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Species Identification of Enterococci via Intergenic Ribosomal PCR

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Accurate species identification of enterococci has become important with the wide prevalence of acquired vancomycin resistance and the presence of less epidemiologically important, inherently vancomycin-resistant enterococci. Using a collection of enterococcal strains, we found that PCR amplification of the intergenic spacer (ITS-PCR) between the 16S and 23S rRNA genes can produce amplicon profiles characteristic of the enterococcus examined. The species examined were group I enterococci (*Enterococcus avium*, *Enterococcus raffinosus*, *Enterococcus malodoratus*, and *Enterococcus pseudoavium*), group II enterococci (*Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus casseliflavus*, *Enterococcus mundtii*, and *Enterococcus gallinarum*), and group III enterococci (*Enterococcus durans* and *Enterococcus hirae*). The enterococcal species in group I, as well as *E. faecalis* and two strains of *E. hirae*, were similar and therefore had to be differentiated from each other by *Sau*3A restriction digests. This produced patterns characteristic of each of these species. The remaining group II and group III enterococcal species produced amplicons characteristic of a particular species except *E. gallinarum*. The PCR products from *E. gallinarum* displayed strain-to-strain heterogeneity in the number and size of amplicons. To further test the utility of this technique, 11 phenotypically aberrant strains which had been assigned species identification based on Facklam and Collins-type strain reactions (R. R. Facklam and M. D. Collins, J. Clin. Microbiol. 27:731–734, 1989) were subjected to ITS-PCR. ITS-PCR of the phenotypically aberrant strains identified six strains with reactions consistent with those of type strains. However, five strains were characterized as follows: two strains originally identified as *E. mundtii* were identified by ITS-PCR as *E. casseliflavus*, one strain originally identified by ITS-PCR as *E. raffinosus* was identified as *E. durans*, one strain originally identified by ITS-PCR as *E. hirae* was identified as *E. faecium*, and one strain originally identified as *E. durans* was identified by ITS-PCR as *E. hirae*. We conclude that amplification of the intergenic 23S and 16S rRNA gene regions of enterococci provides a reliable technique for species identification of enterococci.

Enterococci are recognized as important agents of nosocomially acquired infections such as bacteremia (17). These infections are often difficult to treat due to the increased antibiotic resistance associated with this organism, leaving few therapeutic options (13). As an aid in the management of patients with such infections, species identification can play an important role. For example, determining if two enterococcal isolates recovered from two consecutive blood cultures are the same enterococcal species can be important in the selection of appropriate antibiotics for the treatment of a patient (13, 16). Enterococcal species identification can be a useful tool for epidemiologic surveillance. Species identification of enterococci can determine whether a possible outbreak of vancomycin-resistant enterococci (VRE) is caused by a species such as *Enterococcus faecium*, is due to an intrinsically vancomycin-resistant species such as *Enterococcus gallinarum*, or is caused by an unusual enterococcal species not previously recognized as being vancomycin resistant.

Studies have shown that *Enterococcus faecalis* accounts for 80 to 90% of infections, followed by *E. faecium*, which accounts for 5 to 10% of infections, and the other enterococcal species, which account for the remainder (6, 13). Species identification of enterococci is usually determined phenotypically by using characteristics such as motility, pigment production, and carbohydrate utilization (6, 18). However, this becomes difficult when unusual strains with few characteristic reactions arise and when nonmotile *E. gallinarum* or *Enterococcus casseliflavus* strains are encountered. Dutka-Malen et al. (5) have added the technique of PCR for rapid species identification of *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* based on amplification of the species-specific ligase gene or portion of this gene in these species. However, this technique only identifies the four species mentioned above, leaving the other enterococcal species unidentifiable except by phenotypic means. Therefore, an alternative rapid DNA-based methodology would be an asset to the identification of the remaining enterococci to the species level.

To explore the use of molecular biology for bacterial species identification, previous investigators have used 16S and 23S rRNA sequences as targets for DNA probes and/or sequencing of these genes (2–4, 9, 19). However, due to evolutionary constraints, there is minimal variation in the 16S or 23S rRNA sequences between species of bacteria. Barry et al. (2) and Jensen et al. (8) overcame the problem of minimal variability by examining the 16S-23S rDNA (genes coding for rRNA) intergenic region, referred to as the internally transcribed spacer region (ITS). It was suggested that this segment of DNA would be under minimal selective pressure compared to the selective pressure that rRNA genes would be under and therefore may allow for species identification due to enhanced variability between species within a genus. This has met with some success for bacterial genera such as *Listeria*, *Escherichia*, *Enterobacter*, and *Citrobacter* (8). We have further expanded that work to include the genus *Enterococcus.*

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TABLE 1. Oligonucleotides used in the study

