Streptococcus plurextorum sp. nov., isolated from pigs

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Biochemical and molecular genetic studies were performed on an unknown Gram-positive, catalase-negative, coccus-shaped organism isolated from clinical samples from pigs. On the basis of the results of cellular morphological and biochemical tests, the organism was identified as a streptococcal species. 16S rRNA gene sequence comparisons confirmed its identification as a member of the genus *Streptococcus*, but the organism was distinct from any recognized species of this genus. The closest phylogenetic relative of the unknown organism corresponded to *Streptococcus suis* NCTC 10234^T (97.2 % 16S rRNA gene sequence similarity) and this phylogenetic position was confirmed by analysis of *rpoB* and *sodA* sequences. DNA–DNA hybridization studies showed that the unidentified organism produced a DNA reassociation value of 36.6 % with respect to *S. suis* NCTC 10234^T. The novel bacterium was distinguished from *S. suis* and other *Streptococcus* species using biochemical tests. On the basis of phenotypic and phylogenetic data, the unidentified organism represents a novel species of the genus *Streptococcus*, for which the name *Streptococcus plurextorum* sp. nov. is proposed. The type strain is 1956-02^T (=CECT 7308^T=CCUG 52972^T).

Although streptococci can be isolated as part of the normal flora of the skin, alimentary, respiratory and genitourinary tracts of humans and various animals (Kilian, 1998), some species are well-established as pathogens that cause a variety of diseases such as endometritis, respiratory infections, endocarditis, meningitis, arthritis and mastitis (Chanter, 1997; Köhler, 2007). In recent years, novel streptococcal species isolated from animals have been described (Lawson *et al.*, 2005a, b; Collins *et al.*, 2001, 2002; Vela *et al.*, 2002). In this article, we report the phenotypic and phylogenetic characterization of two strains of an unusual *Streptococcus*-like organism isolated from clinical specimens from pigs. On the basis of the data presented, a novel species of the genus *Streptococcus* is described.

Bacterial strains were isolated in 2002 and 2003 from the lung (strain 1956-02^T) and kidney (strain 1355-03) of two pigs with lesions associated with pneumonia and septicæmia, respectively. The pigs were kept under intensive-

management conditions at different farms. 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; GasPak Plus (BBL) system] conditions.

The novel isolates were Gram-stained and assessed for the presence of catalase. The haemolytic reaction was assessed on Columbia agar containing 5 % defibrinated sheep blood (bioMérieux) incubated aerobically at 37 °C for 24 and 48 h (Facklam & Elliot, 1995). Growth was determined, as recommended by Facklam & Elliot (1995), at 10 and 42 °C in brain heart infusion broth (Difco) supplemented with 3, 4.5 and 6.5 % NaCl, with the pH adjusted to 7.5. Growth in brain heart infusion broth at pH 9.6 was assessed (Facklam & Elliot, 1995). Susceptibility to optochin was determined using commercial (Oxoid) discs as described previously (Ruoff *et al.*, 2003). The Lancefield serological group reaction was determined with the commercial Slidex Streptokit according to the manufacturer's instructions (bioMérieux). The isolates were characterized biochemically using the API Rapid ID 32 Strep, API ZYM and API 50 CH systems (all bioMérieux) according to the manufacturer's instructions. The API 50 CH strips (with CHB suspension medium) were read after incubation for up to 7 days at 37 °C. With the API Rapid ID 32 Strep kit, the phenotypic profiles of the isolates were identical (43072401100) and corresponded to a presumptive

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB* and *sodA* gene sequences of strain 1956-02^T are AM774228, AM774232 and AM774230, respectively; those for strain 1355-03 are AM774229, AM774233 and AM774231, respectively.

A complete neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain CECT 7308^T with respect to *Streptococcus* reference species, is available with the online version of this paper.

identification as *Streptococcus pneumoniae*. However, this species could be differentiated from the novel pig isolates by its ability to produce acid from amygdalin (Kilian, 1998) and by its susceptibility to optochin (Facklam, 2002). Moreover, the novel coccus-shaped organism gave a positive reaction for Lancefield group B antisera. A detailed description of the physiological, biochemical and morphological characteristics of the isolates is given in the species description and in Table 1.

