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Multilocus Sequence Typing Scheme for *Enterococcus faecium*

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A multilocus sequence typing (MLST) scheme has been developed for *Enterococcus faecium*. Internal fragments from seven housekeeping genes of 123 epidemiologically unlinked isolates from humans and livestock and 16 human-derived isolates from several outbreaks in the United States, the United Kingdom, Australia, and The Netherlands were analyzed. A total of 62 sequence types were detected in vancomycin-sensitive *E. faecium* (VSEF) and vancomycin-resistant *E. faecium* (VREF) isolates. VSEF isolates were genetically more diverse than VREF isolates. Both VSEF and VREF isolates clustered in host-specific lineages that were similar to the host-specific clustering obtained by amplified fragment length polymorphism analysis. Outbreak isolates from hospitalized humans clustered in a subgroup that was defined by the presence of a unique allele from the housekeeping gene *purK* and the surface protein gene *esp*. The MLST results suggest that epidemic lineages of *E. faecium* emerged recently worldwide, while genetic variation in both VREF and VSEF was created by longer-term recombination. The results show that MLST of *E. faecium* provides an excellent tool for isolate characterization and long-term epidemiologic analysis.

Vancomycin-resistant *Enterococcus faecium* (VREF) has recently emerged as an important threat in U.S. hospitals (5, 24). In Europe, VREF isolates are found relatively frequently in the community and farm animals, while prevalence in hospitals is generally low (14). The latter observation was explained by the use of the glycopeptide avoparcin as an antimicrobial growth promoter in animal feeding operations.

Several molecular typing schemes have been developed to study the epidemiology of VREF. Of these, pulsed-field gel electrophoresis analysis of genomic restriction fragments has been considered the "gold standard" for the study of hospital outbreaks because of its high degree of isolate differentiation (15, 17, 20, 23). However, due to this high degree of isolate differentiation, pulsed-field gel electrophoresis typing is less suitable for determining the degree of relatedness among epidemiologically unrelated isolates. Recently, amplified fragment length polymorphism (AFLP) analysis was applied as a new method for the typing of VREF (1, 33). AFLP analysis is a robust and fast typing technique with high intra- and inter-experimental reproducibilities and appears to be discriminatory enough for the recognition of hospital outbreaks (1, 32, 33). In addition, AFLP analysis has allowed the detection of associations among different *E. faecium* genetic lineages and different human and animal hosts (33), suggesting the existence of host-specific VREF lineages. Whether this is also true for vancomycin-sensitive *E. faecium* (VSEF) is not known, since VSEF isolates were not included in that study. AFLP typing also disclosed two different human-associated lineages. One lineage comprised epidemic-related isolates recovered from hospitalized patients, while isolates of the other lineage were mainly from nonhospitalized persons. Interestingly, the epidemic lineage was characterized by the presence of the *esp* virulence gene (32). Recently, the existence of host-specific lineages and an epidemic *esp*-bearing lineage was confirmed by ribotyping analysis of VREF isolates (4).

Although AFLP data generated within a given laboratory appear reproducible and were successfully used in identifying clusters of closely related *E. faecium* isolates, the method may be less suitable for global epidemiologic analysis. Variations in band intensities can easily occur and can be a source of ambiguities when curve-based similarity coefficients, such as the Pearson correlation, are used to compare banding patterns (27).

An unambiguous international database of *E. faecium* genetic lineages could be a powerful resource for global epidemiologic study, recognition, and tracking the worldwide inter-hospital spread of virulent, epidemic, and multiresistant clones. The most appropriate technique for such studies is multilocus sequence typing (MLST), as MLST is based on identifying alleles from DNA sequences of internal fragments of housekeeping genes. This technique is preeminently useful for electronic data exchange. MLST has been used successfully for the study of the molecular epidemiology and the exploration of the population structure and evolution of virulence of various bacterial species (6, 8, 10, 11, 18, 22).

In this study, we describe an MLST scheme for *E. faecium* based on the nucleotide sequences of seven housekeeping genes. Previous studies focused only on vancomycin-resistant isolates; therefore, we included vancomycin-sensitive isolates as well. We show that MLST discerns the same main genetic lineages as were previously disclosed by ALFP typing and that VSEF isolates are grouped within these lineages. Furthermore, we show that epidemic VREF isolates have been disseminated

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TABLE 1. Origins and characteristics of *E. faecium* isolates

Origin	No. of isolates								
	Total	VSEF[b]	VREF		From[a]:				
			vanA	*vanB*[c]	NL	Aus	UK	USA	F
Healthy humans	17	7	10	0	17	0	0		
Hospitalized humans[d]	50	14	26	10	21	19	3	4	3
Calves	18	5	13	0	18				
Pigs	9	3	6	0	8		1		
Poultry	24	12	12	0	17	6	1		
Cats or Dogs	5	0	5	0	5				
Total	123	41	72	10	86	25	5	4	3

[a] NL, The Netherlands; Aus, Australia; UK, United Kingdom; USA, United States; F, France.
[b] Except for nine isolates from hospitalized humans and six isolates from poultry in Australia, all VSEF isolates were from The Netherlands.
[c] One *vanB*-carrying isolate was from a hospitalized patient in the United States; the remainder were from Australia.
[d] Isolates from hospitalized humans included 10 outbreak-associated (epidemic) isolates, 29 isolates from clinical sites without a known linkage to outbreaks, and 11 fecal isolates from hospital screenings.

worldwide in the recent past and that recombination plays an important role in the generation of diversity in *E. faecium*.

