DNA sequence heterogeneity in the three copies of the long 16S–23S rDNA spacer of *Enterococcus faecalis* isolates

Volker Gürtler, Rao YuJun, Stephen R. Pearson, Susan M. Bates and Barrie C. Mayall

Author for correspondence: Volker Gürtler. Tel: +61 3 9496 3136. Fax: +61 3 9459 1674.
e-mail: volker@austin.unimelb.edu.au

Department of
Microbiology, Austin &
Repatiation Medical Centre
(Austin Campus), Studley
Road, Heidelberg 3084,
Victoria, Australia

The possibility of intragenic heterogeneity between copies of the long intergenic (16S–23S rDNA) spacer region (LISR) was investigated by specific amplification of this region from 21 *Enterococcus faecalis* isolates. Three copies of the LISR (*rrnA*, *B* and *C*) were demonstrated by hybridization of the LISR to genomic DNA cleaved with *I*-*CeuI* and *SmaI*. When the LISR amplicon was digested with *Tsp509I*, two known nucleotide substitutions were detected, one 4 nt upstream from the 5' end of the *tRNA^{ala}* gene (allele *rrnB* has the *Tsp509I* site and *rrnA* and *C* do not) and the other 22 nt downstream from the 3' end of the *tRNA^{ala}* gene (*rrnC* has the *Tsp509I* site). Sequence differences at these sites were detected at the allelic level (alleles *rrnA*, *B* and *C*) and different combinations of these alleles were designated *Tsp* Types. Using densitometry to analyse bands from electrophoresis gels, the intra-isolate ratios of the separate alleles (*rrnA:rrnB:rrnC*) were determined in each *Tsp* Type: I (0:3:0), II (1:2:0), III (2:0:1), IV (3:0:0), V (2:1:0) and VI (1:1:1). Sequence variation between the three copies of the LISR was confirmed by the detection of at least five other intra-isolate nucleotide substitutions using heteroduplex analysis by conformation-sensitive gel electrophoresis (CSGE) that were not detected by *Tsp509I* cleavage. Perpendicular denaturing gradient gel electrophoresis was capable of resolving homoduplexes; six to seven out of a possible nine curves were obtained in some isolates. In the isolate where seven curves were obtained one or more further nucleotide substitutions, not detected by *Tsp509I* cleavage or CSGE, were detected. On the basis of LISR sequence heterogeneity, isolates were categorized into homogeneous (only one allele sequence present) and heterogeneous (two or three allele sequences present). The transition between homogeneous and heterogeneous LISRs may be useful in studying evolutionary mechanisms between *E. faecalis* isolates.

Keywords: long intergenic 16S–23S rDNA spacer region, heteroduplex, homoduplex, *Enterococcus faecalis*, concerted evolution

INTRODUCTION

Enterococci are opportunistic human pathogens. The two species that usually cause infections, *Enterococcus*

Abbreviations: CSGE, conformation-sensitive gel electrophoresis; DGGE, denaturing gradient gel electrophoresis; SISR and LISR, short and long intergenic (16S–23S rDNA) spacer region, respectively.

The GenBank accession number for the 16S rRNA gene sequence reported in this paper is AF039902. The Institute for Genomic Research (TIGR) *E. faecalis* database accession number for the 23S rRNA gene sequence reported in this paper is 6199.

faecalis and *Enterococcus faecium*, can acquire multiple antibiotic resistance, leaving few therapeutic options. Rapid species identification incorporating the PCR detection of species-specific ligase genes that confer antibiotic resistance have been used to determine the presence of antibiotic-resistant *E. faecalis* and *E. faecium* (Miele *et al.*, 1995; Patel *et al.*, 1997; Bell *et al.*, 1998). Of the two vancomycin resistance phenotypes found in *E. faecalis* and *E. faecium*, VanA (encoded by *vanA*) confers high level resistance to both vancomycin and teicoplanin and VanB (encoded by *vanB*) confers mod-

erate to high level resistance to vancomycin only (Arthur *et al.*, 1996). The origin of *vanA* and *vanB* is unknown and it is likely, from the diversity of types causing outbreaks that acquisition of resistance is due to repeated sporadic events (Bell *et al.*, 1998). Once acquired, it has been shown in numerous outbreaks that nosocomial spread accounts for considerable amplification of antibiotic-resistant enterococci (Dunne & Wang, 1997; Pegues *et al.*, 1997).

The proportion of *E. faecalis* and *E. faecium* clinical isolates amongst the 19 *Enterococcus* species is about 90 and 5–10%, respectively (Facklam & Collins, 1989; Murray, 1990). Most studies have shown more antibiotic-resistant *E. faecium* than *E. faecalis* isolates (Bell *et al.*, 1998; Uttley *et al.*, 1988). The genus has been delineated on the basis of serological and 16S rRNA sequence studies as a genetically distinct but heterogeneous group (Schleifer & Kilpper-Bälz, 1984, 1987). The 16S rRNA sequences have been determined from 17 *Enterococcus* species (Patel *et al.*, 1998). On the basis of 16S rRNA sequencing, *E. faecalis* (Williams *et al.*, 1991; Monstein *et al.*, 1998; Patel *et al.*, 1998) and *E. faecium* (Monstein *et al.*, 1998) formed distinct lineages within the genus.

The number of *rrn* operons in *Enterococcus* species has only been determined for *Enterococcus hirae* in which the number has been estimated as six (Sechi & Daneo-Moore, 1993). The intergenic (16S–23S rDNA) spacer region (ISR) has been used to detect spacer-length polymorphisms by electrophoresis for *Enterococcus* species identification (Tyrrell *et al.*, 1997). In *E. faecalis* there are short and long intergenic (16S–23S) rDNA spacer regions (SISR and LISR, respectively) with a tRNA^{ala} gene present in the LISR (342 bp) and absent in the SISR (240 bp) (Naïmi *et al.*, 1997; Hall, 1994). The only other differences detected were five nucleotide substitutions in the LISR from four *E. faecalis* isolates (Hall, 1994). In this study the aim was to determine the number of LISRs in the *E. faecalis* genome and then use three simple screening techniques for the detection of intragenomic nucleotide substitutions between LISRs from 21 *E. faecalis* isolates.

