Streptococcus tigurinus sp. nov., isolated from blood of patients with endocarditis, meningitis and spondylodiscitis

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Four Gram-stain-positive, catalase-negative, coccus-shaped bacterial strains were isolated from multiple blood cultures of patients with endocarditis, meningitis and spondylodiscitis. The isolates were tentatively identified as viridans streptococci on the basis of phenotypic characteristics. Comparative 16S rRNA gene sequencing studies showed that the organisms were members of the Streptococcus mitis group but did not correspond to any recognized species. The nearest phylogenetic relative was S. mitis ATCC 49456T, with 98.6 % sequence similarity. The representative strain AZ_3aT showed less than 96.8, 97.6, 94.5 and 95.5 % similarity to the phylogenetically most closely related species by recA, rpoB, sodA and groEL gene sequence analysis, respectively. DNA–DNA hybridization analyses showed a low reassociation value of 32.2 % between strain AZ_3aT and S. mitis DSM 12643T. Reassociation values with members of other S. mitis group species ranged from 27.3 to 49.7 %. The G+C content of the DNA was 40.0 mol%. Based on our biochemical and molecular analyses, the isolates represent a novel species, for which the name Streptococcus tigurinus sp. nov. is proposed. The type strain is AZ_3aT (= CCOS 600T = DSM 24864T).

Accurate identification of bacteria within the Streptococcus mitis group remains a challenge, in particular for Streptococcus mitis, S. pneumoniae, S. pseudopneumoniae and S. oralis. Conventional phenotypic methods are limited in providing an accurate identification (Arbique et al., 2004), and sequence analysis of the 5′ part of the 16S rRNA gene is not sufficiently discriminative to differentiate these species, because the sequence similarity is >99 % (Arbique et al., 2004; Kawamura et al., 1995). Several other target genes such as sodA (Kawamura et al., 1999; Poyart et al., 1998), rpoB (Drancourt et al., 2004) and groEL (Glazunova et al., 2009) have been investigated for species differentiation, primarily using type strains, and we recently proposed recA as an alternative target to differentiate S. pneumoniae from other viridans streptococci (Zbinden et al., 2011). Since commensal species such as S. oralis and S. mitis have been recognized as important agents of endocarditis (Douglas et al., 1993; Spellerberg & Brandt, 2011), accurate identification of these bacteria is important.

Recently, we isolated a viridans streptococcal organism from a 74-year-old patient with endocarditis that was present in six out of six blood cultures and was designated strain AZ_3aT. Molecular analyses by 16S rRNA gene sequencing were performed for accurate identification. An identical sequence was also detected in an aortic valve specimen of the patient by direct 16S rRNA gene broad-range PCR (Bosshard et al., 2003). BLAST analysis of the 16S
rRNA gene sequence of strain AZ_3a^T was initially performed using SmartGene software. BLAST searches to reference sequences in the public databases revealed the highest sequence similarity to the sequence of S. mitis ATCC 15914 (GenBank accession no. AY281076), with 99.9% identity; the next best reference sequence was that of the S. mitis type strain, ATCC 49456^T (AY485601), with 98.6% sequence identity. This relatively high sequence demarcation of 1.3% strongly suggested the recognition of a novel streptococcal species and an erroneous assignment of strain ATCC 15914, which was typed in 1977 based solely on phenotypic characteristics (Facklam, 1977). Moreover, the correct species assignment of strain ATCC 15914 was questioned recently by several reports on the basis of analyses of the housekeeping genes zwf and gki (Kiratiris et al., 2005) and the 16S–23S rRNA intergenic spacer region (Tung et al., 2006, 2007). A retrospective analysis of our molecular database revealed three additional clinical streptococcal isolates (AZ_4a, AZ_7a, AZ_10) that displayed the closest 16S rRNA gene sequence similarity to strain ATCC 15914 in the public database. These three isolates were derived from multiple blood cultures of patients with endocarditis, meningitis and spondylodiscitis. Further evaluation by conventional biochemical testing, molecular analyses and DNA–DNA hybridization studies revealed that the four new isolates and strain ATCC 15914 represent a novel species of the S. mitis group.