MATERIALS AND METHODS

Bacterial isolates and bacteriological determinations. A total of 123 *E. faecium* isolates were used for establishing the MLST scheme; the main properties of these isolates are described in Table 1. These isolates were not known to have an epidemiological link (32). Isolates from nonhospitalized humans and animals were derived from the feces of healthy hosts. Isolates from hospitalized humans consisted of representative clinical isolates from hospital outbreaks in the United States, the United Kingdom, The Netherlands, and Australia; isolates from clinical sites not related to outbreaks; and fecal isolates from screenings in hospitals. The samples included previously described vancomycin-resistant isolates (33). Another 16 isolates were derived from hospitalized individuals involved in the outbreaks. Species identification of *E. faecium* was performed by D-alanine:D-alanine ligase (*ddl*) gene-specific PCR (7). Susceptibility testing for vancomycin and determination of *vanA* and *vanB* genes were performed as described earlier (33).

MLST. Seven housekeeping loci were selected for the characterization of *E. faecium* isolates by MLST (Table 2). The choice of these housekeeping genes was based on their putative function, on their use in MLST schemes for other bacterial species (9, 12, 31) and, in most cases, on the availability of sequence data from *E. faecalis*. These loci are separated by at least 160 kb in *E. faecalis* (preliminary sequence data from The Institute for Genomic Research; www.tigr.org). Although the complete sequence of the *E. faecium* genome has not yet been determined, all seven housekeeping genes are located on different *E. faecium* contigs; therefore, these loci are likely to be genetically unlinked. With the exception of the sequences of the primers for D-alanine:D-alanine ligase (*ddl*), the primer sequences for the housekeeping genes were taken from homologues in the *E. faecalis* genome database (The Institute for Genomic Research).

Internal 400- to 600-bp fragments of the following genes were amplified by PCR: *adk* (adenylate kinase), *atpA* (ATP synthase, alpha subunit), *ddl* (D-alanine:D-alanine ligase), *gyd* (glyceraldehyde-3-phosphate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *purK* (phosphoribosylaminoimidazol carboxylase ATPase subunit), and *pstS* (phosphate ATP-binding cassette transporter). Fragments were amplified from bacterial lysates by PCR with the following sets of primers: adk, 5′-TAT GAA CCT CAT TTT AAT GGG-3′, and adk2, 5′-GTT GAC TGC CAA ACG ATT TT-3′; atpA1, 5′-CGG TTC ATA CGG AAT GGC ACA-3′, and atpA2, 5′-AAG TTC ACG ATA AGC CAC GG-5′; ddl1, 5′-GAG ACA TTG AAT ATG CCT TAT G-3′, and ddl2, 5′-AAA AAG AAA TCG CAC CG-3′; gdh1, 5′-GGC GCA CTA AAA GAT ATG GT-3′, and gdh2, 5′-CCA AGA TTG GGC AAC TTC GTC CCA-3′; gyd1, 5′-CAA ACT GCT TAG CTC CAA GG C-3′, and gyd2, 5′-CAT TTC GTT GTC ATA CCA AGC-3′; purK1, 5′-GCA GAT TGG CAC ATT GAA AGT-3′, and purK2, 5′-TAC ATA AAT CCC CCT GTT TY-3′; and pstS1, 5′-TTG AGC CAA GTC GAA GCT GGA G-3′, and pstS2, 5′-CGT GAT CAC GTT CTA CTT CC-3′.

PCR conditions for all amplification reactions were as follows: initial denaturation at 94°C for 3 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and extension at 72°C for 5 min. Reactions were performed in 50-μl volumes with buffers and *Taq* polymerase from SphaeroQ (Leiden, The Netherlands). PCR products were purified with a PCR purification kit from Qiagen Inc. (Hilden Germany) and sequenced with PCR forward or reverse primers, an ABI PRISM Big Dye Cycle Sequencing Ready Reaction kit (Perkin-Elmer,

TABLE 2. Allelic variation in seven housekeeping genes from VSEF and VREF isolates

Locus	Sequence length (bp)	No. of alleles in the following strains[a]:				Avg no. of nucleotide differences between alleles (n = 123)
		All (n = 123)	VSEF (n = 41)	VREF		
				vanA (n = 72)	*vanB* (n = 10)	
gdh	530	12 (9)	11 (9)	5	2	17.0
purK	492	12 (9)	11 (9)	6	2	14.5
pstS	583	17 (12)	14 (11)	5	2	17.6
atpA	556	17 (13)	15 (12)	9	2	11.0
gyd	395	11 (6)	7 (4)	5	2	3.0
adk	437	7 (6)	4 (4)	4	2	2.8
ddl	465	9 (7)	8 (7)	5	2	6.5
Total no. of alleles		85 (62)	70 (56)	39		
Mean allelic diversity		0.6 (0.57)	0.71 (0.64)	0.53		

[a] Values in parentheses are the numbers of alleles determined with the exclusion of the genetically divergent isolates (118 and 37 isolates in the all and VSEF isolate groups, respectively).

