Streptococcus caprae sp. nov., isolated from Iberian ibex (*Capra pyrenaica hispanica*)

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Biochemical and molecular genetic studies were performed on a novel Gram-stain-positive, catalase-negative, coccus-shaped organism isolated from tonsil samples of two Iberian ibexes. The micro-organism was identified as a streptococcal species based on its cellular, morphological and biochemical characteristics. 16S rRNA gene sequence comparison studies confirmed its identification as a member of the genus *Streptococcus*, but the organism did not correspond to any species of this genus. The nearest phylogenetic relative of the unknown coccus from ibex was *Streptococcus porci* 2923-03^T (96.6 % 16S rRNA gene sequence similarity). Analysis based on *rpoB* and *sodA* gene sequences revealed sequence similarity values lower than 86.0 and 83.8 %, respectively, from the type strains of recognized *Streptococcus* species. The novel bacterial isolate was distinguished from *Streptococcus porci* and other *Streptococcus* species using biochemical tests. Based on both phenotypic and phylogenetic findings, it is proposed that the unknown bacterium be classified as representing a novel species of the genus *Streptococcus*, for which the name *Streptococcus caprae* sp. nov. is proposed. The type strain is DICM07-02790-1C^T (=CECT 8872^T=CCUG 67170^T).

There remain many unknown or poorly understood aspects of diseases affecting wild ruminants caused by bacteria and there is even less information on the nature of the bacterial microbiota of these animals despite the potential ecological and evolutionary importance of commensal microbiota. This lack of information also extends to new bacterial species isolated from wild ruminants. In fact, the description of novel bacteria from such sources is extremely poor, with only five species described since 2001: '*Mycoplasma feriruminatoris*' (Jores *et al.*, 2013), *Streptococcus porcorum* (Vela *et al.*, 2011a), *Streptococcus rupicaprae* (Vela *et al.*, 2011b), *Mycobacterium caprae* (Aranaz *et al.*, 1999, 2003) and *Bartonella schoenbuchii* (Dehio *et al.*, 2001). During an investigation into the microbiota present in tonsils of Iberian ibexes (*Capra pyrenaica hispanica*), two *Streptococcus*-like organisms (DICM07-02790-1C^T and DICM07-02658-1A) were isolated from two animals that were hunted on the same day and at the same location (National Game Reserve of Puertos de Tortosa-Beceite, north-eastern Spain). Samples were cultured on Columbia-CNA agar plates (bioMérieux) that were incubated at 37 °C for 24 h under aerobic and anaerobic [with 4–10 % CO_2 using a GasPak Plus (BBL) system] conditions. Biochemical profiles of the isolates determined using the API Rapid ID 32 Strep system (code 21026601110) did not correspond to any valid species. Thus, phenotypic and phylogenetic characterization of both isolates were carried out and, based on the results, a novel species of the genus *Streptococcus* is proposed.

For phylogenetic analysis, a continuous fragment (approx. 1400 bp) of the 16S rRNA gene of the two isolates (DICM07-02790-1C^T and DICM07-02658-1A) was determined from PCR-amplified products, derived using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; positions 1541–1522). The PCR was carried out in a volume of 100 µl, using 2.5 U *Taq* polymerase (Boehringer Mannheim), about 350 ng DNA, 500 nM of each primer and 200 µM dNTPs, in the appropriate buffer. After 2 min denaturation

The GenBank accession numbers for the 16S rRNA, *rpoB* and *sodA* gene sequences of strain DICM07-02790-1C^T are LN833583, LN833584 and LN833585, respectively.

One supplementary table and three supplementary figures are available with the online Supplementary Material.

