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Evaluation of Arbitrarily Primed PCR Analysis and Pulsed-Field Gel Electrophoresis of Large Genomic DNA Fragments for Identification of Enterococci Important in Human Medicine

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The increasing problems encountered with enterococcal nosocomial infections and the intrinsic and acquired resistance of the enterococci to different antimicrobial compounds highlight the need for a rapid identification technique. *Enterococcus faecalis* is readily identified by biochemical tests, but species differentiation within the *Enterococcus faecium* and *Enterococcus gallinarum* species groups is less well established. In the present study, 66 strains representing the most prevalent human enterococci were used to develop a PCR-based species-specific identification protocol. Whole-cell protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used as a reference method for species identification. In addition, the genomic *Sma*I macro-restriction fragment distribution of all of the strains was examined by pulsed-field gel electrophoresis (PFGE). Oligonucleotide D11344-primed PCR was as discriminative as whole-cell protein analysis and resulted in more easily interpreted band patterns. This PCR-based technique allowed identification of clinical isolates by visual examination of the DNA profiles obtained. The inability of both methods to discriminate between *Enterococcus casseliflavus* and *Enterococcus flavescens* brought into question the species status of *E. flavescens*. PFGE did not result in species-discriminative DNA bands or band patterns, but proved to be superior for interpretation of interstrain relationships.

The incidence of nosocomial enterococcal infections has risen to an alarming extent (12). Ample examples of enterococcal endocarditis, bacteremia, urinary tract infection, and neonatal sepsis have been reported (33). *Enterococcus faecium* and *Enterococcus faecalis* are part of the intestinal and fecal flora of humans and animals (7–9, 25), yet they are notorious organisms in human and veterinary medicine for their inherent and acquired resistance to multiple antibiotics (2, 17).

Because of naturally occurring differences in susceptibility, in some cases species level identification is very important in determining the appropriate antibiotic therapy (12, 25), while strain-specific characterization of the bacteria has great value for epidemiologic surveillance (25). However, the phenotypic schemes currently used to identify enterococcal species fail to distinguish several species, especially species in the *Enterococcus gallinarum* and *E. faecium* species groups (6). Alternative identification protocols based on penicillin-binding protein profiles (45), bacteriolytic pattern analysis (29), and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of whole-cell proteins (35) have been developed. The development of new methods involving various DNA-based typing techniques has opened new perspectives for identification of clinical isolates on both the strain level and the species level. In the present study we evaluated two of these DNA-based typing techniques, namely, PCR-based DNA typing in which random or repetitive sequences are used as primers and pulsed-field gel electrophoresis (PFGE) of genomic macro-restriction fragments. Both of these identification methods have been used primarily for epidemiological surveillance of clinical isolates (37, 39, 40), but they appeared to be useful for species level differentiation in a number of genera (11, 14, 15). Since there is a correlation between DNA-DNA hybridization values and whole-cell protein pattern similarity levels (30), the SDS-PAGE technique was used as a reference method for enterococcal species level identification.

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MATERIALS AND METHODS

Collection of strains. A total of 66 strains representing *Enterococcus casseliflavus*, *Enterococcus durans*, *E. faecalis*, *E. faecium*, *Enterococcus flavescens*, *E. gallinarum*, and *Enterococcus hirae* were included in all analyses (Table 1). Reference strains and field isolates were selected so that most enterococcal species were equally represented; the only exception was *E. flavescens*, for which only four strains were available. The field isolates originated from a range of different sources and were isolated over a long period of time. They were kindly supplied by L. A. Devriese (Faculty of Veterinary Medicine, University of Ghent, Ghent, Belgium) and B. Gordts (Laboratory of Clinical Microbiology, Hospital AZ St.-Jan Brugge, Bruges, Belgium) or were our own isolates (University Hospital of Antwerp, Antwerp, Belgium). All field isolates were preliminarily identified in different laboratories by classical biochemical tests (13).

