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Comparison of *Enterococcus raffinosus* with *Enterococcus avium* on the Basis of Penicillin Susceptibility, Penicillin-Binding Protein Analysis, and High-Level Aminoglycoside Resistance

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We reidentified our laboratories' collections of 57 enterococcal isolates previously classified as *Enterococcus avium* by the API Rapid Strep identification system (Analytab Products, Plainview, N.Y.) with the identification criteria recommended by Facklam and Collins (R. R. Facklam and M. D. Collins, J. Clin. Microbiol. 27: 731–734, 1989). Thirty isolates were identified as true *E. avium*, 25 isolates were identified as *E. raffinosus*, and 2 isolates were identified as *E. pseudoavium*. *E. raffinosus* could be differentiated from *E. avium* on the basis of penicillin susceptibility, as follows: MIC for 50% of *E. raffinosus* isolates tested (MIC_{50}), 32 μg/ml; MIC_{90}, 64 μg/ml (range, 4 to 64 μg/ml); *E. avium* MIC_{50}, 1 μg/ml; MIC_{90}, 2 μg/ml (range, 0.5 to 2 μg/ml). No strains produced detectable β-lactamase. Penicillin-binding protein (PBP) analysis of all *E. raffinosus* isolates demonstrated the unique pattern reported previously (M. D. Collins, R. R. Facklam, J. A. E. Farrow, and R. Williamson, FEMS Microbiol. Lett. 57:283–288, 1989); however, a number of newly identified PBPs were noted. Of 25 isolates, 13 had an additional PBP of 77 kDa (designated PBP 6*), while all isolates possessed a 52-kDa PBP (PBP 7) and a 46-kDa PBP (PBP 8). The presence or absence of PBP 6* did not correlate with penicillin susceptibility; however, PBP 7 demonstrated many features suggestive of low penicillin-binding affinity and may represent a possible mechanism for the relative resistance of this species to penicillin, although this hypothesis remains speculative since attempts to develop a penicillin-hypersusceptible *E. raffinosus* mutant were unsuccessful. *E. raffinosus* isolates were significantly more likely to exhibit high-level resistance to kanamycin than *E. avium* strains were ($P < 0.001$; chi-square); however, no strains demonstrated high-level resistance to gentamicin. No trend toward increasing penicillin resistance was noted among this collection of *E. avium* and *E. raffinosus* isolates collected over the past 35 and 14 years, respectively. Relative resistance to penicillin may be a helpful differentiating feature between *E. avium* and *E. raffinosus* when assessment of raffinose metabolism is not possible or is indeterminant.

Resistance to various antimicrobial agents is becoming increasingly widespread among clinical enterococcal isolates (3, 5, 6, 11–13, 15–17, 22, 23). Although *Enterococcus faecalis* and *E. faecium* account for the majority of clinical infections, other, less common species such as *E. avium* and *E. durans* are also known to cause significant disease (7, 14, 20, 23). In 1989 a new species, *E. raffinosus*, was distinguished from the phenotypically similar species *E. avium* by the ability of the former to metabolize raffinose (2, 7), a characteristic not recognized unless detailed biochemical examination is undertaken. A number of recent reports of infections caused by enterococci that are relatively resistant to beta-lactam antibiotics by non-β-lactamase mechanisms have included strains of *E. raffinosus* (19, 21). The potential importance of this newly designated species prompted us to review the collections of organisms in our laboratories which would have been classified previously as *E. avium* in order to identify other clinically useful features which may differentiate between *E. avium* and *E. raffinosus* isolates and to examine the penicillin-binding proteins (PBPs) of representative isolates of each.

