Streptococcus porci sp. nov., isolated from swine sources

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Two unidentified Gram-positive, catalase-negative, coccus-shaped organisms were recovered from pigs and subjected to a polyphasic taxonomic analysis. 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 sequence studies confirmed this identification and showed that the nearest phylogenetic relatives of the unknown cocci were *Streptococcus plurextorum* 1956-02^T and *Streptococcus suis* NCTC 10234^T (97.9 and 96.0 % 16S rRNA gene sequence similarity, respectively). The new isolates were related most closely to *S. suis* CIP 103217^T based on *rpoB* gene sequence analysis (<8 % sequence divergence). DNA–DNA pairing studies showed that one of the unidentified strains (2923-03^T) displayed DNA relatedness values of 26.6 and 27.2 % with *S. plurextorum* CECT 7308^T and *S. suis* NCTC 10234^T, respectively. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown isolates from pigs be classified in the genus *Streptococcus* as members of *Streptococcus porci* sp. nov., with the type strain 2923-03^T (=CECT 7374^T =CCUG 55896^T).

The genus *Streptococcus* represents a large group within the low G + C content Gram-positive bacteria. This taxon has undergone considerable expansion and revision in the past decade; currently over 65 species (Euzéby, 1997; <http://www.bacterio.net>) are recognized, and novel taxa are continually being discovered within this genus (Shewmaker *et al.*, 2007; Takada & Hirasawa, 2007, 2008; Milinovich *et al.*, 2008; Vela *et al.*, 2009). Streptococci have been isolated from a wide range of environments and can be found as part of the normal flora of the skin and alimentary, respiratory and genito-urinary tracts of humans and different animals (Kilian, 1998). Some *Streptococcus* species have been implicated as the causes of different diseases in humans and animals, such as endometritis, respiratory infections, endocarditis, meningitis, arthritis and mastitis (Chanter, 1997; Köhler, 2007).

During routine microbiological diagnosis from different clinical specimens submitted to the Animal Health

Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), two unidentified Gram-positive, coccoid organisms were recovered from the pericardium (strain 2923-03^T) and bronchial lymph nodes (strain 2857-03) of two pigs with lesions of pericarditis and pneumonia, respectively. Strains were isolated on Columbia blood agar plates (bioMérieux) incubated for 24 h at 37 °C under both aerobic and anaerobic [with 4–10 % CO₂ using the GasPak Plus system (BBL)] conditions. On the basis of the phenotypic and phylogenetic results, a novel species of the genus *Streptococcus*, *Streptococcus porci* sp. nov., is proposed.

The new isolates were Gram-stained and assessed for the presence of catalase. Haemolytic reaction was determined on Columbia agar containing 5 % defibrinated sheep blood (bioMérieux) incubated aerobically at 37 °C for 24 and 48 h (Facklam & Elliot, 1995). Determination of growth at 10 and 42 °C, or with 3.0, 4.5 and 6.5 % added NaCl in brain-heart infusion broth (Difco) with the pH adjusted to 7.5, was performed as recommended by Facklam & Elliot (1995). Growth in brain-heart infusion broth (Difco) was assessed at pH 9.6 (Facklam & Elliot, 1995). Lancefield serological group reaction was determined with a commercial Slidex Strepto kit (bioMérieux) by using specific group A, B, C, D, F and G streptococcal latex-agglutinating

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *rpoB* gene sequences of strain 2923-03^T are AM941160 and AM941162, respectively; those for strain 2857-03 are AM941161 and AM941163, respectively.

A supplementary figure showing an extended neighbour-joining tree based on 16S rRNA gene sequences is available with the online version of this paper.

antisera. The isolates were characterized biochemically by using the Rapid ID32 Strep, API 50CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The API 50CH strips using CHB suspension medium were read up to 7 days incubation at 37 °C. The two isolates exhibited almost-identical biochemical char-

acteristics, except for the acidification of L-rhamnose, methyl β -D-xylopyranoside and pullulan (only isolate 2923-03^T was positive for these tests). A detailed description of the physiological, biochemical and morphological characteristics of the isolates is given in the species description and in Table 1.

