

Molecular Typing of Selected *Enterococcus faecalis* Isolates: Pilot Study Using Multilocus Sequence Typing and Pulsed-Field Gel Electrophoresis

Sreedhar R. Nallapareddy,^{1,2} Ruay-Wang Duh,^{1,2,†} Kavindra V. Singh,^{1,2}
and Barbara E. Murray^{1,2,3*}

Division of Infectious Diseases, Department of Internal Medicine,¹ Center for the Study of Emerging and Re-emerging Pathogens,² and Department of Microbiology and Molecular Genetics,³ University of Texas Medical School, Houston, Texas 77030

Received 29 August 2001/Returned for modification 23 November 2001/Accepted 16 December 2001

The present study compared the recently developed multilocus sequence typing (MLST) approach with a well-established molecular typing technique, pulsed-field gel electrophoresis (PFGE), for subspecies differentiation of *Enterococcus faecalis* isolates. We sequenced intragenic regions of three *E. faecalis* antigen-encoding genes (*ace*, encoding a collagen and laminin adhesin; *efaA*, encoding an endocarditis antigen; and *salA*, encoding a cell wall associated antigen) and one housekeeping gene (*pyrC*) of 22 *E. faecalis* isolates chosen largely for their temporal and geographical diversity, but also including some outbreak isolates. MLST analysis of polymorphic regions of these four genes identified 13 distinct sequence types (STs) with different allelic profiles; the composite sequences generated from the four sequenced gene fragments of individual isolates showed 98.3 to 100% identity among the 22 isolates. We also found that the allelic profiles from two sequences, *ace* and *salA*, were sufficient to distinguish all 13 STs of this study. The 13 STs corresponded to 12 different PFGE types, with one previously designated PFGE clone (a widespread U.S. clone of β -lactamase-producing isolates) being classified into two highly related STs which differed at 2 of 2,894 bases, both in the same allele. MLST also confirmed the clonal relationships among the isolates of two other PFGE clonal groups, including vancomycin resistant isolates. Thus, this pilot study with representative *E. faecalis* isolates suggests that, similar to PFGE, the sequence-based typing method may be useful for differentiating isolates of *E. faecalis* to the subspecies level in addition to identifying outbreak isolates.

Enterococci, normal gut commensals, were recognized as a causative agent of endocarditis and urinary tract infections in ca. 1900 and have also been reported as a common cause of nosocomial infections since the 1970s (25, 26). Recently, accumulation of antibiotic resistances has made enterococcal infections a life-threatening clinical challenge, and thus, the methods that distinguish an outbreak from an endogenous strain have become important for designing strategies to prevent and control outbreaks (24, 26). A number of phenotypic or genotypic typing methods (including biochemical typing, serotyping, multilocus enzyme electrophoresis [MLEE], phage typing, insertion sequence element-based typing, pulsed-field gel electrophoresis [PFGE], restriction fragment length polymorphism [RFLP] analysis, ribotyping, repetitive sequence-based PCR, arbitrary primed PCR, and random amplification of polymorphic DNA) have been applied to the epidemiological investigations of *Enterococcus faecalis* (2–4, 6, 14, 18, 22, 28, 35, 40, 47, 48). These molecular and epidemiological studies have provided valuable information and clarified some misconceptions regarding *E. faecalis* infections, such as demonstrating

that some *E. faecalis* infections are caused by nosocomial transmission of outbreak strains rather than arising from the patient's own prehospitalization intestinal flora. Among the known enterococcal molecular typing methods, PFGE has proven to be a highly reproducible and accurate typing method, which can distinguish clonal populations, and hence is considered a "gold standard" for subspecies discrimination of *E. faecalis* clinical isolates. However, the results obtained by PFGE are not readily transportable, making it difficult to compare results among different laboratories and thus limiting studies involving interlaboratory comparisons.

Microbial genome sequencing programs have provided enormous data for easy identification of species-specific genes. This, together with rapid automated DNA sequencing, has generated considerable interest in DNA sequence-based typing methods. A few recent studies have used variable intragenic sequences (flanked by highly conserved regions) of either a single gene or two genes and reported their usefulness in strain differentiation. These include the *hsp65* gene (which encodes a 65-kDa heat shock protein) of *Mycobacterium scrofulaceum*, the *porB* gene (which encodes an outer membrane porin protein) of *Neisseria meningitidis*, and repeat regions of the *spaA* (which encodes protein A) as well as the *coa* (which encodes coagulase) genes of *Staphylococcus aureus* and the *emm* gene (which encodes an M protein) of *Streptococcus pyogenes* (1, 33, 38, 39, 41, 42, 44, 45).

Recently, multilocus sequence typing (MLST) was developed for identification of clonal complexes within bacterial

* Corresponding author. Mailing address: Center for the Study of Emerging and Re-emerging Pathogens, Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical School at Houston, 6431 Fannin St., Houston, TX 77030. Phone: (713) 500-6767. Fax: (713) 500-5495. E-mail: Barbara.E.Murray@uth.tmc.edu.

† Present address: Section of Infectious Diseases, Department of Medicine, Veterans General Hospital-Taipei, Taiwan, Republic of China.

TABLE 1. Sequence-based typing results for various *E. faecalis* isolates in this study

Laboratory name (alternative designation[s]) ^a	Clinical source; origin; yr of isolation or collection	Reference(s)	MLEE type ^b	PFGE type ^b	Sequence type (allelic profile for <i>ace</i> , <i>efaA</i> , <i>pyrC</i> , <i>salA</i>)
TX4002 (OG1RF, ATCC 47077)	Laboratory strain; <1978 ^c	14, 22, 48	25	VIII	ST-1 (A, A, A, A)
TX2621 ^{d,e,f}	Blood; Houston, Tex.; 1996	5		HH6	ST-2 (C, C, B, B)
TX2619 ^{d,e,f}	Urine; Houston, Tex., 1996	5		HH9	ST-3 (B, C, D, E)
TX0855 (BE83)	Urine; Bangkok, Thailand; 1980	14, 28		B-1	ST-4 (E, F, A, A)
TX2783 (B343) ^d	Chicken product; Logrono, Spain; 1998	37		BR-1	ST-5 (D, I, B, A)
TX0614 (E228) ^{g,h}	Urine; Richmond, Va.; 1990	22, 40, 48	20	5	ST-6 (C, E, B, H)
TX0615 (E278) ^{g,h}	Foot ulcer; Richmond, Va.; 1990	40, 48	20	5	ST-6 (C, E, B, H)
TX0616 (E340) ^{g,h}	Urine; Richmond, Va.; 1990	40, 48	20	5	ST-6 (C, E, B, H)
TX0617 (E366) ^{g,h}	Sputum; Richmond, Va.; 1991	40, 48	20	5	ST-6 (C, E, B, H)
TX0638 (DEL) ^g	Wilmington, Del.; 1986	22, 29, 48	20	5	ST-7 (C, E, B, I)
TX0669 (PENN, PA) ^g	Blood; Philadelphia, Pa.; 1983	29, 48	17	5	ST-7 (C, E, B, I)
TX0921 (HH22) ^f	Houston, Tex.; 1981	22, 29, 48	20	5	ST-7 (C, E, B, I)
TX4000 (JH2-2)	Laboratory strain; <1974 ^c	14, 22, 48	17	VII	ST-8 (D, B, B, G)
TX0630 (HG6280) ^{g,i}	Blood; Buenos Aires, Argentina; 1989	22, 27, 29, 48	16	19	ST-9 (G, G, A, C)
TX0631 (HG10528) ^{g,i}	Gastric fluid; Buenos Aires, Argentina; 1989	27, 48	16	19	ST-9 (G, G, A, C)
TX0633 (HG9829) ^{g,i}	Subdiaphragmatic abscess; Buenos Aires, Argentina; 1989	27, 48	16	19	ST-9 (G, G, A, C)
TX0645 (Beirut) ^g	Blood; Beirut, Lebanon; 1989	29, 48	7	7	ST-10 (F, D, E, D)
TX0860 (BE88)	Catheter tip; Bangkok, Thailand; 1980	14, 28, 48	18	B-3	ST-11 (G, H, C, F)
TX2486 ^{d,e}	Urine; Houston, Tex.; 1994	5, 22		1	ST-12 (H, C, B, B)
TX2487a ^{d,e}	Urine; Houston, Tex.; 1994	5		1	ST-12 (H, C, B, B)
TX2490 ^{d,e}	Urine; Houston, Tex.; 1994	5		1	ST-12 (H, C, B, B)
TX0635 (WH245) ^g	Urine; West Haven, Conn.; 1986	29, 34, 48	5	8	ST-13 (I, G, A, D)

