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Genotypic Diversity, Antimicrobial Resistance, and Virulence Factors of Human Isolates and Probiotic Cultures Constituting Two Intraspecific Groups of *Enterococcus faecium* Isolates[▽]

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The intraspecific relationships among a collection of *Enterococcus faecium* isolates comprising probiotic cultures and human clinical isolates were investigated through the combined use of two high-resolution DNA-fingerprinting techniques. In addition, the incidences of antimicrobial resistance and virulence traits were investigated. A total of 128 *E. faecium* isolates from human clinical or nonclinical sources or used as probiotic cultures were subjected to fluorescent amplified fragment length polymorphism (FAFLP) fingerprinting and pulsed-field gel electrophoresis (PFGE) analysis of SmaI macrorestriction patterns. Susceptibilities to 16 antimicrobial agents were tested using broth microdilution, and the presence of the corresponding resistance genes was investigated using PCR. Multiplex PCR was used to detect the presence of the enterococcal virulence genes *asa1*, *gelE*, *cylA*, *esp*, and *hyl*. The results of the study showed that two intraspecific genomic groups (I and II) were obtained in FAFLP analysis. PFGE analysis demonstrated high variability within these two groups but also indicated that some probiotic cultures were indistinguishable and that a number of clinical isolates may be reisolations of commercial probiotic cultures. Compared to group II, which contained the majority of the probiotic isolates and fewer human clinical isolates, higher phenotypic and genotypic resistance frequencies were observed in group I. Two probiotic isolates were phenotypically resistant to erythromycin, one of which contained an *erm*(B) gene that was not transferable to enterococcal recipients. None of the probiotic *E. faecium* isolates demonstrated the presence of the tested virulence genes. The previously reported observation that *E. faecium* consists of two intraspecific genomic groups was further substantiated by FAFLP fingerprinting of 128 isolates. In combination with antimicrobial resistance and virulence testing, this grouping might represent an additional criterion in assessing the safety of new potential probiotic *E. faecium* isolates.

Enterococci are normal inhabitants of the gastrointestinal tracts of both humans and animals. In the human intestine, *Enterococcus faecium* and *Enterococcus faecalis* are the two predominant species (11, 26). On the other hand, enterococci also occur in or are deliberately added to fermented foods, in which they contribute to the organoleptic properties, and have also been used as probiotics (16). According to the FAO/WHO definition, a "probiotic" is a live microorganism that, when administered in adequate amounts, confers a health benefit on the host (57). Enterococci used as probiotics may improve the microbial balance of the intestine or can be used in the treatment of gastroenteritis in humans and animals (13). Enterococcal strains used in food and as probiotics mainly belong to the species *E. faecium* (13).

In contrast to most other genera of the lactic acid bacteria, not all enterococcal species have "generally recognized as safe" status (11). Indeed, enterococci have been recognized as important nosocomial pathogens causing endocarditis, bacteremia, and central nervous system infections, as well as neonatal, respiratory tract, urinary tract, and other infections (25, 26), which may in part be linked to the presence of antibiotic resistance and virulence properties. Resistance of *E. faecium* and *E. faecalis* to therapeutically important antibiotics is emerging, in particular, resistance to the glycopeptides vancomycin and teicoplanin, which is often associated with high-level resistance to aminoglycosides (11). The emergence of vancomycin-resistant enterococci, belonging predominantly to *E. faecium*, has resulted in cases of untreatable infections (28). Antibiotic resistance may confer a selective advantage on enterococci in the hospital environment, thereby supporting their virulence potential (26). In addition, dissemination of antimicrobial resistance genes through clonal expansion and horizontal transmission causes great concern for infectious disease specialists.

The origin of enterococcal pathogenicity has been linked to a range of virulence traits involved in adhesion, translocation, and immune evasion (20, 26). Several putative virulence factors have been identified in enterococci, such as aggregation substance (encoded by *asa1*) (14), cytolysin (encoded by *cylA*) (18), gelatinase (encoded by *gelE*) (40), hyaluronidase (en-

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coded by the *hyl* gene) (36), and enterococcal surface protein (encoded by *esp*) (38).

Among other criteria, the FAO-WHO have recommended that antimicrobial resistance patterns and opportunistic virulence properties should be tested to document the safety of probiotic strains (58). However, because both characteristics are strain specific, molecular strain typing should also be considered for safety assessment of potential probiotics. Previous typing studies (34, 56) have indicated that antibiotic-resistant *E. faecium* isolates from different sources tend to cluster according to their sources and hosts. Based on amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA-PCR analysis, Vancanneyt et al. (47) delineated two intraspecific genomic groups (I and II) among *E. faecium* isolates from various sources. The authors suggested that subclusters of group I could to some extent be correlated with the origins and pathogenicities of the strains. In all of the above-mentioned studies, however, characterization of antibiotic resistance and virulence genes was not performed, and only a few human clinical isolates were investigated.

The aim of the present study was to investigate the intraspecific relationships among a total of 128 *E. faecium* isolates comprising human clinical isolates and commercial probiotic cultures through the combined use of pulsed-field gel electrophoresis (PFGE) and AFLP. In addition, the incidences of antimicrobial resistance and virulence traits were investigated.

MATERIALS AND METHODS

Bacterial isolates. A total of 128 *E. faecium* isolates were collected in the framework of a European Union-funded research project, PROSAFE (48), including 37 isolates from human feces, 79 isolates from different human clinical samples, and 12 isolates commercially used as probiotics, 6 of which were isolated from products with probiotic claims (41) while the remaining 6 were received directly from the manufacturer or the depositor (Table 1). A representative subset of these isolates has been deposited in the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium [http://www.belspo.be/bccm/lmg.htm]). The isolates were routinely grown on Columbia agar (Becton-Dickinson, Sparks, MD) supplemented with 5% defibrinated horse blood at 37°C for 24 h.

Identification and typing. For identification purposes, the isolates were first subjected to fluorescent AFLP (FAFLP) analysis as described below.

