MOLECULAR CHARACTERISATION

Heterogeneity within the gram-positive anaerobic cocci demonstrated by analysis of 16S–23S intergenic ribosomal RNA polymorphisms

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Peptostreptococci are gram-positive, strictly anaerobic bacteria which, although regarded as members of the commensal human microflora, are also frequently isolated from sites of clinical infection. The study of this diverse group of opportunist pathogens has been hindered by an inadequate taxonomy and the lack of a valid identification scheme. Recent re-classification of the Peptostreptococcus family into five distinct genus groups has helped to clarify the situation. However, this has been on the basis of 16S rRNA sequence determinations, which are both time-consuming and expensive. The aim of the present study was to evaluate the use of PCR-amplified ribosomal DNA spacer polymorphisms for the rapid differentiation of the currently recognised taxa within the group of anaerobic gram-positive cocci. A collection comprising 19 reference strains with representatives of each of the 15 species, two close relatives and two of the well-characterised groups, together with 38 test strains was studied. All strains were identified to species group level by phenotypic means. Amplification of the 16S–23S intergenic spacer region (ISR) with universal primers produced distinct banding patterns for all the 19 reference strains and the patterns could be differentiated easily visually. However, of the 38 test strains, less than half could be speciated from ISR analysis alone. Only five groups produced correlating banding patterns for all members tested (*Peptoniphilus lacrimalis*, *P. ivorii*, *Anaerococcus octavius*, *Peptostreptococcus anaerobius* and *Micromonas micros*). For other species, either the type strain differed significantly from other species members (e.g., *A. hydrogenalis*) or there appeared to be considerable intra-species variation (e.g., *A. vaginalis*). Partial 16S rRNA gene sequences for the 'trisimilis' and 'βGAL' groups showed that both are most closely related to the *Anaerococcus* group. This work highlights the heterogeneous nature of a number of *Peptostreptococcus* species and hence the need for still further revision of the taxonomy of this important group of pathogens.

Introduction

Gram-positive anaerobic cocci (GPAC) are widely distributed as part of the normal flora of skin and mucosal surfaces [1] and are most commonly known as the peptostreptococci. However, they may also be regarded as opportunist pathogens as they are frequently isolated from local and systemic infections and have been reported as comprising approximately one quarter of all isolates from anaerobic infection [2–5].

In the majority of diagnostic laboratories, clinical isolates of GPAC are identified only to genus level. However, correct identification of the individual species is essential to determine whether individual taxonomic groups are associated with specific body sites or disease conditions and hence elucidate the pathological importance of individual species. Previous studies of their significance in disease have been hampered by an inadequate taxonomy and the consequent lack of valid identification methods. More recently, this problem has been addressed and a logical identification scheme has been proposed [6], supported by chemotaxonomic data from RNA/DNA analysis, pyrolysis mass spectrometry and cellular carbohydrate analysis and phylogenetic

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data from ribosomal (r)RNA analysis [7–10]. Moreover, this has also led to a reclassification of the group previously known as *Peptostreptococcus* to the effect that only *Pstr. anaerobius* now remains within this genus [11], being the only species within the GPAC closely related to the clostridia [9]. The GPAC include several further genera not included in this study, such as *Sarcina*, *Coprococcus* and *Atopobium*. However, for the purposes of this paper, the names GPAC and peptostreptococci will both be used to describe the strains formerly known as *Peptostreptococcus*, although neither term is now totally correct.

There are currently 15 species belonging to the peptostreptococci, including three newly proposed species, *Peptoniphilus harei*, *P. octavius* and *P. ivorii* [12]. Two other well-defined groups have also been described – the ‘ β GAL’ (β -galactosidase) strains and the ‘*trisimilis*’ group. It has been suggested that these two groups merit species status [6]. However, the taxonomy has not been completely resolved and some of the established groups, e.g., *P. asaccharolyticus*, *P. vaginalis* and the *prevotii/tetradis* group, are known to be heterogeneous. On the basis of 16S rRNA sequence analysis five distinct phylogenetic groups have been identified [9]. These include *Pstr. anaerobius* and two newly proposed genera for the second phylogenetic group, *Micromonas* and *Finegoldia* [13]. Recently, Ezaki *et al.* [14] proposed three new genera for the remaining three groups (see Table 1), namely *Anaerococcus*, *Peptoni-*

philus and *Gallicola* based on peptidoglycan structure and biochemical traits.