Amplicon name	Size of PCR product (bp)	Oligonucleotide	Sequence	Origin (reference)
Van A	1,031	A1	5′-ATGAATAGAATAAAAGTTGCA	1
		A2	5′-TTATCACCCCTTTAACGCT	
Van RS	1,849	R1	5′-ATGAGCGATAAAATACTT	1
		S2	5′-TTAGGACCTCCTTTTATC	
Van HAX	2,607	H1	5′-ATGAATAACATCGGCATTAC	1
		X2	5′-TTATTTAACGGGGAAATC	
Van YZ	1,550	Y1	5′-ATGAAGAAGTTGTTTTTTTTA	1
		Z2	5′-CTTACACGTAATTTATTC	
Van B	457	B	5′-CCCGAATTTCAAATGATTGAAAA	12
		B1	5′-CGCCATCCTCCTGCAAAA	
Van C1 *E. casseliflavus*	822	C1	5′-GGTATCAAGGAAACCTC	5
		C2	5′-CTTCCGCCATCATAGCT	
Van C2 *E. gallinarum*	439	D1	5′-CTCCTACGATTCTCTTG	5
		D2	5′-CGAGCAAGACCTTTAAG	
ITS-RBS	Various	L1	5′-CAAGGCATCCACCGT	8
		G1	5′-GAAGTCGTAACAAGG	

MATERIALS AND METHODS

Bacterial strains. The enterococcal strains used in this study, their sources, and their vancomycin phenotypes prior to ITS-PCR are listed below. Strains from New York City (NYC strains) are enterococcal isolates submitted to the New York Public Health Research Institute from various hospitals throughout New York City and are a gift of Barry Kreiswirth. Strains from the Centers for Disease Control and Prevention (CDC) in Atlanta, Ga., are a gift of Richard Facklam. VGH strains were collected from the Victoria General Hospital, Halifax, Nova Scotia, Canada. *E. faecalis* T-25 is a gift of John Conly, Toronto Hospital, Toronto, Ontario, Canada. Included in this collection are 11 American Type Culture Collection (ATCC) type strains as well as a number of biochemically aberrant strains of enterococci. These aberrant strains differ by one or more phenotypic traits from the prototypic standard phenotypic traits defined by Facklam and Collins (6) for species identification of enterococci (20). VSE strains are vancomycin-susceptible enterococcal isolates. The following enterococcal strains were used in this study. Group I strains were *Enterococcus avium* ATCC 14025 VSE, SS-559 CDC VSE, 353-77 CDC VSE, 2098-75 CDC VSE, 695-80 CDC VSE, 589-85 CDC VSE, 517-87 CDC VSE, and W43 NYC VSE; *Enterococcus raffinosus* ATCC 49427 VSE, SS-1278 CDC VSE, 1962-81 CDC VSE, 887-82 CDC VSE, 1237-84 CDC VSE, 1157-84 CDC VSE, 1399-87 CDC VSE, 512-85 CDC VSE, and W185 NYC VSE; *Enterococcus malodoratus* ATCC 43197 VSE, SS-1226 CDC VSE, 1999-89 CDC VSE, 2000-89 CDC VSE; and *Enterococcus pseudoavium* ATCC 49372 VSE and SS-1277 CDC VSE. Group II strains were *E. faecalis* ATCC 19433 VSE, 144/92 NYC VanA, 187/92 NYC VanA, 251/92 NYC VanA, 290/92 NYC VanA, JH2-2 (7) VSE, and T-25 Toronto VanB; *E. faecium* ATCC 19434 VSE, 137/92 NYC VanA, 141/92 NYC VanA, W1 NYC VanA, W97 NYC VanA, W99 NYC VanA, W229 NYC VanA, W231 NYC VanA, W233 NYC VanA, W235 NYC VanA, W243 NYC VanA, W33-91 NYC VanA, and W14-91 NYC VanA; *E. casseliflavus* ATCC 25788 VanC, 96-12-0917 VGH VanC, 96-16-1221 VGH VanC, 96-34-0619 VGH VanC, 0497 VGH VanC, and W209 NYC VanC; *Enterococcus mundtii* ATCC 43186 VSE, SS-1232 CDC VSE, SS-1233 CDC VSE, SS-1234 CDC VSE, 1491-76 CDC VSE, 236-94 CDC VSE, W73 NYC VanA, W74 NYC VanA, and W186 NYC VSE; and *E. gallinarum* ATCC 49573 VanC, 96-19-1482 VGH VanC, 96-19-1480 VGH VanC, 96-19-0906 VGH VanC, 96-15-0770 VGH VanC, 96-19-0875 VGH VanC, 96-12-0671 VGH VanC, 96-25-1194 VGH VanC, 96-29-0567 VGH VanC, 1550 VGH VanC, 0441 VGH VanC, 2003 VGH VanC, W189 NYC VanC, W211 NYC VanC, and W376 NYC VanC. Group III strains were *Enterococcus durans* ATCC 19432 VSE, SS-661 CDC VSE, SS-497 CDC VSE, SS-1225 CDC VSE, 1880-79 CDC VSE, 2042-91 CDC VSE, and W148 NYC VSE and *Enterococcus hirae* ATCC 8043 VSE, SS-1227 CDC VSE, 662-91 CDC VSE, 663-91 CDC VSE, 2537-93 CDC VSE, 3564-95 CDC VSE, and W225 NYC VanA.

