Streptococcus orisuis sp. nov., isolated from the pig oral cavity

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Five bacterial strains, designated as NUM 1001^T, NUM 1002, NUM 1003, NUM 1004 and NUM 1005, were isolated from the oral cavities of pigs. Colonies grown on mitis salivarius agar were similar in morphology to those of mutans streptococci. The novel isolates were analysed biochemically using the Rapid ID 32 Strep microsystem, subjected to DNA–DNA hybridization with oral streptococci and had their 16S rRNA genes sequenced. On the basis of the phylogenetic and phenotypic evidence obtained, the strains represent a novel species of the genus *Streptococcus*, for which the name *Streptococcus orisuis* sp. nov. is proposed. The type strain is NUM 1001^T (=JCM 14035^T=DSM 18307^T).

The mutans streptococci, comprising seven species and eight different serotypes, are generally accepted as the main bacterial causes of dental caries. *Streptococcus mutans* (serotypes *c*, *e* and *f*) and *Streptococcus sobrinus* (serotypes *d* and *g*) are most commonly isolated from human dental plaque, *Streptococcus criceti* (serotype *a*) has been isolated from hamsters, *Streptococcus rattii* (serotype *b*) and *Streptococcus ferus* (serotype *c*) have been isolated from rats and *Streptococcus downei* (serotype *h*) and *Streptococcus macacae* (serotype *c*) have been isolated from monkeys (Beighton *et al.*, 1981; Hamada & Slade, 1980; Whiley *et al.*, 1988). Recently, a novel species displaying close affinity with mutans streptococci from equine teeth was reported (Collins *et al.*, 2004). The colonization of the oral cavity by mutans streptococci is assumed to be caused by sucrose intake. As pigs are omnivorous animals, they often have the opportunity to ingest sucrose. We investigated the microflora of the porcine oral cavity, focusing on mutans streptococci.

To investigate the oral microflora of pigs, a group of Gram-positive cocci were characterized. Mitis salivarius agar (Difco) is widely used to isolate *S. mutans* as well as other oral streptococcal species. The five mutans streptococcus-like strains selected for this study were randomly chosen from among 117 streptococcal isolates obtained on mitis salivarius agar from the oral cavities of 39 pigs (from two different farms). The strains formed small, raised, adherent colonies with irregular margins. The strains were grown at 37 °C under anaerobic conditions on brain-heart infusion agar (Difco) supplemented with 5 % horse blood. A biochemical analysis was conducted using the Rapid ID32 Strep, API 50 CH and API ZYM (bioMérieux) and Streptogram (Wako) systems according to the manufacturers'

instructions. The colonial formation and biochemical characteristics resembled those of mutans streptococci. Isolates NUM 1001^T, NUM 1002, NUM 1003, NUM 1004 and NUM 1005 were subjected to further taxonomic study.

DNA was extracted from bacterial cultures by using the Promega Genome kit according to the manufacturer's instructions. The G + C contents of the DNAs were determined by HPLC using a method described previously (Hirasawa & Takada, 1994). The DNA G + C contents of strains NUM 1001^T, NUM 1002, NUM 1003, NUM 1004 and NUM 1005 ranged from 42 to 44 mol %, which is similar to that for *S. criceti*. DNA–DNA hybridization was performed according to the microtitration plate method (Ezaki *et al.*, 1989), with minor modifications. A heat-denatured sample of DNA (1 µg) was immobilized in each well of a microplate (Immuno plate II; Nunc) at 30 °C for 2 h. The microplate was dried at 45 °C for 2 h and then photobiotin-labelled heat-denatured probe DNA (0.125 µg per well) was used for the hybridization (incubated at 42.5 °C for 3 h). Other procedures were conducted according to the original instructions. Levels of DNA–DNA hybridization were examined using labelled DNA from strain NUM 1001^T and *S. criceti* ATCC 19642^T against other strains of mutans streptococci. There were very high levels of hybridization among the five isolates and between *S. criceti* strains ATCC 19642^T and OMZ 61 relative to each labelled strain, confirming a relationship at the species level. The highest level of DNA–DNA hybridization was found with *S. criceti* ATCC 19642^T, but the level of relatedness was only 42.5–58.1 %. DNAs from strains of mutans streptococci, namely *S. downei* NCTC 11391^T, *S. sobrinus* NIDR6715, *S. rattii* ATCC 19645^T and *S. mutans* JC2, exhibited relatedness levels of 21.4, 12.8, 7.4 and 7.2 %, respectively, to strain NUM 1001^T. To determine the phylogenetic affinity of each of the clinical isolates, the almost-complete 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NUM 1001^T is AB182324.

Fig. 1. Neighbour-joining phylogenetic tree, derived from 16S rRNA gene sequences, showing the position of strain NUM 1001^T with respect to members of the genus *Streptococcus*. The tree is based on a comparison of approximately 1330 nt. Bootstrap percentages (based on 1000 replicates), where greater than 50 %, are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

was sequenced and subjected to a comparative analysis. The 16S rRNA gene was amplified by using a PCR with primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3'; *Escherichia coli* positions 8–27) and 1525r (5'-AAAGGAGGTGATCCAGCC-3'; *E. coli* positions 1543–1525) according to the method described by Shinoda *et al.* (2000). The PCR products were purified with a Mini Elute gel extraction kit (Qiagen) and