Identification of strains by SDS-PAGE. Strains were grown for 24 h at 37°C on De Man-Rogosa-Sharpe agar (Difco Laboratories, Detroit, Mich.) and were incubated in a microaerobic atmosphere containing 5% O_2, 10% CO_2, and 85% N_2. PAGE of whole-cell proteins, densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis were performed as described by Pot et al. (30). Strains were identified by using a database comprising protein patterns of more than 700 enterococcal strains representing all currently described species (16, 32, 42).

PFGE. All cultures were grown on Columbia agar (GIBCO, Life Technologies, Paisley, Scotland) supplemented with 5% defibrinated horse blood and were incubated at 37°C in the presence of 5% CO_2. One loopful of cells from a culture that had been grown overnight was washed three times in 1 ml of EET buffer (100 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl [pH 8.0]; all of the reagents used were obtained from Sigma Chemical Co. unless otherwise noted). The cells were pelleted and then were resuspended in EC buffer (6 mM Tris-HCl [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% Brij 58, 0.2% deoxycholate, 0.5% *N*-lauroylsarcosine), adjusted to a density of 4×10^9 CFU/ml by turbidimetry, mixed with an equal volume of 1.6% (wt/vol) low-melting-point preparative grade agarose (Bio-Rad Laboratories, Nazareth, Belgium) in EC buffer, and pipetted into a plug mold. The solidified plugs were incubated overnight at 37°C in 0.7 ml of EC lysis buffer (2.88 mg of lysozyme per ml of EC buffer). The lysis buffer was replaced by 1 ml of protein digestion buffer (3.3 mg of pronase E per ml of EET buffer containing 1.6% [wt/vol] SDS), and the preparation was incubated overnight at 37°C. Before restriction digestion, the agarose plugs were washed four times for 1 h in EET buffer, twice for 1 h in $T_{10}E_{0.1}$ buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]), and once for 1 h in the appropriate restriction buffer at room temperature. Macro-restriction of the genomic DNA was carried out overnight at 25°C in 250 μl of fresh restriction buffer containing 25 U

TABLE 1. Enterococcal strains used and their origins

Species	Strain[a]	Designation as received[a,b]	Source of isolation	Year of isolation
E. casseliflavus	LMG 10745^T	ATCC 25788^T	Plant material	
	LMG 12306	SPE 12b	Avian intestine	1992
	LMG 12307	LSC 6	Avian intestine	1992
	LMG 12309	A 22	Avian intestine	1992
	LMG 12310	RS 129a	Bovine intestine	1992
	LMG 12311	S 289	Equine intestine	1992
	LMG 12314	Kat 32	Feline anus	1992
	LMG 12901	NCFB 2310	Plant material (grass silage)	
	LMG 14406	PF6	Equine manure	1993
	LAB 938	14/6	Human feces	1993
E. durans	LMG 10746^T	ATCC 19432^T	Dried milk	
	LMG 12283	SP 2	Avian intestine	1992
	LMG 12285	SP 225	Avian intestine	1992
	LMG 12691^T	CCM 5612^T	Dried milk	
	LMG 12903	NCFB 496		
	LMG 13604t2	A 96	Avian intestine	
	LMG 14197	27382		1993
	LMG 14202	GBg22	Fish (herring)	1993
	LMG 14374	Esc 12	Bovine	1993
	LMG 14763	Sc. 123im		Before 1950
	LMG 16172	V580C	Equine feces	
E. faecalis	LMG 7937^T	DSM 20478^T		
	LMG 9475[c]	DSM 20452	Rodent intestine (rat)	
	LMG 11395	DSM 20371	Human pleural fluid	
	LMG 11636A	12C/S3		Before 1950
	LMG 11734	STR 302	Dog	
	LMG 13596	Ton 609	Pig	
	LMG 14392	266/6974	Japanese nightingale intestine	
	LMG 14409	PF 8	Equine manure	
	LMG 14818t1	STR 761	Parakeet	
	LMG 15073	AL 22	Bovine rumen (deer)	
	LMG 16304	PF 47	Equine manure	
E. faecium	LMG 8148	CCUG 14	Human	1968
	LMG 8149^T	CCUG 542^T		Before 1946
	LMG 9430	ATCC 21053	Sour milk	
	LMG 11422	NCFB 1632		
	LMG 16164	V 201	Equine feces	
	UZA 1-20	1-20	Human leg wound	1993
	UZA 1-9	1-9	Human blood	1993
	UZA 3-26	3-26	Human bile	1993
	UZA 6-8	6-8	Human anus	1993
	LAB 1265	10-14	Human feces	
E. flavescens	LMG 13518^T	CCM 4239^T	Human blood	1992
	LMG 13597t2	GBf12	Plant material (pepper)	
	LMG 16313	CCUG 30568	Human abscess	1992
	LMG 16314	CCUG 30569	Human osteomyelitis	1992
E. gallinarum	LMG 12313	A 5	Avian intestine	1992
	LMG 12904	NCFB 1618		
	LMG 13129^T	ATCC 49573^T	Avian intestine	1978
	LMG 14041	MCV-3	Human	
	LAB 931	UZA 4/1	Human pus, rectum	1993
	LAB 932	UZA 9/4	Human abdominal wound	1993
	LAB 933	UZA 15/4	Human appendix	1993
	LAB 1259	66V	Human feces	
	LAB 1261	9V	Human feces	
	LAB 1262	90V	Human feces	
E. hirae	LMG 6399^T	ATCC 8043^T		
	LMG 11425	NCFB 1648		
	LMG 11492	NCFB 2693		
	LMG 11493	NCFB 2708		1993
	LMG 14200	EVF/87/152	Avian	1993
	LMG 14201	2CTI	Avian	1993

