A new species of oral *Streptococcus* isolated from Sprague–Dawley rats, *Streptococcus orisratti* sp. nov.

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Taxonomic studies were performed on an unusual oral *Streptococcus* strain isolated from Sprague–Dawley rats. The isolates were α -haemolytic, bile-tolerant, aesculin-hydrolytic and unable to grow in 6.5% NaCl. They fermented lactose, sucrose and trehalose. They were distinguished from other recognized species of oral and viridans streptococci by several biochemical characteristics and by Lancefield's group antigen, as well as by unique DNA–DNA hybridization characteristics. 16S rDNA sequence studies confirmed the genealogical distinctiveness of the species. The results of the study demonstrated that the isolates represented a new species of the oral and viridans streptococci. The name *Streptococcus orisratti* sp. nov. is proposed for the new species. The type strain is A63^T (= ATCC 700640^T).

Keywords: Viridans streptococci, taxonomy, DNA–DNA hybridization, rDNA sequencing, rat

INTRODUCTION

Viridans streptococci comprise a large proportion of the normal human oral bacteria and have been associated with dental caries, endocarditis, septicaemia and abscess formation. However, in comparison with β -haemolytic streptococci, the viridans streptococci have relatively low virulence and give poor or variable reactions in serological and conventional physiological tests. Despite the considerable efforts of microbiologists to classify the viridans streptococci (Colman, 1976; Drucker & Melville, 1971; Facklam, 1977; Gilmour *et al.*, 1987; Hardie & Bowden, 1976; Parker & Ball, 1976), there is no universally accepted system and consequently, in most studies, some strains remain unclassified.

The oral viridans streptococci of humans have been studied extensively. The most prevalent streptococci isolated from the human oral cavity are members of the *Streptococcus sanguinis* group, including *S. sanguinis*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus cristatus* and

Streptococcus parasanguinis (Hamada & Slade, 1980; Marsh & Martin, 1992; Willcox, 1996). *Streptococcus salivarius* and *Streptococcus vestibularis* are isolated from the mucous membranes of the oral cavity (Marsh & Martin, 1992). The mutans streptococci are usually isolated from dental plaque (Hamada & Slade, 1980; Marsh & Martin, 1992). There are seven different species of mutans streptococci, including *Streptococcus ratti* and *Streptococcus ferus*, which have been isolated from the oral cavity of rats. Most previous studies (Hamada & Slade, 1980; Larrimore *et al.*, 1983; Loesche, 1986; Rolla *et al.*, 1983; Schilling & Bowen, 1992) have demonstrated that dietary sucrose facilitates the colonization of the mutans streptococci on the teeth of humans, monkeys and rodents, as the mutans streptococci synthesize extracellular polysaccharides from sucrose. During an investigation of the effect of high-sucrose diets on the oral bacteria of Sprague–Dawley rats, a new type of α -haemolytic *Streptococcus* was occasionally isolated. This organism occurred in about 50% of the experimental rat mouths and increased as a percentage of the total streptococci in the mouths of rats fed with a high-sucrose diet (Zhu *et al.*, 1997). In the current study, the physical, biochemical and serological characteristics of the new isolates were examined and compared with other oral streptococci. DNA–DNA hybridization was performed with oral streptococci to determine their

Abbreviations: FTF, fructosyltransferase; GTF, glucosyltransferase; MSA, mitis salivarius agar.

The GenBank accession number for the 16S rDNA sequence of strain A63^T is AF124350.

relatedness. The 16S rDNA sequence of the type strain was also analysed.

METHODS

Bacterial strains. The new strains A63^T, B64, A69 and B610 were isolated from the surfaces of the lower molars of two Sprague–Dawley rats, before and after feeding with high-sucrose diets, by using selective mitis salivarius agar (MSA) plates (Difco). The reference strains used in the study for comparative purposes are listed in Table 1. Stock cultures were maintained at –70 °C in brain/heart infusion broth (Oxoid) containing 0.3% (w/v) yeast extract (BHIY; Difco) supplemented with 30% glycerol.