Applied Biosystems, Foster City, Calif.), and an ABI 3700 DNA sequencer (Perkin-Elmer). Different sequences of a given locus were given allele numbers, and each unique combination of alleles (the allelic profile) was assigned a sequence type (ST).

AFLP analysis and sequence analysis of 16S ribosomal DNA were performed by previously described methods (3, 33). 16S ribosomal DNA sequences were analyzed by a BLAST search of the GenBank database.

Computer analysis of MLST data. Clustering of 123 isolates from the matrix of pairwise similarities between the allelic profiles was performed with BioNumerics software (Applied Maths) by the unweighted pair-group method with arithmetic averages (UPGMA) and the categorical coefficient of similarity.

The MEGA suit of programs (version 2.1; http://www.megasoftware.net) (21) was used to calculate average numbers of nucleotide differences between alleles and to construct gene trees by the neighbor-joining (NJ) method. The significance of branching of the NJ trees was evaluated by bootstrap analysis of 500 computer-generated trees.

Ratios of nonsynonymous to synonymous substitutions were calculated to test the degree of selection operating on a locus by using START (http://www.mlst.net). Allelic diversity was calculated with the equation $[n/(n-1)](1-\Sigma x_i^2)$, where x_i is the frequency of the ith allele and n is the number of isolates (25).

The measure of linkage equilibrium between alleles at the seven housekeeping genes was assessed by calculating the index of association (I_a) with the program at the MLST website (http://www.mlst.net). The I_a is defined as the observed variance in the distribution of allelic mismatches in all pairwise comparisons of the allelic profiles divided by the expected variance in a freely recombining population, minus 1 (30). When the alleles are in linkage equilibrium, the I_a is expected not to deviate significantly from zero. The significance of I_a was estimated by comparing the observed variance obtained from the actual data with the maximum variance calculated from 1,000 data sets under the assumption of the random association of loci. Significant linkage disequilibrium was established when the observed variance obtained from the actual data was greater than the calculated maximum variance after 1,000 randomizations ($P < 0.001$).

Nucleotide sequence accession numbers. The sequences of the alleles from the *E. faecium* housekeeping genes have been given the following GenBank accession numbers: AF443299 to AF443305 (*adk*); AF443306 to AF443322 (*atpA*); AF443323 to AF443331 (*ddl*); AF443332 to AF443343 (*gdh*); AF443344 to AF443354 (*gyd*); AF443355 to AF443367 (*purK*); and AF443368 to AF443384 (*pstS*).

RESULTS

Allelic variation in *E. faecium*. The set of *E. faecium* isolates subjected to MLST analysis comprised 123 epidemiologically unlinked isolates (41 vancomycin sensitive and 72 *vanA* and 10 *vanB* containing) originating from hospitalized and nonhospitalized humans, veal calves, pigs, poultry, dogs, and cats. The number of unique alleles found for each of the seven housekeeping genes ranged from 7 for *adk* to 17 for *pstS* and *atpA* (Table 2). The average number of nucleotide differences between alleles of a given locus varied from 2.8 (for *adk*) to 17.6 (for *pstS*) among the 123 *E. faecium* isolates analyzed. The variations in the sequences extended over the whole stretch of the sequenced portion of each of the seven genes investigated, as shown in Tables 3 and 4 for the *gdh* and *atpA* allelic variants. Most polymorphisms resulted in synonymous substitutions. The ratios of nonsynonymous to synonymous substitutions varied from 0 for *atpA* and *ddl* to 0.07 for *pstS*. The average ratio for all loci was 0.02 (results not shown). These low ratios indicate a very limited contribution of environmental selection to the sequence variations in the seven housekeeping genes used in this study; therefore, these housekeeping genes are assumed to be suitable for a population-genetic study.

Dendrograms of the alleles of the separate housekeeping gene targets are shown in Fig. 1. Interestingly, a clear bifurcation into majority and minority allelic populations was observed for all the genes, except for *atpA*. The alleles of *atpA* also clustered into two populations, but the populations had

TABLE 3. Polymorphic nucleotide sites in the *gdh* locus[a]

gdh allele	No. of strains	Nucleotide at polymorphic site: 3	42	45	60	69	72	105	108	129	135	147	150	156	189	195	207	219	222	237	246	255	264	270	285	297	303	312	360	369	374	375	396	424	432	444	447	450	468	474	481	492	510
1	89	G	G	C	T	T	T	C	G	G	T	G	C	T	A	C	C	T	A	T	A	C	T	G	C	A	A	T	T	C	T	T	T	G	G	A	A	C	G	A	A	C
2	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
11	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.
5	11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
4	1	A	A	.	A	C	C	T	A	A	C	A	T	A	C	A	.	C	T	C	G	.	.	T	T	G	G	.	.	C	.	G	C	C	A	T	G	G	.	.	.	.	.
8	3	A	.	.	A	C	C	T	A	A	C	A	T	A	C	A	.	C	T	C	G	A	.	T	T	G	G	.	.	C	.	G	C	C	A	T	.	G	.	A	C	.	.
9	1	A	.	T	A	C	.	T	A	A	C	A	T	A	C	A	.	C	T	C	G	G	.	T	T	.	G	.	.	C	.	G	C	C	A	T	.	G	.	A	C	.	.
10	1	A	.	.	A	C	C	T	A	A	C	A	T	A	C	C	A	T	C	T	C	G	A	.	T	T	G	G	.	C	.	G	C	C	A	T	.	G	.	A	C	.	.

