Penicillin-Binding Protein 5 Sequence Alterations in Clinical Isolates of Enterococcus faecium with Different Levels of β-Lactam Resistance

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The low-affinity penicillin-binding protein (PBP) 5 is the main β-lactam target and is responsible for resistance to this class of antibiotics in Enterococcus faecium. The PBP 5 variants of 15 clinical isolates (including 8 resistant to vancomycin) with different levels of β-lactam resistance were analyzed. Most of the highly β-lactam–resistant isolates produced small quantities of PBP 5 of low affinity. This was associated with particular amino acid substitutions: an Ala or Ile for Thr-499, a Glu for Val-629, and a Pro for Ser-667. A change of Met-485 to Thr or Ala (adjacent to the conserved SDN box) was observed in isolates with MICs of ampicillin of 64 or 128 µg/mL, respectively. In the 2 most resistant isolates, with MICs of ampicillin of 256 µg/mL, an additional Ser was present just after Ser-466. Thus, particular point mutations in PBP 5 and combinations thereof may lead to high-level β-lactam resistance in E. faecium.

Enterococci are increasingly responsible for nosocomial infections and are the second most common pathogen isolated from hospitalized patients [1]. Ampicillin resistance in Enterococcus faecium is a world-wide therapeutic problem, especially when associated with high-level resistance to aminoglycosides and glycopeptides. Recent evidence suggests that the prevalence of E. faecium (especially multiresistant isolates) is increasing in many hospital centers and that this species is frequently involved in hospital outbreaks [2]. The natural low-level resistance to β-lactam antibiotics observed in enterococci is due to the presence of a high-molecular-weight, low-affinity penicillin-binding protein (PBP) 5 [3–9]. In addition to the three classical conserved motifs, STFK, SDN, and KTG [7, 9], four additional conserved boxes in the C-terminal domain are common in many low-affinity PBP 5 Enterococcus variants and in PBP 2a of Staphylococcus aureus [9]. Higher levels of β-lactam resistance have been observed in E. faecium, and the increased resistance was attributed either to increased quantities of PBP 5 [5, 8, 9] or to point mutations near the three conserved motifs [7, 9].

This study attempted to correlate β-lactam resistance levels, PBP 5 affinities and amounts, and C-terminal mutations. We also explored the extent of PBP variations in a collection of clinical E. faecium isolates from various sources in order to evaluate more fully the potential of this species for the development of resistance to β-lactam antibiotics.

Materials and Methods

Bacterial isolates and growth conditions. Sixteen E. faecium clinical isolates from France, Germany, Hungary, and the United States were studied. Among these, 8 were resistant to vancomycin. By pulsed-field gel electrophoresis, these strains did not appear to be related, with the possible exception of isolates D3 and 25 to CII and 34, respectively. All of the PBP 5 genes of isolates D366, H80721, and EFM-1 were previously sequenced [9]. The isolates were identified by the criteria of Facklam [10] and by the species-specific profiles of the PBPs [11]. The origin of each isolate is shown in table 1. Bacteria were generally grown without shaking at 37°C in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit) until mid-log phase.

Antibiotics and susceptibility tests. Benzylpenicillin, piperacillin, imipenem, and ampicillin were provided by Spécia, Lederle, Merck Sharp & Dohme–Chibret, and Bristol-Myers (all Paris), respectively. The MICs were determined on BHI agar containing 2-fold serially diluted antibiotics. About 10⁴ cfu was applied with a Steers-type replicator, and MICs were read after 18 h at 37°C.

Analysis of PBPs. The technique used for analysis of PBPs was exactly as previously described [8]. [3H]benzylpenicillin (0.66 TBq mmol⁻¹) was provided by Rhône-Poulenc-Rorer Recherche (Vitry-sur-Seine, France) and synthesized at the Service des Molécules Marquées, Commissariat à l’Énergie Atomique (Gif-sur-Yvette, France).

Immunoblot analysis. Immunoblot analysis was done as previously described [16] with an antiserum directed against PBP 5 of E. faecium EFM-1 [9, 13].

