Streptococcus porcorum sp. nov., isolated from domestic and wild pigs

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Seven isolates of an unidentified Gram-stain-positive, catalase-negative, coccus-shaped organism isolated from domestic and wild pigs were characterized by phenotypic and molecular-genetic methods. Based on cellular morphology and biochemical criteria, the isolates were tentatively assigned to the genus *Streptococcus*, although the organisms did not appear to correspond to any recognized species. Comparative 16S rRNA gene sequencing showed that the unknown bacterium was phylogenetically closely related to, but distinct from, *Streptococcus suis* (97.5 % 16S rRNA gene sequence similarity to the type strain). *rpoB* and *sodA* sequence analysis showed minimum interspecies divergence from phylogenetically close 16S rRNA gene sequence-based relatives of 13.8 and 18.6 %, respectively. DNA–DNA hybridization of a strain of the unidentified organism demonstrated 8–18 % reassociation with *S. suis* NCTC 10234^T. The novel bacterium could be distinguished from *S. suis* and other *Streptococcus* species using biochemical tests. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown isolates from domestic and wild animals be assigned to a novel species of the genus *Streptococcus*, *Streptococcus porcorum* sp. nov. The type strain is 682-03^T (=CCUG 58479^T =CECT 7593^T).

The genus *Streptococcus* has undergone significant expansion due to improved phenotypic and molecular identification methods, and includes 65 recognized species at the time of writing (Euzéby, 1997; http://www.bacterio.cict.fr/s/streptococcus.html). During the last decade, most novel streptococcal species have been isolated from animal sources (Collins *et al.*, 2001, 2002; Lawson *et al.*, 2005a, b; Takada & Hirasawa, 2007; Vela *et al.*, 2009). Animal streptococci have been isolated from a wide range of environments (Kilian, 1998) and some of them have been associated with a variety of diseases such as endometritis, respiratory infections, endocarditis, meningitis, arthritis and mastitis (Chanter, 1997; Köhler, 2007). During routine microbiological diagnosis from clinical specimens submitted to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), we have characterized seven *Streptococcus*-like organisms using phenotypic and molecular-genetics methods. The unidentified Gram-stain-positive, coccoid organisms were recovered from pigs (strains 682-03^T, 1606-02 and 1792-03) and wild boar (183-08 and 229-08) in Spain and pigs in Chile (1561-2D2-04 and 1561-9D2-04) (strain designations formed from strain number-year of isolation). The strains were isolated from pleura (1606-02) and lungs (1792-03, 1561-2D2-04 and 1561-9D2-04) of four pigs with lesions of pneumonia, a joint of a pig with arthritis (682-03^T) and tonsils from two apparently healthy wild boars (183-08 and 229-08). Strains were isolated on Columbia blood agar plates (bioMérieux) incubated for 24 h at 37 °C under both aerobic and anaerobic [with 4–10 % CO_2 using the GasPak Plus system (BBL)] conditions. On the basis of the phenotypic and phylogenetic results, a novel species of the genus *Streptococcus* is proposed to accommodate the strains.

In order to determine the phylogenetic relations of the isolates, almost the entire 16S rRNA gene of each isolate

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *sodA* gene sequences of strain 682-03^T are FN643224, FN814308 and FN814309, respectively; those for the 16S rRNA gene sequences of strains 1606/02, 1792/03, 1561-2D2/04, 1561-9D2/04, 183/08 and 229/08 are AJ871184 and FN908165–FN908169, respectively.

A supplementary figure and table are available with the online version of this paper.

