Streptococcus lactarius sp. nov., isolated from breast milk of healthy women

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Three strains of a hitherto-unknown, Gram-stain-positive coccus were recovered from the milk of three non-related healthy women. The isolates shared 99 % 16S rRNA gene sequence similarity with sequences from uncultured members of the *Lactobacillales* and *Streptococcus*. The closest sequence corresponding to a defined species was that of *Streptococcus peroris* GTC 848^T, with a similarity of 98 %. A partial sequence (488 bp) of the *tuf* gene also showed 97 % similarity with that of *S. peroris* CCUG 39814^T. The combined 16S rRNA/*tuf*-based phylogeny revealed that all the isolates grouped in a statistically well-supported cluster separate from *S. peroris*. Enzyme activity profiles as well as fermentation patterns differentiated the novel bacteria from other members of the *Streptococcus mitis* group. Finally, phenotypic, genotypic and phylogenetic data supported the proposal of a novel species of the genus *Streptococcus*, for which the name *Streptococcus lactarius* sp. nov. is proposed. The type strain is MV1^T (=CECT 7613^T =DSM 23027^T).

Breast milk is a source of commensal and potentially probiotic bacteria for the infant gut, including streptococci, staphylococci, bifidobacteria and lactic acid bacteria (Heikkilä & Saris, 2003; Martín *et al.*, 2003; Perez *et al.*, 2007; Jiménez *et al.*, 2008a). At the time of writing, the genus *Streptococcus* consisted of 109 species and 15 sub-species (DSMZ, 2010), most of them belonging to one of the six phylogenetic clusters that have been established based on 16S rRNA gene sequence analysis (Kawamura *et al.*, 1995). Streptococci isolated from human colostrum or milk of healthy women usually belong to the *Streptococcus salivarius* (*S. bovis*, *S. salivarius*) or *Streptococcus mitis* (*S. mitis*, *S. oralis*, *S. parasanguinis*, *S. infantis*, *S. peroris*) groups, as determined by classical phenotypic characteristics and 16S rRNA gene sequencing (Heikkilä & Saris, 2003; Jiménez *et al.*, 2008b; Marín *et al.*, 2009). In addition, streptococcal DNA is also commonly detected in this biological fluid by using molecular techniques such as

PCR-DGGE (Delgado *et al.*, 2008) or quantitative real-time PCR (Collado *et al.*, 2009).

The members of the *mitis* group, which includes 12 species (*S. mitis*, *S. pneumoniae*, *S. pseudopneumoniae*, *S. oralis*, *S. infantis*, *S. australis*, *S. parasanguinis*, *S. sanguinis*, *S. gordonii*, *S. sinensis*, *S. cristatus* and *S. peroris*), have traditionally caused considerable confusion for both clinical microbiologists and taxonomists (Facklam, 2002). They are difficult to identify by using biochemical tests due to a lack of reliable and updated biochemical traits (Kawamura *et al.*, 1998). Furthermore, members of the *mitis* group are closely related to each other genetically, and it is very difficult to differentiate between them even by genetic methods, since some members display more than 99 % 16S rRNA gene sequence similarity and show 40–60 % DNA–DNA relatedness (Kawamura *et al.*, 1995). However, sequencing of other housekeeping genes, such as *sodA*, *rpoB* or *tuf*, has provided useful tools for the identification of species within the genus *Streptococcus* and, in particular, within the *mitis* group (Poyart *et al.*, 1998; Drancourt *et al.*, 2004; Picard *et al.*, 2004). Recently, Bishop *et al.* (2009) developed software based on multilocus sequence analysis and phylogenetic comparisons of seven housekeeping genes for the identification of viridans streptococci. The difficulties in achieving an accurate

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *tuf* gene sequences of strain MV1^T are GU045364 and GU045365, respectively.

A supplementary figure is available with the online version of this paper.

identification among the species of the mitis group may have practical consequences, since this group contains 11 species that are considered prototype commensals of the digestive and upper respiratory tracts, as well as one of the leading human pathogens (*S. pneumoniae*) (Kilian *et al.*, 2008).