Variation due to the rearrangement of blocks of nucleotides (varying in length from 3 to 108 bp) has been found in the ISR of a number of species, including *Clostridium difficile* (Gürtler, 1993), *Staphylococcus aureus* (Gürtler & Barrie, 1995), *Escherichia coli* (Antón *et al.*, 1998; García-Martínez *et al.*, 1996a, b), *Salmonella enterica* (Perez-Luz *et al.*, 1998) and *Vibrio cholerae* (Lan & Reeves, 1998). The number of nucleotide substitutions between intragenomic and inter-isolate copies is small in the ISR sequence blocks of *Staphylococcus aureus* (Gürtler & Barrie, 1995) and *Escherichia coli* (Antón *et al.*, 1998). These studies show that the rearrangement of sequence blocks results in sequence heterogeneity between intragenomic copies of the ISR and sequence conservation of blocks is consistent with concerted evolution (homogenization) of the ISR (Gürtler & Mayall, 1999). Possible mechanisms to

explain these two apparently opposing events within the ISR are relatively frequent horizontal transfer (Perez-Luz *et al.*, 1998) and recombination events between complete *rrn* operons (Liu & Sanderson, 1995). In contrast to the examples listed above, the variation found in copies of the ISR of *E. faecalis* appears to be limited to the presence or absence of the tRNA^{ala} gene and a limited number of intraspecies nucleotide substitutions at which intragenomic differences have not been demonstrated. The aims of this study were to demonstrate intragenomic nucleotide substitutions between copies of the LISR in *E. faecalis* isolates and the relationship of these substitutions to concerted evolution.

METHODS

Bacterial isolates. The 21 *E. faecalis* clinical isolates were divided into two groups on the basis of *van* genotype: (1) *vanB* (see below), 3, 66397, 70726, 86228, 84706, 235, 173, 162; (2) non-*vanA*, *vanB* or *vanC* (see below), 196, 65, 87578, 197, 198, 200, 185, 203, 195, 89349, 84546, 86549, 87631. Those clinical isolates with designations of three numerals or less were provided by Jan Bell (Adelaide) and were isolated from hospitals in Melbourne, Sydney or Adelaide (isolates with the *vanB* genotype were reported in Bell *et al.*, 1998). The remaining clinical isolates were obtained from the Austin & Repatriation Medical Centre (A&RMC).

Phenotypic and genotypic species identification. The *E. faecalis* isolates were identified by phenotypic identification (Vitek and API), PYR (Murex); vancomycin and teicoplanin MICs were tested by Etest (AB BIODISK) according to manufacturer's instructions. All *E. faecalis* isolates were subjected to a multiplex-PCR specific for the *vanA* (primers *vanA* and *vanA1*), *vanB* (primers *vanB* and *vanB1*), *Enterococcus gallinarum vanC1* (primers *vanC1* and *vanC2*) and *Enterococcus casseliflavus vanC2/3* (primers *vanC1* and *vanC2*) ligase genes as described by Miele *et al.* (1995). Four to five colonies were taken directly from agar plates using a straight wire and added directly to the PCR mixture (Patel *et al.*, 1997).

PCR of the LISR. The LISR sequence amplified and the sequences of the primers used are shown in Fig. 1(a); the sequence of EFLGC (5' end 54 nt from the 3' end of the 16S rRNA gene) is specific for the LISR and LR20F (nt 21–38 of the 23S rRNA gene) is identical to region 5 described in Gürtler & Stanisich (1996). A GC-clamp (CGCCCGCCGCGCCCGCGCC) was attached to the 5' end of primer EFLGC to improve detection of nucleotide substitutions by denaturing gradient gel electrophoresis (DGGE) (Sheffield *et al.*, 1989). The PCR conditions have been described previously (Gürtler, 1993) except that AmpliTaq Gold (Perkin-Elmer) was used and four to five colonies were taken directly from agar plates using a straight wire and added directly to the PCR mixture. DNA separated by conformation-sensitive gel electrophoresis (CSGE) and DGGE (see below) was eluted from the polyacrylamide gel by incubation with 20–50 µl TE at 4 °C overnight and 5 µl of this was then subjected to a further round of PCR.

Restriction endonuclease analysis. A 10 µl aliquot of the LISR amplicons was incubated with 1 U *Tsp5091* or *HinfI* (New England Biolabs) for 2 h at 65 °C.

PFGE. For preparation of agarose blocks, digestion with *SmaI*

and separation of DNA fragments, the protocol of Gürtler *et al.* (1991) was followed with some modifications. Bacteria were grown in 10 ml tryptone soya broth (TSB, Oxoid) and placed in 1 mg lysozyme ml⁻¹ and 50 µg lysostaphin ml⁻¹ overnight at 37 °C. Specific digestion of rRNA operons was achieved by digestion of blocks overnight with 10 U of the intron-encoded endonuclease, I-CeuI (Liu *et al.*, 1993). The DNA fragments were separated with pulse times of 5 s for 10 h, 20 s for 10 h and 80 s for 10 h (*Sma*I), and 5 s for 6 h, 20 s for 8 h and 100 s for 16 h (I-CeuI).

Agarose gel electrophoresis. The *vanA*, *B* and *C* ligase gene amplicons were separated on 2% AR grade (IBI) agarose gels and the *Tsp509I* and *HinfI* restriction endonuclease fragments were separated on 2% NuSeive (FMC) plus 2% AR grade agarose gels by loading 3 µl loading buffer (Gürtler, 1993) added to 10 µl PCR mixture.

Southern hybridization. For hybridizations, the protocol of Gürtler *et al.* (1991) was followed with one exception. The PCR product shown in Fig. 1 was digested with *HinfI*; fragments from EFLGC to *HinfI* were purified and labelled with digoxigenin.

Conformation-sensitive gel electrophoresis (CSGE). For CSGE the protocol of Ganguly *et al.* (1993) and Kórkö *et al.* (1998) was followed with some modifications. The DCode apparatus (Bio-Rad) was used and 2 µl loading buffer (Gürtler, 1993) plus 6 µl undigested LISR amplicons were electrophoresed on a 16% 99:1 ratio of acrylamide (Merck) to *N,N'*-diacryloylpiperazine (Sigma) at room temperature for 16 h at 80 V in 1 × TTE (89 mM Tris/HCl, 15 mM taurine, 0.5 mM EDTA) in the lower chamber and 0.25 × TTE in the upper chamber. A total of 6 µl PCR product plus 2 µl loading buffer (Gürtler, 1993) was loaded per well and electrophoresis was performed at a constant 80 V at room temperature for 16 h. After electrophoresis the gels were stained with ethidium bromide or SYBR green I (FMC bioproducts) and visualized on a UV transilluminator.

Denaturing gradient gel electrophoresis (DGGE). For the detection of single base changes by DGGE the protocol of Myers *et al.* (1987) was followed with some modifications. The DCode apparatus (Bio-Rad) was used to perform parallel and perpendicular DGGE with 8% polyacrylamide (37.5:1) in Tris/acetate buffer at pH 8.0 (40 mM Tris/HCl, 20 mM acetate, 1 mM EDTA). The gradient was made with the Bio-Rad model 475 Gradient Delivery System with starting stock solutions of 20% (8% formamide and 1.4 M urea) and 40% (16% formamide and 2.8 M urea). A total of 100 µl PCR product plus 25 µl loading buffer (Gürtler, 1993) was loaded into a single-well preparative comb (or half the amount for a double-well preparative comb) and electrophoresis was performed at constant voltage and temperature (130 V and 60 °C). After electrophoresis the gels were stained with ethidium bromide or SYBR green I and visualized on a UV transilluminator.