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MATERIALS AND METHODS

Strains and identification. Our collection of 57 enterococcal isolates, which had previously been classified as *E. avium*-like, were reidentified by using the API Rapid Strep identification system (Analytab Products, Plainview, N.Y.). Fifty-two strains were previously considered to be *E. avium*, while five strains were identified as *E. raffinosus*, four in an earlier report (21) and one at the Centers for Disease Control, Atlanta, Ga. All strains were clinical isolates that were either collected by our laboratories or referred to us for assessment by other U.S. institutions. Isolates were obtained from a wide variety of clinical sources, including wound, biliary, and blood cultures, through 1990. When possible, the date of original isolation of each strain was noted. All isolates were plated on horse blood agar and examined for alpha- or beta-hemolysis and were identified on the basis of their biochemical profiles, with reactions being read at 4 and 24 h, as recommended by Analytab Products. Raffinose metabolism is identified by the API Rapid Strep identification system; however, Analytab Products does not currently report the species *E. raffinosus* but, instead, lists these raffinose-positive organisms as *E. avium*. The criteria recommended by Facklam and Collins (7) (including raffinose metabolism identified by the API Rapid Strep identifi-

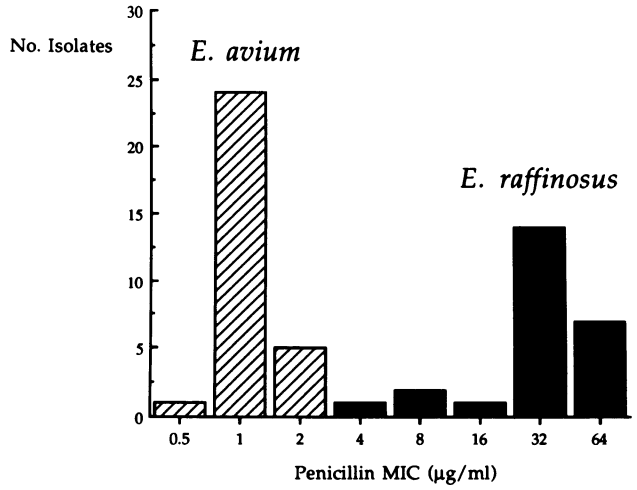

FIG. 1. Susceptibilities of *E. avium* (n = 30) and *E. raffinosus* (n = 25) strains to penicillin.

cation system) were used to distinguish *E. raffinosus* from *E. avium* and from other group 1 enterococci.

Assessment of penicillin and aminoglycoside susceptibilities. Antibiotic susceptibilities were determined by a standard agar dilution technique described by the National Committee for Clinical Laboratory Standards (18) by using Mueller-Hinton agar. Penicillin concentrations were tested in twofold increments from 0.125 to 256 μg/ml. Aminoglycosides (kanamycin and gentamicin) were tested in a similar manner, using concentrations from 0.5 to 4,000 μg/ml. Final inocula of 10^4 CFU per spot were applied to agar plates by using a multiprong inoculating device. Plates were incubated at 35°C and examined for evidence of growth after 18 h. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as controls. High-level resistance to streptomycin (MIC, >2,000 μg/ml) was tested by streaking isolates onto Mueller-Hinton agar containing 2,000 μg of streptomycin per ml, incubating them at 35°C, and examining the cultures for visible growth at 24 h. β-Lactamase production was tested by using nitrocefin disks (Cefinase; BBL Microbiology Systems, Cockeysville, Md.).

Antibiotics and reagents. [^{3}H]benzylpenicillin ethylpiperidinium salt (57.83 mCi/mg) was a generous gift from Merck Sharp & Dohme Research Laboratories, Rahway, N.J., while nonradioactive benzylpenicillin was a gift from Eli Lilly & Co., Indianapolis, Ind. The following aminoglycosides were used: kanamycin (SoloPak Laboratories, Franklin Park, Ill.), streptomycin (Eli Lilly & Co.), and gentamicin (Elkins-Sinn, Inc., Cherry Hill, N.J.). Protein molecular weight standards were purchased from Bio-Rad Laboratories, Rockville Center, N.Y.