While assessing the bacterial diversity of breast milk from healthy woman (Martín *et al.*, 2007; Jiménez *et al.*, 2008a), we isolated streptococci that seemed to belong to the mitis group, but study of the phenotypic and genotypic characteristics and phylogenetic position of strains MV1^T, MV2 and MV3 indicated that these strains represented a novel species of the genus *Streptococcus*.

Isolates MV1^T, MV2 and MV3 were isolated from breast-milk samples collected as described previously (Jiménez *et al.*, 2008b), and each one was obtained from a different (and non-related) healthy woman. The samples had been inoculated on Columbia CNA agar plus 5 % sheep blood, containing colistin and nalidixic acid (CNA; bioMérieux), under anaerobic conditions (MINI-MACS anaerobic workstation; DW Scientific) at 37 °C for 48 h. Preliminary 16S rRNA gene sequence analysis (~450 bp) of the three isolates by PCR amplification with primers plb16 (5'-AGAGTTTGATCCTGGCTCAG-3') and mlb16 (5'-GGCTGCTGGCACGTAGTTAG-3') (Kullen *et al.*, 2000) revealed very high similarity (>98 %) with a sequence from an uncultured *Streptococcus* clone.

To determine the phylogeny relationships of the three isolates, the 16S rRNA gene was amplified and sequenced with primers 7for (5'-AGAGTTTGATYMTGGCTCAG-3') and 1510r (5'-TACGGYTACCTGTTACGACTT-3') (Lane, 1991). In addition, partial amplification and sequencing (488 bp) of the *tuf* gene with primers TufStrep1 (5'-GAAGAATTGCTTGAATTGGTTGAA-3') and Tu-Strep-R (5'-GGACGGTAGTTGTTGAAGAATGG-3') (Collado *et al.*, 2009) was performed.

The DNA sequences of the 16S rRNA and *tuf* genes of the isolates were compared with those available in the NCBI by using the BLASTN algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). DNA sequences belonging to type strains of the genus *Streptococcus* were obtained from the GenBank database (see Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). These sequences were aligned by using the CLUSTAL W method (Thompson *et al.*, 1994) with the MEGA 4 (version 4.0.2) software (Tamura *et al.*, 2007). Phylogenetic trees were constructed based on the neighbour-joining method (Saitou & Nei, 1987). Bootstrapping analysis (1000 replicates) was done to study the stability of the groupings.

The G+C content of strain MV1^T was determined by HPLC following the procedure of Mesbah *et al.* (1989). DNA-DNA hybridization experiments were performed by the chemiluminescent-haemagglutinin method as described by Urdiain *et al.* (2008) using genomic DNA of strains MV1^T, MV2 and MV3 and of *S. peroris* DSM 12493^T and *S.*

Fig. 1. Phylogenetic relationships of strain MV1^T with members of the *S. mitis* group based on comparison of 16S rRNA gene sequences (1452 nt). For clarity, isolates MV2 and MV3, which are 100 % identical to MV1^T in their 16S rRNA gene sequences, are not shown. Nucleotide accession numbers are given in parentheses. The tree was based on the neighbour-joining method. Numbers on the tree indicate bootstrap values calculated for 1000 subsets for branch-points (values greater than 50 % are shown). Bar, 0.005 changes per nucleotide position (0.5 % sequence divergence).

parasanguinis DSM 6778^T. The reference DNA was that of strain MV1^T, and the results are expressed as mean percentage values based on three independent hybridization experiments.

The 16S rRNA and *tuf* gene sequences of isolates MV1^T, MV2 and MV3 were 100 % identical. BLAST analysis of the 16S rRNA gene sequences (1452 bp) showed 99.2 % similarity with sequences from uncultured clones assigned to the *Lactobacillales* and *Streptococcus*. The closest sequence corresponding to a defined species was that of *S. peroris* GTC 848^T, with a similarity of 98.2 %. Similarly, BLAST analysis of the partial sequence (488 bp) of the *tuf* gene showed 97.7 % similarity with that of *S. peroris* CCUG 39814^T.

