Heterogeneity among 16S–23S rRNA intergenic spacers of species within the '*Streptococcus milleri* group'

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The 16S–23S rRNA intergenic spacer has been suggested as a suitable region of the bacterial genome from which to derive useful taxonomic information, particularly with regard to identification at the species level. To investigate this approach as an aid to the identification of the three species comprising the '*Streptococcus milleri* group' (SMG), the spacers of isolates of *Streptococcus intermedius*, *Streptococcus anginosus* and *Streptococcus constellatus* were amplified by PCR and length polymorphisms determined by agarose gel electrophoresis. Phenotypically atypical isolates which had been identified presumptively as belonging to these three species were also included. Spacers from two representatives of each spacer length found within the three SMG species were sequenced. 16S–23S rRNA intergenic spacer length polymorphisms allowed discrimination between *S. anginosus* (350 bp or 450 bp amplification product) and *S. constellatus* (380 bp amplification product), species that are difficult to differentiate phenotypically. *S. intermedius* (330 bp or 450 bp amplification product) and *S. anginosus* (350 bp or 450 bp amplification product) were not reliably distinguished by this method but are phenotypically distinct. Sequencing data demonstrated that the spacers had a central region of highly variable length flanked by conserved regions which included a single tRNA_{Ala} gene. Polymorphism in the length of the 16S–23S spacer determined by PCR provides a rapid and useful adjunct to strain identification for *S. anginosus* and *S. constellatus*, which are not readily differentiated phenotypically.

Keywords: *Streptococcus intermedius*, *Streptococcus anginosus*, *Streptococcus constellatus*, 16S–23S intergenic rRNA spacer, length polymorphism

INTRODUCTION

Comparative sequence analysis of small subunit rRNA genes is currently used to determine phylogenetic relationships between both closely and distantly related bacteria (Woese, 1987) and to derive species-specific DNA probes for strain identification (Stahl & Amann, 1991; Greisen *et al.*, 1994). Within the rRNA operon (*rrn*), the intergenic spacer separating the 16S and 23S rRNA genes has been suggested as a candidate region from which DNA probes

for identification and strain typing may be derived (Barry *et al.*, 1991; Uemori *et al.*, 1992; Kostman *et al.*, 1992; Gurtler, 1993). This region is involved in the processing of pre-rRNA (Young & Steitz, 1978) and may contain coding sequences for tRNA (Morgan *et al.*, 1977; Young *et al.*, 1979; Bacot & Reeves, 1991) but is otherwise considered to be largely non-functional, under minimal selective pressure and therefore likely to contain sequence variations from which taxonomically useful information might be obtained (Barry *et al.*, 1991). Indeed, sequence divergence between different species within a genus has already been reported (Barry *et al.*, 1991; Frothingham & Wilson, 1994; Uemori *et al.*, 1992). Conserved regions within the 16S and 23S genes can be used as targets for primers to amplify the intergenic spacer by PCR (i) in order to determine size polymorphisms on agarose gels

Abbreviations: SMG, '*Streptococcus milleri* group'; T, type strain.

The GenBank accession numbers for the nucleotide sequences reported in this paper are: L36931 [*S.ang*₄₅₀ (strain NCTC 10713^T)]; L36932 [*S.ang*₃₅₀ (isolate Q4039)]; L36933 [*S.con*₃₈₀ (strain NCDO 2226^T)]; L36934 [*S.int*₃₃₀ (strain NCDO 2227^T)]; L36935 [*S.int*₄₅₀ (isolate HW12)].

for use as species specific markers or (ii) for direct sequencing in order to determine the presence of oligonucleotide signatures suitable for primers to be used in a PCR-based identification approach (Barry *et al.*, 1991; McLaughlin *et al.*, 1993).