at 94 °C, the following cycle was repeated 30 times: 1 min denaturation at 94 °C, 65 seconds min annealing at 55 °C and 90 seconds polymerization at 72 °C. The last cycle was followed by 10 min elongation at 72 °C. The amplified product was sequenced bidirectionally using universal primers pA, pH*, antiKK (5′-CGTGCCAGCAGCCGCGGTAAT; positions 517–537) and 3 (5′-GTTGCGCTCGTTGCGGGACT). Comparative sequence analysis revealed 99.9 % similarity between the two isolates, thereby demonstrating their high genealogical relatedness. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (Kim *et al.*, 2012; http://eztaxon-e.ezbiocloud.net/). Sequence searches revealed that the unknown cocci were members of the genus *Streptococcus*, being related most closely to *Streptococcus porci* 2923-03^T (96.6 % 16S rRNA gene sequence similarity). Sequence similarity between isolate DICM07-02790-1C^T and the type strains of other *Streptococcus* species was less than 96.1 %. Sequences of the type strains of all recognized species of the genus *Streptococcus* were retrieved from GenBank and aligned with the newly determined sequence using the program SeqTools (Rasmussen, 2002). Phylogenetic trees were reconstructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987) using the programs SeqTools and TreeView (Page, 1996; Rasmussen, 2002), maximum-parsimony using the software package MEGA version 4 (Kumar *et al.*, 2004) and maximum-likelihood using the PHYML software (Guindon & Gascuel, 2003). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated using Kimura's two-parameter method (Kimura, 1980), and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The parameters in the program PHYML were as follows: input sequences are interleaved, with 500 non-parametric bootstrap analysis, GTR model of nucleotide substitution, four substitution rate categories and fixed Gamma distribution parameter (alpha=2.00). Phylogenetic trees obtained by using the neighbour-joining (Fig. 1) and the other two methods (data not shown) revealed a clear affiliation of the unknown cocci (as exemplified by strain DICM07-02790-1C^T) to the genus *Streptococcus.* It is evident from the phylogenetic tree based on the neighbour-joining algorithm (Fig. 1) that isolate DICM07-02790-1C^T formed a distinct subline, clustering with two species (*S. porci* and *Streptococcus plurextorum*) although bootstrap resampling analysis did not demonstrate a significant association between isolate DICM07-02790-1C^T and the aforementioned species. 16S rRNA gene sequence divergence values of >3.4 % between the novel isolate and the type strain of *S. porci* suggests that it represents a distinct species (Stackebrandt & Goebel, 1994).

Sequence analysis of two housekeeping genes (*sodA* and *rpoB*) was included as a supplement to the 16S rRNA gene sequence analysis. Partial sequences of the *rpoB* (701 bp) and *sodA* (356 bp) genes were amplified using primer pairs d1 and d2 (Poyart *et al.*, 1998) and StreptoF and StreptoR (Drancourt *et al.*, 2004), respectively, and sequenced as described previously (Glazunova *et al.*, 2006). The two isolates shared 99.4 and 99.0 % *rpoB* and *sodA* gene sequence similarity, respectively. When comparing the *sodA* and *rpoB* gene sequences of strain DICM07-02790-1C^T with those of the type strains of *Streptococcus* species available in GenBank, highest sequence similarity was with *Streptococcus suis* CIP 103217^T (83.8 and 86 %, respectively). Evolutionary distances, the resulting trees and bootstrap values were determined as described above. Isolate DICM07-02790-1C^T formed a separate branch from other *Streptococcus* species in the phylogenetic trees inferred based on *rpoB* and *sodA* gene sequence comparisons (Figs S1 and S2, respectively, available in the online Supplementary Material).

The two new isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was determined on Columbia agar containing 5 % defibrinated sheep blood (bioMérieux) incubated aerobically at 37 °C for 24 and 48 h (Facklam & Elliott, 1995). Determination of growth at 15, 22, 30, 37 and 42 °C was performed in brain heart infusion broth (Difco) with the pH adjusted to 7.5 (Facklam & Elliott, 1995). The ability of the isolates to tolerate the presence of 3.5, 4.5 and 6.5 % (w/v) NaCl was assessed as recommended by Facklam & Elliott (1995). The Lancefield serological group reaction was determined with the commercial Slidex Strepto kit (bioMérieux) by using specific group A, B, C, D, F and G streptococcal latex-agglutinating antisera. The isolates were characterized biochemically using the Rapid ID 32 Strep, API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The API 50 CH strips using the CHB suspension medium were read after up to 7 days of incubation at 37 °C. The two isolates exhibited homogeneous phenotypic and physiological characteristics, except in tests for β-galactosidase, valine arylamidase, naphthol-AS-BI-phophohydrolase and α-glucosidase using the API ZYM system (DICM07-02790-1C^T was negative) and acid production of melezitose, ribose, inulin and melibiose using the API 50 CH system (DICM07-02790-1C^T was positive). Discrepant results were detected between the commercial systems for some biochemical tests (raffinose was positive by API CH 50, β-glucuronidase was positive by Rapid ID 32 Strep and alkaline phosphatase was positive by API ZYM). The phenotypic characteristics that differentiate the proposed species from closely related species are shown in Table 1. 16S rRNA gene sequencing has shown that the genus *Streptococcus* comprises a distinct 'species group' that can be separated based on several microbiological tests (Whiley & Hardie, 2009). Characteristics differentiating the newly proposed species from these streptococcal 'species group' members are indicated in Table S1.