Physiological and biochemical tests. The isolates were examined for their morphology, Gram reaction, catalase formation and ability to grow aerobically as well as anaerobically. For examination of haemolysis, strains were grown on 5% (v/v) Columbia sheep-blood-agar plates (Difco) under anaerobic conditions (95% N₂, 5% CO₂) at 37 °C for 18 h. Bacterial tolerance to bile salts was assessed by determining their ability to grow on Columbia blood-agar base supplemented with 10% and 40% Bacto Oxygall (Difco). Salt tolerance of the bacteria was tested in BHIY broth containing 6.5% (w/v) NaCl. The capacity for bacterial growth at 45 and 50 °C was examined in BHIY broth. To determine the growth of the isolates at pH 9.6, the strains were incubated in BHIY broth adjusted to this pH by the addition of 1 M NaOH. The final pH of bacterial cultures was determined after inoculation and incubation (37 °C) of the bacteria in BHIY broth containing an excess of glucose (5%) for 3 d.

The ability of strains to hydrolyse starch was tested by using the method of Paik (1980). Briefly, the organisms were grown on BHIY agar plates supplemented with 2% soluble potato starch (BDH) for 48 h at 37 °C. After flooding the surface of the plate with 1% (w/v) iodine, a colourless zone indicated hydrolysis and a dark blue to purple colour indicated that the starch had not been hydrolysed. API 20 Strep, Rapid ID32 Strep and API 50 CHS test kits were used according to the manufacturer's instructions (bioMérieux Vitek) for the further characterization of the organisms.

Serological reactions. Lancefield group typing was performed with a Streptococcal grouping kit (Oxoid). After growing on Columbia blood-agar plates at 37 °C for 18 h, a loopful of pure culture was emulsified in 0.4 ml Oxoid extraction enzyme and incubated in a water bath (37 °C) for 10 min. The extracts were then used for the Lancefield grouping reaction following the manufacturer's instruction.

Production of glucosyltransferase (GTF) and fructosyltransferase (FTF). For the examination of the production of GTF and FTF, *Streptococcus sobrinus* 6715 was used as the positive control, as this strain is well known to produce GTF and FTF (Willcox *et al.*, 1993). The bacterial strains were grown in BHIY for 18 h and harvested by centrifugation (6000 g, 4 °C, 15 min). Extracellular and cell-bound GTF and FTF activities were assayed with radioactive substrates as described previously (Jacques, 1983; Jacques & Wittenberger, 1981).

DNA isolation. Strains were cultured in 10 ml BHIY at 37 °C for 18 h. The cells were harvested and washed once in TE buffer (50 mM Tris/HCl, pH 7.5; 20 mM EDTA). Chromo-

Table 1. DNA similarity between strain A63^T and other test strains

Strain	Source	DNA similarity (%)
A63 ^T	Rat, oral cavity	100
B64	Rat, oral cavity	100
A69	Rat, oral cavity	98.8
B610	Rat, oral cavity	99.4
<i>S. anginosus</i> NCTC 10713 ^T	Human, throat	14.8
<i>S. bovis</i> ATCC 33317	Cow, dung	27.3
<i>S. bovis</i> 2M2	Human, oral cavity	25.8
<i>S. constellatus</i> NCTC 11325 ^T	Human, purulent pleurisy	6.4
<i>S. criceti</i> E49	Hamster, oral cavity	6.6
<i>S. equinus</i> ATCC 9812 ^T	Human, faeces	18.7
<i>S. gordonii</i> ATCC 10558 ^T	Human, SBE	13.2
<i>S. intermedius</i> NCTC 11324 ^T	Human	21.5
<i>S. mitis</i> NCTC 12261 ^{NT}	Human, SBE	18.7
<i>S. oralis</i> NCTC 11427 ^T	Human, oral cavity	10.0
<i>S. parasanguinis</i> ATCC 15912 ^T	Human, throat	18.0
<i>S. pyogenes</i> NCTC 8198	Human, scarlet fever	27.9
<i>S. rattii</i> FA1	Rat, oral cavity	24.1
<i>S. salivarius</i> ATCC 25975	Human, oral cavity	28.6
<i>S. sanguinis</i> NCTC 7863 ^T	Human, SBE	16.4
<i>S. sobrinus</i> 6715	Human, oral cavity	ND
<i>S. suis</i> ATCC 43765	Pig, throat	20.2