^a As designated in previous studies.

^b The original MLEE and PFGE pattern names in the earlier publication(s) are used.

^c Isolated at unknown time prior to the year shown.

^d Vancomycin resistant isolate.

^e Isolates TX2619, TX2621, TX2486, TX2487a, and TX2490 were from the same hospital.

^f Isolates TX2619 and TX2621 were not tested for vancomycin resistance in the previous study (5) and hence were not reported as vancomycin-resistant strains.

^g β -Lactamase-producing isolate.

^h Isolates TX0614, TX0615, TX0616, and TX0617 were from the same hospital.

ⁱ Isolates TX0630, TX0631, and TX0633 were from the same hospital.

populations and has been used successfully for molecular epidemiological analysis of *N. meningitidis*, *Streptococcus pneumoniae*, *S. aureus*, *S. pyogenes*, and *Campylobacter jejuni* (7, 9, 11, 12, 21). MLST typically characterizes isolates of bacteria using ~400 to 500 bp of intragenic sequences of six to seven housekeeping genes or loci, and thus, MLST is similar in principle to MLEE, but with greater sensitivity due to its ability to detect neutral genetic variations. This method distinguishes strains based on the observed allelic variations in the nucleotide sequences of several loci, rather than the degree of sequence variation in any single gene or locus. MLST and other the DNA sequence-based typing methods have been suggested as offering advantage over other techniques because (i) the data are objective and readily comparable between laboratories, (ii) the data can be stored in a shared central database to provide a broader resource for epidemiological studies, and (iii) evolutionary genetic analyses can be performed. At the same time, DNA sequencing is expensive when compared to PFGE and considerable technical skill and knowledge in sequence analysis is critical for typing the isolates (32).

In the present study, we have evaluated the discriminatory ability of a sequence-based typing method and compared the results to those obtained with PFGE. Internal fragments of three antigen-encoding genes that were detected with *E. faecalis*-infected patient sera and an internal fragment of one pyrimidine biosynthesis gene were used for the MLST analysis.

The MLST generated sequence types were found to be comparable to types or clones identified with PFGE.

MATERIALS AND METHODS

Bacterial isolates. A total of 22 *E. faecalis* isolates from different geographic locations were chosen for this study, primarily because they had been previously studied by us using one or more other typing techniques (Table 1). A collection of eight β -lactamase-producing (Bla⁺) isolates that were recovered from five cities in the United States (four isolates from the same hospital) during an 11-year period (1981 through 1991), three additional Bla⁺ isolates from a single hospital in Argentina (isolated between June and September of 1989), and one Bla⁺ isolate from Lebanon (isolated in 1989) that were previously analyzed by PFGE and MLEE were included (27–29, 34, 48). Six other isolates were vancomycin resistant, including five clinical isolates from a single hospital (of which three isolates were part of a single outbreak) in the United States (5) and one nonclinical isolate collected from Spain isolated from chicken products (37). Two isolates with high-level aminoglycoside resistance were from Thailand (14, 28), and two commonly used laboratory isolates of *E. faecalis* OG1RF (ATCC 47077) and JH2-2 were also included in this study (14, 17, 22, 23, 30).

PFGE. PFGE was performed with some modifications of a previously described method (14, 28). Agarose plugs containing genomic DNA were digested with *Sma*I (Gibco, BRL, Gaithersburg, Md.), and electrophoresis was carried out using a clamped homogeneous electric field (CHEF-DRII device; Bio-Rad Laboratories, Richmond, Calif.), with ramped pulse times beginning with 5 s and ending with 45 s, at 200 V for 26 h. The gels were stained with ethidium bromide (0.4 μ g/ml) for 30 min and photographed. The PFGE patterns were interpreted using the criteria suggested by Tenover et al. (46), with closely and possibly related patterns being designated as belonging to a single clone. PFGE pattern names that were presented in earlier publications are used here.

Genomic DNA isolation; PCR; and DNA sequencing of *ace*, *efaA*, *pyrC*, and

TABLE 2. PCR and sequencing primers used in this study

Gene	ORF size (bp)	Deoxynucleotide primer ^a			Amplicon size (bp)
		Function(s)	Type	Sequence (5'→3')	
<i>ace</i>	2,025 ^c	PCR, sequencing	Forward	GAGCAAAAGTTCAATCGTTGAC	99–120
		PCR, sequencing	Reverse ^d	GTCTGTCITTTCACTTGTTCT	1101–1080
		Sequencing	Forward	CCAAATTGAGCGAGACTATC	510–529
		Sequencing	Forward	CACTGCCGAGTTTGAGC	719–736
		Sequencing	Reverse	GAGAACTATTGGTGATAAGCG	424–404
<i>efaA</i>	924	PCR, sequencing	Forward	CGTTAGCTGCTTGCGGGAATC	47–67
		PCR, sequencing	Reverse	CCATACTACGTTTATCGACAC	781–761
		Sequencing	Forward	CCACAATATTTAACAAGTGCC	364–384
		Sequencing	Forward	CAAACACTACATGAGGAAGCC	537–555
		Sequencing	Reverse	GTTTGTCTTGACCGGCAC	398–380
<i>pyrC</i>	1,284	PCR, sequencing	Forward	CGGGTAGTAAAGCTGCTGC	227–245
		PCR, sequencing	Reverse	CTTCTCCTTCATGCATCACAC	586–566
<i>salA</i>	1,449	PCR, sequencing	Forward	CATTAACAAGCGTAGCGTTG	44–63
		PCR, sequencing	Reverse	GCCTTTTTTCAGGAGTCGTTG	1005–986
		Sequencing	Forward	GATGCTGTCTTAGATGCAG	364–382
		Sequencing	Forward	GCACAACGTTCTGAAGAACAAG	613–634
		Sequencing	Reverse	CACTTGGACATCGCGTGC	339–322

^a *ace*, *efaA*, *pyrC*, and *salA* primers were designed from *E. faecalis* strain V583 database sequences (The Institute for Genomic Research).

