Molecular fingerprinting of isolates of the genus *Peptostreptococcus* using rRNA genes from *Escherichia coli* and *P. anaerobius*

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(Received 10 February 1991; accepted 19 February 1991)

Restriction fragment length polymorphisms (RFLPs) of rRNA genes were evaluated as a tool for intra- and inter-species differentiation of *Peptostreptococcus* isolates. RFLPs from a collection of 20 clinical isolates and five ATCC strains representing five *Peptostreptococcus* spp. (*P. anaerobius*, *P. asaccharolyticus*, *P. magnus*, *P. micros* and *P. prevotii*) were obtained by hybridization of Southern blots of *Hind*III- or *Eco*RI-digested genomic DNA with three probes: probe A, a 0.98 kb *Hind*III fragment with a partial 16S rRNA gene sequence from *P. anaerobius* ATCC 27337; probe B, cloned *Escherichia coli* *rrnB* operon in plasmid pKK3535; and probe C, *E. coli* 16S and 23S rRNA. The hybridization patterns varied, but all yielded RFLPs useful for both intra- and inter-species differentiation. RFLPs of *P. asaccharolyticus* clinical isolates were closely related to each other and differed significantly from those of the ATCC type strains. The profiles of *P. prevotii* differed from those of the other four species studied, and based on the *Hind*III- and *Eco*RI-generated RFLPs, the strains in this species are more heterogeneous than the other four species studied.

Introduction

Most female genital tract infections, including the classic sexually transmitted diseases, are polymicrobial in nature (Garber & Chow, 1989). The most common anaerobes found in these infections are *Peptostreptococcus* and *Bacteroides* spp. (Garber & Chow, 1989; Sweet, 1986). The relative importance of *Peptostreptococcus* spp. in mixed anaerobic infections has been studied (Brook & Walker, 1984) and it has been proposed that these organisms play a synergistic role in polymicrobial female genital tract infections (Garber & Chow, 1989). Besides their frequent association with mixed anaerobic infections, peptostreptococci have also been isolated in pure culture from soft tissue infections (Bougault *et al.*, 1980), suggesting that they could also be the primary aetiological agent of these infections.

Although the importance of the indigenous cervico-vaginal flora both in health and in disease has become increasingly recognized, the pathogenic potential of specific microbes, including *Peptostreptococcus* spp. has been difficult to assess. This is partly due to problems in the sampling, culturing, identification and taxonomic classification of these organisms from clinical specimens

(Brook, 1987; Mardh & Westrom, 1984). In clinical laboratories, anaerobic Gram-positive cocci are often not differentiated at the species level when they are isolated from polymicrobial infections because they are frequently considered either as contaminants due to their presence as normal flora or as less significant pathogens than *Bacteroides* (Brook, 1987; Bougault *et al.*, 1980; Finegold, 1989; Murdoch *et al.*, 1988). Recently, the genus *Peptostreptococcus* has undergone taxonomic reclassification and now includes several species which were previously classified as *Peptococcus* (Ezaki *et al.*, 1983; Moore *et al.*, 1987). The differentiation of *Peptostreptococcus micros* from *P. magnus* based on phosphatase tests and electrophoretic patterns of soluble protein has made it difficult to reliably discriminate between these species (Moore *et al.*, 1987). Although an identification system (RapID ANA, Mercia Diagnostics) based on pre-formed enzyme detection has been used to reliably identify *P. anaerobius* and *P. asaccharolyticus*, this system was unable to distinguish *P. magnus* from *P. micros* (Murdoch *et al.*, 1988).

At present, there is no definitive correlation between specific *Peptostreptococcus* species or strains and any type of clinical infection (Finegold, 1989; Bougault *et al.*, 1980). The role of *Peptostreptococcus* as an emerging, opportunistic or synergistic pathogen in causing specific

Abbreviation: RFLP, restriction fragment length polymorphism.

anaerobic infections remains unclear. In order to determine its specific pathogenic role in causing infections it is necessary to carry out correlational studies of specific species or strains to properly diagnosed infections. This will be possible only if reliable differentiation systems for these strains are first developed. Serotyping and plasmid profile analysis were attempted and were not useful methods for strain differentiation (Smith *et al.*, 1986; Ng & Dillon, 1989). However, molecular typing schemes based on DNA fingerprinting have not been explored. In this study, restriction fragment length polymorphism (RFLP) of rRNA (*rrn*) operons of *Peptostreptococcus* was investigated as a tool for intra- and inter-species differentiation of five *Peptostreptococcus* species.