was sequenced and subjected to comparative analysis as described previously (Vela *et al.*, 2002). Sequences of a large continuous fragment (approx. 1390 bases) of the 16S rRNA genes of two isolates (1606-02 and 682-03^T) and 1000 nt from isolates 1792-03, 1561-2D2-04, 1561-9D2-04, 183-08 and 229-08 were obtained by bidirectional sequencing using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; 1541–1522). Comparative sequence analysis revealed 99.3–100 % sequence similarity between the strains, thereby demonstrating their high genetic relatedness. Sequence searches of GenBank using the program FASTA (Pearson, 1994) revealed that the unknown cocci exhibited the highest 16S rRNA gene sequence similarity with *Streptococcus suis* ATCC 43765^T (97.5 %). The next most closely related type strains were *Streptococcus gordonii* ATCC 10558^T (97.2 % similarity), *S. urinalis* 2285-97^T (97.2 %), *S. lutetiensis* NEM 782^T (97.2 %), *S. infantarius* HDP 90056^T (97.1 %), *S. equinus* CCUG 27302^T and *S. alactolyticus* ATCC 43077^T (97.1 %). These sequences and those of other representative species within the genus *Streptococcus* were retrieved from GenBank and aligned with the newly determined sequences using the program SEQtools (Rasmussen, 2002). Phylogenetic trees were reconstructed according to three different methods, the neighbour-joining algorithm (Saitou & Nei, 1987), performed with the programs SEQtools and TreeView (Page, 1996; Rasmussen, 2002), maximum-likelihood analysis using the PHYML software (Guindon & Gascuel, 2003) and the maximum-parsimony method carried out using the software package MEGA version 3.1 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by Kimura's two-parameter model (Kimura, 1980) and close-neighbour-interchange (search level=2, random additions=100) was applied in maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). Phylogenetic trees obtained by using the neighbour-joining method (Fig. 1) and the other two methods (not shown) revealed a clear affiliation of the unknown cocci (exemplified by strain 682-03^T) to the genus *Streptococcus*. It is evident from Fig. 1 that strain 682-03^T displays a phylogenetic affinity with a subcluster of species consisting of *S. suis*, *Streptococcus porci* and *Streptococcus plurextorum*; however, the branching of the strain at the base of this subcluster was not supported by bootstrap analysis of the neighbour-joining tree (<50 % support) or the other treeing methods (not shown).

Sequence analysis of the *sodA* and *rpoB* genes has been shown to be a useful tool for differentiating streptococci on phylogenetic grounds (Poyart *et al.*, 1998, 2002; Drancourt *et al.*, 2004). Partial sequences of *rpoB* (701 bp) and *sodA* (356 bp) were amplified and sequenced as described previously (Glazunova *et al.*, 2006). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1980) and trees were reconstructed using the

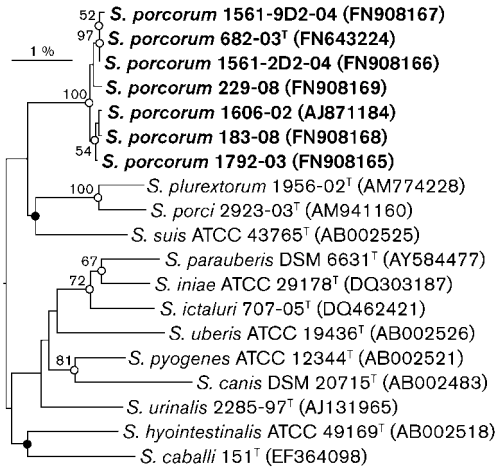

Fig. 1. Phylogenetic tree inferred from 16S rRNA gene sequence comparisons using the neighbour-joining method, showing the relationships of the novel strains (*Streptococcus porcorum* sp. nov.) and their closest phylogenetic neighbours. *Enterococcus faecalis* ATCC 19433^T (not shown) was used as an outgroup to generate the full version of the phylogenetic tree, which included all currently recognized *Streptococcus* species. Bootstrap values (expressed as percentages of 1000 replications) higher than 50 % are given at branching points. Open circles indicate that the corresponding nodes (groupings) were also obtained in maximum-likelihood and maximum-parsimony trees; filled circles indicate that the corresponding nodes (groupings) were also obtained in maximum-parsimony trees. Bar, 1 % sequence divergence.

neighbour-joining method (Saitou & Nei, 1987). Bootstrap values (1000 replicates) were also calculated. The seven isolates showed high sequence similarity in the two gene sequences: 97.5–99.7 % for *rpoB* and 97.7–99.8 % for *sodA*. Strain 682-03^T formed a branch separate from other *Streptococcus* species in phylogenetic trees inferred from *rpoB* and *sodA* gene sequence comparisons (Supplementary Fig. S1, available in IJSEM Online); the highest sequence similarity based on the *rpoB* gene was between strain 682-03^T and *Streptococcus canis* CIP 103223^T (88.5 %), while the highest sequence similarity based on the *sodA* gene was to *S. suis* CIP 103217^T (81.4 %). Glazunova *et al.* (2009) evaluated the discriminatory power of the *rpoB* and *sodA* genes for the identification of strains of 58 species of *Streptococcus* with validly published names and found that the similarity between pairs of species was in most cases between 80 and 90 % for the *rpoB* gene and 70–80 % for the *sodA* gene (Glazunova *et al.*, 2009). These similarities are similar to those obtained between strain 682-03^T and streptococcal species that were phylogenetically closely related on the basis of the 16S rRNA gene. Strain 682-03^T exhibited a mean *rpoB* gene sequence similarity of 84.8 %, ranging from 86.2 % (*S. alactolyticus*) to 83.4 % (*S. gordonii*). The mean sequence similarity for the *sodA* gene was 73.1 %, ranging from 81.4 % (*S. suis*) to 66.7 % (*S. lutetiensis*). Thus, minimum interspecies divergence values

of 13.8 and 18.6 % for the *rpoB* and *sodA* genes, respectively, between strain 682-03^T and it closest relatives based on 16S rRNA gene sequence phylogeny support separate species status for the unidentified catalase-negative cocci from domestic and wild pigs.