DNA sequencing. All sequencing reactions were performed by dideoxy sequencing methods (Sanger *et al.*, 1977) and analysed using an Applied Biosystems model 373 DNA sequencer (Perkin-Elmer).

Data analysis. The multiple sequence alignment was performed using CLUSTAL W (Thompson *et al.*, 1994). Photographs of electrophoresis gels of the *Tsp509I* digests were scanned using an Astra 610S (UMAX) flatbed scanner with an optical resolution of 300 × 600 d.p.i. and then NIH Image 1.59 was used to determine the relative number of pixels cor-

responding to each band (expressed as a percentage of the total number of pixels from all the bands in a lane) from computer scanned photographs of agarose electrophoresis gels. The number of LISR copies (*rrnA*, *B* or *C*) were determined from the experimental data using the formula (relative number of pixels/100) × (337/*b*) × 3, where 337 is the total length (nt) of the PCR fragment amplified, *b* is the size of the band (nt) and 3 is the total copy number of the LISR. For the 111 bp fragment the following correction factor was used: c_b was derived from the band with size *b* nt (when present) such that $c_{225}/(c_{225} + c_{185} + c_{137})$ was used as a multiplication factor on c_{111} . The DNA melt profile (the temperature of the 50% probability that a nucleotide pair is dissociated) was calculated using POLAND (Poland, 1974) in the EGCG package supplied by the Australian National Genomic Information Service (ANGIS).

RESULTS AND DISCUSSION

LISR copy number

The number of copies of the LISR was determined as three using the following two approaches.

(1) The intron-encoded restriction endonuclease I-CeuI only recognizes a 23 bp sequence specific to all 23S rRNA genes producing a number of restriction fragments which correspond to the number of rRNA operons per bacterial genome (Liu *et al.*, 1993). When I-CeuI fragments from seven *E. faecalis* isolates (Table 1) were separated by PFGE and hybridized to the 130 bp LISR-specific *HinfI* fragment (Fig. 1) in all seven isolates three fragments hybridized and one fragment (1.3 Mbp) did not hybridize (Table 1).

(2) To exclude the possibility of partial digestion of genomic DNA by I-CeuI the same experiment was performed substituting *SmaI* for I-CeuI which has more cleavage sites than I-CeuI (15–20 fragments are produced by *SmaI*). In the three isolates studied three (out of a total of 10–15) *SmaI* fragments (with both intra- and inter-isolate length variations) hybridized to the LISR-specific *HinfI* fragment (Table 1). There is no *SmaI* cleavage site in the 23S rRNA gene nor in the 130 bp *HinfI* fragment used as a probe; however, there is one *SmaI* cleavage site at nt 1352–1357 of the 16S rRNA gene which is consistent with the detection of a polymorphic *SmaI* cleavage site past the 3' end of the 23S rRNA gene.

However, the possibility of *rrn* operon duplications resulting in more than three copies of the LISR cannot be excluded in other isolates. There were intra- and inter-isolate differences in the sizes of the hybridized fragments for both *SmaI* and I-CeuI, indicating that rearrangements in the genome or nucleotide substitutions in the *SmaI* or I-CeuI sites had taken place. Rearrangements are consistent with the insertion, deletion or duplication of DNA sequences such as insertion elements, as has been described for other Gram-positive bacteria (Leblond & Decaris, 1998). It is also possible that the *SmaI* and I-CeuI fragment size differences are due to *rrn* operon rearrangements by homologous recombination as has been described for *Salmonella typhi* (Liu & Sanderson, 1995, 1996).

Fig. 1. Sequence alignments (a) and schematic maps (b) of the LISR, amplified using primers EFLGC and LR20F, showing the sequence variants identified in this study (*rrnA*, *B* and *C*). (a) The *Tsp509I*¹⁻³ sites present on each LISR are shown sequentially, double underlined and in lower case [G:A nucleotide substitution converts sequence into a *Tsp509I* site at position 22 (*rrnB*) and at position 118 (*rrnC*)]. Position 118 is also the beginning of the *HinfI* site. The *Tsp509I* sites that would be created by a single nucleotide substitution are shown in lower case alone. The LISR sequences from isolates 617, 775-1, 775-2 and 805 were reported by Hall (1994). The SISR sequences reported by Naimi *et al.* (1997) (accession no. X87182) and Hall (1994) are identical. The SISR sequence is shown to demonstrate the specificity of EFLGC for the LISR sequence. Symbols: *, 617, 805 (accession no. X87186); †, 775-1; ‡, 617; §, 775-1, 775-2, 805; ¶, beginning of the 23S rRNA gene; ·, identical nucleotide; ·, nucleotide absent (gap); ↓, position of the *tRNA*^{ala} gene. The reverse primer (LR20F) sequence is the complement of that shown underlined at the end of the sequence alignment and the forward primer (EFLGC) sequence is shown underlined at the beginning of the alignment. (b) The LISR amplified is shown by the black boxes; narrow boxes on the ends depict the 16S and 23S rRNA genes, respectively. *Tsp509I* sites are marked by vertical lines and the size (nt) of the *Tsp509I* fragments are shown below each allele (horizontal lines show the span of each fragment).

Heterogeneity between the three copies of the LISR

The specific amplification of the LISR was achieved by using primer EFLGC (Fig. 1a) that contains an LISR-specific sequence at the 3' end (Fig. 1a). Amplification of the LISR was done to avoid the pattern complexity introduced by extra restriction fragments from the SISR that do not differentiate isolates any further (results not shown). A single amplification product was obtained in all isolates when analysed by agarose gel electrophoresis or PAGE (results not shown). To determine whether there were intra-isolate sequence differences in the PCR amplification products obtained, representing the three

copies of the LISR, the following three approaches were used:

(i) **Detection of nucleotide substitutions using *Tsp509I*.** To detect differences between *E. faecalis* isolates a restriction enzyme was sought which could differentiate between one or more of the five previously identified nucleotide substitutions in the LISR (Hall, 1994). The sequence differences between *rrnA*, *B* and *C* (identified in this study) at two *Tsp509I* sites (*Tsp509I*¹, nt 21–24; *Tsp509I*², nt 118–121) are shown in Fig. 1(a). The *Tsp509I* cleavage fragment sizes and the location of *Tsp509I*¹⁻³ for alleles *rrnA*, *B* and *C* are shown in Fig.

