Allofustis seminis gen. nov., sp. nov., a novel Gram-positive, catalase-negative, rod-shaped bacterium from pig semen

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An unknown Gram-positive, catalase-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacterium originating from semen of a pig was characterized using phenotypic, molecular chemical and molecular phylogenetic methods. Chemical studies revealed the presence of a directly cross-linked cell wall murein based on L-lysine and a DNA G + C content of 39 mol%. Comparative 16S rRNA gene sequencing showed that the unidentified rod-shaped organism formed a hitherto unknown subline related, albeit loosely, to *Alkalibacterium olivapovlitis*, *Alloiococcus otitis*, *Dolosigranulum pigrum* and related organisms, in the low-G + C-content Gram-positive bacteria. However, sequence divergence values of > 11 % from these recognized taxa clearly indicated that the novel bacterium represents a separate genus. Based on phenotypic and phylogenetic considerations, it is proposed that the unknown bacterium from pig semen be classified as a new genus and species, *Allofustis seminis* gen. nov., sp. nov. The type strain is strain 01-570-1^T (=CCUG 45438^T=CIP 107425^T).

During the course of a routine examination of porcine semen specimens submitted by private artificial insemination centres for bacteriological analysis, an unusual Gram-positive, rod-shaped organism was isolated. Most of the semen specimens from the artificial insemination centres contain bacterial species, the majority of which are environmental organisms such as *Klebsiella* spp., *Serratia* spp. and *Pseudomonas* spp. However, from one of the centres, we occasionally isolate a bacterium that somewhat resembles *Actinomyces*-like or *Arcanobacterium*-like species. Cells are catalase-negative, short, Gram-positive-staining rods that form pin-point colonies on sheep-blood agar. Preliminary biochemical studies, however, indicated that the organism did not conform to any species of these genera. Therefore, a detailed polyphasic taxonomic study was performed in an attempt to identify the unknown organism from porcine semen. In this article, we report the results of this taxonomic investigation and propose that the porcine bacterium be assigned to a new genus as *Allofustis seminis* gen. nov., sp. nov.

Strain 01-570-1^T (=CCUG 45438^T=CIP 107425^T) was isolated from stored porcine semen. The unidentified bacterium was cultured on Columbia blood-agar base

supplemented with 5 % horse blood at 37 °C, under anaerobic conditions. The organism was characterized biochemically using the API Rapid ID 32Strep, API Rapid ID 32A and API ZYM systems according to the manufacturer's instructions (API bioMérieux). Cell-wall murein was prepared by mechanical disruption of cells and acid hydrolysates were analysed as described by Schleifer & Kandler (1972), except that ascending TLC with cellulose sheets was used. Long-chain cellular fatty acids were analysed as described by Kämpfer & Kroppenstedt (1996). The G + C content of DNA was determined by HPLC according to Mesbah *et al.* (1989). The 16S rRNA gene of the isolate was amplified by PCR and sequenced directly using a *Taq* Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the novel isolate were determined by performing database searches. These sequences and those of other known related strains were retrieved from GenBank and aligned with the newly determined sequence using the program DNATools (Rasmussen, 1995). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated with the programs PRETTY and DNADIST (using the Kimura 2-parameter correction) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR and the stability of the groupings was estimated by bootstrap

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCUG 45438^T is AJ410303.

analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The unidentified organism recovered from porcine semen consisted of short, Gram-positive rods. The organism was facultatively anaerobic but grew better under anaerobic conditions and was catalase-negative. Using the API Rapid ID 32Strep system, the unknown organism did not produce acid from any of the carbohydrates tested. Activity was observed for arginine dihydrolase, alkaline phosphatase, alanine-phenylalanine-proline arylamidase, β -glucosidase, glycyl tryptophan arylamidase, *N*-acetyl- β -glucosaminidase, pyroglutamic acid arylamidase and β -mannosidase. All other enzyme tests were negative with this kit and the organism did not hydrolyse hippurate and did not produce acetoin. Using the API Rapid ID 32A system, the organism failed to produce acid from mannose and raffinose, did not reduce nitrate and did not produce indole. The organism displayed activity for alanine arylamidase, alkaline phosphatase, arginine arylamidase, arginine dihydrolase, α -fucosidase, β -glucosidase, glycine arylamidase, histidine arylamidase, proline arylamidase, leucyl glycine arylamidase, leucine arylamidase, *N*-acetyl- β -glucosaminidase, phenyl-alanine arylamidase, pyroglutamic acid arylamidase, serine arylamidase and tyrosine arylamidase. All other enzyme tests in this kit gave negative results. With the API ZYM system, acid phosphatase, alkaline phosphatase, phosphoamidase, leucine arylamidase and valine arylamidase were detected. All other tests in the API ZYM kit were negative. The long-chain cellular fatty acids of the organism were found to be of the straight-chain saturated and monounsaturated types, with C16:0 (32.7%), C16:1 (4.3%), C18:0 (19.3%) and C18:1 ω 9c (40.2%) predominating. Cell wall murein analysis showed the presence of an A1 α murein type: L-Lys-direct. The G+C content of DNA of the porcine bacterium was 39 mol%.

