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 olivapowliticus, 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 01-570-1T (=CCUG 45438T = CIP 107425T).

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 01-570-1T (=CCUG 45438T = CIP 107425T) 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
sis showed the presence of an A1 most closely related to database searches revealed that the unknown organism was genetically position of the unidentified bacterium, its almost or Arcanobacterium identification as belonging to one of the genera DNA of the isolate were incompatible with its provisional leucine arylamidase, oryroglutamic acid arylamidase, proline arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase and b-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, x-fucosidase, c-glucosidase, glycine arylamidase, histidine arylamidase, proline arylamidase, leucyl glycine arylamidase, leucine arylamidase, N-acetyl-c-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 : 1o9c (40.2 %) predominating. Cell wall murein analysis showed the presence of an A1z 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 olivopoliviticus, 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 olivopoliviticus, 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 olivopoliviticus, 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 NFCB 2979T, 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 NFCB 2890T (87.9%), an ovoid-shaped organism associated with human ear infections (Aguirre & Collins, 1992), and Alkalibacterium olivopoliviticus WW2-SN4aT (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.](image-url)
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 A1z-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 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* (Allo. 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 pyrog glutamic 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 A1z (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, cyclohextrin, 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, glycy l tryptophan arylamidase, histidine arylamidase, leucyl glycine arylamidase, leucine arylamidase, phosphoamidase, proline arylamidase, phenylalanine arylamidase, pyrog glutamic 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, χ-glucosidase, β-glucuronidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, lipase C14, χ-mannosidase, chymotrypsin, trypsin and urease is not detected. α-Fucosidase, β-glucosidase and N-acetyl-β-glucosaminidase may or may not be detected. Hipsitter 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 C18:1ω9c. Other chemotaxonomic properties are as described for the genus.

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

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Allofustis seminis</em></th>
<th><em>Alloiococcus otitis</em></th>
<th><em>Dolosigranulum pigrum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>Ovoid</td>
<td>Ovoid</td>
</tr>
<tr>
<td>Relation to air</td>
<td>Facultative anaerobic</td>
<td>Aerobic</td>
<td>Facultative anaerobic</td>
</tr>
<tr>
<td>Acid from:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maltose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Production of:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine dihydrolase</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Murein type</td>
<td>1-Lys-direct</td>
<td>ND</td>
<td>1-Lys–D-Asp</td>
</tr>
</tbody>
</table>

ND, Not determined.
Acknowledgements

We are grateful to Hans Trüper of the University of Bonn for help in coining the genus name.

References