At the present time, the differentiation of the many taxonomic groups within the GPAC relies on the study of phenotypic properties including volatile fatty acid profiles, carbohydrate fermentation tests and the analysis of pre-formed enzyme profiles (PEPs) [6]. In some cases, the examination of typical colonial and cellular morphologies is also required. These conventional methods are time-consuming, labour-intensive and restricted to laboratories that possess the appropriate expertise and equipment.

The analysis of rRNA and, in particular, the intergenic spacer regions (ISR) has been used extensively for the characterisation of a wide range of organisms at both the inter-species and intra-species level [15–18]. In the past, a ribotyping approach has been used successfully in the analysis of five species within the genus previously known as ‘*Peptostreptococcus*’ [19]. However, to date, studies do not appear to have examined the use of genotypic methods for the rapid differentiation of members of the GPAC, including the newly described taxonomic groups. The aim of the current study was to evaluate the use of intergenic rRNA polymorphisms for the rapid differentiation of this diverse group of anaerobic bacteria and to determine whether it represents a suitable alternative or supplement to phenotypic speciation.

Table 1. Type strains and clinical isolates representing the 19 currently recognised species or groups in or closely related to the “*Peptostreptococcus*” genus

*Group	Species	†Strain no.	Status	EMBL sequence accession no.	Previous designation *(RNA group)
I Saccharolytic, butyrate producer, lysine as peptidoglycan diaminoacid	<i>Peptoniphilus lacrimalis</i>	DSM 7455	Type strain	D14141	2
	<i>P. indolicus</i>	DSM 20464	Type strain	D14147	2
	<i>P. harei</i>	DSM 10020	Type strain	Y07839	
	<i>P. asaccharolyticus</i>	DSM 20463	Type strain	D14138	2
	<i>P. asaccharolyticus</i>	‡R7131	Clinical isolate	None	2
	<i>P. ivorii</i>	DSM 10022	Type strain	Y07840	
II Non-saccharolytic, butyrate producer, ornithine as peptidoglycan diaminoacid	<i>Anaerococcus vaginalis</i>	DSM 7457	Type strain	D14146	3
	<i>A. hydrogenalis</i>	DSM 7454	Type strain	D14140	3
	<i>A. prevotii</i>	DSM 20548	Type strain	D14139	3
	<i>A. tetradis</i>	DSM 2951	Type strain	D14142	3
	<i>A. lactolyticus</i>	DSM 7456	Type strain	D14154	3
	<i>A. octavius</i>	DSM11663	Type strain	Y07841	
	‘ β GAL’ group	‡R7149	Clinical isolate	This study	
	‘ <i>Trisimilis</i> ’ group	‡R3262	Clinical isolate	This study	
III Butyrate negative, variable peptidoglycan structures	<i>Gallicola barnesae</i>	DSM 3244	Type species	AB038361	6
	<i>Finegoldia magna</i>	DSM 20470	Type strain	D14149	5
	<i>Micromonas micros</i>	GIFU 7701	Clinical isolate	D14143	4
	<i>Peptococcus niger</i>	DSM 20475	Type species	X55797	
	<i>Peptostreptococcus anaerobius</i>	DSM 2949	Type strain	L04168	1
	<i>Ruminococcus productus</i>	DSM 2950	Type strain	D14144	8

**Peptostreptococcus* new group designations based on Ezaki *et al.* [14] and previous RNA group designations based on Li *et al.* [9].

†DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; GIFU, Gifu University School of Medicine, Gifu, Japan.

‡Designations of the PHLS Anaerobic Reference Unit, Cardiff.

