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Correlation between Phenotypic Characteristics and DNA Relatedness within *Enterococcus faecium* Strains

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We noted that a number of enterococcal strains isolated from human clinical specimens resembled *Enterococcus faecium* but were able to produce acid from glycerol, raffinose, and/or sorbitol, while others failed to form acid from mannitol. An additional concern was that many of these strains with atypical phenotypic characteristics also appeared to acquire vancomycin resistance. In order to determine if such atypical strains were variants of *E. faecium* or new *Enterococcus* species, 35 *E. faecium* or *E. faecium*-like strains (grouped into 10 phenotypes on the basis of the results of the following tests: capacity to form acid from glycerol, mannitol, raffinose, or sorbitol and susceptibility to vancomycin) and four strains of *Enterococcus faecalis* were taken from our culture collection, analyzed for their whole-cell protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and identified to the species level by DNA-DNA reassociation experiments. All *E. faecium*-like strains, including four mannitol-negative variants, conformed to at least two of three DNA-DNA relatedness criteria: they were 70% or more related to the type strain of *E. faecium* at optimal conditions, they had less than 5% divergence within the related sequences, and they had a relatedness of 60% or greater under stringent conditions. The protein profiles of atypical strains were similar to those of typical strains and were easily distinguishable from those of *E. faecalis* and other enterococcal species. The five *E. faecalis* strains were 12 to 16% related to the *E. faecium* type strain. These results indicate that the phenotypic description of *E. faecium* should include all of these variable characteristics.

The enterococcal species were included in the genus *Streptococcus* until 1984, when Schleifer and Kilpper-Balz (12) suggested that a new genus be established. At that time only two species (*Streptococcus faecalis* and *Streptococcus faecium*) were transferred to the new genus *Enterococcus*. Later in 1984, five other previously identified *Streptococcus* species were transferred to the *Enterococcus* genus as *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. malodoratus* (3). Ten additional species have subsequently been either newly described or transferred to the genus *Enterococcus* (4). The complexity of the genus has increased with each additional species, and differentiation of some of the species has been problematic because of the overlap of phenotypic characteristics. The precise differentiation of enterococcal species has taken on additional importance because of the acquisition of resistance traits among strains, especially resistance to glycopeptides. Four phenotypes of glycopeptide resistance have been reported among enterococci: VanA phenotype, with inducible high-level resistance to vancomycin as well as teicoplanin; VanB phenotype, with variable levels of inducible resistance to vancomycin only; and VanC and VanC-like phenotypes, with noninducible low-level resistance to vancomycin (2). The VanA and VanB phenotypes are usually associated with *E. faecium* and *E. faecalis* strains, while the VanC and VanC-like phenotypes are seen in *E. gallinarum* and *E. casseliflavus* strains, respectively. Thus, precise identification of the vancomycin-resistant strains to the species level is very important in the management of patients with enterococcal infections. We have noted that some of the vancomycin-resistant strains resembled *E. faecium*, but these strains possessed one or more atypical phenotypic characteristics.

The aim of the study described here was to determine if the vancomycin-resistant and -susceptible strains with atypical physiological characteristics were variants of *E. faecium* or new *Enterococcus* species.

MATERIALS AND METHODS

Bacterial strains. A total of 39 strains were studied, including 35 *E. faecium* or *E. faecium*-like strains and 4 strains of *E. faecalis*. The strains were taken from the culture collection of the Streptococcus Laboratory, Centers for Disease Control and Prevention. The type strains for *E. faecium*, ATCC 19434, and *E. faecalis*, ATCC 19433, were obtained from the American Type Culture Collection.

Characterization of strains. Strains were tested for their phenotypic characteristics by conventional biochemical tests as described previously (5, 7). The disk diffusion screening test was used to determine the susceptibility or resistance to vancomycin as recommended by Facklam et al. (6). Serogrouping was performed by the Lancefield hot-acid extraction procedure and capillary precipitin tests with anti-group D sera prepared at the Centers for Disease Control and Prevention (8). Strains were also tested with the AccuProbe *Enterococcus* culture confirmation test (Gen-Probe, Inc., San Diego, Calif.).

Analysis of whole-cell protein profiles by SDS-PAGE. Preparation of samples and analysis of whole-cell protein profiles by conventional one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were performed as described by Merquior et al. (10), except that the strains used for protein extraction were grown on brain heart infusion blood agar instead of Columbia blood agar.