For FAFLP analysis, total chromosomal DNA was prepared using a modification of the method described by Pitcher et al. (31). Template preparation was carried out as described previously (19). Essentially, purified genomic DNA was digested by two restriction enzymes, a 4-bp cutter EcoRI and a 6-bp cutter TacI. Small double-stranded DNA molecules (adaptors; 15 to 20 bp) containing one compatible end were ligated to the corresponding "sticky ends" of the restriction fragments. These adaptors served as binding sites for selective amplification with the primer combination E01/T01 (primers extended with an additional A) (19). PCR products were separated according to their lengths on a high-resolution polyacrylamide gel using a DNA sequencer (ABI 377). Fragments that contained an adaptor specific for the restriction half-site created by the 6-bp cutter were visualized due to the 5′ end labeling of the corresponding primer with the fluorescent dye 6-carboxyfluorescein. The resulting electrophoretic patterns were numerically analyzed with Bionumerics software version 4.01 (Applied Maths, Belgium), using the Dice coefficient and unweighted-pair group method using average linkages cluster analysis.

The intraspecific diversity among the collection of *E. faecium* isolates was also studied by PFGE, as previously described (7). Briefly, bacterial cells from an overnight culture were embedded in low-melting-point preparative agarose (Bio-Rad Laboratories, Nazareth, Belgium). After cell wall and protein digestion, the plugs were digested overnight with 30 U of SmaI (MBI Fermentas, St. Leon-Rot, Germany) at 25°C. PFGE was performed with a 1% agarose gel by using a CHEF Mapper apparatus (Bio-Rad Laboratories) in 0.5× Tris-borate-EDTA buffer at 14°C at 6 V/cm. A linearly ramped switching time from 5 to 35 s was applied for 24 h. The DNA band profiles were stained with ethidium bromide, and the image was digitized with the Gel Doc 1000 System (Bio-Rad Laboratories). Conversion, normalization, and further analysis of the DNA band patterns were performed using GelCompar software version 4.0b (Applied Maths, Kortrijk, Belgium) as described previously (33). Similarity between PFGE patterns was expressed using the Dice band-based correlation coefficient.

Phenotypic and genotypic characterization of antimicrobial susceptibility. Antimicrobial susceptibility testing of the isolates was performed using broth microdilution following CLSI guidelines (5) to determine the MICs of the following agents (with the concentration ranges [mg/liter] tested given in parentheses): penicillin (0.032 to 64), ampicillin (0.032 to 64), ampicillin/sulbactam (sulbactam was tested as a fixed concentration of 8 mg/liter; 0.032 to 64), vancomycin (0.125 to 256), teicoplanin (0.125 to 256), gentamicin (1 to 2,048), streptomycin (2 to 4,096), erythromycin (0.016 to 32), clindamycin (0.032 to 32), quinupristin-dalfopristin (Q/D; tested as a 30:70 ratio; 0.032 to 64), oxytetracycline (0.063 to 128), chloramphenicol (0.125 to 256), and linezolid (0.016 to 32). For cotrimoxazole (tested as a 1:19 ratio; 0.25 to 512), MIC breakpoints according to CLSI guidelines for *Staphylococcus* (5) were used. For fusidic acid (0.063 to 128), breakpoints as defined by Toma and Barriault (43) were used, while for trimethoprim (0.25 to 512), European Food Safety Authority guidelines (9) were followed. According to the CLSI guidelines, enterococci are considered naturally resistant to clindamycin. Occasionally, however, strains with MICs in the susceptibility range have been observed (personal observation). Except for sulbactam and linezolid (Pfizer), teicoplanin and Q/D (Sanofi-Aventis), and erythromycin (Abbott), all tested antibiotics originated from Sigma.

For isolates displaying phenotypic resistance to one or more of the tested agents, the presence of the following acquired (and potentially transferable) resistance genes was investigated: the *tet*(M), *tet*(L), *tet*(K), *tet*(O), *tet*(P), *tet*(Q), *tet*(T), *tet*(S), *tet*(W), and *tet*(M) group; *van*(A), *van*(B), *erm*(A), *erm*(B), *erm*(C), *cat*(pC194), *cat*(piP501), *aad*(E), *aph(2″)-aac(6′)*, *aad*(E)-*aph*(A), *vat*(D), and *vat*(E). For this purpose, DNA was isolated using the DNeasy tissue kit (Qiagen), and amplification of the corresponding gene fragments was performed in a DNA Engine Thermal Cycler "PTC-200" (MJ Research), as previously described (22). The following positive control strains were used: *E. faecium* UW 1342 for *vat*(D) (52); *E. faecium* UW 1965 for *vat*(E), *erm*(B), *aad*(E), *aad*(E)-*aph*(A), *cat*(pC194), and *cat*(piP501) (50, 51, 53, 55); *Staphylococcus aureus* 694/01 for *erm*(A), *erm*(C), *tet*(K), *tet*(M), and *aac*(A)-*aph*(D) (39); *E. faecium* UW 1873 for *tet*(L) (54); *E. faecium* BM4147 for *van*(A) (3, 29); *E. faecium* V583 for *van*(B) (29, 35); and *Streptococcus pyogenes* A498 for *tet*(T) (4). For the remaining genes, the following control plasmids were used: pGEM-tet(O) for *tet*(O) (2); pJIR667 for *tet*(P) (23a); pBT-1 for *tet*(Q) (27); pVP2 for *tet*(S) (30); and pGEM-tetW for *tet*(W) (2). The amplification products were detected by electrophoresis in a 1.5% agarose gel and subsequent ethidium bromide staining.

In vitro transfer experiments were performed by conjugation (filter mating) as previously described (22). Possible transconjugants were identified in several steps, selecting for the selective and nonselective markers. Probiotic *E. faecium* isolates representing non-wild-type isolates with acquired antibiotic resistance(s) were used as donors, whereas the well-documented strains *E. faecium* 64/3 and *E. faecalis* JH2-2 were chosen as recipients. Possible transconjugants were further characterized by MIC determination, PCR-based detection of resistance genes, PFGE, and $(GTG)_5$-PCR (15, 21).