Materials and methods

Strains and culture conditions

Type strains ($n = 16$) and clinical isolates ($n = 3$) representing 17 of the currently recognised species or groups in the former *Peptostreptococcus* genus in addition to two closely related strains *Peptococcus niger* and *Ruminococcus productus* were from the collection of the PHLS Anaerobe Reference Unit (ARU), or were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Table 1). Another 38 consecutive clinical GPAC isolates referred to the ARU for identification were also examined by ISR analysis in a single blind test. Strains were cultured on Fastidious Anaerobe Agar (FAA; LabM, Bury) supplemented with horse blood 5% v/v and incubated under anaerobic conditions (CO_2 10%, H_2 10% and N_2 80%) at 37°C. Briefly, species identification was confirmed as follows: strains were examined according to gram-stain morphology, growth characteristics, carbohydrate fermentation and volatile fatty acid profiles by gas-liquid chromatography [20–22]. Strains were also analysed for the presence of a range of preformed enzymes by the rapid ID 32A identification kit (bioMérieux, Lyon, France) according to the manufacturer's instructions. The bacterial suspension for inoculation of the commercial kit was obtained by harvesting 48-h anaerobic growth from the surface of Columbia Blood Agar plates (LabM) to produce a bacterial suspension of McFarland standard 4 turbidity. Differential characteristics for identification were applied as summarised by Murdoch [6].

PCR amplification of 16S–23S rDNA intergenic spacer region

Nucleic acid was prepared by a rapid technique described by de Lamballerie *et al.* [23]. A bacterial suspension of a 48-h culture harvested from the surface of FAA plates was made to MacFarland standard 4; 100 μl of Chelex-100 ion-exchange resin (Biorad Laboratories, Hemel Hempstead) 5% w/v was added and heated at 96°C for 12 min. Samples were then centrifuged at 12 000 g for 15 min and 0.5 μl of the supernate was used as template for the PCR reactions. The extracted genomic DNA mixture was stored at –20°C until required.

Genomic DNA was amplified by PCR with primers complementary to bases 1390–1408 (numbering based on *Escherichia coli* rRNA sequence) of the 16S rRNA (primer A; 5' TTGTACACACCGCCCGTCA 3') and bases 191–206 of the 23S rRNA (primer B; 5' GGTACCTTAGATGTTTCAGTTC 3') [17]. The PCR mix contained 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl_2 , 0.2 mM each deoxynucleoside triphosphate (Roche, Lewes), 35 pmol of each primer and *Taq* polymerase (Promega, Southampton) 1.25 U. Negative controls contained water in place of template DNA. Amplification was performed with a programmable heating block (MJ Research PTC-100, GRI, Braintree) in 25- μl volumes under the following

conditions: an initial denaturation step at 95°C for 6 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were concentrated by heating at 75°C to one third the original volume before electrophoresis. PCR products (typically 10 μl of reaction mixture) were separated on agarose 3% w/v gel – NuSieve (Flowgen, Lichfield): Agarose (Biolone, London) 1:1 – in 1 $\times$ Tris-borate-EDTA buffer, pH 7.5, for 3–4 h at 60 V. Mol. wt markers (1 kb plus; Invitrogen, Paisley) were run in the outer two lanes of each gel. Gels were stained with ethidium bromide by standard procedures and a computerised gel image was recorded with the Gel Doc 1000 system and associated software (BioRad). Each strain was subjected to PCR and profile analysis on at least two separate occasions.