Table 1. Fragment sizes of genomic DNA from *E. faecalis* isolates cleaved with *I-CeuI* and *SmaI*, separated by PFGE and hybridized to the 130 bp *HinfI* LISR-specific fragment

The size of the fragments was estimated from the distance migrated on PFGE using λ DNA concatemers as markers. The sizes obtained were then corrected using a value of 2825 kb for the size of the *E. faecalis* genome (Murray *et al.*, 1993). ND, Not done.

Isolate	<i>I-CeuI</i> (kb)	<i>SmaI</i> (kb)
86549	371, 308, 87	69, 59, 15
87631	813, 417, 263	69, 59, 15
86228	823, 361, 280	620, 69, 15
173	827, 383, 261	ND
197	742, 402, 323	ND
185	692, 476, 285	ND
3	742, 445, 292	ND

1(b). Different combinations of these alleles were found and designated *Tsp* Types (Fig. 2). *Tsp509I*¹ is present in *rrnB* and absent in *rrnA* and *C*, *Tsp509I*² is present only in *rrnC* and *Tsp509I*³ is present in all alleles, isolates and *Tsp* Types (the 111 bp fragment was present at approximately equal intensity).

The nucleotide at position 22 (Fig. 1b) is 'A' in *rrnB* and 'G' in *rrnA* and *C* [the isolates from Hall (1994) with these nucleotides are listed in the Fig. 1(a) legend]. This A:G nucleotide substitution abolishes the *Tsp509I*¹ site (AATT, Fig. 1a) found in *rrnB*. The *Tsp509I*¹ site is only 20 bp from the end of the region amplified, making resolution of this *Tsp509I*¹ restriction fragment difficult. The extra length provided by the 20 bp GC-clamp used in this study allowed easier resolution of this fragment by agarose gel electrophoresis. When *Tsp509I*¹ is also present as in *rrnB*, fragments of 185 bp, 111 bp and 40 bp were obtained corresponding to *Tsp* Type I (Fig. 2a). When *Tsp509I*¹ was absent, as in *rrnA*, fragments of 225 and 111 bp were obtained corresponding to *Tsp* Type IV (Fig. 2d). The *Tsp509I*² site (identified in this study only and designated *rrnC*) was deduced from sizes of the digested fragments derived from the seven possible sites which required a single nucleotide substitution to create a *Tsp509I* site (Fig. 1a). Allele *rrnC* was present in *Tsp* Types III (Fig. 2c) and VI (Fig. 2f).

The *Tsp509I*¹ site (*rrnB*) was also present in *Tsp* Types II (Fig. 2b), V (Fig. 2e) and VI (Fig. 2f). However, in all of these isolates extra restriction fragments of variable intensity were present. When the lengths of these restriction fragments were added, the total length was greater than the length of the undigested amplicon (337 bp). These extra restriction fragments are unlikely to be partial digests because when the formula stated in Methods was used, the copy numbers of *rrnA*, *B* and *C* were the same as when they were deduced from the length of each fragment. Based on the relative number of pixels for each fragment it was found that *Tsp* Types II,

V and VI contain a heterogeneous population of 16S–23S rDNA spacers: *Tsp* Type II has alleles *rrnA* and *B* in a ratio of 1:2 (Fig. 2b); *Tsp* Type III has alleles *rrnA* and *C* in a ratio of 2:1 (Fig. 2c); *Tsp* Type V has alleles *A* and *B* in a ratio of 2:1 (Fig. 2e) and *Tsp* Type VI has alleles *rrnA*, *B* and *C* in a ratio of 1:1:1 (Fig. 2f).

(ii) **Heteroduplex analysis using CSGE.** Further evidence to support the presence of a heterogeneous population of LISR was sought by the use of CSGE to detect heteroduplexes (Fig. 3). If a mixture of amplification products is obtained from an isolate containing nucleotide differences at one or more positions (corresponding to the presence of *rrnA*, *B* or *C*), heteroduplexes will be formed due to mismatches causing conformational changes under the mildly denaturing solvent conditions used in CSGE, the result being migration differences between homoduplexes and heteroduplexes (Ganguly *et al.*, 1993).

The CSGE of the LISR from 11 *E. faecalis* isolates is shown in Fig. 3(a) and a schematic representation of the allele combinations that may produce the observed heteroduplex and homoduplex molecules is shown in Fig. 3(b). The positive strand and negative strand mismatches result in heteroduplexes that run at equivalent mobilities (represented as heteroduplex pairs in Fig. 3b) since CSGE only detects conformation changes and not sequence differences (Ganguly *et al.*, 1993). The variants identified can be explained by the three possibilities shown in Fig. 3(b) using the diversity names listed in Table 2.

(i) **HOMI and II.** All three 16S–23S spacers have identical sequences resulting in a single band corresponding to one homoduplex (Fig. 3a, lane 1), including seven isolates that are *Tsp* Types I (Fig. 2a) and IV (Fig. 2d). The exceptions are those isolates that are *Tsp* Type V (Fig. 3a, lanes 7, 10 and 11) with one band on CSGE (Type a) even though *Tsp509I* cleavage demonstrates a mixture of two different allele sequences (Fig. 2e). These exceptions can be explained if *Tsp509I*¹ (Fig. 1a, 40 bp from 5' end) intra-isolate heterogeneity is not detected by CSGE, consistent with previous studies where single-base mismatches within 50 bp of one end of a heteroduplex were shown not to produce extra heteroduplex bands by CSGE (Ganguly *et al.*, 1993).

(ii) **HETII, and V–VIII.** One allele sequence differs (*rrnA*) from the other two (*rrnB* and *C*) at one nucleotide position or two allele sequences have one nucleotide substitution and the other sequence has another nucleotide substitution, resulting in one most intense homoduplex band [comprising both homoduplexes (AA) and (BB or CC)] and one heteroduplex band [A (B or C)]. These possibilities explain: Fig. 3(a), lane 2 (HETVIII), lane 3 (HETVI; Fig. 5d DGGE predicts three heteroduplexes), lane 5 (HETII), lane 6 (HETVII; two bands were more visible on another gel not shown) and lane 9 (HETV). CSGE Type e has two bands even though *Tsp509I* cleavage shows a mixture of three different allele sequences (*Tsp* Type VI) consistent with (i) above that

Fig. 2. Density profiles of *Tsp509I*-digested PCR products described in Fig. 1 and separated by agarose gel electrophoresis. The six profiles shown (a–f) represent *Tsp509I* Types I–VI, respectively. The isolates representing each *Tsp509I* Type are (a) 3, 196, 65, 66397, 70726, 86228 and 87578, (b) 235, (c) 197, (d) 198, (e) 200, 185, 203, 195 and 89349, and (f) 173, 162 and 84546. The size of *Tsp509I* fragments represented by each of the peaks and the alleles (*rrnA*, *B* and *C*) they correspond to are listed at the top of the figure. Molecular mass markers number V (pBR328 DNA cleaved with *Hae*III) and VI (pBR328 DNA cleaved with *Bgl*I and *Hinf*I) from Boehringer Mannheim were used to determine the sizes of the *Tsp509I* fragments. The copy number of each allele is listed in parentheses after the allele name for each *Tsp509I* Type. The relative number of pixels expressed as a percentage of the total number of pixels for all the peaks in that *Tsp509I* Type is listed above each density peak [mean ± SEM and (no. of determinations; 1–4 *Tsp509I* digestions per isolate)].