Species identification and vancomycin genotype determination. All enterococci used in this study were subjected to the phenotypic identification methods of Facklam and Collins (6). The vancomycin (Sigma Chemical Co., St. Louis, Mo.) MIC was determined by the agar dilution method according to National Committee for Laboratory Standards guidelines (14). All VRE were subjected to the PCR with oligonucleotides specific for the *vanA* and *vanB* ligase genes, the *E. casseliflavus vanC* ligase gene, and the *E. gallinarum vanC* ligase gene. Strains were classified as VanA if the PCR demonstrated the presence of the *vanA* ligase gene and as VanB if the PCR demonstrated the presence of the *vanB* ligase gene. Also, strains were classified as VanC if isolates were identified as *E. casseliflavus* or *E. gallinarum* as determined by pigment production and/or motility and if the PCR demonstrated the presence of the *vanC* ligase gene specific for each species.

DNA preparation. The enterococci were cultured on Trypticase soy agar containing sheep blood (Becton Dickinson and Co., Cockeysville, Md.) and were subsequently cultured in 2 ml of Todd-Hewitt broth (Becton Dickinson and Co.) and harvested after overnight growth. Total DNA was prepared by the guanidium thiocyanate method of Pitcher et al. (15).

PCR. All oligonucleotides used in this study are listed in Table 1. Oligonucleotides A1, A2, R1, S2, H1, X2, Y1, and Z2 were designed on the basis of the nucleotide sequence of the transposon Tn*1546* (1). Oligonucleotides B and B1 are primer sequences specific for the *vanB* ligase gene (12). Oligonucleotides C1 and C2 (specific for the ligase gene of *E. gallinarum*) and oligonucleotides D1 and D2 (specific for the ligase gene of *E. casseliflavus*) are published sequences (5). Oligonucleotides L1 and G1 are the same as those described by Jensen et al. (8). All oligonucleotides were purchased from Gibco BRL, Burlington, Ontario, Canada. PCR amplifications were performed by using a DNA Engine PTC 2000 thermocycler (MJ Research) in 0.5-ml Micro Amp reaction tubes. *Taq* DNA polymerase was purchased from Boehringer Manneheim Canada (Laval, Quebec, Canada). Amplifications were performed in mixtures containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (pH 8.3), 0.15 mM deoxynucleoside triphosphates, and 1.25 U of *Taq* DNA polymerase. The final reaction volume of 0.05 ml contained 50 pmol of each primer and 500 ng of enterococcal DNA purified by the method of Pitcher et al. (15). Thermocycler reaction conditions were as follows: amplicons van A, van RS, van HAX, and van YZ were generated by an initial cycle of 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 74°C for 3 min, after which an extension cycle of 74°C for 7 min was added. Amplicons van C1 and van C2 were generated according to the conditions of Dutka-Malen et al. (5). ITS-PCR amplicons were generated according to the thermocycler conditions published by Jensen et al. (8).

Restriction endonuclease digestions. Amplicons were treated with 1 U of *Sau*3A restriction endonuclease purchased from Gibco BRL for 1 h at 37°C. After incubation, the DNA was subjected to gel electrophoresis.

Electrophoresis. A 10-μl aliquot of the amplicon was combined with 5 μl of loading buffer, and the preparation was electrophoresed on a 6% nondenaturing acrylamide-bisacrylamide (29:1) gel by using the Mini Protean II gel electrophoresis apparatus (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). The gels were stained with ethidium bromide and were photographed on a UV transilluminator.

RESULTS

Species identification and vancomycin genotype determination. All enterococci were initially assigned a species designa-

TABLE 2. Species determination of phenotypically aberrant strains

Phenotypic characterization	Result for the following strain:									
	W43	W73	W74	W97, W99	W148	W185	W186	W209	W225	W33-91
Mannitol	+	+	+	+	−	+[a]	+	+	−[b]	+
Sorbitol	+	−	+[a]	−	−	+[b]	+[a]	+[a]	−	+[a]
Sorbose	+	−	−	+[b]	−	+[b]	+[b]	−	−	−
Arginine	+[b]	+	+	−[b]	+	+	+	+	+	+
Arabinose	+	+	+	−[b]	−	+[b]	+	+	+	+
Raffinose	−	+	+	−	−[b]	+[b]	+	+	−	+[a]
Tellurite	−	−[b]	−[b]	−	−	−	−	−	−	−
Motility	−	−	−	−	−	−	−	+	−	−
Pigment	−	+	+	−	−	−	+	+	−	−
Sucrose	+	+	+	+	+	+[b]	+	+	+	+
Phenotypic identification	*E. avium*	*E. mundtii*	*E. mundtii*	*E. faecium*	*E. durans*	*E. raffinosus*	*E. mundtii*	*E. casseliflavus*	*E. hirae*	*E. faecium*
ITS-PCR identification	*E. avium*	*E. casseliflavus*	*E. casseliflavus*	*E. faecium*	*E. hirae*	*E. durans*	*E. mundtii*	*E. casseliflavus*	*E. faecium*	*E. faecium*

