***Streptococcus australis* sp. nov., a novel oral streptococcus**

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Strains of streptococci were isolated from the mouths of children attending the United Dental Hospital, Sydney, Australia. These strains were analysed biochemically using the Rapid ID32 Strep microsystem, were subjected to DNA–DNA hybridization with other members of the oral streptococci and had their 16S rRNA analysed. On the basis of DNA–DNA hybridization, their nearest relative was *Streptococcus parasanguinis*, whereas, on the basis of 16S rRNA analysis, it was *Streptococcus infantis*. The name *Streptococcus australis* sp. nov. is proposed for the new species. The type strain is AI-1^T (= ATCC 700641^T = NCTC 13166^T).

Keywords: α -haemolytic streptococci, oral cavity, DNA–DNA hybridization, 16S rRNA, *Streptococcus mitis* group

INTRODUCTION

The oral cavity is colonized by a large number of different streptococcal species, mostly belonging to the viridans streptococci. Members of the group have been isolated from all areas of the oral cavity, including the tooth surfaces and epithelia. Members of the largest groups of viridans streptococci, containing the species *Streptococcus mitis* and *Streptococcus sanguinis*, can colonize all areas of the oral cavity and have been associated with dental caries in animal models (Willcox *et al.*, 1985, 1988) and isolated from cases of endocarditis (Dyson *et al.*, 1999).

It has been notoriously difficult to classify the *S. mitis* group using standard biochemical tests (Ezaki *et al.*, 1988), although tests described by Whiley & Beighton (1998) have been used successfully. Recently, two species within the *S. mitis* group have been described, namely *Streptococcus peroris* and *Streptococcus infantis* (Kawamura *et al.*, 1998). These species were isolated predominantly from the oral cavities/pharynges of children, some of whom were suffering from Kawasaki disease.

During microbiological analysis of the saliva of children attending the United Dental Hospital in Sydney, Australia, we isolated strains of streptococci that were able to grow on media containing relatively high (up to 500 mM) concentrations of NaCl or KCl (Willcox *et al.*, 1991). These strains were initially identified as *S.*

mitis (Willcox *et al.*, 1991), but subsequent biochemical analysis and DNA–DNA hybridization (Willcox, 1996) demonstrated that the strains constituted a separate species. From the current study, we present additional data on these strains, including the results of biochemical analyses using the Rapid ID32 Strep microsystem, DNA–DNA hybridization and rRNA gene sequencing.

METHODS

Bacterial strains and physiological and biochemical analyses. Strains were isolated as described previously (Willcox *et al.*, 1991) on media containing relatively high concentrations of NaCl or KCl. The strains were routinely grown on Columbia blood (5%, v/v, defibrinated sheep blood) agar (CBA; Difco) under microaerophilic conditions. Biochemical traits were determined using the Rapid ID32 Strep system (bioMérieux). The ability of strains to grow at 45 °C was measured in brain/heart infusion (Oxoid) containing 0.3% (w/v) yeast extract (BHIY; Difco) and the ability to grow in 6.5% (w/v) NaCl was tested in BHIY.

DNA–DNA hybridization. DNA–DNA hybridization values were obtained under stringent (0.1 × SSC) or relaxed (0.5 × SSC) conditions as described previously (Willcox, 1996; Kusuda *et al.*, 1991). The type strains used in the DNA–DNA hybridization were *Streptococcus parasanguinis* (formerly *Streptococcus parasanguis*; Trüper & de'Clari, 1997) ATCC 15912^T, *Streptococcus gordonii* ATCC 10558^T, *Streptococcus cristatus* (formerly *Streptococcus crista*; Trüper & de'Clari, 1997) ATCC 12479^T, *S. sanguinis* (formerly *Streptococcus sanguis*; Trüper & de'Clari, 1997) NCTC 7863^T, *Streptococcus oralis* NCTC 11427^T and *S. mitis* NCTC 12261^T.

The GenBank accession number for the 16S rRNA sequence (1336 bases) of strain AI-1^T is AF184974.

Table 1. Biochemical characteristics of *S. australis* and other members of the viridans streptococci

Data for *S. peroris* and *S. infantis* are from Kawamura *et al.* (1998).

