Division of the genus *Enterococcus* into species groups using PCR-based molecular typing methods

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Broad-range 16S rDNA PCR (BR-PCR) applied to DNA from 32 clinical enterococcal isolates and 12 other enterococci from a clinical reference collection followed by species-specific hybridization analysis identified 25 strains of *Enterococcus faecalis* and 19 *Enterococcus* species. Randomly amplified polymorphic DNA (RAPD) analysis using UPGMA clustering on the same material revealed four different clusters at a similarity level of 49%. Based on partial 16S rDNA sequence analysis of variable regions V4 and V9, it was possible to divide the 19 type strains specifying the genus *Enterococcus* into 12 different 16S rDNA species groups. The type strain distribution then served as a template for the analysis of the other 44 strains which were assigned to four different species groups (a–d) based on their 16S rDNA motifs. There was good agreement with the RAPD clusters. Species group a was an individual species line containing 25 strains that were identified as *E. faecalis*. Group b also represented an individual species line of 12 strains identified as *E. faecium*. The remaining seven strains that formed species groups c and d could not be fully identified to species by this analysis. It was concluded that BR-PCR of 16S rDNA followed by partial sequence analysis of the PCR products is a reliable technique for the identification and classification of enterococci. Further division of unresolved species groups should be achievable if regions other than V4 and V9 of 16S rDNA are also analysed.

Keywords: *Enterococcus*, 16S rDNA PCR, enterococcal 16S rDNA sequences, enterococcal RAPD analysis

INTRODUCTION

Enterococci are opportunistic human pathogens. The two most important species, *Enterococcus faecalis* and *Enterococcus faecium*, which are considered part of the normal intestinal flora, are among the leading causes of nosocomial infection and may cause severe infections,

including endocarditis and septicaemia with high mortality (Moellering, 1995). Recent studies have revealed that more unusual strains such as *Enterococcus avium*, *Enterococcus durans* and *Enterococcus gallinarum* constitute a significant proportion of enterococci isolated from clinical blood samples (Miele *et al.*, 1995; Perlada *et al.*, 1997).

Enterococci are intrinsically resistant to a number of antimicrobial agents, including cephalosporins. Strains resistant to high levels of aminoglycosides, all β -lactam antibiotics and, more recently, the glycopeptide vancomycin have emerged (Arthur & Courvalin, 1993; Center for Disease Control and Prevention, 1993; Landman *et al.*, 1993). *E. faecium* is more frequently found than *E. faecalis* among multi-resistant strains. The resistance genes are transferable to other pathogens, at least under laboratory conditions (Noble *et al.*, 1992; Poyart *et al.*, 1997) and this is a cause for concern.

Abbreviations: BR-PCR, broad-range PCR; ITS, intergenic spacer; RAPD, randomly amplified polymorphic DNA.

The EMBL accession numbers for the sequences reported in this paper are Y12905–Y12924 for *Enterococcus faecalis* ATCC 19433^T, *E. faecium* ATCC 19434^T, *E. avium* ATCC 14025^T, *E. casseliflavus* ATCC 25788^T, *E. durans* ATCC 19432^T, *E. gallinarum* CCUG 18658^T, *E. malodoratus* CCUG 30572, *E. hirae* ATCC 8043^T, *E. mundtii* CCUG 18656^T, *E. raffinosus* CCUG 29292^T, *E. solitarius* CCUG 29293^T, *E. pseudoavium* CCUG 33310^T, *E. cecorum* CCUG 27299^T, *E. columbae* CCUG 27894^T, *E. saccharolyticus* ATCC 43076^T, *E. dispar* CCUG 33309^T, *E. sulfureus* CCUG 33313^T, *E. seriolicida* CCUG 33312^T, *E. flavescens* CCUG 30567^T and *Streptococcus pyogenes* ATCC 19615^T, respectively.