Continued on following page

TABLE 1—*Continued*

Species	Strain[a]	Designation as received[a,b]	Source of isolation	Year of isolation
	LMG 14260	Esc 110T		1994
	LMG 14488	PF 12	Equine feces	1994
	LMG 14489	94/290	Avian (parrot spleen)	
	LAB 937	UZA 16/3	Human postoperative wound	1993

[a] LMG, Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; LAB, Lactic Acid Bacteria Culture Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; UZA, Universitair Ziekenhuis Antwerpen, Culture Collection of the University Hospital Antwerp, Antwerp, Belgium.

[b] Culture collection strains were obtained from the following international culture collections: ATCC, American Type Culture Collection, Rockville, Md.; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Göteborg, Sweden; NCFB, National Collection of Food Bacteria; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. Strains without culture collection designations are our own isolates.

[c] Originally received as the type strain of *Lactobacillus murinus.*

of *Sma*I (MBI Fermentas, St. Leon-Rot, Germany). The digestion reaction was stopped by adding 0.5 ml of 0.5 M EDTA (pH 8.0), and the plugs were stored at 4°C. The chromosomal restriction fragments were separated by PFGE in a contour-clamped homogeneous electric field MAPPER system (Bio-Rad Laboratories) by loading pieces of the plugs (2 by 5 by 0.5 mm) into the wells of a 1% (wt/vol) Pulsed Field Certified Agarose (Bio-Rad Laboratories) gel that was prepared in 0.5× TBE (45 mM Tris-HCl, 45 mM boric acid, 1 mM EDTA) and sealed with the same agarose. Electrophoresis was performed in a level electrophoresis chamber containing 2 liters of 0.5× TBE equilibrated at 14°C at a constant voltage of 6 V/cm, with pulse times ramping linearly from 5 to 35 s for 24 h.

The *Sma*I-digested genome of *Staphylococcus aureus* NCTC 8325 (37) was used both as a molecular size standard and as an intrinsic gel normalization standard. This standard was prepared by the procedures described above except that lysozyme was replaced by lysostaphin (100 μg/ml of EC buffer).

Typically, each pulsed-field gel consisted of 15 DNA patterns, 3 of which were the normalization standard. The DNA band profiles were stained with ethidium bromide, visualized, and digitized by the Gel Doc 1000 documentation system (Bio-Rad Laboratories). Conversion, normalization, and further analysis of the patterns were performed by using the GelCompar software, version 3.1b (Applied Maths, Kortrijk, Belgium) as described previously (30, 43). The levels of similarity between different PFGE patterns were calculated by using the Dice coefficient (10), and correlation coefficients were calculated by the unweighted pair group method with arithmetic averages.

