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Phylogeny and Identification of Enterococci by *atpA* Gene Sequence Analysis

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The relatedness among 91 *Enterococcus* strains representing all validly described species was investigated by comparing a 1,102-bp fragment of *atpA*, the gene encoding the alpha subunit of ATP synthase. The relationships observed were in agreement with the phylogeny inferred from 16S rRNA gene sequence analysis. However, *atpA* gene sequences were much more discriminatory than 16S rRNA for species differentiation. All species were differentiated on the basis of *atpA* sequences with, at a maximum, 92% similarity. Six members of the *Enterococcus faecium* species group (*E. faecium*, *E. hirae*, *E. durans*, *E. villorum*, *E. mundtii*, and *E. ratti*) showed >99% 16S rRNA gene sequence similarity, but the highest value of *atpA* gene sequence similarity was only 89.9%. The intraspecies *atpA* sequence similarities for all species except *E. faecium* strains varied from 98.6 to 100%; the *E. faecium* strains had a lower *atpA* sequence similarity of 96.3%. Our data clearly show that *atpA* provides an alternative tool for the phylogenetic study and identification of enterococci.

The genus *Enterococcus* comprises 30 validly published species (www.bacterio.cict.fr/e/enterococcus.html) of gram-positive, oxidase- and catalase-negative, non-spore-forming, ovoid bacteria that are arranged in single cells, pairs, or chains. The genus *Enterococcus* was first proposed by Schleifer and Kilpper-Bälz in 1984 (31). Enterococci belong to the lactic acid bacteria, which are part of the clostridial branch of the gram-positive bacteria. The closest phylogenetic neighbors of enterococci are *Tetragenococcus*, *Vagococcus*, *Carnobacterium*, and *Aerococcus* (16, 20). The classification of the enterococci underwent considerable changes in recent years. Since the recognition of *Enterococcus* as a separate genus (31), several new species, e.g., *Enterococcus canis* (4), *E. hermanniensis* (21), and *E. phoeniculicola* (22), have been described as a result of improvements of the methods for their classification. In addition, *E. porcinus* and *E. seriolicida* were reclassified as *E. villorum* (4) and *Lactococcus garvieae* (35), respectively. The phylogenetic relationship of the different species within the genus *Enterococcus* has been determined by comparative sequence analysis of their 16S rRNA genes. Different species groups can be distinguished on the basis of these data (6, 12, 20).

Several molecular biology-based techniques, such as multilocus sequence typing, randomly amplified polymorphic DNA (RAPD) analysis, 16S rRNA gene sequencing, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), and intergenic ribosomal PCR, have been used to identify enterococci to the species and the strain levels (1, 2, 3, 5, 8, 17, 26). AFLP analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) are among the most reliable techniques currently used for *Enterococcus* species identification (39). However, AFLP analysis and SDS-PAGE may present problems concerning reproducibility and data portability. 16S rRNA gene sequences have limited discriminating power for several closely related enterococcal species, e.g., members of the *E. faecium* species group (7, 29, 38). PFGE was found to be superior for interpretation of the interstrain relationships among enterococci but did not result in species-specific discriminative DNA bands (5).

Recent in silico studies based on the whole-genome sequences of different bacterial groups proposed that *atpA* may be an alternative phylogenetic and identification marker for *Enterococcus* (14, 15, 23, 40). *atpA* codes for the α subunit of the bacterial ATP synthase, which functions in ATP synthesis coupled to proton transport (24). The aim of present study was to analyze the usefulness of *atpA* gene sequences for the reliable identification of *Enterococcus* species.

MATERIALS AND METHODS

The strains used in this study are listed in Table 1. The strains were grown on blood agar medium (Columbia agar base) under microaerophilic conditions by using CO_2-Gen (Oxoid Co.) at 37°C for 48 h. All strains included in this study are present in the BCCM/LMG Bacteria Collection at Ghent University (Ghent, Belgium). Bacterial genomic DNA was extracted by the methodology described by Gevers et al. (13).

The sequences of the primers used for amplification and sequencing of *atpA* are listed in Table 2. These primers were designed by using 12 *atpA* gene sequences of lactic acid bacteria, i.e., *E. faecalis* (V583), *Lactobacillus plantarum* (WCFS1), *Lactococcus lactis* subsp. *lactis* (IL-1403), *Streptococcus pneumoniae* (TIGR4 and R6), *Streptococcus agalactiae* (NEM316 and 2603 V/R), *Streptococcus pyogenes* (MGAS8232, SSI-1, MGAS315, and SF370), and *Streptococcus mutans* (UA159), which originated from publicly available data from whole-genome sequencing projects.