Methods

Strains and culture conditions. Twenty *Peptostreptococcus* isolates comprising five species, obtained from the culture collection (courtesy of E. P. Ewan) of the National Laboratory for General Bacteriology (Laboratory Centre for Disease Control, Ottawa), and eight ATCC strains were included in this study (see Table 1). The speciation of the strains was re-confirmed using the VPI system (Holdeman *et al.*, 1977). *Escherichia coli* JM83 was used as host for cloning experiments with the plasmid vector pUC9.

Stock cultures were either stored at -70°C in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol or were lyophilized in 2% (w/v) skim milk. *Peptostreptococcus* were subcultured on BHI agar supplemented with 5% (v/v) sheep blood, vitamin K (1 mg l^{-1}) and 0.1% haemin and incubated for 16–24 h at 37°C under anaerobic conditions (5% CO_2 , 5% H_2 and 90% N_2).

Isolation of chromosomal DNA from *Peptostreptococcus* spp. For isolation of chromosomal DNA, 25 ml Peptone Yeast Extract (PY) broth was inoculated with confluent growth from an entire BHI agar plate and incubated at 37°C anaerobically. After 24 h, the PY broth culture was transferred to 225 ml fresh PY broth and incubated for a further 24 h. Because the peptostreptococcal cell wall is not susceptible to lysozyme alone (Ng & Dillon, 1989), penicillin G (Sigma) in PY broth was added to cultures, at a final concentration of one-sixth of the minimal inhibitory concentration (MIC) of the specific isolate (see Table 1) 2–4 h prior to harvesting, to facilitate lysis. The MICs of penicillin G for ATCC strains were not determined in this study. The concentration of penicillin G to be used for these strains was determined by growing them in 5 ml PY broth containing twofold dilutions (0.002 – 0.02 mg l^{-1}) of the antibiotic. All ATCC strains yielded the highest amount of DNA from cell lysis if $0.004\text{ mg penicillin G l}^{-1}$ was used.

After 2–4 h of incubation, the cells were harvested by centrifugation for 10 min at 10000 r.p.m. in a GSA rotor, Sorvall RC5C centrifuge. They were then resuspended in 5 ml TE (10 mM-Tris, pH 8.0, 1 mM-EDTA) buffer with 25% (w/v) sucrose. Lysozyme (3 mg ml^{-1}) was added, followed, after incubation for 30 min at 37°C , by proteinase K (0.2 mg ml^{-1}) and sodium dodecyl sulphate (SDS; 0.5%, w/v, final concentration), with incubation at 37°C for a further 30 min. The SDS concentration was then increased to 1%. If complete lysis, as judged visually by clearing of cell suspension and increased viscosity, was not achieved, the cells were heated at 55°C until complete lysis was observed. The lysate was deproteinated with isoamyl alcohol/chloro-

Table 1. *Peptostreptococcus* strains (clinical isolates and ATCC strains) used in this study and their susceptibility to penicillin G

Species	LCDC/ATCC strain designation	Strain no. in this study	MIC (mg l^{-1})*
<i>P. anaerobius</i>	A18	P1	1.0
	781233	P2	0.125
	1981-75	P3	0.125
	ATCC 27337		NT
<i>P. asaccharolyticus</i>	A133	PA1	0.063
	AN2-413A	PA2	0.063
	AN2-419	PA3	0.063
	AN2-434C	PA4	0.063
	821758	PA5	0.063
	83-169	PA6	0.25
	ATCC 14963		NT
<i>P. magnus</i>	A95	PM1	0.063
	791273	PM2	0.125
	AN2-970	PM3	0.063
	ATCC 15794		NT
<i>P. micros</i>	A53	PMI1	0.063
	A120	PMI2	0.063
	ATCC33270		NT
<i>P. prevotii</i>	A129	PP1	0.25
	83-125	PP2	0.063
	801478	PP3	0.063
	811556	PP4	0.063
	831883	PP5	0.5
	AN2-253	PP6	0.063
	ATCC 9321		NT
<i>P. indolicus</i>	ATCC 29427		NT
<i>P. productus</i>	ATCC 27340		NT
<i>P. tetradius</i>	ATCC 35098		NT

* MIC was determined using the agar dilution method as described previously (Ng & Dillon, 1989). NT, Not tested.

form (1:24, v/v) extractions and then 0.1 vol. 3 M-sodium acetate was added prior to precipitation with 95% (v/v) ethanol at -20°C . The DNA was further purified on an ethidium bromide/caesium chloride gradient (Sambrook *et al.*, 1989). The purified DNA was resuspended and stored in $0.1 \times \text{SSC}$ (Sambrook *et al.*, 1989) at 4°C .

