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Analysis of Electrophoretic Whole-Cell Protein Profiles as a Tool for Characterization of *Enterococcus* Species

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Abstract. The whole-cell protein-profiling technique was evaluated for identifying *Enterococcus* species. Reference strains, strains from human infections, from animals other than human, and from environmental sources were studied. Whole-cell extracts were obtained by lysozyme treatment and were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and densitometry. Each *Enterococcus* species had a unique and distinguishable whole-cell protein profile. The major differences among species-specific profiles were found in the positions corresponding to 60–40 and 30–20 kDa. Profiles of the same species did not show qualitative variations. Analysis of whole-cell protein profiles was shown to be a relatively simple, easy, and reproducible procedure for the reliable and fast differentiation and identification of the enterococcal species.

The *Enterococcus* genus was officially recognized as a separate genus (split from the *Streptococcus*) in 1984 [28]. Since that time additional species have been added to the genus by recognition of new species and the transfer of species formerly considered as streptococci [2–5, 7, 14, 18, 21, 27, 31]. At this time there are 18 species included in the *Enterococcus* genus. The procedures used to identify the species of enterococci are based on determining physiologic characteristics of the strains with conventional test-tube tests or with chromogenic substrate tests [12, 17]. In several cases only one phenotypic characteristic differentiates one species from another. In addition, some enterococcal strains do not possess the exact phenotypic characteristics of the type strains, and there remains a question of their exact taxonomic status. It is also difficult in some cases to identify some of the species because they do not possess all the characteristics of enterococci. *Enterococcus cecorum, E. columbae,* and *E. saccharolyticus* [7, 27, 31] fail to grow satisfactorily in 6.5% NaCl broth and are pyrrolidonylarylamidase negative, two phenotypic characteristics that all other enterococci have.

Minor differences in physiologic characteristics have also been used to trace strains of *E. faecalis* [20] and *E. faecium* [6]. This procedure is time consuming, costly, and probably unreliable because some of the reactions have test-to-test variation. Although other subtyping systems including serologic, bacteriocin, and phage typing have been proposed to identify differences among enterococcal strains, none of these systems have met with acceptance for epidemiologic studies [25]. These systems are either difficult to reproduce or the reagents are not available for general use.

On the other hand, enterococci are becoming more and more resistant to the commonly used antimicrobials. Some strains have acquired vancomycin resistance, β-lactamase production, and high-level aminoglycoside and β-lactams resistance [24, 25]. Thus the need for reliable tools to accurately identify the species and study the epidemiologic transmission of enterococci in human infections is apparent.

Polyacrylamide gel electrophoresis (PAGE) of whole-cell polypeptides solubilized by treatment with sodium dodecyl sulfate (SDS) has been used to identify and type bacteria [15, 16]. This technique allows the comparative study of large numbers of proteins encoded by a significant portion of the genome and, therefore, has a very high potential for measuring relationships among isolates [10, 11, 15, 29, 30].

We have applied the whole-cell protein profile procedure to enterococcal species, including most of

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the newly recognized ones, to evaluate the discriminatory power of this technique for their identification.

Materials and Methods

Strains. The 16 type strains of *Enterococcus* species used in this study were taken from the collection of the Respiratory Diseases Bacterial Reference Laboratory, Centers for Disease Control and Prevention (CDC). Thirty-eight clinical isolates were selected from the CDC and Universidade Federal do Rio de Janeiro collections. The sources and species identification are listed in Table 1.

Identification. All strains were identified by conventional test methods [12].

Whole-cell protein preparation. Enterococcal strains were grown on Columbia agar (Difco Laboratories, Detroit, Michigan) containing 5% sheep blood for 24 h at 37°C. The samples were prepared by removing the bacterial growth from the surface of each agar plate with a sterile wood spatula-like applicator and suspending the cells in 0.5 ml of a 10 mg/ml aqueous solution of lysozyme (Sigma Chemical Co., St. Louis, Missouri). The suspensions were incubated in a 37°C water bath for 2 h. Whole-cell extracts were obtained by mixing one part of the sample to one part of 0.5 M Tris-hydrochloride, pH 6.8, containing SDS 4% w/v, glycerol 20% v/v, 2-mercaptoethanol 10% v/v, and bromophenol blue 0.001% wt/vol and boiling for 5 min.

SDS–PAGE. Discontinuous SDS–PAGE was performed by a modification of the procedure described by Laemmli [19]. Soluble samples were applied to wells in a 4% acrylamide stacking gel [0.125 M Tris-hydrochloride (pH 6.8)] over a 10% acrylamide separating gel [0.375 M Tris-hydrochloride (pH 8.8)]. The electrophoresis buffer was 25 mM Tris-hydrochloride-0.192 M glycine (pH 8.3). The final concentration of SDS was 0.1% in both gels and in the electrode buffer.

