Streptococcus orisasini sp. nov. and Streptococcus dentasini sp. nov., isolated from the oral cavity of donkeys

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Four Gram-positive, catalase-negative, coccoid isolates that were obtained from donkey oral cavities formed two distinct clonal groups when characterized by phenotypic and phylogenetic studies. From the results of biochemical tests, the organisms were tentatively identified as a streptococcal species. Comparative 16S rRNA gene sequencing studies confirmed the organisms to be members of the genus Streptococcus. Two of the isolates were related most closely to Streptococcus ursoris with 95.6 % similarity based on the 16S rRNA gene and to Streptococcus ratti with 92.0 % similarity based on the 60 kDa heat-shock protein gene (groEL). The other two isolates, however, were related to Streptococcus criceti with 95.0 and 89.0 % similarities based on the 16S rRNA and groEL genes, respectively. From both phylogenetic and phenotypic evidence, the four isolates formed two distinct clonal groups and are suggested to represent novel species of the genus Streptococcus. The names proposed for these organisms are Streptococcus orisasini sp. nov. (type strain NUM 1801T = JCM 17942T = DSM 25193T) and Streptococcus dentasini sp. nov. (type strain NUM 1808T = JCM 17943T = DSM 25137T).

Oral streptococci make up a proportion of the normal flora of the oral cavity in humans and animals. Among oral streptococci, the mutants group is one of the groups of microorganisms responsible for dental caries (Hamada & Slade, 1980; Loesche, 1986). This grouping was proposed because they share similar phenotypic characteristics. Mutans streptococci usually form small, raised, rough, pale blue colonies on Mitis Salivarius Agar (MS; Difco) medium containing 5 % sucrose. The unique colonial morphology of mutans streptococci is due to the production of insoluble glucans from sucrose (Hamada & Slade, 1980). One of the causes of caries is an enzyme, glucosyltransferase (GTase), which is produced by this group of bacteria and catalyses the formation of sticky, water-insoluble glucans from sucrose. Bacteria are adhered by the glucan and continue to produce acid on the tooth surfaces, causing dental caries (Hamada & Slade, 1980; Loesche, 1986). Previously, we reported four novel mutans streptococci, Streptococcus orisuis, Streptococcus dentirousetti, Streptococcus dentapri and Streptococcus ursoris, isolated from the oral cavity of the pig, bat, wild boar and bear, respectively (Takada & Hirasawa, 2007, 2008; Takada et al., 2010; Shinozaki-Kuwahara et al., 2011). Recently Streptococcus troglodytae from a chimpanzee oral cavity was proposed as a member of the mutans group species (Okamoto et al., 2012).

To investigate the oral microflora of donkeys, MS and MS supplemented with bacitracin (0.2 units ml⁻¹) (MSB) were used to isolate Streptococcus mutans, as well as other oral streptococcal species. The four mutants streptococci-like strains on MSB selected for this study were randomly chosen from among 24 isolates of streptococci obtained from the oral cavities of five donkeys from a zoo. The strains were grown on Brain Heart Infusion (Difco) medium supplemented with 1 % yeast extract (BHI-Y; Difco) at 37 °C for 48 h in an atmosphere of 95 % N₂ and 5 % CO₂.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of Streptococcus orisasini NUM 1801T and NUM 1805 and Streptococcus dentasini NUM 1808T and NUM 1818 are AB668377 and AB771789, and AB668378 and AB771790, respectively. The accession numbers for the groEL and gyrB gene sequences of NUM 1801T are AB738937 and AB738939, respectively, and of NUM 1808T are AB738940 and AB738942, respectively.

