Moraxella pluranimalium sp. nov., isolated from animal specimens

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Four unusual Gram-negative, catalase-positive, oxidase-positive, coccus-shaped bacteria isolated from one sheep and three pigs were characterized using phenotypic and molecular genetic methods. On the basis of cellular morphology and biochemical criteria, the isolates were tentatively assigned to the genus *Moraxella*, although the organisms did not appear to correspond to any recognized species. Comparative 16S rRNA gene sequencing studies demonstrated that the isolates represent a novel subline within the genus *Moraxella*. The most closely related species in phylogenetic terms was *Moraxella cuniculi*, with 16S rRNA gene sequence similarity of 97.9% to the type strain CCUG 2154^T, although the DNA–DNA relatedness value was only 29%. The novel isolates were readily distinguished from all recognized *Moraxella* species by means of physiological and biochemical tests. On the basis of molecular genetic and phenotypic evidence, therefore, the four isolates represent a novel species of the genus *Moraxella*, for which the name *Moraxella pluranimalium* sp. nov. is proposed. The type strain is 248-01^T (=CECT 7295^T =CCUG 54913^T).

The genus *Moraxella*, originally described by Lwoff (1939), contains both coccoid and rod-shaped bacteria that are genetically related. At the time of writing, the genus comprises 17 species (Euzéby, 1997; Juni & Bøvre, 2005). Over the past two decades, clinical microbiologists have become increasingly aware of the importance of members of this genus as emerging pathogens. In humans, *Moraxella catharralis* is of clinical relevance, being responsible for respiratory infections, endocarditis and meningitis, especially in immunocompromised patients (Verduin *et al.*, 2002), although other *Moraxella* species have also been implicated (Berrocal *et al.*, 2001). Although various *Moraxella* species have been isolated from animals, only *Moraxella bovis* has been traditionally considered of clinical relevance in veterinary medicine, being responsible for ocular and respiratory infections (Brown *et al.*, 1998; Quinn *et al.*, 1999; Lavin *et al.*, 2000). In this study, we

report the phenotypic and phylogenetic features of a novel bacterial species of the genus *Moraxella*.

The bacterial strains studied, designated 248-01^T, M1364, 1387-02, CD12CA4, were isolated from the brain of a sheep with meningitis (M1364), the nasal turbinate of a healthy pig (248-01^T) and the pleura (1387-02) and peritoneal cavity fluid (CD12CA4; Olvera *et al.*, 2007) of two different pigs with lesions of pleuritis and polyserositis, respectively. Samples were collected and frozen at –40 °C until processing in the laboratory. Strains were isolated on Columbia blood agar plates (bioMérieux) after incubation at 37 °C for 24–48 h under aerobic conditions.

Gram stain, oxidase and catalase tests were performed as described by Barrow & Feltham (1993). DNA hydrolysis was tested by using DNase test agar. Growth in brain heart infusion broth was assessed at 4 and 15 °C for up to 14 days, at 22 °C for up to 7 days and at 30, 37 and 42 °C for 48 h. Growth in the presence of 0.5, 1.5, 3.0, 5.0 and 6.5 % NaCl and under anaerobic (with 4–10 % CO₂) and microaerobic (with 5–15 % O₂ and 5–12 % CO₂)

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 248-01^T is AM884564.

conditions, using the GasPak Plus and CampyPak Plus systems (BBL), respectively, was assessed at 37 °C for 48 h. Growth on MacConkey agar (bioMérieux) and chocolate agar (bioMérieux) was tested at 37 °C for 24 and 48 h. Additional biochemical tests were performed by using the API 20NE, API 20E and API ZYM systems (bioMérieux) according to the manufacturer's instructions (except that the API 20NE incubation was performed at 37 °C for 72 h). The four isolates exhibited similar phenotypic characteristics, except with regard to the assimilation of glucose (isolate CD12CA4 was negative), L-arabinose and D-mannose (only M1364 was positive in both tests), the production of alkaline phosphatase (M1364 and 1387-02 were positive) and growth in presence of 3 % NaCl (248-01^T and CD12CA4 did not grow). Details of the morphological, physiological and biochemical characteristics of the isolates are given in the species description and in Table 1.