Electrophoresis was performed in a Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, California) at a constant current of 20 mA until the bromophenol blue marker had reached the bottom. Whole-cell polypeptide gels were stained with Coomassie brilliant blue R-250 0.125% wt/vol in methanol:acetic acid:water (5:1:4 vol/vol) and destained in the same solvent mixture before being swollen to their original sizes in acetic acid 7% vol/vol. Molecular weight marker polypeptides (Prestained SDS–PAGE Standards–Low Range, Bio-Rad) were run in the same gels to allow the estimation of molecular weights. Visual comparisons of band patterns were made on the wet gels with transillumination. Densitometry tracings were performed on the wet gels with an LKB Ultrascan Laser Densitometer.

Analysis. The average similarity between any two strains of enterococcal polypeptide profiles represented by stained gels was assessed by the Dice coefficient [8]. The average percentage of similarity equals the number of matching bands times two, divided by the total number of bands in both isolates. The calculation of similarity was based on visual comparisons and from peak position data obtained by densitometry.

Results

There was complete correlation between the whole-cell protein profiles and results of conventional biochemical tests identification, since each physiologi-

Table 1. Strains analyzed in this study

Identification number	*Enterococci* species	Source
SS-559	*E. avium*	Type strain
1290-76	*E. avium*	CSF
2499-81	*E. avium*	Blood
SS-1225	*E. durans*	Type strain
CL-572	*E. durans*	Respiratory tract
486-81	*E. durans*	Endometrium
154-82	*E. durans*	Blood
1999-89	*E. malodoratus*	Type strain
SS-1227	*E. hirae*	Type strain
CL-261	*E. hirae*	Animal
CL-262	*E. hirae*	Animal
CL-263	*E. hirae*	Animal
CL-275	*E. hirae*	Animal
CL-292	*E. hirae*	Animal
607-68	*E. hirae*	Animal
SS-1228	*E. gallinarum*	Type strain
CL-236	*E. gallinarum*	Abscess
CL-500	*E. gallinarum*	Ascitic fluid
CL-568	*E. gallinarum*	Ascitic fluid
1606-76	*E. gallinarum*	Wound
1966-78	*E. gallinarum*	CSF
SS-1229	*E. casseliflavus*	Type strain
CL-259	*E. casseliflavus*	Blood
CL-260	*E. casseliflavus*	Animal
CL-265	*E. casseliflavus*	Animal
SS-1232	*E. mundtii*	Type strain
CL-460	*E. mundtii*	Blood
SS-1273	*E. faecalis*	Type strain
CL-231	*E. faecalis*	Wound
CL-244	*E. faecalis*	Endocarditis
CL-333	*E. faecalis*	Abscess
CL-497	*E. faecalis*	Meningitis
CL-278	*E. faecalis*	Animal
CL-282	*E. faecalis*	Animal
CL-474	*E. faecalis*	Environment
CL-477	*E. faecalis*	Environment
SS-1274	*E. faecium*	Type strain
CL-350	*E. faecium*	Urine
CL-376	*E. faecium*	CSF
CL-377	*E. faecium*	Ascitic fluid
CL-276	*E. faecium*	Animal
CL-313	*E. faecium*	Environment
SS-1277	*E. pseudoavium*	Type strain
SS-1278	*E. raffinosus*	Type strain
CL-523	*E. raffinosus*	Blood
CL-585	*E. raffinosus*	Unknown
1097-86	*E. raffinosus*	Teeth
1157-84	*E. raffinosus*	Urine
1167-85	*E. raffinosus*	Urine
SS-1295	*E. dispar*	Type strain
SS-1296	*E. cecorum*	Type strain
SS-1297	*E. saccharolyticus*	Type strain
SS-1310	*E. columbae*	Type strain
SS-1314	*E. sulfureus*	Type strain