Streptococcus anginosus, *S. constellatus* and *S. intermedius* (often collectively referred to as the 'Streptococcus milleri group': SMG) (Whiley & Beighton, 1991) are common inhabitants of the human oral cavity (Whiley *et al.*, 1993) and are of clinical interest due to an association with oral abscesses and more serious purulent infections that may involve the major organs of the body (Gossling, 1988; Whiley *et al.*, 1992). Bentley *et al.* (1991) demonstrated by 16S rRNA sequence analysis that these three species are phylogenetically distinct taxa, grouped together and possessing a common line of descent. However, routine identification of these streptococci is currently only possible using a biochemical scheme that includes fluorogenic substrates for detecting preformed enzymes together with traditional bacteriological tests (Whiley *et al.*, 1990). This approach is particularly suitable for identifying isolates of *S. intermedius*, a phenotypically distinct species when compared with *S. anginosus* and *S. constellatus*. As noted by previous authors (Kilpper-Balz *et al.*, 1984) the latter two species are less readily distinguished from each other by phenotypic tests and present much more of a potential problem to the diagnostic microbiologist. At the present time discrimination between, and therefore identification of, *S. anginosus* and *S. constellatus*, although improved, would be greatly facilitated by a simple DNA-based test.

The aim of this study was to investigate the presence and basis of length polymorphisms within the 16S–23S rRNA intergenic spacers of *S. anginosus*, *S. constellatus* and *S. intermedius* and to determine whether exploitation of this region of the genome represents a suitable alternative or adjunct to the identification of SMG species by phenotypic testing.

METHODS

Bacterial strains. A total of 65 streptococci belonging to the SMG were examined: these had been identified previously as *S. intermedius* (21 isolates), *S. constellatus* (22 isolates) or *S. anginosus* (22 isolates) on the basis of whole genomic DNA–DNA hybridization (Whiley & Hardie, 1989), cluster analysis or using the biochemical scheme of Whiley *et al.* (1990). The strains examined included the type strains NCTC 10713^T (*S. anginosus*), NCDO 2226^T (*S. constellatus*) and NCDO 2227^T (*S. intermedius*). The biochemical profiles (biotypes) of the reference strains and clinical isolates examined in this study are shown in Table 1. Six of the *S. anginosus* isolates possessed a broader fermentation capability than most in being able to produce acid from mannitol (mann+) and raffinose (raff+). Isolates with the latter phenotype have been described before and are frequently isolated from the urogenital tract (Ruoff & Kunz, 1982). Also included amongst the above were two 'atypical-*S. constellatus*' isolates characterized by an inability to hydrolyse aesculin or arginine (biotype c11, isolate F436) or in not producing detectable levels of α -glucosidase or hyaluronidase (biotype c10, isolate FW73), four isolates presumptively identified as *S. intermedius* which did not produce detectable levels of sialidase (sial–) (biotype i5,

isolate 3206393; biotype i6, isolate 3206692; biotype i3, isolates 3209432 and I782) and two isolates of *S. anginosus* in which hyaluronidase activity was detected (biotype a9, isolate AGP37W and biotype i10, isolate MS101).

All cultures were incubated at 37 °C in an anaerobic atmosphere of H₂/CO₂/N₂ (20:10:70, by vol.). Cultivation was carried out in Streptococcus Sugar Base broth (SSB) containing 2% (w/v) proteose peptone (Oxoid), 0.5% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, 0.1% (w/v) Na₂HPO₄, pH 7.6, supplemented with 0.5% (w/v) glucose and 0.25% (w/v) NaHCO₃. Cells were grown in 3 ml cultures to late exponential phase (assessed visually) and harvested by centrifugation. Cells were washed and then resuspended in 0.1 ml TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Lysozyme was added to a final concentration of 5 mg ml⁻¹, and the mixture was incubated at 37 °C for 30 min. The cells were then lysed by adding 0.5 ml GES buffer (5 M guanidium thiocyanate, 0.1 M EDTA, pH 8.0, 0.5% w/v, *N*-laurylsarcosine) and incubated at room temperature for at least 10 min. After extraction with an equal volume of chloroform/isoamyl alcohol (24:1), the DNA was precipitated with 0.54 vols 2-propanol, resuspended, then precipitated with ethanol and finally resuspended in 0.1–0.2 ml TE buffer.