^b Numbering for all the primers is given relative to the start codon (ATG for *ace*, *efaA*, and *pyrC*; TTG for *salA*) for the respective genes.

^c *ace* ORF size represented here is for *E. faecalis* strain V583 (36). *ace* gene size varies in different *E. faecalis* strains due to variation in the number of repeats of the B domain (31).

^d See text for the reverse primer used for the selected strains.

salA. Genomic DNA was extracted from *E. faecalis* isolates freshly streaked from freezer vials and cultured in brain heart infusion broth. Genomic DNA was isolated by the hexadecyltrimethyl ammonium bromide method as described previously (22). We chose internal regions of three *E. faecalis* antigen-encoding genes (*ace*, *efaA*, and *salA*) and one housekeeping gene (*pyrC*) for sequencing. These four loci were chosen primarily because all these genes were well studied in our laboratory (8, 19, 31, 43), and the sequence diversity of the region coding for the A domain of *ace* has been recognized in our earlier study (31). The open reading frame (ORF) sizes of the four chosen loci are listed in Table 2. The internal gene fragments of *ace*, which encode a collagen and laminin adhesin (959 bp [890 bp for one isolate due to a 69-bp deletion] [31, 36]); *efaA*, which encodes an endocarditis antigen (693 bp [20]); *pyrC*, which encodes a dihydroorotase (320 bp [19]); and *salA*, which encodes a cell wall-associated antigen (F. Teng, B. E. Murray, and G. M. Weinstock, unpublished data) (919 or 922 bp, due to a 3-bp in-frame deletion in some isolates) were amplified using the optimized buffer B (1× buffer: 60 mM Tris-HCl [pH 8.5], 15 mM ammonium sulfate, 2 mM MgCl₂) obtained from Invitrogen (San Diego, Calif.). PCR was performed in volumes of 50 μl, with an initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s (72°C for 45 s for *pyrC*) and a final extension of 72°C for 7 min. The PCR primers used for amplification and sequencing of all four genes are listed in Table 2. Our initial attempts with *ace* amplification primers were unsuccessful for three strains, namely, TX2486, TX2487a, and TX2490, subsequently found to be due to variations in the *ace* gene at the reverse primer region. The *ace* amplicon for these strains was amplified using *ace* forward PCR primer (Table 2) and reverse primer with the sequence 5'-ATTTAATTTTGAATTGGTTCCTAAGCAG-3' (located at positions 1896 to 1867, relative to the start codon). The PCR amplicons were purified using the Wizard PCR DNA Cleanup System (Promega Corporation, Madison, Wis.). Sequencing of both strands of the amplified fragments was achieved using an Applied Biosystems Prism 377 automated DNA sequencer using the *Taq* Dye-Deoxy terminator method (PE Applied Biosystems, Foster City, Calif.). Sequences were assembled using SeqMan program of DNASTAR software (Lasergene, Madison, Wis.).

Sequence analysis. Multiple sequence alignments of the 22 isolates for the four gene fragments (*ace*, *efaA*, *pyrC*, and *salA*) were done by the Jotun Hein method (16) using the MegAlign program of DNASTAR software. A 2,894-bp (for 10 strains), 2,891-bp (for 11 strains), 2,822-bp (for one strain) nucleotide composite sequence (derived from four concatenated gene fragments) was also aligned by the MegAlign program. Phylogenetic trees (cladograms) based on the matrix of pairwise sequence divergence were constructed using the MegAlign program.

Allele and sequence type (ST) assignment. In order to identify the nucleotide variation in the above gene fragments, sequences from the different isolates were compared to the corresponding sequences in the well-studied *E. faecalis* strain OG1RF (30). Each gene sequence differing by one or more nucleotides was considered to be a different allele (no weight was given to the degree of sequence divergence between alleles, although the alleles that differed by a single nucleotide were denoted as single nucleotide variants [SNVs]), and the distinct allelic sequences were assigned an arbitrary letter designation in the order of the number of base pairs that varied with respect to the OG1RF sequence. The alleles at the four loci provided the allelic profile, which defined the ST for each isolate. Isolates showing identical allelic profiles in the four gene fragments were assigned to the same ST, and those with a difference in their allelic profile were assigned to a different ST. The relatedness among the strains was analyzed by constructing a dendrogram based on the matrix of pairwise differences in the allelic sequences by the unweighted pair group method with arithmetic averages (UPGMA) method.

RESULTS AND DISCUSSION

Among known phylogenetic typing methods, PFGE and MLST, which are based on multiple sites and loci scattered around the chromosome, have consistently been shown to be capable of discriminating isolates at the subspecies level, although in different ways. PFGE is particularly useful for distinguishing strains circulating within a geographical location (microvariation) and is based on the selected variable regions of the genome (9, 15). MLST is based on variations that accumulate slowly and appears more suitable for long-term and global epidemiology (macrovariation) (9, 10, 15). However, in both methods, the relative rates of nucleotide substitutions and/or recombination occurring in nature set the limitations of phylogenetic analysis, and there are insufficient experiential data to establish these rates for enterococci.

Choice of loci. Since it is impractical to sequence large or multiple regions of the chromosome, we chose loci we had previously studied (*ace*, *efaA*, *pyrC*, and *salA*) and that had

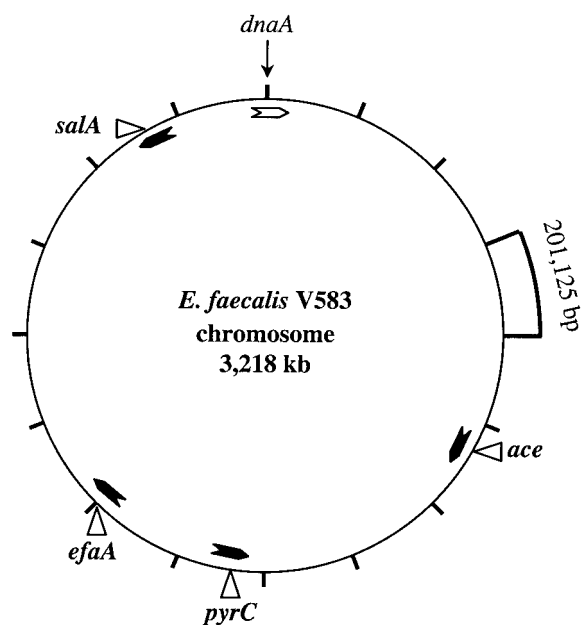


FIG. 1. Chromosomal locations of the sequenced loci. The locations are marked on a constructed physical map of *E. faecalis* V583 using the complete genome sequence (available at <http://www.tigr.org/> [The Institute for Genomic Research]). The 3,218-kb genome is divided into 16 segments, with each segment representing 201,125 bases. The ORF coding for chromosomal replication factor (*dnaA*) was identified based on identity with *dnaA* sequences of *S. pyogenes* and *S. aureus* and positioned at nucleotide one. The arrowheads inside the circle represent the ORF orientations of the four loci and are marked at the respective positions.