*Tsp509I*¹ heterogeneity is not detected by CSGE. Both *Tsp* Types III and VI have *Tsp509I*² and produced very similar CSGE profiles, suggesting that the nucleotide substitution responsible for the creation of *Tsp509I*² was detected within CSGE Types c and e (Fig. 3a, lanes 5 and 9). There is considerable CSGE variability within *Tsp* Type V with three homogeneous isolates (Fig. 3a, lanes 1, 7, 10 and 11) and two heterogeneous isolates (Fig. 3a, lanes 2 and 6).

(iii) HETIV. One allele (*rrnA*) has a single nucleotide substitution, another (*rrnB*) has two or more nucleotide substitutions and the third allele (*rrnC*) has no nucleotide substitutions, resulting in three heteroduplexes with

only one mismatch each (R or S and X or Y; one allele has a correctly matched XY pair) or two allele sequences have two different nucleotide substitutions giving rise to two heteroduplexes with one mismatch and another heteroduplex with two mismatches. Type f (Fig. 3a, lanes 4 and 8) has one most intense central homoduplex band and two heteroduplex bands on CSGE. However, Fig. 3b predicts three bands, thus one of the bands in CSGE Type f is expected to be a mixture of heteroduplex and/or homoduplex molecules. This contrasts to the prediction from the *Tsp* Type which is consistent with the presence of only one DNA sequence that does not differ at the three *Tsp509I* cleavage sites. When the separate bands were cut from the gel, eluted into buffer,

Fig. 3. (a) CSGE of *E. faecalis* isolates and (b) schematic representation of the combination of heteroduplex and homoduplex molecules detected by CSGE. (a) The CSGE and *Tsp509I* Types are listed below the lane numbers. Lanes: 1, 84706; 2, 89349; 3, 235; 4, 3; 5, 197; 6, 200; 7, 203; 8, 3; 9, 162; 10, 185; 11, 195. (b) The plus strand is shown by the thick line and the minus (complementary) strand is shown by the thin line. The two nucleotide substitutions and their locations on one or more alleles are depicted as 'XY' and 'RS'. The three homoduplex molecules for alleles *rrnA*, *B* and *C* are shown at the top and the heteroduplex molecules formed by various combinations of substituted and non-substituted strands are shown at the bottom of the figure in groups of two with equivalent mobilities (one heteroduplex has the nucleotide substitution on the plus strand and in the other it is on the minus strand). (i) The three homoduplex molecules corresponding to alleles *rrnA*, *B* and *C* have identical sequences, therefore no heteroduplex molecules are formed. (ii) One allele (*rrnA*) differs from the other two alleles (*rrnB* and *C*) by a single nucleotide substitution (XY) or two alleles have a single nucleotide substitution (XY) and the other allele (*rrnB*) has a different single nucleotide substitution (RS); both possibilities result in one heteroduplex pair. (iii) One allele (*rrnA*) has a single nucleotide substitution (XY), another allele (*rrnB*) has two nucleotide substitutions (XY and RS) and the third allele has no nucleotide substitutions or one allele (*rrnA*) has one nucleotide substitution (XY), another allele (*rrnB*) has a different nucleotide substitution (RS) and the third allele (*rrnC*) has no nucleotide substitutions; both possibilities result in two heteroduplex pairs (the same number of heteroduplexes are expected with more than two nucleotide substitutions per strand).

amplified and the products separated by CSGE, all bands gave the same band pattern as the original (Fig. 3a, lane 4). In summary, heterogeneity between alleles due to the presence or absence of the *Tsp509I*¹ cleavage site was not detected by CSGE. However, heterogeneity due to at least five other nucleotide substitutions (CSGE Types b, d, f and g) was detected by CSGE.

(iii) Heteroduplex and homoduplex analysis using DGGE. To increase the resolution obtained with CSGE and *Tsp* typing, DGGE was used to resolve homoduplex and heteroduplex molecules generated by amplification of three copies of the LISR. When these sets of duplexes are separated by electrophoresis perpendicular to a linearly increasing gradient of denaturants (urea and formamide) the duplexes will meet a micro-environment that matches exactly the lowest melting domain of the

duplexes and unwind, retarding their mobility (Myers *et al.*, 1987). The small differences in the melting temperature values of the various duplexes will allow a number of bands to be resolved corresponding to the number of different LISR sequences (one to three copies corresponding to one to three homoduplexes and zero to six heteroduplexes, respectively).

The theoretical melting temperatures of four LISR sequences reported by Hall (1994) are shown in Fig. 4. The melting temperatures of LISR sequences from isolates 775-1, 775-2 and 805 (Fig. 4, 68 °C; Fig. 1a, due to 'A' at position 134 and 'A' at position 163) in Domain I are predicted to be less than that from isolate 617 (Fig. 4, 70 °C; Fig. 1a, due to 'C' at position 134 and 'G' at position 163) such that the homoduplex from 617 will migrate more quickly than the homoduplexes from

Table 2. Diversity of the LISR in 21 *E. faecalis* isolates determined by *Tsp509I* cleavage, CSGE and DGGE

Isolate	Location	<i>van</i> *	<i>Tsp509I</i> Type	No. of copies			CSGE Type	DGGE Type	Alleles†	Diversity‡
				<i>rrnA</i>	<i>rrnB</i>	<i>rrnC</i>				
65	Adelaide	S	I	0	3	0	a	1§	1	HOMI
196	Adelaide	S	I	0	3	0	a	1§	1	HOMI
66397	A&RMC	B	I	0	3	0	a	ND	1	HOMI
70726	A&RMC	B	I	0	3	0	a	1§	1	HOMI
86228	A&RMC	B	I	0	3	0	ND	1§	1	HOMI
87578	A&RMC	S	I	0	1	0	a	1	1	HOMI
84546	A&RMC	S	VI	1	1	1	ND	ND	3	HOMI
84706	A&RMC	B	I	0	3	0	a	ND	1	HOMI
198	Adelaide	S	IV	3	0	0	a	1§	1	HOMII
185	Adelaide	S	V	2	1	0	a	1	2	HETI
195	Adelaide	S	V	2	1	0	a	1§	2	HETI
197	Adelaide	S	III	2	0	1	c	2	2	HETII
203	Adelaide	S	V	2	1	0	a	3§	2	HETIII
3	Sydney	B	I	0	3	0	f	6	3	HETIV
162	Melbourne	B	VI	1	1	1	e	ND	3	HETV
173	Melbourne	B	VI	1	1	1	e	2	3	HETV
235	Brisbane	B	II	1	2	0	d	7	2-3	HETVI
200	Adelaide	S	V	2	1	0	b	4	2	HETVII
89349	A&RMC	S	V	2	1	0	g	2	2	HETVIII

ND, Not done (including 86549 and 87631).