A phylogenetic analysis was performed on the basis of 16S rRNA gene sequence comparisons, as described previously (Vela *et al.*, 2002). A large fragment (approx. 1450 bases) of the 16S rRNA gene of both novel isolates was obtained bidirectionally. The 16S rRNA gene sequence analysis revealed 99.6% similarity between the two strains, thereby demonstrating strong genealogical similarity. Sequence searches of GenBank using the FASTA program (Pearson, 1994) revealed that the unknown cocci were phylogenetically most closely related to *Streptococcus suis* NCTC 10234^T (97.2% 16S rRNA gene sequence similarity). These sequences and those of other representative species 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 using three different methods: a neighbour-joining algorithm (Saitou & Nei, 1987), performed with the programs dnaTools and TreeView (Page, 1996); maximum-likelihood analysis performed using PHYLML software (Guindon & Gascuel, 2003); and a maximum-parsimony method carried out using the MEGA software package, version 3.1 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated using the Kimura two-parameter model (Kimura, 1980), whereas the maximum-parsimony analysis was performed using the close-neighbour-interchange search option (search level=2, random additions=100). The stability of the groupings was estimated by means of bootstrap analysis (based on 1000 replications). The phylogenetic trees obtained using neighbour-joining (Fig. 1; an extended version of this tree is presented in Supplementary Fig. S1, available in IJSEM Online) and the other two methods (data not shown) revealed a clear affiliation between the unidentified cocci (as exemplified by strain 1956-02^T) and the genus *Streptococcus*. Strain 1956-02^T formed a distinct subline, clustering within a small subgroup of species (embracing *S. suis*, *Streptococcus entericus* and *Streptococcus acidominimus*), but bootstrap resampling analysis did not demonstrate a strong association between strain 1956-02^T and the aforementioned species. However, the branching position of strain 1956-02^T with respect to its closest phylogenetic relative, *S. suis* NCTC 10234^T, was relatively stable, according to the three tree-making algorithms used in this study (Fig. 1). The unidentified isolates were distinguished from *S. suis* on the basis of phenotypic data (Table 1) and the negative results obtained using a *S. suis*-specific PCR-based assay that targets the glutamate dehydrogenase (*gdh*) gene (Okwumabua *et al.*, 2003).

Table 1. Characteristics useful in differentiating strain 1956-02^T from closely related species of the genus *Streptococcus*

Strains: 1, 1956-02^T; 2, *S. suis* CCUG 7984^T; 3, *S. entericus* CECT 5353^T; 4, *S. acidominimus* CCUG 27296^T. Data for *Streptococcus* species are taken from this study. +, Positive reaction, –, negative reaction.

Characteristic	1	2	3	4
Hydrolysis of:*				
Arginine	–	+	–	–
Hippurate	–	–	–	+†
Production of:*				
β-Glucosidase	–	+	+	+‡
α-Galactosidase	+	+	–	–
β-Glucuronidase	+	+	–	–§
Pyroglutamic acid arylamidase	–	+	–	+
Production of acid from:*				
Mannitol	–	–	–	+
Raffinose	+	+	–	–
Cyclodextrin	–	–	+	–
Glycogen	–	+	+	–
Methyl β-D-glucopyranoside	–	+	+	–
Pullulan	–	+	–	–
Production of:¶				
α-Glucosidase	–	+	–	+
Esterase (C4)	–	+	+	–
Ester lipase (C8)	–	+	+	–
α-Chymotrypsin	–	–	–	+
Acid phosphatase	+	–	+	+
Naphthol-AS-BI-phosphohydrolase	–	–	+	–
Production of acid from:¶				
Mannose	–	+	+	+
N-Acetylglucosamine	–	+	+	+
Arbutin	–	–	+	–
Cellobiose	–	+	+	–
Aesculin	–	+	+	+
Inulin	–	+	–	–
Salicin	+	+	+	–
Starch	–	+	+	–
Gentiobiose	–	–	+	–
Fructose	+	+	+	–
Lancefield antigen	B	D	D	D

*Results obtained using the API Rapid ID 32 Strep kit.

†Negative reaction according to Devriese *et al.* (1999).

‡Negative reaction according to Collins *et al.* (2002).

§Positive reaction according to Devriese *et al.* (1999).

¶Results obtained using the API ZYM kit.

¶Results obtained using the API 50 CH kit.

Additional gene sequence analyses were performed in order to clarify the phylogenetic affinities of the two novel isolates. Partial sequences of the *rpoB* (701 bp) and *sodA* (356 bp) genes were amplified and sequenced as described previously (Poyart *et al.*, 1998; Drancourt *et al.*, 2004). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980) and the resulting

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain 1956-02^T and species of the genus *Streptococcus*. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points. Open circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-likelihood and parsimony trees. The filled circle indicates that the corresponding node was also obtained in maximum-parsimony trees. *Enterococcus faecalis* ATCC 19433^T (not shown) was used as an outgroup. Bar, 1% sequence divergence. A full version of this tree is presented in Supplementary Fig. S1 (available with IJSEM Online).

trees were constructed using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap percentages (based on 1000 replicates) were also calculated. Strains 1956-02^T and 1355-03 shared high levels of sequence similarity with each other: 95.8% for the *sodA* gene and 98.9% for the *rpoB* gene. With regard to related strains, 1956-02^T and 1355-03 showed the highest similarities with sequences from *S. suis* ATCC 43765^T: 85.4–85.8% for *sodA* and 85.1–85.2% for *rpoB*. Strain 1956-02^T formed a separate branch with respect to *S. suis* and other *Streptococcus* species in the phylogenetic tree inferred from the *sodA* (Fig. 2) and *rpoB* (data not shown) gene sequence comparisons.