[a] Reaction is an occasional exception.
[b] Reaction opposite that of the species identified by ITS-PCR.

tion on the basis of the phenotypic identification method of Facklam and Collins (6). Eleven enterococcal strains possessed phenotypic traits that did not allow conclusive species identification to take place (Table 2). All VRE isolates in this group were subjected to the PCR for *vanA*, *vanB*, *E. casseliflavus vanC*, and *E. gallinarum vanC* ligase genes (data not shown). Amplification of the *E. casseliflavus vanA* ligase, *vanB* ligase, and *vanC* ligase genes and the *E. gallinarum vanC* ligase genes identified 19 isolates as VanA, 1 isolate as VanB, and 21 isolates as VanC.

ITS-PCR profiles. To achieve maximum resolution of the ITS-PCR amplicon profiles, it was necessary to use a 6% nondenaturing acrylamide-bisacrylamide gel. Agarose gels of various concentrations failed to adequately resolve the ITS-PCR amplicon profiles. ATCC strains of different enterococcal species gave ITS-PCR patterns different from one another on the basis of amplicon size and the number of amplicons present (Fig. 1). All ATCC enterococcal species (except for *E. gallinarum*) possessed two or three major bands with approximate sizes of 300 to 600 bp and larger minor bands. The ATCC enterococcal strains *E. avium*, *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, and *E. faecalis* produced major amplicon products similar in size, making differentiation difficult. This was overcome by cleaving these ATCC species-specific amplicons with *Sau*3A. After cleavage, the ITS-PCR amplicon pattern on the gel was diagnostic for the ATCC strains *E. raffinosus*, *E. malodoratus*, and *E. faecalis* (Fig. 2). However, both ATCC strains of *E. avium* and *E. pseudoavium* possessed identical *Sau*3A digestion profiles (Fig. 2).

To validate ITS-PCR as a method for identifying species among isolates of enterococci, various strains of known species were collected from a number of centers. All enterococcal strains gave the same or similar ITS-PCR pattern as the ATCC prototype strains (Fig. 3). No variability was seen among strains of the species *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, *E. faecium*, *E. casseliflavus*, and *E. durans* examined (Fig. 3 and 4). *E. avium* 353-77 and 2098-75 possessed profile variability among the poorly amplified fragments; however, the intensely stained major bands were consistent with those of the *E. avium* prototype strain (Fig. 3). The majority of *E. faecalis* and *E. mundtii* isolates possessed two major band products; however, some strains appeared to have doublet bands that ran very close together. This always occurred with the larger major band. *E. gallinarum* and *E. hirae* possessed variability in the sizes and numbers of major band products (Fig. 3 and 4). *E. gallinarum* exhibited the highest degree of variability, ranging

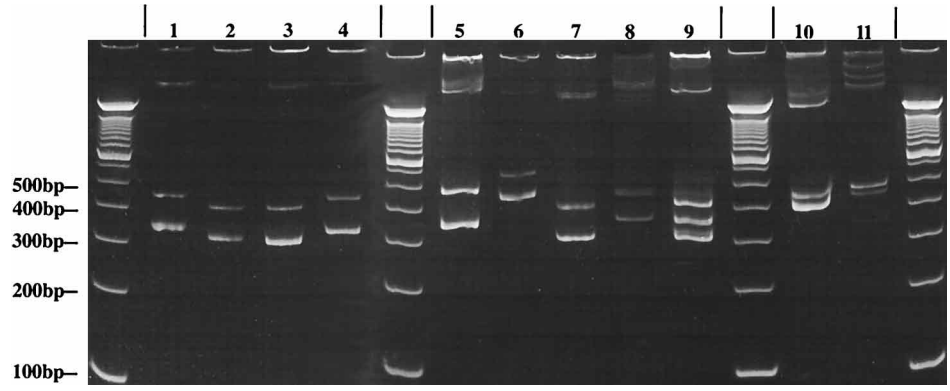

FIG. 1. ITS-PCR profiles of ATCC enterococcal species. Lanes: 1, *E. avium* ATCC 14025; 2, *E. raffinosus* ATCC 49427; 3, *E. malodoratus* ATCC 43197; 4, *E. pseudoavium* ATCC 49372; 5, *E. faecalis* ATCC 19433; 6, *E. faecium* ATCC 19434; 7, *E. casseliflavus* ATCC 25788; 8, *E. mundtii* ATCC 43186; 9, *E. gallinarum* ATCC 49573; 10, *E. durans* ATCC 19432; and 11, *E. hirae* ATCC 8043. The species are divided into groups on the basis of the Facklam and Collins identification scheme (6). The unnumbered lanes are molecular DNA size markers.