[a] Nucleotides present in the variable sites are shown for the first allele. In the other alleles, only the sites that differ are shown. A period means that the site is the same as that in the first allele.

TABLE 4. Polymorphic nucleotide sites in the *atpA* locus[a]

atpA allele	No. of strains	Nucleotide at polymorphic site:																														
		59	62	95	98	107	122	128	176	188	230	239	242	248	254	266	269	284	314	320	323	329	335	347	479	482	485	527	539	542	551
5	19	T	G	C	T	C	A	C	G	T	G	G	C	C	G	C	A	C	T	G	C	G	C	T	A	A	A	A	C	T	A
16	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
10	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
9	12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
13	2	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
8	15	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.
17	1	.	.	.	.	T	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	.
6	1	.	.	.	.	.	.	.	.	C	.	.	T	T	.	.	.	.	.	A	.	A	.	.	.	.	G	.	.	.	.
11	1	.	A	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	G	.	T	.	.
4	17	A	.	.	A	T	G	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	G	G	.	.	C
7	3	A	.	.	A	T	G	.	A	C	T	A	.	T	A	T	C	T	.	.	.	.	.	.	G	G	G	G	.	.	C
3	9	A	.	.	A	T	G	.	A	C	T	A	.	T	A	.	C	T	.	.	.	.	.	.	G	G	G	G	.	.	C
12	1	A	.	.	A	T	G	.	A	C	T	A	.	T	A	.	C	T	C	.	.	.	.	.	G	G	G	G	.	.	C
2	13	A	.	.	A	T	G	.	A	C	T	A	.	T	A	.	C	T	C	.	.	.	.	.	G	G	G	G	.	G	C
14	1	A	.	T	A	T	G	T	A	C	T	A	.	T	A	.	C	T	C	.	.	.	.	.	G	G	G	G	.	.	C
1	24	A	.	T	A	T	G	T	A	C	T	A	.	T	A	.	C	T	C	.	.	.	.	.	G	G	G	G	.	G	C

[a] Nucleotides present in the variable sites are shown for the first allele. In the other alleles, only the sites that differ are shown. A period means that the site is the same as that in the first allele.

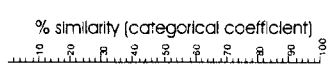
approximately equal numbers of isolates and both comprised VREF and VSEF isolates. The significance of the observed bifuractions is strongly supported by bootstrap analysis. The average numbers of nucleotide differences between the majority and the minority group populations were 30, 29, 33, 10, 4.3, and 4.1 for *purK*, *pstS*, *gdh*, *ddl*, *adk*, and *gyd*, respectively. Only five isolates were shared among the minority allelic populations of these six housekeeping genes. Based on MLST data, these five isolates, four VSEF isolates and one *vanB*-harboring *E. faecium* isolate, are most distantly related to the other isolates, clustering at the bottom of the dendrogram constructed from the allelic profiles (Fig. 2, isolates represented by STs 39, 60, 40, 61, and 62). They differed in six of the seven alleles from all of the remaining isolates investigated in this study. Furthermore, these isolates also clustered separately from the four major lineages determined by AFLP analysis (data not shown). Therefore, these isolates are genetically the most divergent of the isolates analyzed in this study, and much of the allelic variation shown in Table 2 is due to variations in these five genetically divergent isolates.

To test whether these five isolates might have been misdiagnosed as *E. faecium* by the standard bacteriological determination, sequencing of their 16S rRNA genes was undertaken. This analysis revealed that the three isolates with STs 40, 60, and 61 differed in only one residue from both *E. faecium* and *E. durans*, whereas the sequences of isolates with ST 39 and ST 62 were identical to the sequence of *E. durans*. However, species determination by *ddl* gene analysis, a method generally used for enterococci (7, 26), grouped the sequences of the five divergent isolates in a cluster having 98% similarity with all *E. faecium ddl* sequences, well separated (83% similarity) from the *E. durans* sequence (GenBank accession number AF170804) and the *E. hirae* sequence (GenBank accession number U39788) (results not shown). Furthermore, all polymorphisms in the *ddl* alleles were silent, coding for peptide fragments with identical amino acid compositions which differed by 3% from the compositions of the fragments in *E. durans* and *E. hirae*. These data suggest that three of the divergent isolates can be considered *E. faecium* and that classification of two of the isolates is ambiguous.