Two supplementary figures are available with the online version of this paper.
5% CO₂. Biochemical analysis was conducted using the Rapid ID32 Strep, API 50CH and API ZYM systems (bioMérieux) according to the manufacturer’s instructions. The colony forming and biochemical characteristics resembled those of the mutans streptococci group. The four strains (NUM 1801ᵀ, NUM 1808ᵀ, NUM 1805 and NUM 1818) formed colonies similar in morphology to those of mutans-like streptococci on MS plates after 48 h at 37 °C. The phenotypic characteristics that differentiate the proposed species from closely related species and type strains of species of the genus *Streptococcus* are shown in Table 1. Strains NUM 1801ᵀ and NUM 1808ᵀ differed from *Streptococcus pyogenes* with regard to fermentation of raffinose, sorbitol and inulin and hydrolysis of aesculin. Strain NUM 1801ᵀ could be distinguished from *S. ursoris* by melibiose, D-arabitol, D-ribose and inulin fermentation, arginine hydrolysis and differences in bacitracin susceptibility. Strain NUM 1805 had the same characteristics as strain NUM 1801ᵀ. Strain NUM 1808ᵀ differed from *Streptococcus criceti* with regard to melibiose fermentation, sensitivity to bacitracin and serotype. Strain NUM 1818 had the same characteristics as strain NUM 1808ᵀ.

DNA was extracted from bacterial cultures using a Wizard Genomic DNA purification kit (Promega) according to the manufacturer’s instructions. The G+C content of the DNA was determined by HPLC using a method described previously (Hirasawa & Takada, 1994). The DNA G+C content of strains NUM 1801ᵀ and NUM 1805 ranged from 37 to 38 mol% and of strains NUM 1808ᵀ and NUM 1818 ranged from 39 to 40 mol%. To determine the phylogenetic affinity of each of the clinical isolates, almost complete 16S rRNA and housekeeping genes were sequenced and subjected to a comparative analysis. The 16S rRNA gene was amplified by PCR using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AAAGGAGGTGATCCAGGC-3') and several other primers for assembly of the gene sequence according to the method described by Shinoda et al. (2000). The partial groEL and the B subunit of DNA gyrase (gyrB) genes of the isolates were amplified by PCR using forward and reverse primer sets groF (GATGATGTAGATGGTGAA) and groR (CCTGCAACAAATCTTCTT) for the groEL gene and gyrF (GGTTCTTCGGTCGTTAATGC) and gyrY (GTTGATCGGTTCACCTTCAT) for the gyrB gene, respectively. These housekeeping gene primers were designed from the whole genome sequence of *S. mutans* UA159 (AE014133). The PCR amplified rRNA, groEL and gyrB genes from these isolates were purified using Suprec-PCR (Takara), and sequenced with a PRISM 3130 Genetic Analyzer (ABI) using a Big Dye Terminator v1.1 cycle sequencing kit (Life Technologies). The sequence data were analysed using GENETYX ver. 11 genetic information processing software (Genetyx). Previously determined 16S rRNA and housekeeping gene sequences used for comparison in this study were retrieved from the DDBJ, EMBL and GenBank nucleotide sequence databases using BLAST algorithm software. Sequence data were aligned with CLUSTAL W (Thompson et al., 1994). Nucleotide substitution rates ($K_{sub}$ values) were calculated (Kimura, 1980) and the phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou & Nei, 1987). The

| Table 1. Characteristics differentiating *S. orisasisi* sp. nov. and *S. dentasini* sp. nov. from closely related species of the genus *Streptococcus* |
|---------------------------------|---|---|---|---|---|---|---|---|
| **Characteristic**              | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Fermentation of:                |   |   |   |   |   |   |   |   |
| Melibiose                       | – | – | + | + | + | + | + | NR |
| Raffinose                       | + | + | + | + | + | + | + | – |
| D-Sorbitol                      | + | + | + | + | + | + | + | – |
| D-Arabinol                      | + | – | – | NR | – | – | NR | NR |
| D-Tagatose                      | – | + | NR | + | + | NR | NR | NR |
| Methyl β-D-glucopyranoside      | – | – | + | – | – | – | NR | – |
| Inulin                          | – | + | – | + | + | + | + | – |
| Hydrolysis of:                  |   |   |   |   |   |   |   |   |
| Aesculin                        | + | + | + | + | + | + | (+) | – |
| Arginine                        | + | + | + | – | – | – | – | – |
| Sensitivity to bacitracin (2 U ml⁻¹) | – | – | + | – | + | – | – | + |
| DNA G+C content (mol%)          | 37–38 | 39–40 | 33–35 | 41–43 | 42 | 42–44 | 42–44 | 34.5–40 |
| Serovar of mutans group         | NI | d | b | NR | d | a | NR | – |