been shown to be present in all *E. faecalis* strains tested; indeed, hybridization with *ace* and *efaA* probes had proven useful in differentiating *E. faecalis* from other species (8, 43). On the basis of the *E. faecalis* V583 complete genome sequence (available at <http://www.tigr.org/> [The Institute for Genomic Research, Rockville, Md.]), we derived the physical map shown in Fig. 1, which illustrates that the loci chosen were spread around the chromosome and the distance between any two loci is ranged from 328 to 1,355 kb.

PFGE fingerprinting. PFGE with *Sma*I yielded <20 visible bands for each isolate (data not shown). As per the recommendations described by Tenover et al. (46), including closely and possibly related patterns, a total of 12 PFGE types, 3 of which contained three or more isolates, were recognized. Seven of the twelve *Bla*⁺ isolates were classified as belonging to the same clonal group (PFGE pattern 5); two of these strains showed an identical pattern (TX0614 and TX0615), while others (TX0616, TX0617, TX0638, TX0669, and TX0921) showed patterns with three to six band differences, depending on which two isolates were being compared. Among three Argentinean *Bla*⁺ isolates, TX0630 and TX0633 showed an identical PFGE pattern, while TX0631 showed a pattern almost identical to these two (classified as PFGE pattern 19) (27). Similarly, three of the five vancomycin-resistant Houston isolates were classified as clonally related (PFGE pattern 1). These results are in agreement with our earlier studies (5, 14, 18, 22, 28, 29, 37, 48).

MLST typing. The length of the loci used for allele assignment in our MLST scheme was between 959 bp (*ace*) and 320 bp (*pyrC*). The total length of the four sequenced loci of this study approximates the total length of six loci common to several other MLST studies. Variable sites of different alleles of the four sequenced loci are presented in Fig. 2A, and the phylogenetic trees based on the matrix of pairwise divergence in these sequences are shown in Fig. 2B. Nine unique alleles were identified for three of the loci, while only five alleles were identified for the *pyrC* locus, likely because of the smaller region evaluated (Table 3; Fig. 2). Allelic profiles of the four loci identified 13 STs among the 22 isolates (Table 1). A dendrogram drawn from allelic profiles by the UPGMA method demonstrated four (ST-6, ST-7, ST-9, and ST-12) of the 13 STs contain more than one isolate (Fig. 3a). ST-6 and ST-7 as well as ST-2 and ST-12 differed by variation at a single locus.

The percentage of variable sites in a given locus ranged from 1.3 (*efaA*) to 5.6 (*ace*). A 69-bp in-frame deletion was detected in the *ace* locus of a *Bla*⁺ isolate (allele I) from Connecticut. Eight of nine alleles of the *efaA* locus differed by a single nucleotide, and they were designated SNV alleles (Table 3). Similarly, four of the five alleles of the *pyrC* locus are SNV alleles, and the fifth allele has five nucleotide changes (*Bla*⁺ isolate from Lebanon). In the *salA* locus, a 3-bp in-frame deletion was observed in 55% of the isolates. The nucleotide changes which alter the amino acid sequence are indicated as nonsynonymous base substitutions and the nucleotide changes which do not alter the amino acid sequence are indicated as synonymous base substitutions (Fig. 2A).

Composite sequence-based typing. In order to determine the overall divergence of the sequenced gene fragments of the four loci studied, these sequences were spliced together to obtain a concatenated composite sequence for each of the isolates. For calculating the percentage of identity or divergence, in-frame insertions or deletions were not taken into account. A cladogram created from the matrix of pairwise sequence divergence of composite sequences identified 13 phylogenetic lineages (Fig. 3b), and these were identical to the 13 STs obtained from the allelic profiles. The identity between the 22 composite sequences was found to be between 98.3 and 100%. The composite sequence of ST-6 isolates from Richmond, Va. (TX0614, TX0615, TX0616, and TX0617, which showed 100% identity among the four isolates) have 99.93% identity (2,892 of 2,894 bases) with the composite sequence of ST-7 isolates from three different states (TX0638, TX0669, and TX0921, which were in turn 100% identical to each other).

Congruence between PFGE, MLST, and composite sequence-based typing methods. Analysis of the clusters in the phylogenetic trees generated by the methods described above identified 13 different genotypes versus 12 different PFGE types among the 22 *E. faecalis* isolates tested. Both sequence-based typing methods, i.e., MLST with four loci as well as the composite sequence alignment, confirmed the clonal relationships among isolates of PFGE patterns 1, 5, and 19. TX0921 (*Bla*⁺) obtained from Texas in 1981 showed the identical MLST type as the Pennsylvania *Bla*⁺ strain (TX0669) isolated in 1983 and also strain TX0638 (*Bla*⁺) isolated in Delaware in 1986, confirming our previous finding of widespread dispersion of this clone in the United States (29). Sequence-based typing further confirmed that the three *Bla*⁺ clones from South

A

ace

```

1111111111112223333333456666666667777777888999
11347990222255599225013677780434445566772335589446234
286853621235712325188326005761672584737899580728691187

Allele A  ACAAATATGACCCGGTTTCCTGCAGGTACGTGCGATCTGGAAACAGACACCTCC
Allele B  .....TT.....A...C..G.....
Allele C  .....A.....TT.....A...C..G.....
Allele D  ..GC..C...A...C...T..T...C.....A.....GG.....A..
Allele E  .....C.....T....C.T.AA...A.T.A..T...G.....T...
Allele F  ..GC...A.T.T..C...T.....T..C..A.G.....TA..
Allele G  GT.CG...GT.T..C.....T.....A.....G...A..CT.A..
Allele H  ..GC.C.A.....CCT.C..TAA.GA.C...G...AGGGGG.GACT.ATT
Allele I  ***G...GT.T..C.....T.....A.....G...A..CT.A..
Consensus ACAAATATGACCCGGTTTCCTGTAGGTACGTGCAATCTGGGAACAGACACCTCC
SSSSSSSSNNNSNNSSSSSSSSSSSSSSNNNSNNSSSSSSSSNNNSNNNSNN
    
```