Cloning of rRNA genes from *P. anaerobius*. DNA fragments from *Hind*III digestion of *P. anaerobius* ATCC 27337 chromosomal DNA were cloned into the *Hind*III site of pUC9, and transformed into *E. coli* JM83. To confirm that the recombinant clones carried *P. anaerobius* chromosomal DNA, the DNA used for cloning was end-labelled with [γ - ^{32}P]ATP and hybridized to *P. anaerobius* ATCC 27337 using dot-blot hybridization. The positive controls for the dot-blot hybridization included *P. anaerobius* clinical isolates (P1, P2 and P3) and the negative controls included *E. coli* JM83 with pUC9 and the ATCC strains of the other seven *Peptostreptococcus* spp. listed in Table 1. Clones were screened for sequences of rRNA genes (*rrn*) by hybridization with [γ - ^{32}P]ATP end-labelled 16S and 23S rRNA from *E. coli* (Boehringer Mannheim, BMC, Canada). Plasmid DNA was prepared by mini-alkali/SDS lysis (Birnbom & Doly, 1979), subjected to electrophoresis, and transferred to a nylon membrane (Amersham) (Southern, 1975). The three clones (pLK16, pLK51 and pLK54) containing *rrn* operon sequences were further characterized by hybridization with three ^{32}P end-labelled oligonucleotides containing universal prokaryotic 16S

rRNA sequences (BMC) that are complementary to positions 519–536 (primer 1), 907–926 (primer 2) and 1392–1406 (primer 3) of 16S rRNA of *E. coli* (Lane *et al.*, 1975). The sizing of inserts and restriction endonuclease analysis were carried out by agarose gel electrophoresis and using λ *Hind*III digests and 1 kb ladder (BRL, BRL/Gibco Canada) as molecular size markers.

DNA probes and labelling for rRNA genes. To determine the RFLPs of rRNA genes, three probes were compared. Probe A was a *Hind*III fragment from plasmid pLK54 containing a 16S rRNA gene sequence of *P. anaerobius* ATCC 27337. Probe B comprised the 7.2 kb *Bam*HI–*Pst*I fragment from plasmid pKK3535 which contained the entire *rrn* operon of *E. coli* (Brosius *et al.*, 1981). This fragment was digested with *Eco*RI before labelling because preliminary experiments showed that the 7.2 kb *Bam*HI–*Pst*I fragment labelled by random priming did not hybridize to most of the restriction fragments which hybridized to probe A or C, even at the low-stringency conditions. This problem was overcome by restriction of the 7.2 kb fragment with *Eco*RI to produce smaller fragments before labelling. Probe C was a mixture of 16S and 23S rRNA from *E. coli* (BMC). Specific fragments from plasmids pLK54 and pKK3535 were extracted from low-melting-point agarose (BRL). Both probes A and B were labelled non-radiometrically (BMC-DIG) with a random priming labelling kit purchased from BMC. rRNA (probe C) was end-labelled with [γ - 32 P]ATP (Sambrook *et al.*, 1989). In our hands, rRNA probes labelled non-radiometrically using the end-tailing method with terminal transferase (BRL) were not as sensitive as 32 P end-labelled probes.

Fingerprinting by RFLPs. Chromosomal DNA from *Peptostreptococcus* spp. was digested with *Hind*III and *Eco*RI. The digested DNAs were heated at 65 °C to release bound proteins and to prevent the annealing of sticky ends and then fractionated on a 0.8% or 0.7% agarose gel in Tris/acetate buffer (pH 7.8) at 50 mA for 16 h to obtain the restriction endonuclease profiles. The fractionated DNA in agarose gel was visualized by ethidium bromide staining and photographed under UV light. The restriction endonuclease profile determination required numerous agarose gel electrophoreses because the resolution of the large number of fragments was critically dependent on the amount of DNA loaded per lane.