PCR-based typing. DNA was prepared by using the rapid procedure described by Pitcher et al. (27). Two repetitive primers (ERIC 1R and ERIC 2) (44) and three random primers (D14307, D11344, and D8635) (1) were evaluated for their suitability to differentiate enterococci. DNA amplification was performed with a DNA thermal cycler (model 9600 GeneAmp PCR system; Perkin-Elmer, Zaventem, Belgium). Each 100-μl PCR mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 0.2 mM deoxynucleoside triphosphates, 50 pmol of primer ERIC 1R or ERIC 2 or 100 pmol of primer D14307, D11344, or D8635, 0.6 U of Goldstar polymerase (Eurogentec, Seraing, Belgium), and 100 ng of extracted DNA. When primer ERIC 1R or ERIC 2 was used, 40 cycles were performed, each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 25°C, and 2 min of elongation at 74°C. With primers D14307, D11344, and D8635 the amplification process was as follows: an initial four cycles consisting of denaturation at 94°C for 5 min, annealing at 40°C for 5 min, and elongation at 72°C for 5 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min, followed by one cycle consisting of 10 min at 72°C.

After amplification, 25 μl of the amplicon was mixed with 7 μl of loading buffer (50% glycerol, 0.8 mg of bromophenol blue per ml) and electrophoresed in a 1.5% pronarose D1 gel (Sphaero Q, Burgos, Spain) for 3 h at 100 V in 0.5× TBE containing 0.05 mg of ethidium bromide per liter.

The normalization standard, which was applied three times to each gel (the gels contained a maximum of 16 lanes), was the amplification product of *E. faecium* LAB 1265 obtained with primer D14307. Image acquisition and computer analysis were performed as described above for PFGE data acquisition. The levels of similarity between different PCR band patterns were calculated by using the Pearson product moment correlation coefficient (r) (34).

RESULTS

SDS-PAGE. Multiple strains were cultured several times to check the reproducibility of both the protein extraction procedure and the electrophoresis technique. The average r value for duplicate electropherograms was more than 0.95. The r value for reproducibility of the normalization standard was more than 0.95.

After a numerical analysis of the 66 protein electropherograms was performed, six distinct clusters were delineated, as shown in Fig. 1. The reference strains of most of the species grouped in separate clusters; the only exceptions were the *E. casseliflavus* and *E. flavescens* strains, which belonged to the same cluster. Most of the field isolates, which originally were identified by biochemical tests (data not shown), grouped in the appropriate clusters; the only exceptions were isolates LMG 12306, LMG 12307, and LMG 12314, which originally were identified as *E. mundtii*, *E. mundtii*, and *E. gallinarum*, respectively, which grouped in the *E. casseliflavus-E. flavescens* cluster.

Visual inspection of the electropherograms (data not shown) revealed almost identical electrophoretic protein profiles within each cluster, including the *E. casseliflavus-E. flavescens* cluster. The lowest r value between different clusters was more than 0.75.

PCR-based typing. The overall r value for reproducibility of the PCR assay and the running conditions, as estimated by an analysis of duplicate DNA extracts of several strains, was greater than 0.92.

Purified DNAs of all strains were examined in five PCR analyses, and in each analysis a single primer was used. A numerical analysis of the DNA band patterns obtained with each primer was performed. Figure 1 shows the dendrogram obtained with primer D11344 (5′-AGTGAATTCGCGGTGAGATGCCA-3′). The reference strains and field isolates of *E. durans*, *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. hirae* grouped in well-separated clusters, while the *E. casseliflavus* and *E. flavescens* strains formed a single cluster. The differences in the band patterns of the different clusters were substantial (Fig. 2). Visual inspection of the DNA band patterns within each of the clusters revealed small differences between most strains. The level of similarity (r value) between different clusters was always less than 0.40 (Fig. 1).

