Streptococcus dentiloxodontae sp. nov., isolated from the oral cavity of elephants

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A Gram-stain-positive, catalase-negative, coccus-shaped organism was isolated from oral cavity samples collected from healthy elephants. The isolated strain, NUM 2404T, was tentatively identified as a streptococcal species based on the results of biochemical tests. Although a comparative 16S rRNA gene sequence analysis suggested the classification of this organism into the genus Streptococcus, it did not correspond to any recognized species of the genus. Strain NUM 2404T was related most closely to Streptococcus saliviloxodontae NUM 6306T with 95.8% 16S rRNA gene sequence similarity, but the phylogenetic tree reconstructed based on the 16S rRNA gene sequence showed that NUM 2404T clustered with Streptococcus mutans NCTC 10449T and Streptococcus troglodytæ TKU 31T. Comparative sequence analysis based on two housekeeping genes, groEL, which encodes the 60 kDa heat-shock protein, and rpoB, encoding the β subunit of RNA polymerase, of NUM 2404T indicated that it was most closely related to those of Streptococcus orisratti A63T and Streptococcus sobrinus ATCC 33478T with 82.7% and 85.1% sequence similarities, respectively. On the basis of genotypic and phenotypic differences, it is proposed that the novel isolate be classified in the genus Streptococcus as representative of a novel species, Streptococcus dentiloxodontae sp. nov. The type strain is NUM 2404T (=JCM 19284T=DSM 27381T).

The genus Streptococcus embraces a broad range of Gram-positive, facultatively anaerobic, catalase-negative cocci arranged in chains or pairs (Okamoto et al., 2013). A large number of streptococcal species colonize the oral cavities of animals and humans. Oral streptococci have been divided into four major groups, designated the mutans, salivarius, anginosus and mitis groups (Whiley & Beighton, 1998). Some oral streptococcal species are known to form characteristic colonies on sucrose-containing agar due to the synthesis of extracellular polysaccharides (Whiley & Beighton, 1998). By focusing on this characteristic, several streptococci from various animal oral cavities have been identified (Takada & Hirasawa, 2007, 2008, 2010; Shinozaki-Kuwahara et al., 2011; Takada et al., 2013; Okamoto et al., 2013; Saito et al., 2015). Several novel Streptococcus species, namely Streptococcus oriloxodontæ, Streptococcus loxodontisalivarius and Streptococcus saliviloxodontae, have recently been isolated from elephant oral cavities (Shinozaki-Kuwahara et al., 2014; Saito et al., 2014). We herein describe an additional novel species belonging to the mutans streptococcal group, isolated from the elephant oral cavity.

To examine the oral microflora of elephants, mitis salivarius (MS) agar (Becton Dickinson) was used to isolate mutans Streptococcus-like species. Strain NUM 2404T was obtained from samples taken from the oral cavities of six elephants in a zoo as previously described (Saito et al., 2014). Strain NUM 2404T formed small, raised, adherent colonies that had irregular margins on MS agar after incubation at 37°C for 48 h under an atmosphere of 95.0% N2 and 5.0% CO2 in an anaerobic chamber. A biochemical analysis was conducted using the Rapid ID32 Strep, API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer’s instructions. Cells were Gram-stain-positive and catalase-negative. Four supplementary figures are available with the online Supplementary Material.

Abbreviations: ML, maximum-likelihood; MLSA, multi-locus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, groEL, rpoB, gyrB and sodA gene sequences of Streptococcus dentiloxodontae NUM2404T are AB828596, LC114466, LC114467, LC114468 and LC114469, respectively.

Four supplementary figures are available with the online Supplementary Material.
activity was negative. Colonies on brain heart infusion (BHI; Becton Dickinson) medium supplemented with 5.0 % sheep blood agar after at 37 °C for 48 h in the anaerobic chamber were non-haemolytic. Susceptibility to bacitracin as assessed by the agar disc diffusion method using commercial bacitracin discs (2 U; Becton Dickinson) according to the manufacturer’s instructions was positive. Although colony formation resembled that of the mutans group streptococci, the biochemical characteristics of the isolated strain did not correspond to any recognized Streptococcus species. The main differences in the characteristics of strain NUM 2404T and other related streptococcal species are shown in Table 1. Strain NUM 2404T was distinguished from \textit{S. saliviloxodontae} NUM 6306T by its ability to ferment pullulan and methyl β-D-glucopyranoside and to produce alkaline phosphatase, naphthol-AS-BI-phosphohydrolase and urease. The novel isolate was subjected to further genetic studies.

