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Characterization of Clinical Isolates of β-Lactamase-Negative, Highly Ampicillin-Resistant *Enterococcus faecalis*

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We analyzed the penicillin-binding protein (PBP) profiles of two clinical isolates of *Enterococcus faecalis* for which ampicillin MICs were 32 and 64 μg/ml. Six PBPs were detected in both isolates, demonstrating an apparently increased amount of PBP 5 and decreased penicillin binding of PBPs 1 and 6. These results suggest that ampicillin resistance in the clinical isolates of *E. faecalis* described could be associated with alterations in different PBPs.

Enterococci have become an increasingly important cause of nosocomial infections. In addition, they are intrinsically resistant to a large number of antimicrobial agents, and they also show a remarkable ability to acquire new mechanisms of resistance (10). The relative resistance of *Enterococcus faecalis* to penicillin and ampicillin is a characteristic feature of these species and appears to be due to low affinity of the penicillin-binding proteins (PBPs) (2). In 1983, resistance to β-lactams due to β-lactamase production was reported in *E. faecalis* (11), and in 1990, we reported the existence of ampicillin-resistant, β-lactamase-negative *E. faecalis* (MIC, ≥16 μg/ml) (4). Here, we characterize two clinical isolates of β-lactamase-negative, ampicillin-resistant *E. faecalis* (MIC, ≥32 μg/ml) recovered in our hospital.

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Both strains were isolated from urine. Identification was performed by conventional methods (5) and confirmed with the API 20 STREP system (BioMérieux, S.A., Marcy l'Etoile, France). Testing for β-lactamase was performed with a nitrocefin disk (BBL Cefinase; Becton Dickinson Microbiology Systems, Cockeysville, Md.). Antimicrobial susceptibility was determined by the National Committee for Clinical Laboratory Standards agar dilution technique (12) on Mueller-Hinton agar (Oxoid, Unipath Spain, S.A.) with an inoculum of approximately 10^4 CFU per spot. Testing for high-level resistance to streptomycin and gentamicin utilized Mueller-Hinton agar plates containing the aminoglycosides at 2,000 and 500 μg/ml, respectively. Ampicillin was obtained from Beecham Pharmaceuticals, Bristol, Tenn.; imipenem was from Merck Sharp & Dohme, West Point, Pa.; vancomycin was from Eli Lilly & Co., Indianapolis, Ind.; gentamicin was from Schering, Kenilworth, N.J.; and penicillin G and streptomycin were purchased from Sigma Chemical Company, St. Louis, Mo.

Analysis of PBPs and saturation assays were done as follows. Exponentially growing cells were harvested by centrifugation and washed with 10 mM potassium phosphate buffer (pH 7). Membranes from both ampicillin-resistant *E. faecalis* isolates and an ampicillin-susceptible strain (*E. faecalis* ATCC 29212) were obtained by previously described methods (14) with the addition of mutanolysin (final concentration, 108 μg/ml) and lysozyme (final concentration, 8 mg/ml). Radioactive penicillin (tritiated benzylpenicillin) was a gift from Merck, Sharp & Dohme Laboratories, Rahway, N.J. The radioactivity concentration was 8.9 mCi/ml (77.8 mCi/mg). The product was dried and resuspended in an adequate volume of 50 mM sodium phosphate to obtain a solution five times more concentrated. A quantity of 5 μl of this solution corresponded to a final penicillin concentration in the assay of 52 μg/ml. Aliquots of 50 μl of a membrane suspension (8 mg of protein per ml) were incubated for 15 min at 37°C with increasing concentrations of tritiated benzylpenicillin (1.3, 13, 26, and 52 μg/ml). With penicillin at 52 μg/ml, any possible weakly reacting PBP should be detectable. Binding was stopped by isotopic dilution with benzylpenicillin at a concentration of 2 mg/ml, and PBPs were detected after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent fluorography (14).

We reviewed the microbiology laboratory records from January 1989 to December 1995 to study the evolution of ampicillin resistance among all of the *E. faecalis* isolates recovered in our hospital. We also reviewed the clinical records of the patients from whom β-lactamase-negative, highly ampicillin-resistant *E. faecalis* was isolated. During the study period, we recovered two highly ampicillin-resistant clinical isolates of *E. faecalis* (MIC, ≥32 μg/ml). To date, we have not found any β-lactamase-positive enterococcus strain. The ampicillin-resistant strains were isolated in 1989 and 1994 from two different patients, both males, who were hospitalized in two different wards. Both patients had a urinary tract infection and had been previously treated with ampicillin. The MICs of different antimicrobial agents against the two *E. faecalis* isolates were 64 (penicillin), 32 and 64 (ampicillin), and 16 (imipenem) μg/ml. Both isolates had high-level resistance to gentamicin and streptomycin and were β-lactamase negative and susceptible to vancomycin. However, the isolates were different on the basis of antibiograms: one was erythromycin resistant, clindamycin resistant, norfloxacin susceptible, and ciprofloxacin susceptible, and the other was erythromycin susceptible, clindamycin resistant, norfloxacin resistant, and ciprofloxacin resistant. Preliminary analysis by random amplified polymorphic DNA fingerprinting suggested that they were genetically different (data not shown).

