Streptococcus dentapri sp. nov., isolated from the wild boar oral cavity

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Four Gram-stain-positive, catalase-negative, coccoid-shaped isolates were obtained from the oral cavities of wild boars and characterized by phenotypic and phylogenetic studies. On the results of biochemical tests, the organisms were tentatively identified as a streptococcal species.

Comparative 16S rRNA gene sequencing studies confirmed that the organisms are members of the genus *Streptococcus*, with *Streptococcus equi* subsp. *equi* ATCC 33398^T as their closest phylogenetic relative (94.7% similarity). DNA–DNA hybridization analysis showed that the isolates displayed less than 10% relatedness to *Streptococcus equi* subsp. *equi* DSM 20561^T. From the phylogenetic and phenotypic evidence, the four isolates represent a novel species of the genus *Streptococcus*, for which the name *Streptococcus dentapri* sp. nov. (type strain NUM 1529^T = JCM 15752^T = DSM 21999^T) is proposed.

Oral streptococci make up a proportion of the normal flora of the oral cavity in humans and animals. Among oral streptococci, the mutans group is one of the groups of micro-organisms responsible for dental caries (Hamada & Slade, 1980; Loesche, 1986). This group was proposed because its members share similar phenotypic characteristics. One of the causes of caries is an enzyme, glucosyltransferase (GTase), which is produced by this group of bacteria. GTase catalyses the formation of sticky water-insoluble glucans from sucrose. The glucans adhere oral bacteria to teeth and so lead to the formation of dental plaque. The bacteria continue to produce acid on the tooth surfaces and cause dental caries (Hamada & Slade, 1980; Loesche, 1986). Previously, we reported a novel mutans streptococci, *Streptococcus orisuis*, isolated from the oral cavity of a pig (Takada & Hirasawa, 2007). GTase gene (*gtf*) studies and rat caries experiments involving *S. orisuis* have been performed (Shinozaki-Kuwahara *et al.*, 2008; Yamaguchi, 2008). *Streptococcus dentirousetti* (Takada & Hirasawa, 2008), isolated from the bat oral cavity, showed a *gtf* gene sequence similar to that

of *S. orisuis* (unpublished GenBank accession no. AB355819). The colonization of the oral cavity by mutans streptococci is assumed to result from sucrose intake. As wild boars are omnivorous animals, they will probably have the opportunity to ingest sucrose. We investigated the microflora of the oral cavities of wild boars, focusing on mutans streptococci.

To investigate the oral microflora of wild boars, mitis salivarius agar (MS agar; Difco) and MS agar supplemented with 0.2 U bacitracin ml⁻¹ (MSB agar) are widely used to isolate *Streptococcus mutans* as well as other oral streptococcal species. The four mutans streptococcus-like strains on MSB agar selected for this study were chosen randomly from 38 isolates of streptococci obtained from the oral cavities of eight generally healthy wild boars (three from Aomori and five from Tokushima prefectures in Japan). The strains were grown on brain heart infusion (BHI; Difco) agar supplemented with 5% horse blood at 37 °C in an atmosphere of 95% N₂ and 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 1529^T and NUM 1530–NUM 1532) formed small, raised adherent colonies with irregular margins and dark-blue colour on MS agar plates after 48 h at 37 °C.

Abbreviation: GTase, glucosyltransferase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NUM 1529^T is AB469560.

DNA–DNA hybridization values for strain NUM 1529^T and related streptococci are available as supplementary material with the online version of this paper.

Mutans streptococci usually form small, raised, rough, pale-blue colonies on MS agar, which contains 5 % sucrose. The unique colonial morphology of mutans streptococci is due to the production of insoluble glucans from sucrose (Hamada & Slade, 1980). Colonies of the isolates grown on MS agar were similar in morphology to those of mutans-like streptococci. A water-insoluble material was produced following incubation with sucrose, and the culture supernatant was analysed by colorimetric assay (Van Handel, 1967; Fukushima *et al.*, 1981). An adhesive, water-insoluble glucan was synthesized from sucrose by GTase, which was produced by strain NUM 1529^T.

DNA was extracted from bacterial cultures using the 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 G+C content of strains NUM 1529^T and NUM 1530–NUM 1532 ranged from 39 to 41 mol%. To determine the phylogenetic affinity of each of the isolates, almost the entire 16S rRNA gene of each strain was sequenced and subjected to a comparative analysis, as described previously (Takada & Hirasawa, 2008). Previously determined 16S rRNA gene sequences used for comparisons in this study were retrieved from the DDBJ, EMBL and GenBank nucleotide sequence databases. Sequence data were aligned with CLUSTAL W (Thompson *et al.*, 1994) and the alignment was corrected by manual inspection. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura, 1980) after gaps and unknown bases were eliminated. The phylogenetic tree was constructed by employing the neighbour-joining method (Saitou & Nei, 1987).

Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the reliability of the topologies. Strain NUM 1529^T exhibited high 16S rRNA gene sequence similarities with members of the pyogenic group (*S. equi* subsp. *equi* ATCC 33398^T, 94.7%; *S. equi* subsp. *zooepidemicus* ATCC 43072^T, 94.0%) and the mutans group (*S. orisuis* NUM 1001^T, 94.1%; *S. ferus* ATCC 33477^T, 93.9%; *S. ratti* ATCC 19645^T, 93.7%; *S. macacae* ATCC 35911^T, 92.9%). A neighbour-joining tree depicting the phylogenetic position of strain NUM 1529^T within the genus *Streptococcus* is shown in Fig. 1. The topology of this phylogenetic tree, together with comparative 16S rRNA gene sequence analysis, gave divergence values of <7 % between the novel isolate and its nearest recognized relatives and mutans streptococci group. The results of phylogenetic analysis show strain NUM 1529^T grouping with many of the organisms considered members of the mutans group (Fig. 1).

DNA–DNA hybridization was performed according to the microtitre plate method (Ezaki *et al.*, 1989) with minor modifications (Takada & Hirasawa, 2007). DNA–DNA relatedness was examined using labelled DNA from strain NUM 1529^T with related type strains (Supplementary Table S1, available in IJSEM Online). There were very high levels of relatedness among the four isolated strains, confirming a relationship at the species level. DNA–DNA relatedness values between strain NUM 1529^T and closely related type strains of *Streptococcus* species showed low levels of relatedness (less than 12 %).

Mutans streptococci have eight different serotypes, *a–h*, based on the cell-surface carbohydrate antigens (Hamada

Fig. 1. Phylogenetic tree showing the positions of strain NUM 1529^T and related members of the genus *Streptococcus*, based on 16S rRNA gene sequences and constructed with the neighbour-joining method. Bootstrap percentages (>50 %) based on 1000 resamplings are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

& Slade, 1980; Whiley & Beighton, 1998). Two more serotypes, *k* and *p*, have recently been proposed (Nakano *et al.*, 2004; Takada *et al.*, 2008). The serotype of strain NUM 1529^T 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–Randall-extract antigens (Rantz & Randall, 1955) were prepared from cells of overnight BHI cultures. Immunodiffusion experiments with strain NUM 1529^T identified only the serotype *c* antibody. No cross-reactive precipitin bands were formed with sera prepared against other serotypes. These results suggest that the novel isolate NUM 1529^T should be grouped in serotype *c*. The presence of Lancefield carbohydrate antigens was also tested (streptococcal grouping kit; Oxoid), and none were detected.

The phenotypic characteristics that differentiate the proposed species from closely related species are shown in Table 1. Based on the distinct phenotypic and genotypic characteristics and the phylogenetic evidence, we believe that the four isolates warrant classification in a novel species of the genus *Streptococcus*, as a member of the mutans streptococci, for which the name *Streptococcus dentapri* sp. nov. is proposed.

Description of *Streptococcus dentapri* sp. nov.

Streptococcus dentapri (den.tap'ri. L. n. *dens*, -tis tooth; L. masc. n. *aper*, *apri* wild boar; N.L. gen. n. *dentapri* from the tooth of a wild boar).

Table 1. Characteristics differentiating strain NUM 1529^T from closely related species of the genus *Streptococcus*

Taxa: 1, *Streptococcus dentapri* sp. nov. NUM 1529^T; 2, *S. equi* subsp. *equi* DSM 20561^T; 3, *S. equi* subsp. *zooepidemicus* DSM 20727^T; 4, *S. orisuis* NUM 1001^T; 5, *S. ferus*; 6, *S. rattii*; 7, *S. macacae*. Data in columns 1–4 were obtained in this study; data in columns 5–7 were taken from Whiley & Beighton (1998). NA, Not applicable.

Characteristic	1	2	3	4	5	6	7
Fermentation of:							
Lactose	–	–	+	+	+	+	+
Mannitol	+	–	–	+	+	+	+
Melibiose	–	–	–	+	–	+	–
Raffinose	–	–	–	+	–	+	+
Sorbitol	+	–	+	+	+	+	+
Trehalose	+	–	–	+	+	+	+
Hydrolysis of:							
Aesculin	+	–	–	+	–	+	–
Arginine	–	+	+	–	–	+	–
Voges–Proskauer test	+	–	–	+	+	+	+
Lancefield antigen	None	C	C	None	None	None	None
Serovar of mutans group	<i>c</i>	NA	NA	<i>d</i>	<i>c</i>	<i>b</i>	<i>c</i>

Cells are Gram-stain-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. The cells form small, dark-blue, crinkled colonies on MS agar. Facultatively anaerobic and catalase-negative. No Lancefield carbohydrate antigens are detected. With Rapid ID32 Strep and API 50CH, produces acid from glucose, fructose, D-mannose, D-mannitol, D-sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose and trehalose, but not from galactose, lactose, D-ribose, raffinose, melibiose or tagatose. With Rapid ID32 Strep and API ZYM, alanine-phenylalanine-proline arylamidase, α - and β -glucosidases, acid and alkaline phosphatases, leucine, valine and cystine arylamidases and naphthol-AS-BI-phosphohydrolase are produced. Arginine dihydrolase, β -glucuronidase, α - and β -galactosidases and *N*-acetyl- β -glucosaminidase are not produced. Voges–Proskauer test is positive. Hippurate hydrolysis test is negative. Not susceptible to bacitracin. A water-insoluble glucan is produced by glucosyltransferase from sucrose. The serotype is type *c*. The DNA G + C content of the type strain is 39.9 mol%.

The type strain is NUM 1529^T (=JCM 15752^T =DSM 21999^T), isolated from the oral cavity of a wild boar.

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