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Modification of Penicillin-Binding Protein 5 Associated with High-Level Ampicillin Resistance in *Enterococcus faecium*

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High-level ampicillin resistance in *Enterococcus faecium* has been shown to be associated with the synthesis of a modified penicillin-binding protein 5 (PBP 5) which had apparently lost its penicillin-binding capability (R. Fontana, M. Aldegheri, M. Ligozzi, H. Lopez, A. Sucari, and G. Satta. Antimicrob. Agents Chemother. 38:1980–1983, 1994). The *pbp5* gene of the highly resistant strain *E. faecium* 9439 was cloned and sequenced. The deduced amino acid sequence showed 77 and 54% homologies with the PBPs 5 of *Enterococcus hirae* and *Enterococcus faecalis*, respectively. A gene fragment coding for the C-terminal part of PBP 5 containing the penicillin-binding domain was also cloned from several *E. faecium* strains with different levels of ampicillin resistance. Sequence comparison revealed a few point mutations, some of which resulted in amino acid substitutions between SDN and KTG motifs in PBPs 5 of highly resistant strains. One of these converted a polar residue (the T residue at position 562 or 574) of PBP 5 produced by susceptible and moderately resistant strains into a nonpolar one (A or I). This alteration could be responsible for the altered phenotype of PBP 5 in highly resistant strains.

The evolution of resistance to β-lactam antibiotics in enterococci has been favored by the properties of a low-affinity penicillin-binding protein (PBP 5) which is a normal component of the PBP pattern of these bacteria and which is able to substitute for the functions of the susceptible PBPs when they are inhibited by β-lactams (1, 2, 8–10). Overproduction of PBP 5 is associated with the acquisition of resistance to all β-lactams, including penicillin and ampicillin, which are normally active against enterococci at therapeutic concentrations.

In *Enterococcus hirae*, the mutation responsible for the resistance phenotype has been mapped in a genetic element (*psr*) which is located 1 kb upstream of the *pbp5* gene and which negatively controls the expression of this gene (16).

Recently, acquisition of high-level ampicillin resistance in *Enterococcus faecium* has been found to be associated with the overproduction of a PBP 5 with a reduced β-lactam affinity (7). In the study described here we analyzed the gene coding for PBP 5 of a clinical isolate (*E. faecium* 9439) that has a very high level of resistance to ampicillin. We have also determined the sequence of a gene fragment coding for the C-terminal penicillin-binding domains of PBPs 5 of *E. faecium* strains with different levels of ampicillin resistance and have identified a number of mutations causing amino acid substitutions whose locations suggest that they could be responsible for the mutated phenotype of PBP 5 in highly resistant strains.

MATERIALS AND METHODS

Bacterial strains. The *E. faecium* strains used in the present study were clinical isolates and laboratory mutants described in previous studies (7, 15). Strains 9439 and 26 were highly resistant to ampicillin (MIC, 128 μg/ml). Strains 6036 and 28R were moderately resistant (MIC, 32 μg/ml). Strain 28R was obtained from the ampicillin-susceptible strain 28S (MIC, 0.5 μg/ml) by exposure to increasing penicillin concentrations (15). *E. faecium* strains were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.). *Escherichia coli* strains were grown in Luria-Bertani medium supplemented with ampicillin at 50 μg/ml.

Cloning techniques. Procedures for the construction, isolation, and analysis of plasmids and for transformation were based on those of Sambrook et al. (18). The chromosomal DNAs of the *E. faecium* strains were prepared by lysis of bacterial cells with lysozyme in the presence of sodium dodecyl sulfate (SDS) and proteinase K. The restriction enzymes were purchased from Boehringer, Mannheim, Germany. For hybridization experiments the probe DNA was labeled with digoxigenin-11-dUTP, and the DNA hybrids were detected colorimetrically with alkaline phosphatase-coupled sheep antidigoxigenin antibodies as specified by the manufacturer (Boehringer).

PCR amplification. Degenerate oligonucleotide primers based on the conserved amino acid sequences of the C-terminal part of PBPs 5 of *E. hirae* (5) and *Enterococcus faecalis* (19) were constructed and were used to amplify gene fragments coding for this region. At the 5′ end, the forward primer of each pair had a *Bam*HI site and the reverse primer had an *Eco*RI site. The amplification reaction was performed as described by Piras et al. (17), and the fragments were subsequently cloned into pUC18.