Multiplex PCR virulence genes. Multiplex PCR for the detection of the virulence genes *asa1*, *gelE*, *cylA*, *esp*, and *hyl* was performed as described previously (49). Briefly, each 50-μl PCR mixture consisted of 5 μl of bacterial suspension; 0.1 μM primers for the detection of *asa1*, *gelE*, and *hyl*; 0.2 μM primers for the detection of *cylA* and *esp*; 25 μl HotStarTaq Master Mix (Qiagen, Germany); and an additional 1.0 mM $MgCl_2$. PCR was performed in a GeneAmp PCR System 9600 (Perkin Elmer, Wellesley, MA). An initial activation step at 95°C for 15 min, during which the HotStarTaq DNA polymerase was activated, was followed by 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min), and extension (72°C for 1 min), followed by 1 cycle consisting of 10 min at 72°C. The PCR products were electrophoresed in a 1.5% pronarose D1 gel (SphaeroQ, Burgos, Spain) for 1 h at 150 V in 0.5× Tris-borate-EDTA containing 0.05 mg/liter ethidium bromide (positive and negative controls were included in each set of amplifications) (49). A 100-bp DNA ladder (Invitrogen, Merelbeke, Belgium) was used as molecular size marker.

Statistical analysis. Student's *t* test was used for statistical analysis. A P value of <0.05 was considered statistically significant.

RESULTS

Identification and typing. All strains were identified at the species level as *E. faecium* by FAFLP (data not shown). The dendrogram obtained from numerical analysis of digitized

TABLE 1. *E. faecium* isolates included in this study

PRSF no.	Other strain no.	Depositor[a]	Origin[b]	Source; geographical origin; yr of isolation	FAFLP group	PFGE group
PRSF-E001	6254	H. Dupont	H	Peritonitis; France	I	32a′
PRSF-E002	21771	H. Dupont	H	Peritonitis; France	II	45a
PRSF-E003	22183	H. Dupont	H	Peritonitis; France	I	30a
PRSF-E004	31505	H. Dupont	H	Peritonitis; France	I	9c
PRSF-E005	37215	H. Dupont	H	Peritonitis; France	I	37b
PRSF-E006	43169	H. Dupont	H	Peritonitis; France	I	28a
PRSF-E007	44849; LMG 24169	H. Dupont	H	Peritonitis; France	I	37a
PRSF-E008	45414	H. Dupont	H	Peritonitis; France	II	82a
PRSF-E009	45780	H. Dupont	H	Peritonitis; France	I	38a
PRSF-E010	46741	H. Dupont	H	Peritonitis; France	I	48a
PRSF-E011	47271	H. Dupont	H	Peritonitis; France	I	25a
PRSF-E012	59910	H. Dupont	H	Peritonitis; France	II	27a
PRSF-E013	65233	H. Dupont	H	Peritonitis; France	I	37a
PRSF-E015	74254	H. Dupont	H	Peritonitis; France	I	98a
PRSF-E016	75133; LMG 24170	H. Dupont	H	Peritonitis; France	I	32a
PRSF-E017	78601	H. Dupont	H	Peritonitis; France	I	18a
PRSF-E018	83123	H. Dupont	H	Peritonitis; France	I	19c
PRSF-E019	90056	H. Dupont	H	Peritonitis; France	I	15a
PRSF-E020	960018; LMG 24171	UZA	H	Blood; Belgium; 2000	I	31a
PRSF-E021	960050	UZA	H	Blood; Belgium; 2000	I	19b
PRSF-E022	1–20	UZA	H	Blood; Belgium; 1995	I	16a
PRSF-E023	1–9	UZA	H	Blood; Belgium; 1995	I	17a′
PRSF-E024	11/4	UZA	H	Blood; Belgium; 1995	I	23a
PRSF-E025	13/1; LMG 24172	UZA	H	Blood; Belgium; 1995	II	26b
PRSF-E026	13/11	UZA	H	Blood; Belgium; 1995	II	74a
PRSF-E027	13/5	UZA	H	Blood; Belgium; 1995	II	26a
PRSF-E028	14/1	UZA	H	Blood; Belgium; 1995	I	94a
PRSF-E029	17/7	UZA	H	Blood; Belgium; 1995	II	101a
PRSF-E030	18/2	UZA	H	Blood; Belgium; 1995	I	35a
PRSF-E031	18/7	UZA	H	Blood; Belgium; 1995	I	93a
PRSF-E032	2/5	UZA	H	Blood; Belgium; 1995	I	49a
PRSF-E033	20/8; LMG 23226	UZA	H	Blood; Belgium; 1995	I	22a
PRSF-E034	22/1; LMG 23227	UZA	H	Blood; Belgium; 1995	I	92a
PRSF-E035	3–26; LMG 23228	UZA	H	Blood; Belgium; 1995	I	95a
PRSF-E036	4/10; LMG 23229	UZA	H	Blood; Belgium; 1995	I	17a
PRSF-E037	4/19; LMG 23230	UZA	H	Blood; Belgium; 1995	I	17a
PRSF-E038	5/5; LMG 23231	UZA	H	Blood; Belgium; 1995	I	36b
PRSF-E039	6–8; LMG 23232	UZA	H	Blood; Belgium; 1995	II	83a
PRSF-E040	7/6; LMG 23233	UZA	H	Blood; Belgium; 1995	I	41a
PRSF-E041	8/1; LMG 23234	UZA	H	Blood; Belgium; 1995	I	4a
PRSF-E042	9/4; LMG 23235	UZA	H	Blood; Belgium; 1995	II	44a
PRSF-E043	01SE05 LMG 23236	UA	H	Fecal flora; Belgium; 1997	I	20b
PRSF-E044	01VHM19; LMG 23237	UA	H	Fecal flora; Belgium; 1996	II	66a
PRSF-E045	02bVHM05; LMG 23238	UA	H	Fecal flora; Belgium; 1997	II	12a
PRSF-E046	02VHM03; LMG 23239	UA	H	Fecal flora; Belgium; 1996	I	100a
PRSF-E047	04VHM06; LMG 23240	UA	H	Fecal flora; Belgium; 1996	II	59a
PRSF-E048	04VHM09; LMG 23241	UA	H	Fecal flora; Belgium; 1996	II	88a
PRSF-E049	04VWK14; LMG 23242	UA	H	Fecal flora; Belgium; 1997	II	2a
PRSF-E050	06VHM11; LMG 23243	UA	H	Fecal flora; Belgium; 1996	II	87a
PRSF-E051	06VWK04; LMG 23244	UA	H	Fecal flora; Belgium; 1997	II	76c
PRSF-E052	07SS01; LMG 23245	UA	H	Fecal flora; Belgium; 1997	I	22b
PRSF-E053	07TB04; LMG 24173	UA	H	Fecal flora; Belgium; 1997	I	23b
PRSF-E054	09SS01; LMG 24174	UA	H	Fecal flora; Belgium; 1997	II	9b
PRSF-E055	09VHM05	UA	H	Fecal flora; Belgium; 1997	II	85a
PRSF-E057	10SS05	UA	H	Fecal flora; Belgium; 1997	I	3a
PRSF-E058	11T	UA	H	Fecal flora; Belgium; 1996	I	97a
PRSF-E059	126T	UA	H	Fecal flora; Belgium; 1996	I	89a
PRSF-E060	13/13	UA	H	Blood; Belgium; 1995	II	17a′
PRSF-E061	162V	UA	H	Fecal flora; Belgium; 1996	I	7a
PRSF-E062	175V	UA	H	Fecal flora; Belgium; 1996	I	53a
PRSF-E063	24/16	UA	H	Blood; Belgium; 1995	I	47a
PRSF-E064	24/19	UA	H	Blood; Belgium; 1995	II	103a′
PRSF-E065	302V	UA	H	Fecal flora; Belgium; 1996	I	68a
PRSF-E066	325T	UA	H	Fecal flora; Belgium; 1996	I	69a
PRSF-E067	360V	UA	H	Fecal flora; Belgium; 1996	I	8a′
PRSF-E068	39771a	H. Dupont	H	Peritonitis; France	I	14a
PRSF-E069	398T	UA	H	Fecal flora; Belgium; 1996	I	96a