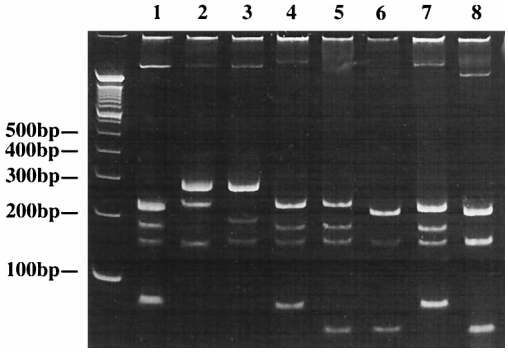

FIG. 2. *Sau*3A-digested ITS-PCR amplicons of ATCC species possessing nearly identical ITS-PCR amplicon patterns and strains W43 and W148. Lanes: 1, *E. avium* ATCC 14025; 2, *E. raffinosus* ATCC 49427; 3, *E. malodoratus* ATCC 43197; 4, *E. pseudoavium* ATCC 49372; 5, *E. faecalis* ATCC 19433; 6, *E. hirae* 2537-93; 7, W43; and 8, W148. The unnumbered lane is molecular DNA size markers.

from two or more major bands, with the smallest band remaining constant from strain to strain. Because of the high degree of similarity displayed by *E. avium*, *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, *E. faecalis*, and *E. hirae* 2537-93 and 3564-95, their ITS-PCR amplicons were digested with *Sau*3A to generate a more diagnostic pattern for each species (data not shown). The *Sau*3A patterns generated distinguished between species and were the same for each strain within a particular species (data not shown). This indicates that no sequence variability in relation to *Sau*3A cleavage sites occurred from strain to strain among the strains of the species *E. avium*, *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, *E. faecalis*, and *E. hirae* examined.

Interestingly, there was variability in the number and size of high-molecular-weight minor bands running near the top of the gel. Some strains of *E. mundtii* and *E. hirae* possessed multiple faintly staining minor products in this region; however, these large minor amplicons were not present in every *E.*