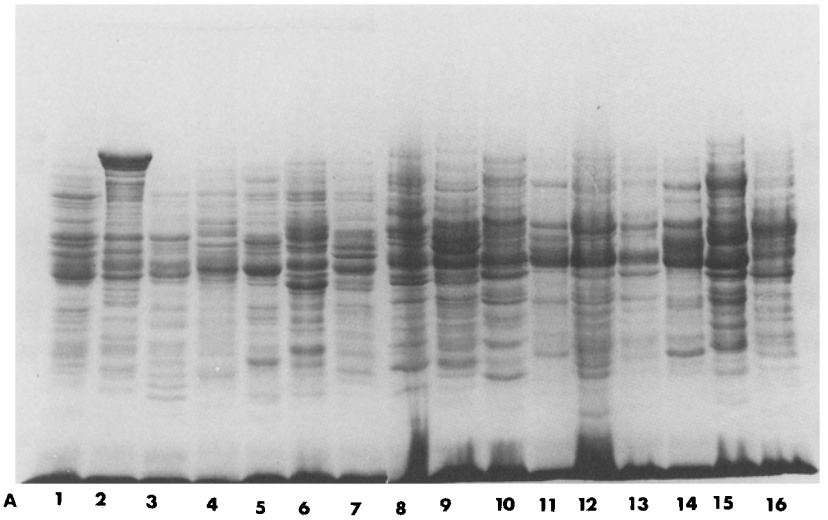

Fig. 1. SDS–PAGE patterns of whole-cell protein extracts of *Enterococcus* species. Lanes A, molecular ratio standards; **1.** SS-1314 *E. sulfureus*; **2.** SS-1310 *E. columbae*; **3.** SS-1297 *E. saccharolyticus*; **4.** SS-1296 *E. cecorum*; **5.** SS-1295 *E. dispar*; **6.** SS-1278 *E. raffinosus*; **7.** SS-1277 *E. pseudoavium*; **8.** 1999-89 *E. malodoratus*; **9.** SS-1274 *E. faecium*; **10.** SS-1273 *E. faecalis*; **11.** SS-1232 *E. mundtii*; **12.** SS-1229 *E. casseliflavus*; **13.** SS-1228 *E. gallinarum*; **14.** SS-1227 *E. hirae*; **15.** SS-1225 *E. durans*; **16.** SS-559 *E. avium*.

cally defined species corresponded to a distinct banding pattern. The SDS–PAGE of whole-cell extracts of the 16 type strains of *Enterococcus* species are shown in Fig. 1. The corresponding densitometer tracings were different and easily distinguishable from each other. Each species had a unique banding pattern, and each profile had 30–35 visually detectable bands. The major differences between species were identified in the 60–40 kDa and 20–30 kDa regions.

Different strains of the same species had similar protein profiles, as shown in Fig. 2. Minor quantitative differences were observed among profiles of the same species, but no qualitative differences were found. The species-specific protein profiles were the same regardless of the geographic region, isolation sites, or sources of the strains. Analysis of duplicate protein extracts obtained from cells grown independently under identical conditions showed identical banding patterns.

Estimation of the Dice coefficients of similarity confirmed the distinctive nature of the species-specific profiles. Average percentage of similarity values ranged from 13.3% to 74.6% (Table 2), indicating low levels of similarity among isolates of different species. The highest level of similarity was between *E. hirae* and *E. durans* (74.6%). The lowest was between *E. faecalis* and *E. avium*(13.3%).

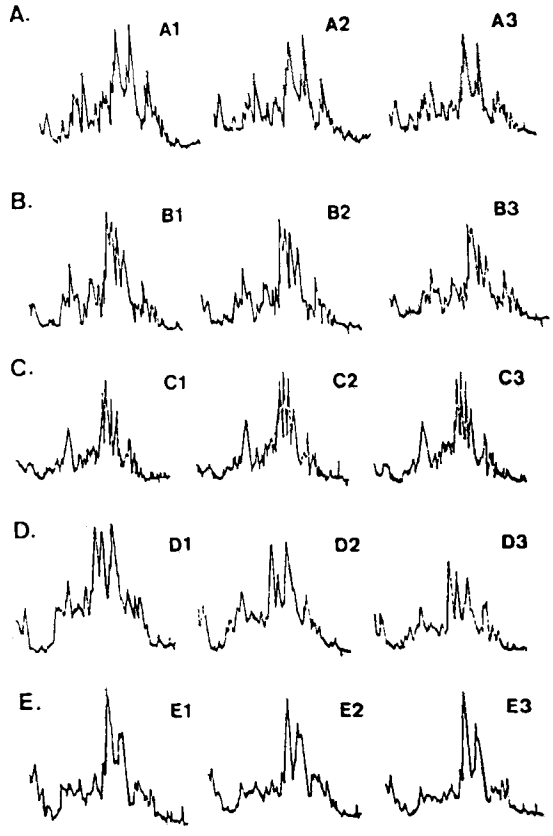

Fig. 2. Densitometer tracings showing similarity of whole-cell protein profiles among strains belonging to the same enterococcal species. **A.** *E. faecalis*, strains CL-231 (A1), CL-246 (A2), and CL-278 (A3); **B.** *E. faecium*, strains CL-276 (B1), CL-313 (B2), and CL-350 (B3); **C.** *E. hirae*, strains CL-261 (C1), CL-262 (C2), and CL-263 (C3); **D.** *E. raffinosus*, strains CL-523 (D1), CL-585 (D2), and 1154-84 (D3); **E.** *E. casseliflavus*, strains CL-259 (E1), CL-269 (E2), and CL-265 (E3).