directly sequenced using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (ABI PRISM, model 373A; Applied Biosystems) by Takara Bio (Mie, Japan). The closest known relatives of the novel isolates were identified by performing database searches. Their 16S rRNA gene sequences were compared with those available in the DNA Data Bank of Japan (Mishima, Japan) using BLAST software. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) and phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987). The topologies of the trees were evaluated by performing a bootstrap analysis of the sequence data, using CLUSTAL W software (Thompson *et al.*, 1994). Sequence similarity values were calculated manually. The highest levels of 16S rRNA gene sequence similarity to strain NUM 1001^T were obtained with *S. criceti* ATCC 19642^T and *S. downei* NCTC 11391^T (97.8 and 94.1 %, respectively). Despite the relatively high levels of sequence similarity among *S. criceti* ATCC 19642^T, *S. downei* NCTC 11391^T and NUM 1001^T compared with the levels of similarity among representatives of the other species within the mutans streptococci, the results show that the pig strains are sufficiently dissimilar from *S. criceti* and *S. downei* to warrant separate species status. A tree constructed by neighbour-joining, depicting the phylogenetic affinity of strain NUM 1001^T with members of the genus *Streptococcus*, is shown in Fig. 1.

Serotypes were 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 brain-heart infusion agar as described previously. Fig. 2 shows the double immunodiffusion patterns obtained with the Rantz-Randle extract antigens and various antibodies. In immunodiffusion experiments with strain NUM 1001^T, cross-reactive precipitin bands were formed with typing

Fig. 2. Double immunodiffusion analyses of antigen preparations with various antisera. Wells designated a, b, c, d and e contain anti-NUM 1001^T serum, anti-NUM 1001^T absorbed with cells of *S. criceti* HS-6 (serotype a) serum, anti-NUM 1001^T absorbed with cells of *S. sobrinus* NIDR6715 (g) serum, anti-NUM 1001^T absorbed with cells of *S. sobrinus* OMZ-176 (d) serum and anti-OMZ-176 serum, respectively. Antigens designated 1, 2, 3 and 4 were extracted from cells of NUM 1001^T, HS-6, OMZ-176 and NIDR6715, respectively.

Table 1. Characteristics that differentiate species within the mutans streptococci

Taxa: 1, strain NUM 1001^T (*S. orisuis* sp. nov.); 2, *S. mutans*; 3, *S. ferus*; 4, *S. criceti*; 5, *S. sobrinus*; 6, *S. rattii*; 7, *S. downei*; 8, *S. macacae*. Data are from Whiley & Beighton (1998) and this study. All of the taxa are positive for fermentation of mannitol and for production of insoluble glucan from sucrose and all give positive results in the Voges–Proskauer test.

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

sera prepared against mutans streptococci strains of serotype *a*, *d* or *g* (Fig. 2, left panel) but not *c*, *e* or *f*. The specific antiserum obtained by absorbing antiserum raised against novel isolate NUM 1001^T with cells of serotype *a* and *g* reacted with NUM 1001^T and a serotype *d* strain (Fig. 2, centre panel). The antiserum raised against NUM 1001^T or serotype *d* cells reacted identically with NUM 1001^T and serotype *d* (Fig. 2, right panel). The antiserum obtained by absorbing antiserum raised against NUM 1001^T with cells of serotype *d* did not react with NUM 1001^T. These results suggest that isolate NUM 1001^T should be grouped within serotype *d*.

Strain NUM 1001^T isolated from the porcine oral cavity is serologically unique. Biochemically, too, it is readily distinguishable from all other previously described mutans streptococci (Hamada & Slade, 1980). The phenotypic characteristics that differentiate strain NUM 1001^T from other mutans streptococci are shown in Table 1. Therefore, on the basis of the distinct phenotypic characteristics and biochemical and molecular genetic data obtained in this study, strain NUM 1001^T warrants classification within a novel species of the genus *Streptococcus*, and a member of the mutans streptococci, for which the name *Streptococcus orisuis* sp. nov. is proposed.

Description of *Streptococcus orisuis* sp. nov.

Streptococcus orisuis (o.ri.su'is. L. gen. n. *oris* of the mouth; L. gen. n. *su*is of a pig; N.L. gen. n. *orisuis* from/of the mouth of a pig).

Cells are Gram-positive, non-spore-forming cocci, 0.5–0.75 µm in diameter, that occur in pairs or in short chains.

Colonies on blood agar are small and white, 0.75–1.0 mm in diameter and non-haemolytic at 37 °C. On mitis salivarius agar, colonies are small, dark blue and crinkled. Facultatively anaerobic and catalase-negative. No Lancefield carbohydrate antigens (streptococcal grouping kit; Oxoid) are detected. According to API systems, strains produce acid from D-glucose, galactose, D-fructose, D-mannose, mannitol, sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, D-raffinose, β-gentiobiose, methyl β-D-glucopyranoside and D-tagatose. Alanine phenylalanine proline arylamidase, β-glucosidase, α-glucosidase, α-galactosidase, acid phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, naphthol-AS-BI-phosphohydrolase and glucosyltransferase are produced. Arginine dihydrolase, β-glucuronidase, alkaline phosphatase, β-galactosidase and *N*-acetyl-β-glucosaminidase are not produced. Voges–Proskauer test is positive. Hippurate hydrolysis test is negative. Resistant to bacitracin. An adhesive, insoluble glucan is produced from sucrose. The bacterium is cariogenic when monoassociated with specific pathogen-free rats. The serotype is type *d*. The DNA G + C content of the type strain is 43 mol %.

The type strain, NUM 1001^T (=JCM 14035^T=DSM 18307^T), was isolated from clinical specimens from the oral cavities of pigs.

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