Streptococcus ursoris sp. nov., isolated from the oral cavities of bears

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Three Gram-positive, catalase-negative, coccus-shaped organisms were isolated from the oral cavities of bears. The isolates were tentatively identified as a streptococcal species based on the results of biochemical tests. Comparative 16S rRNA gene sequencing studies confirmed that the organisms were members of the genus *Streptococcus*, but they did not correspond to any recognized species of the genus. The nearest phylogenetic relative of the new isolates was *Streptococcus rattus* ATCC 19645^T (98.6%), however, DNA–DNA hybridization analysis showed that the isolates displayed less than 15% DNA–DNA relatedness with the type strain of *S. rattus*. Colonies of the novel strains grown on mitis salivarius agar showed an extracellular polysaccharide-producing colony morphology. Based on phenotypic and phylogenetic evidence, it is proposed that the novel isolates are classified in the genus *Streptococcus* as *Streptococcus ursoris* sp. nov. The type strain of *S. ursoris* is NUM 1615^T (=JCM 16316^T=DSM 22768^T).

The genus *Streptococcus* embraces a broad range of Gram-positive, facultatively anaerobic, catalase-negative, chain-forming and coccus-shaped organisms. A large number of streptococcal species colonize the oral cavities of animals and humans and these oral streptococci have been divided into five major groups (Facklam, 2002). Among oral streptococci, the mutans group is responsible for dental caries (Hamada & Slade, 1980). These bacteria are known to form characteristic colonies on mitis salivarius (MS) agar plates demonstrating extracellular polysaccharide synthesis. In humans, it is proposed that water-insoluble glucan, which is synthesized by the action of glucosyl-transferase (GTF) from sucrose as the substrate, is one of the most important cariogenic factors (Hamada & Slade, 1980). Several kinds of mutans streptococcal species, which can synthesize glucans, have been isolated from the oral cavities of various animals, for example, hamster, rat, monkey, pig, bat and wild boar (Beighton *et al.*, 1981; Whaley & Beighton, 1998; Takada & Hirasawa, 2007, 2008, 2010). Bears are known to be omnivorous animals and may have the chance to ingest sucrose in the form of sweet fruits, such as berries, and in honey. Dental caries have been reported in the oral cavities of wild bears (Manville, 1990). In this study, three previously unidentified strains isolated from the oral cavity of Japanese black bears (*Ursus*

thibetanus japonicus) and capable of glucan synthesis on MS agar were characterized.

Examination of the oral microflora of bears was performed on MS agar (BD Difco). MS agar is widely used to isolate strains of *Streptococcus mutans* as well as other oral streptococcal species. Three streptococci-like strains (NUM 1610, NUM 1615^T and NUM 1619) obtained from samples from the oral cavities of wild bears were isolated on MS agar. The isolated strains formed small, raised, adherent colonies with irregular margins. The cocci stained as Gram-positive. The strains were grown on brain heart infusion (BHI, BD Difco) agar supplemented with 5% horse blood at 37 °C under an atmosphere of 95% N₂ and 5% CO₂ with non-haemolytic activity. Catalase activity was found to be negative. The organisms were characterized biochemically using the Rapid ID32 Strep, API 50 CH and API ZYM systems according to the manufacturer's instructions (bioMérieux). The colony formation resembled that of members of the mutans streptococcus group, although the biochemical characteristics did not correspond to any recognized species of the genus *Streptococcus*. The new isolates were subjected to further genetic studies.

Serological analyses of isolates were conducted by agar-gel immunodiffusion methods using rabbit antisera raised against reference strains of mutans streptococci prepared previously (Hirasawa *et al.*, 1980; Takada *et al.*, 1984). Rantz-Randall extract antigens were prepared from cells cultured overnight on BHI as described previously (Rantz & Randall, 1955). Immunodiffusion experiments with isolate NUM 1615^T showed that no cross-reacted precipitin bands formed with any sera prepared against mutans

Abbreviations: GTF, glucosyltransferase; MS, mitis salivarius.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *groEL* gene sequences of strain NUM 1615^T are AB501126 and AB541991, respectively.