PCR mixtures were composed of 33.5 μl sterile MilliQ water, 5.0 μl PCR buffer (10×), 5.0 μl deoxynucleoside triphosphates (2 mM each), 0.5 μl forward primer (atpA-20-F; 50 μM); 0.5 μl reverse primer (atpA-27-R), 0.5 μl AmpliTaq DNA polymerase (1 U/μl), and 5.0 μl template DNA (0.01 μg/μl). PCR was performed with a GeneAmp PCR system 9600 thermocycler (Applied Biosystems). The thermal program consisted of (i) 5 min at 95°C; (ii) 3 cycles of 1 min

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TABLE 1. Enterococcal strains used in this study

Enterococcal species	Strain no.	Place of isolation	Yr of isolation	Source
E. asini	LMG 18727^{T}			Donkey (*Equus asinus*), cecum
E. avium	LMG 16309	Sweden	1995	Human, sacral wound
	LMG 10744^{T}			Human, faeces
	LMG 12900			Human
	LMG 15118			
E. canis	LMG 12316^{T}	Belgium	1991	Dog, anus
	LMG 21545	Belgium	2000	Dog, anal swabs
	LMG 21555	Belgium	2000	Dog, anal swabs
E. casseliflavus	LMG 10745^{T}			Plant material
	LMG 16286	Belgium	1990–1991	Horse
	LMG 14406	Belgium		Horse manure
E. cecorum	LMG 12902^{T}	Belgium		Chicken, caecum
	LMG 17333	Switzerland	1995	44-year-old woman
E. columbae	LMG 11740^{T}	Belgium		Pigeon
	LMG 12296	Belgium		Pigeon, intestine
	LMG 11747	Belgium		Pigeon
E. dispar	LMG 13521^{T}	United States		Human, synovial fluid
E. durans	LMG 10746^{T}			Dried milk
	LMG 16193			
	LMG 20231	Ireland	1999	Farm, human feces
	LMG 16888			Human, blood
	LMG 16892			Human with septic infection, synovium
E. faecalis	LMG 7937T			
	LMG 17122	Belgium		Pig
	LMG 16301	Belgium	1990–1991	Horse
	LMG 20925	Italy	1971	Raw milk
	LMG 8222			Urine
	LMG 11207			Young chicken, intestine
	LMG 11396			Abscess
	LMG 13566			
E. faecium	LMG 11423T			
	LMG 8147	Sweden	1968	
	LMG 16198	Sweden	1995	Human newborn, umbilical catheter
	LMG 16270	Belgium	1995	Dog
	LMG 20730	The Netherlands	1995	Human, wound
	LMG 20721	Belgium	1997	Human, feces
	LMG 20789	Ireland	1994	Cheddar cheese
	LMG 20769	Greece	1995	Feta cheese
	LMG 20760	Greece	1995	Feta cheese
	LMG 20943	Ireland	1997	Human, faeces
	LMG 20772	Greece	1995	Feta cheese
	LMG 20635	Germany	1990	Swiss cheese salad
	LMG 20909	Italy	1997	Scamorza cheese
	LMG 20630	Burkina Faso	1995	Dawadawa
	LMG 20908	Italy	1997	Scamorza cheese
	LMG 20768	Greece	1995	Feta cheese
E. flavescens	LMG 13518^{T}	Italy	1985	Human suffering from septicemia, blood
	LMG 16313	Italy		Patient suffering from lung cancer, postsurgical abscess
	LMG 16314	Italy		Patient suffering from posttraumatic osteomyelitis of the frontal bone, purulent discharge
E. gallinarum	LMG 13129^{T}			Chicken, intestine
	LMG 12904			
	LMG 16204	Sweden	1995	Human, blood
	LMG 12290	Belgium		Chicken, intestine
	LMG 16197	Belgium		Pheasant
E. gilvus	LMG 21841^{T}	Canada	1998	Patient with cholecystitis, bile
	LMG 13601	Belgium		Tonsil

Continued on following page

TABLE 1—*Continued*

Enterococcal species	Strain no.	Place of isolation	Yr of isolation	Source
E. haemoperoxidus	LMG 19487^T	Czech Republic		Service water
E. hermanniensis	LMG 12317^T	Belgium	1991	Dog, tonsil
	LMG 21989	Finland		Modified atmosphere-packaged broiler legs
E. hirae	LMG 6399^T			
	LMG 20637	South Africa	1996	Ostrich, colon
	LMG 11492			Chicken, crop
	LMG 14260	Belgium		Bovine, mastitis
E. italicus	LMG 22039^T	Italy	2001	Toma cheese
E. malodoratus	LMG 10747T			Gouda cheese
	LMG 15718	Belgium		Pig
	LMG 13601			
	LMG 12302	Belgium	1991	Cat, tonsil
E. moraviensis	LMG 19486^T	Czech Republic	1996	Spring Hájek
E. mundtii	LMG 10748^T			Soil
	LMG 20649	Italy	1997	*Tapes decussatus* (shellfish)
	LMG 20698	Italy	1995	Grass silage
E. pallens	LMG 21842^T	Canada	2000	Peritoneal dialysate from a patient with peritonitis
E. phoeniculicola	LMG 22471^T			
E. pseudoavium	LMG 11426^T	France		Cow, udder
	LMG 11732			
	LMG 20707	The Netherlands	1996	Milk (cow)
E. raffinosus	LMG 12888^T			Blood
	LMG 12172t2			
E. ratti	LMG 21828^T	United States	1992	Neonatal rat with diarrhea
	LMG 21829	United States		Rat
E. saccharolyticus	LMG11427^T			Straw bedding
E. saccharominimus	LMG 21727^T	Belgium	2002	Contaminant of pasteurized cow's milk
	LMG 22196	Romania	2002	Raw cow's milk
	LMG 22197	Morocco	1999	White soft cheese
E. solitarius	LMG 12890^T			Ear exudate
E. sulfureus	LMG 13084^T			Plant material
E. villorum	LMG 12287^T	Canada		Piglet, intestine
	LMG 17496	United States		Pig, intestine
	LMG 19177	United States	1981	2-day-old piglet
	LMG 19179	Korea		Pig, intestine