* Genotype as defined in Methods. B, *vanB*; S, non-*vanA*, B or C.

† Number of sequence-variable alleles.

‡ With respect to *Tsp509I* Type, no. of copies of *rrnA*, B or C, CSGE Type and DGGE Type. HOM, homogeneous; HET, heterogeneous.

§ Parallel DGGE for screening and all others perpendicular DGGE for greater resolution (no. of bands or curves, respectively, is shown).

Fig. 4. Predicted DNA melt profile showing the temperature of the 50% probability (vertical axis) that the LISR sequences are denatured at the corresponding nucleotide positions (range shown on the horizontal axis corresponds to the region amplified as shown in Fig. 1a) for isolates 617, 775-1, 775-2 and 808 (nt substitutions shown in Fig. 1a). The GC-clamp is depicted at the beginning of the horizontal axis by a dark box from nt -20 to 0.

775-1, 775-2 and 805. When an isolate contains heterologous LISR sequences (one equivalent to the sequence in isolate 617 and the other equivalent to the sequences in isolates 775-1, 775-2 and 805), mismatches at positions 134 (C:A) and 165 (G:A) are expected, resulting in

heteroduplexes that will migrate more quickly than the homoduplexes. A similar situation occurs in Domain II at a higher melting temperature [75 and 78 °C due to 'A' in 617 and 805 and 'G' in 775-1 and 775-2 at position 22 (Figs 1a and 4); 78 and 80 °C due to 'A' in 617, 805,

Fig. 5. Perpendicular DGGE of CSGE Types a, b, d and f. The direction of electrophoretic migration is from top to bottom and the direction of decreasing denaturant concentration (40–20%) is from left to right in each panel. Electrophoresis was performed at a constant temperature of 60 °C. (a) *Tsp509I* Type I, CSGE Type a, isolate 185. (b) *Tsp509I* Type III, CSGE Type c, isolate 197. (c) *Tsp509I* Type I, CSGE Type f, isolate 3. (d) *Tsp509I* Type II, CSGE Type d, isolate 235.

775-1 and G in 775-2 at position 38 (Figs 1a and 4)] corresponding to a higher denaturant concentration at which strand separation occurs. The final feature illustrated in Fig. 4 is the GC-clamp at the 5' end of the PCR product that is the synthetically added highest temperature melting domain making the detection of most of the nucleotide substitutions in Domain II possible; without the GC-clamp, sequence-dependent gel migration will be lost upon complete strand separation (Myers *et al.*, 1985a, b).

When a fragment of a diploid eukaryotic gene with a single-base pair loop-out or nucleotide substitution on one chromosome is separated by perpendicular DGGE, a total of four curves are expected – two for the two possible homoduplexes and two for the two possible heteroduplexes (Lerman *et al.*, 1986; Myers & Maniatis, 1986). However, both the identity of the unpaired base and the sequence of the flanking base pairs influence the degree of destabilization and therefore the resolution of homoduplex and heteroduplex denaturation melting curves (Ke & Wartell, 1995). In those isolates that were HOMI or HOMII (Table 2) only one allele sequence was predicted (*rrnB* or *rrnA*, respectively). When DGGE was used to resolve HOMI (Fig. 5a) and HOMII (not shown) one curve was obtained as expected. In the isolate that was HETII (Table 2), two allele sequences were predicted giving rise to the possibility of two homoduplexes and two heteroduplexes. The result

obtained demonstrates two curves (Fig. 5b) as opposed to the expected four curves; the right-hand curve probably corresponds to the two homoduplexes and the left-hand curve to the two heteroduplexes. In those isolates that were HETIV and V (Table 2) three different allele sequences were predicted (*rrnA*, B and C) giving rise to the possibility of three homoduplexes (AA, BB, CC) and six heteroduplexes (AB, AC, BA, BC, CB, CA). Therefore a maximum of nine curves were expected on perpendicular DGGE. The result obtained demonstrates only six curves (Fig. 5c), which may be due to the complementary heteroduplexes (AB and BA, AC and CA, BC and CB) migrating together as one curve; the two heteroduplexes on the right and the one homoduplex on the left in each of the three groups of two curves. In those isolates that were HETVI only two allele sequences were predicted. However, using perpendicular DGGE seven curves were obtained (Fig. 5d), suggesting the presence of further nucleotide substitutions not detected by *Tsp509I* cleavage or CSGE.

For CSGE Types c, f and d (Fig. 5b, c and d), when DNA corresponding to separate curves was eluted and subjected to a further round of PCR and perpendicular DGGE, the same number of curves as the original were obtained (not shown), suggesting each curve contained combinations of duplex molecules. The determination of the number and type of nucleotide substitutions requires the sequencing of DNA in DGGE (and CSGE)

bands that contain only one type of duplex. The optimization of DGGE conditions may help to overcome these problems. A recent report has added a porosity gradient in addition to the denaturing gradient to achieve enhanced resolution of homoduplex bands (Cremonesi *et al.*, 1999).

In summary, *Tsp509I* cleavage, CSGE and perpendicular DGGE have characterized isolates into three clear categories on the basis of LISR sequence diversity (Table 2): (i) HOMI and II, only one allele sequence present; (ii) HETII and V–VIII, two allele sequences present; (iii) HETIV, three allele sequences present. To our knowledge this is the first demonstration of the use of CSGE and perpendicular DGGE for the characterization of a DNA sequence present in more than two copies per genome. DGGE or CSGE may be a faster and cheaper alternative to sequencing for screening large numbers of *E. faecalis* isolates for intragenomic LISR sequence heterogeneity. The results presented here demonstrate the increased sensitivity of DGGE (compared with *Tsp509I* cleavage and CSGE) for the detection of sequence variants in a mixture of DNA sequences present in more than two copies per genome.

Sequence diversity in the three copies of the *E. faecalis* LISR

Two homogeneous LISR Types (Table 2, HOMI and HOMII) and eight heterogeneous Types (HETI–VIII) were demonstrated by *Tsp509I* cleavage, CSGE and DGGE. For the homogeneous Types (Table 2, HOMI and HOMII) no sequence differences were detected between copies of the LISR using the three approaches. However, HOMI differs from HOMII by at least the presence of a *Tsp509I*¹ site in all copies. In the heterogeneous Types (Table 2, HETI–VIII) the *Tsp509I*¹ site is present (in at least one copy and less than three copies) in conjunction with a number of other nucleotide substitutions.