*11–89% of strains are positive.*
Isolates NUM 1801\textsuperscript{T} and NUM 1805 showed the highest (92\%) based on the 16S rRNA gene and to that of \textit{Streptococcus ratti} (92\%) based on the \textit{groEL} gene. Isolates NUM 1808\textsuperscript{T} and NUM 1818 were determined to be related most closely to the type strain of \textit{S. cetic} (95.0 and 89\%) based on the 16S rRNA and \textit{groEL} genes, respectively. Sequence divergence between the two groups of isolates was 6.7–6.8\% for the 16S rRNA gene and 23.7–24.0\% for the \textit{groEL} gene. Analysis of partial 16S rRNA gene sequences of strains NUM 1801\textsuperscript{T} and NUM 1808\textsuperscript{T} showed less than 97\% similarity, the threshold value for defining different species, with the most closely related species (Stackebrandt & Goebel, 1994). The trees reconstructed based on the 16S rRNA and the \textit{groEL} gene sequences by the neighbour-joining method, depicting the phylogenetic affinity of strains NUM 1801\textsuperscript{T} and NUM 1808\textsuperscript{T} with members of the genus \textit{Streptococcus}, are shown in Fig. 1 (an extended version of this tree is available as Fig. S1 in IJSEM Online) and Fig. 2, respectively. The phylogenetic tree based on housekeeping gene sequences, such as the \textit{gyrB} gene, was similar to those based on partial 16S rRNA gene sequences (Fig. S2). The \textit{gyrB} gene sequence similarities of NUM 1801\textsuperscript{T} (473 bp) and NUM 1808\textsuperscript{T} (471 bp) to the type strains of \textit{S. ratti} and \textit{S. cetic} were 88\% and 79\%, respectively.

Mutans streptococci have eight different serotypes, \textit{a}–\textit{h}, based on cell surface carbohydrate antigens (Hamada & Slade, 1980; Whiley & Beighton, 1998). Furthermore, two serotypes, \textit{k} and \textit{p}, have recently been proposed as new antigens, (Nakano \textit{et al.}, 2004; Takada \textit{et al.}, 2008). The serotype was determined with an agar gel immunodiffusion test using rabbit antisera raised against reference strains prepared as described previously (Hirasawa \textit{et al.}, 1980; Takada \textit{et al.}, 1984). Rantz-Randall extract antigens were prepared from cells cultured in BHI overnight (Rantz & Randall, 1955). Immunodiffusion experiments with strains of the novel isolate, NUM 1808\textsuperscript{T}, identified the serotype \textit{d} antibody. However, the antigen from strain NUM1801\textsuperscript{T} showed that no cross-reactive precipitin bands were formed with sera prepared against any mutants streptococci serotype strains.

Mutans streptococci possess some GTases and produce water-insoluble glucans by incubation with sucrose and the culture supernatant. GTase gene (\textit{gtf}) studies and rat caries experiments involving \textit{S. orisuis} have been performed (Shinozaki-Kuwahara \textit{et al.}, 2008; Yamaguchi, 2008). The isolates NUM 1801\textsuperscript{T} and NUM 1808\textsuperscript{T} also synthesized water-insoluble glucans from sucrose.

Results of 16S rRNA gene sequence similarity calculations and phylogenetic analysis (Figs 1 and S1) clearly indicated that strains NUM 1801\textsuperscript{T}, NUM 1805, NUM 1808\textsuperscript{T} and NUM 1818 belong to the genus \textit{Streptococcus}. Phylogenetic analysis (Figs 2 and S2) and differential phenotypic properties (Table 1) distinguished strains NUM 1801\textsuperscript{T} and NUM 1805, and NUM 1808\textsuperscript{T} and NUM1818 from recognized species of the genus \textit{Streptococcus}. The clinical

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**Fig. 1.** Phylogenetic tree showing the positions of strains NUM 1801\textsuperscript{T} and NUM 1808\textsuperscript{T} and related species of the genus \textit{Streptococcus}. The tree was reconstructed by the neighbour-joining method based on 16S rRNA gene sequences. Numbers on branches indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50\%. Bar, 0.01 substitutions per nucleotide position.
isolates warrant classification as two novel species of the genus *Streptococcus* for which the names *Streptococcus orisasini* sp. nov. and *Streptococcus dentasini* sp. nov. are proposed.