PCR amplification of 16S rRNA genes for cloning and sequencing

16S rRNA amplification of strains belonging to the 'βGAL' and 'trisimilis' groups was performed with PCR primers 63f and 1387r as described by Marchesi *et al.* [24]. PCR products were ligated into the pCR2.1 TOPO vector (Invitrogen) according to the manufacturer's instructions and transformed into Top10 competent *Escherichia coli* cells (Invitrogen). Blue-white screening of transformants was done on Luria-Bertani agar containing kanamycin 50 mg/L and top spread with 40 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) 20 g/L. DNA for sequencing was prepared from clones with Wizard plus SV minipreps (Promega) and two clones from each strain were sequenced. Both strands were sequenced on an automated laser fluorescence sequencer (ABI 377; PE Applied Biosystems) with M13 primers giving double coverage of >1 kb of 16S rDNA for phylogenetic analysis. The 16S rRNA gene sequences for the 'βGAL' and 'trisimilis' groups were compared with those of the EMBL database (release 69) [25] by FASTA3 [26, 27] at the European Bioinformatics Institute (<http://www.ebi.ac.uk>) and with those of the Ribosomal Database Project (<http://www.rdp.cme.msu.edu>) by SEQUENCE MATCH [28] to identify closely related sequences. 16S rRNA gene sequence alignments were done with CLUSTALW [29]. Evolutionary distances were calculated with the Jukes-Cantor algorithm [30] and phylogenetic trees were determined by the neighbour-joining method [31] with TREECON for Windows [32].

Results

The species identity of each reference and test strain was determined by the phenotypic methods described previously [33] (results not shown). Amplification of the ribosomal intergenic spacer regions yielded rela-

tively simple banding patterns of one to four bands for each test strain (Fig. 1a). Distinct profiles were obtained for most of the 19 taxonomic groups tested (summarised in Fig. 1b) except for those of *A. lactolyticus* and the 'βGAL' group strain R7149 which were very similar. However, the use of appropriate reference markers allowed all banding patterns to be easily discernible by eye. Banding patterns were found to be reproducible in repeat tests on at least two

occasions and, for two strains, in repeated tri-weekly subculture for 2 months (results not shown). However, although each reference strain gave a unique banding pattern, the test strains could not all reliably be distinguished by this method. Of the 38 test strains examined blind, less than half (39%) could be speciated from ISR analysis alone (Table 2). Only five groups produced identical banding patterns for all members tested – *P. lacrimalis*, *P. ivorii*, *A. octavius*, *Pstr.*

Fig. 1. (a) Profiles generated by PCR amplification of the 16S–23S rRNA intergenic spacer region from type strains of the former *Peptostreptococcus* genus. Lanes L, mol. wt marker sizes in bp. The numbered lanes are **1**, *A. vaginalis* (DSM 7457^T); **2**, *A. hydrogenalis* (DSM 7454^T); **3**, *A. prevotii* (DSM 20548^T); **4**, *A. tetradius* (DSM 2951^T); **5**, *A. lactolyticus* (DSM 7456^T); **6**, *A. octavius* (DSM 11663^T); **7**, 'trisimilis' group (R3262); **8**, 'βGAL' group (R7149); **9**, *Peptoniphilus lacrimalis* (DSM 7455^T); **10**, *P. indolicus* (DSM 20464^T); **11**, *P. harei* (DSM 10020^T); **12**, *P. asaccharolyticus* (R7131); **13**, *P. asaccharolyticus* (DSM 20463^T); **14**, *P. ivorii* (DSM 10022^T); **15**, *Gallicola barnesae* (DSM 3244^T); **16**, *Finegoldia magna* (DSM 20470^T); **17**, *M. micros* (GIFU 7701); **18**, *Peptococcus niger* (DSM 20475^T); **19**, *Peptostreptococcus anaerobius* (DSM 2949^T); **20**, *Ruminococcus productus* (DSM 2950^T). **(b)** Schematic representation of the profiles shown in (a).

Table 2. Comparison of 16S–23S intergenic spacer region (ISR) profiles of GPAC strains of the same species

Strains	Test organisms of the same species producing identical ISR analysis profiles	Number of test strains with identical ISR profiles	Total number of strains of the same species tested
<i>M. micros</i>	GIFU 7701, *R13385, *R13133	3	3
<i>Pstr. anaerobius</i>	DSM 2949, R12911, R12913	3	3
<i>P. ivorii</i>	DSM 10022, R7067, R11403	3	3
<i>P. lacrimalis</i>	DSM 7455, R4755	2	2
<i>P. asaccharolyticus/harei</i>		0	6
<i>A. vaginalis</i>		0	5
<i>A. hydrogenalis</i>	R7114, R7112	2	3
<i>A. prevotii/teradius</i>		0	5
<i>A. tetradius</i>		0	5
<i>A. octavius</i>	DSM 11663, R8862	2	2
' β GAL' group	R7149, R2679, R5366, R5563, R6790	5	7
'Trisimilis' group		0	2
<i>Finegoldia magna</i>		0	3
<i>Ruminococcus productus</i>		0	2
Total	20	20	51

For abbreviations see footnote of Table 1.

