NOTE

***Corynebacterium testudinoris* sp. nov., from a tortoise, and *Corynebacterium felinum* sp. nov., from a Scottish wild cat**

¹ School of Food Biosciences,
University of Reading,
Reading RG6 6AP, UK

² SAC Veterinary Science
Division, Inverness, UK

³ Culture Collection,
Department of Clinical
Bacteriology, University of
Göteborg, Sweden

Matthew D. Collins,¹ Lesley Hoyles,¹ Roger A. Hutson,¹ Geoffrey Foster²
and Enevold Falsen³

Author for correspondence: Matthew D. Collins. Tel: +44 118 935 7226. Fax: +44 118 926 7917.
e-mail: M.D.Collins@reading.ac.uk

Two unknown Gram-positive, rod-shaped bacteria isolated from a tortoise and a Scottish wild cat were subjected to a polyphasic taxonomic analysis. Chemical analysis revealed the presence of straight-chain and monounsaturated fatty acids and short-chain mycolic acids in the two isolates consistent with the genus *Corynebacterium*. Comparative 16S rRNA gene sequencing confirmed that the unknown isolates were members of the genus *Corynebacterium*, with the two organisms displaying greater than 3% sequence divergence from each other and from established species of the genus. The unknown *Corynebacterium* isolates were readily distinguished from each other and from all recognized species of the genus by biochemical tests. Based on phylogenetic and phenotypic evidence, it is proposed that the unknown organisms from a tortoise and a cat be classified in the genus *Corynebacterium* as *Corynebacterium testudinoris* sp. nov. and *Corynebacterium felinum* sp. nov., respectively. The respective type strains of *C. testudinoris* and *C. felinum* are CCUG 41823^T and CCUG 39943^T.

Keywords: *Corynebacterium testudinoris*, *Corynebacterium felinum*, wild cat, tortoise, 16S rRNA

The genus *Corynebacterium* is one of the largest genera of Gram-positive asporogenous bacteria within the *Actinobacteria*. Corynebacteria are found in a broad variety of habitats such as soil, plant material, food products and marine and animal sources. During the past decade, a very considerable number of new corynebacterial species have been described (see for example Collins *et al.*, 1999; Fernández-Garayzábal *et al.*, 1997, 1998; Funke *et al.*, 1997, 1998; Riegel *et al.*, 1995). Most of these novel species have come to light primarily as the result of the availability of more precise molecular diagnostic tools (e.g. 16S rRNA gene sequencing) and improved phenotypic criteria (e.g. miniaturized tests and improved databases). The vast majority of these newly described organisms have been recovered from human clinical and veterinary sources, where they occur as cutaneous or mucocutaneous contaminants, whereas others represent

hitherto-unrecognized pathogens (Funke *et al.*, 1997). In the course of an on-going study of unusual or taxonomically problematic *Actinobacteria* from animal sources, we have used molecular chemical and molecular genetic approaches to aid the characterization of two *Corynebacterium*-like organisms recovered from the mouth of a tortoise and a dead Scottish wild cat. Based on the reported findings, we propose two further new species of the genus *Corynebacterium*, *Corynebacterium testudinoris* sp. nov. and *Corynebacterium felinum* sp. nov.

The unidentified rod-shaped organism designated M935/96/4^T (CCUG 41823^T) was isolated from necrotic lesions in the mouth of a tortoise. The unknown organism was isolated along with *Escherichia coli*, a *Streptococcus* species and a *Pseudomonas* species. Strain M714/95/5^T (CCUG 39943^T) was isolated from a Scottish wild cat (*Felis sylvestris*) that had died from feline influenza. Both unidentified rods were cultured on Columbia blood agar base supplemented with 5% defibrinated sheep blood at 37 °C in air plus 5% CO₂. The strains were bio-

The GenBank accession numbers for the 16S rRNA gene sequences of strains CCUG 41823^T and CCUG 39943^T are AJ295841 and AJ401282, respectively.

chemically characterized by using the API CORYNE and API ZYM systems according to the manufacturer's instructions (API bioMérieux). Cell-wall murein was prepared by mechanical disruption of cells and complete acid hydrolysates, analysed as described by Schleifer & Kandler (1972). Fatty acid methyl esters were prepared and analysed as described by Kämpfer & Kroppenstedt (1996). The presence of mycolic acids was investigated by GLC analysis of trimethylsilylated derivatives (TMS-MAME) (Klatte *et al.*, 1994). The 16S rRNA genes of the isolates were 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). A phylogenetic tree was constructed according to the neighbour-joining method and the stability of the groupings was estimated by bootstrap analysis (Felsenstein, 1989).

The two unidentified isolates recovered from the mouth of a tortoise and a dead wild cat consisted of Gram-positive, non-motile, non-spore-forming, catalase-positive, diphtheroid-shaped cells. With commercially available API systems, the tortoise bacterium produced acid from glucose, maltose, ribose and sucrose but not from lactose, glycogen, mannitol, *N*-acetyl- β -glucosamine or D-xylose. Reactions for acid phosphatase (weak), ester lipase C-8 (weak), esterase C-4, leucine arylamidase and β -glucosidase were positive. No activity was detected for alkaline phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -fucosidase, lipase C14, α -mannosidase, valine arylamidase, pyrazinamidase, pyrrolidonyl arylamidase, phosphoamidase, chymotrypsin, trypsin or urease. Different results for α -glucosidase production were obtained with the two commercial kits. Using the API ZYM kit, α -glucosidase was detected, but in the API CORYNE system, in which the enzyme activity is checked at a higher pH, α -glucosidase was not detected. The tortoise bacterium hydrolysed aesculin but did not hydrolyse gelatin. In contrast, the cat bacterium produced acid from glucose, maltose and ribose but not from lactose, mannitol, sucrose, *N*-acetyl- β -glucosamine or D-xylose. Reactions for α -glucosidase, pyrazinamidase, pyrrolidonyl arylamidase and leucine arylamidase were positive but no activity was detected for alkaline phosphatase, acid phosphatase, chymotrypsin, ester lipase C-8, esterase C-4, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, α -fucosidase, lipase C14, α -mannosidase, phosphoamidase, valine arylamidase or trypsin. The organism did not hydrolyse aesculin, urea or gelatin and did not reduce nitrate. In terms of their overall morphological and biochemical characteristics, the unidentified isolates somewhat resembled corynebacteria but they did not correspond to any recognized species of this genus.