DNA sequencing. The denatured double-stranded DNA plasmids carrying the cloned fragments were sequenced by the dideoxy-ribonucleotide-chain termination reaction with an AutoRead Sequencing Kit (Pharmacia, Uppsala, Sweden) and an ALF DNA sequencer (Pharmacia-LKB, Uppsala, Sweden). The DNA sequences and the derived protein sequences were analyzed and compared with published sequences of similar genes by using DNASIS and PROSIS software from Hitachi Software Engineering Co., Brisbane, Calif. Errors arising from the PCR were eliminated by sequencing at least two independent clones for each strain. The nucleotide sequence data reported here will appear in the EMBL,

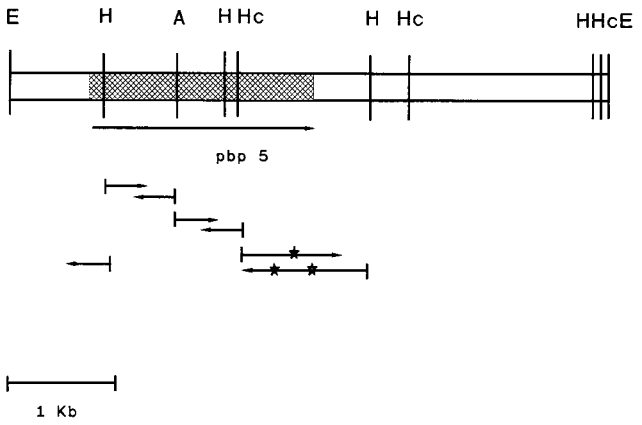

FIG. 1. Restriction map of the *pbp5* gene of *E. faecium* 9439 and sequencing strategy. Restriction sites: E, *Eco*RI; H, *Hin*dIII; A, *Acc*I; Hc, *Hin*cII. All of the fragments were cloned in pUC18 and were sequenced by using M13 universal or synthetic primers (★).

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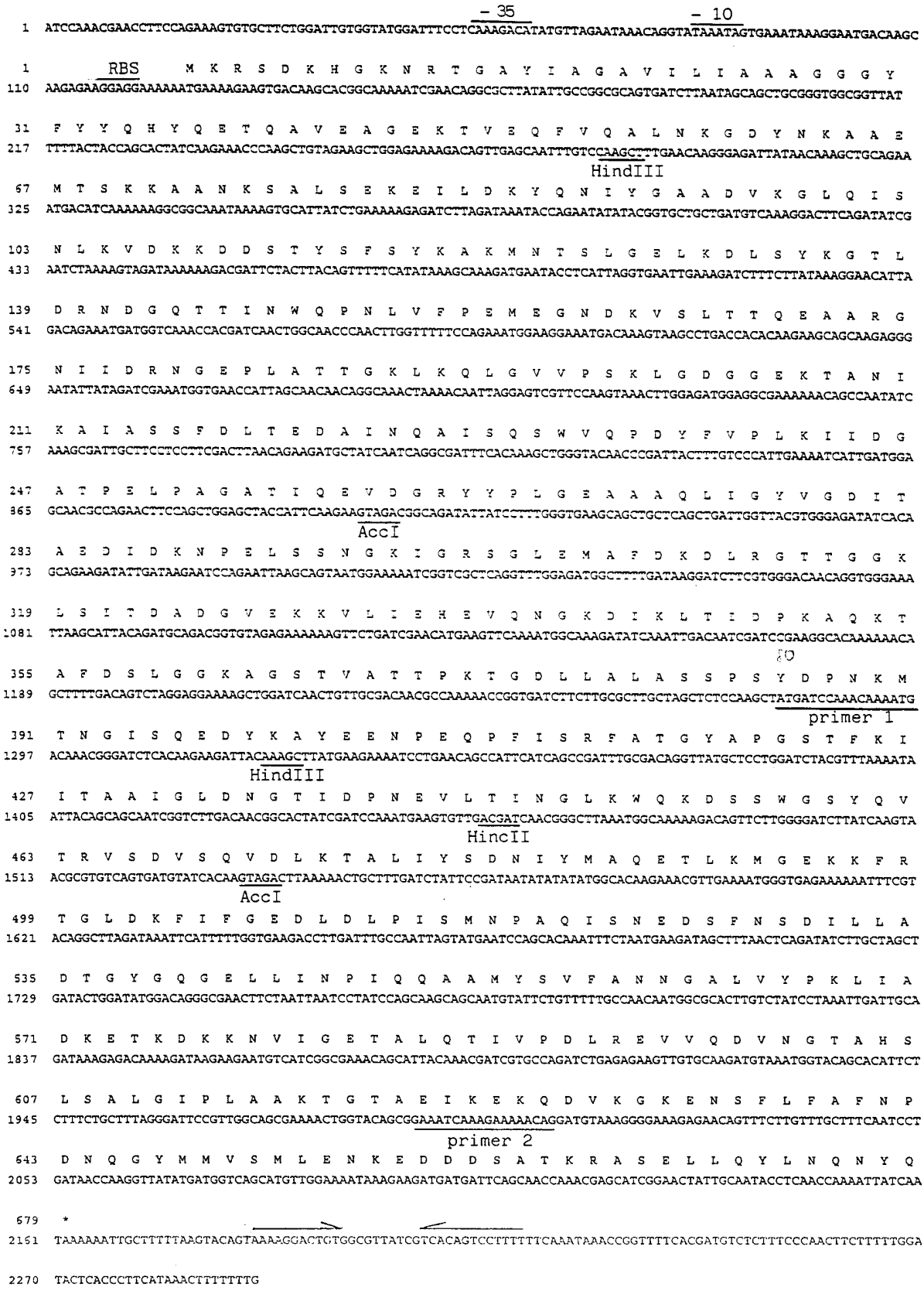