PCR. PCR was performed with GeneAmp PCR core reagents, 2.5 mM MgCl₂, and AmpliTaq polymerase (Perkin Elmer Cetus). Primers used for determining size polymorphisms were A: 5'-GAAGTCGTAACAAGGTA(AG)CCGT-3' and B: 5'-TGCCAAGGCATCCACC-3' (nucleotides in parentheses are degenerate nucleotide positions). The 5'-end of primer A is located at position 1491 of the 16S rRNA gene and the 5'-end of primer B 23 bp downstream from the 5'-end of the 23S rRNA gene (*E. coli* numbering). Reaction conditions were 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with 30 cycles on a Hybaid thermal reactor with plate control. Primer concentration was 1 µg ml⁻¹. PCR amplifications were analysed on 1.5% (w/v) agarose gels.

DNA–DNA reassociation. This was carried out by the S1-nuclease method of Crosa *et al.* (1973) using reference [³H]DNA from type strains NCDO 2226^T (*S. constellatus*), NCDO 2227^T (*S. intermedius*) and NCTC 10713^T (*S. anginosus*) as described previously (Whiley & Hardie, 1989). DNA purification for these experiments was by the method of Garvie (1976). Reassociation experiments were carried out under stringent (75 °C) conditions and all hybridizations were performed in triplicate. Controls included a homologous reassociation as a positive (100%) control and calf thymus DNA as a negative (0%) control. For each test DNA the degree of sequence homology was expressed as a percentage of the homologous control after correction for self reassociation in the negative control.

DNA sequencing. PCR products, generated with primers C: 5'-CTTGACACACCGCCCGTCA-3' and D: 5'-CCACGTCC-TTC(AT)TCG(CG)CT-3', [5' biotin] (nucleotides in parentheses are degenerate nucleotide positions) under the same conditions as described earlier, were purified and the strands separated using paramagnetic beads conjugated with Streptavidin (Dynabeads M-280 Streptavidin, Dynal) according to the manufacturer's instructions. The 5'-end of primer C is located at position 1389 of the 16S rRNA gene and the 5'-end of primer D at position 44 downstream of the 5'-end of the 23S rRNA gene (*E. coli* numbering). The biotin-labelled strand and the unlabelled strand were purified separately and used as templates for sequencing using primers A and B, respectively. Sequencing was performed by chain termination using a Sequenase version 2.0 kit and protocol (USB). Sequences were

Table 1. Biotypes examined

Abbreviations: Amyg, amygdalin; Lac, lactose; Mann, mannitol; Raff, raffinose; Aesc, aesculin; Arg, arginine; β -DFuc, β -D-fucosidase; β -NAGal, β -N-acetylgalactosaminidase; Sial, sialidase; β -NAGlu, β -N-acetylglucosaminidase; β -Gal, β -galactosidase; α -Gal, α -galactosidase; α -Glu, α -glucosidase; β -Glu, β -glucosidase; Hyal, hyaluronidase. *n*, Number of strains/isolates.