Continued on following page

TABLE 1—*Continued*

PRSF no.	Other strain no.	Depositor[a]	Origin[b]	Source; geographical origin; yr of isolation	FAFLP group	PFGE group
PRSF-E070	406T	UA	H	Fecal flora; Belgium; 1996	I	8a
PRSF-E072	615V; LMG 24175	UA	H	Fecal flora; Belgium; 1996	I	8b
PRSF-E073	7532b; LMG 24176	H. Dupont	H	Peritonitis; France	I	98d
PRSF-E074	80A	UA	H	Fecal flora; Belgium; 1996	I	105a
PRSF-E075	8489b	H. Dupont	H	Peritonitis; France	I	51a
PRSF-E076	88156a	H. Dupont	H	Peritonitis; France	I	13b
PRSF-E077	95-04-3212	UA	H	Fecal flora; Belgium; 1995	I	95b
PRSF-E078	95-12-6002	UA	H	Fecal flora; Belgium; 1995	I	90a
PRSF-E079	95-12-6037	UA	H	Fecal flora; Belgium; 1995	I	6b
PRSF-E080	95-12-6076; LMG 24177	UA	H	Fecal flora; Belgium; 1995	I	7b
PRSF-E081	9727/1700	UA	H	Urine; Belgium; 1997	I	98c
PRSF-E083	A.N.07/27/99	UA	H	Wound; Belgium; 1999	I	36a
PRSF-E084	D.A.072999	UA	H	Wound; Belgium; 1999	I	19e
PRSF-E085	M4/05/0203	UZA	H	Blood; Belgium; 1994	II	86a
PRSF-E086	M4/06/0908; LMG 24178	UZA	H	Blood; Belgium; 1994	II	62a
PRSF-E087	M5/01/0874; LMG 24179	UZA	H	Blood; Belgium; 1995	I	24a
PRSF-E088	M5/04/0316	UZA	H	Blood; Belgium; 1995	I	19b′
PRSF-E089	M5/04/0363	UZA	H	Blood; Belgium; 1995	I	31b
PRSF-E090	M5/04/0408; LMG 24180	UZA	H	Blood; Belgium; 1995	I	6a
PRSF-E092	M5/10/0447	UZA	H	Blood; Belgium; 1995	I	21a
PRSF-E093	M6/02/0521; LMG 24181	UZA	H	Blood; Belgium; 1996	II	57a
PRSF-E094	M6/02/0956	UZA	H	Blood; Belgium; 1996	I	24b
PRSF-E095	M6/03/1007	UZA	H	Blood; Belgium; 1996	I	9a
PRSF-E097	S.E.072899	UA	H	Wound; Belgium; 1999	I	19d
PRSF-E098	V.K.07/27/99	UA	H	Wound; Belgium; 1999	II	19b′
PRSF-E099	V.K.072999; LMG 24182	UA	H	Wound; Belgium; 1999	I	21b
PRSF-E100	VL.F.080499; LMG 24183	UA	H	Wound; Belgium; 1999	I	19a
PRSF-E101	W.R.032400; LMG 24184	UA	H	Wound; Belgium; 2000	I	25b
PRSF-E102		D38-P18	P	Probiotic product	II	75a
PRSF-E103		D29-P07	P	Probiotic product	II	57b
PRSF-E104		D29-P29	P	Probiotic product	II	62b
PRSF-E105		D42-P17	P	Probiotic product	II	63a
PRSF-E106		D28-P06	P	Probiotic product	II	76b
PRSF-E107		D27-P05	P	Probiotic product	II	63a
PRSF-E110	UW 1952	RKI	H	Peritonitis	II	43a
PRSF-E113	UW 2434	RKI	H	Blood	I	Bad profile
PRSF-E114	UW 2493	RKI	H	Blood	I	Bad profile
PRSF-E115	UW 2742	RKI	H	Liver	I	61a
PRSF-E116	UW 3181	RKI	H	Blood	I	46a
PRSF-E117	D05		PHA	Fecal flora; Sweden; 1968	II	63a
PRSF-E121	LMG 20734	LMG/BCCM	H	Fecal flora; The Netherlands; 1995	I	42a
PRSF-E122	LMG 20735	LMG/BCCM	H	Fecal flora; The Netherlands; 1995	I	11a
PRSF-E123	LMG 20939	LMG/BCCM	H	Fecal flora; Ireland; 1997	II	56a
PRSF-E124	LMG 20946	LMG/BCCM	H	Fecal flora; Ireland; 1997	II	56a
PRSF-E125	CCUG 31387	CCUG	H	Blood; Sweden; 1993	I	39a
PRSF-E126	CCUG 32171	CCUG	H	Blood; Sweden; 1993	I	58a
PRSF-E127	CCUG 32655	CCUG	H	Blood; Sweden; 1994	II	50a
PRSF-E128	CCUG 34324	CCUG	H	Blood; Sweden; 1995	I	51c
PRSF-E129	CCUG 45539	CCUG	H	Blood; Sweden; 2001	I	13a
PRSF-E130	CCUG 29743	CCUG	H	Blood; Sweden; 1992	I	50b
PRSF-E131	CCUG 36838	CCUG	H	Blood; Sweden; 1996	II	1a
PRSF-E132		D04	PH	Fecal flora; Italy; 1977	II	63a
PRSF-E133		D04	PH	Fecal flora; Italy; 1977	II	63a
PRSF-E134		D10	PH	Non-human; Canada; 1988	II	76a
PRSF-E140		D15	PH	Animal; United Kingdom	I	29a
PRSF-E142	H 437	P. Moreillon	H	Blood; Switzerland; 1996	I	10a
PRSF-E144	03WS09	C. Vael	H	Fecal flora	I	20a
PRSF-E146	01SS07	C. Vael	H	Fecal flora	II	60a
PRSF-E149	C68	L. Rice	H	Fecal flora	I	40a
PRSF-E162	FH 02001		H	Fecal flora; France; 2002	I	5a
PRSF-E164	LMG 23514	D50	PHA	Dairy product, cheese; Italy	II	63a