Supplementary figures and a supplementary table are available with the online version of this paper.

Table 1. Characteristics that differentiate strain NUM 1615^T from closely related streptococcal species

Taxa: 1, NUM 1615^T; 2, *S. ratti* ATCC 19645^T; 3, *S. devriesei* CCUG 47155^T; 4, *S. mutans* NCTC 10449^T; 5, *S. ferus* ATCC 33477^T; 6, *S. macacae* NCTC 11558^T. Data for taxa 2, 4, 5 and 6 are from Whiley & Beighton (1998). Data for taxon 3 are from Collins *et al.* (2004) and the present study. All taxa give a positive reaction in the Voges–Proskauer test and are positive in tests for the hydrolysis of aesculin and the fermentation of mannitol and sorbitol. +, Positive; –, negative; ND; not detected.

Characteristic	1	2	3	4	5	6
Hydrolysis of:						
Arginine	+	+	–	–	–	–
Fermentation of:						
Inulin	–	+	+	+	+	–
Melibiose	+	+	+	+	–	–
Raffinose	+	+	+	+	–	+
D-Ribose	+	–	–	–	–	–
Starch	–	–	–	–	+	–
Sensitivity to bacitracin (2 U ml ^{–1})	+	–	+	–	+	+
Serovar(s)	ND	<i>b</i>	ND	<i>c, e, f</i>	<i>c</i>	<i>c</i>
GTF activity	+	+	–	+	+	+
DNA G + C content (mol%)	33–35	41–43	42	36–38	43–45	35–36
Source	Bear	Rat, human	Horse	Human	Rat	Monkey

streptococci strains of serotypes *a* to *h*, suggesting that strain NUM 1615^T could not be grouped with any known serotype of *mutans* streptococci. The Lancefield grouping test was performed using the Streptococcal grouping kit (Oxoid). No Lancefield carbohydrate antigens were detected from strain NUM 1615^T. Strain NUM 1615^T was able to synthesize adhesive water-insoluble glucan from sucrose as the substrate and showed sucrose-dependent colonization by the action of GTF enzymes. The phenotypic characteristics that differentiated the new strains from closely related streptococcal species are shown in Table 1.

To determine the G + C contents, DNA–DNA relatedness and 16S rRNA gene sequences of the isolated strains, chromosomal DNA was extracted and purified as described previously (Shiroza *et al.*, 1998). Measurement of the G + C content of the DNA was carried out by HPLC according to the method of Hirasawa & Takada (1994). The G + C contents of the three strains ranged from 33 to 35 mol% and were similar to those previously determined for *Streptococcus macacae* and *S. mutans*. To assess the

phylogenetic position of the newly isolated strains, their 16S rRNA genes were amplified by PCR using primers described previously by Takada & Hirasawa (2007), directly sequenced with an ABI PRISM 3130 Genetic Analyzer using a Big Dye Terminator v1.1 Cycle Sequencing kit (Life Technologies Co.) and then subjected to a comparative analysis. The closest known relatives of the new isolates were determined by performing DDBJ/EMBL/GenBank database searches. The 16S rRNA gene sequences were compared using BLAST algorithms. Distance matrices were determined following the assumptions described by Kimura (1980). Phylogenetic trees were constructed according to the neighbour-joining method (Saitou & Nei, 1987) and the topology of the tree was estimated by bootstrap analysis using the CLUSTAL W program (Thompson *et al.*, 1994). Highest sequence similarities were obtained with *Streptococcus ratti* ATCC 19645^T (98.6%). The next most closely related strains were *Streptococcus devriesei* CCUG 47155^T (96.0%) and *S. mutans* NCTC 10449^T (95.1%), both of which were below the 97% threshold value for the definition of separate species. The phylogenetic tree constructed by the