Species and (biotypes)	<i>n</i> =	Acid from:				Hydrolysis of:		Production of:									
		Amyg	Lac	Mann	Raff	of:		β - DFuc	β - NAGal	Sial	β - NAGlu	β -Gal	α -Gal	α -Glu	β -Glu	Hyal	
						Aesc	Arg										
<i>S. intermedius</i>																	
(i1)*	13	+	+	—	—	+	+	+	+	+	+	+	—	+	+	+	
(i2)	3	—	+	—	—	+	+	+	+	+	+	+	—	+	—	+	
(i3)	2	+	+	—	+	+	+	+	+	—	+	+	—	+	+	+	
(i4)	1	+	+	—	—	+	+	+	+	+	+	+	—	+	—	+	
(i5)	1	—	+	—	—	+	—	+	+	—	+	+	—	+	+	+	
(i6)	1	+	+	—	—	+	+	+	+	—	+	+	—	+	+	+	
<i>S. constellatus</i>																	
(c1)	4	—	—	—	—	+	+	—	—	—	—	—	—	+	—	+	
(c2)	3	—	—	—	—	—	+	—	—	—	—	—	—	+	—	+	
(c3)	3	+	—	—	—	+	+	—	—	—	—	—	—	+	—	+	
(c4)	4	+	+	—	—	+	+	—	—	—	—	—	—	+	—	+	
(c5)†	2	—	+	—	—	+	+	—	—	—	—	—	—	+	—	+	
(c6)	1	—	+	+	—	+	+	—	—	—	—	—	—	+	—	+	
(c7)	1	—	—	—	—	+	+	—	—	—	—	—	—	—	—	+	
(c8)	1	—	+	—	—	+	+	—	—	—	—	—	—	—	—	+	
(c9)	1	—	+	—	—	—	+	—	—	+	—	—	—	+	—	+	
(c10)	1	—	+	—	—	+	+	—	—	—	—	—	—	—	—	—	
(c11)	1	—	—	—	—	—	—	—	—	—	—	—	+	+	—	+	
<i>S. anginosus</i>																	
(a1)	10	+	+	—	—	+	+	—	—	—	—	—	—	—	+	—	
(a2)	2	+	+	—	—	+	+	—	—	—	—	—	—	—	—	—	
(a3)	2	+	+	+	+	+	+	—	—	—	—	—	—	+	+	—	
(a4)	2	+	+	+	+	+	+	—	—	—	—	+	+	+	+	—	
(a5)	1	+	+	+	+	+	+	—	—	—	—	+	—	+	+	—	
(a6)‡	1	+	+	—	+	+	+	—	—	—	—	—	—	—	+	—	
(a7)	1	+	+	+	+	+	—	—	—	—	—	—	—	+	+	—	
(a8)	1	+	—	—	—	+	+	—	—	—	—	—	+	—	+	—	
(a9)	1	+	+	—	—	+	+	—	—	—	—	—	—	+	+	+	
(a10)	1	+	+	—	—	+	+	—	—	—	—	+	—	+	+	+	

* Biotype includes NCDO 2227^T.

† Biotype includes NCDO 2226^T.

‡ Biotype includes NCDO 10713^T.

read on a sonic digitizer and processed with PC/gene software (Intelligenetics). Spacers from two representative strains of each spacer length found within the three species were sequenced.

RESULTS

Size polymorphisms within the 16S–23S rRNA spacer regions

All PCR amplifications carried out using primers A and B resulted in the detection of single bands on agarose gels. Fragment sizes determined by comparison against molecular size markers (123 bp ladder, Gibco BRL) are

summarized in Table 2. Representative examples of the 16S–23S amplification products are shown in Fig. 1. Ambiguous nucleotide positions on the sequencing gels that could reflect heterogeneity between different copies of the spacer were not observed.

Twenty isolates previously identified as *S. constellatus* yielded a 380 bp PCR product. Two isolates designated as 'atypical *S. constellatus*' (FW73 biotype c10 and F436 biotype c11) differed by giving 450 bp and 300 bp PCR products, respectively. All 13 mann–, raff–, hyal– (biotypes a1, a2, a8) isolates of *S. anginosus* examined together with the type strain NCTC 10713^T (biotype a6)

Table 2. Size polymorphisms of the PCR products within SMG species, determined using primers A and B

Species	Biotypes	Size of PCR product (bp)	No. examined (n)	Spacer designation
<i>S. constellatus</i>	c1 → c9	380	20	<i>S.con</i> ₃₈₀
'Atypical- <i>S. constellatus</i> ' (FW73)	c10	450	1	
'Atypical- <i>S. constellatus</i> ' (F436)	c11	300	1	
<i>S. anginosus</i>	a1, a2, a6, a8	450	14	<i>S.ang</i> ₄₅₀
<i>S. anginosus</i> (mann +, raff +)	a3 → a5, a7	350	6	<i>S.ang</i> ₃₅₀
'Atypical- <i>S. anginosus</i> ' (AGP37-W, MS101)	a9, a10	380	2	
<i>S. intermedius</i>	i1, i2, i4	450	10	<i>S.int</i> ₄₅₀
<i>S. intermedius</i>	i1, i2	330	7	<i>S.int</i> ₃₃₀
'Atypical- <i>S. intermedius</i> ' (I782, 3206393, 3206692)	i3, i5, i6	380	3	
'Atypical- <i>S. intermedius</i> ' (3209432)	i3	330	1	