Fractionated genomic DNA in an agarose gel was transferred to a nylon membrane (Amersham). The membrane-bound DNAs were hybridized with probes B and C at 50 °C and with probe A at 63 °C. For 32 P-labelled probe C, the hybridization solution contained 5 $\times$ SSC, 0.5% SDS and 5 $\times$ Denhardt's solution (Sambrook *et al.*, 1989). For non-radioactive probes A and B, the hybridization solution was prepared according to the instructions of the manufacturer (BMC). These hybridization conditions were determined by preliminary experiments which showed that probe A could be used under higher stringency than probes B and C. Although probe C (32 P-labelled 16S and 23S rRNA) hybridized well with *P. anaerobius* DNA at 63 °C, it failed to hybridize with DNA from other *Peptostreptococcus* spp. unless the hybridization temperature was lowered to 50 °C.

The sizes of the chromosomal DNA fragments which hybridized with the probes were determined by using λ DNA digested with *Hind*III, *Kpn*I and *Xho*I as molecular size markers. The mean sizes from at least two gels were obtained and recorded. For schematic representation of RFLPs, fragment sizes were transformed to a log₁₀ scale (Prolat *et al.*, 1990). This method minimized the error in sizing large fragments with low mobility in 0.8% agarose gels and also provided easier visual comparison of fragments due to the non-linear mobility of various size fragments in agarose gel electrophoresis. The variation in the intensities of bands due to either difference in the degree of homology between the probe and the target DNA or the presence of doublets not resolved in agarose gel will not be observed in the schematic representation of RFLPs.

Results

Cloning and identification of 16S rRNA from *P. anaerobius*

Three of 67 recombinant plasmids, designated pLK16, pLK51 and pLK54, contained inserts that hybridized with a mixture of 16S and 23S rRNA (probe C). Labelled chromosomal DNA of *P. anaerobius* ATCC 27337 hybridized with the positive controls of *P. anaerobius* strains but did not hybridize with the negative controls of *E. coli* and the other seven *Peptostreptococcus* spp. This probe also hybridized with the insert DNA in plasmids pLK16, pLK51 and pLK54, thus confirming the origin of the insert. The size of the *Hind*III insert from both pLK16 and pLK51 was 0.84 kb and that of the insert from pLK54 was 0.98 kb. The three *Hind*III inserts also cross-hybridized with each other, indicating that they contained similar sequences or gene(s).

The clones were hybridized with primers 1 to 3 to establish which 16S rRNA conserved regions they contained. The *Hind*III inserts from pLK16, pLK51 and pLK54 hybridized with primer 3 (see Fig. 1, lanes F–H); none of them hybridized with primer 1, and only pLK54 hybridized with primer 2. Thus, all three plasmids contained partial 16S rRNA sequence and pLK54 carried more 16S rRNA sequences than the smaller plasmids (data not shown). From the coordinates of *E. coli* 16S rRNA sequences, pLK54 was estimated to contain about half of the 16S rRNA gene.

Restriction endonuclease analysis of pLK16, pLK51 and pLK54 showed that each of the *Hind*III inserts contained a single *Sma*I site. Each of the 0.84 kb *Hind*III inserts from pLK16 and pLK51 comprised a 0.45 kb and a 0.39 kb *Sma*I fragment, while the 0.98 kb insert from pLK54 comprised 0.59 and 0.39 kb *Sma*I fragments (see Fig. 2). There were no internal sites within the *Hind*III inserts in these clones for endonucleases *Acc*I, *Bam*HI, *Bgl*II, *Bst*EII, *Eco*RI, *Hind*II, *Sal*I, *Sst*I, *Sst*II and *Tth*III. Therefore, the restriction endonuclease sites of the 16S rRNA gene of *P. anaerobius* ATCC 27337 are different from those of the 16S rRNA gene of *E. coli* in pKK3535, which contains *Bst*EII, *Eco*RI, *Sal*I, *Sst*II and *Tth*III sites besides the *Sma*I site (Brosius *et al.*, 1981). From the restriction endonuclease analysis data, it was concluded that the clones pLK54 and pLK16 were derived from two different *rrn* operons on the same chromosome of *P. anaerobius* ATCC 27337 but pLK16 and pLK51 were probably from the same *rrn* operon. Plasmid pLK54 was selected as one of the probes for the determination of RFLPs of *rrn* operons of *Peptostreptococcus* isolates because it contained more 16S rRNA sequence.