To establish the phylogenetic affinities of the unknown isolates, their 16S rRNA gene sequences were determined as described previously (Vela *et al.*, 2005) and were subjected to a comparative analysis. Pairwise analysis of the almost-complete sequences (1395 nt) determined revealed 99.3–99.9 % sequence similarity among the four strains. Sequence searches of GenBank performed using the program FASTA (Pearson, 1994) showed that the isolates were most closely related to members of the genus *Moraxella*. The isolates exhibited the highest levels of 16S

rRNA gene sequence similarity with *Moraxella cuniculi* CCUG 2154^T (97.9 % sequence similarity). These sequences and those of strains of representative species within the genus *Moraxella* with validly published names were retrieved from GenBank and aligned with the newly determined sequences by using the program DNATools (Rasmussen, 1995). Phylogenetic trees were constructed according to three different algorithms: neighbour joining (Saitou & Nei, 1987), with the programs DNATools and TreeView (Page, 1996), maximum likelihood, with PHYL software (Guindon & Gascuel, 2003), and maximum parsimony, with the software package MEGA version 3.1 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated using Kimura's two-parameter model (Kimura, 1980); close-neighbour interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by using bootstrap analysis (based on 1000 replications). The phylogenetic trees obtained using neighbour joining (Fig. 1) and the other two methods (data not shown) revealed a clear affiliation between the isolates (exemplified by strain 248-01^T) and the genus *Moraxella*: the novel strains were positioned as a separate branch within the genus. It is evident from Fig. 1 that strain 248-01^T is phylogenetically related to *M. cuniculi*. Bootstrap resampling revealed a strong association (100 %) between the novel isolates and the aforementioned species.

Table 1. Characteristics useful in differentiating strain 248-01^T from recognized *Moraxella* species isolated from animals

Strains: 1, 248-01^T; 2, *M. cuniculi* CCUG 2154^T; 3, *M. bovis* CCUG 2133^T; 4, *M. canis* CCUG 8415^T; 5, *M. caprae* CCUG 33296^T; 6, *M. caviae* CCUG 2132^T; 7, *M. equi* CCUG 4950^T; 8, *M. ovis* CCUG 354^T; 9, *M. boevis* CCUG 35435^T; 10, *M. bovoculi* CCUG 52049^T. Phenotypic data for the known *Moraxella* species were taken from the database of the Culture Collection of the University of Göteborg, Sweden (<http://www.ccug.se>). +, Positive reaction; –, negative reaction; w, weak reaction; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10
Morphology*	C	C	R	C	R	C	R	C	SR	C
Tolerance of NaCl at:										
3 %	–	–	+	+	+	+	+	+	+	+
5 %	–	–	–	+	+	–	–	+	–	+
Growth at 42 °C	–	+	–	+	–	+	–	+	–	–
Haemolysis	–	–	+	+	+	+	–	–	+	–
Nitrate reduction	–	–	+	+	–	–	–	+	+	+
Assimilation of glucose	+	–	ND	–	ND	ND	–	ND	ND	–
Production of:										
DNase	–	–	–	+	–	–	–	–	–	–
Gelatinase	–	–	+	–	–	–	+	–	–	w
Alkaline phosphatase	–	–	–	+	–	+	+	+	–	+
Acid phosphatase	–	+	–	–	–	–	–	–	–	–
Esterase (C4)	+	+	+	+	+	–	+	+	+	+
Lipase (C14)	–	–	–	–	–	–	–	–	+	–
Naphthol-AS-BI phosphohydrolase	+	–	–	–	–	–	–	–	–	–
DNA G + C content (mol%)†	46.4	44.6	42.5	49.6	41.5	46.9	ND	45.9	41.0	ND

*C, Coccus; R, rod; SR, short rod.

†Data are from this study and from Bøvre (1984), Rossau *et al.* (1991) and Kodjo *et al.* (1995, 1997).

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain CECT 7295^T with respect to closely related taxa. Bootstrap percentages (based on 1000 replications) >50% are given at the branching points. Filled circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-parsimony tree; open circles indicate that the corresponding nodes (groupings) were also obtained in the maximum-likelihood and maximum-parsimony trees. The sequence of *Acinetobacter calcoaceticus* DSM 30006^T was used as an outgroup. Bar, 1% sequence divergence.