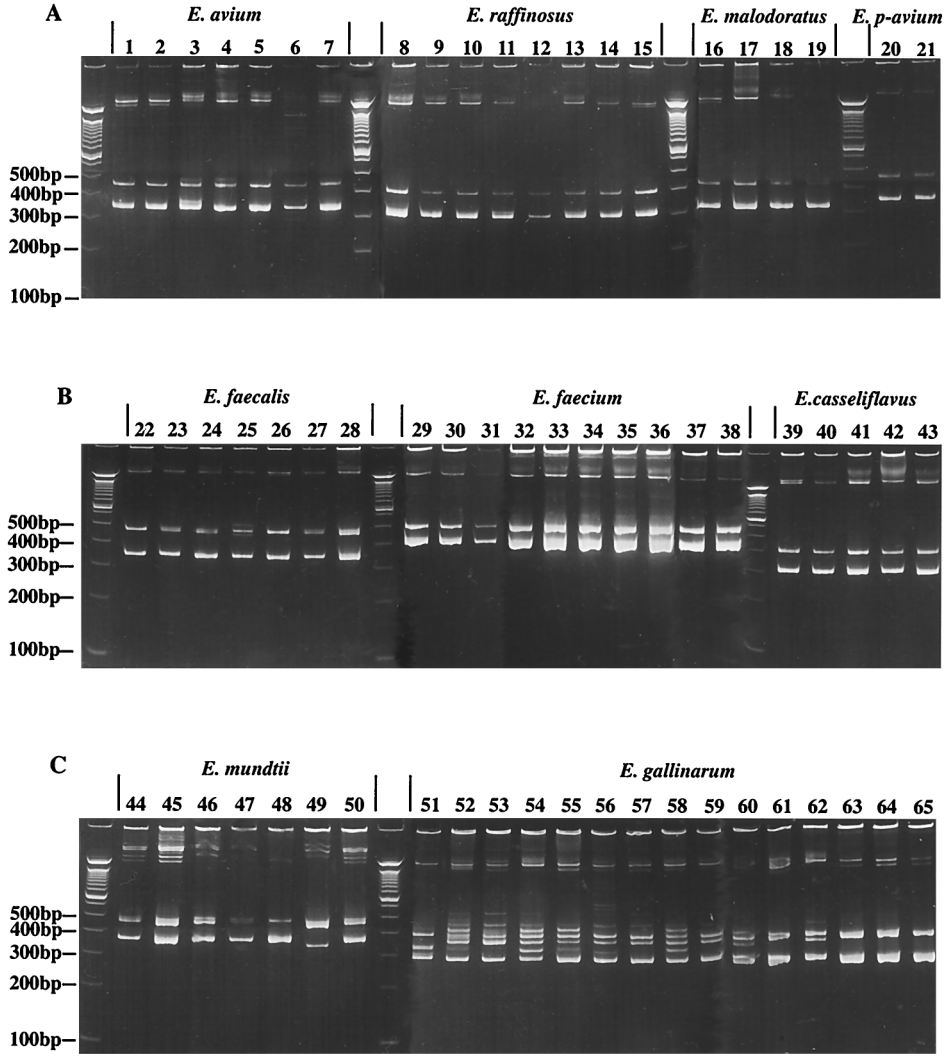

FIG. 3. ITS-PCR profiles of enterococci within a particular species. (A) Group I enterococci. (B and C) Group II enterococci. The species names are listed above each grouping. The numbered lanes are (A) Lanes: 1, ATCC 14025; 2, SS-559; 3, 353-77; 4, 2098-75; 5, 695-80; 6, 589-85; 7, 517-87; 8, ATCC 49427; 9, SS-1278; 10, 1962-81; 11, 887-82; 12, 1237-84; 13, 1157-84; 14, 1399-87; 15, 512-85; 16, ATCC 43197; 17, SS-1226; 18, 1999-89; 19, 2000-89; 20, ATCC 49372; 21, SS-1277. (B) Lanes: 22, ATCC 19433; 23, 144/92; 24, 187/92; 25, 251/92; 26, 290/92; 27, JH2-2; 28, T-25; 29, ATCC 19434; 30, 137/92; 31, 141/92; 32, W1; 33, W229; 34, W231; 35, W233; 36, W235; 37, W243; 38, W14-91; 39, ATCC 25788; 40, 96-12-0917; 41, 96-16-1221; 42, 96-34-0619; and 43, 0497. (C) Lanes: 44, ATCC 43186; 45, SS-1232; 46, SS-1233; 47, SS-1234; 48, 1491-76; 49, 236-94; 50, W186; 51, ATCC 49573; 52, 96-19-1482; 53, 96-19-1480; 54, 96-19-0906; 55, 96-15-0770; 56, 96-19-0875; 57, 96-12-0671; 58, 96-25-1194; 59, 96-29-0567; 60, 1550; 61, 0441; 62, 2003; 63, W189; 64, W211; and 65, W376. The unnumbered lanes are molecular DNA size markers.

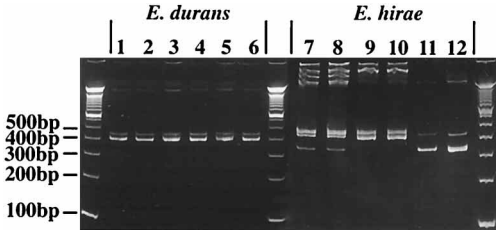

FIG. 4. ITS-PCR profiles of group III enterococci (*E. durans* and *E. hirae*). The species names are listed above the numbered lanes. Lanes: 1, ATCC 19432; 2, SS-661; 3, SS-497; 4, SS-1225; 5, 1880-79; 6, 2042-91; 7, ATCC 8043; 8, SS-1227; 9, 662-91; 10, 663-91; 11, 2537-93; and 12, 3564-95. The unnumbered lanes are molecular DNA size markers.

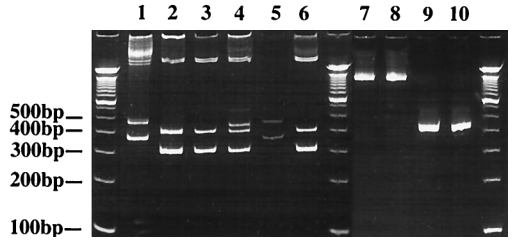

FIG. 5. Strain identification of isolates W73, W74, W186, and W209 and identification of *vanA* and *vanC* amplicons in strains W73 and W74. Lanes: 1, *E. mundtii* ATCC 43186; 2, *E. casseliflavus* ATCC 25788; 3, strain W73; 4, strain W74; 5, strain W186; 6, strain W209; 7, *vanA* amplicon of W73; 8, *vanA* amplicon of W74; 9, *vanC* amplicon of W73; 10, *vanC* amplicon of W74. The unnumbered lanes are molecular DNA size markers.

mundtii and *E. hirae* strain examined (Fig. 3 and 4). However, these products may possibly be used in providing additional confirmation of a species identification which is based on the strongly staining major 300- to 600-bp bands. The nature of these weakly staining large fragments is unclear. We found these large fragments to occur in an inconsistent manner, and hence, they are not particularly useful in making enterococcal species identification.