SBE, Subacute bacterial endocarditis; ND, not determined.

somal DNA was then isolated from all the test strains as described previously (Willcox, 1996). Briefly, the cells were resuspended and incubated in 350 µl TE buffer containing mutanolysin (125 units) at 60 °C for approximately 2 h. After lysing cells by the addition of 20 µl 20% (w/v) SDS, 5 µl 20 mg proteinase K ml⁻¹ was added and incubated at 37 °C for 1 h. Protein was then precipitated by the addition of 200 µl saturated NaCl solution and removed by centrifugation. The residual RNA was removed by incubating with RNase at 37 °C for 1 h. The DNA was further purified by adding an equal volume of equilibrated phenol, next with an equal volume of phenol/chloroform (1:1) and finally with an equal volume of chloroform. The DNA was precipitated by addition of 10 µl 1.0 M sodium acetate followed by 0.6 vols propan-2-ol.

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

DNA-DNA hybridization. DNA-DNA hybridization was based on the method described by Kusuda *et al.* (1991) and Willcox (1996) using the enhanced chemiluminescence (ECL) gene detection system (Amersham). Briefly, DNAs of all test strains were dissolved in water, boiled for 5 min and then immediately chilled. The single-strained DNA was spotted in equal amounts (10 µg for each strain) onto nitrocellulose membranes. After pre-treating in hybridization buffer at 42 °C for 1 h, the membrane was reacted with a peroxidase-labelled DNA probe of the new isolate A63^T overnight at 42 °C. The membrane was transferred to detection reagents that included hydrogen peroxide, luminol and enhancer to produce chemiluminescence and then exposed to hyperfilm-ECL autoradiography film (Amersham). The hybridization was carried out under 'relaxed' conditions in 4.38 g sodium chloride l⁻¹, 2.2 g sodium citrate l⁻¹ buffer, pH 7.0 (0.5 × SSC). The percentage similarity between the labelled DNA and the test-strain DNAs was determined by measuring the density of the dark spots formed on the developed film with a laser densitometer (LKB 2222-020 UltroScan XL).

16S rDNA amplification. The oligonucleotide primers used in this study were synthesized by Auspep (Victoria, Australia). Sequences of the primers corresponded to those within the 16S rDNA gene of *S. sanguinis* (Whiley *et al.*, 1990a). The 5' primer 5'-CTGGCTCAGGACGAACG-3' corresponded to nucleotides 18–34 of the 16S rDNA. The 3' primer 5'-GCCCCGGAACGT ATTCAC-3' corresponded to nucleotides 1375–1392 (Whiley *et al.*, 1990a). 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], 50 pmol of each primer and 2.5 units *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). 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 resuspended in 20 µl distilled water for direct sequencing.

16S rDNA sequencing and sequence analysis. The amplified 16S rDNA products were sequenced in the Prince Alfred Macromolecular Analysis Centre, University of Sydney, by using standard dye-terminator chemistry. The sequences were then aligned and the homology values were determined by using the WEBANGIS program (Australian National Genomic Information Services). The unrooted phylogenetic tree was generated by using the neighbour-joining method (ENEIGHBOUR algorithm) in the WEBANGIS software.