Table 1. Characteristics useful in differentiating *Streptococcus porci* sp. nov. from other streptococci isolated from pigs

Strains: 1, 2923-03^T (*S. porci* sp. nov.); 2, *S. plurextorum* 1956-02^T; 3, *S. suis* CCUG 7984^T; 4, *S. porcinus* CCUG 27628^T; 5, *S. dysgalactiae* subsp. *equisimilis* CCUG 36637^T; 6, *S. alactolyticus* CCUG 27297^T; 7, *S. bovis* CCUG 34832^T; 8, *S. orisuis* CCUG 55425^T; 9, *S. ferus* CCUG 34834^T; 10, *S. thoraltensis* CCUG 32906^T; 11, *S. hyointestinalis* CCUG 27888^T; 12, *S. hyovaginalis* CCUG 37866^T. Phenotypic data are taken from this study, from Devriese *et al.* (1997), Takada & Hirasawa (2007), Vela *et al.* (2009) and from the CCUG database (<http://www.ccug.se>). +, Positive reaction; –, negative reaction; NG, non-groupable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
API Rapid ID 32 Strep												
Hydrolysis of arginine	–	–	+	+	+	–	–	–	–	+	–	–
Production of:												
β -Glucosidase	+	–	+	+	–	+	+	+	+	+	+	–
β -Glucuronidase	–	+	+	+	+	–	–	–	–	+	–	+
α -Galactosidase	+	+	+	–	–	+	+	–	–	+	+	–
Alkaline phosphatase	–	–	–	+	+	–	–	–	–	–	–	+
Acetoin	–	–	–	+	–	+	+	–	+	+	+	+
Glycyl-tryptophan arylamidase	+	+	+	–	–	–	–	–	–	–	–	–
Production of acid from:												
D-Ribose	–	–	–	+	+	–	–	–	–	+	–	+
D-Mannitol	–	–	–	+	–	+	–	+	+	+	–	+
D-Sorbitol	–	–	–	+	–	–	–	–	+	+	–	+
D-Lactose	+	+	+	+	–	–	+	+	+	+	–	+
Trehalose	+	+	+	+	+	–	–	+	+	+	–	+
Raffinose	+	+	+	–	–	+	+	+	–	+	–	–
Pullulan	+	–	+	+	+	–	–	–	+	+	–	–
Melibiose	+	–	–	–	–	–	+	–	–	–	–	–
Methyl β -D-glucopyranoside	–	–	+	+	–	+	+	+	+	+	–	–
API ZYM												
Production of:												
Esterase (C4)	–	–	+	–	–	+	–	–	–	–	–	+
Esterase lipase (C8)	–	–	+	–	–	–	–	–	–	–	–	+
Valine arylamidase	–	–	–	–	–	+	–	–	+	+	–	+
Cystine arylamidase	–	–	–	–	+	+	–	–	–	–	–	+
Acid phosphatase	–	+	–	+	+	–	+	–	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	–	–	–	+	+	–	–	+	–	–	–	+
β -Galactosidase	+	+	+	–	–	–	–	–	–	–	–	+
α -Glucosidase	+	–	+	+	+	+	+	–	+	+	+	+
N-Acetyl- β -glucosaminidase	–	–	+	–	–	–	–	–	–	–	–	+
API 50 CH												
Production of acid from:												
L-Arabinose	+	–	–	–	–	–	+	–	–	+	–	–
D-Xylose	+	–	–	–	–	–	–	–	–	+	–	–
Amygdalin	–	–	–	+	–	+	+	+	+	+	+	+
Cellobiose	+	–	+	+	–	+	+	+	+	+	+	+
Inulin	–	–	+	–	–	–	+	+	–	+	–	–
Starch	+	–	+	–	–	–	+	–	–	–	–	–
Glycogen	+	–	+	–	–	–	+	–	–	–	–	–
Gentiobiose	–	–	–	+	–	+	+	+	+	+	+	–
Lancefield antigen	B	B	D	V	C	D	D	D	C	NG	NG	NG

A phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis as described previously (Vela *et al.*, 2002). A large, continuous fragment (approx. 1390 bases) of the 16S rRNA gene of the two isolates was obtained bidirectionally. This analysis revealed that the two isolates had the same 16S rRNA gene sequence (100% similarity). Sequence searches of GenBank using the program FASTA (Pearson, 1994) revealed that the unknown cocci were phylogenetically related most closely to *Streptococcus plurextorum* 1956-02^T and *Streptococcus suis* NCTC 10234^T (97.9 and 96.0% 16S rRNA gene sequence similarity, respectively). These sequences and those of other representative species with validly published names within the genus *Streptococcus* were retrieved from GenBank and aligned with the newly determined sequence by using the program DNATools (Rasmussen, 1995). Phylogenetic trees were constructed according to three different methods: the neighbour-joining algorithm (Saitou & Nei, 1987), performed with the programs DNATools and TreeView (Page, 1996), a maximum-likelihood analysis using PHYLML 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 the Kimura two-parameter method (Kimura, 1980), and close-neighbour interchange (search level=2, random additions=100) was applied in maximum-parsimony analysis. Stability of the groupings was estimated by bootstrap analysis (1000 replications). Phylogenetic trees obtained by using the neighbour-joining algorithm (Fig. 1; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online) and the other two methods (data not shown) revealed a clear affiliation of the unknown cocci (as exemplified by strain 2923-03^T) to the genus *Streptococcus*, and placed the novel bacterium as a separate branch within this genus. It is evident from Fig. 1 that strain 2923-03^T is related closely to *S. plurextorum*. Bootstrap-resampling analysis shows a strong association (100%) between the unknown swine bacterium and the aforementioned species.

Additional gene sequence analyses were carried out in order to clarify the phylogenetic affinities of the two novel isolates. Partial sequences (701 bp) of the *rpoB* gene were amplified and sequenced as described by Drancourt *et al.* (2004). Evolutionary distances were calculated by using the Kimura two-parameter method (Kimura, 1980) and the resulting trees were constructed by using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kumar *et al.*, 2004) and maximum-likelihood (Guindon & Gascuel, 2003) methods. Bootstrap values (1000 replicates) were also calculated. Strains 2923-03^T and 2857-03 showed high sequence similarity (96.6%) in their *rpoB* gene sequences. The highest sequence similarities for these strains were with that of *S. suis* CIP 103217^T (91.3–91.8%). Strain 2923-03^T formed a separate branch from *S. suis* and other *Streptococcus* species in the phylogenetic tree inferred from *rpoB* gene sequence comparisons (Fig. 2).

Fig. 1. Phylogenetic tree inferred from 16S rRNA gene sequence comparison using the neighbour-joining method, showing the relationships of *Streptococcus porci* sp. nov. with other *Streptococcus* species. *Enterococcus faecalis* ATCC 19433^T was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at branching points. ○ indicates that the corresponding nodes (groupings) are also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 1% sequence divergence. Supplementary Fig. S1 (available in IJSEM Online) presents a full version of the phylogenetic tree, which includes a wider sample of *Streptococcus* species.

DNA–DNA hybridization experiments were carried out between the two isolates (strains 2923-03^T and 2857-03) and between strain 2923-03^T and its nearest phylogenetic neighbours, *S. plurextorum* CECT 7308^T and *S. suis* NCTC 10234^T. Genomic DNA was isolated by using a French pressure cell (Thermo Spectronic) and was 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 multi-cell changer and a temperature controller with *in situ* temperature probe (Varian). The DNA–DNA hybridization study showed 81.7% DNA relatedness between the two new isolates, demonstrating that they are members of the same species (Wayne *et al.*, 1987). DNA–DNA reassociation values between strain 2923-03^T and *S. plurextorum* CECT 7308^T and *S. suis* NCTC 10234^T were 26.6 and 27.2%, respectively, clearly confirming that the new isolates constitute a separate species (Wayne *et al.*, 1987).