Infection control and epidemiological studies require simple means of identification and typing of clinical isolates. No phenotypic criteria are available which unequivocally separate the genus *Enterococcus* from other Gram-positive, catalase-negative cocci (Devriese *et al.*, 1993). Delineation of the genus was founded on serological studies and 16S rRNA sequences (Schleifer & Kilpper-Bälz, 1984) which, together with nucleic acid hybridization analysis, characterized enterococci as a genetically distinct but heterogeneous group (Schleifer & Kilpper-Bälz, 1987). The genus *Enterococcus* was divided into the *faecium*, *avium* and *gallinarum* groups (reviewed by Facklam & Sahm, 1995) while *E. faecalis* forms a distinct lineage (Williams *et al.*, 1991). Division of the genus *Enterococcus* into new species groups based on PCR amplification of the intergenic spacer (ITS) between the 16S and 23S rRNA genes was suggested recently (Naïmi *et al.*, 1997; Tyrell *et al.*, 1997).

Nineteen species (Devriese *et al.*, 1993; Facklam & Sahm, 1995) are presently included in the genus *Enterococcus*, of which *E. faecalis* and *E. faecium* comprise about 90 and 5–10%, respectively, of the isolates in a typical clinical setting (Facklam & Sahm, 1995). Various molecular biological techniques, such as intergenic ribosomal PCR (Naïmi *et al.*, 1997; Tyrell *et al.*, 1997), PCR-based glycopeptide resistance genotyping (Dutka-Malen *et al.*, 1995; Miele *et al.*, 1995), randomly amplified polymorphic DNA (RAPD) analysis (Berg *et al.*, 1994; Issack *et al.*, 1996; Power, 1996; Descheemaeker *et al.*, 1997), restriction enzyme analysis (Hall *et al.*, 1992), PFGE (Murray *et al.*, 1990; Goering & Winters, 1992), ribotyping (Kostman *et al.*, 1995; Woodford *et al.*, 1993) and plasmid profiling (Boyce *et al.*, 1992), have been used to study the intragenic relationships and also for 'fingerprinting' when studying nosocomial transmission of enterococcal strains.

Broad-range PCR (BR-PCR) amplification (Chen *et al.*, 1989), using conserved universal 16S rDNA PCR amplification primers, is another powerful tool to study phylogenetic relationships between bacteria (Stahl & Amann, 1991; Relman *et al.*, 1992; Amann *et al.*, 1995). Recently, we reported the use of universal 16S rDNA primers for BR-PCR amplification of selected variable regions V3, V4 and V9 of bacterial 16S rDNA (Monstein *et al.*, 1996; Tiveljung *et al.*, 1995, 1996). Direct sequencing of such BR-PCR products is nearly always highly informative for determining genus affiliation and sometimes species.

The purpose of this investigation was to evaluate the discriminatory power of PCR-based methods, including BR-PCR-hybridization and DNA sequence analysis of 16S rDNA BR-PCR-amplified products, for typing of enterococci in comparison with RAPD analysis.

METHODS

Enterococcal strains and culture conditions. A reference collection of clinical isolates of enterococci (Malmö reference collection) originally characterized by conventional phenotyping using API 20 strep and RAPD analysis, including enterococcal type strains, was received from A. K. Petersson,

Department of Microbiology, University Hospital, Lund. This comprised a set of 12 strains which were numbered HJ 1–HJ 12 (HJ 1, *E. raffinosus* AKP; HJ 2, *E. faecalis* V583; HJ 3, *E. casseliflavus* B77; HJ 4, *E. gallinarum* B41; HJ 5, *E. faecium* B36; HJ 6, *E. faecalis* ATCC 19433^T; HJ 7, *E. faecalis* B8; HJ 8, *E. faecium* B4; HJ 9, *E. faecalis* ATCC 29212; HJ 10, *E. gallinarum* B95; HJ 11, *E. faecalis* B1; HJ 12, *E. faecium* B5). The enterococcal clinical isolates in this investigation were obtained between January and May 1996 from routine clinical specimens at the University Hospital, Linköping. They were putatively identified as enterococci by Gram staining, colony morphology and a positive bile aesculin reaction, and consecutively labelled HJ 13–HJ 45. *E. faecalis* ATCC 29212 (HJ 22) and *E. faecium* ATCC 35607 (HJ 21) were included as blind controls in the study. The 19 enterococcal type strains were obtained from the culture collection at the University of Gothenburg, Sweden. All strains were cultured on blood agar plates or in Brain Heart Infusion (Difco) broth (BHI) overnight at 37 °C and stored frozen in 20% (v/v) glycerol in BHI at –70 °C.