When primer D14307 (5′-GGTTGGGTGAGAATTGCACG-3′) was used, only the *E. gallinarum* strains were unequivocally identified at the species level by the presence of one unique amplimer (data not shown). Strains of the other species were not identified at the species level. Primers D8635, ERIC 1R, and ERIC 2 generated very diverse DNA band patterns for each of the species and proved to be useful for strain discrimination but not for species differentiation.

PFGE. The reproducibility between different restriction digests and electrophoretic runs for the same genome was 100%, as determined by the Dice coefficient (data not shown).

In general, *Sma*I produced 13 to 23 distinct genomic DNA fragments in all of the strains studied. Cluster analysis and visual inspection of the restriction profiles obtained revealed that the following strains had identical patterns: LMG 16313,

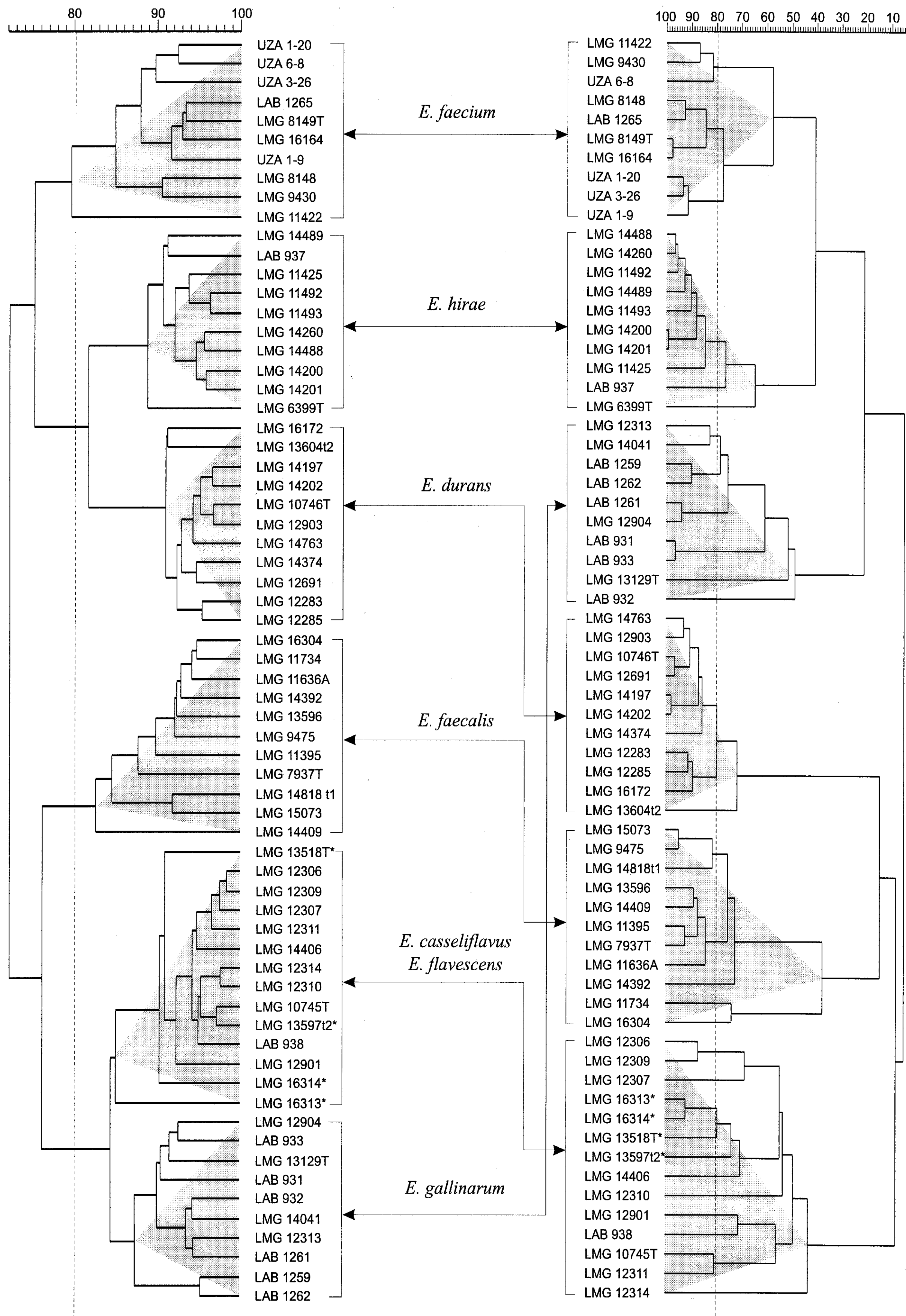

FIG. 1. Dendrograms derived from unweighted pair group average linkage of *r* values for protein electropherograms (left side) and D11344-primed PCR patterns (right side) of 66 enterococcal strains. *E. flavescens* strains are indicated by asterisks. The vertical dotted line indicates the 80% similarity level for comparative purposes.