**Description of Streptococcus orisasini** sp. nov.

*Streptococcus orisasini* (or.is.as.i’ni. L. gen. n. oris of the mouth; L. gen. n. asini of a donkey; L. gen. n. orisasini from the mouth of a donkey).

Cells are Gram-positive, non-spore-forming cocci occurring in pairs or short chains. Colonies on blood agar are small and white, 0.75–1.0 mm in diameter and non-haemolytic at 37 °C for 24 h. Forms small, dark blue, crinkled colonies on MS. Facultatively anaerobic and catalase negative. No Lancefield carbohydrate antigens (*Streptococcal grouping kit; Oxoid*) are detected. Using Rapid ID32 Strep and API 50CH, produces acid from glucose, galactose, fructose, lactose, D-mannose, D-mannitol, D-sorbitol, N-acetylgalactosamine, amygdalin, arbutin, aesculin, inulin, salicin, cellobiose, maltose, sucrose, D-tagatose, dulcitol, D-arabitol, raffinose and trehalose, but not from D-ribose, rhamnose, methyl β-D-glucopyranoside, melibiose, melezitose, L-arabinose or glycogen. In Rapid ID32 Strep and API ZYM, alanine phenylalanine proline arylamidase, α-glucosidase, acid phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase are produced. Arginine dihydrolase, alkaline phosphatase, β-glucuronidase, β-glucosidase, α-galactosidase, β-galactosidase and N-acetyl-β-glucosaminidase are not produced. Voges–Proskauer positive. Hippurate hydrolysis negative. Not susceptible to bacitracin. A water-insoluble glucan is produced from sucrose by glucosyltransferase. The DNA G+C content is 37–38 mol%.

The type strain is NUM 1801T (=JCM 17942T=DSM 25193T), isolated from clinical specimens from donkey oral cavities.

**Description of Streptococcus dentasini** sp. nov.

*Streptococcus dentasini* (den.ta.si’ni. L. gen. n. dens -entis a tooth; L. gen. n. asini of a donkey; L. gen. n. dentasini from the tooth of a donkey).

Cells are Gram-positive, non-spore-forming cocci occurring in pairs or short chains. Colonies on blood agar are small and white, 0.75–1.0 mm in diameter and non-haemolytic at 37 °C for 24 h. Forms small, dark blue, crinkled colonies on MS. Facultatively anaerobic and catalase negative. No Lancefield carbohydrate antigens (*Streptococcal grouping kit; Oxoid*) are detected. Using Rapid ID32 Strep and API 50CH, produces acid from glucose, galactose, fructose, lactose, D-mannose, D-mannitol, D-sorbitol, N-acetylgalactosamine, methyl β-D-glucopyranoside, amygdalin, arbutin, aesculin, inulin, salicin, cellobiose, maltose, sucrose, D-tagatose, dulcitol, D-arabitol, raffinose and trehalose, but not from D-ribose, rhamnose, methyl β-D-glucopyranoside, melibiose, melezitose, L-arabinose or glycogen. In Rapid ID32 Strep and API ZYM, alanine phenylalanine proline arylamidase, acid phosphatase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase are produced. Arginine dihydrolase, β-glucuronidase, α-galactosidase, β-galactosidase, β-glucosidase, valine arylamidase, cystine arylamidase and N-acetyl-β-glucosaminidase are not produced. Voges–Proskauer and hippurate hydrolysis positive. Not susceptible to bacitracin. A water-insoluble glucan is produced from sucrose by glucosyltransferase. Serotype d. The DNA G+C content is 39–40 mol%.
The type strain is NUM 1808T (=JCM 17943T = DSM 25137T), isolated from clinical specimens from donkey oral cavities.

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References