Genetic diversity of VSEF and VREF isolates. Among the 123 epidemiologically unlinked isolates investigated, 62 STs were found; the majority of these, 44 STs, were represented by single isolates (Fig. 2). The types most frequently encountered were ST 17 (17 isolates), ST 6 (14 isolates), ST 4 (9 isolates), and ST 16 (6 isolates). Isolates with ST 16 and ST 17, which shared six of the seven housekeeping alleles, were from hospitalized individuals. All ST 16 isolates and two of the ST 17 isolates were representatives from outbreaks; the remaining ST 17 isolates were taken from clinical sites and were not known to have an epidemiological link. Three of the 14 ST 6 isolates were from feces derived from hospitalized humans and were not outbreak related. The remaining ST 6 isolates originated from healthy humans (six isolates) and pigs (five isolates). All ST 4 isolates originated from calves (Fig. 2).

Thirty-six different STs were found among 41 VSEF isolates, and 31 were found among 82 VREF isolates. VSEF and VREF isolates sharing the same ST were present only among the five allelic types ST 4, ST 5, ST 6, ST 17, and ST 44; however, the majority of the isolates belonging to these STs (40 of 47 isolates) were vancomycin resistant. The most prevalent allelic type, ST 17, was the only one comprising both *vanA*- and *vanB*-carrying isolates (Fig. 2). Although the number of VSEF isolates ($n = 41$) examined was considerably smaller than the number of *vanA*-carrying *E. faecium* isolates ($n = 72$), the VSEF isolates contributed disproportionately more to the allelic diversity of the whole sample of isolates analyzed: 70 different alleles were present among the vancomycin-sensitive isolates, and only 39 were identified among the *vanA*-carrying isolates. Consistent with this difference is the higher mean allelic diversity value of the vancomycin-sensitive isolates (0.71) than of the *vanA*-carrying isolates (0.53) (Table 2). Even after exclusion of the five divergent isolates, the vancomycin-sensitive isolates were genetically more diverse than the van-

FIG. 1. Dendrograms showing the genetic relationships among the allele sequences of individual loci used for MLST. The trees were established by analysis of the allele sequences from the seven housekeeping loci by the NJ method. Numbers of VSEF and VREF strains in the different clusters are given to the right of the dendrograms. The scale bar indicates five nucleotide differences. Dots indicate the alleles associated with allele *purK6*. Bootstrap values of greater than 90% are indicated.