<i>efaA</i>		<i>pyrC</i>		<i>salA</i>	
	23346		2223		34444555677778888889
	55744741		23782891		774668115324560677781
	516881164		58656069		281899392941923778905
Allele A	CCTCCGTGC	Allele A	CTGCAGGT	Allele A	TCTAGCGTGCCACCA***GA
Allele BT	Allele BA..	Allele B	A.....CA.A.C...***..
Allele CA..T	Allele CG...	Allele C	...AT..A...TT...***..
Allele DC.T	Allele DA.	Allele D	...AT..A...TT.T***..
Allele E	T...A..T	Allele E	TCAT...C	Allele ET..A.A.C...GTG..
Allele FA.C.T	Consensus	CTGCAAGT	Allele F	.T...TA.A..T...GTG..
Allele G	.TC.....T		SSSSSSNS	Allele G	..AC....A..A..A.GTGA.
Allele H	.TCG....T			Allele H	.T...T..ATA.C...GTG..
Allele I	.TC..A.AT			Allele I	.T...T..ATA.C..TGTG.T
Consensus	CCTCCATGT			Consensus	TCTAGTGTACACCCA***GA
	SSSNSSSS				SSSSSSSSSSSSSSNNNS

FIG. 2. Allelic variation of the four sequenced loci. (A) Variable sites identified in all the four gene fragments. The nucleotide sites that are identical in all the alleles are not shown. The nucleotides present in each of the variable sites of allele A (*E. faecalis* OG1RF) are shown. Only those sites that differ are shown for all the other alleles. The position of each variable site within the sequenced fragment is shown in the numbers above the nucleotide, read vertically. The consensus sequence is shown on the bottom. The variations that are synonymous (S) and nonsynonymous (N) are also shown. (B) Cladograms of the four loci sequenced. Phylogenetic trees were based on the matrix of pairwise sequence divergence in the sequences (generated by the Jotun Hein method of the DNASTAR software package). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

America and a single isolate from Lebanon as well as the single isolate from Connecticut were different from each other.

Although there was an agreement among all three analyses, one apparent exception was the recognition of two different STs (containing multiple isolates) by sequence-based typing among isolates characterized as belonging to PFGE pattern 5. As pointed out earlier, four of these seven *Bla*⁺ PFGE type 5 isolates (ST-6) obtained from Richmond, Va., in 1990 to 1991 are identical at all four loci, while the other three PFGE type 5 *Bla*⁺ isolates (isolated 5 to 10 years earlier in different states) were classified as ST-7, which differs only by variation of two synonymous bases at the *salA* locus (i.e., these are single-locus variants [SLVs]); all other isolates differed from these *Bla*⁺ isolates by two or more bases at this locus, as well as differing at other loci. While most MLST studies that deal with six or more loci would have clustered these single locus variants as a single clonal group, we did not do so because of the smaller number of loci studied. The ST-6 and ST-7 group of *Bla*⁺ isolates has previously been considered clonally related based

on PFGE and repetitive sequence-based PCR (22). This difference may be related to the use of a somewhat broader assignment of PFGE types (i.e., up to even six fragment differences were seen, depending on which two isolates were compared), while counting even a single base change in assigning the alleles of MLST.

Among other possibly related STs, three vancomycin-resistant outbreak isolates (ST-12) from Houston, Tex. (1994) are SLVs of another Houston vancomycin resistant 1996 isolate (ST-2), the only other SLV of this study. However, the ST-2 isolate has only 98.9% overall composite sequence identity with ST-12 isolates due to 33 scattered base changes in the *ace* locus. These vancomycin-resistant SLVs also show a relatively similar PFGE pattern when compared to the other isolates of this study. This, together with the identity at three of the four loci, raises the possibility that the extensive differences in *ace* may be due to horizontal exchange, as has been suggested to occur during conjugative transposition (49).

The other possibly related composite sequences were those

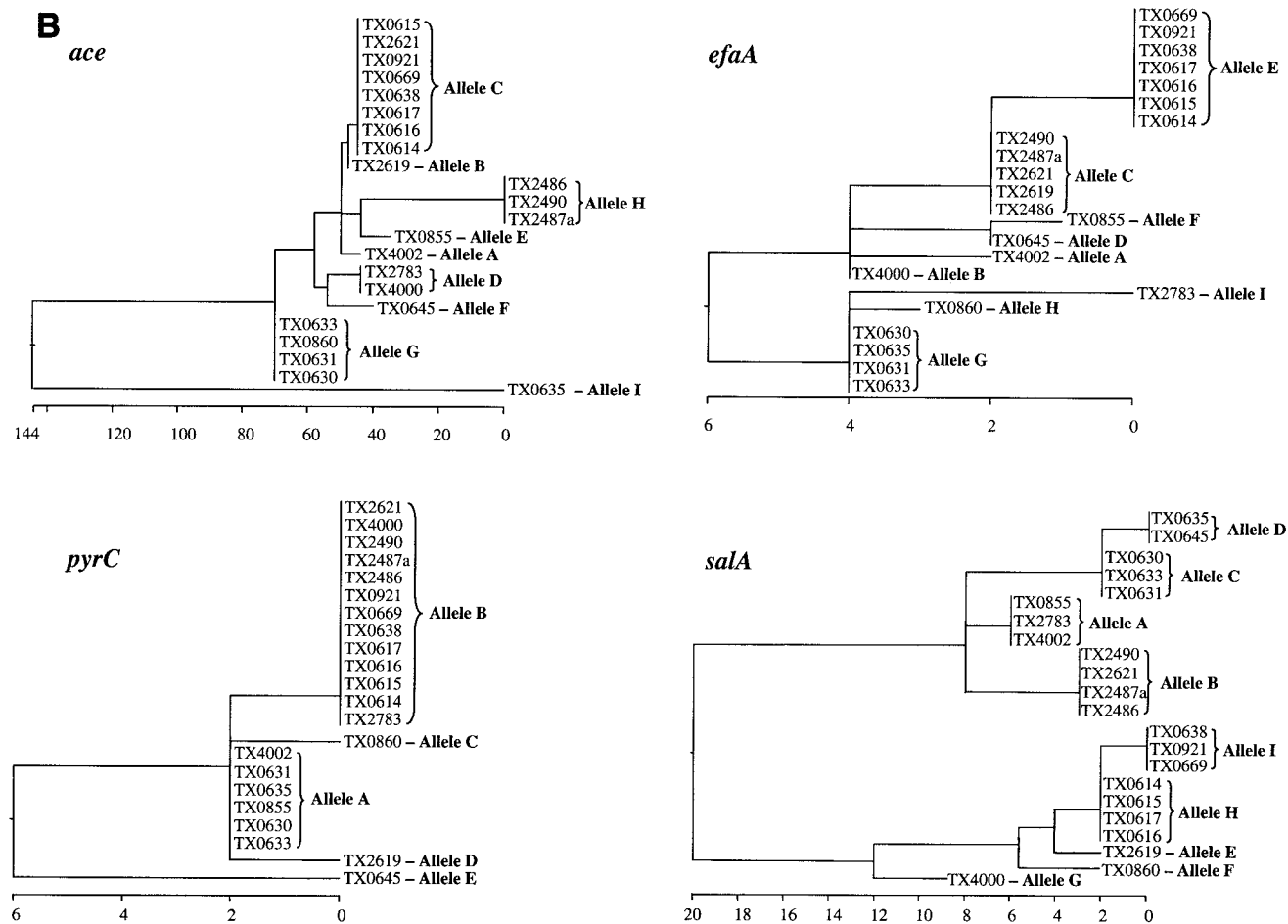


FIG. 2—Continued.

of the Connecticut *Bla*⁺ ST-13 isolate and the South American *Bla*⁺ ST-9 isolates; these differed by a 69-bp deletion at the *ace* locus and a single base change at the *salA* locus. However, the PFGE patterns of these two STs differed substantially.