DNA reassociation studies. The strains used for DNA extraction were grown in 2 liters of Todd-Hewitt broth at 37°C for 18 to 20 h with gentle shaking. Cells were harvested by centrifugation and were suspended in 50 ml of TS buffer (50 mM Tris buffer [pH 8.0] containing 12.5% sucrose) plus 20 ml of lysozyme solution (10 mg/ml) and 30 ml of 50 mM EDTA solution. After incubation for 30 min at 37°C, 330 μl of proteinase K solution (25 mg/ml) was added, and the suspension was incubated for 30 min at 37°C. Lysis was completed by the addition of 10 ml of 20% SDS solution. The procedures to purify DNA for the determination of DNA relatedness in free solution by the hydroxyapatite hybridization method have been described previously (1). The DNAs were labeled enzymatically in vitro with [^{32}P]dCTP by using a nick translation reagent kit (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) as directed by the

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TABLE 1. Phenotypes (biotypes) of *E. faecium* strains

Biotype	Physiological test result					Strains included in each biotype
	Acid formation in[a]:				Susceptibility to vancomycin[b]	
	Man	Gly	Raf	Sor		
1	+	−	−	−	S	SS-442, 387-89, 451-89, 300-91, ATCC-19434
2	+	−	−	−	R	2648-90, 126-91, 2401-91
3	+	−	+	−	S	2337-80, 2180-82, 591-83, 427-84, 383-89, 401-91
4	+	−	+	−	R	1085-90, 1938-90, 2058-90
5	+	−	+	+	S	SS-960, 631-90, 632-90, E-45-88, E-81-88
6	+	−	+	+	R	2167-90, 2539-90, 2504-91, 2527-91
7	+	+	+	+	S	1639-93
8	+	+	+	+	R	1193-91, 1148-92, 1308-92
9	+	+	−	−	R	1739-89
10	−	−	−	−	S	954-82, 1120-83, 1308-89, 1697-92

[a] Man, mannitol; Gly, glycerol; Raf, raffinose; Sor, sorbitol.
[b] S, susceptible; R, resistant.

manufacturer. DNA hybridization experiments were performed at 55°C for optimal DNA reassociation. Some of the strains, including all of those with the lowest relative binding ratios (RBRs) under the optimal conditions, were also tested at the stringent DNA reassociation temperature of 70°C. Levels of divergence (percent *D*) within related sequences were determined by assuming that each degree of heteroduplex instability, compared with the melting temperature of the homologous duplex, was caused by approximately 1% of unpaired bases. Percent *D* was calculated to the nearest 0.5%.

RESULTS

All enterococcal strains used in the study had the following characteristics: they were gram-positive cocci, were catalase negative and bile esculin positive, produced leucine aminopeptidase and L-pyrrolidonylarylaminidase, did not produce gas from *Lactobacillus* Man, Sharp, Rogosa broth, grew in broth containing 6.5% NaCl, and grew at 10 and 45°C. All strains were positive with the AccuProbe *Enterococcus* genetic probe.

On the basis of the diversity of the five physiological characteristics listed in Table 1, 10 phenotypes were identified among the *E. faecium* or *E. faecium*-like strains. All but four of these strains were arginine and mannitol positive (see exceptions below); sorbose, pyruvate, and tellurite negative; formed acid from arabinose; and were nonmotile and nonpigmented which presumptively identified them as *E. faecium* or *E. faecium*-like. The four mannitol-negative variant strains included in the study were also negative for sucrose, while all other strains produced acid from sucrose. Three of these four strains were isolated from blood and one was isolated from gallbladder.

The results of DNA-DNA hybridization studies, shown in Table 2, indicate that *E. faecium* and *E. faecium*-like strains of all 10 phenotypes belong to the same species. Except for three strains, all strains were 71% or more related to the type strain of *E. faecium* at optimal reassociation conditions (55°C), were 62% or more related under stringent conditions (70°C), and had a *D* of 4% or less in the related sequences. The three strains giving RBRs under 70% (59, 66, and 67%) at 55°C were found to have RBRs of 73, 70, and 70% at 70°C and *D* of 0.5, 2.5, and 1.0%, respectively. Conversely, the four *E. faecalis* strains were 12 to 16% related to the *E. faecium* type strain.

Resistance to vancomycin had no effect on the extent of DNA relatedness among strains. RBRs ranged from a low of 70% to a high of 98% for vancomycin-resistant strains and from 62 to 99% for vancomycin-susceptible strains.