The conservation (homogenization corresponding to HOMI and HOMII) of LISR sequences in *E. faecalis* may be explained by concerted evolution (Dover, 1982) where the number of intraspecies differences between genes is less than the number of interspecies differences (Zimmer *et al.*, 1980). Many more heterogeneous Types were detected (HETI–VIII) and could be explained by (i) base pair substitutions (including single and compensatory), (ii) genetic rearrangements (deletions, duplications, translocations and inversions) and (iii) horizontal transfer of DNA sequences from other species. Possible mechanisms by which these events could occur are homologous (i, ii and iii) and non-homologous (iii) recombination, random nucleotide substitutions (i) or by other events such as transposition.

A number of previous studies point to sequence heterogeneities in the 16S rDNA gene of some clostridial species; one *HpaII* site within the 16S rRNA gene was present on most alleles in *Clostridium bifermentans*, present on a minority of alleles in *Clostridium sordelli*

and absent in *Clostridium difficile* (Gürtler *et al.*, 1991). Sequence differences were documented between two different copies of the 16S rRNA gene in *Mycobacterium celatum* (Reischl *et al.*, 1998) and *Mycobacterium* sp. isolates (Ninet *et al.*, 1996). Differences have also been shown in *Escherichia coli* (Cilia *et al.*, 1996) and in *Thermobispora bispora* (Wang *et al.*, 1997). In these species and in *E. faecalis*, evidence for the proposal that the ISRs do not differ much from the 16S rRNA genes at the level of isolated nucleotide substitutions (Perez-Luz *et al.*, 1998; Antón *et al.*, 1998) could be obtained by comparing the intragenomic sequence heterogeneity of these two regions. The level of insertion and deletion of sequence blocks found in the ISR has not been found in the 16S rRNA gene (e.g. Gürtler & Mayall, 1999; Antón *et al.*, 1998; Perez-Luz *et al.*, 1998).

The intra-isolate sequence conservation of ISR sequence blocks (Gürtler & Mayall, 1999), the insertion and deletion of some ISR sequence blocks and the intragenomic sequence diversity of LISR in *E. faecalis* demonstrated by this study must be explained in any mechanism invoking homologous recombination of complete *rrn* operons to explain concerted evolution of the ISR. In *E. faecalis* isolates, sequence diversity of the LISR may involve homologous recombination of regions susceptible to nucleotide substitutions within the spacer and subsequent fixation of nucleotide substitutions during homogenization. The three approaches described in the present study (*Tsp509I* cleavage, CSGE and DGGE) show considerable potential for determining the role of concerted evolution in LISR sequence diversity in *E. faecalis* isolates with known evolutionary relationships.

ACKNOWLEDGEMENTS

We thank Drs Tim Littlejohn and Bruno Gaeta from ANGIS for help with DNA sequence data analysis. We are grateful to Jan Bell and Drs Alex Padiglione and Lindsay Grayson, for supplying us with bacterial isolates. We thank two anonymous reviewers for their comments.

REFERENCES

- Antón, A. I., Martínez-Murcia, A. J. & Rodríguez-Valera, F. (1998). Sequence diversity in the 16S–23S intergenic spacer region (ISR) of the rRNA operons in representatives of the *Escherichia coli* ECOR collection. *J Mol Evol* 47, 62–72.
- Arthur, M., Reynolds, P. & Courvalin, P. (1996). Glycopeptide resistance in enterococci. *Trends Microbiol* 4, 401–407.
- Bell, J. M., Paton, J. C. & Turnidge, J. (1998). Emergence of vancomycin-resistant enterococci in Australia – phenotypic and genotypic characteristics. *J Clin Microbiol* 36, 2187–2190.
- Cilia, V., Lafay, B. & Christen, R. (1996). Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Mol Biol Evol* 13, 451–461.
- Cremonesi, L., Carrera, P., Fumagalli, A. & 7 other authors (1999). Validation of double gradient denaturing gradient gel electrophoresis through multigenic retrospective analysis. *Clin Chem* 45, 35–40.