Discussion

Analysis of whole-cell protein profiles was shown to be a relatively simple and reproducible procedure for differentiation of *Enterococcus* species. Dice coeffi-

Table 2. Average percentage of similarity (Dice coefficients) of enterococcal strains shown in Fig. 1

Strains compared	SS-559	SS-1225	1999-89	SS-1227	SS-1228	SS-1229	SS-1232	SS-1273	SS-1274	SS-1277	SS-1278	SS-1295	SS-1296	SS-1297	SS-1310	SS-1314
SS-559		47.8	58.8	45.7	49.2	52.7	36.9	13.3	30.7	51.5	64.8	36.1	28.1	40.0	36.6	38.9
SS-1225			46.1	74.6	58.1	49.3	58.1	47.2	67.7	44.4	52.9	46.4	26.2	50.8	23.5	37.7
1999-89				41.2	54.0	58.0	44.4	38.3	72.7	71.9	60.9	45.7	29.0	41.1	34.7	37.1
SS-1227					58.5	52.8	64.6	48.0	70.8	38.1	50.7	36.1	46.9	41.7	36.6	44.4
SS-1228						62.7	53.3	28.6	53.3	49.2	51.5	38.8	30.5	33.8	27.3	47.8
SS-1229							68.6	31.2	47.7	47.1	52.1	45.9	27.3	41.7	35.6	37.8
SS-1232								45.7	56.6	42.6	48.5	56.7	50.9	40.0	36.4	41.8
SS-1273									42.8	42.2	28.9	44.2	51.9	57.9	57.9	50.6
SS-1274										68.8	30.3	53.7	47.4	30.8	36.4	38.8
SS-1277											47.8	41.2	34.0	33.3	32.3	41.2
SS-1278												46.6	36.9	45.1	38.9	32.9
SS-1295													39.9	44.4	32.9	40.5
SS-1296														50.0	55.9	39.4
SS-1297															42.2	52.8
SS-1310																41.1
SS-1314																

cients of the densitometry tracing of the gels removed the subjectivity of the procedure and confirmed the visual observations. This procedure will be potentially useful in assisting in the identification of strains that do not have phenotypic characteristics identical to the type strains of each species. All the clinical strains tested in this study had physiologic characteristics identical to the type strains. Moreover, the reproducibility of the SDS–PAGE technique for enterococci characterization was confirmed by the analysis of duplicate protein extracts: cells grown independently had similar banding patterns. Preliminary studies from our laboratory have also shown that the profiles were unaffected by growth in and on different commonly used culture media (data not shown). It will be necessary to test strains that have atypical characteristics but are still considered to be a member of the species (sorbitol-positive *E. faecium*, arginine-negative *E. faecalis*, etc.). Strains that belong to the *Enterococcus* genus but cannot be identified by physiologic characteristics will also have to be tested. The results of these tests will support efforts to place atypical strains into the proper taxonomic identification and to help define limits of phenotypic characterization.

Although protein profiles also represent phenotypic characteristics, they are considered to provide an excellent approximation of a microorganism's genome information, being rather simpler and faster to obtain than analysis of genotypic characteristics. Hook *et al.* [15] reported protein profiles as having a high degree of correlation with DNA RFLP analysis, 5S RNA base sequence analysis, and DNA-DNA hybridization for several bacterial species. Our data generated from SDS-PAGE profiles of enterococci also indicate that certain species are more closely or distantly related to each other. For example, *E. faecalis*, *E. saccharoliticus*, *E. cecorum*, *E. columbiae*, and *E. sulfureus* were found to be more distantly related to most of the other enterococcal species, in agreement with information obtained from analysis of genetic characteristics [21]. On the other hand, analysis of whole-cell protein profiles and densitometry tracings did not allow for differentiation of enterococci at subspecific level. Perhaps complementary analysis with other techniques such as Western blot will be more definitive and applicable for strain-to-strain differentiation for epidemiological studies [22]. For this purpose, analysis of genomic DNA has also been successfully used in recent years for typing enterococci, especially *E. faecalis* and *E. faecium* [9, 23, 26].

One major advantage of the whole-cell protein profile analysis over conventional physiologic tests is that, once the bacteria are isolated and identified to the genus level, protein extracts can be prepared and SDS–PAGE results determined in one day. In contrast, the physiologic tests need to be incubated for a minimum of 7 days. At the current time the rapid identification procedures, such as API 20S, RapidStrep, and RapID Strep systems are accurate only for *E. faecalis* [1, 13, 32]. Identification of all other species requires additional conventional tests.

In conclusion, our results show that analysis of soluble whole-cell proteins can be used to discriminate the most common species of *Enterococcus* isolates from human and nonhuman sources. It provides an additional tool for the characterization and for distinguishing between species that are usually hard to differentiate by physiologic tests. Application of this procedure as a routine method for enterococcal characterization requires standardization of refer-

ence banding patterns. In addition, a data bank of reference protein profiles could be constructed with which the protein profile of any unknown isolate could be compared.

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