ST	No. of strains	AFLP group	Host	Presence of VanA/B	Presence of esp	allelic profile adk	atpA	ddl	gyd	gdh	purK	pstS	country
1	2	D	C	A	no	5	8	4	1	5	7	1	2 NL
2	1	B	C	A	no	5	8	4	9	5	7	1	1 NL
3	1	D	C	A	no	5	8	4	1	5	7	12	1 NL
43	1	D	C	S	no	5	3	4	1	5	7	1	1 NL
4	9	D	C	8 A, 1 S	no	5	8	4	1	1	3	1	9 NL
41	2	D,B	C	S	no	5	8	4	1	5	3	1	2 NL
42	1	A	H	S	no	5	17	4	1	1	13	1	1 NL
59	1	A	H	S	no	5	14	4	1	10	6	15	1 NL
5	5	A	2 HF, 1 H, 2 S	4 A,1 S	no	5	5	2	1	1	9	1	5 NL
6	14	A	3 HF, 6 H, 5 S	13 A, 1S	no	5	4	2	1	1	9	1	13 NL, 1 UK
7	1	A	H	A	no	5	4	2	1	1	2	1	1 NL
44	2	A	HF, C	1 A, 1 S	no	5	3	2	1	12	9	1	2 NL
45	1	A	S	S	no	5	5	5	1	1	6	1	1 NL
46	1	A	HF	S	no	6	5	2	1	1	11	14	1 NL
47	1	A	H	S	no	5	9	2	1	1	11	14	1 NL
8	3	B	P	A	no	1	5	2	1	1	6	7	3 NL
9	2	B	H, P	A	no	1	5	2	1	6	6	7	1 NL, 1 UK
11	1	B	P	A	no	1	5	2	1	7	6	1	1 NL
10	1	B	P	A	no	1	5	2	1	1	6	1	1 NL
12	1	B	P	A	no	1	5	2	1	6	6	1	1 NL
13	3	B	P	A	no	1	9	2	7	1	6	7	3 NL
14	1	B	P	A	no	1	9	2	1	7	6	7	1 NL
26	1	B	P	A	no	1	9	2	1	1	6	1	1 NL
48	1	B	P	S	no	1	9	2	1	1	6	16	1 NL
49	1	B	S	S	no	1	15	2	1	12	6	1	1 NL
50	1	B	HF	A	no	1	15	1	1	6	6	1	1 NL
51	1	B	P	S	no	1	16	2	2	7	6	1	1 NL
52	1	B	HF	S	no	1	12	9	1	6	3	13	1 NL
53	1	B	P	S	no	1	3	2	1	6	3	7	1 NL
54	1	B	H	S	no	1	2	9	1	6	6	11	1 NL
16	6	5 C, B	HO	A	yes	1	1	2	1	1	1	1	1 USA, 5 NL
17	17	C	2 HO, 15 HC	5A, 9 B, 3S	12 yes, 4 no, 1 n.d.	1	1	1	1	1	1	1	2 UK, 2 F, 2 USA, 11 Aus
31	1	n.d.	HC	S	no	3	1	3	1	1	1	1	1 Austr.
18	2	C	HO, HC	A	1 yes, 1 no	1	7	1	5	1	1	1	1 UK, 1 Austr.
19	1	C	HF	A	no	1	7	1	1	1	1	11	1 NL
20	1	C	HO	A	yes	1	5	1	8	1	1	6	1 USA
22	3	C	HC	A	no	1	2	3	1	1	2	1	3 NL
33	1	n.d.	HC	S	n.d.	1	2	3	1	1	2	5	1 Austr.
24	1	C	Dog	A	no	1	2	3	1	1	7	1	1 NL
55	1	C	P	S	no	1	2	1	1	1	2	1	1 NL
23	1	C	Dog	A	no	7	2	3	1	1	2	1	1 NL
21	2	C	HC, Cat	A	no	1	9	3	1	1	2	1	2 NL
25	1	B	HC	A	no	1	9	3	1	1	6	1	1 F
56	1	B	H	S	no	1	5	3	1	1	6	1	1 NL
32	2	C	HC, P	S	no	1	3	3	1	1	2	1	1 NL/ 1 Austr.
35	2	n.d.	P	S	n.d.	1	3	3	1	1	6	1	2 Austr.
34	1	n.d.	P	S	n.d.	1	3	3	2	1	2	1	1 Austr.
29	1	n.d.	HC	S	n.d.	1	4	5	1	1	3	1	1 Austr.
57	1	B	HC	S	no	1	4	3	1	1	3	1	1 NL
36	1	n.d.	P	S	n.d.	1	2	3	3	2	2	1	1 Austr.
27	2	C	C, Dog	A	no	1	2	7	1	5	7	1	2 NL
28	1	C	Cat	A	no	1	2	7	1	5	6	1	1 NL
37	1	n.d.	P	S	n.d.	1	9	7	1	5	7	8	1 Austr.
30	1	n.d.	HC	S	n.d.	1	5	3	2	1	6	2	1 Austr.
58	1	B	P	S	no	1	5	3	2	11	6	16	1 NL
15	1	B	H	A	no	4	9	2	1	7	9	7	1 NL
38	1	B	HC	S	n.d.	1	2	5	4	3	5	4	1 Austr.
39	**1**	**R**	**HC**	**B**	**n.d.**	**2**	**6**	**6**	**3**	**4**	**4**	**3**	**1 Austr.**
60	**1**	**R**	**H**	**S**	**no**	**6**	**13**	**8**	**11**	**8**	**8**	**10**	**1 NL**
40	**1**	**R**	**P**	**S**	**n.d.**	**6**	**10**	**8**	**6**	**8**	**8**	**10**	**1 Austr.**
61	**1**	**R**	**H**	**S**	**no**	**6**	**13**	**8**	**6**	**8**	**8**	**17**	**1 NL**
62	**1**	**R**	**HF**	**S**	**no**	**6**	**11**	**8**	**10**	**9**	**10**	**9**	**1 NL**

comycin-resistant isolates, although the difference in mean allelic diversity decreased after exclusion of the divergent isolates (values of 0.64 and 0.53 for the vancomycin-sensitive and vancomycin-resistant isolates, respectively) (Table 2).

There were only 10 *vanB*-carrying isolates in the collection; 9 originated from Australia and 1 was from the United States. Nine of the isolates had the most prevalent allelic type, ST 17 (Fig. 2). The remaining isolate was one of the five genetically divergent isolates described above.

Epidemiology of genetic lineages. The results of the clustering by UPGMA of the allelic profiles are shown in Fig. 2. Four major groups, containing isolates with >40% similarity in allelic profiles, were discernible; a residual group included the five genetically most divergent isolates. Groups were designated MLST lineages A through D (Fig. 2). These lineages corresponded well with the previously established genogroups A through D obtained by clustering based on AFLP typing of *E. faecium* (33). Interestingly, the relationship between lineages and host specificity was found not only among the vancomycin-resistant isolates, as described previously, but also among the vancomycin-sensitive isolates (Fig. 2). Lineage D isolates were mainly from calves (16 of 18 isolates), lineage A isolates were mainly from nonhospitalized humans and pigs (17 of 25 isolates), lineage B isolates were mainly from poultry (15 of 20 isolates), and lineage C isolates were from hospitalized humans (37 of 47 isolates) and a variety of sources, such as dogs, cats, and poultry (10 of 47 isolates).

All four major lineages contained both vancomycin-sensitive and vancomycin-resistant isolates. Isolates from humans were distributed among all lineages; however, isolates related to hospital outbreaks in The Netherlands, Australia, the United Kingdom, and the United States all clustered in a genetically closely related subgroup of lineage C with ST 16, 17, 18, or 20 (Fig. 2, lineage C1). The *esp* gene, which has been associated with virulence and epidemicity in hospitals (29, 32), was uniquely present in isolates with these STs (Fig. 2). The remaining isolates in subgroup C1 (STs 19 and 31) were sporadic isolates from patients in different hospitals and were not known to be related to an outbreak. Both isolates lacked the *esp* gene, a finding consistent with the idea that *esp* is a requisite for epidemicity. All isolates belonging to subgroup C1 shared allele *purK1*, which was not found among any of the other isolates. This result confirms the previous observation that this *purK* allele invariably was correlated with the presence of *esp* in vancomycin-resistant isolates (32).