at 95°C, 2 min 15 s at 55°C, and 1 min 15 s at 72°C; (iii) 30 cycles of 35 s at 95°C, 1 min 15 s at 55°C, and 1 min 15 s at 72°C; and (iv) a final 7 min at 72°C. An annealing temperature of 58°C was used as an exception for a few strains. Amplifications were qualitatively and quantitatively checked by RESult 1% LE Agarose (Biozym, The Netherlands) gel electrophoresis, with SmartLadder included as a reference. Positive PCR results gave a product with the expected size (ca. 1,100 bp), and the products were purified by using the Nucleofast 96 PCR cleanup membrane system (Macherey-Nagel, Germany). Subsequently, 3.0 μl of the purified and concentration-normalized PCR product was mixed with 1.0 μl ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Mix (version 3.1; Applied Biosystems), 3.0 μl sequencing primer (4 μM), 1.5 μl dilution buffer (5×), and 1.5 μl MilliQ water. The primers listed in Table 2 were used for sequencing. The thermal program consisted of 30 cycles of 15 s at 96°C, 1 s at 35°C, and 4 min at 60°C. Sequencing products were purified with a Montage SEQ_{96} sequencing reaction cleanup kit (Millipore). Purified products from the sequencing reactions were recovered in 20 μl of injection solution and mixed with 20 μl deionized formamide. Sample preparation was assisted by use of a Tecan Genesis Workstation 200 (Tecan, Switzerland). Subsequently, separation of the DNA fragments was performed in an ABI PRISM 3100 genetic analyzer (Applied Biosystems). Sample injection was performed for 20 s at 1.25 kV. Each run was performed at 50°C for 6,500 s at 0.1 mA and 12.2 kV. Raw sequence data were transferred to Factura 1.2or6 and AutoAssembler software 1.4.0 (Applied Biosystems), with which consensus sequences were determined by using the six reads. Consensus sequences were imported into BioNumerics 3.0 software (Applied Maths, Belgium), with which a similarity matrix and phylogenetic trees were created on the basis of the maximum-parsimony and neighbor-joining method (30). The reliability of the groups was evaluated by bootstrap analysis with 1,000 resamplings. The 16S rRNA gene sequence data were obtained from EMBL. SplitsTree decomposition analysis was done with software available on the web (http://bibiserv.techfak.uni-bielefeld.de/splits/) (18), while the G+C content, the ratio of the mean number of synonymous substitutions per synonymous site/mean number of nonsynonymous substitutions per nonsynonymous site (d_s/

TABLE 2. Amplification and sequencing primers used in this study

Primer name	Sequence (5′→3′)[a]	Position
atpA-20-F	TAYRTYGGKGAYGGDATYGC	97
atpA-22-F	GCWCCYGGTRTYATGCARCG	397
atpA-23-R	CGYTGCATRAYACCRGGWGC	397
atpA-24-F	GATGAYYTWTCAAARCAAGC	781
atpA-25-R	GCTTGYTTTGAWARRTCATC	781
atpA-27-R	CCRCGRTTHARYTTHGCYTG	1219

[a] R, A or G; Y, T or C; W, A or T; H, A or T or C; D, G or A or T; K, G or T.

d_n) and Sawyer's test were calculated by using the software package START, which was obtained from (http://pubmlst.org/software/analysis/start/) (19).

Nucleotide sequence accession numbers. The EMBL accession numbers of the *atpA* gene sequences are AJ843267 to AJ843313, AJ843315 to AJ843325, AJ843329 to AJ843338, AJ843340, AJ843341, AJ843343, and AJ843345 to AJ843372.