DNA–DNA hybridization experiments were carried out between strain 682-03^T and its nearest phylogenetic neighbour, *S. suis* NCTC 10234^T, and between strain 682-03^T and strains 229-08 and 1792-03. Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out in 2× SSC at 68 °C by the Identification Service of the DSMZ (Braunschweig, Germany) using the method described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) with a model Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA–DNA hybridization study between the three new isolates showed 80–92.4 % DNA relatedness between them, demonstrating that they are members of the same species (Wayne *et al.*, 1987). The DNA–DNA reassociation between strain 682-03^T and *S. suis* NCTC 10234^T was 8–18 %, clearly confirming that the new isolates constitute a separate species (Wayne *et al.*, 1987).

The G+C content of the DNA of representative isolate 682-03^T was determined at the DSMZ by using the HPLC method of Mesbah *et al.* (1989). The G+C content of strain 682-03^T was 38.9 mol%.

The seven 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 10, 22, 30, 37 and 42 °C and with 3, 4.5 and 6.5 % added NaCl in brain heart infusion broth (Difco) at pH 7.5 was performed 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. Results from API 50 CH strips using CHB suspension medium were followed for 7 days of incubation at 37 °C. The isolates exhibited almost identical biochemical characteristics, except for the acidification of raffinose (isolates 1561-2D2-04 and 1561-9D2-04 were negative) and inulin (isolates 183-08 and 229-08 were positive) and production of α-galactosidase (isolates 1606-02 and 1561-9D2-04 were positive) and acetoin (isolates 229-08 and 1561-9D2-04 were positive). The phenotypic characteristics that differentiate the proposed species from closely related species are shown in Table 1.

16S rRNA gene sequencing has revealed that the genus *Streptococcus* includes distinct 'species groups' that can be separated based on several microbiological tests (Whiley & Hardie, 2009). Characteristics that differentiate the proposed novel species from these streptococcal 'species groups' are indicated in Supplementary Table S1. The new isolates could also be distinguished from other *Streptococcus* species isolated from pigs by several phenotypic characteristics such as the haemolytic reaction in blood agar and identification of Lancefield group antigens,

Table 1. Characteristics useful in differentiating *Streptococcus porcorum* sp. nov. from other streptococci that are phylogenetically closely related based on 16S rRNA gene tree topology using the neighbour-joining method

Strains: 1, *S. porcorum* 682-03^T; 2, *S. porci* 2923-02^T; 3, *S. plurextorum* 1956-02^T; 4, *S. suis* CCUG 7984^T; 5, *S. pyogenes* CCUG 4207^T (included as a representative of the type species of the genus). +, Positive reaction; −, negative reaction. NG, non-groupable. Lancefield serological group reaction was determined using group A-, B-, C-, D-, F- and G-specific streptococcal latex agglutinating antisera (bioMérieux). Phenotypic data were obtained in this study.

Characteristic	1	2	3	4	5
Lancefield antigen	NG	B	B	D	A
Hydrolysis of arginine	−	−	−	+	+
Production of:					
β-Glucosidase	+	+	−	+	−
β-Glucuronidase	−	−	+	+	−
α-Galactosidase	−	+	+	+	−
Glycyl-tryptophan arylamidase	−	+	+	+	−
Esterase (C4)	−	−	−	+	+
Esterase lipase (C8)	−	−	−	+	−
Alkaline phosphatase	−	−	−	−	+
Acid phosphatase	+	−	+	−	+
β-Galactosidase	−	+	+	+	−
α-Glucosidase	−	+	−	+	+
Naphthol-AS-BI-phosphohydrolase	−	−	−	−	+
N-Acetyl-β-glucosaminidase	−	−	−	+	−
Pyroglutamic acid arylamidase	−	−	−	+	+
Valine arylamidase	−	−	−	−	+
Cystine arylamidase	−	−	−	−	+
Production of acid from:					
Pullulan	−	+	−	+	+
Raffinose	+	+	+	+	−
Melibiose	−	+	−	−	−
Methyl β-D-glucopyranoside	+	−	−	+	+
L-Arabinose	−	+	−	−	−
D-Xylose	−	+	−	−	−
Amygdalin	+	−	−	−	+
Cellobiose	+	+	−	+	+
Inulin	−	−	−	+	−
Starch	−	+	−	+	+
Glycogen	−	+	−	+	+
Gentiobiose	+	−	−	−	−