Analysis of PBPs. Labeling of PBPs in whole cells was performed as described previously (25), with some modifications, as follows. Exponential-phase organisms were incubated with 100 μg of [^{3}H]benzylpenicillin per ml (2, 9, 24) at 37°C for 90 min prior to the termination of the reaction with excess unlabeled penicillin. In other experiments, binding was examined in samples taken after 15, 30, 45, 60, or 90 min of incubation. Competition studies were performed as described by Fontana et al. (8), with the following modifications. Whole cells were incubated with 0, 3.2, 32, 320, or 640 μg of nonradioactive penicillin per ml for 60 min before being washed, incubated with [^{3}H]benzylpenicillin for 90 min, and then processed. Deacylation rates were assessed by a method similar to that described previously (10), except that after standard incubation of strains with 100 μg of [^{3}H]benzylpenicillin per ml for 90 min, specimens were flooded with 6 mg of nonradioactive penicillin per ml and sampled after 0, 30, 60, 90, 120, and 150 min of further incubation at 37°C.

Polyacrylamide gel electrophoresis and detection of PBPs. PBPs were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis and fluorography as described previously (25, 26). Separating gels consisted of 8% acrylamide and 0.13% bisacrylamide. Exposure times for fluorographs were 3 to 6 days at −70°C. Fluorographs were scanned with an LKB Ultrascan XL laser densitometer (LKB, Bromma, Sweden), to assess PBP band absorbance, and this optical density was plotted graphically.

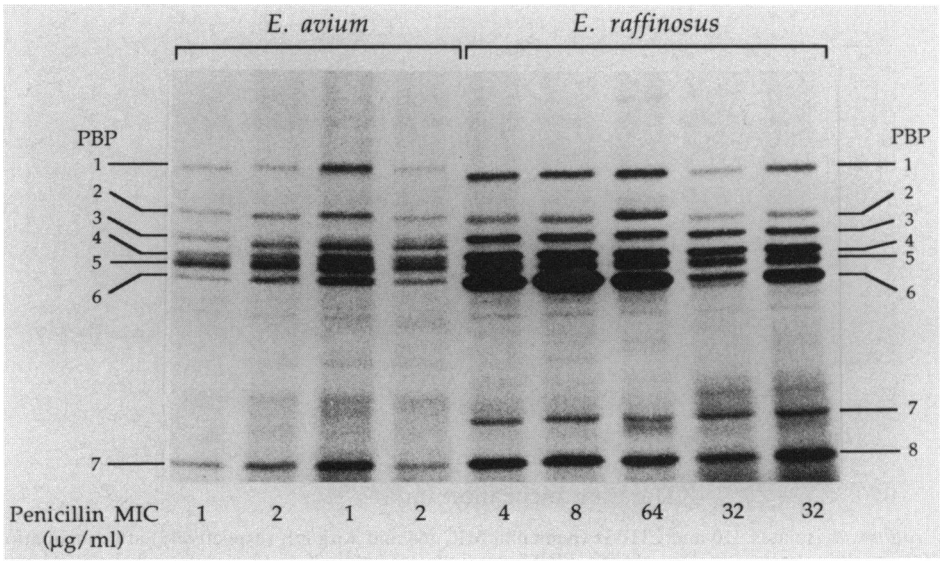

FIG. 2. PBPs of four *E. avium* strains and five *E. raffinosus* strains (8% acrylamide gel). Note PBP 7 (52 kDa) in all *E. raffinosus* isolates.

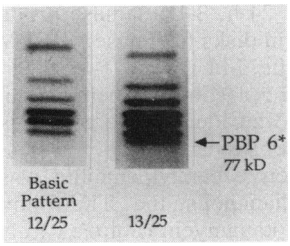

FIG. 3. PBP patterns (70- to 120-kDa range) of *E. raffinosus*. The basic pattern (2) was noted in 12 of 25 strains, while 13 strains had an additional 77-kDa PBP (PBP 6*). No correlation was noted between the presence or absence of PBP 6* and the susceptibility in *E. raffinosus* to penicillin.