Fig. 1. Phylogenetic tree of members of the genus *Streptococcus* inferred from comparison of 16S rRNA gene sequences by the neighbour-joining method. Bar, 0.01 substitutions per nucleotide position. An extended version of this tree is available as Supplementary Fig. S1.

neighbour-joining method (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online) revealed a clear affiliation between strain NUM

1615^T and the genus *Streptococcus*. The phylogenetic trees constructed by the neighbour-joining and maximum-parsimony methods (see Supplementary Fig. S2 in IJSEM

Fig. 2. Phylogenetic tree based on 757 bp of *groEL* gene fragment sequences constructed by using the neighbour-joining method. Bootstrap values >50 % calculated for 1000 subsets are shown at branch-points. Bar, 0.01 substitutions per nucleotide position.

Online) showed a similar evolutionary process for strain NUM 1615^T and *S. rattii* ATCC 19645^T. A phylogenetic tree (Fig. 2) constructed by the neighbour-joining method with partial sequences of the *groEL* (757 bp) gene (Glazunova *et al.*, 2009) also confirmed the phylogenetic placement of representative strains within the genus *Streptococcus*.

DNA–DNA hybridization experiments were performed as previously described (Takada & Hirasawa, 2007). DNA–DNA relatedness was examined by labelled DNA from strain NUM 1615^T with unlabelled single-stranded DNA from related streptococci (see Supplementary Table S1 in IJSEM Online). The three new isolates showed DNA–DNA relatedness values of >70% with each other and therefore these results confirmed that the isolates belonged to the same species (Johnson, 1973). Since the three isolates exhibited high degrees of relatedness, their relationship at the strain level was confirmed. *S. rattii* ATCC 19645^T showed a high 16S rRNA gene sequence similarity to strain NUM 1615^T, but the DNA–DNA relatedness value between the two strains was only 13.3%. The DNA from other streptococci, such as *S. devriesei* and *S. mutans*, showed low levels of DNA–DNA relatedness to strain NUM 1615^T (all <32%).

Therefore, based on phenotypic and phylogenetic criteria, it is considered that strains NUM 1615^T, NUM 1610 and NUM 1619, isolated from wild bears, warrant classification as a novel species of the genus *Streptococcus*, for which the name *Streptococcus ursoris* sp. nov. is proposed.

Description of *Streptococcus ursoris* sp. nov.

Streptococcus ursoris (urs.o'ris. L. n. *ursus* -i bear; L. n. *os oris* mouth; N.L. gen. n. *ursoris* of the mouth of a bear).

Cells stain Gram-positive, are non-spore-forming cocci, 0.5–0.7 µm in diameter and occur in pairs or short chains. Colonies are white, non-haemolytic and 0.75–1.0 mm in diameter after incubation on blood agar at 37 °C for 24 h. On MS agar, colonies appear small, dark blue and crinkled. Facultatively anaerobic and catalase-negative. Lancefield carbohydrate antigens are not detected. Acid is produced from D-glucose, D-fructose, D-galactose, D-mannose, mannitol, sorbitol, N-acetylglucosamine, salicin, cellobiose, maltose, lactose, sucrose, trehalose, raffinose, D-tagatose D-ribose and melibiose, but not from D-arabinose, D-xylose, glycogen, inulin or starch. Aesculin is hydrolysed, but hippurate is not. Voges–Proskauer test is positive. Enzyme activities are detected for arginine dihydrolase, alanyl-phenylalanyl-proline arylamidase, β-glucosidase, α-glucosidase, α-galactosidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, valine arylamidase, cystine arylamidase and leucine arylamidase. β-Galactosidase, β-glucuronidase, alkaline phosphatase, N-acetyl-β-glucosaminidase and urease are not produced. Sensitive to bacitracin. In BHI broth, there is no apparent growth at 45 °C and the GTF enzyme is produced.

The type strain, NUM 1615^T (=JCM 16316^T=DSM 22768^T), was isolated from clinical specimens obtained

from the oral cavity of a bear. The DNA G + C content of the type strain is 34 mol%.

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