Fig. 1. Identification of 16S rRNA sequences in recombinant plasmids. The *Hind*III inserts in pLK16, pLK51 and pLK54 in 1% agarose gel (lanes A–E) were transferred to nylon membrane and hybridized with 32 P end-labelled primer 3 which contained consensus *E. coli* 16S rRNA sequences (lanes F–I). Lanes: A, λ *Hind*III markers (23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb); B and I, pUC9 digested with *Hind*III; C and H, 0.84 kb *Hind*III fragment of pLK16; D and G, 0.84 kb *Hind*III fragments of pLK51; E and F, 0.98 kb *Hind*III fragment of pLK54.

Chromosomal DNA fingerprinting by RFLP

The fragment sizes of DNA fragments generated by *Hind*III digestion of the eight ATCC strains as well as the 20 clinical isolates differed. *Hind*III digestion of *P. indolicus* ATCC 29427, *P. tetradius* ATCC 35098 and *P. productus* ATCC 27340 produced fragments greater than 30 kb in size while digestion of the other species with *Hind*III generated fragments smaller than 23 kb. *Hind*III digestion of *P. prevotii* ATCC 9321 produced the smallest fragments (less than 10 kb) among the *Peptostreptococcus* isolates studied (data not shown). The *Eco*RI fragments resolved in 0.8% agarose gels for all the ATCC strains were larger than those obtained with *Hind*III digestion. Although the DNA fragments ranging in size between 3 and 30 kb resolved well in 0.8% agarose in TA buffer, the resolution of smaller fragments (less than 3 kb) was poor due to diffusion of the DNA. Therefore, differences between strains were only noted for the larger fragments and complete restriction endonuclease profiles of the

Fig. 2. Agarose gel electrophoresis of *Hind*III/*Sma*I-restricted plasmid DNA. Lanes: A, pLK54; B, pLK51; C, pLK16; D, 1 kb molecular size markers. The sizes of the *Hind*III/*Sma*I fragments are indicated on the left.

numerous *Hind*III or *Eco*RI were not recorded. This presented more difficulty in comparison of *Eco*RI restriction endonuclease profiles because they contained more large fragments than *Hind*III digests. The resolution of the *Hind*III and *Eco*RI fragments also depended on the amount of DNA loaded. Therefore, restriction endonuclease profiles will be of limited use for the differentiation of *Peptostreptococcus* isolates.

The determination of the RFLP pattern of *rrn* operons was evaluated as a tool for inter- and intra-species differentiation of *Peptostreptococcus* isolates. The non-radiometric labelling method showed better resolution than 32 P-labelled probes for fragments of similar size because the hybridization bands were sharper. For example, the 2.8 and 2.95 kb *Hind*III fragments of PA1 and PA3 (Fig. 3, lanes A and C) were observed as double bands when hybridized with non-radioactive probe B but

Fig. 3. Comparison of RFLPs generated by non-radioactive and radioactive probes. Two Southern blots of *P. asaccharolyticus* DNA digested with *Hind*III were hybridized with non-radioactive probe B (lanes A–C) and radioactive probe C (lanes D–F). Lanes: A and D, PA1; B and E, PA2; C and F, PA3. The sizes of λ *Hind*III markers are shown on the left and right. The bands in lane A were distorted during electrophoresis.

as a single band for the radioactive probe C (Fig. 3, lanes D and F).

The RFLPs of the 20 isolates from five *Peptostreptococcus* spp. produced by hybridization of either *Eco*RI- or *Hind*III-digested chromosomal DNA with probes A (*Hind*III insert of pLK54), B (*Bam*HI–*Pst*I fragment of pKK3535) or C (mixture of 16S and 23S rRNA) are summarized in the schematic representation in Figs 4 and 5, respectively. The sizes of the fragments reported in this study were means of two to four determinations.

The differences between runs for small fragments (under 5 kb) did not vary more than 0.5 kb; however, larger fragments (>10 kb) varied as much as 2–3 kb, corresponding to a millimetre difference in the measurement of mobility between gels. To overcome this problem, reference ATCC strains were included in each gel to allow accurate alignment of fragments of the same size. For the schematic representation (Figs 4 and 5) of the common bands, the sizes are the means of the bands from all the strains.