Results of the analysis of whole-cell protein profiles (represented in Fig. 1) correlated with those of DNA relatedness. Apart from quantitative differences, all *E. faecium* or *E. faecium*-like strains had similar protein profiles, which were easily distinguishable from those of *E. faecalis* and the other enterococcal species.

DISCUSSION

E. faecium is the second most frequent enterococcal species isolated from human infections (5, 9, 11, 14), and it is recognized as one of the most physiologically heterogeneous species among the enterococci. Several special antimicrobial resistance traits, including high-level resistance to aminoglycosides, penicillin, and, more recently, vancomycin, have increasingly been associated with *E. faecium* strains (9). The emergence of multiple antimicrobial agent-resistant *E. faecium* is so remarkable that the microorganism has been considered the "nosocomial pathogen of the 1990s" (13).

Until recently strains that are now recognized as members of new enterococcal species were identified as *E. faecium*. Therefore, the available descriptions of this species are sometimes conflicting or unclear, especially in relation to physiological tests involving carbohydrate fermentation. On the other hand, a number of enterococcal strains isolated from human clinical specimens resemble *E. faecium* but do not exhibit the biochemical characteristics of the majority of the strains belonging to this species. Typical strains of *E. faecium* produce acid from arabinose and mannitol and fail to produce acid from glycerol, raffinose, and sorbitol (5). *E. faecalis* strains typically produce acid from glycerol, mannitol, and sorbitol but not from arabinose. Upon acquiring resistance to vancomycin, some *E. faecium* strains also appeared to acquire the capacity to produce acid from glycerol, raffinose, and/or sorbitol. This apparent overlapping of phenotypic characteristics caused concern about the proper identification of the species.

In the widely used conventional test scheme for the identification of enterococcal species, proposed by Facklam and Collins (5) and recently revised (7), a positive reaction for mannitol is considered one of the key tests for inclusion in enterococcal group II. This group is composed of some of the most common species, including *E. faecium*. Therefore, the finding of mannitol-negative strains that otherwise fit the characteristics of *E. faecium* or that fit the characteristics of related species that are differentiated according to the results of one single key test leads to confusion, since it raises questions as to which physiological characteristics should be considered most definitive of a given species and should be used to rule out other possible identifications. Additionally, all mannitol-nega-

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TABLE 2. Relationship between phenotypic characteristics and relative reassociation DNA binding within *E. faecium*

Source of unlabeled DNA	Phenotype	Labeled DNA from *E. faecium* ATCC 19434		
		RBR at 55°C	*D* (%)[a]	RBR at 70°C
E. faecium				
SS-442	1	97	1.0	ND[b]
387-79		99	0.0	ND
451-89		97	0.0	ND
300-91		67	1.0	70
2648-90	2	95	0.5	ND
126-91		72	0.5	75
2401-91		81	0.0	ND
2337-80	3	85	0.0	ND
2180-82		71	3.5	64
591-83		71	4.0	70
427-84		71	3.5	64
383-89		96	0.0	93
401-91		72	4.0	62
1805-90	4	89	0.5	93
1938-90		83	0.5	ND
2058-90		59	0.5	73
SS-960	5	99	0.0	94
631-90		93	0.0	ND
632-90		94	0.5	ND
E-45-88		86	1.0	ND
E-81-88		91	0.5	ND
2167-90	6	81	0.5	86
2539-90		91	0.5	ND
2504-91		88	1.0	ND
2527-91		85	0.5	86
1639-93	7	93	0.0	ND
1193-91	8	66	2.5	70
1148-92		89	0.0	ND
1308-92		89	0.0	86
1739-89	9	98	1.0	ND
954-82	10	86	2.0	79
1120-83		85	2.0	80
1308-89		87	1.0	ND
1697-92		83	3.0	79
E. faecalis				
ATCC 19433	NA[c]	13	ND	ND
V 583	NA	14	ND	ND
A 256	NA	12	ND	ND
SS-499	NA	16	ND	ND

[a] Calculated to the nearest 0.5%.
[b] ND, not determined.
[c] NA, not applicable.