Fig. 1. PCR amplification products. Lanes: B and C, *S. constellatus* (*S.con*₃₈₀); E and G, *S. anginosus* (*S.ang*₄₅₀); F and I, *S. anginosus* (*S.ang*₃₅₀); J and K, *S. intermedius* (*S.int*₃₃₀); L, *S. intermedius* (*S.int*₄₅₀); D, phenotypically atypical isolate FW73 (biotype c11); M, phenotypically atypical isolate 3206393 (biotype i5); H, negative control lane; A and N, molecular size markers of 246, 369, 492, 615 and 738 bp (123 bp ladder, Gibco BRL).

were characterized by a 450 bp PCR product and all six mann +, raff +, hyal – (biotypes a3, a4, a7) isolates of this species tested differed from the former by the production of a shorter, 350 bp fragment. Isolates identified presumptively as hyaluronidase-producing *S. anginosus* (AGP37-W and MS101, biotypes a9 and a10) both gave a 380 bp PCR product. Isolates of *S. intermedius* were divided into two groups yielding either a longer, 450 bp ($n = 10$), or shorter, 330 bp ($n = 8$), PCR product, respectively. In addition, three sial – isolates that had been presumptively assigned to *S. intermedius* (3206393 biotype i5; 3206692 biotype i6; and I782 biotype i3) were distinguished from other *S. intermedius* isolates by the production of a 380 bp band. The fourth sial – isolate

examined (3209432, biotype i3) was included in the 330 bp group.

DNA–DNA reassociations

'Atypical-*S. constellatus*' isolates (F436 and FW73), '(sial –) *S. intermedius*' isolates (3206393 and I782) together with hyaluronidase-producing *S. anginosus* isolates AGP37-W and MS101 were examined by DNA–DNA reassociation. Of these, only I782 could be assigned with confidence to one or other of the SMG species; 88 % similarity was shared with *S. intermedius* type strain NCDO 2227^T compared to 30 % and 42 % with type strains NCTC 10713^T (*S. anginosus*) and NCDO 2226^T

Table 3. DNA–DNA reassociations of atypical isolates

Numbers show the range for each set of replicates with the mean in parentheses.

Unlabelled DNA from:	Percentage reassociation with reference [³ H]DNA from:		
	NCTC 10713 ^T	NCDO 2226 ^T	NCDO 2227 ^T
NCTC 10713 ^T	100	34–39 (37)	46–58 (50)
NCDO 2226 ^T	28–33 (30)	100	12–33 (21)
NCDO 2227 ^T	36–46 (39)	43–55 (48)	100
Atypical-<i>S. constellatus</i>			
F436	36–41 (38)	5–8 (7)	26–42 (33)
FW73	45–52 (47)	18–26 (21)	26–28 (27)
Atypical-<i>S. anginosus</i>			
AGP37-W	9–7 (8)	1–3 (2)	8–20 (13)
MS101	20–28 (24)	41–48 (45)	43–48 (46)
Atypical-<i>S. intermedius</i>			
I782	25–33 (30)	35–47 (42)	78–97 (88)
3206393	33–39 (36)	34–42 (39)	44–54 (48)

(*S. constellatus*), respectively. None of the remaining isolates tested demonstrated sufficient similarity with these type strains to be assigned to a species, with all giving less than 60% DNA reassociation with the reference DNAs (Table 3). All of these isolates were subsequently re-examined by biochemical testing in order to check the original identifications and gave identical results to those obtained previously.

DNA sequencing of 16S–23S spacer regions

Sequencing of the 16S–23S spacers was carried out on two representatives for each size of PCR product obtained from all three species. Aligned sequences are shown in Fig. 2. The 16S–23S spacers were determined to be either 270 bp (short) or 391/392 bp (long) in length for *S. intermedius*, 319 bp for *S. constellatus* and either 388/391 bp (long) or 295 bp (short) for *S. anginosus*. All sequences contained a single tRNA_{Ala} gene as described previously for *S. pneumoniae* (Bacot & Reeves, 1991).

