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Vancomycin Susceptibility and Identification of Motile Enterococci

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Thirty-seven clinical isolates of *Enterococcus gallinarum* and *Enterococcus casseliflavus* and three type or reference strains of the species were studied with respect to vancomycin susceptibility and key identification characteristics. With the exception of one clinical isolate of *E. casseliflavus* (MIC, 4 μg/ml), MICs of vancomycin were 8 to 32 μg/ml. The type strain of *E. gallinarum*, NCDO 2313, and five of the clinical isolates had similar penicillin-binding protein profiles and shared 90 to 100% DNA homology. Two isolates, identified as *E. gallinarum* by conventional tests, were shown to be non-pigment-producing *E. casseliflavus* on the basis of penicillin-binding protein profile and DNA homology. The type and reference strains of *E. casseliflavus*, ATCC 25788 and ATCC 25789, were nonmotile in our experiments. However, both shared 65 to 100% DNA homology with each other and with five clinical isolates of *E. casseliflavus*. These data suggest that the MICs of vancomycin observed for strains of *E. gallinarum* and *E. casseliflavus* are higher than those usually associated with other enterococci and may be a common property of these species. Additionally, pigment production and motility may occasionally be misleading criteria for definitive identification of these organisms.

The first report of vancomycin-resistant *Enterococcus gallinarum* was published in 1988 (8). Recent data from the Centers for Disease Control suggest that *E. gallinarum* is less susceptible to vancomycin than other enterococci (11). Including the type strains, we have examined 23 isolates of *E. gallinarum* and 17 isolates of *Enterococcus casseliflavus*, all but one of which were more resistant to vancomycin than other enterococci, with MICs of vancomycin ranging from 8 to 32 μg/ml.

Bacterial strains. *E. gallinarum* strains used were NCDO 2313^T (2, 4); ATCC 35038 (said to be the same as NCDO 2313); three former clinical isolates, UCLA I, UCLA II, and SC I; 11 additional isolates from liver transplant patients in Pittsburgh, Pa. (LT strains); and 9 isolates (G strains) from patients at the University of Chicago. The three former clinical isolates have been described previously (12) and were isolated from an intra-abdominal abcess, bile, and blood at the University of California at Los Angeles and the Medical University of South Carolina, respectively. We also obtained ATCC 35038, said to be a copy of *E. gallinarum* NCDO 2313, from the American Type Culture Collection, Rockville, Md. *E. casseliflavus* ATCC 25788 and 25789 were also obtained from the American Type Culture Collection. *E. casseliflavus* from the University of Chicago Medical Center (C strains) and from Pittsburgh (LT strains) as well as the type strains used and the corresponding MICs are described below. The Pittsburgh isolates have also been previously described (5). The Chicago isolates were isolated from patient specimens submitted to the University of Chicago Clinical Microbiology Laboratories. *Enterococcus faecium* D61 (penicillin susceptible) (13), CDC 3-74 (9), *Enterococcus durans* ATCC 19432 (9), and *Enterococcus faecalis* JH2-2 (6) have been previously described. *Enterococcus raffinosus* 785B and *E. faecium* 1006D (both penicillin resistant) were recently described (1).

Characterization of strains. The enterococci were characterized by the methods of Facklam and Collins (3). The 30°C motility test was the best method for distinguishing *E. gallinarum* and *E. casseliflavus* from *E. faecium* and *E. faecalis*. *E. casseliflavus* was distinguished from *E. gallinarum* on the basis of the production of a yellow pigment (3). Strains LT31 and G13 were classified as *E. gallinarum* because they were motile but nonpigmented. Both ATCC strains of *E. casseliflavus* were nonmotile in our experiments.