To determine the ability of ITS-PCR to identify phenotypically aberrant enterococcal strains to which species names had been tentatively assigned, three *E. faecium* strains (W97, W99, and W33-91), three *E. mundtii* strains (W73, W74, and W186), and one strain each of *E. hirae* (W225), *E. avium* (W43), *E. casseliflavus* (W209), *E. raffinosus* (W185), and *E. durans* (W148) were subjected to ITS-PCR. These strains had proven to be difficult to identify to the species level due to one or more biochemical reactions or motility characteristics not being consistent with the Facklam and Collins-type strain reactions (6) (Table 2). ITS-PCR identified strain W186 as *E. mundtii* and strain W209 as *E. casseliflavus* (Fig. 5) and *E. faecium* strains as *E. faecium* (Fig. 6). However, ITS-PCR identified strains W73 and W74 as *E. casseliflavus* and not *E. mundtii* (Fig. 5), strain W185 as *E. durans* and not *E. raffinosus* (Fig. 6), and strain W225 as *E. faecium* and not *E. hirae* (Fig. 6). Interestingly, W74 displayed variability in its ITS-PCR profile compared to that of *E. casseliflavus* ATCC 25788; however, the ITS-PCR profile most closely resembled that of *E. casseliflavus* as opposed to that of the other pigmented enterococcus, *E. mundtii*. Due to the nearly identical ITS-PCR patterns exhibited by W43 and W148 in relation to those of *E. avium*, *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, *E. faecalis*, and some strains of *E. hirae*, it was necessary to digest these two ITS-PCR amplicons with *Sau*3A. After digestion, the ITS-PCR profiles of W43 and W148 were identical by molecular size and the number of bands to those of *E. avium* and *E. hirae*, respectively (Fig. 2).

Identification of genes mediating vancomycin resistance in W73 and W74. Of the enterococcal species whose phenotypic identification differed from the ITS-PCR identification, only W73 and W74 had high-level vancomycin resistance. To determine if W73 and W74 possessed all genes necessary for the VanA resistance phenotype, a PCR for these genes was done. Both W73 and W74 possessed *vanR*, *vanS*, *vanH*, *vanX*, *vanY*, and *vanZ* genes (data not shown) and the *vanA* gene (Fig. 5), indicating that they are VanA isolates. To further substantiate that W73 and W74 belonged to the species *E. casseliflavus*, PCR was performed by using species-specific C1-C2 and D1-D2 oligonucleotide primer pairs (Table 1). Only the *E. casseliflavus vanC2* ligase gene was amplified, proving that W73 and W74 are *E. casseliflavus* strains that have acquired the VanA gene cluster (Fig. 5).

DISCUSSION

ITS-PCR is able to identify to the species level the majority of enterococci in the Facklam and Collins (6) group I, group II, and group III typing scheme (excluding *Enterococcus flavescens*, *E. dispar*, and *E. faecalis* [variant] which were not tested). The biochemical relatedness of each grouping is mirrored in the ITS-PCR profiles of the enterococci tested. The group I enterococci have similar or identical ITS-PCR profiles. Group II enterococci also have similar ITS-PCR profiles; however, the majority can be distinguished from each other. The group III enterococci, *E. durans* and *E. hirae* ATCC 8043, SS-1227, 662-91, and 663-91, have ITS-PCR profiles unlike those found in strains in groups I and II. Due to the high degree of similarity within the group I ITS-PCR profiles, *E. faecalis* and the remaining *E. hirae* strains, strains 2537-93 and 3564-95, it is difficult to distinguish between these species. This was overcome somewhat by *Sau*3A restriction endonuclease cleavage. *E. avium* and *E. pseudoavium* were readily distinguishable from *E. raffinosus*, *E. malodoratus*, *E. faecalis*, and *E. hirae* when the ITS-PCR products were cleaved with *Sau*3A (Fig. 2). However, we were unable to distinguish between *E. avium* and *E. pseudoavium* ITS-PCR profiles either before or after *Sau*3A digestion (Fig. 1 and 2). This suggests that very little, if any, variability exists in the intergenic space between these two species. It is plausible that these two species recently diverged from one common *E. avium* or *E. pseudoavium* species. When

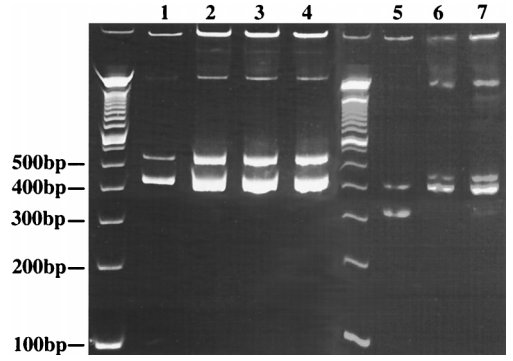

FIG. 6. ITS-PCR profiles of phenotypically aberrant enterococcal strains. Lanes: 1, *E. faecium* ATCC 19434; 2, W97; 3, W225; 4, W33-91; 5, *E. raffinosus* ATCC 49427; 6, *E. durans* ATCC; and 7, W185. The unnumbered lanes are molecular DNA size markers.

one considers the negligible prevalence of *E. pseudoavium* among clinical isolates, the inability of ITS-PCR to distinguish between these species is clinically insignificant.