RESULTS AND DISCUSSION

Primers atpA-20-F and atpA-27-R enabled the amplification and final comparison of a 1,102-bp *atpA* fragment of all *Enterococcus* species, which corresponded to 73.5% of the coding region of this gene. The mean ± standard deviation G+C content of the *atpA* genes of the 91 enterococcal strains (43% ± 2%) was consistent with the average G+C content for the total genome of enterococci (20, 28). Correlations and regression curves between pairwise similarities of the *atpA* and the 16S rRNA gene sequences were made by using Pearson's product-moment correlation coefficient. The results showed a significant correlation ($R = 0.7$) between the results obtained with both genes. The *atpA* and 16S rRNA genes had a logarithmic relationship and best fit a polynomial regression of the second degree (Fig. 1). Analysis of the regression curve between the *atpA* and the 16S rRNA gene sequences clearly shows that *atpA* is a phylogenetic marker for enterococci, but it also revealed that *atpA* gene sequences are much more discriminatory than 16S rRNA gene sequences. 16S rRNA gene sequence similarities above 97% and 99% corresponded to *atpA* gene sequence pairwise similarities above 74% and 84%, respectively (Fig. 1). SplitsTree decomposition analysis on the basis of the results for all 91 enterococcal strains resulted in a star-like tree (fit = 56). In addition, Sawyer's test based on the maximum condensed fragments ($P = 1$) did not show evidence of gene conversion in any of the strains. The d_s/d_n ratio for *atpA* for the whole panel of strains was 25.7, suggesting that this locus is under neutral selective pressure. Overall, these analyses did not show evidence of horizontal gene transfer of the *atpA* gene sequences of the *Enterococcus* strains.

The neighbor-joining tree created by use of the *atpA* gene sequences revealed distinct clusters within the genus *Entero-*

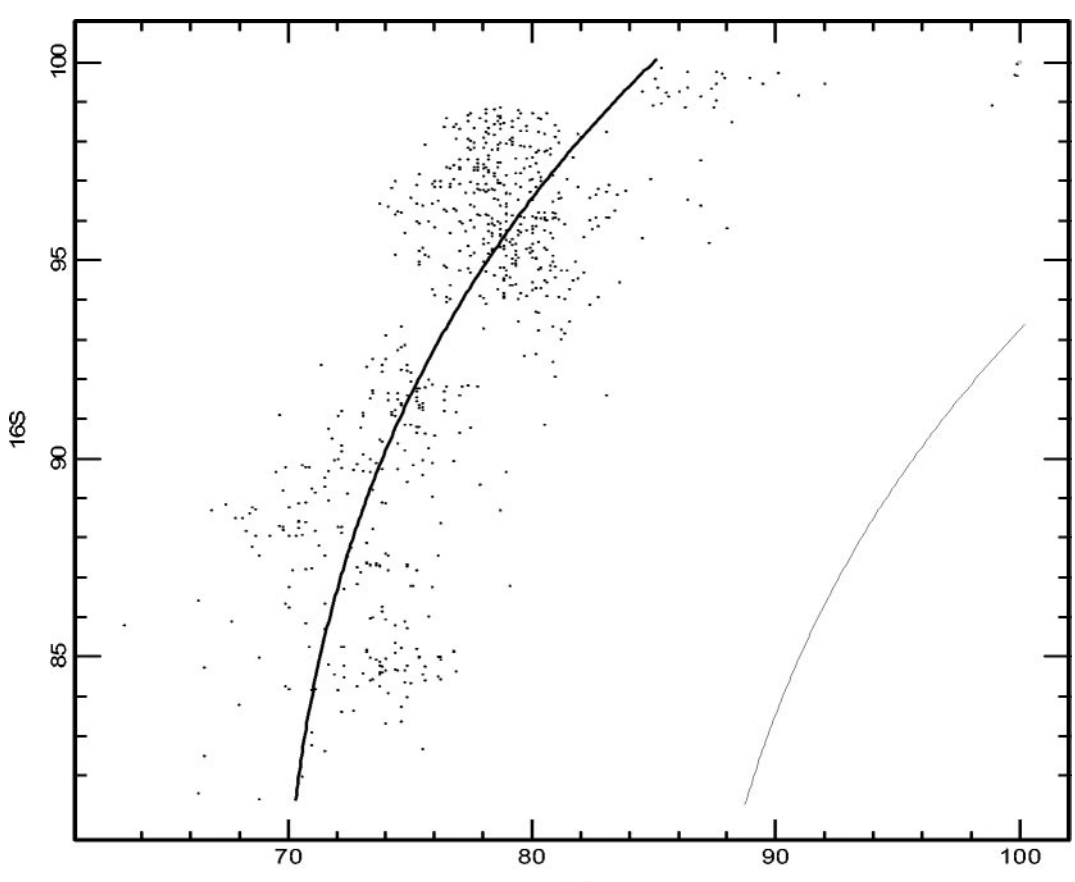

FIG. 1. Regression curve between *atpA* and 16S rRNA pairwise gene sequence similarity.

coccus (Fig. 2). Members of the *E. avium*, *E. faecalis*, *E. casseliflavus*, *E. cecorum*, and, with the exception of *E. canis*, *E. faecium* species groups (20) cluster together in the phylogenetic trees created from both the *atpA* and the 16S rRNA gene sequences. However, the 16S rRNA gene sequences were much less discriminatory for differentiating closely related enterococcal species within the various species groups. Different topologies between the *atpA* and 16S rRNA phylogenetic trees between different species groups were often observed. Maximum values of *atpA* sequence similarity of 92% were observed among the species groups.

