NOTE

Characterization of a novel Gram-positive, catalase-negative coccus from horses: description of *Eremococcus coleocola* gen. nov., sp. nov.

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Two strains of an unknown Gram-positive, catalase-negative, facultatively anaerobic coccus originating from the reproductive tract of horses were characterized by phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequencing studies demonstrated that the two strains constitute a new subline within the lactic-acid group of bacteria, close to, but distinct from, *Abiotrophia defectiva*, *Globicatella sanguinis* and close relatives. The unknown bacterium was readily distinguished from other described Gram-positive, catalase-negative cocci by biochemical tests and electrophoretic analysis of whole-cell proteins. On the basis of phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium be classified as *Eremococcus coleocola* gen. nov., sp. nov. The type strain of *Eremococcus coleocola* is CCUG 38207^T.

Keywords: *Eremococcus coleocola* gen. nov., sp. nov., taxonomy, phylogeny, 16S rRNA, horse

The Gram-positive, catalase-negative cocci constitute a phenotypically heterogeneous group of organisms within the *Clostridium* subphylum of the Gram-positive bacteria. In the 1986 edition of *Bergey's Manual of Systematic Bacteriology*, only six genera of Gram-positive, catalase-negative cocci were listed (Sneath *et al.*, 1986). During the past decade, the number of described genera and species of Gram-positive, catalase-negative cocci has increased considerably. Some of these new taxa have resulted from the taxonomic reorganization of existing genera, such as *Enterococcus* and *Lactococcus* from the genus *Streptococcus*; *Oenococcus* and *Weissella* from the genus *Leuconostoc*; and *Tetragenococcus* from the genus *Pediococcus* (Collins *et al.*, 1990, 1993b; Dicks *et al.*, 1995; Schleifer & Kilpper-Bälz, 1984; Schleifer *et al.*, 1985). However, many of the recently delineated taxa were previously unknown, e.g. *Alloiococcus* (Aguirre & Collins, 1992b), *Dolosigranulum* (Aguirre *et al.*, 1993), *Facklamia* (Collins *et al.*, 1997), *Globicatella* (Collins *et al.*, 1992), *Ignavigranum* (Collins *et al.*, 1999) and *Helcococcus* (Collins *et al.*, 1993a), and

their recognition has been largely facilitated by the availability of much-improved taxonomic frameworks, which in turn have stemmed from the use of phenotypic and molecular genetic methodologies in concert. Despite this rapid expansion in the number of described taxa, there is a growing awareness that many other genera and species of Gram-positive, catalase-negative cocci remain to be discovered, particularly from human and animal sources. In this study we describe the characteristics of an unknown α -haemolytic coccus isolated from the reproductive tract of two horses. On the basis of the results of a polyphasic taxonomic study, we describe yet another new taxon, *Eremococcus coleocola* gen. nov., sp. nov., within the Gram-positive, catalase-negative cocci.

Strain M1832/95/2^T was isolated in mixed culture with a *Corynebacterium* species from a thoroughbred cross horse. This was a follow-up sample for a profuse vaginal discharge that had failed to resolve following treatment. The initial sample had yielded a pure growth of *Gardnerella vaginalis*. Strain M923/98/1 was isolated from a clitoral fossa swab of a different but apparently healthy thoroughbred mare, which was submitted as a pre-service check. Strains M1832/95/2^T and M923/98/1 have been deposited in the Culture

The GenBank accession number for the 16S rRNA gene sequence of *Eremococcus coleocola* strain CCUG 38207^T is Y17780.

Collection of the University of Göteborg (CCUG) under accession numbers 38207^T and 39488, respectively. Both strains were cultured on Columbia agar (Difco) supplemented with 5% horse blood at 37 °C, in air plus 5% CO₂. The strains were characterized biochemically by using the API Rapid ID32S and API ZYM systems according to the manufacturer's instructions (API bioMérieux). PAGE analysis of whole-cell proteins was performed as described by Pot *et al.* (1994). For densitometric analysis, normalization and interpretation of protein patterns, the GELCOMPARGCW 3.0 software package (Applied Maths) was used. The cell-wall murein structure of strain CCUG 38207^T was determined by the method of Schleifer & Kandler (1972), except that ascending TLC on cellulose sheets (Merck) was used. The G+C content of DNA of strain CCUG 38207^T was determined as described by Garvie (1978). The 16S rRNA genes of the isolates were amplified by PCR and directly sequenced by 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 new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences by using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated by using the programs PRETTY and DNADIST (using Kimura's two-parameter correction) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). Parsimony analysis was also performed on the same dataset (Felsenstein, 1989).