RESULTS

General characteristics

After incubating for 3 d on MSA plates, colonies of the new isolate were approximately 0.5–1.0 mm in diameter, rough or heaped. On blood-agar plates, strains produced small, white, rough colonies with α-haemolysis. After three or four subcultures, the colonies changed to a smooth colony type. Adherent growth to the vessel wall in broth culture was observed. The cells were Gram-positive cocci in pairs or in short to moderate chains (three to six cells per chain). The isolates were catalase-negative and grew well under both anaerobic and aerobic conditions. Growth occurred on 10 and 40% bile agar plates and also in broth of pH 9.6 and at 37 and 45 °C but not at 50 °C. There was no growth in broth with 6.5% NaCl. The terminal pH in glucose-containing broth media was 4.3–4.4.

Biochemical characteristics

Table 2 shows the biochemical characteristics of the new species, reference strains *Streptococcus suis* ATCC 43765, bovine *Streptococcus bovis* ATCC 33317, human *S. bovis* 2M2, *S. ratti* FA1 and *Streptococcus pyogenes* NCTC 8198. For all four strains of the new species (Table 2), aesculin and starch were hydrolysed but arginine was not utilized. Acetoin, GTF and FTF were not produced. The isolates were negative for pyrrolidonylaryl amidase, α-galactosidase, β-glucuronidase, β-galactosidase and alkaline phosphatase. Acid was produced from lactose, trehalose, inulin, raffinose, glycogen, amygdalin, galactose, glucose, fructose, mannose, glucosamine, arbutin, salicin, cellobiose, maltose, melibiose and sucrose. Acid was not produced from ribose, L-arabinose, mannitol, sorbitol or melezitose. The strains gave a positive reaction for the Lancefield group A antigen and negative reactions for the other grouping antigens.

API Rapid Strep identification

The biochemical profile of the isolates in API 20 Strep (4040473) was interpreted by the API LAB computer system as an unacceptable one. The presumptive identification included *S. bovis* II (34.2% identity), *S. bovis* I (34.0%), *Streptococcus pneumoniae* (11.9%), *S. sanguinis* (9.9%) and *S. suis* II (7.5%). The identification obtained by using the Rapid ID32 Strep kit

Table 2. Characteristics that differentiate *S. oristratti* sp. nov. from related streptococci

All four strains of *S. oristratti* showed the same characteristics by the tests shown. ND, Not determined.

Characteristic	<i>S. oristratti</i>	<i>S. suis</i> ATCC 43765	Bovine <i>S. bovis</i> ATCC 33317	Human <i>S. bovis</i> 2M2	<i>S. rattii</i> FA1	<i>S. pyogenes</i> NCTC 8198
Hydrolysis of:						
Arginine*	—	+	—	—	+	+
Aesculin*	+	+	+	+	+	—
Hippurate*	—	—	—	—	—	—
Starch	+	+	+	+	+	+
Production of:						
Acetoin*	—	—	+	+	+	—
Glucan	—	—	—	+	+	—
Fructan	—	—	—	+	+	—
Activity of:*						
Pyrrolidonylarylamidase	—	—	—	—	—	+
α -Galactosidase	—	+	+	—	+	—
β -Glucuronidase	—	+	—	—	—	—
β -Galactosidase	—	+	—	—	—	—
Alkaline phosphatase	—	—	—	—	—	+
Leucine arylamidase	+	+	+	+	+	+
Growth in/at:						
6.5 % NaCl	—	—	—	—	—	ND
pH 9.6	+	ND	—	—	ND	ND
45 °C	+	+	+	+	—	ND
10 % Bile	+	+	+	+	+	ND
40 % Bile	+	+	+	+	—	ND
Fermentation of:*						
Ribose	—	—	—	—	—	—
L-Arabinose	—	—	+	—	—	—
Mannitol	—	—	—	+	+	—
Sorbitol	—	—	—	—	+	—
Lactose	+	+	+	+	+	+
Trehalose	+	+	—	+	+	+
Inulin	+	+	+	+	—	—
Raffinose	+	+	+	+	+	—
Glycogen	+	+	+	+	—	+
Amygdalin	+	+	+	+	—	+
Lancefield antigen	A	D	D	—	—	A

* Data obtained from commercially available kits (API 20 Strep, Rapid ID32 Strep and API 50 CHS).