The cell wall murein structure and low G+C content of DNA of the isolate were incompatible with its provisional identification as belonging to one of the genera *Actinomyces* or *Arcanobacterium*. Therefore, to investigate the phylogenetic position of the unidentified bacterium, its almost complete 16S rRNA gene sequence was determined. Sequence database searches revealed that the unknown organism was most closely related to *Alkalibacterium olivapovlenticus*,

Alloiococcus otitis, *Carnobacterium* species, *Dolosigranulum pigrum*, *Enterococcus* species, *Granulicatella* species and related catalase-negative organisms. *Actinomyces* and other high-G+C-content bacteria revealed very low levels of similarity to the isolate (data not shown). A tree constructed using the neighbour-joining method showing the phylogenetic relationships of the unidentified bacterium is illustrated in Fig. 1 and demonstrates that the isolate possesses an affinity with *Alkalibacterium olivapovlenticus*, *Alloiococcus otitis* and *Dolosigranulum pigrum*. This association was confirmed by maximum-parsimony analysis.

From the comparative 16S rRNA gene sequence analysis, it is evident that the unidentified catalase-negative, rod-shaped organism represents a hitherto unknown taxon. Phylogenetically, the novel bacterium is related to the low-G+C *Clostridium* subphylum of the Gram-positive bacteria and forms an association with a cluster of organisms that includes *Alkalibacterium olivapovlenticus*, *Alloiococcus otitis*, *Dolosigranulum pigrum* and some uncultured bacteria associated with the sheep mite *Psoroptes ovis*. The unidentified isolate formed a distinct subline branching at the periphery of this rRNA cluster. The branching of the unknown organism at the base of this group was supported by a bootstrap resampling value of 90% (Fig. 1). In terms of sequence similarities, the unknown organism displayed 88.5% sequence similarity to *Dolosigranulum pigrum* NCFB 2975^T, a coccus-shaped organism associated with human clinical sources (Aguirre *et al.*, 1993). A comparable level of sequence similarity (88.4%) was also displayed between the porcine bacterium and some uncultured bacteria (16S rDNA accession numbers AF124034 and AF124041) associated with sheep scab mites (Hogg & Lehane, 1999). Marginally lower levels of similarity were exhibited to *Alloiococcus otitis* NCFB 2890^T (87.9%), an ovoid-shaped organism associated with human ear infections (Aguirre & Collins, 1992), and *Alkalibacterium olivapovlenticus* WW2-SN4a^T (87.9%), a rod-shaped alkaliphile recovered from wash waters of edible olives (Ntougias & Russell, 2001). From its branching position in the tree and sequence divergence values of approximately 11–12% from these genera, it is clear that the porcine bacterium merits classification at a similar taxonomic rank (i.e. genus). It is pertinent to note that the separateness of the novel

Fig. 1. Unrooted tree based on 16S rRNA showing the phylogenetic relationships of *Allofustis seminis* gen. nov., sp. nov. Bar, 1% sequence divergence.