The two novel strains were characterized by pulsed-field gel electrophoresis profiling of their genomic DNAs, after digestion with the restriction enzyme *Sma* I, according to previous specifications (Vela *et al.*, 2003). Similarities between restriction endonuclease digestion profiles were

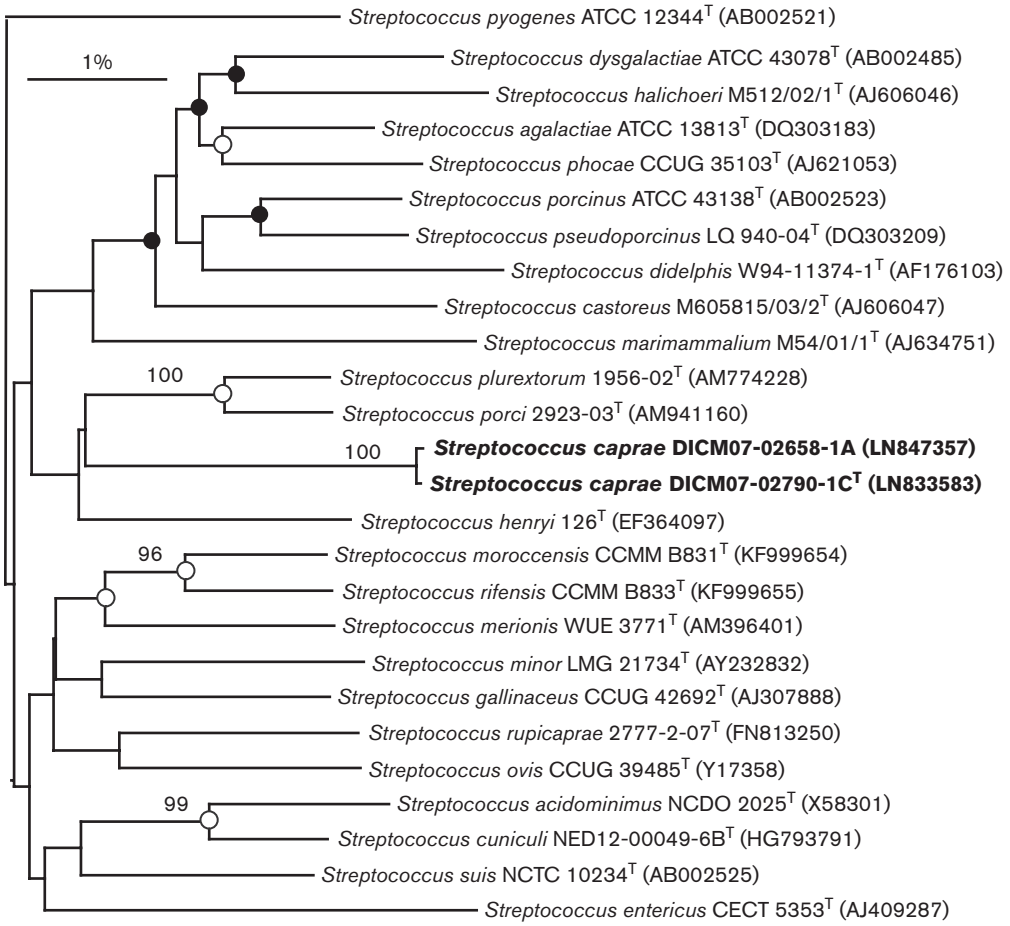

Fig. 1. Neighbour-joining phylogenetic tree inferred from comparison of 16S rRNA gene sequences of strains DICM07-02790-1C^T and DICM07-02658-1A and related members of the genus *Streptococcus*. *Enterococcus faecalis* ATCC 19433^T was used as the outgroup (not shown). Filled circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-parsimony tree. Open circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-likelihood and maximum-parsimony trees. Bootstrap values (expressed as a percentage of 1000 replications) higher than 50 % are given at branch points. The different branches were supported by the results of the other two algorithms. Bar, 1 % sequence divergence.