Seven members of the *E. faecium* species group (*E. faecium*, *E. canis*, *E. hirae*, *E. durans*, *E. villorum*, *E. mundtii*, and *E. ratti*) showed >98.8% 16S rRNA gene sequence similarity, but highest values of only 89.9% *atpA* gene sequence similarity were observed. The *atpA* gene sequence analysis did not cluster *E. canis* as a member of this species group but showed that it occupied a distinct branch with 80.2% similarity in its *atpA* gene sequence compared to those of *E. asini* 18727^T and *E. dispar* 13521^T. In order to evaluate the intraspecies *atpA* gene sequence similarity, multiple strains of each species and, in particular, 16 well-characterized strains of *E. faecium* were included. Some of the strains were previously extensively studied by AFLP analysis, RAPD analysis, PFGE, and DNA-DNA hybridization studies (37). The phylogenetic tree of the *atpA* gene revealed two subclusters within the *E. faecium* strains with 0.9% and 3.7% sequence divergence, respectively. The two subclusters obtained did not correspond to the two genomic groups, delineated on the basis of AFLP and RAPD analyses by Vancanneyt et al. (37). Homan et al. (17) confirmed an intraspecies *atpA* sequence similarity of about 97% in *E. faecium*. It has also been concluded that horizontal gene transfer plays a role in the long-term evolution of *E. faecium* (17). SplitsTree decomposition analysis of our 16 *E. faecium* strains showed a net-like tree (fit = 78), suggesting that recombination may indeed be an important mechanism in the evolution of the species *E. faecium*.

Within the *E. faecalis* species group, the tree of the *atpA* gene revealed two subclusters, i.e., *E. faecalis* and *E. moraviensis*-*E. haemoperoxidus*. *E. faecalis* was more distantly related to *E. moraviensis* (85%) and *E. haemoperoxidus* (83.3%), and these results confirm the 16S rRNA gene sequencing data. It is worth mentioning that *E. haemoperoxidus* LMG 19487^T and *E. moraviensis* LMG 19486^T have 99.4% 16S rRNA gene sequence similarities but only 92% *atpA* gene sequence similarity. This result further emphasizes the discriminatory power of *atpA* gene sequence analysis.

Analogous congruence between the *atpA* gene- and 16S rRNA gene-based phylogenetic trees was obtained for the other species groups, although the topology of the species within the groups may have been different. Within the *E. avium* species group, all species occupied distinct positions with at a maximum of 89.5% *atpA* gene sequence similarity. The closest neighbors of *E. avium* were *E. malodoratus* (89.5%), *E. gilvus* (87.6%), *E. pseudoavium* (86.9%), *E. hermanniensis* (86.4%), *E. raffinosus* (85.1%), and *E. pallens* (80.5%). Two distinct subclusters were found within the *E. casseliflavus* species group, i.e., *E. gallinarum* and *E. casseliflavus*-*E. flavescens*. *E. casseliflavus* LMG 10745^T and *E. flavescens* LMG 13518^T were highly related to each other (98.9% *atpA* sequence similarity). The corresponding 16S rRNA (27), $sodA_{int}$ (29), *ddl* (25), and *vanC* (9) gene sequences of the *E. casseliflavus* and *E. flavescens* type strains were almost identical (98.8%, 98%, 99.5%, and 96% sequence similarities, respectively). This confirms that *E. flavescens* is not a separate species but, in fact, should be classified as *E. casseliflavus*, in accordance with the findings of previous studies (5, 36). The two species had at a maximum 85.7% gene sequence similarity to *E. gallinarum*. The *E. cecorum* species group comprises *E. cecorum* and *E. columbae*. The type strains of both species had at a maximum 88.1% *atpA* gene sequence similarity.

The recently described species *E. saccharominimus* LMG 21727^T and *E. italicus* LMG 22039^T were highly related, having about 100% 16S rRNA gene sequence similarity and an analogous *atpA* sequence similarity, which suggests that *E. saccharominimus* and *E. italicus* might be synonymous species (10, 39). The 16S rRNA gene-based phylogenetic tree showed that *E. saccharolyticus* LMG 11427^T, *E. sulfureus* LMG 13084^T, *E. saccharominimus* LMG 21727^T, and *E. italicus* LMG 22039^T group in one species group (data not shown), whereas on the basis of the *atpA* gene-based tree, *E. saccharolyticus* LMG 11427^T and *E. sulfureus* LMG 13084^T occupied distinct branches. Also, *E. phoeniculicola* LMG 22471^T and *E. solitarius* LMG 12890^T constituted distinct branches. *E. solitarius* is phylogenetically more closely related to *Tetragenococcus* than to the other enterococci (11, 20).