Bacterial strains of other genera. *Helicobacter pylori* CCUG 17874^T, *Streptococcus pyogenes* ATCC 19615, *Lactococcus lactis* ATCC 19435^T, *Bordetella pertussis* CCUG 30873, *Escherichia coli* ATCC 25922 and clinical isolates identified as *Chlamydia trachomatis*, *Mobiluncus* sp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Actinomyces* sp., *Acinetobacter* sp. and coagulase-negative *Staphylococcus* sp. were used in this study. All identifications of clinical isolates were established at the University Hospital, Linköping using routine procedures.

Preparation of crude cell extract for RAPD-PCR. Cells from 1 ml of an overnight culture were collected by centrifugation, washed twice in 1 ml sterile distilled water and finally resuspended in 200 µl sterile distilled water. The cells were disintegrated by vigorous shaking with 10 glass beads (0.2 mm diam.) in each tube using an Eppendorf mixer 5432 for 45 min at 4 °C. The disrupted cells were pelleted by centrifugation and 1 µl of the supernatant fluid was used for the RAPD-PCR reaction.

BR-PCR amplification and Southern blot analysis. Bacterial DNA was extracted according to a recently described procedure (Monstein *et al.*, 1996), which was developed for the isolation of DNA from both Gram-positive and -negative bacteria and intended for use in 16S rDNA BR-PCR amplifications, and was quantified spectrophotometrically at 260/280 nm.

The universal 16S rDNA PCR primers pJB-1 and p13B, which amplify an approximately 440 bp fragment of the 16S rRNA gene between positions 959 and 1397 (located within the second half of the 16S rDNA gene), including variable regions V3, V4 and V9 (nomenclature from Gray *et al.*, 1984), were used. BR-PCR amplifications were carried out under conditions described previously (Monstein *et al.*, 1996; Tiveljung *et al.*, 1995, 1996). PCR-amplified 16S rDNA products (8 µl) were subjected to agarose gel electrophoresis as described by Tiveljung *et al.* (1995). The products were stained with ethidium bromide and documented photographically. [γ -³²P]ATP 5'-end-labelling of hybridization probes *E. faecalis* V9 (5' TGCATGACCTCGCGGTC 3'), *E. faecium* V9 (5' AGCTTAGCCTCGCGACT 3') at positions 1247–1268 and the universal BR-PCR primer pJB-1 (5' ATTCGATGCAACGCGAAGAACCCTTACC 3') was performed as described previously using [γ -³²P]ATP (Tiveljung *et al.*, 1995). For Southern blot analysis, PCR products were capillary-transferred onto a Hybond-N membrane (Amersham) and fixed to the membrane by cross-linking in a UV box (Stratagene 1800).

Hybridization conditions, using [γ - 32 P]ATP 5'-end-labelled hybridization probes, were as described by Tiveljung *et al.* (1995). Subsequently, filters were washed in $1 \times$ SSC/0.1% SDS, followed by $0.5 \times$ SSC/0.1% SDS and $0.25 \times$ SSC/0.1% SDS for 3×20 min in each wash at 50°C ($1 \times$ SSC = 150 mM NaCl, 15 mM sodium citrate). After each washing step, membranes were exposed to Amersham MP X-ray films at -80°C using a DuPont intensifier screen for 12–14 h.

Between each hybridization step, the filters were recycled by boiling in sterile water/0.5% SDS for 2–5 min and exposed overnight to ensure that the hybridized probe was stripped off prior to a new hybridization step. To ascertain the specificity of the PCR amplification, negative control (PCR mix, without DNA template) and positive control (PCR mix with *Escherichia coli* DNA template) experiments were included.