Vancomycin susceptibilities were determined by diluting the antibiotics through agar, as recommended by the National Committee for Clinical Laboratory Standards, except that brain heart infusion agar replaced Mueller-Hinton agar (10). Teicoplanin susceptibilities were determined on the basis of either agar dilution MICs, as described above, or macrobroth dilution, as recommended by the National Committee for Clinical Laboratory Standards (10). The results are as follows. Vancomycin MICs for *E. gallinarum* ATCC 35038 and NCDO 2313 were 2 and 16 mg/liter, respectively; the MICs for 22 other *E. gallinarum* strains ranged from 8 to 32 mg/liter. MICs for *E. casseliflavus* ATCC 25788, ATCC 25789, and C7 were 16, 16, and 4 mg/liter, respectively. MICs for 14 other strains ranged from 8 to 16 mg/liter. Of the *E. gallinarum* strains, only ATCC 35038 was susceptible to vancomycin, whereas its presumed copy, NCDO 2313, was more resistant. All *E. casseliflavus* strains but one (C7) (MIC, 4 μg/ml) had vancomycin MICs ranging from 8 to 32 μg/ml. All strains were susceptible to ≤0.5 mg of teicoplanin per liter (data not shown). To further confirm our identifications, we used penicillin-binding protein (PBP) profiling and DNA homology.

PBP profiles. Realizing that PBP profiles correlated well with species identification in the genus *Enterococcus* (13), we examined the PBP profiles of some of our enterococcal strains essentially by the method of Williamson et al. (13). Overnight cultures were diluted to obtain an optical density at 650 nm of 0.25. One milliliter was withdrawn and centrifuged (16,000 × *g* for 5 min at room temperature). The pellet was suspended in 50 μl of ice-cold 50 mM phosphate buffer,

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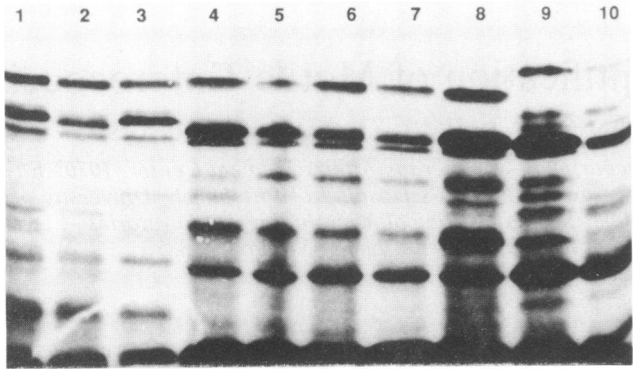

FIG. 1. PBP profiles of enterococcal strains. Lane 1, *E. casseliflavus* LT52; lane 2, *E. casseliflavus* LT62; lane 3, *E. casseliflavus* LT31; lane 4, *E. gallinarum* LT40; lane 5, *E. gallinarum* UCLA I; lane 6, *E. gallinarum* UCLA II; lane 7, *E. gallinarum* SC I; lane 8, *E. gallinarum* NCDO 2313; lane 9, *E. faecium* D359; lane 10, *E. faecium* D61.

TABLE 1. Homology of enterococcal DNA

Source of unlabelled DNA	% Homology with labelled DNA from[a]:	
	UCLA I	ATCC 25788
E. gallinarum		
NCDO 2313	100.0	ND
UCLA I	100.0	9.0
UCLA II	95.5	ND
SC I	92.5	ND
LT40	111.8	ND
G9	92.8	12
E. casseliflavus		
ATCC 25788	ND	100.0
ATCC 25789	ND	100.0
LT52	19.5	92.0
LT62	20.4	80.0
LT31	21.5	66.0
C7	15	74
G13	19	65
E. faecium CDC 3-74	16.0	ND
E. durans ATCC 19432	7.0	ND

[a] Values represent the average of two determinations. ND, not done.