Polymerase chain reaction (PCR) experiments and DNA sequencing. DNA was prepared from 20 mL of an exponential-phase culture by standard methods [17]. A 794-bp pbp5fm fragment from E. faecium encoding the three conserved motifs (STFK, SDN, and KTG) and the entire 3’ terminus [9] was amplified by PCR using the oligonucleotide primers 5’-CGGATCTCTACAAGAAGAT-3’ and 5’-TTATTGATAATTTTGGTT-3’, corresponding to positions 1176–1194 and 2037–2020, respectively. Reactions were performed with a DNA thermal cycler (PHC-2; Techne, Cambridge, UK): 45 1-min cycles at 92°C, 2 min of annealing at 52°C, and 2 min of extension at 72°C. Amplified
Table 1 lists the quantities of PBP 5 of all 16 isolates analyzed and their MICs of ampicillin.

**PBP 5 variants and relationships between variance sequences and affinity for benzylpenicillin.** The amino acid sequences of the C-terminal domains of different low-affinity PBP 5 variants from 13 isolates of *E. faecium* were compared with those of the known low-affinity PBP 5 variants of *E. faecium* D63R, D366, EFM-1, H80721 [9], and 9439. The

Table 1. MICs of different β-lactam antibiotics and quantity of PBP 5 of different β-lactam–resistant *E. faecium* clinical isolates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillin (µg/mL)</th>
<th>Ampicillin (µg/mL)</th>
<th>Piperacillin (µg/mL)</th>
<th>Imipenem (µg/mL)</th>
<th>Quantity of PBP 5* isolate</th>
<th>Origin of isolate</th>
<th>References</th>
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<tr>
<td>BM4107²</td>
<td>4</td>
<td>2</td>
<td>16</td>
<td>4</td>
<td>+</td>
<td>France</td>
<td>[12]</td>
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<tr>
<td>D359</td>
<td>8</td>
<td>4</td>
<td>32</td>
<td>8</td>
<td>+++</td>
<td>France</td>
<td>[8]</td>
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<tr>
<td>EFM-4²</td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>+++</td>
<td>France</td>
<td>[13]</td>
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<tr>
<td>D366²</td>
<td>32</td>
<td>16</td>
<td>64</td>
<td>16</td>
<td>+++</td>
<td>France</td>
<td>[14]</td>
</tr>
<tr>
<td>D444</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td>32</td>
<td>+++</td>
<td>France</td>
<td>[8]</td>
</tr>
<tr>
<td>D3²</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td>128</td>
<td>+</td>
<td>France</td>
<td>This study</td>
</tr>
<tr>
<td>CHI²</td>
<td>128</td>
<td>64</td>
<td>256</td>
<td>128</td>
<td>+</td>
<td>Hungary</td>
<td>This study</td>
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<tr>
<td>AR9</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>128</td>
<td>+</td>
<td>France</td>
<td>This study</td>
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<tr>
<td>6024²</td>
<td>128</td>
<td>64</td>
<td>256</td>
<td>64</td>
<td>+</td>
<td>France</td>
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</tr>
<tr>
<td>EFM-1</td>
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<td>256</td>
<td>128</td>
<td>+</td>
<td>France</td>
<td>[13]</td>
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<tr>
<td>7090⁻²</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td>32</td>
<td>+</td>
<td>Germany</td>
<td>[15]</td>
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<tr>
<td>34</td>
<td>256</td>
<td>128</td>
<td>512</td>
<td>256</td>
<td>+</td>
<td>Germany</td>
<td>[6]</td>
</tr>
<tr>
<td>25</td>
<td>256</td>
<td>128</td>
<td>512</td>
<td>256</td>
<td>+</td>
<td>Germany</td>
<td>[6]</td>
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<tr>
<td>A888</td>
<td>256</td>
<td>128</td>
<td>512</td>
<td>512</td>
<td>+</td>
<td>USA</td>
<td>This study</td>
</tr>
<tr>
<td>6885²</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>-¹</td>
<td>USA</td>
<td>This study</td>
</tr>
<tr>
<td>H80721¹</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
<td>+</td>
<td>USA</td>
<td>[9]</td>
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* Estimated by immunoblot.
² Resistant to vancomycin (VAN A class).
³ Below limit of detection.

pBP5fm fragments were purified with a PCR purification kit (High Pure; Boehringer Mannheim, Mannheim, Germany) and sequenced with a sequencing kit (Promega, Madison, WI) according to the manufacturers’ recommendations. Mutations were verified by nucleotide sequencing in at least two independent experiments.