RESULTS

All 57 strains examined in this study demonstrated alpha-hemolysis on horse blood agar and were categorized as *E. avium* by the API Rapid Strep identification system. Because 24 of these strains metabolized both arabinose and raffinose, we subsequently classified them as *E. raffinosus*. Thirty strains metabolized arabinose, but not raffinose, and were classified as true *E. avium*. One strain (2 BLD) repeatedly demonstrated variable raffinose metabolism and, thus, could not be definitively identified on the basis of biochemical criteria (see below). Two other strains (2992-2 and SF 188) did not metabolize either arabinose or raffinose, and hence, we classified them as *E. pseudoavium* (7) and excluded them from further analysis.

Penicillin susceptibility. *E. avium* and *E. raffinosus* isolates could be differentiated on the basis of their susceptibilities to penicillin (Fig. 1). The MICs of penicillin for all *E. avium* isolates were ≤2 μg/ml, with an MIC for 50% of isolates tested (MIC_{50}) of 1 μg/ml and an MIC_{90} of 2 μg/ml (MIC range, 0.5 to 2 μg/ml). The MICs of penicillin for all *E. raffinosus* isolates were ≥4 μg/ml, with an MIC_{50} of 32 μg/ml and an MIC_{90} of 64 μg/ml (MIC range, 4 to 64 μg/ml). The MICs of penicillin for the strain with an uncertain identification (2 BLD) was 32 μg/ml, and this strain was later identified as *E. raffinosus* on the basis of its pattern of PBPs. None of the 57 study strains produced detectable β-lactamase.

Analysis of PBPs. All *E. raffinosus* isolates and four representative strains of *E. avium* were examined for their patterns of PBPs to determine possible correlates of the identified differences in susceptibilities to penicillin. All four *E. avium* isolates had a PBP pattern identical to that described previously (24). *E. raffinosus* isolates had PBP patterns distinct from that of *E. avium* (Fig. 2). Although this pattern of *E. raffinosus* PBPs was similar to that reported earlier (2), a number of additional features not described previously were noted. The basic PBP pattern reported previously by Collins et al. (2) was seen in all 25 isolates (Fig. 3). In 13 isolates, however, an additional PBP of 77 kDa was noted (designated PBP 6*; Fig. 3) which did not appear to be especially low in penicillin-binding affinity since it was well visualized after 45 to 60 min of incubation. Furthermore, one isolate had both PBP 6* and an additional PBP of 102 kDa. No correlation could be identified between the presence or absence of these additional PBPs or the intensity of other PBPs and penicillin susceptibility.

In addition to these findings, two newly recognized PBPs of 52 and 46 kDa (designated PBP 7 and PBP 8, respectively) were a notable feature of *E. raffinosus*. Of particular interest was the 52-kDa PBP (PBP 7) which was found in all *E. raffinosus* but no *E. avium* isolates and which demonstrated features suggestive of low penicillin-binding affinity. Studies of various durations of incubation (four strains) demonstrated that [^{3}H]benzylpenicillin binding to PBP 7 began to appear only after 30 min of incubation, with optimal visualization of PBP 7 noted after 90 min of incubation (Fig. 4). Low penicillin-binding affinity was further suggested by competition studies. Blocking by nonradioactive penicillin of subsequent [^{3}H]benzylpenicillin-PBP binding was noted for all PBPs (particularly at a concentration 10× the MIC); however, PBP 7 was blocked to a far lesser extent and was therefore visibly labeled with [^{3}H]benzylpenicillin at this concentration (Fig. 5). To investigate the possibility that,