To investigate how MLST performs with epidemiologically related isolates, we analyzed 16 additional isolates (not belonging to the sample of 123 isolates) involved in different outbreaks in the United States, The Netherlands, Australia, and the United Kingdom and belonging to STs 16, 17, 18, and 20.

TABLE 5. I_as for *vanA*-carrying and VSEF isolates

Level at which I_a was calculated	I_a for:				
	Entire sample			Sample without 5 divergent isolates	
	All (n = 123)	*vanA* (n = 72)	VSEF (n = 41)	All (n = 118)	VSEF (n = 37)
Strains	1.07[a]	0.77[a]	0.91[a]	0.83[a]	0.38[b]
STs	0.79[a]	0.41[b]	0.79[a]	0.44[b]	0.21[b]

[a] The P value was <0.001; the I_a deviates significantly from zero (linkage disequilibrium); see Materials and Methods.
[b] The P value was >0.05; the I_a does not deviate significantly from zero (no evidence for linkage); see Materials and Methods.

The analysis revealed that all isolates involved in a particular outbreak exhibited the same allelic profile as the representative isolate (data not shown).

Evidence for recombination in *E. faecium*. As described above, the gene tree of the *atpA* alleles is not congruent with the trees of the other loci. The noncongruence between the gene trees suggests a weak linkage between the *atpA* alleles and the other loci, and this characteristic is indicative of a role for recombination in the generation of divergence in *E. faecium*. The poor linkage between alleles from different loci is directly obvious from Fig. 2 and is further illustrated for *purK6* in Fig. 1. The latter figure shows that allele *purK6* is associated with several alleles from the other loci. A more quantitative analysis of the association between alleles from different loci was performed by calculating the I_a (30). Significant linkage disequilibrium was detected when all 123 isolates or STs were included in the analysis (I_as, 1.07 and 0.79, respectively) (Table 5). Also, after exclusion of the five genetically most divergent isolates, the alleles of the 118 remaining isolates were found to be in linkage disequilibrium (I_a, 0.83; $P < 0.001$). However, no evidence for linkage was detected when the analysis was performed at the level of STs (I_a, 0.44; $P > 0.05$) (Table 5). Analysis of VSEF isolates and *vanA*-containing isolates separately showed that, at the level of STs, there was no evidence for linkage between loci of the *vanA*-containing isolates (I_a, 0.41; $P > 0.05$). Loci of the VSEF isolates were in linkage disequilibrium at the levels of both isolates and STs. However, no significant linkage between loci was detected at both isolate and ST levels when the most divergent isolates were excluded from the VSEF group (Table 5). These observations imply that horizontal transfer of DNA plays an important role in the generation of genetic variations in both VSEF and *vanA*-containing isolates.

An approximate estimation of the relative contributions of recombination and mutation to clonal divergence was made by using the method and criteria described by Feil et al. (13).

FIG. 2. Dendrogram (categorical, UPGMA) showing the genetic relatedness among the STs of *E. faecium*. The following data are included: ST; number of strains with the same ST; AFLP group (genogroup determined by AFLP analysis; R, non-A, -B, -C, or -D); host origin (C, calf; H, human; HF, fecal sample from a hospitalized human; HC, clinical sample from a hospitalized human, not outbreak related; HO, clinical sample from a hospitalized human, outbreak related; S, pig; P, poultry); presence of *vanA* or *vanB* (A, *vanA* resistant strain; B, *vanB* resistant strain; S, sensitive strain); presence of the *esp* gene; and country (NL, The Netherlands; UK, United Kingdom; USA, United States; Aus or Austr., Australia). The dendrogram is divided by dotted lines into a number of lineages, labeled A to D, similar to the genogroups detected by AFLP analysis (33). Genetically divergent isolates are indicated in bold. n.d., not determined.

Clonal complexes consisting of STs that differed in one or two alleles and that were part of the UPGMA lineages were made, and the contribution of recombination or mutation was calculated. It was estimated that recombinational exchange generated new alleles at a frequency fivefold higher than point mutation, while single nucleotide sites were 24 times more likely to change through recombination than through mutation (results not shown). The per-site recombination-to-mutation ratio was about one-half the ratio for *Streptococcus pneumoniae* and one-fourth the ratio for *Neisseria meningitidis* (13).

