

## NOTE

***Helcococcus ovis* sp. nov., a Gram-positive organism from sheep**M. D. Collins,<sup>1</sup> E. Falsen,<sup>2</sup> G. Foster,<sup>3</sup> L. R. Monasterio,<sup>1</sup> L. Dominguez<sup>4</sup> and J. F. Fernandez-Garazabal<sup>4</sup>Author for correspondence: M. D. Collins. Tel: +44 118 935 7226. Fax: +44 118 926 7917.  
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**Two strains of a hitherto undescribed Gram-positive, catalase-negative, facultatively anaerobic coccus isolated from sheep were characterized by phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequencing studies demonstrated that the unknown strains were genealogically highly related and constitute a new line close to, but distinct from, *Helcococcus kunzii*. The unknown bacterium was readily distinguished from *H. kunzii* by biochemical tests and electrophoretic analysis of whole-cell proteins. Based on phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium be classified as *Helcococcus ovis* sp. nov. The type strain of *Helcococcus ovis* is CCUG 37441<sup>T</sup>.**

**Keywords:** *Helcococcus ovis*, taxonomy, phylogeny, 16S rRNA

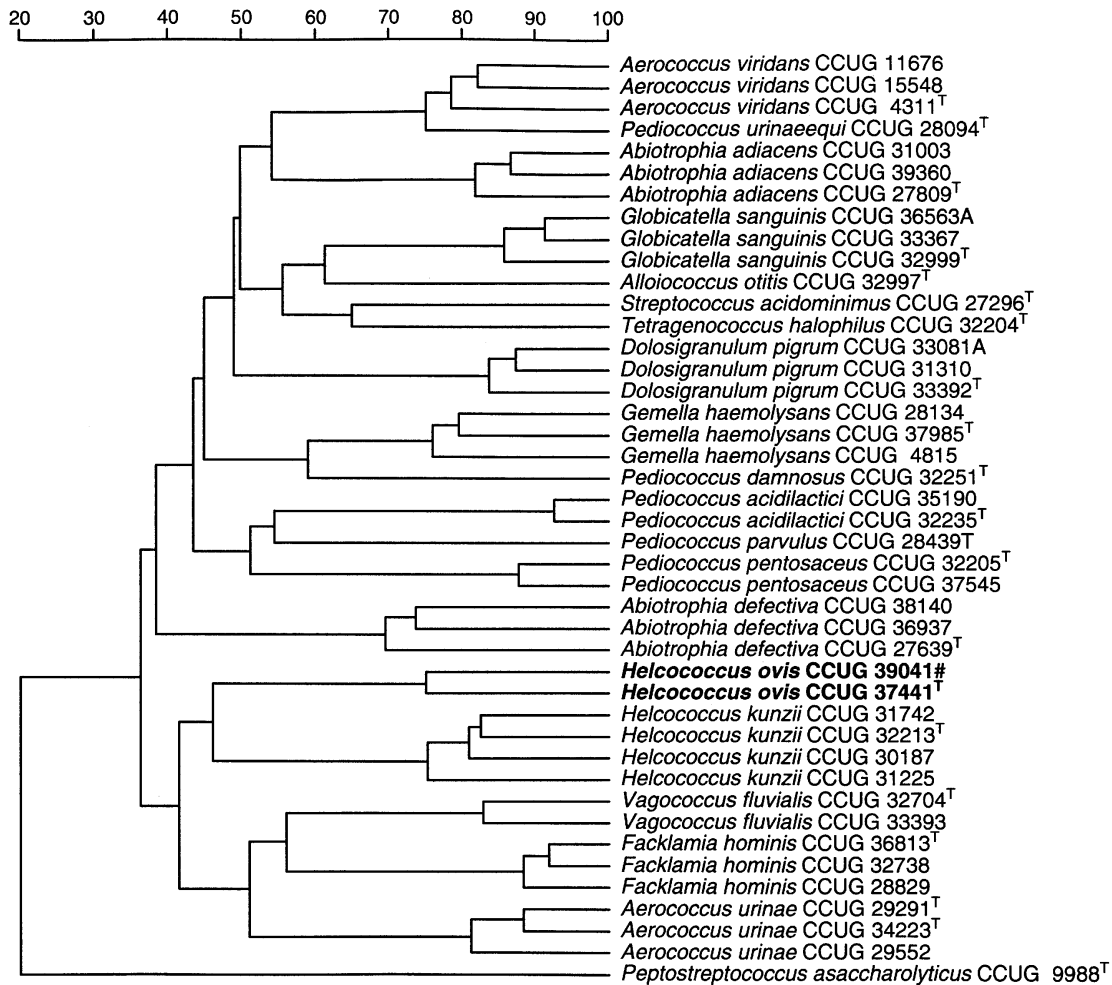
*Helcococcus kunzii* was described in 1993 as a new genus and species of catalase-negative, facultatively anaerobic, Gram-positive cocci (Collins *et al.*, 1993). *H. kunzii* has been isolated in mixed cultures from clinical specimens (lower-extremity wounds and breast masses) (Caliendo *et al.*, 1995) and has recently been reported as the sole organism from an infected sebaceous cyst (Peel *et al.*, 1997). *H. kunzii* represents one of a plethora of new catalase-negative, Gram-positive cocci causing and/or associated with animal, particularly human, disease described in recent years [e.g. *Alloiococcus otitis* (Aguirre & Collins, 1992), *Dolosigranulum pigrum* (Aguirre *et al.*, 1993), *Facklamia hominis* (Collins *et al.*, 1997), *Globicatella sanguinis* (Collins *et al.*, 1992)]. Most of these new taxa have become apparent as a result of improved taxonomies of the Gram-positive, catalase-negative cocci, in particular through the use of 16S rRNA gene sequencing as a diagnostic tool, and due to an increasing recognition by clinical microbiologists of the possible role of these organisms as potential pathogens. In the course of a study of catalase-negative, Gram-positive cocci associated with animal disease, we have characterized two isolates of a hitherto unknown coccus from sheep. The two isolates were recovered in mixed cultures from geographically separated animals and shown to comprise a new subline within the genus *Helcococcus*. Based on the results of a polyphasic