(profile 20072061111) was *S. bovis* II (61.1 %), *S. bovis* I (23.0 %) and *S. suis* II (15.7 %). When using the API 50 CHS system, the isolate was identified as *S. suis* II (98.7 %).

DNA studies

The C+G content of DNA of the isolates was 39.6–43.5 mol %. Labelled DNA from the new isolate A63^T was used as the probe for DNA–DNA hybridization. The results of DNA hybridization (Table 1) showed high similarity values (98.8–100 %) within the strains of the new species. However, DNA from various other oral streptococci showed sufficiently low DNA similarity values to the labelled

DNA (Table 1; DNA similarity ranging from 6.4 to 28.6 %) to warrant distinguishing the new species from other species of *Streptococcus*. DNA from the Lancefield group A *Streptococcus* species *S. pyogenes* NCTC 8198 had only 27.9 % relatedness. DNA from the phenotypically similar species *S. suis* ATCC 43765, *S. bovis* ATCC 33317 and human *S. bovis* 2M2 had 20.2, 27.3 and 25.8 % similarity, respectively.

The 16S rDNA sequence of strain A63^T was determined for 1337 bases. The unrooted phylogenetic tree (Fig. 1) demonstrated that the new strain was located in the cluster of the mutans streptococci group. The strain was clearly a phylogenetically distinct taxon on the basis of the sequence data, although there was a loose phenotypic affinity between the new species and

Fig. 1. Unrooted tree showing the phylogenetic relationships of *S. orisratti* to some other streptococci. Accession numbers of the sequences are shown.

S. ratti. The overall sequence homology to the nearest relative strain *S. ratti* for a continuous stretch of 1265 bases was 94.1%, corresponding to 75 nucleotide differences. This number of nucleotide differences is consistent with discrete species.

DISCUSSION

According to the biochemical characteristics, the most closely related *Streptococcus* species to the new isolates was *S. suis* (Table 2), as members of both species were Voges-Proskauer-negative, aesculin-positive, trehalose-positive, failed to grow in the presence of 6.5% NaCl and did not show β -haemolysis on sheep

blood agar (Tarradas *et al.*, 1994). *S. suis* is an important emerging cause of meningitis, pneumonia, septicaemia and arthritis in young piglets (Touil *et al.*, 1988; Windsor, 1977) and it has also been implicated in different types of infections in other animals such as ruminants and horses (Devriese *et al.*, 1990; Higgins *et al.*, 1990). In addition, meningitis caused by *S. suis* in humans has been reported (Arends & Zanen, 1988; Chau *et al.*, 1983). Both the tonsils and the nose have been shown to be carrier sites of *S. suis* in pigs. In our study, new isolates were distinguished biochemically and serologically from *S. suis* (Table 2) by the absence of arginine hydrolysis, the presence of Lancefield group A antigen, low DNA-hybridization similarity values (Table 1) and the unique 16S rDNA sequence.

Another bacterium, *S. bovis*, appeared to resemble the new species. *S. bovis* also hydrolysed aesculin, produced acid from lactose, sucrose, raffinose, amygdalin and glycogen and did not grow in 6.5% NaCl. *S. bovis* is commonly isolated from the alimentary tract of cows, sheep and other ruminants and has been shown to cause endocarditis in elderly people (Hoppe & Lerner, 1974; Moellering *et al.*, 1974). In taxonomic studies, it has been shown that many bovine strains, including the type strain, are not typical of *S. bovis* strains derived from humans (Knight & Shlaes, 1985). Two biotypes of *S. bovis* derived from humans have been recognized. Biotype I ferments mannitol, hydrolyses starch and produces glucan from sucrose, whereas biotype II (or *S. bovis* variant) gives negative results in all these tests (Facklam, 1972; Knight & Shlaes, 1985). However, the bovine type strain hydrolysed starch but failed to form acid from mannitol. The new isolates were more similar to the bovine type strain but differed in having no ability to produce acetoin and in the presence of the Lancefield group A antigen. Additionally, there was no significant genetic similarity between the new species and *S. bovis*.