We report in this study on the identification and phylogenetic positioning of all enterococcal species, which are not clearly distinguishable by their 16S rRNA gene sequences. The 16S rRNA gene is very useful for discriminating the main groups of enterococci, i.e., the *E. avium*, *E. casseliflavus*, *E. cecorum*, *E. faecalis*, and *E. faecium* species groups; but it fails to discriminate closely related species, i.e., the members of *E. faecalis* and *E. faecium* species groups. Consequently, all currently known *Enterococcus* species were clearly differentiated on the basis of their *atpA* sequences (Fig. 2). At the interspecies level, the *atpA* gene sequence similarity was always a maximum of 92% for all species. In order to evaluate the *atpA* gene sequence variation at the intraspecies level, we included several representative strains of each *Enterococcus* species. These strains were selected on the basis of AFLP analysis, SDS-PAGE of whole-cell proteins, and, if they were available, other polyphasic data and represent the known heterogeneity of *Enterococcus* species. With the exception of the *E. faecium* strains, strains of the same species had 98.6 to 100% *atpA* gene sequence similarity; the *E. faecium* strains had 96.3% *atpA* gene sequence similarity. We may therefore conclude that strains of a single species will have at least 96.3% *atpA* sequence similarity. The use of protein-coding gene sequence data for the determination of genomic relatedness at the intra- and interspecies levels has recently been advocated because of its advantages over the banding pattern techniques, i.e., reproducibility and portability (33, 40). For determination of relatedness at the interspecies level, DNA-DNA hybridization presents several inconveniences; i.e., few laboratories can execute this technique, the method is the slowest and the most problematic for the description of species, and the DNA-DNA hybridization data are not cumulative (34). AFLP analysis randomly samples the whole genome and better differentiates closely related strains. At the strain level, *atpA* gene sequence

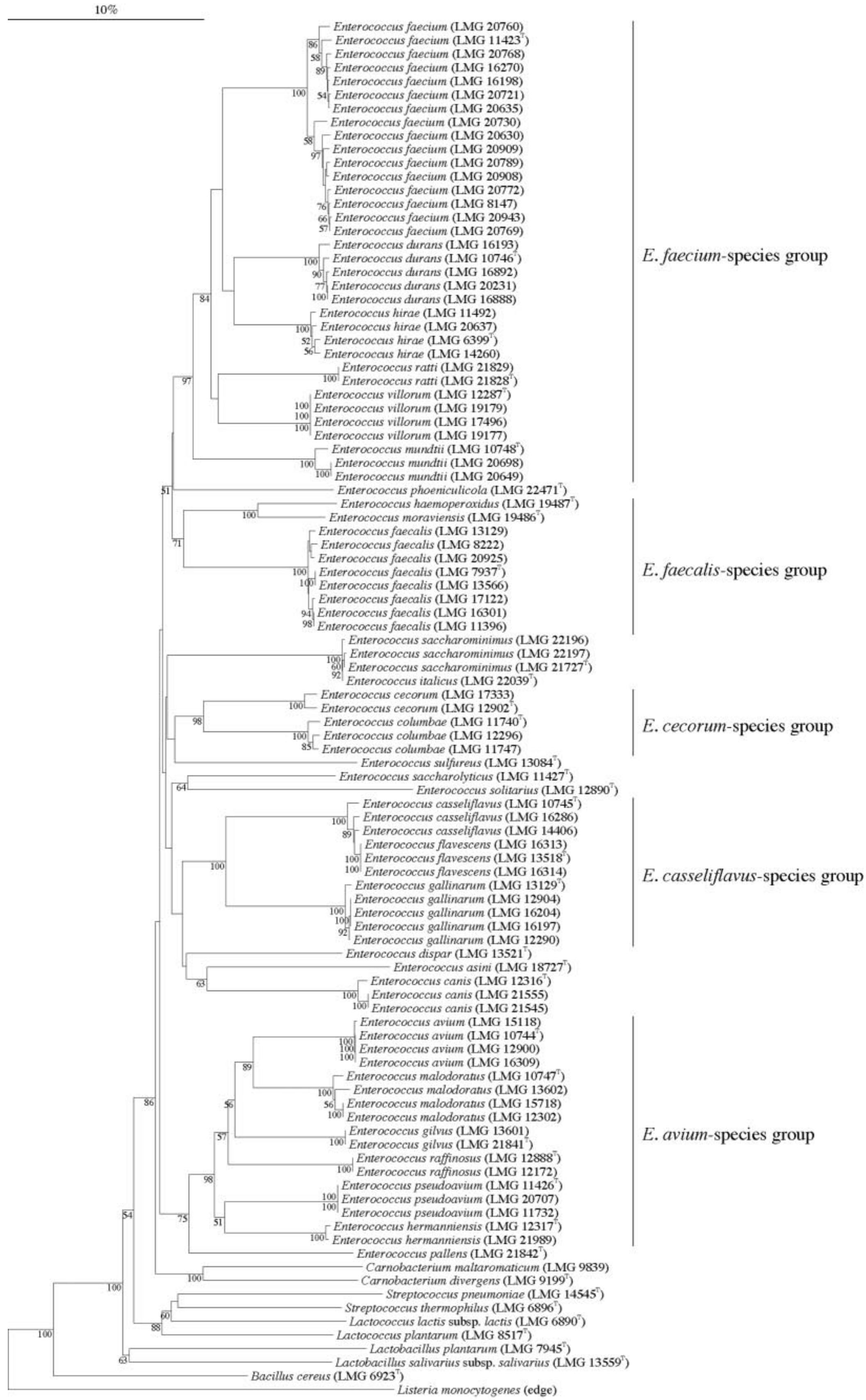

FIG. 2. Neighbor-joining tree based on the *atpA* gene sequences of 91 enterococcal strains. Bootstrap values after 1,000 repetitions are indicated. *Carnobacterium piscicola*, *Carnobacterium divergens*, *Lactococcus plantarum*, *Lactococcus lactis* subsp. *lactis*, *Streptococcus pneumoniae*, *Streptococcus thermophilus*, *Lactobacillus plantarum*, *Lactobacillus salivarius* subsp. *salivarius*, *Bacillus cereus*, and *Listeria monocytogenes* were included as outgroups.