pH 7.0, containing 0.5 μg of [^{3}H]benzylpenicillin per ml (specific activity, 26 mCi/mg; kindly provided by Merck, Sharpe & Dohme, Rahway, N.J.) and incubated at 37°C for 30 min to completely saturate the PBPs. The samples were placed in ice, and 500 μl of unlabelled benzylpenicillin (Sigma Chemical Co., St. Louis, Mo.) (4 mg/ml) was added. After recentrifugation, the pellets were resuspended in 20 μl of ice-cold phosphate buffer containing lysozyme (500 μg/ml) and mutanolysin (500 μg/ml) in 0.1% Triton X-100 (all from Sigma Chemical Co.). The samples were incubated at 37°C until complete lysis was observed visually. After the addition of 5 μl of a mixture of sodium dodecyl sulfate, Coomassie blue, and β-mercaptoethanol, the samples were boiled for 2 min and then kept at −20°C. The PBPs were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% acrylamide and 0.08% *N,N'*-bisacrylamide) followed by fluorography by the method of Williamson et al. (13), except that the films were not prefogged. The results are shown in Fig. 1. The *E. gallinarum* strain NCDO 2313 had a profile similar to those of the three vancomycin-resistant clinical isolates. These profiles were also clearly distinct from that of *E. faecalis* (data not shown), *E. faecium* (penicillin-susceptible [D61] and -resistant [D359] strains), and *E. raffinosus* (data not shown). LT31 and G13 (data for G13 not shown), identified as *E. gallinarum* on the basis of motility and lack of pigment, had a PBP profile resembling those of *E. casseliflavus* LT52 and LT62. *E. casseliflavus* ATCC 25788 and ATCC 25789 had PBP patterns resembling those of LT52 and LT62 as well (data not shown). These results suggested that LT31 and G13 might be nonpigmented strains of *E. casseliflavus*.

DNA homology of *E. gallinarum* and *E. casseliflavus*. DNA was extracted from *E. gallinarum* and *E. casseliflavus* by methods previously described (9). Labeling with [^{3}H]thymidine triphosphate was conducted by nick translation (9). DNA homologies were determined by using S1 nuclease in solution under optimal conditions for hybridization (9). Under our conditions, 50% homology is considered to be consistent with identity at the species level. The results are presented in Table 1. With labelled DNA from one of the *E. gallinarum* clinical isolates (UCLA I), there was 93 to 100% homology with *E. gallinarum* NCDO 2313 and all the clinical isolates identified by conventional methods as *E. gallinarum* except LT31 and G13. DNAs from none of the *E. gallinarum* strains were homologous (0 to 11%) to labelled DNA from ATCC 35038 (data not shown). On the other hand, the type strain of *E. casseliflavus*, ATCC 25788, was 65 to 100% homologous to all *E. casseliflavus* strains, including LT31 and G13.

One of the early reports of enterococcal vancomycin resistance involved *E. gallinarum* (8). In a study recently performed at the Centers for Disease Control (11), the investigators noted that six *E. gallinarum* strains were more resistant to vancomycin than other enterococcal species. Our vancomycin MICs are consistently one dilution tube higher than those reported by Swenson et al., possibly because they used broth dilution with Mueller-Hinton medium and we used agar dilution through brain heart infusion medium. In general, our data are consistent with their observations and further suggest that decreased susceptibility to vancomycin is also a property of the other motile species, *E. casseliflavus*. We have also shown that ATCC 35038, which is susceptible to vancomycin (MIC, 2 μg/ml) and which is said to be a copy of NCDO 2313, is not *E. gallinarum* and should not be used as a reference strain by investigators until the problem is resolved. On the basis of the PBP and physiological profiles, we believe the strain to be *Enterococcus faecalis*. We have communicated our results to the American Type Culture Collection, and they have withdrawn ATCC 35038 from circulation.

According to Facklam and Collins (3), the primary difference between *E. casseliflavus* and *E. gallinarum* is the production of yellow pigment by the former species. However, pigment production may not be a stable property, as is illustrated by strains LT31 and G13. PBP profiles and DNA homology results for these nonpigmented, motile, vancomycin-resistant strains demonstrated that they were *E. casseliflavus*. Similarly, the *E. casseliflavus* ATCC strains were nonmotile but showed DNA homology and PBP profiles consistent with those of the motile clinical isolates to which they were compared. These data indicated that pigment production and motility may not always be reliable traits on which to base identification of *E. gallinarum* and *E. casseliflavus*. Better criteria may be established as more strains

are collected and studied. Because MICs of vancomycin for these species are generally higher than those for other enterococci, a scheme for their definitive identification in clinical microbiology laboratories is needed.

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