RAPD-PCR. RAPD-PCR was carried out in a 50 μl reaction mix using Boehringer Mannheim standard PCR buffer (pH 8.3, 1.5 mM MgCl_2), 0.2 mM dNTPs (Perkin-Elmer), 5 mM random primer (5' ACGCGCCCT 3', synthesized by Scandinavian Gene Synthesis, Köping, Sweden) and 2.5 units *Taq* polymerase (Boehringer Mannheim). The reaction mix was overlaid with mineral oil and the following cycle program was used: 94°C for 45 s, 30°C for 2 min and 72°C for 60 s for four cycles, followed by 94°C for 5 s, 36°C for 30 s and 72°C for 30 s for 26 cycles (the extension step was increased by 1 s for every new cycle). The PCR reaction was terminated at 72°C for 10 min and thereafter cooled to 4°C .

Gel electrophoresis. PCR products (20 μl) were applied to a 1.5% (w/v) agarose gel followed by electrophoresis in TB buffer (89 mM boric acid, 23 mM H_3PO_4 , 2.5 mM EDTA, pH 8.3) at room temperature for about 2.5 h at 100 V. A DNA molecular mass standard (VI, 0.5 μg ; Boehringer Mannheim) was used. Gels were stained in ethidium bromide for 5 min and thereafter washed for 10 min. The PCR products were visualized at 302 nm with a UV transilluminator (UVP) and photographed.

Reading of RAPD patterns, numerical analysis and evaluation of reproducibility. Photographic negatives were scanned into a computer using a flatbed scanner (UMAX) at a resolution of 200 d.p.i. The gel images were then converted to GelCompar format (Applied Maths). Size markers which were included on each gel were used for normalization and all further analysis of the gels was done using GelCompar 4.0. The gel traces were analysed using the Pearson product moment correlation coefficient (r) and the unweighted pair group method with arithmetic means (Romersburg, 1984). The reproducibility test of RAPD analysis was evaluated running all strains in duplicate (separate cell preparations and PCR trials of the same strain) on different gels, followed by UPGMA cluster analysis (Romersburg, 1984).

Partial 16S rDNA sequence analysis of BR-PCR products. PCR products (clinical isolates HJ 1–HJ 44 and type strains HJ 46–HJ 64) were subjected to DNA sequence analysis using 5'-fluorescein-labelled broad-range 16S rDNA primers and an automatic DNA sequencer (ALF, Pharmacia). DNA sequences were analysed using a DNA managing program (R. Staden, Edinburgh, UK; 4th edition, 1994) and a SunSparc Station 5. Automatic DNA sequencing of the clinical isolates (HJ 1–HJ 44) was supplied by the Department of Forensic Serology, University Hospital, Linköping. Partial 16S rDNA sequences of the clinical isolates were compared with the DNA sequences of the 19 enterococcal type strains (Devriese *et al.*, 1993; Facklam & Sahm, 1995) which were assessed by manual DNA sequencing of the BR-PCR-amplified products as described previously (Tiveljung *et al.*, 1995, 1996).

RESULTS

RAPD analysis of enterococcal clinical isolates

Band patterns obtained using RAPD analysis on enterococcal crude DNA extracts, including enterococcal isolates HJ 1–HJ 44 and enterococcal type strains *E. faecalis* ATCC 19433^T, *E. faecium* ATCC 19434^T and *E. gallinarum* CCUG 18658^T are shown in Fig. 1. Several species-specific bands could be observed. The similarity between the strains was evaluated by computerized densitometric analysis of these RAPD profiles as described in Methods. Using UPGMA clustering (Romersburg, 1984), 43 of the 44 isolates were allocated to four different clusters (1–4) at a similarity level of 49% (Fig. 1), arbitrarily chosen for defining species. Cluster 1 contained two enterococcal clinical isolates, two *E. gallinarum* reference strains and *E. gallinarum* CCUG 18658^T. In cluster 2, two enterococcal clinical isolates (HJ 39 and HJ 1) clustered together at a similarity level of 52%. Cluster 3 contained *E. faecium* ATCC 19434^T, eight enterococcal clinical isolates and five reference strains. Cluster 4 could be divided into subclusters 4A and 4B, merging at a similarity level of 70%. Subcluster 4A contained *E. faecalis* ATCC 19433^T and four reference strains of which one was *E. faecalis* ATCC 19433^T, included as a control in the Malmö reference collection (see Methods). Subcluster 4B consisted of 18 enterococcal clinical isolates and *E. faecalis* ATCC 29212 in duplicate (HJ 9 from the Malmö reference collection and HJ 22 which were included as blind controls among our clinical isolates).