Based on cell surface carbohydrate antigens, the mutans streptococcal group has ten serotypes, \textit{a}–\textit{h}, \textit{k} and \textit{p} (Nakano \& Ooshima, 2009). Serological analyses of isolates were conducted using agar gel immunodiffusion methods with rabbit antisera raised against the reference strains of mutants streptococci prepared previously (Hirasawa \textit{et al.}, 1980; Takada \textit{et al.}, 1984), and were performed by a PCR using serotype-specific primers (Nakano \& Ooshima, 2009). These results suggested that isolate NUM 2404\textsuperscript{T} should be grouped within serotype \textit{k}. The Lancefield grouping test was performed using the Streptococcal grouping kit (Oxoid). No Lancefield carbohydrate antigens were detected in strain NUM 2404\textsuperscript{T}. Several streptococcal species are known to secrete water-insoluble polysaccharides such as glucosyltransferase (Whiley \& Beighton, 1992). The novel strain did not correspond to any recognized \textit{Streptococcus} species. The main differences in the characteristics of the isolated strain were: (i) initial denaturation at 98 °C for 2 min; and (ii) 30 cycles consisting of 98 °C for 10 s, 60 °C for 10 s and 68 °C for 1 min. Additional primers for the determination of 16S rRNA gene sequences were as described previously (Hiraishi, 1992). The amplified PCR product was sequenced with an ABI PRISM 3130 Genetic analyser using a Big Dye Terminator ver 3.1 cycle sequencing kit (Life Technologies) according to the manufacturer’s instructions. The sequence data obtained were analysed using DNASTAR software (Hitachi Solutions), and previously determined 16S rRNA and housekeeping gene sequences used for comparisons in this study were retrieved from the GenBank database. The identification of closest phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were performed using the EzTaxon-e server (Kim \textit{et al.}, 2012). An analysis of 16S rRNA gene sequences showed that strain NUM 2404\textsuperscript{T} belonged to the genus \textit{Streptococcus} with 95.8 % similarity to that of \textit{S. saliviloxodontae} NUM 6306T. This value was less than the 98.6 % threshold value for defining different species (Stackebrandt

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Table 1. Characteristics that differentiate strain NUM 2404\textsuperscript{T} from closely related streptococcal species

Strains: 1, NUM 2404\textsuperscript{T}; 2, \textit{S. saliviloxodontae} NUM 6306\textsuperscript{T} (data from Saito \textit{et al.}, 2014 and the present study); 3, \textit{S. mutans} NCTC 10449\textsuperscript{T} (Whiley \& Beighton, 1998); 4, \textit{S. orisratti} A63\textsuperscript{T} (Zhu \textit{et al.}, 2000 and the present study). All are positive for hydrolysis of esculin, activities of alanine phenylalanine-proline arylamidase and leucine arylamidase, and acid production from glucose, fructose, mannose, amygdalin, cellobiose, maltose, sucrose, trehalose and raffinose. All strains are negative for hydrolysis of arginine, activities of β-galactosidase, β-glucuronidase, pyrogalactosidase, N-acetyl-β-glucosaminidase, glycyrrhizin, β-phenylpropionate arylamidase, esterase lipase, lipase, β-galactosidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, and acid production from glyceral, erythritol, D-arabinose, D-xylene, L-xylene, adonitol, rhamnose, dulcitol, inositol, mannitol, sorbitol, melezitose, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol and gluconate. NI, Not identified; NG, non-groupable.