bacterium is strongly supported by phenotypic considerations. The isolate does not match any known Gram-positive organism phenotypically. The unidentified organism differs from *Alloiococcus otitis* by forming rod-shaped cells, growing anaerobically and by numerous biochemical reactions. Cells of *Alloiococcus otitis* are ovoid in shape and the species is nutritionally very fastidious and does not grow under anaerobic conditions (Aguirre & Collins, 1992; Miller *et al.*, 1996). Similarly, the isolate is incompatible with belonging to the genus *Dolosigranulum* in that it forms rod-shaped cells, synthesizes an A1 α -type L-Lys-direct cell wall murein and fails to produce acid from a variety of carbohydrates. By contrast, cells of *Dolosigranulum pigrum* are ovoid in shape, possess an L-Lys-D-Asp-type murein and ferment a variety of carbohydrates (Aguirre *et al.*, 1993). The isolate also differs from *Dolosigranulum pigrum* in numerous other biochemical traits (Table 1) and differs markedly from *Alkalibacterium olivapovlenticus* in not being alkaliphilic or psychrotolerant. *Alkalibacterium olivapovlenticus* is an obligate alkaliphile, growing within a pH range of ~8.5–10.8, with an optimum at pH ~9–10 (Ntougias & Russell, 2001). Based on sequence divergence values of > 11 % from its nearest phylogenetic relatives and the distinct subline formed by the novel bacterium, in concert with its quite distinct phenotypic characteristics, we are of the opinion that the unknown bacterium from porcine semen merits assignment to a new genus and species, for which the name *Allofustis seminis* gen. nov., sp. nov. is proposed.

Description of *Allofustis* gen. nov.

Allofustis (Al.lo.fus'tis. Gr. prefix *allo* the other; L. masc. n. *fustis* stick; N.L. masc. n. *Allofustis* the other stick or rod).

Cells are Gram-positive, non-spore-forming rods. Facultatively anaerobic and catalase-negative. Arginine dihydrolase, leucine arylamidase and pyroglutamic acid arylamidase are produced. Indole-negative. Nitrate is not reduced. Voges-Proskauer-negative. The long-chain cellular fatty acids are of the straight-chain saturated and monounsaturated types.

Cell wall murein is based on L-Lys variation A1 α (type L-Lys-direct). The type species is *Allofustis seminis*. The G+C content of genomic DNA of the type species is 39 mol%.

Description of *Allofustis seminis* sp. nov.

Allofustis seminis (sem.in'is. L. n. *semen* seed; N.L. gen. n. *seminis* of semen).

Cells stain Gram-positive and are rod-shaped. Non-spore-forming. β -Haemolytic reaction on sheep blood. Facultatively anaerobic; grows well on chocolate agar anaerobically or in CO₂, at 37 °C. Catalase-negative. Acid is not produced from L-arabinose, D-arabitol, cyclodextrin, glycogen, lactose, maltose, mannitol, mannose, melibiose, melezitose, pullulan, raffinose, D-ribose, sorbitol, sucrose, D-tagatose or trehalose. Arginine dihydrolase, arginine arylamidase, acid phosphatase, alkaline phosphatase, alanine arylamidase, alanine-phenylalanine-proline arylamidase, glycine arylamidase, glycyl tryptophan arylamidase, histidine arylamidase, leucyl glycine arylamidase, leucine arylamidase, phosphoamidase, proline arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, β -mannosidase, serine arylamidase, tyrosine arylamidase and valine arylamidase are produced. Activity for α -arabinosidase, esterase C-4, ester lipase C8, α -galactosidase, β -galactosidase, β -galactosidase-6-phosphate, α -glucosidase, β -glucuronidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, lipase C14, α -mannosidase, chymotrypsin, trypsin and urease is not detected. α -Fucosidase, β -glucosidase and N-acetyl- β -glucosaminidase may or may not be detected. Hippurate and aesculin are not hydrolysed. Indole and acetoin are not produced and nitrate is not reduced. The major long-chain cellular fatty acids are C16:0, C16:1, C18:0 and C18:1 ω 9c. Other chemotaxonomic properties are as described for the genus.

The type strain is strain 01-570-1^T (= CCUG 45438^T = CIP 107425^T).

Table 1. Characteristics that are useful in differentiating *Allofustis seminis* gen. nov., sp. nov. from *Alloiococcus otitis* and *Dolosigranulum pigrum*

Characteristic	<i>Allofustis seminis</i>	<i>Alloiococcus otitis</i>	<i>Dolosigranulum pigrum</i>
Cell shape	Rods	Ovoid	Ovoid
Relation to air	Facultative anaerobic	Aerobic	Facultative anaerobic
Acid from:			
Maltose	—	—	+
Sucrose	—	—	+
Production of:			
Arginine dihydrolase	+	—	—
β -Galactosidase	—	+	+
Murein type	L-Lys-direct	ND	L-Lys-D-Asp

ND, Not determined.

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