The G + C content of the DNA of one representative isolate (strain 2923-03^T) was determined at the DSMZ by using the HPLC method of Mesbah *et al.* (1989). The DNA G + C content of strain 2923-03^T was 41.5 mol%.

Based on phylogenetic and phenotypic criteria, it is clear that the unidentified catalase-negative cocci merit clas-

Fig. 2. Phylogenetic tree inferred from comparison of *rpoB* gene fragment sequences, showing the relationships of *Streptococcus porci* sp. nov. with other *Streptococcus* species. *Enterococcus faecalis* ATCC 19433^T was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) >50% are given at branching points. ● indicates that the corresponding node (grouping) is also obtained in maximum-parsimony trees; ○ indicates that the corresponding nodes (groupings) are also obtained in both maximum-likelihood and maximum-parsimony trees. Bar, 1% sequence divergence.

sification as members of a novel species of the genus *Streptococcus*, for which the name *Streptococcus porci* sp. nov. is proposed. The two isolates were recovered from different animals and in different farms located in different provinces of Spain. Therefore, it is very unlikely that the isolates could have any epidemiological clonality. Tests that are useful in differentiating *S. porci* from those streptococcal species that have been isolated from pigs or have clinical relevance for this animal species are shown in Table 1. Only one isolate of *S. porci* was isolated in pure culture from the heart of a pig with pericarditis, which precludes any conclusions about the possible pathogenic role for pigs of this novel species of *Streptococcus*.

Description of *Streptococcus porci* sp. nov.

Streptococcus porci (por'ci. L. gen. n. *porci* of a pig).

Cells are Gram-positive, non-spore-forming cocci, 0.3–0.5 µm in diameter, occurring in pairs or short chains of three to eight cells. 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. Cells react to streptococcal group B antisera and are able to grow at 37 °C and at pH 9.6, but do not grow at 10 or 42 °C or in the presence of 6.5% NaCl. With the API 50CH and Rapid ID32 Strep kits (bioMérieux), cells are able to produce acid from D-lactose, D-glucose, D-fructose, salicin, maltose, melibiose, D-galactose, D-xylose, D-mannose, N-acetylglucosamine, trehalose, starch, cellobiose, arbutin, raffinose and sucrose, but not from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-arabitol, D-arabitol, gentiobiose, D-mannitol, inositol, dulcitol, melezitose, inulin, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, L-sorbose, turanose, D-lyxose, xylitol, D-fucose, L-fucose, amygdalin, sorbitol, aesculin, 2-keto-gluconate, 5-ketogluconate, cyclodextrin or tagatose. Cells are able to produce acid from glycogen and L-arabinose

using the API 50CH system. The type strain produces acid from L-rhamnose, methyl β-D-xylopyranoside (API 50CH) and pullulan (Rapid ID32 Strep). β-Galactosidase, α-glucosidase, leucine arylamidase (API ZYM), α-galactosidase, β-glucosidase (API ZYM and Rapid ID32 Strep), alanine-phenylalanine-proline arylamidase and glycyl-tryptophan 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, cystine arylamidase, trypsin, acid phosphatase, α-chymotrypsin (API ZYM), alkaline phosphatase, β-glucuronidase (API ZYM and Rapid ID32 Strep), β-mannosidase or pyroglutamic acid arylamidase (Rapid ID32 Strep). Arginine, hippurate and urea are not hydrolysed (Rapid ID32 Strep). Acetoin is not produced (Rapid ID32 Strep). The DNA G+C content of the type strain is 41.5 mol%.

The type strain, 2923-03^T (=CECT 7374^T=CCUG 55896^T), was isolated from the heart of a pig with pericarditis. Full range of habitat is not known.

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