based on visual comparisons of the band patterns of strains run in the same gel. The strains displayed two different pulsed-field gel electrophoresis restriction profiles (Fig. S3).

Overall, the results of the present study demonstrate that the characteristics of the unidentified catalase-negative cocci match those described for the genus *Streptococcus* and they should be assigned to this genus. Phylogenetic, genotypic and phenotypic differences clearly demonstrate that the strains merit classification as representing a novel species, for which the name *Streptococcus caprae* sp. nov. is proposed.

Description of *Streptococcus caprae* sp. nov.

Streptococcus caprae (ca′prae. L. gen. n. *caprae* of the goat).

Cells are Gram-stain-positive, non-spore-forming cocci, 0.5–1 μm in diameter, occurring in pairs or chains commonly over more than eight cells long. Colonies on blood agar are small, circular and non-pigmented, 0.75–1.0 mm in diameter and α-haemolytic at 37 °C. Cells are facultatively anaerobic, catalase-negative and non-motile. Does not react with Lancefield group A, B, C, D, F and G antisera. Cells are able to grow at 22, 30, 37 and 42 °C but do not grow at 15 °C. Growth occurs in the presence of 3.5, 4.5 and 6.5 % (w/v) NaCl. Cells are able to produce acid from D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, sucrose, methyl α-D-glucopyranoside and trehalose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-arabitol, D-arabitol, starch, glycogen, pullulan, gentiobiose, methyl β-D-glucopyranoside, D-mannitol, D-sorbitol, inositol,

Table 1. Characteristics useful in differentiating strain DICM07-02790-1C^T from other phylogenetically closely related streptococci based on the 16S rRNA tree topology using the neighbour-joining method

Strains: 1, DICM07-02790-1C^T; 2, *S. porci* 2923-02^T; 3, *S. plurextorum* 1956-02^T. Data are taken from this study. +, Positive reaction; −, negative reaction; NG, non-groupable against Lancefield grouping antisera.

Characteristic	1	2	3
API Rapid ID 32 Strep results			
Production of:			
β-Glucosidase	+	+	−
α-Galactosidase	−	+	+
Production of acid from:			
Glycogen	−	+	−
Methyl β-D-glucopyranoside	+	−	−
Pullulan	−	+	−
Melibiose	−	+	−
API ZYM results			
Production of:			
α-Glucosidase	−	+	−
Esterase C4	+	−	−
Ester lipase C8	+	−	−
Acid phosphatase	+	−	+
API 50 CH results			
Production of acid from:			
Mannose	+	+	−
D-Xylose	−	+	−
L-Arabinose	−	+	−
N-Acetylglucosamine	+	+	−
Arbutin	+	+	−
Cellobiose	+	+	−
Aesculin	+	−	−
Starch	−	+	−
Gentiobiose	+	−	−
Lancefield antigen*	NG	B	B

*Lancefield serological group reaction using group A-, B-, C-, D-, F- and G-specific streptococcal latex-agglutinating antisera (bioMé-rieux).

dulcitol, methyl α-D-mannopyranoside, turanose, D-lyxose, xylitol, D-fucose, L-fucose, 2-ceto-gluconate, 5-ceto-gluconate, cyclodextrin or tagatose. Variable results were obtained from D-ribose, melibiose, inulin and melezitose (the type strain is positive). Esterase C4, esterase lipase C8, leucine arylamidase, acid phosphatase, β-glucosidase, glycyl-tryptophan arylamidase and alanine–phenyl-alanine–proline arylamidase are detected. No activity is detected for lipase C14, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, pyroglutamic acid arylamidase, β-mannosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase. Variable results were obtained from valine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase (the type strain is negative). Arginine, hippurate and urea are not hydrolysed and acetoin is not produced.