The two isolates were ovoid in shape and formed single cells, pairs or short chains. They stained Gram-positive and were α -haemolytic on both sheep- and horse-blood agar. The isolates were facultatively anaerobic and catalase- and oxidase-negative. They did not grow on Difco Bacto bile aesculin agar. The strains failed to grow at 10 °C but produced growth at 42 °C. They produced weak growth in 6.5% NaCl but not in 10% NaCl. Using commercial API systems, both strains failed to produce acid from the following carbohydrates tested: D-arabitol, L-arabinose, cyclodextrin, glycogen, lactose, mannitol, melibiose, melezitose, methyl β -D-glucopyranoside, pullulan, raffinose, ribose, sucrose, sorbitol, tagatose, trehalose and D-xylose. Weak acid production was, however, observed from *N*-acetylglucosamine. Activities of arginine dihydrolase, pyroglutamic acid arylamidase, esterase C-4 (weak reaction) and ester lipase C-8 (weak reaction) were detected in both strains. Neither of the strains displayed activity for alanine-phenylalanine-proline

Fig. 1. Similarity dendrogram based on whole-cell protein patterns of *Eremococcus coleocola* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

arylamidase, leucine arylamidase, valine arylamidase, acid phosphatase, alkaline phosphatase, α -fucosidase, α -galactosidase, β -galactosidase, β -galacturonidase, β -glucosidase, β -glucuronidase, glycine-tryptophan arylamidase, α -mannosidase, β -mannosidase, lipase C14, chymotrypsin, trypsin or pyrazinamidase. Urease activity was variable. Both strains hydrolysed hippurate but did not hydrolyse aesculin, gelatin or starch. PAGE analysis of whole-cell proteins confirmed the close phenotypic resemblance between the two horse isolates. The two strains formed a tight cluster that was quite separate from all other Gram-positive, catalase-negative reference organisms examined (Fig. 1). An examination of the cell wall of strain CCUG 38207^T revealed that the unknown coccus possessed a directly cross-linked murein based on L-lysine [type A1 α , according to the nomenclature of Schleifer & Kandler (1972)]. This murein structure is found in a number of Gram-positive, catalase-negative coccus-shaped organisms, such as aerococci (Aguirre & Collins, 1992a), *Alloiococcus otitis* (Aguirre & Collins, 1992b), *Globicatella sanguinis* (Collins *et al.*, 1992) and *Abiotrophia defectiva* (Collins *et al.*, 1997), but serves to distinguish the unknown coccus from *Dolosigranulum pigrum* (Aguirre *et al.*, 1993), *Facklamia hominis* (Collins *et al.*, 1997) and *Facklamia ignava* (Collins *et al.*, 1998), which possess an L-lys-D-asp (type A4 α) cell wall. To investigate the genealogical affinity between the two

Fig. 2. Unrooted tree showing the phylogenetic relationships of *E. coleocola* and some 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.

isolates and their relationship with other Gram-positive, catalase-negative taxa, comparative 16S rRNA gene sequence analyses were performed. The almost complete gene sequences (> 1400 nucleotides) of the two horse strains were determined and pairwise analysis revealed 99.8% 16S rRNA identity. This close genealogical affinity between the isolates was consistent with the results of whole-cell protein PAGE analysis and their close biochemical resemblance. Sequence searches of GenBank and RDP databases revealed that the unknown isolates were phylogenetically most closely associated with the lactic-acid group of bacteria, particularly *Abiotrophia defectiva*, *Globicatella sanguinis* and *Facklamia* species. A tree constructed by the neighbour-joining method depicting the phylogenetic affinity of the unknown coccus as exemplified by strain CCUG 38207^T is shown in Fig. 2. The coccus formed a distinct line branching between *Abiotrophia defectiva* and *Globicatella sanguinis*. The results of the neighbour-joining analysis were confirmed by parsimony analysis.

It is evident from the findings of this investigation that the two strains isolated from mares represent a hitherto unknown bacterium within the Gram-positive, catalase-negative cocci. Phylogenetically, the organism represents a new line within a cluster of organisms that includes *Abiotrophia defectiva*, *Globicatella*, *Ignavigranum* and *Facklamia*. The coccus from horses exhibits approx. 8–9% 16S rRNA sequence divergence from the aforementioned taxa, which, together with tree-branching considerations, indicates that the unknown bacterium represents a new taxon at the level of genus. Although the new bacterium shows an apparently closer phylogenetic affinity to *Globicatella sanguinis* than to other organisms, it can readily be distinguished from the latter by its inability to ferment a broad range of carbohydrates. By contrast, *Globicatella sanguinis* is highly saccharolytic (Collins *et al.*,

1992). In terms of its limited ability to ferment carbohydrates, the unknown coccus more closely resembles *Ignavigranum ruoffiae*, *Facklamia hominis* and *Facklamia ignava* (Collins *et al.*, 1997, 1998, 1999). The new coccus, however, differs from *Ignavigranum ruoffiae* in hydrolysing hippurate and being leucine arylamidase-negative. The horse bacterium can be distinguished from *Facklamia hominis* by its failure to produce alanine-phenylalanine-proline arylamidase, α -galactosidase, β -galactosidase and leucine arylamidase. Similarly, it differs from *Facklamia ignava* in being arginine dihydrolase-positive and alanine-phenylalanine-proline arylamidase-negative. Additionally, the unknown coccus contains a directly cross-linked murein based on L-lysine (type A1 α), in contrast to the A4 α L-lys-D-aspartate wall type found in *Facklamia hominis* and *Facklamia ignava*. On the basis of both phenotypic and phylogenetic findings, we therefore consider that the unknown coccus from horses merits classification within a new genus, and the name *Eremococcus coleocola* gen. nov., sp. nov. is proposed. Tests that serve to distinguish *Eremococcus coleocola* from related Gram-positive, catalase-negative cocci are given in Table 1.