### Results

**Susceptibility testing.** The MICs for the different clinical isolates (table 1) ranged from 4 to 512 µg/mL for penicillin G. The MICs of imipenem were very close to those of penicillin G for all isolates, but the MICs of ampicillin were generally 2-fold lower.

**Examination of PBPs and immunoblotting.** The PBPs of all isolates were labeled with [3H]benzylpenicillin at concentrations ≤70 µg/mL in an attempt to saturate the low-affinity PBP 5. The PBP patterns of 4 selected isolates are shown in figure 1A. While saturation of the PBP 5 variants of D359 and EFM4 (figure 1A) and other isolates with MICs of ampicillin <64 µg/mL (data not shown) was obtained at ≤70 µg/mL, this did not occur with PBP 5 variants of AR9 and 25 (figure 1A) or for isolates with MICs ≥64 µg/mL, which were barely labeled (data not shown). To avoid the problem of quantifying PBP 5 only by radiolabeling with penicillin, which could underestimate the amount of the PBP if its affinity were very low, we used immunoblotting with an anti–PBP 5 antiserum. Figure 1B shows results obtained with the 4 isolates mentioned above. Although quantities of PBP 5 were low in AR9 and 25, they were appreciably higher in D359 and EFM4. Of interest, immunoblotting did not detect PBP 5 in strain 6885 (data not shown).

* Figure 1. A. Autoradiograph of PBPs of *E. faecium* isolates D359, EFM-4, AR9, and 25 labeled with [3H]benzylpenicillin at 70 µg/mL. B. Immunoblots of membrane proteins of same isolates probed with polyclonal antibodies raised against PBP 5. MICs of ampicillin for isolates are in parentheses.
Figure 2. Amino acid (aa) alignments of C-terminal domains of different low-affinity PBP 5 variants from *E. faecium* clinical isolates. PBP 5 sequences of *E. faecium* D366, EFM-1, H80721, and D63r are from Zorzi et al. [9]. *E. faecium* 9439 is from Ligozzi et al. [7]. Only additional aa or those that differ from consensus are shown for individual PBP 5. Boxes 6–9, conserved sequences according to Piras et al. [18]. Boxes A–D, conserved motifs specific for low-affinity PBPs [9]. △ and ▲, aa positions where Ligozzi et al. [7] found differences from consensus in resistant isolates 9439 and 26. *aa position discussed in text.
latter was described by Ligozzi et al. [7] (figure 2). From the alignment of the primary structures, a consensus sequence was obvious. The STFK tetrad and the SDN and KTG triads, known to be part of the active-site cavity, are shown, respectively, in boxes 6–8 [18]. The conserved amino acid motifs (designated A–D) seemed to be specific for the low-affinity PBPs. The amino acid sequences of PBP 5 from E. faecium D359, EFM4, and D366 were very like the sequence of the susceptible strain BM4107. While no difference in the PBP 5 affinity for penicillin was found between these isolates, the larger quantity of this protein present in D359, EFM4, and D366 appeared to be correlated with MICs ≤16 μg/mL (table 1). The PBP 5 variants of these isolates had three substitutions in common: Ala or Ile for Thr-499, Glu for Val-629 located 2 aa after the conserved motif D, and a Pro for Ser-667 near the C-terminus of PBP 5 boxes 6–8 [18]. The conserved amino acid motifs (designated amino acid sequences of PBP 5 from on PBP 5 affinity and 574 (Ile for Thr) observed in the PBP 5 variant of another 572, 626, and 663 in the low- and high-level ampicillin-resistance for Val substitution at aa 586 was found in some of our most Nevertheless, the presence just after Ser-466 of an Asp in A888, 25, and 34, 2 US isolates (H80721 and 6885) from different locations (Shlaes D, personal communication), showed 2-fold increases in MICs of penicillin, ampicillin, and imipenem, although all had an Ala-485 instead of Met or Thr. However, the 2 US isolates had, upstream of SDN, an additional Ser inserted just after Ser-466, and strain 6885 had a further Asp inserted just after Leu-433. Since various substitutions were found at aa 572, 626, and 663 in the low- and high-level ampicillin-resistant isolates, it is likely that they have per se no direct effect on PBP 5 affinity.

