* R40; Efl5, PBP5fl of *E. faecalis* 56R; Sa2′, PBP2′ of *S. aureus*; Ec2, PBP2 of *E. coli*. Cons, conserved residues in the five low-affinity PBPs. Gaps represented by dots were introduced in the sequences to obtain better alignment. □, strict identities between the consensus and the PBP2 sequence of *E. coli*; ●, amino acids of the consensus different from those of PBP2 of *E. coli*; ✱, active-site serine of the STXK box 6. (1) to (9), conserved boxes according to Piras et al. (40). (A), (B), (C), and (D), conserved motifs specific for the low-affinity PBPs. ⮟, Met-485 residue of PBP5fm of *E. faecium* D63r and D366 substituted for by Thr in strain EFM-1 and Ala in strain H80721.


Membrane anchor
N-terminal extension
N-terminal extension
N-terminal domain
(1)
(2)
(3)
(4)
N-terminal domain
C-terminal domain
(5)
(6)
(7)
(A)
(B)
(C)
(8)
(D)
(9)
Efm5
Eh3r
Eh5
Efl5
Sa2'
Cons
Ec2

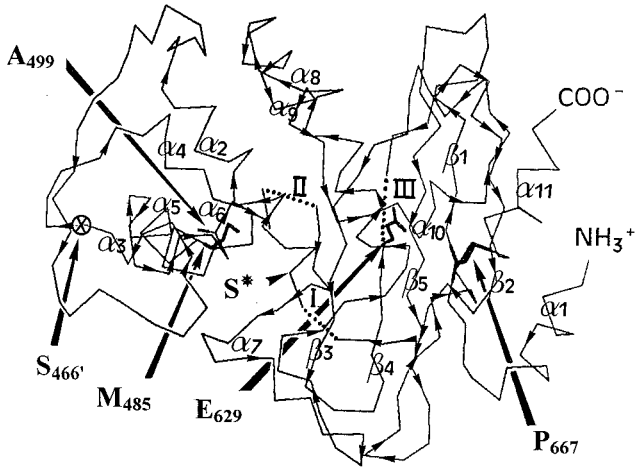


FIG. 6. Tentative tridimensional representation of the C-terminal domain of PBP5fm. The positions and side chains (thick lines) of the modified amino acids in the PBP5fm mutants are indicated by arrows. The peptides (I, II, and III) of unknown conformation are represented by dotted lines (· · · · ·). S*, active-site serine. ⊗, position of the additional Ser-467′ present in PBP5fm of H80721.

strains, the region in which it was inserted could be of importance, because it was shown that an aspartic residue and no other amino acid residues or a tripeptide, Trp-Tyr-Thr, was added in a very similar position in PBP2 of *Neisseria gonorrhoeae* (6) or in PBP2b of *S. pneumoniae* (51), respectively. Residue Ala-499 at the C-terminal end of the α6 helix, although closer to the active-site cavity, is less likely to influence the PBP5fm affinity for benzylpenicillin, because it seems to point away from the active-site cavity. In contrast, residue Met-485 was located in the first turn of the α5 helix (Fig. 6 and 7), and its side chain should protrude into the active site. It could therefore influence the binding of benzylpenicillin by PBP5fm. This residue occurred in the third position downstream from the conserved SXN triad, which was shown to have a structural as well as a functional role, in the active site of different penicillinases and DD-peptidases (27–29). The possible importance of residue Met-485 was strengthened by the progressive decrease in affinity of PBP5fm from D63r and D366 (k_{+2}/K 20 and 17 M^{-1} s^{-1}, respectively) to EFM-1 (k_{+2}/K = 1.5) and H80721 ($k_{+2}/K < 1.3$), in parallel with the progressive decrease of the length of the side chain of residue 485 (Fig. 7) from Met in D63r and D366 to Thr in EFM-1 and Ala in H80721. It is interesting to note that in different penicillin-resistant strains of *S. pneumoniae* which had a low-affinity PBP2b, a Thr residue immediately after the SXN motif of PBP2b was changed to Ala (10). While this work was being submitted, different amino acid substitutions in the region between the SDN and KTG boxes were described in the sequence of other low-affinity PBP5fms (34). Interestingly, while no substitution was found at position 485, residue Met-426 adjacent to the STFK motif was replaced by residue Ile. This suggests that the affinity of PBP5fm could be decreased through different amino acid substitutions in different locations.

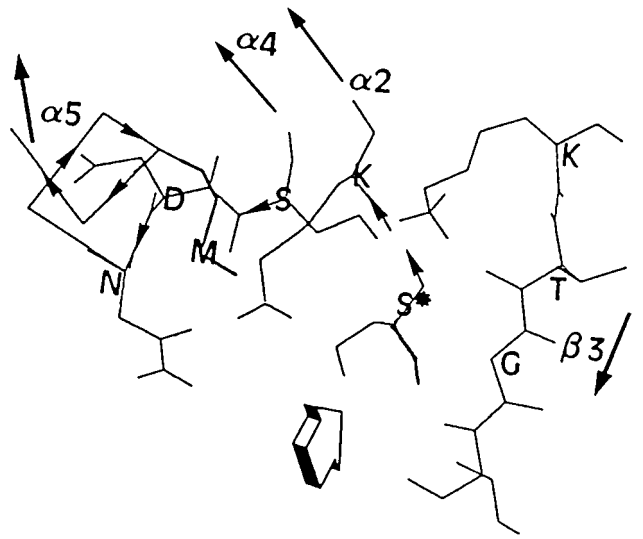


FIG. 7. Enlarged view of the active site (indicated by the large open arrow) where the conserved motifs SDN and KTG and the active serine (S*) are represented. M, side chain of the Met-485 residue pointing into the active site.

Another structural feature of the low-affinity PBPs should be mentioned. While the nine conserved boxes identified previously (13, 40) were clearly distinguished in the low-affinity PBPs and PBP2 of *E. coli* (Fig. 5), four additional conserved boxes (A, B, C, and D in Fig. 5) seemed to be specific for the low-affinity PBPs. They were located in the C-terminal domain and were absent in the other high-molecular-mass PBPs, which have a higher affinity for benzylpenicillin, such as PBP2 of *E. coli*, PBP2x of *S. pneumoniae*, and PBP3 of *N. gonorrhoeae* (13, 19, 20, 40). At this stage, it is difficult to assign a particular role to these motifs in the poor binding capacity of the low-affinity PBPs, particularly since motifs A (AQIS) and B (ILLADXGYG) are part of peptide II, for which no conformation could be proposed (Fig. 6). Both motifs might partially or totally close one side of the active-site cavity. In addition, the Asp D residue in motif B (underlined in the sequence given above) could have the same catalytic importance as Asp-447 in PBP2 of *E. coli* (19, 20, 40). As for motif D, EXKXKQ, it immediately followed the K(T/S)G triad (box 8) which forms the right side of the active-site cavity of the penicillin-recognizing active-serine enzymes (Fig. 7). Amino acid substitutions in these four conserved motifs as well as in position 485 by site-directed mutagenesis should yield additional information on their respective contribution to the low affinity of these enterococcal PBPs.

ACKNOWLEDGMENTS

This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Services fédéraux des affaires scientifiques, techniques et culturelles (PAI no. 19) and the Fonds de la Recherche Scientifique Médicale (contract no. 3.4531.92) and by grants from the Institut National de la Santé et de la Recherche Médicale (Paris, France) (CRE930603 and CRI950601).

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