The novel isolates had a sequence similarity lower than 98% with respect to members of the genus *Moraxella*, a value that falls below the cut-off point (98.7%) specified by Stackebrandt & Ebers (2006) for the delineation of genomic species. Nevertheless, DNA–DNA reassociation experiments were performed between the relevant strain (248-01^T) and its closest phylogenetic neighbour (*M. cuniculi* CCUG 2154^T). DNA was extracted and purified according to the method of Marmur (1961). DNA–DNA hybridization studies were carried out using the competition procedure of the membrane method of Johnson (1994), described in detail by Arahal *et al.* (2001). Hybridization experiments were carried out under optimal conditions, at a temperature of 49.5 °C, which is within the limits of validity for the filter method (De Ley & Tijtgate, 1970). The percentage of hybridization was calculated as described by Johnson (1994). Three independent determinations were carried out for each experiment and the results reported are means. The DNA–DNA reassociation value between strain 248-01^T and *M. cuniculi* CCUG 2154^T was 29%, clearly confirming that the novel isolate represents a separate species (Wayne *et al.*, 1987).

The G+C content of the DNA of strain 248-01^T was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C content of strain 248-01^T was 46.4 mol%, which is within the range reported for members of the genus *Moraxella* (Juni & Bøvre, 2005).

Therefore, on the basis of phenotypic, phylogenetic and genotypic data, it is clear that the four strains isolated from pigs and a sheep merit classification within a novel species of the genus *Moraxella*, for which the name *Moraxella pluranimalium* sp. nov. is proposed. Tests that are useful in differentiating *M. pluranimalium* sp. nov. from its closest

phylogenetic relative and from other *Moraxella* species isolated from animals are shown in Table 1.

Description of *Moraxella pluranimalium* sp. nov.

Moraxella pluranimalium (plu.ra.ni.ma'li.um. L. adj. *plus*, *pluris* many; L. gen. pl. n. *animalium* of animals; N.L. gen. pl. n. *pluranimalium* of/from many animals).

Cells are Gram-negative cocci (1.5–2.0 µm in diameter). Non-spore-forming. Catalase-positive, oxidase-positive and aerobic. Colonies are circular, non-pigmented, smooth and entire and do not produce a diagnostic odour on Columbia blood agar after 48 h incubation at 37 °C. Growth occurs at 22–37 °C but not at 42, 15 or 4 °C. Growth is enhanced in 5–10% CO₂ (demonstrating capnophily) on 5% sheep blood agar and chocolate agar. Growth does not occur on MacConkey agar. Growth occurs in brain–heart infusion broth containing 0.5 or 1.5% NaCl, but not with 5.0 or 6.5% NaCl. Growth with 3% NaCl is variable (strain 248-01^T does not grow). Colonies are non-haemolytic. With the API 20E and API 20NE kits, aesculin, gelatin and urea are not hydrolysed. Indole and acetoin are not produced (API 20E). Nitrate is not reduced (API 20NE). Acid is not produced from D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin or L-arabinose (API 20E). Assimilates glucose and malate, but not citrate, phenyl acetate, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, maltose, gluconate, caprate or adipate (API 20NE). Ester lipase (C8), esterase (C4), leucine arylamidase and naphthol-AS-BI-phosphohydrolase are detected (API ZYM). Production of alkaline phosphatase is variable (strain 248-01^T is negative). Valine arylamidase, lipase (C14), cystine arylamidase, acid phosphatase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, α-glucosidase, N-acetyl-β-glucosa-

minidase, β -glucosidase, β -glucuronidase, α -galactosidase, β -galactosidase, α -mannosidase, α -fucosidase, α -chymotrypsin and trypsin are not detected (API ZYM). The clinical significance is unclear. Strains have been isolated from pigs and a sheep. The DNA G + C content of the type strain is 46.4 mol% (T_m).

The type strain, 248-01^T (=CECT 7295^T=CCUG 54913^T), was isolated from the nasal turbinate of an apparently healthy pig. Additional strains of the species are CD12CA4 (=CECT 7296 =CCUG 54914), M1364 and 1387-02.

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