[a] BCCM/LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium; CCUG, Culture Collection University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; P. Moreillon, CHUV, Centre de Collection de Type Microbien, Centre Hospitalier Universitaire Vaudois, Institut de Microbiologie, Université de Lausanne, Lausanne, Switzerland; D*x*, probiotic strain collected from company *x*; D*x*-P*y*, probiotic strain collected from product *y* from company *x*; UA, University of Antwerp, Antwerp, Belgium; UZA, University Hospital Antwerp, Antwerp, Belgium; C. Vael, ZNA, Antwerp, Belgium; L. Rice, Research and Medical Services, Louis Stokes Cleveland Veterans Affairs Medical Center, and Department of Medicine, Case Western Reserve University, Cleveland, OH; RKI, Robert Koch Institute, Wernigerode Branch, Wernigerode, Germany; H. Dupont, CHU D'Amiens, France.

[b] P, strain used commercially as a probiotic; PH, strain used commercially as a probiotic for human consumption; PHA, strain used commercially as a probiotic for human and animal consumption; H, human isolate.

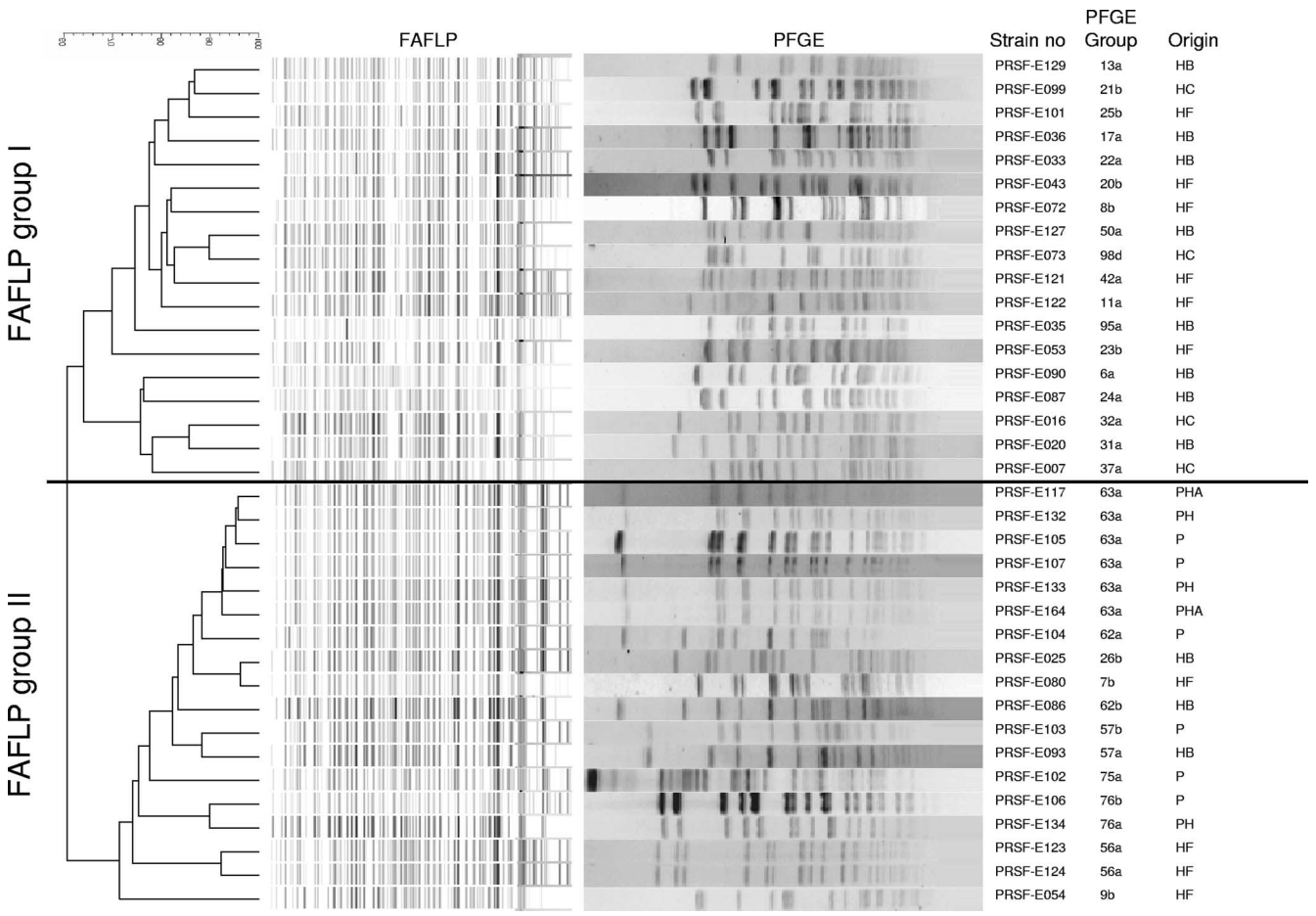

FIG. 1. Dendrogram based on numerical analysis of FAFLP patterns obtained with the primer combination E01/T01 with the corresponding SmaI PFGE patterns of selected *E. faecium* isolates. The dendrogram was constructed using the unweighted-pair group method using average linkages and the band-based Dice similarity coefficient. P, probiotic; PH, probiotic human consumption; PHA, probiotic human and animal consumption; HB, human blood isolate; HC, human clinical isolate; HF, human fecal isolate.

FAFLP generated with primer combination E01/T01 clearly showed the presence of two intraspecific genomic groups (denoted I and II) among the set of 128 *E. faecium* isolates. The delineation of these two groups is shown for a selection of isolates in Fig. 1. A total of 87 *E. faecium* isolates belonged to FAFLP group I, whereas 41 *E. faecium* isolates belonged to FAFLP group II. FAFLP group I consisted of 86 isolates of human origin (25 fecal and 61 clinical) and 1 probiotic culture. In FAFLP group II, 30 isolates were of human origin (12 fecal and 18 clinical), and 11 isolates were received as probiotic cultures.

Cluster analysis and visual inspection of the PFGE profiles revealed high variability within the two genomic FAFLP groups. Based on the criterion that isolates exhibiting a maximum of six band position differences (42) in their respective PFGE patterns belonged to the same PFGE group, a total of 25 PFGE groups containing more than 1 isolate and 59 single isolates were recognized. In case no differences in band number and position were observed upon visual inspection, isolates were considered indistinguishable. The PFGE patterns of a selection of isolates are shown in Fig. 1. A total of 13 groups (i.e., groups 13, 17, 19, 21, 24, 25, 26, 31, 32, 37, 50, 51, and 98) contained only clinical isolates, 3 groups (i.e., groups 8, 20, and 56) contained