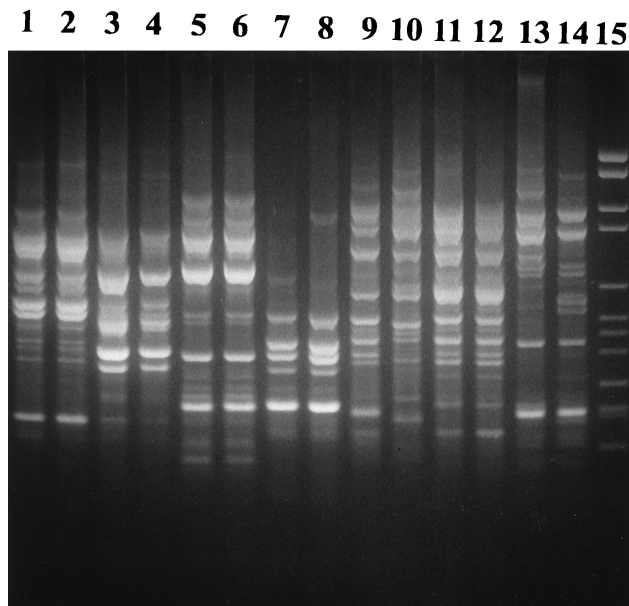

FIG. 2. DNA amplification patterns obtained with primer D11344 for *E. durans* LMG 10746^T (lane 1) and LMG 12691^T (lane 2), *E. hirae* LMG 14201 (lane 3) and LMG 14200 (lane 4), *E. faecium* LMG 8149^T (lane 5) and LMG 16164 (lane 6), *E. gallinarum* LAB 933 (lane 7) and LAB 931 (lane 8), *E. casseliflavus* LAB 938 (lane 9) and LMG 12309 (lane 10), *E. flavescens* LMG 16314 (lane 11) and LMG 16313 (lane 12), and *E. faecalis* LMG 11395 (lane 13) and LMG 7937^T (lane 14). The molecular weight marker (lane 15) (catalog no. 1062590; Boehringer Mannheim) contained 154-, 220-, 234-, 298-, 394-, 453-, 517-, 653-, 1,033-, 1,230-, 1,766-, and 2,176-bp double-stranded DNA fragments.

LMG 16314, and LMG 13518^T (T = type strain) (*E. flavescens*); LMG 14200 and LMG 14201 (*E. hirae*); LMG 12691^T and LMG 10746^T (*E. durans*); and LMG 11492 and LMG 11493 (*E. hirae*). LAB 1259 and LAB 1262 (*E. gallinarum*) were identified as closely related strains that conformed to the definition of Tenover et al. (37). After numerical comparison, species-specific clusters were not obtained, although several *E. gallinarum* strains grouped together because of the presence of multiple poorly resolved bands in the lower fragment region (<200 kb) (data not shown).