DISCUSSION

One of the main objectives of this study was to provide a reference scheme for the typing of *E. faecium* to allow unambiguous comparison of data between different laboratories. We determined the degree of allelic variations in seven housekeeping genes of *E. faecium* by using a sample of 123 isolates originating from human and animal sources in various countries. The degree of isolate differentiation by MLST appears adequate for use in epidemiological investigations, as the number of different types obtained by MLST was comparable to that obtained by AFLP analysis, a technique with proven discriminatory power for VREF isolates (1, 32, 33). Further confirmation of the classification of VREF isolates by AFLP analysis and MLST was recently provided by ribotyping analysis (4). Genogrouping of *E. faecium* isolates by MLST was highly congruent with grouping by AFLP analysis. In previous studies with AFLP analysis, the majority of VREF isolates were grouped into four different lineages, designated genogroups A through D (33). The majority of the isolates investigated by MLST also were grouped into four corresponding lineages. In contrast to previous studies, this study included VSEF isolates, and the majority of these isolates belonged to one of the four lineages established by typing of VREF isolates. The VSEF isolates were overall genetically more diverse than the *vanA*-carrying isolates. However, this difference was largely due to a few genetically highly divergent isolates not belonging to lineages A through D.

Assuming that the dissemination of the *vanA* gene among *E. faecium* started with the introduction of vancomycin and related antibiotics about only 40 years ago implies that *vanA* was rapidly disseminated among the four main lineages of *E. faecium*. Dissemination of vancomycin resistance in *E. faecium* has been extensively described and is mediated by the intercellular spread of self-transferable plasmids and conjugative transposons harboring vancomycin resistance genes (2).

In this study, we included only representative isolates from hospital outbreaks and excluded other isolates that were epidemiologically related to any of these outbreaks. Nevertheless, 78% (64 of 82) of the VREF isolates shared allelic profiles, whereas only 22% (9 of 41) of the VSEF isolates did so. Although the number of isolates included in this study was small, these data suggest strongly that vancomycin-resistant isolates recently spread epidemically. Because isolates with shared allelic profiles originated from different countries and continents, this dissemination is worldwide.

It was previously shown that epidemic strains are exclusively found within AFLP genogroup C, share allele *purK1*, and carry the *esp* gene, irrespective of the country of origin (32). In the current study, all epidemic VREF strains were found in MLST lineage C1, which is characterized by allele *purK1*, and all share the *esp* gene, confirming the classification based on AFLP analysis. The remaining hospitalized human-derived strains present in lineages A, B, and C were taken from clinical sites or obtained from fecal screenings and did not have a known relationship to outbreaks. Most likely, a subpopulation of lineage C gave rise to an epidemic VREF subpopulation, equipped with virulence factors such as *esp*, enabling *E. faecium* to preferentially colonize and spread in hospitalized human patients worldwide (28, 29, 32).

The previously observed relationship between VREF genogroup and host was confirmed in this study. In addition, this study indicates that this relationship holds true for VSEF as well. This result is not unexpected, because the introduction of the mobile vancomycin resistance genes into the *E. faecium* population would not be expected to affect a preexisting specific host-bacterium relationship, which presumably is the result of long-term coevolution of the bacterium and host.

Single-locus phylogenetic trees were noncongruent, suggesting that recombination plays an important role in the generation of diversity of the *E. faecium* population. Consistently, based on I_as between alleles, random association between gene loci was detected at the level of STs but not at the level of isolates, suggesting that *E. faecium* has an epidemic population structure (30). Because the VSEF population was in linkage equilibrium at the level of all isolates (when divergent isolates were excluded), the epidemic population structure seems to be mainly due to lineages in the resistant population. These results suggest that horizontal transfer of genetic information in *E. faecium* played a role in long-term evolution, while clonal lineages emerged and disseminated recently as a consequence of antibiotic pressure. Confirmation of the importance of recombination for the generation of alleles in housekeeping genes from *E. faecium* was derived from a sequence-based approach described by Feil et al. (13). Enterococci contain several efficient systems for exchanging genetic information, including pheromone-responsive plasmids, broad-host-range plasmids, and conjugative transposons. Horizontal transfer of resistance genes in enterococci with these systems is well documented (16). In addition, horizontal transfer of *tuf* housekeeping genes encoding elongation factor Tu, involved in protein synthesis, among enterococci was recently reported (19).

The *E. faecium* MLST scheme developed in this study provides a universal and portable method for isolate typing and addressing long-term epidemiological questions. Laboratories worldwide can build a virtual isolate collection that will enable disclosure of the emergence of epidemic clones that may be disseminated among different hospitals in different countries and continents. By comparison of patient-related information with molecular genetic data, novel genetic lineages with specific properties relating to pathogenicity and epidemiology may be revealed.

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ERRATA

Stx2 Subtyping of Shiga Toxin-Producing *Escherichia coli* Isolated from Cattle in France: Detection of a New Stx2 Subtype and Correlation with Additional Virulence Factors

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Volume 39, no. 9, p. 3060–3065, 2001. Page 3065: References 11 to 29 should be numbered 10 to 28, respectively.

Multilocus Sequence Typing Scheme for *Enterococcus faecium*

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Volume 40, no. 6, p. 1963–1971. Page 1964, column 2: The sequences for the primers gyd1 and purK2 should read as follows: for gyd1, "5′-CAA ACT GCT TAG CTC CAA TGG C-3′," and for purK2, "5′-TAC ATA AAT CCC GCC TGT TTY-3′."