Results from a prior study of MLEE found general agreement for most isolates tested by both PFGE and MLEE (Table 1). The MLEE study assigned the same ET to six of seven *Bla*⁺ isolates (all ST-6 isolates and two of three ST-7 isolates of this study), consistent with the PFGE pattern assignments. However, one ST-7 *Bla*⁺ isolate (TX0669, also PFGE type 5) was assigned a different ET, which was also the ET of Chile isolates in that study as well as another isolate JH2-2 (TX4000), which differed by PFGE (type VII) and MLST (ST-8). These various results, taken together, suggest the possibility of cross-contamination in the MLEE study and misassignment of the ET type of TX0669.

The phylogenetic trees of both the MLST (linkage distance derived from allelic variation [Fig. 3a]) and the composite sequence alignment (linkage distance derived from sequence divergence [Fig. 3b]) confirmed the similar clustering of clonal and related isolates, in addition to differentiating the non-related (as defined by other techniques) isolates. However, an apparent difference was observed in interrelationships of possibly related isolates such as ST-2 and ST-12 that differed

extensively at a single locus. The difference in the two schemes is related to the different criteria that were used to generate linkage distances, i.e., in MLST, for a given locus, equal weight was given for an allele with a single base change or multiple base changes, while in the composite sequence-based typing weight was given to every base change. At this stage, we are not

TABLE 3. Genetic variation in four sequenced loci

Gene	Fragment size (bp)	No. of alleles	No. of variable sites	No. of synonymous ^a base substitutions	SNV ^b alleles
<i>ace</i>	959 ^c	9	54	34	B, C
<i>efaA</i>	693	9	9	8	A, B; B, C, D; C, E; D, F; G, H
<i>pyrC</i>	320	5	8 ^d	7	A, B, C, D
<i>salA</i>	919, 922 ^e	9	21 ^f	14	C, D

^a Synonymous base substitutions are nucleotide changes which did not result in amino acid change.

^b SNVs are alleles that differ by a single nucleotide.

^c In strain TX0635, a 69-nt in-frame deletion was observed.

^d Five of eight variable sites were observed in a single allele.

^e A 3-bp in-frame deletion is observed in 12 of 22 strains.

^f Of 21 variable sites, 18 nt are base substitutions and the remaining 3 nt are deletions.

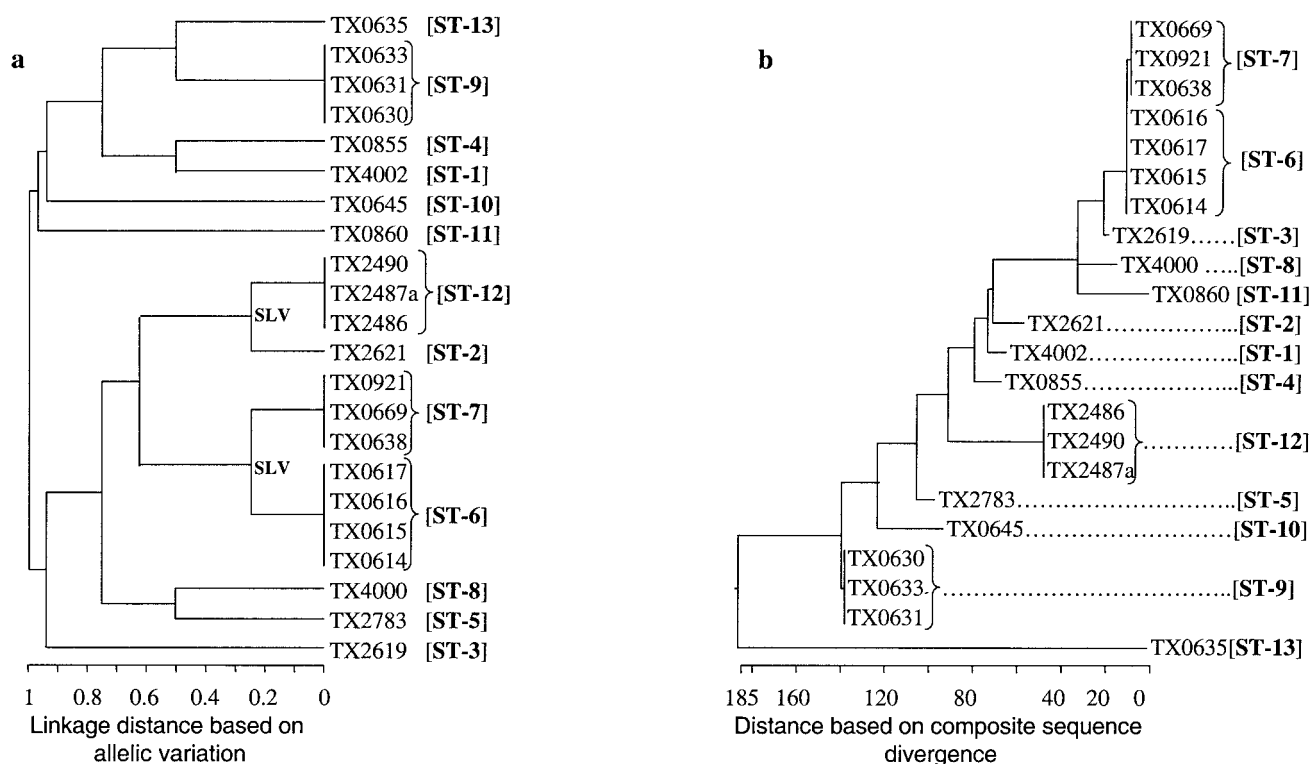


FIG. 3. Phylogenetic trees of genetic relationships among 22 *E. faecalis* strains based on sequences of four gene fragments. (a) The dendrogram is based on the matrix of pairwise differences in the allelic sequences as determined by the UPGMA method. Linkage distances are indicated by scale at the bottom. (b) The cladogram is based on the matrix of pairwise sequence divergence in the concatenated composite sequences (generated by the Jotun Hein method of the DNASTAR software package). The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

able to draw any conclusion on the epidemiological significance of standard MLST approach versus the composite sequence approach due to our inadequate knowledge on relative rates of mutations/recombinations in enterococci.