only fecal isolates, and 5 groups (i.e., groups 6, 9, 22, 23, and 95) contained both clinical and fecal isolates. High similarity was observed between a number of isolates used commercially as probiotics. These isolates belonged to PFGE groups 57, 62, 63, and 76, of which group 63 contained six isolates received from five different depositors (i.e., D04, D05, D27, D42, and D50) with indistinguishable SmaI macrorestriction profiles (i.e., PRSF-E133, PRSF-E132, PRSF-E164, PRSF-E105, PRSF-E117, and PRSF-E107). In PFGE groups 62 and 57, a probiotic isolate clustered with a clinical isolate (i.e., PRSF-E103 with PRSF-E093 and PRSF-E104 with PRSF-E086) but could not be considered indistinguishable based on two band differences (Fig. 1). In PFGE group 76, fecal isolate PRSF-E051 clustered with the two probiotic isolates PRSF-E106 and PRSF-E134, but these isolates were also not considered indistinguishable (Fig. 1).

Phenotypic and genotypic characterization of antimicrobial susceptibilities. Using broth microdilution, phenotypic resistances to 13 antimicrobial agents were determined, after which the genetic basis of the observed resistance was investigated (Table 2). All isolates were susceptible to linezolid, but only 15

TABLE 2. Frequencies of antimicrobial resistance and virulence genes in 128 *E. faecium* isolates

Phenotypic resistance or virulence gene	No. (%) with indicated phenotypic resistance or virulence gene				Antibiotic resistance gene	No. (%) with indicated phenotypic resistance that also possess the indicated antibiotic resistance gene			
	Isolates (n = 128)	Probiotics (n = 12)	Human isolates			Isolates	Probiotics	Human isolates	
			Nonfecal[a] (n = 79)	Fecal (n = 37)				Nonfecal[a]	Fecal
Penicillin	37 (28)		35 (44)	2 (3)	—[b]				
Ampicillin	37 (28)		35 (44)	2 (3)	—				
Ampicillin/sulbactam	36 (28)		35 (44)	1 (3)	—				
Tetracycline	59 (46)		41 (52)	18 (49)	*tet*(M)	57 (97)		39 (68)	18 (32)
					tet(L)	41 (69)		29 (71)	12 (29)
					tet(K)	1 (2)		1 (100)	0 (0)
Vancomycin	19 (15)		3 (4)	16 (43)	*van*(A)	18 (95)		3 (17)	15 (93)
					van(B)	1 (5)		0 (0)	1 (100)
Teicoplanin	18 (14)		3 (4)	15 (41)	*van*(A)	18 (100)		3 (17)	15 (83)
Erythromycin	62 (48)	2 (17)	42 (53)	18 (49)	*erm*(A)	1 (2)		1 (100)	0 (0)
					erm(B)	51 (82)	1 (2)	36 (71)	14 (27)
Chloramphenicol	5 (4)		3 (4)	2 (3)	*cat*(pC194)	10 (?)	7 (70)	3 (30)	
					cat(piP501)	11 (?)		7 (64)	4 (36)
Streptomycin	46 (36)		37 (47)	9 (24)	*aad*(E)	14 (30)	7 (50)	7 (50)	
					aad(E)-*aph*(A)	28 (61)	26 (93)	2 (7)	
Gentamicin	6 (5)		5 (6)	1 (3)	*aph*(2″)-*aac*(6′)	6 (100)		5 (83)	1 (17)
Quinupristin-dalfopristin	16 (13)		9 (11)	7 (19)	*vat*(D)	3 (19)		2 (67)	1 (33)
Linezolid									
Fusidic acid	81 (63)	11 (92)	47 (59)	23 (62)					
Virulence genes									
asa1									
gelE									
cylA									
esp	11 (9)		11 (14)						
hyl									
esp+*hyl*	1 (1)			1 (3)					
None	116 (90)	12 (100)	68 (86)	36 (97)					

[a] Sterile sites (blood, liver, others), wound fluid, urine.
[b] —, no PCR performed.

