Streptococcus rubneri sp. nov., isolated from the human throat

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The novel, Gram-stain-positive, ovoid, lactic acid bacterial isolates LMG 27205, LMG 27206, LMG 27207T and MRI-F 18 were obtained from throat samples of healthy humans. 16S rRNA gene sequence analyses indicated that these isolates belong to the genus *Streptococcus*, specifically the *Streptococcus mitis* group, with *Streptococcus australis* and *Streptococcus mitis* as the nearest neighbours (99.45 and 98.56 % 16S rRNA gene sequence similarity to the respective type strains). Genotypic fingerprinting by fluorescent amplified fragment length polymorphism (FAFLP) and pulsed-field gel electrophoresis (PFGE), DNA–DNA hybridizations, comparative sequence analysis of *pheS*, *rpoA* and *atpA* and physiological and biochemical tests revealed that these bacteria formed a taxon well separated from its nearest neighbours and other species of the genus *Streptococcus* with validly published names and, therefore, represent a novel species, for which the name *Streptococcus rubneri* sp. nov. is proposed, with LMG 27207T (=DSM 26920T) as the type strain.

The streptococci are lactic acid bacteria that belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacilales* and family *Streptococcaceae*. Comparative 16S rRNA gene sequence analysis clusters species of the genus *Streptococcus* into six main species groups (Bentley et al., 1991; Kawamura et al., 1995) that are referred to as the *anginosus, bovis, mitis, mutans, pyogenes* and *salivarius* species groups (Kawamura et al., 1995). The *mitis* group currently includes *Streptococcus australis*, *S. cristatus*, *S. gordoni*, *S. infantis*, *S. lactarius*, *S. massiliensis*, *S. mitis*, *S. oligofermentans*, *S. oralis*, *S. parasanguinis*, *S. peroris*, *S. pseudopneumoniae*, *S. pneumoniae*, *S. sanguinis*, *S. sinensis* and *S. tigirinus* (Kawamura et al., 1999; Hoshino et al., 2005; Glazunova et al., 2006; Naser, 2006; Martin et al., 2011; Zbinden et al., 2012), and combines the *Streptococcus mitis* and *Streptococcus sanguinis* groups reported by Facklam (2002). However, assignment of *Streptococcus massiliensis* to the *mitis* group is based on partial sequences of housekeeping genes, and not on a 16S rRNA gene sequence comparison, which allocates it to the *mutans* group (Glazunova et al., 2006, 2010).

Isolates LMG 27205, LMG 27206, LMG 27207T and MRI-F 18 were obtained during an investigation of the microbial populations associated with the throats of healthy human volunteers taking part in a probiotics study (Seifert et al., 2011). Isolates LMG 27205 and MRI-F 18 were taken from throat swabs of the same individual on different sampling occasions, while LMG 27206 and LMG 27207T originated from throat swabs of different individuals participating in the study. Streptococci were isolated on Columbia CNA 5 % sheep blood agar (Becton Dickinson) and were purified by repeated streaking using the same medium. Cells were Gram-stained and observed by microscopy.

The nearly complete 16S rRNA gene sequences of all four isolates were determined as follows. DNA was extracted according to the method of Pitcher et al. (1989), as modified for Gram-positive bacteria as described by Björkroth & Korkeala (1996). PCR products were purified...
and commercially sequenced at GATC Biotech (Constance, Germany) as described previously (Kostinek et al., 2005). The four isolates had nearly identical 16S rRNA gene sequences (99.79 % similarity). Similarity calculations for the 16S rRNA gene sequence of strain LMG 27207T were done using EzTaxon (Kim et al., 2012) and revealed the highest similarity to the corresponding genes of the type strains of S. australis and S. mitis (sequence similarities of 99.45 and 98.56 %, respectively).

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The mothur software package version 1.28.0 (Schloss et al., 2009) and the corresponding SILVA SSURef 102 database (Pruesse et al., 2007) were used to align and trim the almost-complete 16S rRNA gene sequence of strain LMG 27207T (1457 bp) and the sequences of the type strains of all species of the S. mitis group with validly published names. These sequences (1331 bp) were imported into the software package MEGA version 5.10 (Tamura et al., 2011) and the evolutionary history was inferred by using the maximum-likelihood method based on Tamura’s three-parameter model. The tree with the highest log-likelihood is shown in Fig. 1. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the corresponding branches. A discrete gamma distribution was used to model evolutionary rate differences among sites and allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site. Sequence similarity calculations determined using the mothur software package indicated that the closest relative of strain LMG 27207T is S. australis ATCC 700641T (99.4 %). Lower sequence similarities (<98.6 %) were found towards other species of the S. mitis group.