Evaluation of minimum number of loci needed for subtyping. We also examined the possibility that the sequence variation of fewer loci might be adequate for subspecies typing. The allelic profiles from two antigenic genes, namely, *ace* and *salA*, were found to be sufficient to distinguish all 13 STs. Similarly, the allelic profiles from a second pair of genes, *ace* and *efaA*, distinguished the 12 STs that match with the 12 PFGE types. These results indicate that the combined mutation rates of the *ace*, *efaA*, and *salA* polymorphic regions are comparable to the overall chromosome mutation rates that were detected by PFGE. Strains with different *ace* genes may have the same *salA* or *efaA* genes, or vice versa, as a result of differences in the rates of selection pressure or horizontal recombination as suggested for *por* and *opa* genes of *Neisseria gonorrhoeae* (50). Similar to this idea, others have also applied sequence typing to one or two genes and successfully differentiated most isolates of *N. meningitidis*, *N. gonorrhoeae*, *S. aureus*, and *S. pyogenes* (1, 33, 38, 39, 41, 42, 45, 50). A recent MLST study also used an antigen-encoding gene, in addition to housekeeping genes, to distinguish a meningococcal outbreak (13).

We also identified the most polymorphic regions by examining the distribution of variable sites in the sequenced frag-

ments of all four loci. Although the variations in *ace* are distributed evenly in the complete sequenced region, omission of 200 bases at the 3' end would not have affected the number of *ace* alleles identified. Designing the *ace* reverse PCR primer in the region between bp 910 and 880 (with reference to the start codon) would allow amplification of a PCR product in all 22 strains, thus resolving the problem of using different reverse amplification primers for some isolates (see Materials and Methods). Similarly, the first 377 bp of the sequenced region of *salA* has only one nucleotide variation, and omission of this 377-bp *salA* sequence did not affect the allele profile of this study.

In summary, we studied both micro- and macrovariation of *E. faecalis* isolates by sequencing three antigen-encoding genes and one housekeeping gene. Our results demonstrated that this sequence-based typing method was comparable to PFGE typing in differentiating *E. faecalis* at the subspecies level, including identification of outbreak isolates. DNA sequencing of *ace* and *salA* gene fragments appears to be as efficient as sequencing of all four genes for distinguishing isolates included in this study.

ACKNOWLEDGMENT

This work was supported by NIH grant AI47923 from the Division of Microbiology and Infectious Diseases to B. E. Murray.