Intra-specific heterogeneity. No sequence heterogeneity was found in the short spacer of *S. anginosus* (isolates Q4039 and K481) or the short spacer of *S. intermedius* (strain NCDO 2227^T and isolate 10a). Sequences of the two representative long 16S–23S spacers examined

in *S. intermedius* (isolates HW12 and GN556) and in *S. constellatus* (strain NCDO 2226^T and isolate UNS55) each had a single base difference, while the long spacer of *S. anginosus* exhibited a greater degree of genetic heterogeneity with differences at seven positions between NCTC 10713^T and SL15 (Fig. 2). The main difference between the long and short spacers of *S. anginosus* was the presence of a 97 bp insertion/deletion at position 184 together with nine single base differences (Fig. 2). In contrast, the short and long spacers of *S. intermedius* were identical for the first 137 bp and final 110 bp but the overall difference in length was not accountable for by a single insertion/deletion event as there was considerable sequence divergence in the central section of the spacer.

Inter-specific heterogeneity. All spacer variants from the three species share a high degree of sequence homology for the first 132 bp and the last 101 bp of their lengths. In the central region there is both sequence and length heterogeneity, precluding any useful calculation of percentage homology. However, the two spacers showing greatest similarity are the long variants of *S. intermedius* and *S. anginosus* with only ten mismatched bases and five bases deleted/inserted. The short spacers in these species are less similar to each other, with the long *S. intermedius* spacer having less sequence in common with the short *S. intermedius* spacer than it does with either *S. anginosus* spacer variant. The spacer sequence of *S. constellatus* is distinct from the sequences of both the other two species.

DISCUSSION

The results of this study demonstrate that the 16S–23S rRNA intergenic spacers within the SMG contain sufficient interspecific length polymorphisms and intra-specific conservation to serve as a valuable adjunct to biochemical testing for discriminating between *S. anginosus* and *S. constellatus*. This is especially useful in view of the close phenotypic similarity of these two species. In contrast, however, the presence of virtually identical long spacers in a large proportion of isolates of *S. intermedius* and *S. anginosus* shows that this approach is of little value in differentiating these two species compared to their relatively straightforward identification on the basis of phenotypic criteria. It is of interest that isolates of the same species have been shown here to possess spacers of different length although this has been reported previously for *Pseudomonas cepacia* (Kostman *et al.*, 1992) and within species of *Bacillus* (Gottlieb & Rudner, 1985). However, no evidence was obtained in the present study to suggest that there was heterogeneity between rRNA operons of a single isolate (intra-genomic heterogeneity in spacer length was observed previously within *E. faecalis*; Hall, 1994).

The potential use of 16S–23S spacer length determination for identification is further illustrated by the results obtained with phenotypically atypical isolates; the problem of identifying biochemically atypical isolates of *S. anginosus* and *S. constellatus* has been highlighted previously (Kilpper-Balz *et al.*, 1984) and remains despite some improvement in the discrimination between these two