analysis is less discriminatory than AFLP analysis; strains with identical *atpA* sequences had 70 to 93% AFLP pattern similarities. Although it is not valuable for species differentiation, PFGE appeared to be superior for interpretation of intraspecies relationships (5). We conclude that *atpA* sequence analysis can be used as an alternative to currently used techniques for the identification and phylogenetic analysis of clinically important enterococcal species. In addition, our data may be useful for the rapid detection of *Enterococcus* by using, e.g., real-time PCR.

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REFERENCES

1. **Angeletti, S., G. Lorino, G. Gherardi, F. Battistoni, M. De Cesaris, and G. Dicuonzo.** 2001. Routine molecular identification of enterococci by gene-specific PCR and 16S ribosomal DNA sequencing. J. Clin. Microbiol. **39:** 794–797.
2. **Baele, M., P. Baele, M. Vaneechoutte, V. Storms, P. Butaye, L. A. Devriese, G. Verschraegen, M. Gillis, and F. Haesebrouck.** 2000. Application of tRNA intergenic spacer PCR for identification of *Enterococcus* species. J. Clin. Microbiol. **38:**4201–4207.
3. **Behr, T., C. Koob, M. Schedl, A. Mehlen, H. Meier, D. Knopp, E. Frahm, U. Obst, K. Schleifer, R. Niessner, and W. Ludwig.** 2000. A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. Syst. Appl. Microbiol. **23:**563–572.
4. **De Graef, E. M., L. A. Devriese, M. Vancanneyt, M. Baele, M. D. Collins, K. Lefebvre, J. Swings, and F. Haesebrouck.** 2003. Description of *Enterococcus canis* sp. nov. from dogs and reclassification of *Enterococcus porcinus* Teixeira et al. 2001 as a junior synonym of *Enterococcus villorum* Vancanneyt et al 2001. Int. J. Syst. Evol. Microbiol. **53:**1069–1074.
5. **Descheemaeker, P., C. Lammens, B. Pot, P. Vandamme, and H. Goossens.** 1997. Evaluation of arbitrarily primed PCR analysis and pulsed-field gel electrophoresis of large genomic DNA fragments for identification of enterococci important in human medicine. Int. J. Syst. Bacteriol. **47:**555–561.
6. **Devriese L. A., M. Baele, and P. Butaye.** 2002. The genus *Enterococcus.* In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and W. Stackebrandt (ed.), The prokaryotes, 3rd ed. Springer, Heidelberg, Germany. (Electronic edition, http://link. springer.de/link/service/books/10125/.)
7. **Devriese, L. A., M. Vancanneyt, P. Descheemaeker, M. Baele, H. W. Van Landuyt, B. Gordts, P. Butaye, J. Swings, and F. Haesebrouck.** 2002. Differentiation and identification of *Enterococcus durans*, *E. hirae* and *E. villorum.* J. Appl. Microbiol. **92:**821–827.
8. **Domig, K. J., H. K. Mayer, and W. Kneifel.** 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. Int. J. Food Microbiol. **88:**165–188.
9. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33:**24–27.
10. **Fortune, M. G., G. Ricci, D. Mora, and P. L. Manachini.** 2004. Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov. Int. J. Syst. Evol. Microbiol. **54:**1717–1721.
11. **Franz, C. M., W. H. Holzapfel, and M. E. Stiles.** 1999. Enterococci at the crossroads of food safety? Int. J. Food Microbiol. **47:**1–24.
12. **Franz, C. M., M. E. Stiles, K. H. Schleifer, and W. H. Holzapfel.** 2003. Enterococci in foods—a conundrum for food safety. Int. J. Food Microbiol. **88:**105–122.
13. **Gevers, D., G. Huys, and J. Swings.** 2001. Applicability of rep-PCR fingerprinting for identification of Lactobacillus species. FEMS. Microbiol. Lett. **205:**31–36.
14. **Gevers, D., K. Vandepoele, C. Simillon, and Y. Van de Peer.** 2004. Gene duplication and biased functional retention of paralogs in bacterial genomes. Trends Microbiol. **12:**148–154.
15. **Harris, J. K., S. T. Kelley, G. B. Spiegelman, and N. R. Pace.** 2003. The genetic core of the universal ancestor. Genome Res. **13:**407–412.
16. **Holzapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger.** 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr. **73:**365S–373S.
17. **Homan, W. L., D. Tribe, S. Poznanski, M. Li, G. Hogg, E. Spalburg, J. D. Van Embden, and R. J. Willems.** 2002. Multilocus sequence typing scheme for *Enterococcus faecium*. J. Clin. Microbiol. **40:**1963–1971.