tests that are very useful for differentiating streptococci in clinical laboratories. Thus, *Streptococcus porcinus* and *Streptococcus dysgalactiae* subsp. *equisimilis* are β-haemolytic and react with different Lancefield group antisera (Facklam, 2002) and *Streptococcus orisuis*, *Streptococcus ferus* and *Streptococcus dentapri* are non-haemolytic (Baele *et al.*, 2003; Takada & Hirasawa, 2007; Takada *et al.*, 2010). *Streptococcus thoraltensis*, *Streptococcus hyovaginalis* and *Streptococcus hyointestinalis* are also α-haemolytic and do not react with Lancefield group A, B, C, D, F or G streptococcal antisera. However, these species are acetoin- (Voges–Proskauer test) and alkaline phosphatase-positive (Devriese *et al.*, 1988, 1997), while the proposed novel species is negative for both tests.

Based on the data presented, we consider the new isolates to represent a novel *Streptococcus* species, for which the name *Streptococcus porcorum* sp. nov. is proposed. Isolates were recovered from different animals in different years and different geographical regions of two countries. Therefore, it is very unlikely that the isolates could have any epidemiological clonality. Given that two of the isolates were obtained from apparently healthy animals and only two isolates from clinical samples were isolated in pure culture from the lungs of two pigs with pneumonia, it is not possible to reach conclusions about any pathogenic role of this *Streptococcus* species towards domestic or wild animals.

Description of *Streptococcus porcorum* sp. nov.

Streptococcus porcorum (por.co′rum. L. n. *porcus* -*i* a hog, pig; L. gen. pl. n. *porcorum* of/from pigs).

Cells are Gram-stain-positive, non-spore-forming cocci, 0.5–1 μm in diameter, occurring in pairs or in chains, commonly over 15 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. No Lancefield carbohydrate antigens (Slidex Strepto kit; bioMérieux) are detected. Cells are able to grow at 10, 22, 30, 37 and 42 °C but do not grow in the presence of 3, 4.5 or 6.5 % NaCl. With the API 50CH and Rapid ID32 Strep kits, acid is produced from lactose, D-glucose, D-fructose, trehalose, maltose, methyl β-D-glucopyranoside, D-galactose, D-mannose, *N*-acetylglucosamine, arbutin, amygdalin, aesculin, salicin, cellobiose, gentiobiose and sucrose but not from melibiose, L- or D-arabinose, D-xylose, starch, glycerol, erythritol, D-ribose, L-xylose, D-adonitol, L- or D-arabitol, glycogen, D-mannitol, inositol, dulcitol, melezitose, L-rhamnose, methyl α-D-mannopyranoside, pullulan, methyl α-D-glucopyranoside, L-sorbose, turanose, methyl β-D-xylopyranoside, D-lyxose, xylitol, D- or L-fucose, D-sorbitol, 2-ketogluconate, 5-ketogluconate, cyclodextrin or tagatose. Most strains produce acid from raffinose (Rapid ID32 Strep; type strain positive) but not from inulin (API 50CH; type strain negative). Leucine arylamidase, acid phosphatase (API ZYM), β-glucosidase and alanine-phenylalanine-proline arylamidase (Rapid ID32 Strep) are detected. No activity is detected for *N*-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, α-mannosidase, α-fucosidase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, β-galactosidase, α-glucosidase, cystine arylamidase, trypsin, α-chymotrypsin (API ZYM), alkaline phosphatase, β-glucuronidase (API ZYM and Rapid ID32 Strep), glycyl-tryptophan arylamidase, β-mannosidase or pyroglutamic acid arylamidase (Rapid ID32 Strep). Arginine, hippurate and urea are not hydrolysed (Rapid ID32 Strep). Most strains do not produce α-galactosidase or acetoin (Rapid ID32 Strep; type strain negative for both tests).

The type strain, 682-03^T (=CECT 7593^T =CCUG 58479^T), was isolated from a joint of a pig with arthritis. Full range of habitats is not known. The DNA G+C content of the type strain is 38.9 mol%.

Acknowledgements

This work was funded by the Spanish Ministry of Education and Science (MEC) (projects FAU 2006-00011-CO2-02 and AGL2009-14303-C02-01). The authors thank Professor J. P. Euzéby, Ecole Nationale Vétérinaire, Toulouse, for advice on the Latin species name, A. Casamayor for technical assistance and Juncal Fernández-Garayzábal for her assistance with the English reviewing of the manuscript.

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