<i>S.int</i> ₃₃₀	CTAAGGAAAATACGGAAGACATTGAGTTCGTTTTGTTAGTTTGTAGAGG	50
<i>S.int</i> ₄₅₀	CTAAGGAAAATACGGAAGACATTGAGTTCGTTTTGTTAGTTTGTAGAGG	50
<i>S.ang</i> ₄₅₀	CTAAGGAAAAaCGGAAGCATTGG--TCGTCATGTTTAGTTTGTAGAGG	48
<i>S.ang</i> ₃₅₀	CTAAGGAAAA--CGGAAGCATTGAGTTGTCTTGTAGTTTGTAGAGG	48
<i>S.con</i> ₃₈₀	CTAAGGAAAATCGGAAGCATTGAGTTCGTTTTGTTAGTTTGTAGAGG	50
	*****	
<i>S.int</i> ₃₃₀	TCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTCACGCAGGA	100
<i>S.int</i> ₄₅₀	TCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTCACGCAGGA	100
<i>S.ang</i> ₄₅₀	TCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTCACGCAGGA	98
<i>S.ang</i> ₃₅₀	TCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTCACGCAGGA	98
<i>S.con</i> ₃₈₀	TCTTGTGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTCACGCAGGA	100
	*****	
<i>S.int</i> ₃₃₀	GGTCAGCGGTTTCGATCCCGTAGGCTCCATTAGGATA-----	137
<i>S.int</i> ₄₅₀	GGTCAGCGGTTTCGATCCCGTAGGCTCCATTAGGATAGAATCCTACTGAA	150
<i>S.ang</i> ₄₅₀	GGTCAGCGGTTTCGATCCCGTAGGCTCCATTAGGATAGAATCCTACTGAA	148
<i>S.ang</i> ₃₅₀	GGTCAGCGGTTTCGATCCCGTAGGCTCCATTAGGATAGAATCCTACTGAA	148
<i>S.con</i> ₃₈₀	GGTCAGCGGTTTCGATCCCGTAGGCTCCATTAT-----CTAGGATG	141
	*****	
<i>S.int</i> ₃₃₀	-----TGGAATTC-----	146
<i>S.int</i> ₄₅₀	CTTAATAAAGAAGTGAAGTTGAATACGAGACTAACTTCTTAGGAAAAAa	200
<i>S.ang</i> ₄₅₀	CTTAATAAAGAAGTGAAGTTGAATACGAGACTAACTTCTTAGGAAAAAa	197
<i>S.ang</i> ₃₅₀	CTTAATAAAGAAGTTGAAGTTGAATACGAGACTAACTTCTTAGGAAAAAa	184
<i>S.con</i> ₃₈₀	CAAA-----GAAATGAGATTCGT---TCAACTAGATAAGATAG	178
	*****	
<i>S.int</i> ₃₃₀	-----CCTTTAGTTGATGA-----	160
<i>S.int</i> ₄₅₀	GATAAACTTCCTTGTGTTTCATGGAACACAGCGTCAGTTTCCTATTTCCT	250
<i>S.ang</i> ₄₅₀	GATAAACTTCCTTGTGTTTCATGGAACACARCGTCAGTTTCCTATTTCCT	247
<i>S.ang</i> ₃₅₀	GATAAACTTCCTTGTGTTTCATGGAACACARCGTCAGTTTCCTATTTCCT	184
<i>S.con</i> ₃₈₀	GATAG-----GTGTT-----CAACGGGT-----	196
	*****	
<i>S.int</i> ₃₃₀	-----TATCCTAAAGT-AGCACA	177
<i>S.int</i> ₄₅₀	ACAGAAGTTTTCGCAAGCGAACCGTCTCTTTATATCCTAAAGT-AGCACA	299
<i>S.ang</i> ₄₅₀	ACAGAAGTTTTCGCAAGCGAACCGTCTCTTTATATCCTAAAGT-AGCACA	297
<i>S.ang</i> ₃₅₀	-----ATCCTAAATTTAGCACA	201
<i>S.con</i> ₃₈₀	AAAGAAGTTGA-ACA--CGAACC-----TAATTAGCCCCA	227
	*****	
<i>S.int</i> ₃₃₀	TTGAAAATTGAATAACGATATCAAATAGTAACAAGAAAATAAACCGAAAA	227
<i>S.int</i> ₄₅₀	TTGAAAATTGAATAACGATATCAAATAGTAACAAGAAAATAAACCGAAAA	349
<i>S.ang</i> ₄₅₀	TTGAAAATTGAATAACGATATCAAATAGTAACAAGAAAATAAACCGAAAA	347
<i>S.ang</i> ₃₅₀	TTGAAAATTGAATAACGATATCAAATAGTAACAAGAAAATAAACCGAAAA	251
<i>S.con</i> ₃₈₀	TTGAAAATTGAATAACGATATCAAATAGTAACAAGAAAATAAACCGAAAA	276
	*****	
<i>S.int</i> ₃₃₀	CGCTGTGAATATTAATGAGTTTATGACTGAAAGGTCAAAA-TAA	270
<i>S.int</i> ₄₅₀	CGCTGTGAATATTAATGAGTTTATGACTGAAAGGTCAAAA-TAA	392
<i>S.ang</i> ₄₅₀	CGCTGTGAATATTAATGAGTTTATGACTGAGAGGTCAAAAATAA	391
<i>S.ang</i> ₃₅₀	CGCTGTGAATATTAATGAGTTTATGACTGAGAGGTCAAAAATAA	295
<i>S.con</i> ₃₈₀	CGCTGTGAATATTAATGAGTTTATGACTGAAAGGTCgAAAAATAA	319
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Fig. 2. Alignment of DNA sequences of 16S–23S rRNA intergenic spacers: *S. intermedius*₄₅₀ (long) [isolates HW12 (accession no. L36935) and GN556], *S. intermedius*₃₃₀ (short) [strain NCDO 2227 (accession no. L36934) and isolate 10a], *S. anginosus*₄₅₀ (long) [strain NCTC 10713 (accession no. L36931) and isolate SL15], *S. anginosus*₃₅₀ (short) [isolates Q4039 (accession no. L36932) and KR481] and *S. constellatus*₃₈₀ [strain NCDO 2226 (accession no. L36933) and isolate UNS55]. The tRNA_{Ala} gene deduced by comparison with the tRNA_{Ala} gene of *S. pneumoniae* (Bacot & Reeves, 1991) is shown boxed. Positions in the alignments that are preserved in all sequences examined are indicated by *. Gaps introduced to maintain alignment are indicated by -. Differences within spacer types are: *S.int*₄₅₀, a at 200 is A (HW12) or - (GN556); *S.ang*₄₅₀, R at 7 is A (NCTC 10713) or G (SL15), a at 11 and 12 is A (NCTC 10713) or - (SL15), R at 226 is G (NCTC 10713) or A (SL15), Y at 236 is C (NCTC 10713) or T (SL15), t at 245 is T (NCTC 10713) or - (SL15), W at 289 is T (NCTC 10713) or A (SL15); *S.con*₃₈₀, g at 312 is G (UNS55) or - (NCTC 2226).