- Dover, G. (1982). Molecular drive: a cohesive mode of species evolution. *Nature* **299**, 111–117.
- Dunne, W. M., Jr & Wang, W. (1997). Clonal dissemination and colony morphotype variation of vancomycin-resistant *Enterococcus faecium* isolates in metropolitan Detroit, Michigan. *J Clin Microbiol* **35**, 388–392.
- Facklam, R. R. & Collins, M. D. (1989). Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. *J Clin Microbiol* **27**, 731–734.
- Ganguly, A., Rock, M. J. & Prockop, D. J. (1993). Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* **90**, 10325–10329.
- García-Martínez, J., Martínez-Murcia, A. J., Rodríguez-Valera, F. & Zorraquino, A. (1996a). Molecular evidence supporting the existence of two major groups in uropathogenic *Escherichia coli*. *FEMS Immunol Med Microbiol* **14**, 231–244.
- García-Martínez, J., Martínez-Murcia, A., Antón, A. I. & Rodríguez-Valera, F. (1996b). Comparison of the small 16S to 23S intergenic spacer region (ISR) of the rRNA operons of some *Escherichia coli* strains of the ECOR collection and *E. coli* K-12. *J Bacteriol* **178**, 6374–6377.
- Gürtler, V. (1993). Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S–23S rDNA spacer regions. *J Gen Microbiol* **139**, 3089–3097.
- Gürtler, V. & Barrie, H. D. (1995). Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S–23S rDNA spacer regions: characterization of spacer sequences. *Microbiology* **141**, 1255–1265.
- Gürtler, V. & Mayall, B. C. (1999). rDNA spacer rearrangements and concerted evolution. *Microbiology* **145**, 2–3.
- Gürtler, V. & Stanisich, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
- Gürtler, V., Wilson, V. A. & Mayall, B. C. (1991). Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J Gen Microbiol* **137**, 2673–2679.
- Hall, L. (1994). Are point mutations or DNA rearrangements responsible for the restriction fragment length polymorphisms that are used to type bacteria? *Microbiology* **140**, 197–204.
- Ke, S. H. & Wartell, R. M. (1995). Influence of neighboring base pairs on the stability of single base bulges and base pairs in a DNA fragment. *Biochemistry* **34**, 4593–4600.
- Körkkö, J., Annunen, S., Pihlajamaa, T., Prockop, D. J. & Ala-Kokko, L. (1998). Conformation sensitive gel electrophoresis for simple and accurate detection of mutations: comparison with denaturing gradient gel electrophoresis and nucleotide sequencing. *Proc Natl Acad Sci USA* **95**, 1681–1685.
- Lan, R. & Reeves, P. R. (1998). Recombination between rRNA operons created most of the ribotype variation observed in the seventh pandemic clone of *Vibrio cholerae*. *Microbiology* **144**, 1213–1221.
- Leblond, P. & Decaris, B. (1998). Chromosome geometry and intraspecific genetic polymorphism in Gram-positive bacteria revealed by pulsed-field gel electrophoresis. *Electrophoresis* **19**, 582–588.
- Lerman, L. S., Silverstein, K. & Grinfeld, E. (1986). Searching for gene defects by denaturing gradient gel electrophoresis. *Cold Spring Harbor Symp Quant Biol* **51**, 285–297.
- Liu, S. L. & Sanderson, K. E. (1995). Rearrangements in the genome of the bacterium *Salmonella typhi*. *Proc Natl Acad Sci USA* **92**, 1018–1022.
- Liu, S. L. & Sanderson, K. E. (1996). Highly plastic chromosomal organization in *Salmonella typhi*. *Proc Natl Acad Sci USA* **93**, 10303–10308.
- Liu, S. L., Hessel, A. & Sanderson, K. E. (1993). Genomic mapping with I-CeuI, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. *Proc Natl Acad Sci USA* **90**, 6874–6878.
- Miele, A., Bandera, M. & Goldstein, B. P. (1995). Use of primers selective for vancomycin resistance genes to determine *van* genotype in enterococci and to study gene organization in VanA isolates. *Antimicrob Agents Chemother* **39**, 1772–1778.
- Monstein, H. J., Quednau, M., Samuelsson, A., Ahnér, S., Isaksson, B. & Jonasson, J. (1998). Division of the genus *Enterococcus* into species groups using PCR-based molecular typing methods. *Microbiology* **144**, 1171–1179.
- Murray, B. E. (1990). The life and times of the Enterococcus. *Clin Microbiol Rev* **3**, 46–65.
- Murray, B. F., Singh, K. V., Ross, R. P., Heath, J. D., Dunny, G. M. & Weinstock, G. M. (1993). Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. *J Bacteriol* **175**, 5216–5223.
- Myers, R. M. & Maniatis, T. (1986). Recent advances in the development of methods for detecting single-base substitutions associated with human genetic diseases. *Cold Spring Harbor Symp Quant Biol* **51**, 275–284.
- Myers, R. M., Fischer, S. G., Lerman, L. S. & Maniatis, T. (1985a). Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res* **13**, 3131–3145.
- Myers, R. M., Fischer, S. G., Maniatis, T. & Lerman, L. S. (1985b). Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acids Res* **13**, 3111–3129.
- Myers, R. M., Maniatis, T. & Lerman, L. S. (1987). Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods Enzymol* **155**, 501–527.
- Naïmi, A., Beck, G. & Branlant, C. (1997). Primary and secondary structures of rRNA spacer regions in enterococci. *Microbiology* **143**, 823–834.
- Ninet, B., Monod, M., Emler, S., Pawlowski, J., Metral, C., Rohner, P., Auckenthaler, R. & Hirschel, B. (1996). Two different 16S rRNA genes in a mycobacterial strain. *J Clin Microbiol* **34**, 2531–2536.
- Patel, R., Uhl, J. R., Kohner, P., Hopkins, M. K. & Cockerill, F. R., 3rd (1997). Multiplex PCR detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* genes in enterococci. *J Clin Microbiol* **35**, 703–707.
- Patel, R., Piper, K. E., Rouse, M. S., Steckelberg, J. M., Uhl, J. R., Kohner, P., Hopkins, M. K., Cockerill, F. R., 3rd & Kline, B. C. (1998). Determination of 16S rRNA sequences of enterococci and application to species identification of nonmotile *Enterococcus gallinarum* isolates. *J Clin Microbiol* **36**, 3399–3407.
- Pegues, D. A., Pegues, C. F., Hibberd, P. L., Ford, D. S. & Hooper, D. C. (1997). Emergence and dissemination of a highly vancomycin-resistant *vanA* strain of *Enterococcus faecium* at a large teaching hospital. *J Clin Microbiol* **35**, 1565–1570.
- Perez-Luz, S., Rodríguez-Valera, F., Lan, R. & Reeves, P. R. (1998). Variation of the ribosomal operon 16S–23S gene spacer region in

representatives of *Salmonella enterica* subspecies. *J Bacteriol* **180**, 2144–2151.

Poland, D. (1974). Recursion relation generation of probability profiles for specific-sequence macromolecules with long-range correlations. *Biopolymers* **13**, 1859–1871.

Reischl, U., Feldmann, K., Naumann, L., Gaugler, B. J., Ninet, B., Hirschel, B. & Emler, S. (1998). 16S rRNA sequence diversity in *Mycobacterium celatum* strains caused by presence of two different copies of 16S rRNA gene. *J Clin Microbiol* **36**, 1761–1764.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Schleifer, K. H. & Kilpper-Bälz (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol* **34**, 31–34.

Schleifer, K. H. & Kilpper-Bälz, R. (1987). Molecular and chemotaxonomic approaches to the classification of Streptococci, Enterococci and Lactococci: A review. *Syst Appl Microbiol* **10**, 1–19.

Sechi, L. A. & Daneo-Moore, L. (1993). Characterization of intergenic spacers in two *rrn* operons of *Enterococcus hirae* ATCC 9790. *J Bacteriol* **175**, 3213–3219.

Sheffield, V. C., Cox, D. R., Lerman, L. S. & Myers, R. M. (1989). Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to

genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* **86**, 232–236.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tyrrell, G. J., Bethune, R. N., Willey, B. & Low, D. E. (1997). Species identification of enterococci via intergenic ribosomal PCR. *J Clin Microbiol* **35**, 1054–1060.

Uttley, A. H., Collins, C. H., Naidoo, J. & George, R. C. (1988). Vancomycin-resistant enterococci. *Lancet* **1**, 57–58.

Wang, Y., Zhang, Z. & Ramanan, N. (1997). The actinomycete *Thermobispora bispora* contains two distinct types of transcriptionally active 16S rRNA genes. *J Bacteriol* **179**, 3270–3276.

Williams, A. M., Rodrigues, U. M. & Collins, M. D. (1991). Intrageneric relationships of Enterococci as determined by reverse transcriptase sequencing of small-subunit rRNA. *Res Microbiol* **142**, 67–74.

Zimmer, E. A., Martin, S. L., Beverley, S. M., Kan, Y. W. & Wilson, A. C. (1980). Rapid duplication and loss of genes coding for the alpha chains of hemoglobin. *Proc Natl Acad Sci USA* **77**, 2158–2162.

Received 2 December 1998; revised 5 March 1999; accepted 18 March 1999.