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Multiple sequence alignments via CLUSTAL W was performed using the MEGA 5.2 software (Tamura et al., 2011). Phylogenetic trees were reconstructed by the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) and maximum-likelihood (ML) methods using MEGA 5.2. Evolutionary distances for the NJ and ML methods were computed using the Tamura–Nei model (Tamura & Nei, 1993), and the Subtree-Pruning-Regrafting algorithm (Liò & Goldman, 1998) was used for the MP method. The NJ phylogenetic tree reconstructed based on 16S rRNA gene sequences revealed a clear affiliation between strain NUM2404T and the genus Streptococcus. Strain NUM 2404T consistently clustered with Streptococcus mutans and Streptococcus troglodytae, while S. saliviloxodontae was located in the streptococci salivarius group (Fig. 1; an extended version of this tree is not shown) methods showed a similar tree topology for strain NUM 2404T and S. saliviloxodontae with mutans group streptococci (Fig. 3). In these two phylogenetic trees, S. saliviloxodontae was also located in the salivarius group. In addition to groEL and rpoB, partial sequences of the gyrB gene, which encodes the B subunit of DNA gyrase, and sodA, encoding the superoxide dismutase, of strain NUM 2404T were amplified and sequenced as previously described (Glazunova et al., 2009) and used for a multi-locus sequence analysis (MLSA). An additional tree was reconstructed on the basis of the concatenated sequences of the four housekeeping genes by the NJ method. This phylogenetic tree by MLSA indicated that strain NUM2404T clustered with mutans group streptococci and diverged at a relatively early branch in the cluster (Fig. S3). DNA–DNA hybridization was performed according to the microtitre plate method as previously described (Takada & Hirasawa, 2007). DNA–DNA relatedness was examined by using labelled DNA from strain NUM 2404T with unlabelled single-stranded DNA from related streptococci. The DNA from reference species S. saliviloxodontae NUM 6306T, S. mutans NCTC 10449T and S. orisratti A63T showed low levels of DNA–DNA relatedness to strain NUM 2404T, 20.9, 28.5 and 31.5 %, respectively. Arbitrarily primed (AP)-PCR analyses for genotyping of newly proposed species and other streptococci were carried out as described previously with primers

![Phylogenetic tree of bacteria belonging to the genus Streptococcus inferred from a comparison of 16S rRNA gene sequences by the NJ method. Lactococcus lactis subsp. lactis NCDO 604T was used as an out-group. Numbers on the tree indicate bootstrap values calculated for 1000 subsets; only values >50% are shown. Bar, 0.01 substitutions per nucleotide position.](image-url)
OPA-3, OPA-7 and OPA-13, respectively (Tabchoury et al., 2008). The novel isolate NUM 2404\textsuperscript{T} and other related streptococcal species such as \textit{S. saliviloxodontae} NUM 630\textsuperscript{T}, \textit{S. mutans} NCTC 10449\textsuperscript{T} and \textit{S. orisratti} A63\textsuperscript{T} produced different fingerprint patterns using the above three primers (Fig. S4). On the basis of phenotypic and

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**Fig. 2.** Phylogenetic tree based on partial sequences of the \textit{groEL} gene, which encodes the 60 kDa heat-shock protein, of strain NUM 2404\textsuperscript{T} and closely related species by the NJ method. Numbers on the tree indicate bootstrap values (%) calculated for 1000 subsets; only values >50\% are shown. Bar, 0.1 substitutions per nucleotide position.

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**Fig. 3.** Phylogenetic tree based on partial sequences of the \textit{rpoB} gene, encoding the \(\beta\) subunit of RNA polymerase, of strain NUM 2404\textsuperscript{T} and closely related species by the NJ method. Numbers on the tree indicate bootstrap values (%) calculated for 1000 subsets; only values >50\% are shown. Bar, 0.1 substitutions per nucleotide position.
The results of the Voges–Proskauer test are negative. Tests for enzyme activities by the API ZYM system show positive reactions with alkaline phosphatase, but not with naphthol-AS-BI-phosphohydrolase, valine arylamidase and acid phosphatase, and not with esterase, esterase lipase, lipase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, β-mannosidase or α-fucosidase. Tests for enzyme activities by RapidID32 strips reveal positive reactions with β-glucosidase and α-l-fucosidase, but not with arginine dihydrolase, gelacyltryptophane arylamidase or urease. Susceptibility to bacitracin (2 U) is positive and water-insoluble polysaccharide synthesis from sucrose is detected by the action of secreted extracellular enzymes.

The type strain, NUM 2404T (=JCM 19284T=DSM 27381T), was isolated from clinical specimens of the elephant oral cavity. The DNA G+C content of the type strain is 42.4±1.29 mol%.

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References


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