FIG. 2. Nucleotide sequence of the *pbp5* gene of *E. faecium* 9439 and the deduced amino acid sequence of PBP 5. The primers used for PCR amplification, the putative −35 and −10 regions, and a ribosome-binding site (RBS) are underlined. The asterisk denotes the TAA stop codon. The arrows indicate the position of the putative transcription termination signal. Single-letter codes for amino acids are used.

GenBank, and DDBJ nucleotide sequence databases under the accession number X92687.

RESULTS AND DISCUSSION

Cloning of *pbp5* gene of *E. faecium* 9439. As a first step in the cloning procedure, we attempted amplification of the chromosomal DNA of *E. faecium* 9439 using PCR and several degenerate primers derived from published sequences of *pbp5* of *E. hirae* (5) and *E. faecalis* (19). A pair of these gave a 729-bp amplification product which was cloned into pUC18 and partially sequenced. The results showed a very strong similarity of the *E. faecium* 9439 fragment to the corresponding fragment of the *pbp5* gene of *E. hirae*. The digoxigenin-labeled fragment was found to hybridize with a single 5.6-kb band of *Eco*RI-digested *E. faecium* DNA. A size-selected library of *Eco*RI-digested and alkaline phosphatase-treated pUC18 was transformed into competent *E. coli* cells, and the cells were plated onto medium containing ampicillin (50 μg/ml). The resulting colonies were screened by colony hybridization with the digoxigenin-labeled probe. The plasmid (pEF5) in one clone which produced a positive signal was found to contain a 5.6-kb *Eco*RI fragment. The position of the hybridizing region within this fragment was determined by Southern hybridization of enzyme restriction digests (Fig. 1). To prove that this insert contained the *pbp5* gene, membranes of transformed *E. coli* cells were isolated and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then Western blot (immunoblot) analysis with anti-PBP 5 antibodies (15). An immunoreactive band with an apparent molecular size of 75 kDa was produced by the clone (data not shown).

By using the strategy depicted in Fig. 1, sequencing of the pEF5 insert yielded an open reading frame which started at position 128 with an ATG codon and which terminated at position 2161 with a TAA stop codon (Fig. 2). A possible promoter sequence was located upstream of the putative coding region and was significantly similar to −35 and −10 consensus promoter sequences. A possible ribosome-binding site was located from nucleotides 116 to 121. The TAA stop codon is followed by a palindromic region typical of many prokaryotic terminators. The open reading frame of the *pbp5* gene of *E. faecium* 9439 contained 2,037 nucleotides and coded for 678 amino acid residues. The calculated relative molecular weight was 73,699, which is only slightly lower than the molecular mass of 75 kDa determined by SDS-PAGE.