Initially, due to the appearance of the rough and heaped colonies on MSA plates, the new isolates were characterized as members of the mutans streptococci group. However, more detailed biochemical experiments indicated that they differed from *S. ratti* and *S. ferus*, as they failed to ferment mannitol or sorbitol and had no GTF or FTF activities (Table 2). Moreover, the new isolates reacted with Lancefield group A antisera and had distinct 16S rDNA sequences (Fig. 1).

The new isolates possessed the Lancefield group A antigen. This antigen also occurs in some members of the '*Streptococcus milleri*' group (with others possessing the C, F or G antigen; Gossling, 1988; Whaley *et al.*, 1990b). There are three related species in the '*S. milleri*' group, *Streptococcus anginosus*, *Streptococcus intermedius* and *Streptococcus constellatus* (Whaley & Beighton, 1991; Whaley & Hardie, 1989). Members of these species have been isolated from the human oral cavity, where they have been implicated in dental caries (Drucker & Melville, 1971) and dental

abscess (Williams *et al.*, 1983). However, the new isolates were differentiated from the '*S. milleri*' group by fermentation of inulin and raffinose, absence of arginine hydrolysis and having significantly low DNA similarity values towards them (Table 1).

It has been demonstrated that the Lancefield A antigenic determinant consists of a branched rhamnose chain with a terminal *N*-acetylglucosamine (Hardie, 1986); therefore the current results imply that similar residues may be integral parts of the cell wall of the new isolates. Group A streptococci colonize the nasopharyngeal mucosa of humans, leading either to an inflammatory response followed by clinical illness or to an asymptomatic carrier state. Group A *S. pyogenes* strains are almost always β -haemolytic. The new species was α -haemolytic and was biochemically and genetically distinct from the classic group A streptococci.

On the basis of their physical, biochemical, serological and genetic characteristics, we concluded that the isolates represented a new species of oral *Streptococcus*, for which we propose the name *Streptococcus orisratti* sp. nov. This new species was isolated from the mouths of Sprague–Dawley rats. Care should be taken when Sprague–Dawley rats are used as a model to study the effect of oral bacteria on dental caries, as the new species is morphologically similar to members of the mutans streptococci on MSA plates. Further studies should be undertaken to evaluate the cariogenic potential of this isolate.

Description of *Streptococcus orisratti* sp. nov.

Streptococcus orisratti (o.ris.rat'ti. L. gen. n. *oris* of the mouth; L. gen. masc. n. *ratti* of the rat; M.L. gen. n. *orisratti* of the mouth of the rat).

Cells are facultatively anaerobic, non-motile, catalase-negative, Gram-positive cocci that occur in pairs or short chains. Colonies are white, circular or irregular, 0.5–1.0 mm in diameter and α -haemolytic on sheep-blood agar. Growth occurs at 45 °C, in 40% bile and at pH 9.6, but not in the presence of 6.5% NaCl or at 50 °C. Ferments galactose, glucose, fructose, mannose, glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, starch and glycogen, but not arabinose, mannitol, sorbitol or melezitose. The final pH in glucose-containing culture is 4.3–4.4. Aesculin and starch are hydrolysed but arginine is not. GTF and FTF are not produced. *S. orisratti* belongs to Lancefield group A. The G+C content of DNA is 39.6–43.5 mol%. Source: the tooth surface of laboratory rats. The type strain is A63^T (= ATCC 700640^T).

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