DISCUSSION

Enterococci were traditionally considered to be endogenous in origin and not primary pathogens (17). However, these organisms are well-known opportunistic secondary pathogens which are difficult to eradicate because of their inherent and acquired resistance to several antibiotics. With this perspective, *E. faecalis* and *E. faecium* have recently gotten much attention because of their high levels of resistance to several antimicrobial agents, including glycopeptides. Controlling the spread of such multiply resistant organisms is very important (25, 38). PFGE is considered the molecular typing tool of choice for identification of clonal outbreaks (37). However, with certain infections, especially endocarditis, proper identification of enterococci to the species level is important because of species-specific differences in susceptibility to β-lactam antibiotics and glycopeptides (24). *E. faecalis* is easily differentiated from other enterococci by its tolerance to 0.04% tellurite (13). However, biochemical differentiation between *E. faecium*, *E. durans*, and *E. hirae* on the one hand and *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* on the other hand is very difficult because of the overlap in phenotypic characteristics (6).

In the present study we evaluated PCR-based DNA typing and genome macro-restriction fragment analysis for their ability to identify strains belonging to these species at the species level. Reference strains and several field isolates, identified by biochemical tests, were chosen so that each of the species was represented equally. For *E. flavescens*, only four strains were included. Strains that had various origins and were isolated over a long period of time were selected in order to obtain a diverse collection.

Whole-cell protein electrophoresis as identification standard. Within many gram-positive and gram-negative genera, there is agreement between the results obtained in DNA-DNA hybridization experiments and the results obtained by whole-cell protein electrophoresis (41). Indeed, for the most part strains with very similar whole-cell protein patterns exhibit high levels of DNA homology and therefore belong to the same species (3, 18–20, 31, 35). Teixeira et al. (35) demonstrated that this agreement exists in the genus *Enterococcus* as well. We therefore used SDS-PAGE of whole-cell proteins as the reference method for species level identification (5).

PCR-based typing. As a first approach, 27 different oligonucleotides were evaluated to determine their suitability to type a restricted set of *Enterococcus* strains (4). In the present study, the following five primers were used to examine all of the strains: random primers D11344, D14307, and D8635, which were originally used to type *Helicobacter pylori* strains (1), and repetitive primers ERIC 1R and ERIC 2 (22, 44). A single primer, D11344, proved to be useful for species-specific characterization. A second primer, D14307, allowed straightforward identification of all *E. gallinarum* strains by the presence of one unique dense amplimer. However, species-specific characterization of the other strains was not possible. Finally, primers D8635, ERIC 1R, and ERIC 2 did not produce species-specific band patterns, but primer D8635 was useful for characterization of individual strains (data not shown).

Numerical analysis of the D11344-primed band patterns and whole-cell protein analysis revealed identical clusters of strains (Fig. 1). The reproducibility of the PCR technique was somewhat lower ($r = 0.92$) than the reproducibility of the SDS-PAGE technique ($r > 0.95$); this was mainly due to the less reproducible PCR technique (23). Because of the extremely low levels of similarity between the different clusters (Fig. 1), this lower reproducibility did not influence the identification results. Each DNA band pattern is characterized by several dense bands which are present in all of the strains of the same species. Interstrain variation was observed only in the minor bands (Fig. 2). The DNA band patterns of *E. casseliflavus* (Fig. 2, lanes 9 and 10) and *E. flavescens* (Fig. 2, lanes 11 and 12) are similar and contain no species-specific dense bands.

Because of the simplicity of the DNA band patterns, visual interpretation and, consequently, identification of unknown enterococcal isolates by the PCR technique are feasible. In contrast, the electrophoretic protein profiles of the different species are complex and fairly similar (data not shown), making visual comparisons of the profiles difficult and dependent on the experience of the researcher. With this technique, computer-based image acquisition and analysis are virtually indispensable, although suitable equipment is not widely available in routine laboratories.