The phylogeny inferred from the 16S rRNA gene sequence using the neighbour-joining method showed that isolates MV1^T, MV2 and MV3 formed a new subline within the *Streptococcus* species that make up the mitis group, being grouped with *S. peroris* GTC 848^T (Fig. 1). Bootstrap resampling (96 %) showed that the strain MV1^T and *S. peroris* GTC 848^T branches were statistically significant. The phylogeny inferred from the *tuf* gene sequence showed a clustering of strain MV1^T and *S. peroris* CCUG 39814^T, with a bootstrap value of 98 % (Supplementary Fig. S1). However, the topology of the tree was slightly different from that reconstructed with the 16S rRNA sequence. Therefore, sequences of the 16S rRNA and *tuf* genes (1940 nt) were concatenated. Phylogenetic trees inferred after compilation of the two sequences showed higher bootstrap support than individual phylogenetic analysis of the 16S rRNA and *tuf* genes (not shown).

Since the new taxon contains more than a single strain, and these strains share more than 97 % 16S rRNA gene

sequence similarity, DNA–DNA hybridization was carried following the recommendations of Tindall *et al.* (2010). Intraspecific hybridization assays, performed with strains MV1^T, MV2 and MV3, gave values greater than 70 %, thus including these strains in the same genomic species (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). The relative binding ratios of strains MV2 and MV3 to strain MV1^T were 98.7 % (± 0.7 %) and 92.9 % (± 0.8 %), respectively. Interspecific hybridization of strain MV1^T with *S. peroris* DSM 12493^T and *S. parasanguinis* DSM 6778^T was 57.4 % (± 0.1 %) and 63.6 % (± 0.7 %), respectively. Reciprocal hybridization experiments using genomic DNA of *S. peroris* DSM 12493^T as the template gave a value of 56.1 % (± 1.0 %) with strain MV1^T. These results confirm that the three isolates MV1^T, MV2 and MV3 represent a novel species. The G + C content of strain MV1^T was 41.2 mol%.

Colonies of the novel strains grew on Columbia sheep- or horse-blood agar plates after 2 days of incubation. The colonies were about 0.3–0.5 mm in diameter, circular, flat, greyish with a smooth surface, non-pigmented and α -haemolytic. However, under anaerobic conditions, haemolysis was visible only after the plates had been taken out of the anaerobic chamber for more than 1 h. The cells were coccoid, usually occurring as pairs or short chains, Gram-positive after staining, non-spore-forming and non-motile. They were facultatively anaerobic, but grew better under anaerobic conditions on CNA agar plates. The three strains showed a clumping phenotype when they were inoculated in brain heart infusion broth.

Substrate utilization, the fermentation/oxidation profile, acid production, activities of constitutive enzymes and some other physiological characteristics were examined using Rapid ID32 Strep and API ZYM strips (bioMérieux) according to the manufacturer's instructions. Being α -haemolytic and negative in the Voges–Proskauer test (no production of acetoin), the novel taxon can be placed in the mitis group (Whiley & Hardie, 2009). In addition, some of the results obtained using these phenotypic assays can be used to differentiate strains MV1^T, MV2 and MV3, which showed identical phenotypes, from closely related species belonging to the mitis group (Table 1). Using the Rapid ID32 Strep kit, the three strains produced a numerical profile of 74012601110 which, according to the manufacturer's database, corresponds to *S. parasanguinis*. Optochin sensitivity and bile solubility was tested as described previously (Whatmore *et al.*, 2000), while latex agglutination was assessed using the Slide Pneumo kit (bioMérieux), which comprises a latex suspension sensitized with antisera to 83 pneumococcal capsular serotypes. The results, which were identical for strains MV1^T, MV2 and MV3, are shown in Table 1.

Based on phenotypic, genotypic and phylogenetic characteristics, we suggest that the strains studied represent a novel species of the genus *Streptococcus*, for which the name *Streptococcus lactarius* sp. nov. is proposed.