taxonomic study, a new species, *Helcococcus ovis*, is described.

Two isolates (designated S840-96-1<sup>T</sup> and Sat 1) of an unknown Gram-positive coccus were recovered from sheep. Strain S840-96-1<sup>T</sup> (=CCUG 37441<sup>T</sup>) was isolated following a post-mortem examination of an adult male sheep from Scotland, UK. The strain was recovered together with *Arcanobacterium pyogenes* from lung, liver and spleen. Both organisms were recovered as heavy growth on Columbia agar (Difco) supplemented with 7% citrated sheep blood but we are unable to ascribe clinical significance to the isolate. The second isolate, Sat 1 (=CCUG 39041), was recovered from milk of a sheep in Spain suffering from subclinical mastitis. The strain was isolated together with a *Staphylococcus* sp. on Columbia blood agar (bioMérieux) where it grew only at the periphery of the staphylococcal colonies. Initially, it was not possible to grow the strain independently of the *Staphylococcus* sp. However, after repeated subculturing on Columbia blood agar under microaerophilic conditions (candle jar), the isolate lost its dependency on the *Staphylococcus* sp. for growth. Since staphylococci can cause mastitis in sheep, we do not know the clinical significance of the unknown isolate.

Both strains were routinely cultured on Columbia sheep blood agar (bioMérieux) at 37 °C. The strains were biochemically characterized by using the API rapid ID32 strep and API ZYM systems according to the manufacturer's instructions (bioMérieux). PAGE

The GenBank accession number for the 16S rRNA gene sequence of strain S840-96-1<sup>T</sup> (= CCUG 37441<sup>T</sup>) is Y16279.