**Discussion**

The high-level resistance to β-lactams of E. faecium clinical isolates may pose major epidemiologic and therapeutic problems, especially when associated with resistance to glycopeptides. In this context, it should be noted that the 2 isolates that showed the highest MICs of β-lactams (6885 and H80721) were also resistant to vancomycin.

The level of resistance to ampicillin of isolates D359, EFM-4, and D366 (4–16 μg/mL) and D63r (16 μg/mL [9]), in which the C-terminal PBP 5 domains resemble that of susceptible strain BM4107 (MIC of ampicillin, 2 μg/mL), may be explained by the greater quantities of the low-affinity PBP 5 (9) and this study). As previously described for different strains [3, 5, 6, 8], in the remaining isolates higher MICs were associated with a further decrease in affinity of PBP 5 for penicillin with no apparent increase in the quantity of PBP 5, except in E. faecium D344. Klare et al. [6] reported similar findings for isolates 25 and 34, although they did not use anti–PBP 5–antiserum and no PBP 5 mutations were searched for.

Unlike the PBP 5 of most susceptible isolates, the PBP 5 variants of the 12 isolates with MICs >16 μg/mL and that of E. faecium 9439 (MIC of ampicillin, 128 μg/mL; figure 2) [7] had changes at aa 499, 629, and 667. These substitutions, even though far from the active site [9], could, in addition to the presence of the conserved motifs A–D specific for the low-affinity PBPs, play a role in the higher MICs observed. However, the increased levels of resistance to ampicillin correlated best with a substitution at aa 485, located three amino acids after the SDN triad. A change from Met-485 to Thr was observed in isolates with MICs of ampicillin of 64 μg/mL as was a change from Met-485 to Ala in isolates with MICs of 128 μg/mL. Different insertions in the region between the conserved STFK and SDN boxes are involved in decreased affinity of PBP 2 of *Neisseria gonorrhoeae* and with an increased resistance to penicillin of *Streptococcus pneumoniae* via PBP 2b [19, 20]. An additional Ser was present just after Ser-466 in H80721 associated with an additional Asp located eight amino acids after the STFK motif in 6885. Thus, these mutations associated with the change from Met-485 to Ala could be responsible for a further decrease in the affinity of PBP 5 for β-lactams and consequently for the higher MICs observed. Nevertheless, the presence just after Ser-466 of an Asp in A888 and of a Ser in AR9, also associated with a change at aa 485 with no further increase in their MICs, may not favor this hypothesis.

In contrast to the findings of Ligozzi et al. [7] for *E. faecium* 9439, which has no change at aa 485 (figure 2), no substitution was found in our resistant isolates immediately adjacent to the conserved STFK tetrad nor at aa 562. However, a similar Leu-for-Val substitution at aa 586 was found in some of our most resistant isolates. Other substitutions at aa 558 (Val for Ala) and 574 (Ile for Thr) observed in the PBP 5 variant of another strain of *E. faecium* 26 with a MIC of ampicillin of 128 μg/mL [7] were not found in PBP 5 of our very resistant isolates.

In conclusion, this study of several highly β-lactam–resistant *E. faecium* isolates from different countries supports and extends our previous observation [9] that very high MICs of β-lactam antibiotics are associated with low amounts of PBP 5 with decreased affinity, which relates to the presence of different amino acid substitutions, in particular at aa 485 near the conserved SDN triad. However, since the complete PBP 5 genome of these isolates has not been sequenced, we cannot exclude that other mutations present in the N-terminal, non–penicillin-binding domains could also contribute to the high level of resistance observed in these isolates.

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References