DNA–DNA hybridization experiments were carried out between the two isolates (strains 1956-02^T and 1355-03) and between strain 1956-02^T and its closest phylogenetic neighbour, *S. suis* NCTC 10234^T. Genomic DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by means of chromatography on

hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was performed in 2× SSC at 68 °C by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) using the method described by De Ley *et al.* (1970) and the modifications described by Huß *et al.* (1983). A Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer was used along with a temperature controller with an *in situ* temperature probe (Varian). DNA–DNA hybridization between the two novel isolates demonstrated 81.6% DNA relatedness, showing that they are members of the same species (Wayne *et al.*, 1987). The DNA–DNA reassociation value between strain 1956-02^T and *S. suis* NCTC 10234^T was 36.6%, clearly confirming that the novel isolates constitute a separate species (Wayne *et al.*, 1987).

The G + C content of the DNA of a representative isolate (strain 1956-02^T) was determined at the Deutsche

Fig. 2. Phylogenetic tree, based on *sodA* gene-fragment sequences, showing the relationships between strain 1956-02^T and species of the genus *Streptococcus*. Bootstrap percentages (based on 1000 replications) >50% are shown at branching points. *E. faecalis* ATCC 19433^T (not shown) was used as an outgroup. Bar, 1% sequence divergence.

Sammlung von Mikroorganismen und Zellkulturen using the HPLC method of Mesbah *et al.* (1989). The G+C content of the type strain was found to be 42.1 mol%.

It is clear from the results of this study conducted using a polyphasic taxonomic approach, that the unidentified catalase-negative cocci represent a novel streptococcal species. Phylogenetically, these bacteria isolated from pigs form a distinct subline within the genus *Streptococcus* and exhibit a loose association with *S. suis*. Sequence analysis of the *sodA* and *rpoB* genes and biochemical profiling also demonstrated that the isolates were distinct from all of the currently recognized streptococcal species. Therefore, on the basis of both phylogenetic and phenotypic criteria it is evident that the unidentified cocci merit classification as a novel species of the genus *Streptococcus*, for which the name *Streptococcus plurextorum* sp. nov. is proposed. The two isolates were recovered from different animals, in different farms located in different provinces of Spain and in different years. Therefore, it is very unlikely that the isolates could have any epidemiological clonality. Tests that are useful in differentiating *S. plurextorum* sp. nov. from the most closely related species (as found in the neighbouring phylogenetic tree based on 16S rRNA gene sequences) are shown in Table 1.

Description of *Streptococcus plurextorum* sp. nov.

Streptococcus plurextorum (plu.rex.to'rum. L. comp. pl. *plures* more, several, many; L. pl. n. *exta* -orum entrails; N.L. gen. pl. n. *plurextorum* of several internal organs).

Cells are Gram-positive cocci arranged in short chains. Non-motile. Facultatively anaerobic and catalase- and oxidase-negative. Colonies are non-pigmented, circular and 1 mm in diameter after 24 h on blood agar and produce an α -haemolytic reaction. Growth occurs at 37 °C but not at 10 or 45 °C. Growth does not occur at pH 9.6 or in broth containing 6.5% NaCl. Resistant to optochin. Reacts with Lancefield group B antisera. With the API 50 CH and API Rapid ID 32 Strep kits, cells are able to produce acid from D-lactose, D-glucose, D-fructose, maltose, trehalose, raffinose and sucrose but not from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, L-arabitol, D-arabitol, gentiobiose, D-ribose, melibiose, D-mannitol, inositol, dulcitol, melezitose, pullulan, inulin, methyl α -D-mannopyranoside, starch, methyl β -D-glucopyranoside, N-acetylglucosamine, cellobiose, L-rhamnose, L-sorbose, turanose, D-lyxose, xylitol, D-fucose, L-fucose, D-mannose, glycogen, amygdalin, arbutin, sorbitol, 2-ketogluconate, cyclodextrin or tagatose. Delayed acid production occurs for salicin and 5-ketogluconate (API 50 CH) after 7 days incubation. Acid production from D-galactose and D-xylose is variable (API 50 CH). β -Galactosidase, acid phosphatase, leucine arylamidase (API ZYM), β -glucuronidase, α -galactosidase, alanine-phenylalanine-proline arylamidase and glycyl-tryptophan arylamidase (API Rapid ID 32 Strep) are detected. No activity is detected for alkaline phosphatase, N-acetyl- β -glucosaminidase,

naphthol-AS-BI-phosphohydrolase, α -glucosidase, α -mannosidase, α -fucosidase, esterase (C4), ester lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin or α -chymotrypsin (API ZYM), β -glucosidase, β -mannosidase or pyroglutamic acid arylamidase (API Rapid ID 32 Strep). Arginine, hippurate and urea are not hydrolysed (API Rapid ID 32 Strep). Acetoin is not produced (Rapid ID 32 Strep).

The type strain, 1956-02^T (=CECT 7308^T=CCUG 52972^T), was isolated from the lungs of a pig with pneumonia. The full habitat is not known. The DNA G+C content of the type strain is 42.1 mol%. Strain 1355-03 (=CECT 7307=CCUG 52971) is a reference strain.

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