The streptococcal strains ATCC 15914, AZ_3a^T, AZ_4a, AZ_7a and AZ_10 were grown on Columbia agar plates containing 5% defibrinated sheep blood (bioMérieux) at 37 °C under aerobic conditions and in liquid culture using brain heart infusion broth (Becton Dickinson). Type strains S. pneumoniae DSM 20566^T, S. mitis DSM 12643^T, S. oralis DSM 20627^T, S. pseudopneumoniae CIP 108659^T and Streptococcus infantis CIP 105949^T were obtained from the DSMZ and the Institut Pasteur as indicated. Strain ATCC 15914 was purchased from the American Type Culture Collection.

To determine the phylogenetic affinity of the isolates, almost all the 16S rRNA gene of each strain was sequenced and subjected to a comparative analysis. A large continuous fragment was obtained using universal primers BAK11w (5'-AGTTTGTAGCMTGGGTAGCAG-3'; positions 10–27, Escherichia coli numbering) and Bact-1525a (5'-AAGGAGGTGATCCACRC-3'; positions 1541–1525). Cycling parameters included an initial denaturation for 5 min at 95 °C, 40 cycles of 1 min at 94 °C, 1 min at 48 °C and 1 min at 72 °C, and a final extension for 10 min at 72 °C. The amplicons were sequenced bidirectionally with an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems). Multiple alignment of the sequences was performed with the CLUSTAL V program (Higgins et al., 1992) (MEGALIGN Lasergene version 7; DNASTAR). Comparative 16S rRNA gene sequence analysis revealed 99.8–100% sequence similarity between isolates AZ_3a^T, AZ_4a, AZ_7a and AZ_10 and strain ATCC 15914, thereby demonstrating their high genealogical relatedness. Sequences of the type strains of members of the S. mitis group were retrieved from GenBank and the neighbour-joining method (Saitou & Nei, 1987) was used to reconstruct a phylogenetic tree. Strain AZ_3a^T formed a distinct branch within the S. mitis group (Fig. 1) and revealed a clear affiliation to the genus Streptococcus (an extended version of this tree is available in IJSEM Online as Fig. S1). It is evident from Fig. 1 that strain AZ_3a^T displays a phylogenetic affinity to a cluster of species consisting of S. mitis, S. pseudopneumoniae, S. pneumoniae, S. oralis and S. infantis. Sequence similarity values were calculated by using SmartGene software. Strain AZ_3a^T displayed the highest sequence similarity to S. mitis ATCC 49456^T (98.6%); the next most closely related strains were S. infantis ATCC 700779^T (98.5%), S. pseudopneumoniae ATCC BAA-960^T (98.3%), S. pneumoniae ATCC 33400^T (98.2%) and S. oralis ATCC 35037^T (98.1%).

Additional gene sequence analyses were carried out in order to analyse the phylogenetic affinities of the new isolates and strain ATCC 15914 in more detail. A 313 bp fragment of the recA gene was amplified with primers recA 2F and recA 5R for sequence analysis as described previously (Zbinden et al., 2011). Isolates AZ_3a^T, AZ_4a, AZ_7a and AZ_10 and strain ATCC 15914 displayed an intraspecies variability of 93.0–100%. The highest recA sequence similarity of strain AZ_3a^T was observed with S. oralis DSM 20627^T (96.8%). A phylogenetic tree (Fig. S2) reconstructed by the neighbour-joining method with partial sequences of the recA gene confirmed the phylogenetic placement of the representative strain within the genus Streptococcus.