Characteristic	<i>S. australis</i> GTC 848 ^T	<i>S. peroris</i> GTC 849 ^T	<i>S. infantis</i> GTC 849 ^T	<i>S. oralis</i> NCTC 11427 ^T	<i>S. sanguinis</i> NCTC 7863 ^T	<i>S. gordonii</i> ATCC 10558 ^T	<i>S. parasanguinis</i> ATCC 15912 ^T	<i>S. cristatus</i> ATCC 12479 ^T	<i>S. mitis</i> NCTC 12261 ^T
Production of:									
β -Glucosidase	—	—	—	—	+	+	+	—	—
α -Galactosidase	—	—	—	—	—	—*	+	—	+
Alkaline phosphatase	+	+	—	+	—	+	+	—	—
β -Galactosidase	—	—	+	—	—	—	+	—	—
<i>N</i> -Acetyl β -glucosaminidase	—	—	+	—	—	—	+	—	—
Glycyl-tryptophan arylamidase	+	—	+	+	+	—	+	+	+
β -Mannosidase	—	—	—	—	—	+	—	—	—
Acidification of:									
Lactose	+	+	+	+	+	+	+	+	+
Trehalose	—	—	—	+	+	+	+	+	—
Pullulan	+	—	+	—	+	—	—	—	+
Melibiose	—	—	—	—	—	—	+	—	—
Methyl β -D-glucopyranoside	—	—	—	—	—	+	—	—	—
Tagatose	—	—	+	—	—	+	+	—	—
Hydrolysis of arginine	+	—	—	—	+	+	+	+	—

* Opposite reaction in API 20 Strep test kits (Willcox, 1996).

Table 2. DNA–DNA hybridization values for *S. australis* and other members of the viridans streptococci

Abbreviations: (R), relaxed hybridization conditions; (S), stringent hybridization conditions; ND, not determined.

Strain	DNA–DNA hybridization (%)										
	1 (R)	1 (S)	11 (R)	11 (S)	12	13	14*	15*	16*	17*	18*
1. <i>S. australis</i> AI-1 ^T	100	100	15	7	2	5	10	0	0	0	3
2. <i>S. australis</i> AI-2	100	100	11	11	0	0	0	0	0	0	4
3. <i>S. australis</i> AI-3	80	75	11	11	0	0	10	0	5	0	10
4. <i>S. australis</i> AI-4	100	97	7	2	9	0	0	0	2	0	6
5. <i>S. australis</i> AI-5	80	79	7	7	7	5	0	0	4	0	8
6. <i>S. australis</i> AI-6	97	90	33	4	5	0	0	0	4	0	5
7. <i>S. australis</i> AI-8	80	70	11	11	0	7	0	0	2	0	0
8. <i>S. australis</i> AI-13	98	98	30	4	2	0	0	0	0	0	4
9. <i>S. australis</i> AI-15	96	97	19	20	0	0	0	0	5	0	9
10. <i>S. australis</i> AI-22	98	98	20	4	0	3	0	0	0	0	26
11. <i>S. parasanguinis</i> ATCC 15912 ^T	53	40	100	100	3	0	13	0	0	21	0
12. <i>S. infantis</i> JCM 10157 ^T	4	ND	ND	ND	100	15	ND	ND	ND	ND	ND
13. <i>S. peroris</i> JCM 10158 ^T	15	ND	ND	ND	23	100	ND	ND	ND	ND	ND
14. <i>S. cristatus</i> NCTC 12479 ^T	3	ND	1	ND	ND	ND	100	0	14	0	0
15. <i>S. sanguinis</i> NCTC 7863 ^T	5	ND	4	ND	ND	ND	20	100	7	0	13
16. <i>S. gordonii</i> ATCC 10558 ^T	4	ND	2	ND	ND	ND	0	0	100	25	11
17. <i>S. mitis</i> NCTC 12261 ^T	5	ND	4	ND	ND	ND	0	0	2	100	91
18. <i>S. oralis</i> NCTC 11427 ^T	5	ND	1	ND	ND	ND	0	0	15	45	100

* Results from Willcox (1996).

Determination of DNA G+C content. Base compositions (G+C content) were determined for the proposed new species by the thermal melting method (T_m ; Johnson, 1981). The A_{260} of the sample was recorded continually while the sample was heated from 50 to 95 °C in increments of 1.0 °C min⁻¹. A reference DNA sample from *Escherichia coli* O157 was prepared.