ITS-PCR was able to identify to the species level a collection of phenotypically aberrant clinical enterococcal isolates. ITS-PCR identified a biochemically active enterococcus, strain W185, as *E. durans* and not *E. raffinosus*. ITS-PCR further proved its ability to identify enterococcal species by determining that W73 and W74 were nonmotile *E. casseliflavus* VanA strains and not *E. mundtii* VanA strains as was originally determined phenotypically. These two isolates were collected in New York City in 1991; however, because they were identified as *E. mundtii*, nothing more was done to characterize these isolates. ITS-PCR also determined that W148 was *E. hirae* and not *E. durans*. These two species may be difficult to distinguish from each other phenotypically; however, ITS-PCR resolves this problem readily, as was evident with the strains examined in this study (Fig. 2).

One of the more interesting results from this study was the finding of the high degree of variability evident in the ITS profile of *E. gallinarum*. Because each major species band probably represents the ITS region between one copy of 16S rDNA and one copy of 23S rDNA, these results suggest that strains of *E. gallinarum* possess at least two or more distinct forms of ITS which differ from each other by their nucleotide compositions. The reason for this heterogeneity is unclear. Perhaps it is due to the number of copies of tRNA located in the ITS region. Previous investigators have shown that one or more tRNA genes can be present in the 16S-23S rDNA ITS region of *Escherichia coli* (10, 11). The *E. gallinarum* ITS region may be an analogous situation in that increasing numbers of tRNA genes may account for the increase in size of ITS-PCR bands. Another possibility may be that the increase in size is due to non-tRNA filler. However, it is unclear why *E. gallinarum* would possess a heterogeneous group of ITS-PCR profiles but all other enterococci examined would appear to be fairly constant.

The limited variability of ITS-PCR profiles seen in some isolates, as opposed to no variability, is not wholly unexpected. If a wider sampling of enterococcal strains worldwide were undertaken, this variability would undoubtedly be increased. However, this is also the case with the phenotypic characterization of strains. Phenotypically aberrant strains can always be found, as was the case in this study. Yet laboratories rely on phenotypic characterization as their primary method of identifying enterococcal strains.

Previous investigators have used the sequences of the ligase genes from *E. faecium*, *E. faecalis*, *E. gallinarum*, and *E. casseliflavus* to construct species-specific primers (5). These are the most common enterococcal species isolated clinically, and their rapid identification is important in relation to vancomycin resistance. However, eight primers are required to perform this assay (provided that no preliminary data such as pigment or motility are known). Also, if no PCR product is achieved with any of the four primer sets, it is unclear whether it is because the isolate is not *E. faecium*, *E. faecalis*, *E. gallinarum*, or *E. casseliflavus* or whether the PCR did not work. ITS-PCR overcomes these potential shortcomings by using only one primer pair for all enterococcal species used in this study, and ITS-PCR must always generate a product, regardless of the enterococcal species for which this assay is described.

When ITS-PCR is to be used to identify the species of an unknown enterococcal isolate, the following must be taken into consideration. The ITS-PCR amplicons for *E. faecium*, *E. gallinarum*, *E. durans*, and most strains of *E. hirae* are quite distinct, and therefore, it should be unnecessary to have to compare their ITS-PCR amplicon profiles to known ITS-PCR amplicon profiles of ATCC enterococcal species, provided that molecular size markers are used to determine the size of the ITS-PCR amplicons. *E. mundtii* and *E. casseliflavus* species are the only two pigmented species. When their ITS-PCR profiles are compared, the ITS-PCR products from *E. mundtii* are of a larger size than the ITS-PCR products of *E. casseliflavus*. Therefore, their differentiation should be a simple matter of comparing ITS-PCR amplicon sizes. The remaining species, *E. avium*, *E. raffinosus*, *E. malodoratus*, *E. pseudoavium*, *E. faecalis*, and some strains of *E. hirae*, possess very similar ITS-PCR amplicon profiles, making their differentiation more difficult. This is overcome by cleavage of these amplicons with *Sau*3A, generating amplicon profiles diagnostic for each species. However, a species name can be assigned with confidence only when the ITS-PCR amplicons from the aforementioned ATCC species are digested with *Sau*3A and run on the same gel with the unknown enterococcal species, as was done for Fig. 2. The total time to the identification of an enterococcal isolate by ITS-PCR and *Sau*3A restriction digestion is approximately 10 h.

Because of the necessity of performing ITS-PCR with the ATCC enterococcal species, which need to be used as a comparison for the unknown enterococcal strain being examined, and the expertise required for PCR, this method is not feasible for most routine clinical microbiology laboratories. Rather, it is a methodology that could be used for epidemiologic purposes such as during VRE outbreaks and/or in reference laboratories to identify problematic strains. Also, we do not advocate the use of ITS-PCR in place of phenotypic identification of enterococci but, rather, see it used in conjunction with phenotypic tests to definitively identify an unknown enterococcal isolate.

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