Fig. 1. Neighbour-joining phylogenetic tree based on partial 16S rRNA gene sequences (>1300 bp), showing relationships among strain AZ_3a^T (Streptococcus tigurinus sp. nov.) and related species within the S. mitis group. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points. Published sequences used were from the GenBank database. Bar, 0.01 substitutions per nucleotide position. An extended version of this tree is available as Fig. S1.
Sequence analysis of the rpoB, sodA and groEL genes has also been demonstrated to be useful for phylogenetic differentiation of streptococcal species (Drancourt et al., 2004; Glazunova et al., 2009; Poyart et al., 1998). Partial sequences of rpoB, sodA and groEL were obtained following amplification using primers StreptoF and StreptO (Drancourt et al., 2004), d1 and d2 (Poyart et al., 1998) and streptogroELd and streptogroELr (Glazunova et al., 2009), respectively. Isolates AZ_3aT, AZ_4a, AZ_7a and AZ_10 and strain ATCC 15914 shared 89.5–95.7 % sequence similarity for the rpoB gene, 91.0–98.8 % for sodA and 93.2–95.3 % for groEL. When comparing the rpoB, sodA and groEL gene sequences of strain AZ_3aT with representative reference sequences from species of the S. mitis group available in GenBank, strain AZ_3aT formed a branch separate from other S. mitis group species in phylogenetic trees inferred from rpoB, sodA and groEL gene sequence comparisons (Figs S3, S4 and S5). Strain AZ_3aT exhibited the highest sequence similarity with the type strain of Streptococcus oligofermentans (97.6, 94.5 and 95.5 %, respectively) based on rpoB, sodA, and groEL. By analyses of housekeeping gene sequences, we observed heterogeneity within the four new isolates and strain ATCC 15914. Comparable intraspecies variability for housekeeping genes (e.g. rpoB, gdh and recA) has been reported for closely related S. mitis group species, e.g. S. oralis and S. mitis (Hoshino et al., 2005; Kilian et al., 2008; Nielsen et al., 2009; Sistek et al., 2012). This indicates a limited potential of these genes for species assignment within the S. mitis group. In contrast, analyses of the 16S rRNA gene showed high similarity (99.8–100 %) among the four new isolates (AZ_3aT, AZ_4a, AZ_7a and AZ_10) and strain ATCC 15914.

The four new isolates and strain ATCC 15914 were characterized phenotypically in detail. Strains were Gram-stained and assessed for the presence of catalase. Haemolytic reaction was determined on Columbia agar plates containing 5 % defibrinated sheep blood (bioMérieux) incubated at 37 °C under aerobic conditions for 24 h. Growth was determined at 25–45 °C as well as in brain heart infusion broth containing 6.5 % NaCl. Biochemical data were obtained by using API 20 Strep, API 50 CH and Rapid ID 32 STREP kits (bioMérieux). Identification was performed according to the instructions of the manufacturer. API 50 CH strips using CHB/E suspension medium were read after 24 and 48 h of incubation at 37 °C. The isolates, including strain ATCC 15914, exhibited almost identical biochemical characteristics, except for the acidification of trehalose (isolate AZ_3aT was negative). Phenotypic characteristics that differentiate the proposed species from closely related species are shown in Table 1.

Analysis of the new isolates and strain ATCC 15914 by the VITEK 2 colorimetric card (bioMérieux) revealed identification as S. mitis/S. oralis. In addition, analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was performed with a Microflex LT mass spectrometer (Bruker Daltonik) using the MALDI Biotyper software package (version 3.0) with reference database V.3.1.2.0 (Bruker Daltonik). Sample preparation was done using the ethanol/formic acid extraction protocol according to the manufacturer’s instructions. For all isolates including strain ATCC 15914, MALDI-TOF MS analysis yielded scores of ≥2.2 with S. pneumoniae, suggesting identification at the species level. However, accurate differentiation within the S. mitis group by MALDI-TOF MS has been shown to be limited, and such results should be interpreted with caution (Ferroni et al., 2010; van Veen et al., 2010). The VITEK and MALDI-TOF commercial identification systems might be useful as a screening method for assignment of the unknown organism to the S. mitis group, but not for accurate species identification.

DNA–DNA hybridization studies were performed with strain AZ_3aT and the type strains of its nearest phylogenetic neighbours. Cells were disrupted by using a French pressure cell (Thermo Spectronic) and genomic DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashon et al. (1977). DNA–DNA hybridization was carried out by the Identification Service of the DSMZ under optimal conditions (2 × SSC at 66 °C) as described by De Ley et al. (1970) under consideration of the modifications described by Huß et al. (1983) by using a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an

### Table 1. Biochemical characteristics of S. tigrinus sp. nov. and the closest related species within the S. mitis group