16S rRNA amplification, sequencing and sequence analysis. The oligonucleotide primers used in this study were synthesized by Auspep (Victoria, Australia). The sequences of the primers corresponded to those within the 16S rRNA gene of *S. sanguinis* (Whiley *et al.*, 1990). The 5' primer 5'-CTGGCTCAGGACCAACG-3' corresponded to nucleotides 18–34 of the 16S rRNA and the 3' primer 5'-GCCCGGAACGTATTAC-3' corresponded to nucleotides 1375–1392. A PCR was performed in a 100 µl reaction containing template DNA (approx. 50 ng), 2 µl dNTP mix (10 mM each), 10 µl 10× reaction buffer [Tris/HCl, KCl, (NH₄)₂SO₄, 3 mM MgCl₂, pH 8.7], 60 pmol each primer and 2.5 U *Taq* DNA polymerase (Qiagen). The samples were first denatured for 3 min at 94 °C and then subjected to 35 cycles of amplification in an FTS-320 thermal cycler (Corbett Research). The parameters for the amplification cycles were denaturation for 1 min at 94 °C, annealing of primers for 2 min at 53 °C and primer extension for 1.5 min at 72 °C. After the last cycle, the PCR tubes were incubated for 7 min at 72 °C. On completion, the sample was extracted with 0.6 vols propan-2-ol, precipitated with 2.5 vols ethanol and then resuspended in 20 µl distilled water.

The amplified 16S rDNA products were sequenced, using standard dye-terminator chemistry, by the Prince Alfred Macromolecular Analysis Centre, The University of Sydney. The sequences were aligned and the similarity levels were

obtained by using the WEBANGIS computer program (Australian National Genomic Information Services). The ECLUSTALW program in WEBANGIS was used to align the sequences. After bootstrap resampling of the alignment, the phylogenetic distances were calculated using the neighbour-joining method (using the algorithm of NEIGHBOR) and the consensus tree was generated from 100 resulting trees by using the ECONSENSE program in WEBANGIS.

RESULTS

General characteristics

After incubation on CBA plates for 3 d, colonies of the novel species were approximately 0.5–1.0 mm in diameter, rough or raised and white with α-haemolysis. The cells were Gram-positive cocci grouped in pairs or short chains. The isolates were catalase-negative, non-spore-forming and non-motile. The strains grew at 45 °C but not in 6.5% NaCl broth.

Biochemical characteristics

Table 1 demonstrates the biochemical characteristics that differentiate the novel species from closely related species. Strains of all species tested produced alanine-phenylalanine-proline arylamidase and acidified media containing lactose, maltose and sucrose. No strain was able to produce β-glucuronidase, acetoin (Voges–Proskauer reaction), pyroglutamic acid arylamidase or urease. No strain acidified media containing ribose, mannitol, sorbitol, glycogen, melezitose, L-arabinose

Fig. 1. Unrooted consensus tree showing the phylogenetic position of *S. australis* with respect to selected members of the genus *Streptococcus* based on 16S rRNA sequences. *S. parasanguinis* was used as the outgroup. The numbers at the branching points are bootstrap values. Strain designations, GenBank accession numbers and 16S rRNA similarities (%) to *S. australis* are listed after species names.

or D-arabinose. Strains were unable to hydrolyse hippurate.

G + C content of DNA and DNA–DNA hybridization

The G + C content of strain AI-1^T was 43.5 ± 1.2 mol%. DNA–DNA hybridization studies demonstrated that all strains of the novel species had greater than 79% homology within the group. The species with the next highest homology was *S. parasanguinis*, showing 40% homology under stringent conditions (Table 2).

16S rRNA analysis

The 16S rRNA sequence for strain AI-1^T was determined for 1336 bases. An unrooted phylogenetic tree (Fig. 1) demonstrated that the new strain was located in the cluster corresponding to the *S. mitis* group. The overall similarity to the nearest relative was 97.9% (to *S. infantis*) (Fig. 1).