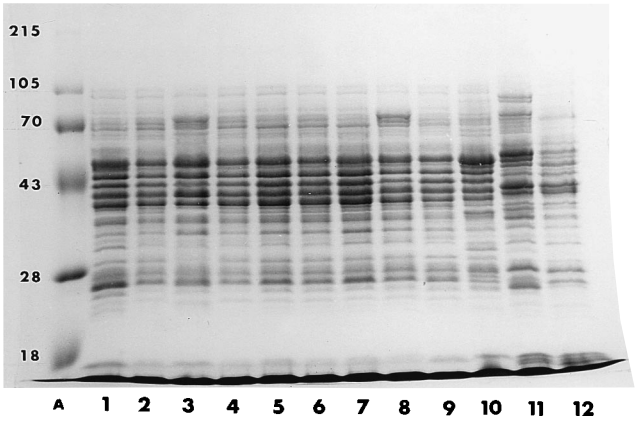

FIG. 1. SDS-PAGE patterns of whole-cell protein extracts of representative strains used in the study. Lanes: A, molecular ratio standards; 1, ATCC 19434, *E. faecium* biotype 1; 2, 126-91, *E. faecium* biotype 2; 3, 427-84, *E. faecium* biotype 3; 4, 2058-90, *E. faecium* biotype 4; 5, 632-90, *E. faecium* biotype 5; 6, 2167-90, *E. faecium* biotype 6; 7, 1639-93, *E. faecium* biotype 7; 8, 1193-91, *E. faecium* biotype 8; 9, 1739-89, *E. faecium* biotype 9; 10, 1697-92, *E. faecium* biotype 10; 11, ATCC 19433, *E. faecalis*; 12, A 256, *E. faecalis*. Numbers on the left are molecular masses (in kilodaltons).

tive strains analyzed in the present study were sucrose negative, which is another unusual characteristic among *E. faecium* strains and may also contribute to misidentification. Therefore, if the results of the arabinose test are considered variable, strains with such a physiological profile would be virtually indistinguishable from *E. durans*. To circumvent this problem, we recommend that results of arabinose tests be considered without exceptions. We have not yet found or know of the description of arabinose-negative variants of *E. faecium* or arabinose-positive variants of *E. durans*. According to our observations, at this point, the results of arabinose tests, in association with other tests like those for mannitol, sorbitol, and arginine, are the most reliable means of differentiating between variant strains of *E. faecium*, *E. durans*, and other physiologically related species isolated from human sources.

We have recently shown that the analysis of whole-cell protein profiles is a reliable tool for the differentiation and identification of enterococcal species (10). However, only typical strains have been tested to date. The agreement between results of DNA relatedness experiments and SDS-PAGE protein profiles obtained in the present study indicates that protein profiles can be used to accurately characterize physiologically atypical strains of *E. faecium*, including mannitol-negative variants.

The results of the present study demonstrate that the *E. faecium* strains examined, although physiologically diverse, constitute a taxon, according to the homogeneity of the whole-cell protein profiles and levels of DNA relatedness. Some strains can even acquire the capacity to ferment carbohydrates usually associated with *E. faecalis*, such as glycerol and sorbitol. All of them, however, conformed to at least two of the three DNA-DNA relatedness criteria that should be considered essential for inclusion in a species: relatedness of 70% or greater under optimal conditions, divergence of less than 5% within related sequences, and relatedness of 60% or greater under stringent conditions (1, 15).

On the basis of these results, the phenotypic description of *E. faecium* should include all of these variable characteristics. The inclusion of mannitol-negative strains within *E. faecium* will necessitate a revision in the species identification scheme for the enterococci. Facklam and Collin's (5) test scheme for the identification of group III enterococcal species should be modified to include asaccharolytic variants of *E. faecium* (because they were also negative for sucrose, glycerol, sorbitol, and raffinose). We also recommend that the arabinose test be added to the key reactions for group III (sucrose, raffinose, and pyruvate) in order to distinguish *E. faecium* from *E. durans* and other related species. *E. faecium* variants should be positive,

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while *E. durans* as well as other species included in group III should be negative.

The results of the present study will broaden the description and allow for a more accurate identification of isolates belonging to an enterococcal species with a remarkable capacity to acquire different mechanisms and patterns of resistance to antimicrobial agents. Information generated by a more precise identification may contribute to improvement in patient care, especially considering that the phenotypic patterns of some of the variants seem to be closely related to certain antimicrobial resistance markers, such as resistance to vancomycin. It is possible that some of the phenotypic markers may prove to be useful as clues to help in the detection of resistant strains and to allow investigators to follow the transmission of resistant strains.

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