The type strain, DICM07-02790-1C^T (=CECT 8872^T=CCUG 67170^T), was isolated from the tonsil of a Spanish ibex. The full range of habitats is not known. DICM07-02658-1A is a second strain of the species.

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References

Aranaz, A., Liébana, E., Gómez-Mampaso, E., Galán, J. C., Cousins, D., Ortega, A., Blázquez, J., Baquero, F., Mateos, A. & other authors (1999). *Mycobacterium tuberculosis* subsp. *caprae* subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. *Int J Syst Bacteriol* **49**, 1263–1273.

Aranaz, A., Cousins, D., Mateos, A. & Domínguez, L. (2003). Elevation of *Mycobacterium tuberculosis* subsp. *caprae* Aranaz *et al.* 1999 to species rank as *Mycobacterium caprae* comb. nov., sp. nov. *Int J Syst Evol Microbiol* **53**, 1785–1789.

Dehio, C., Lanz, C., Pohl, R., Behrens, P., Bermond, D., Piémont, Y., Pelz, K. & Sander, A. (2001). *Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer. *Int J Syst Evol Microbiol* **51**, 1557–1565.

Drancourt, M., Roux, V., Fournier, P. E. & Raoult, D. (2004). *rpoB* gene sequence-based identification of aerobic Gram-positive cocci of the genera *Streptococcus, Enterococcus, Gemella, Abiotrophia*, and *Granulicatella. J Clin Microbiol* **42**, 497–504.

Facklam, R. & Elliott, J. A. (1995). Identification, classification, and clinical relevance of catalase-negative, gram-positive cocci, excluding the streptococci and enterococci. *Clin Microbiol Rev* **8**, 479–495.

Glazunova, O. O., Raoult, D. & Roux, V. (2006). *Streptococcus massiliensis* sp. nov., isolated from a patient blood culture. *Int J Syst Evol Microbiol* **56**, 1127–1131.

Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696–704.

Jores, J., Fischer, A., Sirand-Pugnet, P., Thomann, A., Liebler-Tenorio, E. M., Schnee, C., Santana-Cruz, I., Heller, M. & Frey, J. (2013). *Mycoplasma feriruminatoris* sp. nov., a fast growing *Mycoplasma* species isolated from wild Caprinae. *Syst Appl Microbiol* **36**, 533–538.

Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., Park, S. C., Jeon, Y. S., Lee, J. H. & other authors (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716–721.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Poyart, C., Quesne, G., Coulon, S., Berche, P. & Trieu-Cuot, P. (1998). Identification of streptococci to species level by sequencing the gene encoding the manganese-dependent superoxide dismutase. *J Clin Microbiol* **36**, 41–47.

Rasmussen, S. W. (2002). SeqTools, a Program Suite for Sequence Analysis. Copenhagen: Carlsberg Laboratory.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Vela, A. I., Goyache, J., Tarradas, C., Luque, I., Mateos, A., Moreno, M. A., Borge, C., Perea, J. A., Domínguez, L. & Fernández-Garayzábal, J. F. (2003). Analysis of genetic diversity of *Streptococcus suis* clinical isolates from pigs in Spain by pulsed-field gel electrophoresis. *J Clin Microbiol* **41**, 2498–2502.

Vela, A. I., Sánchez, V., Mentaberre, G., Lavín, S., Domínguez, L. & Fernández-Garayzábal, J. F. (2011a). *Streptococcus porcorum* sp. nov., isolated from domestic and wild pigs. *Int J Syst Evol Microbiol* **61**, 1585–1589.

Vela, A. I., Mentaberre, G., Marco, I., Velarde, R., Lavín, S., Domínguez, L. & Fernández-Garayzábal, J. F. (2011b). *Streptococcus rupicaprae* sp. nov., isolated from a Pyrenean chamois (*Rupicapra pyrenaica*). *Int J Syst Evol Microbiol* **61**, 1989–1993.

Whiley, R. A. & Hardie, J. M. (2009). Genus I. *Streptococcus* Rosenbach 1884, 22^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 3, 2nd edn., pp. 655–711. Edited by P. de Vos, G. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K. H. Schleifer & W. B. Whitman. New York: Springer.