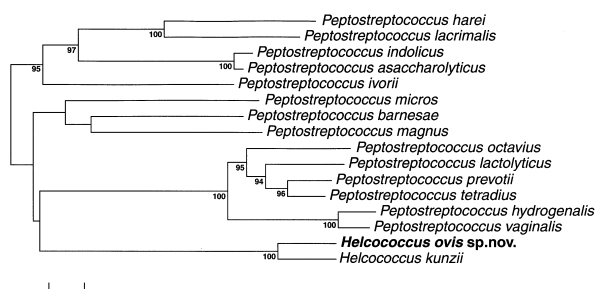


**Fig. 1.** Similarity dendrogram based on whole-cell protein patterns of *Helcococcus ovis* sp. nov. and some other species. Levels of correlation are expressed as percentages of similarity for convenience.

of whole-cell proteins was performed as described previously (Pot *et al.*, 1994). For densitometric analysis, normalization and interpretation of protein patterns the GELCOMP GCW 3.0 software package (Applied Maths, Kortrijk, Belgium) was used. The DNA base composition of strain S840-96-1<sup>T</sup> was determined by thermal denaturation ( $T_m$  method) as described by Garvie (1978). Phylogenetic analysis was performed by comparative 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30–1521 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR using conserved primers close to the 3' and 5' ends of the gene. The PCR products were purified using a Prep-A-Gene kit (Bio-Rad) according to the manufacturer's instructions and directly sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolates were determined by performing sequence database searches. These sequences and those of other known related strains

were retrieved from the GenBank or Ribosome Database Project (RDP) databases and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and approximately 100 bases at the 5' end of the rRNA were omitted from further analyses because of alignment ambiguities. A continuous stretch of 1320 bases was used for distance matrix analysis. A distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The two isolates from sheep were ovoid in shape and formed single cells, pairs or short-chains. Both strains were Gram-positive, catalase-negative, oxidase-negative, facultative anaerobes which produced small pinhead colonies on blood agar. They were non-haemolytic. Both isolates produced acid but no gas



**Fig. 2.** Unrooted tree showing the phylogenetic relationships of *Helcococcus ovis* sp. nov. and some other low-G + C-content, Gram-positive bacteria. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Bar, 1% sequence divergence.

from glucose. Production of acid from cyclodextrin, glycogen and maltose was variable. Neither strain produced acid from D-arabitol, L-arabinose, inulin, lactose, mannitol, melibiose, melezitose, methyl β-D-glucopyranoside, pullulan, raffinose, ribose, sorbitol, starch, sucrose, trehalose, tagatose or D-xylose. Both strains displayed alkaline phosphatase, acid phosphatase, β-glucuronidase and leucine arylamidase activities. Activity for cystine arylamidase, β-galactosidase and phosphoamidase was variable. Neither of the isolates displayed alanyl-phenylalanine-proline arylamidase, glycyl-tryptophan arylamidase, arginine dihydrolase, chymotrypsin, α-fucosidase, esterase C4, ester lipase C8, β-glucosidase, lipase C14, α-mannosidase, β-mannosidase, trypsin, valine arylamidase, pyroglutamic acid arylamidase or N-acetyl-β-glucosaminidase activity. Both isolates failed to

hydrolyse aesculin, gelatin, urea and hippurate, did not reduce nitrate, and were Voges-Proskauer-negative. They were vancomycin-sensitive. The biochemical affinity between the clinical isolates was confirmed by PAGE analysis of whole-cell proteins in which the two strains formed a robust and distinct cluster at about 75% similarity, which was quite separate from all other Gram-positive, catalase-negative reference organisms examined (Fig. 1). The nearest relative of the unknown bacterium on PAGE analysis was *H. kunzii*.

To establish the phylogenetic affinities of the isolates, their partial 16S rRNA gene sequences were examined. A large fragment consisting of > 1400 nucleotides was determined from each strain and no base differences were detected (i.e. 100% 16S rRNA gene sequence similarity) thereby demonstrating their close genealogical relatedness. Sequence searches of GenBank and RDP databases revealed that the unknown organism was phylogenetically most closely associated with the *Clostridium* subphylum of the Gram-positive bacteria. Comparative sequence analyses showed the unknown coccus was most closely related to *H. kunzii* (96% 16S rRNA gene sequence similarity). High sequence relatedness was also shown with *Peptostreptococcus* species (approx. 82–86% 16S rRNA gene sequence similarity). Treeing analysis confirmed the close affinity between the unknown coccus from sheep and *H. kunzii* with the relationship supported by a bootstrap resampling value of 100%. A tree depicting the relationship between the unknown coccus, *Helcococcus kunzii* and some *Peptostreptococcus* species is shown in Fig. 2.