DNA sequence analysis of enterococcal type strains

In previous studies we were able to show that hybridization probes, derived from variable regions V3, V4 and V9, could be used for the detection and identification of bacterial BR-PCR-amplified products using the universal 16S rDNA primers pJB-1 and p13B (Monstein *et al.*, 1996; Tiveljung *et al.*, 1995, 1996). BR-PCR-amplified products from the 19 enterococcal type strains were subjected to DNA sequence analysis. Based on these partial 16S rDNA sequences between positions 1110 and 1180, and 1240 and 1300, covering variable regions V4 and V9, the 19 enterococcal type strains could be divided into 12 different 16S rDNA species groups (Table 1). Variable region V9 (1240–1300) of *E. faecalis*, *E. faecium* and other enterococci revealed significant DNA sequence differences which allowed the delineation of an *E. faecalis*-specific sequence (5' GACCGCGAGGTCATGCA 3', 1262–1278; Fig. 2a). The corresponding *E. faecium* sequence, 5' AGTCGCGAGGCTAAGCT 3', was shared by most other enterococcal type strains (Fig. 2a). However, based on nucleotide sequence differences in variable region V4 derived from the 19 type strains, it was possible to assign a species-specific DNA sequence to *E. faecium* (Fig. 2b). In *E. faecium*, nucleotides C and A occurred at positions 1137 and 1154, respectively, of variable region V4, whereas the other *Enterococcus* type strains had T and G or T and A at the corresponding positions (Fig. 2b).

Fig. 1. Dendrogram obtained using RAPD analysis (clusters 1, 2, 3, 4A and 4B) and the identification results obtained using 16S rDNA hybridization and partial 16S rDNA sequencing. The hybridization species groups are: a, *E. faecalis*; b, other enterococci. The 16S rDNA species groups are: a, *E. faecalis*; b, *E. faecium*; c, *E. casseliflavus*, *E. gallinarum*, *E. flavescentis* and *E. dispar*; d, *E. avium*, *E. raffinosus* and *E. pseudoavium*.

Tabulation of the DNA sequence data in the V4 and V9 regions between positions 1110 and 1180, and 1240 and 1300 (Fig. 2) allowed further division of the 19 type strains into eight individual species lines and four species groups (Table 1).

BR-PCR amplification and Southern blot analysis of PCR products

DNA isolated from all enterococcal strains and strains of other genera used in this study was successfully amplified using the universal 16S rDNA BR-PCR primers pJB-1 and p13B, yielding a PCR product of approximately 440 bp as expected. Based on the different DNA sequences, motifs found within variable

region V9 in *Enterococcus* spp., an *E. faecalis* V9 species-specific hybridization probe (5' GACCGCGAGGTC-ATGCA 3') and another hybridization probe (5' AGT-CGCGAGGCTAAGCT 3'), representing the corresponding *E. faecium* sequence (and also most other species of the genus *Enterococcus*; Fig. 2) were synthesized. Using hybridization conditions as described in Methods, we found that the *E. faecalis* V9 hybridization probe and the V9 hybridization probe representing other enterococcal species hybridized in a mutually exclusive fashion to the BR-PCR-amplified products of enterococci (Fig. 3), i.e. the *E. faecalis* probe would not hybridize with DNA from the other enterococci tested, whereas the *E. faecium* probe hybridized with DNA from all other enterococcal strains in the study except *E. faecalis*. In a parallel experiment, DNA from *E. faecalis*