18. **Huson, D. H.** 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics **14:**68–73.
19. **Jolley, K. A., E. J. Feil, M. S. Chan, and M. C. Maiden.** 2001. Sequence type analysis and recombinational tests (START). Bioinformatics **17:**1230–1231.
20. **Klein, G.** 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. Int. J. Food Microbiol. **88:**23–31.
21. **Koort, M. K., T. Coenye, P. Vandamme, A. Sukura, and J. Björkroth.** 2004. *Enterococcus hermanniensis* sp. nov., from atmosphere packaged broiler meat and canine tonsils. Int. J. Syst. Evol. Microbiol. **54:**1823–1827.
22. **Law-Brown, J., and P. R. Meyers.** 2003. *Enterococcus phoeniculicola* sp. nov., a novel member of the enterococci isolated from the uropygial gland of the red-billed Woodhoopoe, *Phoeniculus purpureus.* Int. J. Syst. Evol. Microbiol. **53:**683–685.
23. **Lerat, E., V. Daubin, and N. A. Moran.** 2003. From gene trees to organismal phylogeny in prokaryotes: the case of the gamma-proteobacteria. PLoS Biol. **1:**101–109.
24. **Muller, V., and G. Gruber.** 2003. ATP synthases: structure, function and evolution of unique energy converters. Cell. Mol. Life Sci. **60:**474–494.
25. **Navarro, F., and P. Courvalin.** 1994. Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens.* Antimicrob. Agents Chemother. **38:**1788–1793.
26. **Ozawa, Y., P. Courvalin, and M. Gaiimand.** 2000. Identification of enterococci at the species level by sequencing of the genes for D-alanine:D-alanine ligases. Syst. Appl. Microbiol. **23:**230–237.
27. **Patel, R., K. E. Piper, M. S. Rouse, J. M. Steckelberg, J. R. Uhl, P. Kohner, M. K. Hopkins, F. R. Cockerill, and B. C. Kline.** 1998. Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. J. Clin. Microbiol. **36:** 3399–3407.
28. **Paulsen, I. T., L. Banerjei, G. S. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:** 2071–2074.
29. **Poyart, C., G. Quesnes, and P. Trieu-Cuot.** 2000. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. J. Clin. Microbiol. **38:**415–418.
30. **Saitou, N., and M. Nei.** 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
31. **Schleifer, K. H., and R. Kilpper-Bälz.** 1984. Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. Int. J. Syst. Bacteriol. **34:**31–34.
32. **Sedgley, C. M., S. L. Lennan, and D. B. Clewell.** 2004. Prevalence, phenotype and genotype of oral enterococci. Oral Microbiol. Immunol. **19:**95–101.
33. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. Grimont, P. Kampfer, M. C. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology. Int. J. Syst. Evol. Microbiol. **52:**1043–1047.
34. **Stackebrandt, E.** 2003. The richness of prokaryotic diversity: there must be a species somewhere. Food Technol. Biotechnol. **41:**17–22.
35. **Teixeira, L. M., V. L. Merquior, M. C. Vianni, M. G. Carvalho, S. L. Fracalanzza, A. G. Steigerwalt., D. J. Brenner, and R. R Facklam.** 1996. Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida.* Int. J. Syst. Bacteriol. **46:**664–668.
36. **Teixeira, L. M., M. G. Carvalho, V. L. Merquior, A. G. Steigerwalt, M. G. Teixeira, D. J. Brenner, and R. R. Facklam.** 1997. Recent approaches on the taxonomy of the enterococci and some related microorganisms. Adv. Exp. Med. Biol. **418:**397–400.
37. **Vancanneyt, M., A. Lombardi, C. Andrighetto, E. Knijff, S. Torriani, K. J. Bjorkroth, C. M. Franz, M. R. Foulquie Moreno, H. Revets, L. De Vuyst, J. Swings, K. Kersters, F. Dellaglio, and W. H. Holzapfel.** 2002. Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. Appl. Environ. Microbiol. **68:**1381–1391.
38. **Vancanneyt, M., C. Snauwaert, I. Cleenwerck, M. Baele, P. Descheemaeker, H. Goossens, B. Pot, P. Vandamme, J. Swings, F. Haesebrouck, and L. A. Devriese.** 2001. *Enterococcus villorum* sp. nov., an enteroadherent bacterium associated with diarrhoea in piglets. Int. J. Syst. Evol. Microbiol. **51:**393–400.
39. **Vancanneyt, M., M. Zamfir, L. A. Devriese, K. Lefebvre, K. Engelbeen, K. Vandemeulebroecke, M. Amar, L. De Vuyst, F. Haesebrouck, and J. Swings.** 2004. *Enterococcus saccharominimus* sp. nov., from dairy products. Int. J. Syst. Evol. Microbiol. **54:**2175–2179.
40. **Zeigler, D. R.** 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int. J. Syst. Evol. Microbiol. **53:**1893–1900.