In order to study the relatedness between the four isolates and their nearest phylogenetic neighbours in more detail, fluorescent amplified fragment length polymorphism (FAFLP) and pulsed field gel electrophoresis (PFGE) of genomic DNA were performed. FAFLP fingerprinting of whole genomes was performed as described previously (Sistek et al., 2012). The resulting electrophoretic patterns were analysed using the Gene Mapper 4.0 software package (Applied Maths), and normalized tables of peaks were transferred into the BioNumerics version 5.1 software package (Applied Maths). The FAFLP fingerprints of LMG 27205, LMG 27206, LMG 27207T and MRI-F 18 formed a single cluster, well separated from the fingerprints of strains of S. australis and S. parasanguinis, which were the nearest neighbours, and from other species belonging to the S. mitis group (Fig. 2). In addition, the cluster showed three distinct DNA fingerprint types (with LMG 27205 and MRI-F 18 forming a single type), suggesting that LMG 27205, LMG 27206, LMG 27207T and MRI-F 18 represent at least three different strains.

PFGE using the restriction enzyme Smal was also used to investigate the clonality of the four isolates. PFGE was done

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**Fig. 1.** Molecular phylogenetic analysis of the 16S rRNA genes by the maximum-likelihood method based on Tamura’s three-parameter mode. Bootstrap percentages based on 1000 replications are shown at branch points. Bar, 1 % sequence divergence.
as described by Huch et al. (2008). The results again showed that LMG 27205 and MRI-F 18 had highly similar fingerprints, while LMG 27206 and LMG 27207 showed distinct fingerprints (Fig. S1, available in IJSEM Online). Together, the PFGE and FAFLP results showed that LMG 27205 and MRI-F 18, which were isolated from the same individual on different occasions, appear to be clonally related and probably represent reisolates of the same strain.

Alternative marker genes such as the superoxide dismutase gene (sodA) (Glazunova et al., 2006; Poyart et al., 1998, 2002), the endoribonuclease P gene (Täpp et al., 2003), recN (Glazunova et al., 2010) and the genes encoding the alpha subunits of phenylalanyl-tRNA synthase (pheS), RNA polymerase (rpoA) and ATP synthase (atpA) have been used as tools with high discriminatory power for species delineation of streptococci and other lactic acid bacteria. In the present study, we used the primers listed in Table S1 for amplification and sequencing of the pheS, atpA and rpoA genes as described by Naser (2006). The primer combinations pheS-21-F/pheS-23-R, rpoA-21-F/rpoA-23-R and atpA-20-F/atpA-26-R amplified the target genes of most strains. Where necessary, alternative primer combinations for rpoA (rpoA-20-F/rpoA-22-R) were used. Amplification conditions and sequencing reactions were as described by Naser et al. (2005a, b). SeaView version 4 was used to concatenate the pheS, rpoA and atpA gene sequences of all four isolates and those of S. parasanguinis, S. australis and S. mitis reference strains (Gouy et al., 2010). The software package MEGA version 5.0 (Tamura et al., 2011) was used to align the translated concatenated gene sequences and to analyse the nucleotide sequences. The statistical reliability of tree topologies was evaluated by bootstrapping analysis based on 1000 tree replicates. The neighbour-joining and maximum-parsimony trees (not shown) revealed topologies similar to that obtained in a phylogenetic tree reconstructed following the maximum-likelihood approach (Fig. 3). Pairwise sequence similarity calculations of the concatenated pheS, rpoA and atpA gene sequences demonstrated that the taxon represented by isolates LMG 27205, LMG 27206, LMG 27207 and MRI-F 18 was readily distinguished from its nearest neighbours; the closest relative of strain LMG 27207 was S. parasanguinis LMG 14537T, with which it shared 98% concatenated sequence similarity. Lower sequence similarities (<90.1%) were found towards the remaining species of the genus Streptococcus (data not shown).