Table 1. Comparison of *Enterococcus* species group identifications proposed in this study and elsewhere

Facklam & Sahm (1995)	Tyrell <i>et al.</i> (1997)	Williams <i>et al.</i> (1991)	This study	
			Species	16S rDNA species group
Group I	Group 1	Avium group		
<i>E. avium</i>	<i>E. avium</i>	<i>E. avium</i>	<i>E. avium</i>	d
<i>E. malodoratus</i>	<i>E. malodoratus</i>	<i>E. malodoratus</i>		
<i>E. raffinosus</i>	<i>E. raffinosus</i>	<i>E. raffinosus</i>	<i>E. raffinosus</i>	d
<i>E. pseudoavium</i>	<i>E. pseudoavium</i>	<i>E. pseudoavium</i>	<i>E. pseudoavium</i>	d
Group II	Group 2	Faecium group		
<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>		
<i>E. faecalis</i>	<i>E. faecalis</i>			
<i>E. mundtii</i>	<i>E. mundtii</i>	<i>E. mundtii</i>		
<i>E. gallinarum</i>	<i>E. gallinarum</i>		<i>E. gallinarum</i>	c
<i>E. casseliflavus</i>	<i>E. casseliflavus</i>		<i>E. casseliflavus</i>	c
<i>E. flavescens</i>			<i>E. flavescens</i>	c
		<i>E. durans</i>		
		<i>E. hirae</i>		
			<i>E. dispar</i>	c
Group III	Group 3			
<i>E. durans</i>	<i>E. durans</i>		<i>E. durans</i>	e
<i>E. hirae</i>	<i>E. hirae</i>			
<i>E. dispar</i>				
<i>E. faecalis</i> (var.)			<i>E. mundtii</i>	e
Group IV				
			<i>E. hirae</i>	f
			<i>E. malodoratus</i>	f
<i>E. sulfureus</i>				
		Gallinarum group		
		<i>E. gallinarum</i>		
		<i>E. casseliflavus</i>		
Atypical species		Individual species lines	Individual species lines	
<i>E. saccharolyticus</i>		<i>E. saccharolyticus</i>	<i>E. saccharolyticus</i>	g
<i>E. cecorum</i>		<i>E. cecorum</i>	<i>E. cecorum</i>	h
<i>E. columbae</i>		<i>E. columbae</i>	<i>E. columbae</i>	i
		<i>E. faecalis</i>	<i>E. faecalis</i>	a
			<i>E. faecium</i>	b
			<i>E. sulfureus</i>	j
			<i>E. solitarius</i>	k
			<i>E. seriolicida</i>	l

and *E. faecium* type strains and from strains of other genera was BR-PCR-amplified and hybridized using enterococcus-specific V9 hybridization probes. As shown in Fig. 4, no cross-hybridization with BR-PCR-amplified products from other bacteria included as negative controls was observed. The *E. faecium* probe also hybridized to *Carnobacterium alterfunditum* (data

not shown) which, however, grows at 16–20 °C and has no clinical relevance.

DNA sequence analysis of *Enterococcus* clinical isolates

Partial 16S rDNA sequences from all enterococcal clinical isolates and reference strains were established by

Fig. 2. Alignment of variable regions V9 (a) and V4 (b) of partial 16S rDNA sequences from 19 *Enterococcus* type strains. Gaps represent base deletions and dashes represent homology to the *E. faecalis* type strain. The location of the *E. faecalis*- and *E. faecium*-specific V9 sequence used as hybridization probe is indicated. Numbering is according to Gray *et al.* (1984). 16S rDNA sequences were obtained by sequencing BR-PCR-amplified products in our laboratory.

means of automatic DNA sequence analysis of BR-PCR-amplified products. Twenty-five clinical isolates showed the *E. faecalis*-specific V9 sequence motif 5' CGCT-

AGACCGCGAGGTCATGCA 3' (designated 16S rDNA species group a). Twelve clinical isolates were identified as *E. faecium* (16S rDNA species group b, Fig.