*These two strains occasionally produced a second larger fragment not produced by the type strain.

Fig. 2. Profiles generated by PCR amplification of 16S–23S rRNA intergenic spacer region from type and test strains of the *Peptostreptococcus* genus. *M. micros* (lanes 1–3), *P. anaerobius* (lanes 4–6), *P. ivorii* (lanes 7–9), *P. lacrimalis* (lanes 10–11) and *A. octavius* (lanes 12 and 13). Lanes L, mol. wt marker sizes in bp. The numbered lanes are as follows: 1, GIFU 7701; 2, *R13385; 3, *R11403; 4, DSM 2949; 5, R12911; 6, R12913; 7, DSM 10022^T; 8, R7067; 9, R11403; 10, DSM7455; 12, DSM11663; 13, R8862. *These two strains occasionally produced a second larger fragment not produced by the type strain.

anaerobius and *Micromonas micros* (Fig. 2) although both *M. micros* test strains occasionally produced a second larger fragment not produced by the type strain. For other species, either the type strain differed significantly from other species members (e.g., *A. hydrogenalis*) or there appeared to be considerable intra-species variation (e.g., *A. vaginalis*). For example, all five *A. vaginalis* strains tested gave different and, therefore, unique banding patterns (Fig. 3) whereas in contrast, the majority (5/7) of test organisms from the 'βGAL' group gave identical banding patterns (Fig. 3).

16S rRNA gene analysis of two 'βGAL' and two 'trisimilis' group strains confirmed their assignment to the peptostreptococci. Percentage sequence identity was >92% over 1346 nucleotides for all four sequences. About 900 bp of sequence was used for the multiple alignment. The dendrogram (Fig. 4) containing all the currently available sequences for members of the peptostreptococci also shows that both groups are most closely related to the newly defined group II *Anaerococcus* sp. [14]. Surprisingly, members from the same group (i.e., 'trisimilis' or 'βGAL' as identified phenotypically) although closely related from 16S rRNA analysis, did not fall within exactly the same branch of the tree, suggesting a greater degree of within-group divergence at the DNA level than originally thought. This is also seen in ISR analysis of these strains

where differing profiles were produced for both of the 'βGAL' and 'trisimilis' strains sequenced.

Discussion

The results of the present study demonstrate that it is possible to differentiate certain members of the 'Peptostreptococcus' genus by analysis of 16S–23S ribosomal DNA ISR. It is interesting that almost all the peptostreptococci yielded spacers of different lengths, although this has been shown previously for other species such as *Pseudomonas cepacia* [34] and members of the *Streptococcus milleri* group [35]. Simple and distinct profiles were obtained for each of the 19 taxa tested and profiles could be easily discriminated visually without the need for computer-assisted analysis. The method, which does not require a lengthy DNA extraction step was found to be rapid with very little 'hands-on' time required and profiles were produced in 6 h from a pure culture of the test organism. In contrast, approximately 48 h were needed to perform the phenotypic methods required for differentiation. Hence, while the 'hands-on' time for the two methods was approximately the same (c. 80 min), the time per strain if multiple isolates were tested was more favourable for the genotypic method.

Fig. 3. Profiles generated by PCR amplification of 16S–23S rRNA intergenic spacer region from type and test strains of the *Peptostreptococcus* genus. *A. vaginalis* (lanes 1–5) and the 'βGAL' group (lanes 6–12). Lanes L, mol. wt marker sizes in bp. The numbered lanes are as follows: 1, DSM 7457^T; 2, R3238; 3, R5152; 4, R5337; 5, R6041; 6, R7149; 7, R2679; 8, R4989; 9, R5366; 10, R5563; 11, R5797; 12, R6790.