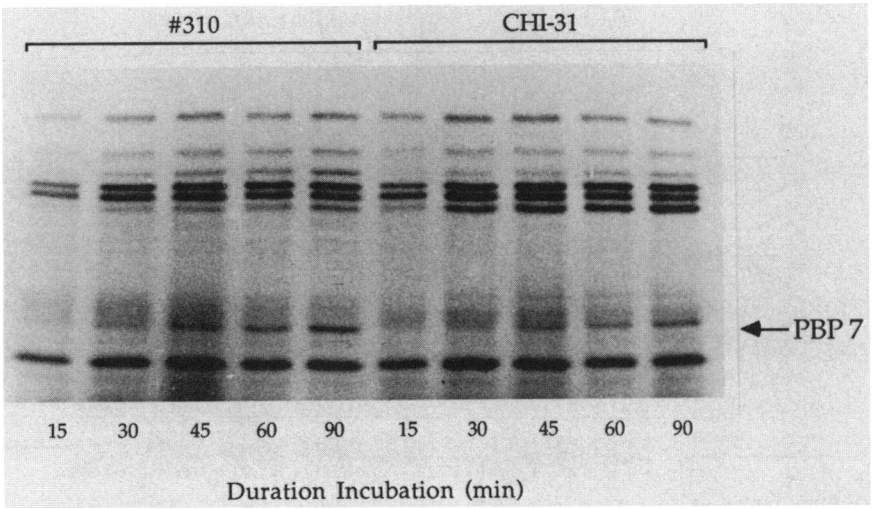

FIG. 4. PBPs of *E. raffinosus* isolates 310 and CHI-31 (penicillin MIC, 64 and 4 μg/ml, respectively) after incubation of whole cells with [^{3}H]benzylpenicillin (100 μg/ml) for various lengths of time. PBP 7 was first noted after 30 to 45 min of incubation and was optimally visualized after 90 min of incubation with [^{3}H]benzylpenicillin.

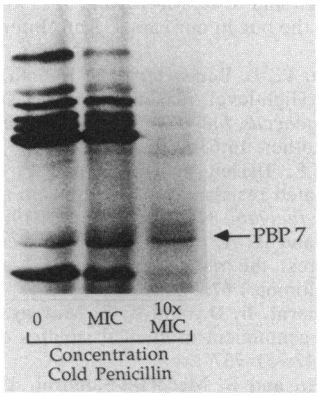

FIG. 5. PBPs of *E. raffinosus*: nonradioactive penicillin and [^{3}H] benzylpenicillin competition study (isolate CHI-31). Specimens were first incubated with variable concentrations (0×, 1×, and 10× the MIC) of nonradioactive penicillin for 60 min before being washed and incubated with 100 μg of [^{3}H]benzylpenicillin per ml for 90 min. Note the blocking of [^{3}H]benzylpenicillin binding to PBPs, with relative sparing of PBP 7.

rather than being related to low penicillin-binding affinity, the prominence of PBP 7 in these studies may have been due to a rapid rate of unlabeled penicillin deacylation in PBP 7 relative to that in other PBPs, we undertook deacylation studies in a number of isolates. These demonstrated only an 11 to 12% decrease in PBP 7–[^{3}H]benzylpenicillin binding after an additional 150 min of incubation. This represented a rate of decline similar to that noted for other PBPs in the same strain (Fig. 6). Attempts by a number of methods to develop an *E. raffinosus* mutant which was hypersusceptible to penicillin were unsuccessful. Thus, it was not possible to document further the potential role of PBP 7 (or other proteins) in the mediation of penicillin resistance.

Trends in antibiotic susceptibility. We assessed whether there was any trend among the species *E. avium* and *E. raffinosus* toward an increase in resistance to penicillin over