It is evident from the results of this study that the unknown coccus from sheep represents a hitherto

**Table 1.** Characteristics useful for differentiating *Helcococcus ovis* sp. nov. and *Helcococcus kunzii*

Data from tests conducted in this study; n, number of strains examined.

Character	<i>Helcococcus ovis</i> sp. nov. (n = 2)	<i>Helcococcus kunzii</i> (n = 6)
Acid produced from:		
Lactose	–	+
Pullulan	–	v
Trehalose	–	+
Hydrolysis of:		
Aesculin	–	+
Starch	–	v
Production of:		
Alkaline phosphatase	+	–
β-Glucosidase	–	+
β-Glucuronidase	+	–
Leucine arylamidase	+	–
Pyroglutamic acid arylamidase	–	+
N-Acetyl-β-glucosaminidase	–	+

undescribed bacterium. The coccus formed a distinct line and displayed a specific affinity with *H. kunzii*. A 16S rRNA gene sequence divergence value of approximately 4% clearly demonstrates that the coccus is a new member of the genus *Helcococcus* and warrants separate species status. The genus *Helcococcus* currently contains a single species, *H. kunzii* (Collins *et al.*, 1993). This species was originally encountered in mixed culture in human clinical specimens (wounds, breast masses) and, more recently, has been reported as the sole isolate from an infected sebaceous cyst (Peel *et al.*, 1997). The two *Helcococcus* isolates characterized in this study were both recovered from sheep, one during post-mortem examination and the other from milk of a sheep with subclinical mastitis. Both strains were recovered in combination with other organisms known to cause disease in sheep and we are unable to ascribe any clinical significance to the unknown coccus. The unknown bacterium can be readily distinguished from *H. kunzii* by the tests shown in Table 1. Therefore, based on both phenotypic and phylogenetic findings, we believe that the unknown coccus merits classification as a new species of the genus *Helcococcus*, for which the name *Helcococcus ovis* sp. nov. is proposed.

#### Description of *Helcococcus ovis* sp. nov.

*Helcococcus ovis* (o'vis. L. gen. n. *ovis* of a sheep).

Gram-positive cocci, which occur singly, in pairs or short chains. Non-pigmented, non-motile and non-haemolytic. Catalase-negative. Facultatively anaerobic. Acid but not gas is produced from glucose. Acid may or may not be formed from cyclodextrin, glycogen and maltose. Acid is not produced from D-arabitol, L-arabinose, inulin, lactose, mannitol, melzitose, melibiose, methyl  $\beta$ -D-glucopyranoside, pullulan, raffinose, ribose, sorbitol, starch, sucrose, tagatose or D-xylose. Alkaline phosphatase, acid phosphatase,  $\beta$ -glucuronidase and leucine arylamidase are produced. Alanine-phenylalanine-proline arylamidase, glycyl-tryptophan arylamidase, arginine dihydrolase, chymotrypsin, esterase C4, ester lipase C8,  $\alpha$ -fucosidase,  $\alpha$ -galactosidase,  $\beta$ -glucosidase, valine arylamidase, lipase C14,  $\alpha$ -mannosidase,  $\beta$ -mannosidase, trypsin and N-acetyl- $\beta$ -glucosaminidase activity is not detected. Activity for cystine arylamidase,  $\beta$ -galactosidase and phosphoamidase is variable. Aesculin, gelatin, hippurate and urea are not hydrolysed. Nitrate is not reduced to nitrite. Voges-Proskauer-negative. Vancomycin-sensitive. Isolated from sheep. Habitat not known. The type strain is S840-96-1<sup>T</sup> (CCUG

37441<sup>T</sup>). The G+C content of the type strain is 29 mol% ( $T_m$  method).

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