Streptococcus dentirousetti sp. nov., isolated from the oral cavities of bats

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Seventy-three strains of streptococci were isolated from the bat oral cavity. The colonies of strains grown on mitis salivarius agar were similar in morphology to those of mutans-like streptococci. The novel strains were analysed biochemically using the Rapid ID32 Strep microsystem and were subjected to DNA–DNA hybridization with other oral streptococci and to 16S rRNA gene sequence analysis. Based on phylogenetic and phenotypic evidence, it is proposed that these isolates be classified as *Streptococcus dentirousetti* sp. nov. The type strain of *Streptococcus dentirousetti* sp. nov. is NUM 1303^T (=JCM 14596^T=DSM 18963^T).

The oral cavities of many animals are colonized by a large number of streptococcal species belonging to the mutans group. This grouping was proposed based on similar phenotypic characteristics. It includes eight species and eight different serotypes, which are accepted as the main bacteria responsible for dental caries. *Streptococcus mutans* (serotypes *c*, *e* and *f*) and *Streptococcus sobrinus* (*d* and *g*) are most commonly isolated from human dental plaque, *Streptococcus criceti* (*a*) has been isolated from hamsters, *Streptococcus rattii* (*b*) and *Streptococcus ferus* (*c*) have been isolated from rats, *Streptococcus downei* (*h*) and *Streptococcus macacae* (*c*) have been isolated from monkeys, and *Streptococcus orisuis* has been isolated from pigs (Beighton *et al.*, 1981; Hamada & Slade, 1980; Takada & Hirasawa, 2007; Whiley *et al.*, 1988). A novel species from equine teeth, *Streptococcus devriesei*, that displayed close affinity with mutans streptococci has also been reported (Collins *et al.*, 2004). The colonization of mutans streptococci in the oral cavity is assumed to be caused by sucrose intake. As fruit bats eat sweet fruits, they have the opportunity to take in sucrose. The microflora of the bat oral cavity was investigated in this study, focusing on mutans streptococci.

To investigate the oral microflora of bats, mitis salivarius agar (MS; Difco), which is widely used to isolate *S. mutans* as well as other oral streptococcal species, was used. The five mutans streptococci-like strains grown on MS agar that were selected for this study (NUM 1303^T, NUM 1308,

NUM 1312, NUM 1320 and NUM 1325) were chosen randomly from 73 streptococcal isolates obtained from the oral cavities of five bats from a zoo. The strains formed small, raised, adherent colonies that were irregular in margin. The strains were grown on brain–heart infusion agar (BHI; Difco) supplemented with 5 % horse blood at 37 °C under anaerobic conditions. Biochemical analysis was conducted using the Rapid ID32 Strep, API 50CH and API ZYM systems (bioMérieux) and Streptogram (Wako) according to the manufacturers' instructions. Colony formation and biochemical characteristics resembled those of the mutans streptococci group. The five isolates were subjected to further taxonomic study.

DNA was extracted from bacterial cultures using the Promega Genome kit 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 1303^T, NUM 1308, NUM 1312, NUM 1320 and NUM 1325 was 41–43 mol%. DNA–DNA hybridization was performed according to the microtitration plate method (Ezaki *et al.*, 1989) with minor modifications (Takada & Hirasawa, 2007). DNA–DNA relatedness was examined using labelled DNA from NUM 1303^T and *S. downei* NCTC 11391^T against mutans streptococci (see Supplementary Table S1, available in IJSEM Online). There were very high levels of relatedness among the five isolated strains, confirming a relationship at the species level. *S. downei* had relatively high relatedness in terms of DNA–DNA hybridization compared with other test strains, but relatedness was less than 70 % against strain NUM 1303^T. DNA from strains of the mutans streptococci *S. sobrinus*, *S. criceti*, *S. orisuis* and *S. mutans* had low levels of DNA–DNA relatedness (all less than 50 %) with strain NUM 1303^T.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NUM 1303^T is AB259061.

Phylogenetic trees derived from 16S rRNA gene sequences of strain NUM 1303^T and closely related streptococci constructed by the neighbour-joining and maximum-parsimony methods and a table giving DNA–DNA hybridization data for the five novel isolates and mutans streptococci are available with the online version of this paper.

To determine the phylogenetic affinity of each of the clinical isolates, almost the entire 16S rRNA gene was sequenced and subjected to a comparative analysis. The 16S rRNA genes of the isolates were amplified by PCR and sequenced directly using a Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (ABI PRISM model 373A; Applied Biosystems). The closest known relatives of the isolates were identified by performing database searches. Their 16S rRNA gene sequences were compared with those available at the DDBJ (<http://www.ddbj.nig.ac.jp>) using BLAST algorithms. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) and phylogenetic trees were constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987). Topology of the trees was evaluated by performing bootstrap analysis of the sequence data using CLUSTAL W software (Thompson *et al.*, 1994). Highest sequence similarity (99.0 %) was shown with *S. downei* NCTC 11391^T. The next most closely related strain was *S. sobrinus* ATCC 33478^T, with 96.8 % similarity, which is less than 97 %, the threshold value for defining different species. A tree constructed by the NJ method depicting the phylogenetic affinity of representative strains within the genus *Streptococcus* is shown in Fig. 1. Two phylogenetic trees of *Streptococci* closely related to strain NUM 1303^T constructed by the NJ method (see Supplementary Fig. S1 in IJSEM Online) and maximum-parsimony method (see Supplementary Fig. S2) showed similar evolutionary

processes for NUM 1303^T and *S. downei*. These phylogenetic trees were based on 16S rRNA gene sequences and would approximate the actual evolution process.