(12%) out of 128 isolates were susceptible to all agents tested. Overall, the highest phenotypic resistance frequencies were observed for erythromycin (62 of 128 isolates; 48%) and oxytetracycline (59 of 128 isolates; 46%). In Fig. 2, the distribution of antimicrobial resistances in FAFLP groups I and II is depicted. Resistance to gentamicin, penicillin, vancomycin, and teicoplanin was observed only in group I, and at least twice as many isolates ($P < 0.001$) in group I were resistant to the other tested antibiotics (Q/D, ampicillin, ampicillin/sulbactam, streptomycin, erythromycin, trimethoprim, cotrimoxazole, oxytetracycline, and chloramphenicol) compared to the isolates in group II. On the other hand, a larger number of group II isolates (31 out of 41 isolates; 76%) were resistant to fusidic acid compared to group I (50 out of 87 isolates; 57%), although the difference was not statistically significant ($P = 0.09$).

The majority of the isolates (87 out of 113 isolates; 77%) displaying phenotypic resistance also possessed the corresponding antibiotic resistance gene (Table 2). Overall, out of the 59 tetracycline-resistant *E. faecium* isolates, the *tet*(M) and *tet*(L) genes were detected in 57 (97%) and 41 (69%) human isolates, respectively. Out of the 53 tetracycline-resistant isolates in group I, 51 (96%) carried the *tet*(M) gene and 37 (69%) carried the *tet*(L) gene. In group II, all six tetracycline-resistant isolates carried the *tet*(M) gene and four (67%) carried the *tet*(L) gene. The *tet*(K) gene was detected in only one human isolate belonging to group I, which also harbored the *tet*(L) gene. In all isolates displaying phenotypic resistance to chloramphenicol, streptomycin, and gentamicin, corresponding genes could be detected (Table 2). On the other hand, 11 nonfecal isolates and three fecal isolates possessed a *cat* gene, while none of these were classified as phenotypically resistant to chloramphenicol. All 18 vancomycin- and teicoplanin-resistant isolates of group I carried the *van*(A) gene. In addition, one isolate with phenotypic resistance to vancomycin but not to teicoplanin also contained the *van*(B) gene. The following genes were not detected: the *tet*(O), *tet*(P), *tet*(Q), *tet*(T), *tet*(S), *tet*(W), and *tet*(M) group; *erm*(C); and *vat*(E).

Among the probiotic isolates, 11 out of 12 (92%) displayed phenotypic resistance to fusidic acid. Two out of these 11 isolates, PRSF-E105 and PRSF-E140, were also resistant to erythromycin, but only isolate PRSF-E140 carried an *erm*(B) gene. Using filter mating, transfer of *erm*(B) from PRSF-E140 to recipients *E. faecium* 64/3 and *E. faecalis* JH2-2 could not be detected (data not shown).

Multiplex PCR virulence genes. A total of 12 out of 128 (9%) *E. faecium* isolates were positive for the enterococcal surface protein gene *esp*, including the fecal isolate PRSF-E149 from a hospitalized patient, which also contained the hyaluronidase gene, *hyl*. Of the remaining 11 (9%) *esp*-positive *E. faecium* isolates, 7 were blood isolates and 4 were wound isolates. More specifically, a total of 7 out of 87 (8%) isolates in FAFLP group I demonstrated the presence of the *esp* gene, of which 1 isolate also harbored the *hyl* gene, whereas in group II, the number of isolates positive for *esp* was slightly higher (5

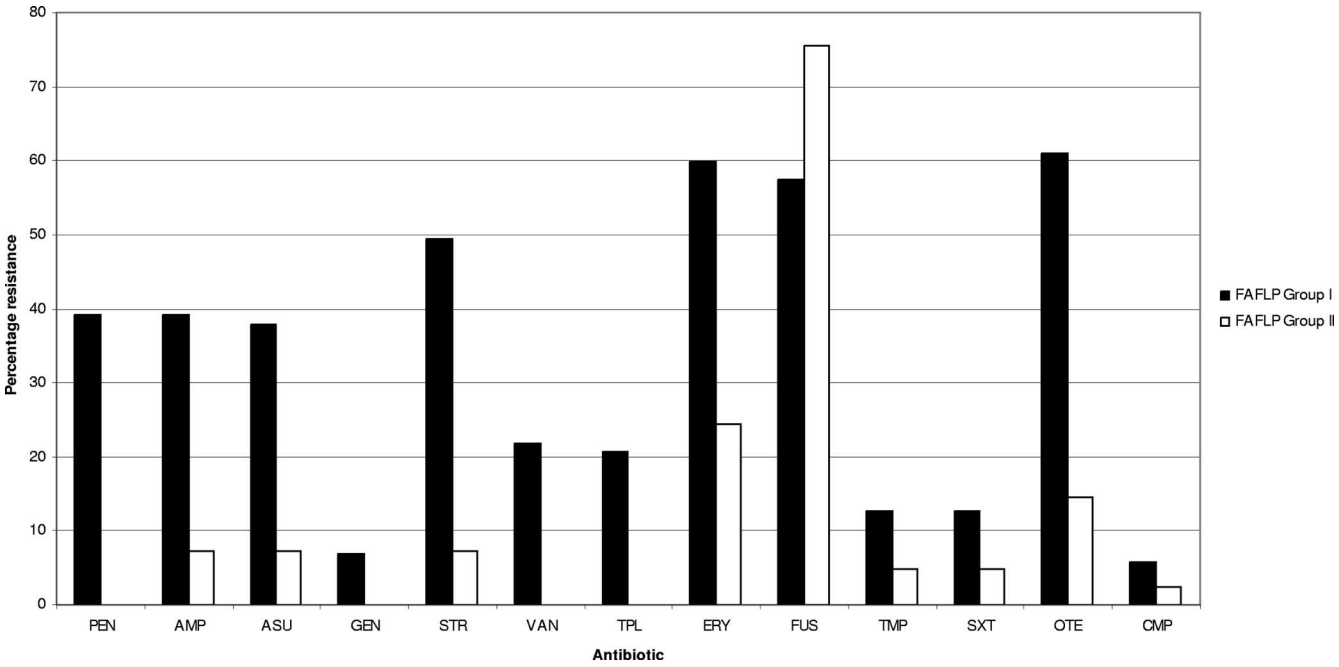

FIG. 2. Antibiotic resistances in FAPLP groups I and II. PEN, penicillin; AMP, ampicillin; ASU, ampicillin-sulbactam; GEN, gentamicin (only high-level resistance was reported); STR, streptomycin (only high-level resistance was reported); VAN, vancomycin; TPL, teicoplanin; ERY, erythromycin; FUS, fusidic acid; TMP, trimethoprim; SXT, cotrimoxazole; OTE, oxytetracycline; CMP, chloramphenicol.

out of 41; 12%). None of the probiotic isolates possessed any of the virulence factors tested. Table 2 gives an overview of the presence of virulence genes in the *E. faecium* isolates tested.