Amino acid sequence homology among PBPs 5 of different enterococcal species. The alignment of the deduced amino acid sequence of PBP 5 of strain 9439 with those of *E. hirae* ATCC 9790 (16) and *E. faecalis* (19) showed that the PBP 5 of *E. faecium* 9439 was 77% similar to that of *E. hirae* and 54% similar to that of *E. faecalis*. The homology increased to 84 and 63%, respectively, when the C-terminal penicillin-binding domains of both proteins were compared. This part of the protein contains the active site-defining motifs characteristic of the penicilloyl serine transferase family, i.e., in the order indicated, the tetrad S*XXK (where S* is the active site serine), the triad SDN (or analog), and the triad KT(S)G (or analog) (11). These results confirmed that *E. hirae* is more closely related to *E. faecium* than to *E. faecalis*, as also suggested by the biochemical profiles of the two species (6). The similarity among the PBPs 5 of the three species was also to be expected on the basis of the demonstrated cross-reactivity with antibodies raised against *E. hirae* PBP 5 (15).

Comparison of the PBP 5 penicillin-binding domains of strains with different susceptibilities to ampicillin. By using the primers underlined in Fig. 2, the gene fragment coding for the amino acid sequence containing the active site-defining motifs of strain 28S (ampicillin-susceptible), strains 28R and 6036 (moderately resistant), and strain 26 (highly resistant) was amplified and cloned in pUC18. Nucleotide sequence analysis and comparison of the derived amino acid sequences revealed that the strain producing a PBP 5 which could be labeled with radioactive penicillin (that is, the PBPs 5 of strains 28S, 28R and 6036) had an identical sequence in the penicillin-binding domain apart from the substitution of an M residue at position 426 (M-426) to an I residue in strain 6036 (Fig. 3). In the PBP 5 of the highly resistant strain 9439, the same M-426-to-I substitution as in strain 6036 and two additional substitutions, T-562 to A and V-586 to L, occurring between the SDN and KTG motifs, were found. The penicillin-binding domain of PBP 5 of strain 26 carried two substitutions: A-558 to V and T-574 to I. The pattern of the amino acid substitutions occurring in the resistant strains suggested that the region from positions 558 to 586 might play an important role in the β-lactam binding site of PBP 5, since changes in this region occurred in both the highly resistant strains, whereas the P-395-to-S substitution only occurred in strain 9439. In addition, both the T-562-to-A and T-574-to-I substitutions may remodel the penicillin-binding domain, since in both cases the original polar amino acid was replaced by a nonpolar one (A or I). Interestingly, a T-to-A substitution just adjacent to the KSG motif has been associated with a further decrease in β-lactam affinity of the mosaic PBP 2x of *Streptococcus pneumoniae* and the acquisition of cefotaxime resistance (3, 12, 14).

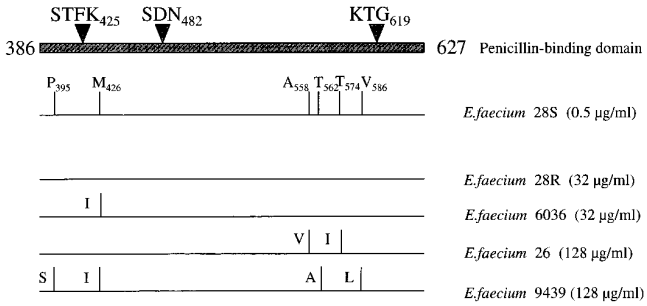

FIG. 3. Amino acid substitutions in the PBP 5 penicillin-binding domain (amino acids 386 to 627) of *E. faecium* strains with different levels of ampicillin resistance. The three homology boxes (STFK with the active-site serine-SDN and KTG) are indicated above. In the sequence of strain 28S, the positions at which resistant strains are altered are indicated. The MICs of ampicillin are indicated in parentheses.

The acquisition of resistance through alterations affecting the penicillin-binding domain is well documented (4, 13, 20, 21). In *S. pneumoniae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, these alterations occur through recombinational events that recruit portions of the homologous gene from related species and result in the creation of mosaic genes coding for hybrid PBPs with a low affinity for penicillin (4, 20, 21). Although the effects of these changes on resistance or PBP 5 affinity have not been demonstrated, the present study suggests that in enterococci the low-level natural susceptibility to β-lactams may evolve to high-level resistance through the combination of multiple mutations which increase the synthesis of the low-affinity PBP 5 and which decrease the affinity of this protein for β-lactams.

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