Table 1. Biochemical characteristics that differentiate the novel strains from other members of the mitis group

Strains: 1, *S. australis* ATCC 700641^T; 2, *S. peroris* DSM 12493^T; 3, *S. infantis* ATCC 700779^T; 4, *S. lactarius* sp. nov. MV1^T (strains MV2 and MV3 gave identical results); 5, *S. oralis* ATCC 35027^T; 6, *S. sanguinis* ATCC 10556^T; 7, *S. gordonii* ATCC 10558^T; 8, *S. parasanguinis* ATCC 15912^T; 9, *S. cristatus* ATCC 12479^T; 10, *S. mitis* ATCC 49456^T; 11, *S. sinensis* CCUG 48488^T. Results for *S. peroris* DSM 12493^T and *S. lactarius* MV1^T were determined in this study by using the Rapid ID 32 Strep kit under the same culture conditions. Data for other reference strains were taken from Willcox *et al.* (2001) and Hoshino *et al.* (2005). ND, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Fermentation of:											
Lactose	+	+	+	+	+	+	+	+	+	+	–
Trehalose	–	–	–	–	+	+	+	+	+	–	–
Pullulan	+	–	+	–	–	+	–	–	–	+	–
Melibiose	–	–	–	–	–	–	–	+	–	–	–
Methyl β -D-glucopyranoside	–	–	–	–	–	–	+	–	–	–	–
Tagatose	–	–	–	–	–	–	+	+	–	–	–
Production of:											
β -Glucosidase (aesculin)	–	–	–	+	–	+	+	+	–	–	–
α -Galactosidase	–	–	–	–	–	–	–	+	–	+	–
Alkaline phosphatase	+	+	–	+	+	–	+	+	–	–	–
β -Galactosidase	–	–	+	–	–	–	–	+	–	–	–
N-Acetyl- β -glucosaminidase	–	–	–	+	–	–	–	+	–	–	–
Glycyl-tryptophan arylamidase	+	–	+	+	+	+	–	+	+	+	–
β -Mannosidase	–	–	–	–	–	–	+	–	–	–	–
Hydrolysis of arginine	+	–	–	+	–	+	+	+	+	–	–
Optochin sensitivity	ND	ND	ND	–	ND	ND	ND	ND	ND	–	ND
Bile solubility	ND	ND	ND	–	ND	ND	ND	ND	ND	–	ND
Pneumococcal latex agglutination	ND	ND	ND	–	ND	ND	ND	ND	ND	–	ND

Description of *Streptococcus lactarius* sp. nov.

Streptococcus lactarius (lac.ta'ri.us. L. masc. adj. *lactarius* of or belonging to milk, referring to the isolation of the first strains from human breast milk).

Cells are Gram-positive after staining and are non-motile, non-spore-forming cocci, 1.0–1.3 μ m in diameter, occurring as pairs or short chains. Colonies on Columbia sheep- or horse-blood agar plates after 2 days of incubation are about 0.3–0.5 mm in diameter, circular, flat, greyish, non-pigmented and α -haemolytic. Grows under aerobic or anaerobic conditions but, under anaerobic conditions, haemolysis is visible only when the plates have been taken out of the anaerobic chamber for more than 1 h. Hydrolyses hippurate. Voges–Proskauer-negative. Exhibits the following enzyme activities: arginine dihydrolase, β -glucosidase, alkaline phosphatase, alanine phenylalanine proline arylamidase and glycyl tryptophan arylamidase.

Lacks β -glucuronidase, α -galactosidase, pyrrolidonyl arylamidase, *N*-acetyl- β -glucosaminidase and β -mannosidase activities. β -Galactosidase activity is detected using *p*-nitrophenyl β -D-galactopyranoside as the substrate, but not with 2-naphthyl β -D-galactopyranoside. Positive for aesculin hydrolysis. Acid is produced from lactose, maltose and sucrose, but not from ribose, mannitol, sorbitol, trehalose, raffinose, glycogen, pullulan, melibiose, tagatose, melezitose, L-arabinose, D-arabitol, cyclodextrin or methyl β -D-glucopyranoside.

The type strain, MV1^T (=CECT 7613^T =DSM 23027^T), was isolated from breast milk of a healthy woman.

Acknowledgements

This work was supported by the FUN-C-FOOD (Consolider-Ingenio 2010) and AGL2007-62042 projects from the Ministerio de Educación y Ciencia (Spain). We thank Esther Jiménez for her skilful assistance in the purification of genomic DNA of *S. lactarius* strains.

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