species (Whiley *et al.*, 1990). That virtually all the presumptively identified, phenotypically atypical isolates tested here had spacer lengths different from those found in typical members of each of the three species suggests that they are genetically distinct. The results of DNA–DNA pairing experiments confirm this. However, the verification by DNA–DNA hybridization of sialidase-negative isolate 1782 as *S. intermedius* despite the production of a 380 bp PCR band with this isolate and the presence in isolates MS101, AGP-37-W, 3206393 and FW73 of PCR products with lengths coincidental with those found in *S. constellatus*, *S. intermedius* and *S. anginosus* demonstrates that 16S–23S rRNA intergenic spacer length determination is better employed only as an adjunct to biochemical identification rather than as a substitute; the true taxonomic position of these atypical isolates and the degree of spacer length polymorphism present within other species of streptococci outside the SMG have yet to be determined.

The demonstration of the shorter (350 bp) spacer only in isolates of *S. anginosus* possessing a broader fermentation

capability could be taken to suggest that these isolates constitute a separate subspecies or even species within the SMG. This evidence supports that from previous DNA–DNA hybridization experiments (Whiley & Hardie, 1989) that included some isolates of this phenotype and which also indicated that *S. anginosus* may be a sufficiently genetically heterogeneous species to warrant further division. Clearly, the relationships between isolates currently included in *S. anginosus* requires clarification. In contrast, no phenotypic differences were detected between isolates of *S. intermedius* having long or short spacer variants.

Previous studies using DNA–DNA hybridization and 16S rRNA partial sequence analysis have shown that *S. intermedius* and *S. constellatus* are more closely related to each other than to *S. anginosus* (Kilpper-Balz *et al.*, 1984; Bentley *et al.*, 1991). Although no formal comparisons of spacer sequences have been attempted here, visual examination of the data reveals that these relationships are not reflected by the results obtained here where the highest degree of overall spacer sequence similarity is shared

between the long spacers of *S. anginosus* and *S. intermedius*. Interestingly, however, the highest similarity within the first 56 and last 99 bp (i.e. omitting the highly variable central region and the tRNA_{Ala} gene) is shared between *S. intermedius* and *S. constellatus*. This suggests that rearrangements in the central region are relatively recent and obscure the true ancestry of these species: one might postulate that the 450 bp spacer is ancestral and that the 330 bp spacer type within *S. intermedius* and the 350 bp spacer type within *S. anginosus* arose by separate deletion events.

The degree of interspecies spacer heterogeneity revealed in this study, particularly between *S. anginosus* and *S. constellatus*, indicates that the same approach may be suitable for inclusion in identification schemes for other recently described species within the genus.

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