DISCUSSION

The results presented demonstrate that the strains isolated from the saliva of children attending the United Dental Hospital of Sydney should be regarded as a separate species of the oral streptococci, for which we propose the name *Streptococcus australis* sp. nov. DNA–DNA hybridization studies demonstrated that *S. parasanguinis* has the greatest homology in terms of DNA–DNA hybridization, but this homology only reaches 53% under non-stringent conditions. Analysis of the 16S rRNA demonstrated that the nearest neighbour was a recently described species, *S. infantis*. Both *S. infantis* and *S. australis* form a cluster within the *S. mitis* group, on the basis of 16S rRNA similarities. However, the species differ with respect to the production of alkaline phosphatase, β -galactosidase and *N*-acetyl β -glucosaminidase, the acidification of tagatose and the hydrolysis of arginine. In addition, the species *S. australis*, *S. peroris* and *S. infantis* were not closely related in DNA–DNA hybridization tests ($\leq 15\%$ under non-stringent conditions). Of note was the fact that there were non-reciprocal hybridization values between strains AI-1^T and ATCC 15912^T under stringent and non-stringent conditions. This may have been due to experimental error owing to the use of light emission to measure hybridization; whilst all efforts were made to control it, the timing of the reaction and detection is critical. The technique is robust at the extremes of hybridization, showing close and distant relationships.

Previous results have demonstrated that strains of *S. australis* were able to bind α -amylase from human saliva (70% positive) and produce neuraminidase, were unable to react with Lancefield grouping sera and had a peptidoglycan linkage of Lys–Ala–Gly (Willcox, 1996). Using fluorescently labelled synthetic compounds, we demonstrated that *S. australis* was able to produce β -galactosidase, *N*-acetylglucosaminidase and *N*-acetylgalactosaminidase, but was unable to produce neuraminidase (Willcox *et al.*, 1995). Demonstration of the production of β -galactosidase and *N*-acetylglucosaminidase with fluorescently labelled substrates was at odds with the results obtained with Rapid ID32 Strep kits, probably demonstrating the effect of different substrates. Indeed, this has been shown previously for different substrates for β -galactosidase activity (Kawamura *et al.*, 1998). Within the *S. mitis* group, the species *S. oralis* and *S. mitis* are able to produce glucosyltransferases and *S. mitis* is able to produce fructosyltransferases. However, *S. australis* cannot produce either of these activities (Willcox *et al.*, 1995) and this may be another useful distinguishing characteristic for this species.

Interestingly, both *S. infantis* and *S. australis* were isolated from the mouths of children. In the case of *S. infantis*, the children were between 3 months and 3 years old and many had Kawasaki disease (Kawamura *et al.*, 1998). The children from whom *S. australis* was isolated (from the mouth) were between 8 and 12 years

old and showed no underlying diseases. Perhaps *S. infantis* and *S. australis* are early colonizers of the oral cavity; the fact that we have never isolated *S. australis* from the mouths of adults (data not shown), together with the absence of reports of *S. infantis* isolated from the mouths of adults, indicates that these species are replaced as part of the oral microbiota over time. Further investigations are needed to determine the habitat and residence time of these streptococci in the mouths of children and adults.

Description of *Streptococcus australis* sp. nov.

Streptococcus australis (aus.tra'lis. L. gen. n. *australis* of the south, southern, relating to the region in which the organism was isolated).

Cells are Gram-positive, non-motile, non-sporulating, catalase-negative cocci. Colonies grow to diameters of approximately 0.5–1.0 mm on blood agar plates and the cells form short chains. Facultatively anaerobic. α -Haemolytic on Columbia blood (sheep) agar plates. The biochemical analysis was performed using Rapid ID32 Strep kits. All strains produced alkaline phosphatase, alanine-phenylalanine-proline arylamidase and glycyl-tryptophan arylamidase, acidified media containing lactose, pullulan, maltose and sucrose and hydrolysed arginine. No strain was able to produce β -glucosidase, β -glucuronidase, α -galactosidase, acetoin (Voges–Proskauer reaction), pyroglutamic acid arylamidase, *N*-acetyl β -glucosaminidase, β -mannosidase or urease. No strain acidified media containing ribose, mannitol, sorbitol, trehalose, raffinose, glycogen, melibiose, melezitose, L-arabinose, D-arabinose, methyl β -D-glucopyranoside or tagatose. Strains were unable to hydrolyse hippurate. The production of β -galactosidase was dependent on the substrate used [either 2-naphthyl β -D-galactopyranoside (–) or *p*-nitrophenyl β -D-galactopyranoside (+)]. Strains have a peptidoglycan linkage type of Lys–Ala–Gly and do not produce neuraminidase (Willcox, 1996). The G + C content of the DNA is 43.5 ± 1.2 mol%. The strains were isolated from the saliva of humans. The type strain is AI-1^T (= ATCC 700641^T = NCTC 13166^T).

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