DNA–DNA hybridizations were performed between strain LMG 27207 and the type strains of S. australis (LMG 21714T) and S. parasanguinis (LMG 14537T). DNA for these hybridization experiments and for determination of the DNA base composition was extracted and purified using the method described by Gevers et al. (2001) with minor modifications (use of proteinase K after enzymic lysis, of a 10 mM Tris/25 mM EDTA buffer to dissolve the extracted DNA and of an additional DNA extraction step after RNase treatment). DNA–DNA hybridizations (four replications) were performed in the presence of 50% 

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**Fig. 2.** Fluorescent amplified fragment length polymorphism (FAFLP) DNA fingerprints of strains of *Streptococcus rubneri* sp. nov. and species of the *S. mitis* group. The dendrogram was derived from unweighted pair-group cluster analysis of the fingerprints, with levels of linkage expressed as Dice similarity coefficients.
formamide at 37 °C using a modification (Goris et al., 1998; Cleenwerck et al., 2002) of the microplate method described by Ezaki et al. (1989). Reciprocal reactions (A × B and B × A) were performed for each DNA pair, and the variation was within the limits of this method (Goris et al., 1998). Strain LMG 27207T exhibited DNA–DNA relatedness below the species level (<70%; Wayne et al., 1987; Stackebrandt et al., 2002) towards S. australis LMG 21714T (54 ± 5.5%, mean ± standard deviation) and S. parasanguinis LMG 14537T (38 ± 0.5%), confirming that it indeed represents a novel species.

The DNA base composition of LMG 27207T was determined by HPLC as described by Mesbah et al. (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and Escherichia coli DNA were used as calibration reference and control, respectively. The DNA G + C content of strain LMG 27207T was 40.9 mol%.

Selected phenotypic characteristics of the novel taxon represented by strains LMG 27205, LMG 27206 and LMG 27207T and other streptococci belonging to the mitis group are shown in Table 1. The novel taxon can be distinguished from all and most other members of this group by its β-haemolytic activity on Columbia 5% sheep blood agar and raffinose utilization, respectively. Furthermore, none of the species of this group can ferment melibiose except for S. parasanguinis (Martin et al., 2011) and isolates LMG 27205 and MRI-F 18. Apart from these traits, there are no further phenotypic characteristics of the novel taxon that allow clear discrimination of the novel taxon from the other members of the mitis group (see Table 1).

In conclusion, the results of this polyphasic study demonstrate that strains LMG 27205, LMG 27206, LMG 27207T and MRI-F 18 represent a novel species of the genus Streptococcus that can be distinguished both genotypically and phenotypically from its nearest phylogenetic neighbours.

We propose to classify these micro-organisms as Streptococcus rubneri sp. nov.

**Description of Streptococcus rubneri sp. nov.**

Streptococcus rubneri (rub’ner.i. N.L. masc. gen. n. rubneri in honour of Max Rubner, 2 June 1854–27 April 1932, a German medical doctor and professor of hygiene, after which the Max Rubner-Institut in Karlsruhe, Germany, was named, and where the strains investigated in this study were isolated).

Cells are Gram-stain-positive, non-spore-forming cocci. Colonies grown on Columbia CNA 5% sheep blood agar at 37 °C are 1–3 mm in diameter, raised with an entire margin. The colour is opaque white and the zone of β-haemolysis is about 2 mm. Grows in brain heart broth at 25, 30 and 37 °C, some strains grow in this medium at 45 °C. Does not produce gas in brain heart medium and does not grow at pH 9.6. Facultatively anaerobic and catalase-negative. Acetoin is not produced from glucose (Voges–Proskauer test). Does not hydrolyse arginine, aesculin, hippurate or starch and is negative in tests for pyrrolidonyl arylamidase, α-D-galactosidase, β-D-galactosidase, β-D-glucosidase, β-D-glucuronidase and alkaline phosphatase. Positive for leucine aminopeptidase. Does not produce acid from glycerol, erythritol, D- or L-arabinose, D-ribose, D- or L-xyllose, D-adenitol, methyl β-D-xylolside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, trehalose, inulin, melezitose, starch, glycogen, xyitol, gentiobiose, D-lyxose, D-tagatose, D- or L-fucos, D- or L-arabinol or 2- or 5-ketogluconate. Utilization of D-sorbitol and melibiose is variable. Capable of acid production from D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, salicin, cellobiose, maltose, lactose, sucrose and raffinose.

The type strain, LMG 27207T (=DSM 26920T), was isolated from a human throat in Germany in 2007 and has a DNA G + C content of 40.9 mol%.
Table 1. Biochemical characteristics that differentiate the novel strains from other members of the mitis group

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*For S. rubneri LMG 27207⁷, acid production from melibiose and d-sorbitol was negative.

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References