Figure 1 shows the PBP saturation profiles of both ampicillin-resistant isolates (B and C) and one susceptible *E. faecalis* strain (A) and the PBP pattern of *Escherichia coli* K-12. Both resistant strains had six PBPs. In comparison with the PBP

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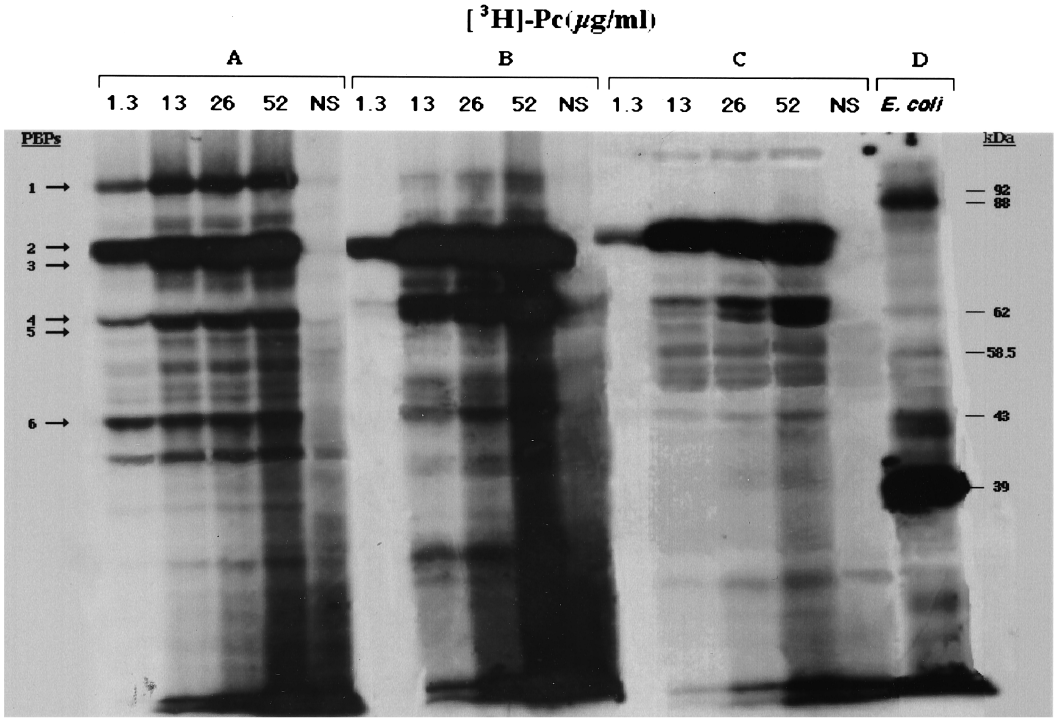

FIG. 1. PBP saturation profiles of three *E. faecalis* strains. A, ampicillin-susceptible *E. faecalis* ATCC 29212; B, ampicillin-resistant *E. faecalis* isolated in 1994; C, ampicillin-resistant *E. faecalis* isolated in 1989; D, PBP profile of *E. coli* K-12. The values above the lanes are the concentrations of tritiated penicillin (Pc) used (1.3, 13, 26, and 52 μg/ml). NS, nonspecific binding (PBPs saturated with unlabeled penicillin).

pattern of *E. coli* K-12 (rightmost lane), these profiles show PBP 1, with a molecular mass of greater than 92 kDa; PBPs 2, 3, 4, and 5, with molecular masses between 60 and 88 kDa; and PBP 6, with a molecular mass of around 43 kDa. Both resistant strains had an apparently increased amount of PBP 5. Binding of PBP 1 was apparently decreased in our resistant strains. The same was true of PBP 6, which also appeared to bind penicillin less well; however, it is not known whether affinity or synthesis of the PBPs is affected, and their contribution to resistance is also not known. Comparison with isogenic strains was not possible because our strains were resistant clinical isolates and not laboratory-obtained resistant mutants. In the unrelated susceptible strain (Fig. 1, lane A), PBP 5 was not hyperproduced and PBPs 1 and 6 were saturated with low penicillin concentrations.

Although we reported in 1990 (4) the existence of clinical isolates of β-lactamase-negative, highly ampicillin-resistant *E. faecalis*, characterization of these strains was not performed. Recently, other investigators in Spain have reported the existence of a clinical isolate of *E. faecalis* exhibiting high-level resistance to ampicillin (MIC, >16 μg/ml) in the absence of β-lactamase production (9). The results of this study confirm that high-level ampicillin resistance among β-lactamase-negative *E. faecalis* strains is a very uncommon event; however, the fact that these isolates also showed high-level resistance to gentamicin and streptomycin raises concerns about the emergence of these isolates. High-level resistance to penicillin or ampicillin in *E. faecium* results in loss of the synergistic bactericidal activity usually observed when gentamicin is combined with penicillin (2, 3); the same phenomenon would probably occur with *E. faecalis*.

Our results suggest that ampicillin resistance in the two clinical isolates of *E. faecalis* described could be associated with alterations in different PBPs, including increased production and low affinity of PBPs. Previous studies have demonstrated that low-affinity PBPs of *E. faecalis* (PBPs 1, 4, 5, and 6) are involved in β-lactam resistance (1, 7, 13, 15). Studies in which resistant mutants were selected in vitro after exposure of susceptible *E. faecalis* to penicillin show that resistance was associated with increased amounts of low-affinity PBPs and that hyperproduction of PBP 5 could lead to increases in resistance to penicillin in *E. faecalis* (1, 8, 15). As the existence of clinical isolates resistant to ampicillin has not been reported before, we do not know if other clinical isolates will show the same alterations as ours. However, modifications of the PBP profiles of laboratory mutant strains are comparable to the PBP profiles of our resistant clinical isolates. Moreover, the increased amount of PBP 5 observed in ampicillin-resistant *E. faecalis* has also been described in strains of *E. faecium* exhibiting moderate levels of ampicillin resistance (6).

The existence of clinical isolates of β-lactamase-negative, highly ampicillin-resistant *E. faecalis* raises new concerns about the treatment of severe enterococcal infections. Considering the emergence of these new pathogens, we suggest susceptibility testing of all enterococcal isolates.

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