Fig. 4. Dendrogram showing the gram-positive anaerobic cocci based on 16S rRNA sequences. Group designations are as described by Ezaki *et al.* [14]. Boxed strains are those sequenced in this study. ^T indicates type strain. Sequence accession numbers are given before the strain name. Bootstrap analysis was performed 1000 times, the results of which are indicated in the figure.

Moreover, the costs for the phenotypic method were markedly greater. In addition, species identification by conventional phenotypic methods relies on expensive, specialist equipment such as gas-liquid chromatography, whereas genotypic-based differentiation may be carried out in a laboratory with basic molecular biology facilities.

A notable advantage of the genotypic method is the ability to differentiate easily between *P. asaccharolyticus* and *P. harei*, which is difficult by phenotypic methods. These species yield similar PEPs and the same volatile fatty acid and carbohydrate fermentation profiles [6]. Hence, conventional differentiation relies on slightly differing cellular and colonial morphologies, which can be an unsatisfactory basis for identification. Fig. 1 shows that the banding pattern of *P. harei* and that of the *P. asaccharolyticus* type strain (indole positive) are very different from each other (producing one and three bands, respectively). In contrast, the banding pattern of *P. harei* was very similar to that of an indole-negative *P. asaccharolyticus* strain (ARU7131) routinely used both for confirming identification of phenotypically atypical strains and for differentiation of phenotypically similar strains. Both strains produced only one band of similar size, the difference between the bands being only just discernible by eye.

It has been suggested that the relatively low 16S rRNA sequence homologies and the results of numerical taxonomic studies are such that the different species of '*Peptostreptococcus*' are, in fact, more likely to represent different genera [7–10]. The *prevotii/tetradius* group is particularly interesting, as strains assigned to this group have been shown to cluster close together in genotypic studies [7, 12]. However, phenotypically they represent a diverse group with a range of PEP profiles [36]. Indeed, in the present study the banding patterns were widely different for eight test strains and two type strains examined (Table 2). There is a particular need to further investigate this group as they represent one of the more frequently isolated taxa. Analysis of ISR polymorphisms generated by restriction endonuclease digestion might prove useful in this respect. Regardless of the eventual taxonomic designations of these organisms, the implied and documented heterogeneity of the current taxa highlights the need for further study of the diversity within the groups [19]. Only then will it be possible to validly investigate the correlation between taxonomically distinct groups and the site of infection in relation to clinical significance.

This is the first publication of 16S rRNA sequences of '*trisimilis*' and ' β GAL' group strains. The results for ' β GAL' strain R7149 correlate well with those of Collins *et al.*, which placed two unnamed representative strains from the ' β GAL' group into the *Clostridium* cluster XIII [7] with both showing closest homology to *A. lactolyticus* as did strain R7149. In contrast, the second strain to be sequenced in this study, R4989, was

most closely related to *A. octavius* and from ISR analysis appeared to be atypical of the group as a whole. Furthermore, the two '*trisimilis*' strains R3262 and R5622 sequenced here for the first time appeared more divergent from one another than even the ' β GAL' group strains, showing closest homology to *A. tetradius* and *A. octavius*, respectively, despite producing identical ISR profiles.

The high pathogenic potential of members of the '*Peptostreptococcus*' genus has been documented [2–5]. The approach used in this study represents a valuable, rapid method for the differentiation of some of the '*Peptostreptococcus*' groups. Certainly it provides a rapid and useful adjunct to identification of these species, which are often not readily differentiated phenotypically. If the ISR method is validated by application to a much larger number of clinical isolates the approach could prove useful in a clinical laboratory and assist studies of the predictive value of the identification of the peptostreptococci. It may also ultimately prove most useful as a screening method to identify and select atypical strains for further study and sequencing.

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