PFGE. Visual comparison of all of the contour-clamped homogeneous electric field electrophoresis patterns confirmed the tendencies found in the *Sma*I restriction fragment patterns described by Donabedian et al. (11), in which *E. faecalis* strains were characterized by always having their largest fragment

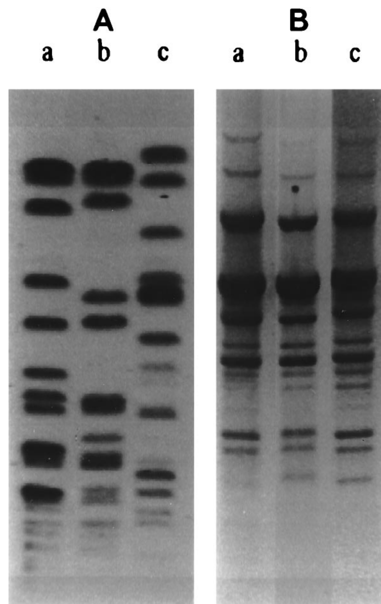

FIG. 3. Discrepancy between the PFGE patterns of three *E. faecium* strains (A) and the D8635-primed PCR patterns of the same strains (B). Identical PCR patterns did not necessarily result in identical PFGE patterns. Lane a, *E. faecium* LMG 16164; lane b, *E. faecium* LMG 8148^T; lane c, *E. faecium* LMG 8149.

larger than 400 kb and *E. gallinarum* strains were characterized by having all of their *Sma*I fragments smaller than 200 kb. This was not obvious when the fragment distribution in the *Sma*I pulsed-field patterns of a large number of strains was studied. For example, *E. gallinarum* strains were indeed characterized by having their largest *Sma*I fragment always smaller than 200 kb (11), but several *E. casseliflavus* strains also had this characteristic. In *E. hirae* and *E. durans* strains, as well as in *E. faecalis* (11), the largest *Sma*I fragment was larger than 400 kb (11). As expected, a numerical analysis of the PFGE patterns obtained revealed no species-specific band patterns. Only the PFGE patterns of several *E. gallinarum* strains grouped together due to multiple poorly resolved bands in the small-fragment region. Other electrophoretic conditions should be considered for optimal electrophoretic separation of *Sma*I restriction fragments of *E. gallinarum* strains, as proposed by Donabedian et al. (11).

Although not valuable for species differentiation, PFGE appeared to be superior for interpretation of interstrain relationships, which confirmed numerous previous reports (21, 24, 38). The identical PFGE patterns obtained were confirmed by identical PCR patterns, independent of the primer used. However, identical PCR patterns did not necessarily result in identical PFGE patterns (Fig. 3).

***E. casseliflavus* and *E. flavescens*.** Neither protein analysis nor PCR-based typing was able to differentiate between *E. casseliflavus* and *E. flavescens*. Several authors have previously noted the striking similarities between these species. When the glycopeptide resistance genotypes were studied, the two species could not be differentiated (12). Furthermore, the high level of similarity between the ligase genes of *E. casseliflavus* and *E. flavescens* was described by Navarro and Courvalin (26) as not being consistent with assigning clinical isolates of *E. casseliflavus* and *E. casseliflavus* to two different species. Surprisingly, three of the four strains studied by Pompei et al. (28) are highly related organisms (if not a single clone), as demonstrated by the identical profiles generated in the *Sma*I restriction analysis and the PCR-based typing analysis performed with primers other than D11344 (data not shown). Moreover, the results of recently conducted DNA reassociation experiments demonstrated that *E. flavescens* and *E. casseliflavus* constitute a single species (36) and are in contrast to the DNA-DNA hybridization values reported by Pompei et al. (28). Phenotypically, *E. flavescens* differs from *E. casseliflavus* in its negative ribose reaction and its failure to produce alpha-hemolysis on sheep blood (28). These data suggest that *E. flavescens* should be considered an *E. casseliflavus* biovar rather than a separate species.

In conclusion, PCR in which primer D11344 was used was as discriminative at the species level as SDS-PAGE analysis of whole-cell proteins. In addition, the former method is technically less demanding, and identification of strains by visual examination is feasible. Like the results of other studies (12, 26, 36), our data do not support the species status of *E. flavescens*. PFGE proved to be superior for differentiation of individual strains but generated little information at the species level.

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