Interspecies differentiation of the five *Peptostreptococcus* spp. based on RFLP was observed by comparing the profiles obtained by using probe A, B or C. Four *P. anaerobius* strains had a common 6.1 kb *Eco*RI band (Fig. 4) and a common 3.5 kb *Hind*III band (Fig. 5) when probe A hybridized to the fractionated DNA. Species-specific bands were also observed for *P. asaccharolyticus*, *P. magnus* and *P. micros*, but not for *P. prevotii* (Figs 4 and 5). Although *P. prevotii* showed no intraspecies homogeneity, the RFLPs of *P. prevotii* strains were distinct from those of the other four species studied.

Intraspecies differentiation of strains was observed for all the *Peptostreptococcus* spp. studied. The four *P. anaerobius* strains had unique as well as common bands in their RFLP patterns generated by the hybridization of *Eco*RI fragments with probe A. For example, *P. anaerobius* P1 had a 7.65 kb *Eco*RI band instead of the 7.2 kb *Eco*RI band of *P. anaerobius* ATCC 27337 (Fig. 4). The size of some of the unique bands varied only slightly between strains (compare the 4.95 kb *Eco*RI band from the ATCC 27337 profile and the 5.1 and 5.0 kb bands of P2 and P3, respectively: Fig. 4). In order to observe this slight difference, the digested DNA from these strains was fractionated side by side in each gel. *P. asaccharolyticus* isolates PA1, PA3, PA5 and PA6 showed very closely related RFLP profiles (Figs 4 and 5). By contrast, the RFLPs of *P. asaccharolyticus* from our culture collection differed significantly from ATCC 14963 (Figs 4 and 5), making it difficult to establish a genetic relationship between the clinical isolates and the ATCC strain. Analysis of RFLP profiles from *P. magnus* also showed that strains from our collection were more closely related to each other than to *P. magnus* ATCC 15794. However, unlike the *P. asaccharolyticus* profiles, common bands were observed in both *Eco*RI- and *Hind*III-generated *P. magnus* RFLP profiles of clinical isolates and ATCC strain (Figs 4 and 5). As mentioned previously, *P. prevotii* strains contained only unique bands in their RFLP profiles (Figs 4 and 5).

Comparison of probes

The three rRNA probes used in this study differed in size, ranging from about 1 kb (probe A, partial 16S

Fig. 4. Schematic representation of RFLPs of rRNA genes generated by hybridization of *Eco*RI fragments of chromosomal DNA from *Peptostreptococcus* isolates with probe A, B or C. |, Band present in the RFLPs of all three probes (A, B and C); |, band present in RFLPs of probes A and B; |, band present in RFLPs of probes B and C; |, band found in the RFLP of probe B only. ▼, on top margins, indicates common bands present between strains within the same species. The sizes of the fragments in Figs 4 and 5 were means of at least two determinations as described in Results.

Fig. 5. Schematic representation of RFLPs of rRNA genes generated by hybridization of *Hind*III fragments of genomic DNA from *Pentostreptococcus* isolates with probe A, B or C. Symbols are as for Fig. 4.

rRNA gene from *P. anaerobius*) to 7.2 kb (probe B, the entire rRNA operon from *E. coli*). Therefore, the number of bands for each RFLP profile generated by using different-sized probes would vary depending on the location and number of *Hind*III or *Eco*RI sites within the rRNA genes. From our data (Figs 4 and 5), the number of bands generally increased with more rRNA sequences in the probe, indicating that the additional bands were due to the additional 23S rRNA in probe C or 23S and 5S rRNA sequences in probe B. In a few cases (8.5 kb *Hind*III fragment of *P. anaerobius*; 5.55 kb *Eco*RI fragment of *P. anaerobius* P1; and the 8.5 kb, 7.0 kb, 5.9 kb and 5.0 kb *Eco*RI fragments of *P. asaccharolyticus* PA4) additional bands for probe C as compared to probe B were observed but not included in Figs 4 and 5. The presence of these additional bands could be due either to the specificity of the probes which allowed hybridization of sequences with a higher percentage of mismatch or to differences in the sequences of rRNA from different operons of the *E. coli* chromosome. However, there are cases where no difference was observed between probes. For example, the same RFLPs were observed when the *Eco*RI fragments of *P. magnus* strains were hybridized with one of the three probes (Fig. 4).