Fig. 3. Southern blot analysis of 16S rDNA BR-PCR products. Membranes were hybridized with a 5' end-labelled *E. faecalis* V9 antisense probe (A) and a 5' end-labelled *E. faecium* probe (B). BR-PCR products were visualized by hybridization using the 5' end-labelled bacterial universal BR-PCR primer pJB-1 (C). Membranes were exposed on Amersham MP X-ray film at -80°C using intensifier screens for appropriate times (usually overnight). Lanes: 1–12, strains from the Malmö reference collection; 13–44, clinical isolates from the University Hospital, Linköping; 45–52, *Enterococcus* type strains (*E. casseliflavus*, 45; *E. durans*, 46; *E. raffinosus*, 47; *E. faecalis*, 48; *E. faecium*, 49; *E. gallinarum*, 50; *E. hirae*, 51; *E. mundtii*, 52. N, negative control; EC, *Escherichia coli*.

2). The remaining seven clinical isolates revealed different DNA sequence motifs at positions 1240–1300 in variable region V9 (designated 16S rDNA species groups c and d; Figs 1 and 2). In 16S rDNA species group c, the two clinical isolates showed the V9 DNA sequence motif 5' TGCGAAGTCGCGAGGCTAAGCT 3' which was present in the type strains representing *E. faecium*, *E.*

Fig. 4. Ethidium-bromide-stained agarose gel (a) and Southern blot analysis (b) of BR-PCR-amplified products from *E. faecalis* (lanes 1 and 9), *Chlamydia trachomatis* (2), *Helicobacter pylori* (3), *Mobiluncus* sp. (4), *Escherichia coli* (5), *Streptococcus pyogenes* (6), *Lactococcus lactis* (7), *E. faecium* (8), negative PCR control (PCR mix without DNA template) (10), 100 bp DNA size ladder (Pharmacia) (11), *Bordetella pertussis* (12), *Listeria monocytogenes* (13), *Staphylococcus aureus* (14), *Actinomyces* sp. (15), *Acinetobacter* sp. (16), coagulase-negative *Staphylococcus* sp. (17) and *L. lactis* (18). Primers used and the exposure of the membranes on X-ray film are as described in the legend to Fig. 3.

casseliflavus, *E. gallinarum*, *E. saccharolyticus*, *E. dispar* and *E. flavescens*. 16S rDNA species group d (Figs 1 and 2) represents one clinical isolate which had the V9 DNA sequence motif 5' CGCGAAGTCGCGAGGCTAAGCT 3', present in *E. avium*, *E. durans*, *E. mundtii*, *E. raffinosus* and *E. pseudoavium*, (Fig. 2a). Variable region V9-specific DNA sequence motifs corresponding to the remaining enterococcal type strains were not detected among the clinical isolates. Alignment of the partial 16S rDNA sequences of the enterococcal clinical isolates (Fig. 1), revealed complete sequence identity within each 16S rDNA species group examined, i.e. 100% similarity in the sequences with no difference in any base between positions 959 and 1397 (data not shown).

DISCUSSION

Twenty-four clinical isolates and reference strains were clearly identified as *E. faecalis* by RAPD analysis comprising cluster 4 (Fig. 1). Based on the partial 16S rDNA sequences, these twenty-four strains, together with an additional strain, HJ 7 which belonged to RAPD cluster 3 (Fig. 1), were designated as 16S rDNA species group a (Fig. 1). RAPD clusters 4A and 4B and 16S rDNA species group a consisting of *E. faecalis* strains were in good agreement. However, strain HJ 7 (*E. faecalis* by biotyping and 16S rDNA analysis; Fig. 1) clustered together with the *E. faecium* strain in RAPD