<table>
<thead>
<tr>
<th>Species:</th>
<th>1, S. tigrinus sp. nov. (results for isolates AZ_3aT, AZ_4a, AZ_7a and AZ_10 and strain ATCC 15914); 2, S. mitis; 3, S. oralis; 4, S. pneumoniae; 5, S. pseudopneumoniae (results for strain CIP 108659T); 6, S. infantis; 7, S. pyogenes. Data for S. tigrinus sp. nov. and S. pseudopneumoniae were obtained in this study; data for S. mitis, S. oralis, S. pneumoniae, S. infantis and S. pyogenes are from Whaley &amp; Hardie (2009).</th>
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<td>Inulin</td>
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<td>Starch</td>
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<td>Hydrolysis of arginine</td>
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<td>Production of:</td>
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<td>N-Acetyl-β-glucosaminidase</td>
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<td>+</td>
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<td>Alkaline phosphatase</td>
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<td>α-D-Galactosidase</td>
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<td>Glycyl-tryptophan arylamidase</td>
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in situ temperature probe (Varian). The DNA–DNA hybridization studies (performed in duplicate) showed that the strain that was most closely related to strain AZ_3aT was S. oralis DSM 20627T, with 49.7 ± 2.2% relatedness. The next most closely related strains displayed relatedness values of 40.9 ± 4.2% (S. pneumoniae DSM 20566T), 35.4 ± 4.6% (S. pseudopneumoniae CIP 108659T), 32.2 ± 4.2% (S. mitis DSM 12643T) and 27.3 ± 4.0% (S. infantis CIP 105949T). These reassociation values were well below 70%, indicating that strain AZ_3aT is distinct and separate from the closest related species (Wayne et al., 1987). The genomic DNA G + C content of strain AZ_3aT was determined by using the HPLC method of Mesbah et al. (1989) as 40.0 mol%.

On the basis of the hybridization results and the above-described genetic and phenotypic analyses, we propose the description of a novel species, Streptococcus tigurinus sp. nov., with the type strain AZ_3aT, and reassignment of strain ATCC 15914 to this species. Analysis by 16S rRNA gene sequencing facilitates accurate identification of S. tigurinus sp. nov. In view of its association with serious clinical manifestations, e.g. endocarditis, meningitis and spondylodiscitis, further epidemiological analyses are needed in order to describe in detail the natural pathogenic potential of this novel species.

**Description of Streptococcus tigurinus sp. nov.**

*Streptococcus tigurinus* (ti.gu.ri’nus. L. masc. adj. *tigurinus* of or pertaining to Tigurum, a district in Helvetia, the modern Zurich, the region where the bacterial species was first recognized).

Gram-stain-positive, non-motile, non-spore-forming cocci, 0.5–1.0 μm in diameter, arranged in chains. Surface colonies on sheep blood agar are circular, smooth, white to greyish, convex, α-haemolytic and 0.5–1.0 mm in diameter after 24 h of incubation at 37 °C under an aerobic atmosphere. Facultatively anaerobic; no enhancement of growth in 5% CO₂. Growth occurs at 25–42 °C but not in 6.5% NaCl broth. Catalase-negative. Produces leucine aminopeptidase and alanyl-phenylalanyl-proline arylamidase but not α-D-galactosidase, alkaline phosphatase, pyrrolidonyl arylamidase, β-glucuronidase, β-glucosidase, pyrogallol-α-naphthylamide, β-mannosidase, urease, glycol-tryptophan arylamidase or N-acetyl-β-glucosaminidase. Production of β-D-galactosidase depends on the substrate used [either resorufin β-D-galactopyranoside (positive) or 2-naphthyl β-D-galactopyranoside (negative)]. Fermentation of starch, pullulan, lactose, D-galactose, D-glucose, D-fructose, D-mannose, maltose, raffinose and sucrose is observed, but D-ribose, arabinose, D-mannitol, D-sorbitol, α-cyclodextrin, arabitol, glycogen, melibiose, melezitose, methyl β-D-glucopyranoside, D-tartarose and inulin are not fermented. Fermentation of trehalose is variable (positive for most strains; type strain AZ_3aT negative). L-Arginine, hippurate and ascelin are not hydrolysed. The Voges–Proskauer reaction is negative.

The type strain is AZ_3aT (=CCOS 600T =DSM 24864T), isolated from human blood. The DNA G + C content of the type strain AZ_3aT is 40.0 mol%. Additional members of the species are isolates AZ_4a, AZ_7a and AZ_10 and strain ATCC 15914 (originally identified as *S. mitis*).

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**References**


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