DISCUSSION

A broad collection of *E. faecium* isolates from different human origins (sterile sites, wound fluid, urine, and fecal origin), as well as commercial probiotic cultures, was included in the study. PFGE analysis of SmaI macrorestriction profiles, which is considered to be the gold standard for genotyping of enterococci (1, 23, 44), was used to determine the strain diversity among human clinical isolates and commercial probiotic cultures of *E. faecium*. A number of probiotic *E. faecium* isolates (Fig. 1) from different producers of probiotic products did not differ by a single band in PFGE using SmaI, indicating that they belonged to the same PFGE clone. Although, this is the reference method for typing enterococci, use of a second restriction enzyme or multilocus sequence typing (17) could provide even stronger evidence. In a few other cases, human *E. faecium* isolates from sterile body sites and feces were highly related to but not indistinguishable from a specific probiotic isolate. Provided that additional PFGE analyses with other restriction enzymes could further substantiate these results, this may suggest that some human isolates in the studied collection may be reisolations of commercial isolates. Similar conclusions were formulated by Vancanneyt et al. (46) for (potentially) probiotic *L. rhamnosus* isolates.

In line with a previous study (47), FAFLP analysis revealed the presence of two intraspecific genomic groups in *E. faecium*. Vancanneyt and colleagues (47) delineated two genomic groups among a collection of 78 *E. faecium* isolates from various human, animal, and food origins on the basis of randomly amplified polymorphic DNA-PCR and AFLP analyses. Four isolates were tested in both studies and were also allocated to the same genomic groups, i.e., PRSF-E122 and PRSF-E123 to group I and PRSF-E124 and PRSF-E125 to group II. In contrast to the former study (47), in which all human clinical isolates belonged to group I, our results showed that both FAFLP groups contained clinical isolates, which might be explained by the larger number of clinical isolates investigated. However, it should be mentioned that there might be a selection bias for the clinical isolates, as the majority of the isolates in our study were isolated in Belgium. In comparison, the 19 clinical isolates included in the study by Vancanneyt et al. (47) mainly originated from The Netherlands ($n = 8$) but also included isolates from Ireland, Belgium, Italy, and Germany. Notably, all but one (PRSF-E140) of the probiotic cultures belonged to FAFLP group II, which contained considerably fewer clinical isolates than FAFLP group I.

Although the enterococcal isolates in this study were in general susceptible to clinically relevant antibiotics, such as vancomycin, teicoplanin, gentamicin, and linezolid, comparison of the two intraspecific groups showed that the isolates in FAFLP group I displayed higher resistance frequencies to all agents tested except fusidic acid (Fig. 2). Possibly, these differences reflect strain origin and selective pressure, because antibiotic resistance was mainly observed in isolates of human origin, irrespective of the FAFLP grouping. The probiotic *E. faecium* isolates were highly susceptible to all tested antimicrobials except fusidic acid, resistance to which was demonstrated in a high percentage (92%) of probiotic isolates. However, fusidic acid has relatively poor activity against enterococci (6, 43), and the MICs of the enterococcal strains tested in the

present study were located around the breakpoint. Importantly, two probiotic *E. faecium* isolates were phenotypically resistant to erythromycin, one of which (PRSF-E140) carried an *erm*(B) gene that was not transferable to enterococcal recipients. The previously reported involvement of *msr*, *mef*, or *vga* genes in erythromycin efflux or other resistance mechanisms (10, 32) might explain the erythromycin resistance phenotype that lacked the *erm*(B) gene. We speculate that probiotic cultures belonging to FAFLP group II may display a better safety record than probiotic members of FAFLP group I (i.e., PRSF-E140), because overall fewer phenotypic and genotypic resistances were detected in isolates from the former group. Furthermore, it is relevant to mention that, based on the descriptive information received from the original strain depositors, PRSF-E140 was the only probiotic *E. faecium* culture of animal origin included in this study. After the presence of acquired antibiotic resistance genes is verified, their transferabilities also need to be investigated in terms of safety evaluation of probiotic bacteria.

Overall, the virulence determinants present were highly similar in the human isolates in both groups. None of the *E. faecium* isolates in either genomic group was found to be positive for *asa1*, *gelE*, and *cylA* genes. Likewise, previous studies of *E. faecium* did not demonstrate the presence of any of these genes (8, 24, 37, 49). None of the probiotic *E. faecium* isolates, which mainly clustered in group II, contained any of the virulence genes tested. To date, *esp* and *hyl* genes have mainly been detected in clinical *E. faecium* isolates (8, 12, 24, 36, 49).

In order to obtain further insights into the evolutionary history and biological importance of intraspecific groups I and II in *E. faecium*, it would be interesting to challenge current FAFLP fingerprinting data with sequence-based approaches, such as multilocus sequence typing (17) and multilocus variable-number tandem-repeat analysis (45).

In conclusion, whole-genome FAFLP fingerprinting confirmed the previously reported intraspecific subdivision of *E. faecium* into two genomic groups. Although the virulence genes present were similar in both groups, FAFLP group II differed from group I because it contained only a minority of clinical isolates and because fewer antibiotic resistances were detected. Combined with phenotypic and genotypic assays investigating the presence of (transferable) antibiotic resistance and virulence traits, this intraspecific genomic grouping might be useful to document the safety records of new probiotic candidates of *E. faecium*.

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