analysis at a similarity level of 55%. HJ 7 did not show the RAPD pattern expected for *E. faecalis* (Fig. 1). Instead it revealed an additional band that dominated the RAPD profile. This band is not identical to the band located at a similar position in the *E. faecium* strains (Fig. 1). Such aberrant dominating bands may arise due to the presence of plasmids carrying antibiotic resistance genes. Indeed, strain HJ 7 (*E. faecalis* B8, Malmö reference collection) harbours a plasmid-borne *vanB* gene which encodes vancomycin resistance (data not shown). All strains allocated to *E. faecium* 16S rDNA species group b showed the typical *E. faecium* RAPD pattern (Fig. 1). Strains HJ 4, HJ 10 (from the Malmö reference collection) and strains HJ 43 and HJ 44 (clinical isolates from the University Hospital, Linköping), were located in 16S rDNA species group c together with *E. gallinarum* CCUG 18658^T in cluster 1 (Fig. 1). Strain HJ 3, which is from the Malmö reference collection and typed as *E. casseliflavus* B77, was a poor performer in RAPD analysis. In spite of this, the strain had the same partial 16S rDNA sequences as other members of 16S rDNA species group c. Similarly, strains HJ 39 and HJ 1 merging together at a similarity level of 52% and comprising cluster 2, were representatives of 16S rDNA species group d (Fig. 1). It should be noted that only two strains were allocated to cluster 2 by RAPD analysis and no type strain was included. RAPD clustering with only a few representatives may not be significant and, therefore, classification of the rare clinical enterococcal isolate may be simpler and more precise from its 16S rDNA sequence covering variable regions V9 and V4. Clearly, RAPD and 16S rDNA sequence analysis are both powerful molecular typing methods for identification of enterococci in clinical isolates. However, due to lack of variation, neither method would seem ideal for subtyping clinical isolates for epidemiological purposes.

Based on phenotypic characteristics (reviewed by Facklam & Sahm, 1995), or genotypic characteristics such as reverse transcriptase DNA sequence analysis of 16S rRNA (Williams *et al.*, 1991) or ITS PCR (Tyrell *et al.*, 1997; Naïmi *et al.*, 1997), various suggestions for the division of the genus *Enterococcus* into species groups have been put forward (for details see Table 1). It is generally acknowledged that neither biotyping methods (phenotypic characteristics) nor ITS PCR allow simple unequivocal separation of *E. faecalis*, *E. faecium* and other less frequently encountered enterococcal species (Table 1; Facklam & Sahm, 1995; Tyrell *et al.*, 1997). The clinical importance of this is obvious since recent studies have revealed that more unusual enterococcal strains such as *E. gallinarum*, *E. casseliflavus*, *E. durans* and *E. avium* may constitute a significant proportion of clinical isolates (Miele *et al.*, 1995; Perlada *et al.*, 1997; this study). According to our results based on BR-PCR-derived partial 16S rDNA sequence analysis, the genus *Enterococcus* may be divided into 12 different 16S rDNA species groups consisting of eight individual species lines (distinct lineages) and four cluster-groups (Fig. 1, Table 1). Admittedly, our division of the genus based on

partial 16S rDNA sequence information covering variable regions V3, V4 and V9 does not exploit the full potential of this technique. The results of Williams *et al.* (1991) based on reverse transcriptase sequences suggest that BR-PCR amplification of one or two additional part-sequences in other regions of the 16S rRNA genes may provide a complete division of the genus *Enterococcus* into single species lines. BR-PCR sequencing is a fast method and highly reliable sequences are usually obtained. The 100% similarity in part-sequences observed within the 16S rRNA groups of the clinical isolates included in the present study indicates that automated DNA sequence analysis of part-sequences might be a powerful technique for the typing of enterococci.

In conclusion, we have shown the potential use of RAPD analysis, partial 16S rDNA sequence analysis, including variable regions V4 and V9, and *Enterococcus*-specific 16S rDNA hybridization analysis in the identification and classification of enterococci from clinical isolates. As judged from the present data, partial 16S rDNA sequence analysis, including variable regions V4 and V9, appears to be a more definitive means of typing enterococci, although several 16S rDNA species groups were unresolved due to lack of variation within the V4 and V9 regions. On the other hand, due to its simplicity and slightly higher discriminating power, RAPD analysis might be better suited for studying the relationship between large numbers of strains, for example in the investigation of nosocomial infection epidemiology.

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