REFERENCES

1. Beall, B., R. Facklam, T. Hoenes, and B. Schwartz. 1997. Survey of *emm* gene sequences and T-antigen types from systemic *Streptococcus pyogenes* infection isolates collected in San Francisco, California; Atlanta, Georgia; and Connecticut in 1994 and 1995. *J. Clin. Microbiol.* **35**:1231–1235.
2. Caprioli, T., F. Zaccour, and S. S. Kasatiya. 1975. Phage typing scheme for group D streptococci isolated from human urogenital tract. *J. Clin. Microbiol.* **2**:311–317.
3. Chiew, Y. F., and L. M. Hall. 1998. Comparison of three methods for the molecular typing of Singapore isolates of enterococci with high-level aminoglycoside resistances. *J. Hosp. Infect.* **38**:223–230.
4. Cocconcelli, P. S., D. Porro, S. Galandini, and L. Senini. 1995. Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett. Appl. Microbiol.* **21**:376–379.
5. Coque, T. M., J. F. Tomayko, S. C. Rieke, P. C. Okhuysen, and B. E. Murray. 1996. Vancomycin-resistant enterococci from nosocomial, community, and animal sources in the United States. *Antimicrob. Agents Chemother.* **40**:2605–2609.
6. 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.
7. Dingle, K. E., F. M. Colles, D. R. Wareing, R. Ure, A. J. Fox, F. E. Bolton, H. J. Bootsma, R. J. Willems, R. Urwin, and M. C. Maiden. 2001. Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**:14–23.
8. Duh, R. W., K. V. Singh, K. Malathum, and B. E. Murray. 2001. *In vitro* activity of 19 antimicrobial agents against enterococci from healthy subjects and hospitalized patients and use of an *ace* gene probe from *Enterococcus faecalis* for species identification. *Microb. Drug Resist.* **7**:39–46.
9. Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. *J. Clin. Microbiol.* **38**:1008–1015.
10. Enright, M. C., and B. G. Spratt. 1999. Multilocus sequence typing. *Trends Microbiol.* **7**:482–487.
11. Enright, M. C., and B. G. Spratt. 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* **144**:3049–3060.
12. Enright, M. C., B. G. Spratt, A. Kalia, J. H. Cross, and D. E. Bessen. 2001. Multilocus sequence typing of *Streptococcus pyogenes* and the relationships between *emm* type and clone. *Infect. Immun.* **69**:2416–2427.
13. Feavers, I. M., S. J. Gray, R. Urwin, J. E. Russell, J. A. Bygraves, E. B. Kaczmarek, and M. C. Maiden. 1999. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. *J. Clin. Microbiol.* **37**:3883–3887.
14. Gordillo, M. E., K. V. Singh, and B. E. Murray. 1993. Comparison of ribotyping and pulsed-field gel electrophoresis for subspecies differentiation of strains of *Enterococcus faecalis*. *J. Clin. Microbiol.* **31**:1570–1574.
15. Goulding, J. N., J. V. Hookey, J. Stanley, W. Olver, K. R. Neal, D. A. A. Ala'Aldeen, and C. Arnold. 2000. Fluorescent amplified-fragment length polymorphism genotyping of *Neisseria meningitidis* identifies clones associated with invasive disease. *J. Clin. Microbiol.* **38**:4580–4585.
16. Hein, J. 1990. Unified approach to alignment and phylogenies. *Methods Enzymol.* **183**:626–645.
17. Jacob, A. E., and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. *J. Bacteriol.* **117**:360–372.
18. Kuhn, I., L. G. Burman, S. Haeggman, K. Tullus, and B. E. Murray. 1995. Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. *J. Clin. Microbiol.* **33**:2812–2817.
19. Li, X., G. M. Weinstock, and B. E. Murray. 1995. Generation of auxotrophic mutants of *Enterococcus faecalis*. *J. Bacteriol.* **177**:6866–6873.
20. Lowe, A. M., P. A. Lambert, and A. W. Smith. 1995. Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. *Infect. Immun.* **63**:703–706.
21. Maiden, M. C., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. USA* **95**:3140–3145.
22. Malathum, K., K. V. Singh, G. M. Weinstock, and B. E. Murray. 1998. Repetitive sequence-based PCR versus pulsed-field gel electrophoresis for typing of *Enterococcus faecalis* at the subspecies level. *J. Clin. Microbiol.* **36**:211–215.
23. Miranda, A. G., K. V. Singh, and B. E. Murray. 1992. Determination of the chromosomal size of three different strains of *Enterococcus faecalis* and one strain of *Enterococcus faecium*. *DNA Cell Biol.* **11**:331–335.
24. Mundy, L. M., D. F. Sahn, and M. Gilmore. 2000. Relationships between enterococcal virulence and antimicrobial resistance. *Clin. Microbiol. Rev.* **13**:513–522.
25. Murray, B. E. 1990. The life and times of the enterococcus. *Clin. Microbiol. Rev.* **3**:46–65.
26. Murray, B. E. 2000. Vancomycin-resistant enterococcal infections. *N. Engl. J. Med.* **342**:710–721.
27. Murray, B. E., H. A. Lopardo, E. A. Ruboglio, M. Frosolono, and K. V. Singh. 1992. Intrahospital spread of a single gentamicin-resistant, beta-lactamase-producing strain of *Enterococcus faecalis* in Argentina. *Antimicrob. Agents Chemother.* **36**:230–232.
28. Murray, B. E., K. V. Singh, J. D. Heath, B. R. Sharma, and G. M. Weinstock. 1990. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. *J. Clin. Microbiol.* **28**:2059–2063.
29. Murray, B. E., K. V. Singh, S. M. Markowitz, H. A. Lopardo, J. E. Patterson, M. J. Zervos, E. Ruboglio, G. M. Eliopoulos, L. B. Rice, F. W. Goldstein, S. G. Jenkins, G. M. Caputo, N. Nasnas, L. S. Moore, E. S. Wong, and G. Weinstock. 1991. Evidence for clonal spread of a single strain of beta-lactamase-producing *Enterococcus (Streptococcus) faecalis* to six hospitals in five states. *J. Infect. Dis.* **163**:780–785.
30. Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock. 1993. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J. Bacteriol.* **175**:5216–5223.
31. Nallapareddy, S. R., K. V. Singh, R.-W. Duh, G. M. Weinstock, and B. E. Murray. 2000. Diversity of *ace*, a gene encoding a microbial surface component recognizing adhesive matrix molecules, from different strains of *Enterococcus faecalis* and evidence for production of Ace during human infections. *Infect. Immun.* **68**:5210–5217.
32. Olive, D. M., and P. Bean. 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* **37**:1661–1669.
33. Oliveira, D. C., I. Crisostomo, I. Santos-Sanches, P. Major, C. R. Alves, M. Aires-de-Sousa, M. K. Thege, and H. de Lencastre. 2001. Comparison of DNA sequencing of the protein A gene polymorphic region with other molecular typing techniques for typing two epidemiologically diverse collections of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **39**:574–580.
34. Patterson, J. E., K. V. Singh, and B. E. Murray. 1991. Epidemiology of an endemic strain of beta-lactamase-producing *Enterococcus faecalis*. *J. Clin. Microbiol.* **29**:2513–2516.
35. Pryce, T. M., R. D. Wilson, and J. K. Kulski. 1999. Identification of enterococci by ribotyping with horseradish-peroxidase-labelled 16S rDNA probes. *J. Microbiol. Methods* **36**:147–155.
36. Rich, R. L., B. Kreikemeyer, R. T. Owens, S. LaBrenz, S. V. Narayana, G. M. Weinstock, B. E. Murray, and M. Hook. 1999. Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. *J. Biol. Chem.* **274**:26939–26945.
37. Robredo, B., C. Torres, K. V. Singh, and B. E. Murray. 2000. Molecular analysis of Tn1546 in *vanA*-containing *Enterococcus* spp. isolated from humans and poultry. *Antimicrob. Agents Chemother.* **44**:2588–2589.
38. Sacchi, C. T., A. P. Lemos, A. M. Whitney, C. A. Solari, M. E. Brandt, C. E. Melles, C. E. Frasch, and L. W. Mayer. 1998. Correlation between serological and sequencing analyses of the PorB outer membrane protein in the *Neisseria meningitidis* serotyping system. *Clin. Diagn. Lab. Immunol.* **5**:348–354.
39. Saunders, N. A., G. Hallas, E. T. Gaworzewska, L. Metherell, A. Efstratiou, J. V. Hookey, and R. C. George. 1997. PCR-enzyme-linked immunosorbent assay and sequencing as an alternative to serology for M-antigen typing of *Streptococcus pyogenes*. *J. Clin. Microbiol.* **35**:2689–2691.
40. Seetulsingh, P. S., J. F. Tomayko, P. E. Coudron, S. M. Markowitz, C. Skinner, K. V. Singh, and B. E. Murray. 1996. Chromosomal DNA restriction endonuclease digestion patterns of beta-lactamase-producing *Enterococcus faecalis* isolates collected from a single hospital over a 7-year period. *J. Clin. Microbiol.* **34**:1892–1896.
41. Shopsis, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehm, S. Naidich, and B. N. Kreiswirth. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **37**:3556–3563.
42. Shopsis, B., M. Gomez, M. Waddington, M. Riehm, and B. N. Kreiswirth. 2000. Use of coagulase gene (*coa*) repeat region nucleotide sequences for typing of methicillin-resistant *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **38**:3453–3456.
43. Singh, K. V., T. M. Coque, G. M. Weinstock, and B. E. Murray. 1998. *In vivo* testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologs for species identification. *FEMS Immunol. Med. Microbiol.* **21**:323–331.
44. Swanson, D. S., X. Pan, and J. M. Musser. 1996. Identification and subspecific differentiation of *Mycobacterium scrofulaceum* by automated sequencing of a region of the gene (*hsp65*) encoding a 65-kilodalton heat shock protein. *J. Clin. Microbiol.* **34**:3151–3159.
45. Tang, Y. W., M. G. Waddington, D. H. Smith, J. M. Manahan, P. C. Kohner, L. M. Highsmith, H. Li, F. R. Cockerill III, R. L. Thompson, S. O. Montgomery, and D. H. Persing. 2000. Comparison of protein A gene sequencing with pulsed-field gel electrophoresis and epidemiologic data for molecular

- typing of methicillin-resistant *Staphylococcus aureus*. J. Clin. Microbiol. **38**: 1347–1351.
46. Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33**:2233–2239.
 47. Thorisdottir, A. S., L. L. Carias, S. H. Marshall, M. Green, M. J. Zervos, C. Giorgio, L. A. Mermel, J. M. Boyce, A. A. Medeiros, H. Fraimow, and L. B. Rice. 1994. IS6770, an enterococcal insertion-like sequence useful for determining the clonal relationship of clinical enterococcal isolates. J. Infect. Dis. **170**:1539–1548.
 48. Tomayko, J. F., and B. E. Murray. 1995. Analysis of *Enterococcus faecalis* isolates from intercontinental sources by multilocus enzyme electrophoresis and pulsed-field gel electrophoresis. J. Clin. Microbiol. **33**:2903–2907.
 49. Torres, O. R., R. Z. Korman, S. A. Zahler, and G. M. Dunny. 1991. The conjugative transposon Tn925: enhancement of conjugal transfer by tetracycline in *Enterococcus faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E. faecalis*. Mol. Gen. Genet. **225**:395–400.
 50. Viscidi, R. P., J. C. Demma, J. Gu, and J. Zenilman. 2000. Comparison of sequencing of the *por* gene and typing of the *opa* gene for discrimination of *Neisseria gonorrhoeae* strains from sexual contacts. J. Clin. Microbiol. **38**: 4430–4438.