Description of *Eremococcus* gen. nov.

Eremococcus (E.re.mo.coc'cus. Gr. adj. *eremos* lonely; Gr. n. *coccus* a grain, berry; M.L. n. *Eremococcus*, a lonely or isolated coccus, referring to its distinct phylogenetic position).

Eremococcus cells are Gram-positive cocci, some of which may be elongated, that occur singly, in pairs or in short chains. α -Haemolytic on sheep- and horse-blood agar. Facultatively anaerobic and catalase-negative. Does not grow at 10 °C or in 10% NaCl. Acid but no gas is produced from glucose. Acid is not produced from most other sugars. Arginine dihydrolase and pyroglutamic acid arylamidase are produced. Leucine arylamidase and alkaline phosphatase are not produced. Hippurate is hydrolysed. Voges-Proskauer negative. The G + C content of DNA of the type strain of the type species is 40 mol%. The cell wall murein type is L-lysine-direct (A1 α). The type species of the genus is *Eremococcus coleocola*.

Description of *Eremococcus coleocola* sp. nov.

Eremococcus coleocola (co'le.o.co.la. Gr. n. *colea* vagina; L. masc. suffix *-cola* inhabitant; M.L. *coleocola* inhabitant of the vagina, referring to the isolation of the type strain).

Cells consist of Gram-positive cocci, some of which may be elongated, occurring singly, in pairs or in short chains. Colonies are pin-point, shiny, entire, circular, convex and non-corroding on blood agar after 24 h. α -Haemolytic on horse- and sheep-blood agar. Facultatively anaerobic and catalase-negative. Grows at 42 °C but not at 10 °C. Grows weakly in 6.5% NaCl but not in 10% NaCl. Does not grow on Difco Bacto

Table 1. Characteristics that differentiate *Eremococcus coleocola* gen. nov., sp. nov. from its nearest phylogenetic relatives

Biochemical tests were carried out using API Rapid ID32S and ZYM systems. v, Variable; ND, not determined.

Characteristic	<i>A. defectiva</i>	<i>I. ruoffiae</i>	<i>F. hominis</i>	<i>F. ignava</i>	<i>G. sanguinis</i>	<i>E. coleocola</i>
Production of acid from:						
Glycogen	+	—	—	—	+	—
Lactose	v	—	—	—	v	—
Mannitol	—	v	—	—	+	—
Melibiose	—	—	—	—	+	—
Methyl β -D-glucopyranoside	—	—	—	—	v	—
Pullulan	+	—	—	—	v	—
Raffinose	v	—	—	—	+	—
Ribose	—	—	—	—	+	—
Sorbitol	—	—	—	—	v	—
Sucrose	+	v	—	—	+	—
Trehalose	+	—	—	—	+	—
Hydrolysis of hippurate	—	—	+	+	+	+
Production of:						
Alanine-phenylalanine-proline arylamidase	v	—	+	+	+	—
Arginine dihydrolase	—	+	+	—	—	+
α -Galactosidase	+	—	+	—	+	—
β -Galactosidase	+	—	+	—	+	—
Urease	—	+	v	—	—	v
Leucine arylamidase	+	+	+	—	ND	—
Murein type	A1 α	A1 α	A4 α	A4 α	A1 α	A1 α

aesculin agar. Acid is produced from glucose. Acid is not produced from L-arabinose, D-arabitol, cyclodextrin, glycogen, lactose, mannitol, maltose, methyl β -D-glucopyranoside, melibiose, melezitose, pullulan, D-raffinose, D-ribose, sorbitol, sucrose, tagatose, trehalose or D-xylose. Weak acid production from N-acetylglucosamine. Arginine dihydrolase, pyroglutamic acid arylamidase, esterase C-4 (weak reaction) and ester lipase C-8 (weak reaction) activities are detected. Urease activity is variable. Alanine-phenylalanine-proline arylamidase, leucine arylamidase, valine arylamidase, acid phosphatase, alkaline phosphatase, α -fucosidase, α -galactosidase, β -galactosidase, β -galacturonidase, β -glucosidase, β -glucuronidase, glycine-tryptophan arylamidase, α -mannosidase, β -mannosidase, lipase C14, chymotrypsin, trypsin and pyrazinamidase activities are not detected. Hippurate is hydrolysed. Aesculin, gelatin and starch are not hydrolysed. Voges-Proskauer negative. Nitrate is not reduced. The G+C content of DNA of the type strain is 40 mol %. The cell wall murein type is L-lysine-direct (A1 α). Isolated from horse vaginal and clitoral fossa swabs. The type strain of *Eremococcus coleocola* is CCUG 38207^T.

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