Discussion

The determination of RFLPs based on rRNA genes has been previously applied to differentiate species and strains of mycoplasmas and revealed genotypic heterogeneity among *Acholeplasma laidlawii* and *Mycoplasma hominis* strains but homogeneity among *M. pneumoniae* strains (Yogev *et al.*, 1988). This study demonstrates that it is possible to use this method to differentiate *Peptostreptococcus* isolates also. By this technique, *P. anaerobius*, *P. magnus* and *P. micros* isolates could be grouped as separate species. This corresponded to results based on identification using conventional methods. Thus, chromosomal DNA fingerprinting by RFLP is potentially useful either for confirming the identity of phenotypically atypical strains of these three *Peptostreptococcus* spp. or for differentiating phenotypically similar *P. magnus* and *P. micros* isolates (Moore *et al.*, 1987). The RFLPs of *P. asaccharolyticus* showed that the clinical isolates of this species were more similar to each other than to the type strain *P. asaccharolyticus* ATCC 14963. This result agrees with DNA homology studies which showed that the clinical isolates were more homogeneous among themselves than to *P. asaccharolyticus* ATCC 14963 (Moore *et al.*, 1986). Thus, we conclude that, for the molecular typing of *Peptostreptococcus* isolates, the choice of ATCC 14963 as a reference strain is undesirable. The heterogeneity of the RFLPs based on *Eco*RI

and *Hind*III digests of chromosomal DNA from *P. prevotii* isolates suggested that this species probably comprised strains that were genetically more diverse than the other four species studied. This is supported by the relative heterogeneity of pre-formed enzyme patterns of *P. prevotii* (Murdoch *et al.*, 1988).

The chromosomal DNA of *Peptostreptococcus* species contained many *Hind*III and *Eco*RI sites. Because the fragments generated were small they could be resolved without pulsed-field electrophoresis apparatus. However, the numerous fragments produced by these digestions presented problems in recording and comparison. Thus, the use of restriction endonuclease profiles alone cannot be recommended for use in molecular typing of *Peptostreptococcus* isolates. Hybridization of *Eco*RI and *Hind*III fragments with probes for *rrn* operons reduced the number of fragments, thereby making comparisons easier. The determination of RFLP patterns is therefore, a better method than restriction endonuclease analysis for molecular typing of peptostreptococci.

Although all the three probes produced useful RFLPs for the differentiation of peptostreptococci, both probes A (*Hind*III fragment from pLK54) and B (7.2 kb *Bam*HI-*Pst*I fragment of pKK3535), in contrast to probe C (16S and 23S rRNA from *E. coli*), provided better resolution of fragments of related sizes using the non-radiometric labelling method as compared to the radiometric method. Probe A is probably a better choice than probe B for further use in peptostreptococcal typing because it contained cloned 16S rRNA gene sequences from *P. anaerobius* which were more similar to the 16S rRNA gene sequences from other peptostreptococci than to those from *E. coli*. This was reflected by the ability of probe A to hybridize with DNA fragments containing 16S rRNA gene sequences at higher stringency conditions (63 °C). Furthermore, probe A did not require an extra *Eco*RI digest of fragments prior to labelling, a step required for probe B. As a result, it was less dependent on hybridization conditions or on the actual size of labelled probes to obtain reproducible results.

This study shows that the determination of RFLPs of *rrn* operons is a potentially useful method for the identification and differentiation of *Peptostreptococcus* species, especially when used in conjunction with other taxonomic methods. It would be interesting to compare this method with other identification schemes such as pre-formed enzyme detection in the distinction of *P. magnus* and *P. micros* (Murdoch *et al.*, 1988). However, one limitation of the RFLP method for taxonomic or identification purposes is the lack of standardized methods for recording RFLPs (Grimont & Grimont, 1986) for the establishment of databanks. This also presents a problem in comparing data from different gels

or between laboratories. Therefore, RFLP determination may be limited to differentiating small numbers of bacterial strains such as those involved in outbreaks or from patients with treatment failure or relapses. For the determination of RFLP of *rrn* operons of *Peptostreptococcus* species, the cloned partial 16S rRNA *Hind*III fragment was more efficient than probes containing *E. coli* rRNA sequences.

We thank Dr J. Brosius for supplying pKK3535 and D. Auger and C. Galvis for their technical assistance.

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