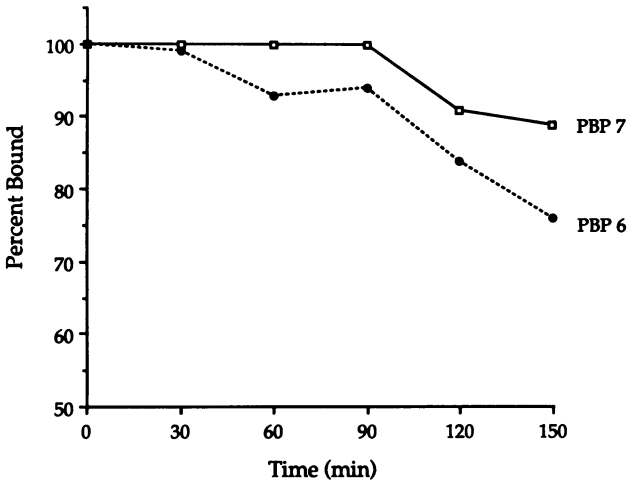

FIG. 6. *E. raffinosus* deacylation study (isolate CHI-31). Note only an 11% decrease in PBP 7–[^{3}H]benzylpenicillin binding after prolonged incubation with excess nonradioactive penicillin. This decrease was comparable to that noted for other PBPs of *E. raffinosus* (PBP 6 also shown for comparison).

TABLE 1. Aminoglycoside resistance among strains of *E. avium* and *E. raffinosus*

Species (no. of strains)	No. (%) of strains with high-level resistance[a] to:				
	Streptomycin alone	Kanamycin alone	Streptomycin and kanamycin	Gentamicin	None
E. avium (30)	0	0	3 (10)	0	27[b] (90)
E. raffinosus (25)	2 (8)	11 (44)	3 (12)	0	9[b] (36)

[a] Aminoglycoside MIC, >2,000 μg/ml.
[b] Significant difference, $P \leq 0.001$ (chi-square).

recent years by comparing the original date of isolation of study strains with their susceptibilities to penicillin. *E. avium* strains, which were isolated from nine sources over the 35-year period since 1955, demonstrated no change in penicillin susceptibility over this period. Similarly, strains of *E. raffinosus*, all of which were isolated during the 14 years since 1976 (seven sources), demonstrated no apparent trend toward increasing penicillin resistance over recent years.

The prevalence of high-level aminoglycoside resistance (MIC, >2,000 μg/ml) among strains of *E. avium* and *E. raffinosus* are shown in Table 1. *E. raffinosus* was significantly more likely to demonstrate such resistance than *E. avium* was ($P \leq 0.001$; chi-square). No high-level resistance to gentamicin was noted in either species.

DISCUSSION

Of the 57 strains examined in this study, all *E. raffinosus* isolates could be distinguished from the *E. avium* isolates on the basis of higher levels of resistance to penicillin. However, because some strains of the former species were inhibited by penicillin at concentrations which were only one dilution above the greatest MIC for *E. avium*, it seems probable that occasional isolates of either species might be difficult to classify unequivocally on the basis of their levels of susceptibility to penicillin alone. Despite recent reports suggesting increasing resistance to beta-lactam antibiotics among some clinical enterococcal isolates (19, 21), we were unable to identify any recent trend toward increasing resistance to penicillin among this collection of 25 strains of *E. raffinosus* isolated during the past 14 years. Whether there has been a recent increase in the rate of isolation of this species, however, cannot be answered from the results of this study. It is noteworthy that, in parallel with higher levels of resistance to penicillin, high-level resistance to kanamycin was also found more commonly in *E. raffinosus*. Of the seven strains for which the penicillin MIC was ≥64 μg/ml, six had high-level resistance to kanamycin only. The higher percentage of *E. raffinosus* strains with high-level resistance to kanamycin but not streptomycin is also of interest, in contrast to the case with *E. faecalis*, in which single high-level aminoglycoside resistance is almost always to streptomycin only (1, 4). While the PBP patterns of the *E. raffinosus* isolates studied included all bands described previously (2), additional PBP bands were observed in our isolates. A 77-kDa PBP (PBP 6*) was detected in 13 of 25 isolates, but its presence or absence did not correlate with the level of susceptibility to penicillin, nor did the relative intensities of other PBP bands provide such a correlation. The use of 8% acrylamide gels permitted the identification in all *E. raffinosus* strains of low-molecular-weight PBPs not described earlier (2). PBP 7 (52 kDa) demonstrated characteristics of a low-affinity PBP, which may play a role in the relative

penicillin resistance of this species. Unfortunately, because we were unable to derive a penicillin-hypersusceptible mutant strain, the precise role of this protein remains speculative.

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