Serotype was determined with an agar-gel immunodiffusion test using rabbit antisera raised against reference strains prepared as described previously (Hirasawa *et al.*, 1980; Takada *et al.*, 1984). Rantz-Randle extract antigens were prepared from cells cultured overnight on BHI as described previously. Immunodiffusion experiments with isolate NUM 1303^T showed that cross-reactive precipitin bands were formed with typing sera prepared against mutans streptococci strains of serotype *a*, *d* and *g*, but not *c*, *e*, *f* or *h*. The specific antiserum obtained by absorbing antiserum raised against isolate NUM 1303^T with cells of serotype *a* and *g* reacted with NUM 1303^T and a serotype *d* strain. The antiserum raised against NUM 1303^T or serotype *d* cells reacted identically with NUM 1303^T and serotype *d*. The antiserum obtained by absorbing antiserum raised against NUM 1303^T with cells of serotype *d* did not react with NUM 1303^T. These results suggest that isolate NUM 1303^T should be grouped in serotype *d*.

An adhesive water-insoluble glucan was synthesized from sucrose by glucosyltransferase, which was produced by strain NUM 1303^T. The phenotypic characteristics that differentiate the proposed novel species from other mutans streptococci are shown in Table 1. Therefore, based on the distinct phenotypic characteristics of these novel

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain NUM 1303^T in the genus *Streptococcus*. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1330 nt. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points greater than 50 %. Bar, 0.01 substitutions per nucleotide position.

Table 1. Characteristics that differentiate species within the mutans streptococci

Species: 1, *S. dentirousetti* sp. nov.; 2, *S. downei*; 3, *S. sobrinus*; 4, *S. orisuis*; 5, *S. mutans*; 6, *S. ferus*; 7, *S. criceti*; 8, *S. rattii*; 9, *S. macacae*. Data are from Whiley & Beighton (1998), Takada & Hirasawa (2007) and this study. All of the species are positive for fermentation of mannitol, production of insoluble glucan from sucrose and the Voges–Proskauer test.

Characteristic	1	2	3	4	5	6	7	8	9
Fermentation of:									
Melibiose	–	–	–	+	+	–	+	+	–
Raffinose	–	–	+	+	+	–	+	+	+
Sorbitol	–	–	+	+	+	+	+	+	+
Hydrolysis of:									
Aesculin	+	–	+	+	+	–	+	+	–
Arginine	–	–	–	–	–	–	–	+	–
Inulin	–	+	–	+	+	+	+	+	–
Sensitive to bacitracin (2 U ml ^{–1})	+	+	–	–	–	+	+	–	+
Serovar	<i>d</i>	<i>h</i>	<i>d, g</i>	<i>d</i>	<i>c, e, f</i>	<i>c</i>	<i>a</i>	<i>b</i>	<i>c</i>
DNA G + C content (mol%)	41–43	41–42	44–46	42–44	36–38	43–45	42–44	41–43	35–36
Source	Bat	Monkey	Human	Pig	Human	Wild rats	Hamster, human, wild rats	Rat, human	Monkey

coccus-shaped isolates and the molecular chemical and molecular genetic evidence, it is believed that strains NUM 1303^T, NUM 1308, NUM 1312, NUM 1320 and NUM 1325 warrant classification as members of a novel species of the genus *Streptococcus* and of the mutans streptococci, for which the name *Streptococcus dentirousetti* sp. nov. is proposed.

Description of *Streptococcus dentirousetti* sp. nov.

Streptococcus dentirousetti (den.ti.rou.set'ti. L. gen. n. *dentis* of the tooth; N.L. gen. masc. n. *rousetti* off/from *Rousettus*, a genus of fruit-eating bats; N.L. gen. n. *dentirousetti* from/of the tooth of bats of the genus *Rousettus*).

Cells are Gram-positive, non-spore-forming cocci, 0.5–0.75 µm in diameter, 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. Forms small, dark blue, crinkled colonies on MS agar. Facultatively anaerobic and catalase-negative. No Lancefield carbohydrate antigens (Streptococcal grouping kit; Oxoid) are detected. Strains produce acid from D-glucose, D-galactose, D-fructose, D-mannose, D-mannitol, N-acetylglucosamine, aesculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose and D-tagatose. Alanine phenylalanine proline arylamidase, β-glucosidase, α-glucosidase, α-galactosidase, acid phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase are produced. Arginine dihydrolase, β-glucuronidase, alkaline phosphatase, β-galactosidase and N-acetyl-β-glucosaminidase are not produced. Positive for the Voges–Proskauer test. Negative for the hippurate hydrolysis test. Susceptible to bacitracin. An adhesive insoluble glucan is produced by glucosyltransferase from sucrose. Cariogenic when mono-associated with germ-free rats. The serotype is type *d*. The DNA G + C content of the type strain is 42 mol%.

The type strain, NUM 1303^T (=JCM 14596^T=DSM 18963^T), was isolated from clinical specimens from the bat oral cavity.

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