Cell-wall analysis revealed the presence of meso-diaminopimelic acid within the two isolates, consistent with their provisional assignment to the genus *Corynebacterium*. The cellular fatty acids of the tortoise

Fig. 1. Unrooted tree based on 16S rRNA showing the phylogenetic relationships of *Corynebacterium testudinoris* sp. nov. and *Corynebacterium felinum* sp. nov. Bootstrap values (expressed as percentages of 500 replications) are given at the branching points. Bar, 1% sequence divergence.

isolate CCUG 41823^T consisted of C_{14:0} (2.5%), C_{16:0} (43.6%), C_{16:1} (8.7%), C_{18:0} (2.5%) and C_{18:1} ω 9c (42.7%), whilst those of isolate CCUG 39943^T from a cat consisted of C_{14:0} (1%), C_{15:0} (2%), C_{16:0} (38%), C_{16:1} ω 9c (13%), C_{17:0} (2%), C_{17:1} (2%), C_{18:0} (14%) and C_{18:1} ω 9c (28%), which again resembled corynebacteria. Tuberculostearic acid was not present in either of the isolates. TLC analysis of whole-cell methanolysates and GLC analysis of trimethylsilylated derivatives revealed the presence of short-chain mycolic acids C30 to C36 (major component C32:0) and C32 to C36 (major component C32:0) in the tortoise and cat strains, respectively, confirming their identification as true *Corynebacterium* species. In order to ascertain the phylogenetic positions of the unknown isolates, their almost complete 16S rRNA gene sequences were determined and subjected to a comparative analysis. Sequence database searches revealed that the unknown bacteria were phylogenetically members of the genus *Corynebacterium*. Treeing analysis clearly demonstrated that the two unidentified isolates were phylogenetically separate from each other and from all described *Corynebacterium* species. A tree constructed by the neighbour-joining method, showing the nearest phylogenetic relatives of the unknown organisms, is shown in Fig. 1. The two strains formed distinct sublines associated with a small group of species, which included *Corynebacterium argenteoratense*, *Corynebacterium diphtheriae*, *Corynebacterium kutscheri*, *Corynebacterium pseudotuberculosis*, *Corynebacterium ulcerans* and *Corynebacterium vitruverminis*.

The results of the polyphasic taxonomic investigation clearly showed that the unknown rod-shaped organisms isolated from necrotic mouth lesions of a tortoise and a dead wild cat represent hitherto unrecognized species within the genus *Corynebacterium*. Phylogenetically, the two isolates formed distinct lines within a cluster of species that includes *C. diphtheriae*, the type species of the genus. The two animal isolates clustered together with a bootstrap re-sampling value of 89% (500 tree replications), but pairwise analysis

Table 1. Characteristics that differentiate *C. felinum* and *C. testudinoris* from their nearest phylogenetic relatives

Biochemical reactions were determined with the API CORYNE system. Characteristics are scored as: +, positive; +(-), most strains positive; -(+), most strains negative; -, negative; v, variable. REV CAMP, Reverse Christie-Atkins-Munch-Petersen reaction.

Characteristic	<i>C. felinum</i>	<i>C. testudinoris</i>	<i>C. argentoratense</i>	<i>C. diphtheriae</i>	<i>C. kutscheri</i>	<i>C. pseudotuberculosis</i>	<i>C. ulcerans</i>	<i>C. vitæruminis</i>
Major cellular fatty acids	C _{16:0} , C _{18:1}	C _{16:0} , C _{18:1}	C _{16:0} , C _{18:1}	C _{16:0} , C _{16:1}	C _{16:0} , C _{18:1}	C _{16:0} , C _{16:1}	C _{16:0} , C _{16:1}	C _{16:0} , C _{16:1} , C _{18:1}
Lipophilism	-	-	-	v	-	-	-	-
Nitrate reduction	-	+	-	+(-)	v	v	-	+
Urease	-	-	-	-	+	+	+	+
Hydrolysis of aesculin	-	+	-	-	+	-	-	+
Pyrazinamidase	+	-	+	-	v	-	-	+
Alkaline phosphatase	-	-	v	-	-	v	+	v
Pyrrolidonyl arylamidase	+	-	-	-	+	-	-	+
Production of acid from:								
Maltose	+	+	-	+	+	+	+	+
Sucrose	-	+	-	-(+)	+	v	-(+)	+
Other characteristics						REV CAMP	REV CAMP	Yellow pigmented

showed that the isolates represent closely related, albeit distinct, species (96.9% 16S rRNA sequence similarity based on a comparison of 1310 bp). *C. vitæruminis* displayed highest sequence relatedness to the cat bacterium (97%), with *C. diphtheriae* and *C. ulcerans* showing slightly lower levels of similarity (96.8–96.9%). These latter two species also exhibited high sequence relatedness (96.6–96.7%) to the unidentified tortoise bacterium, whereas *C. vitæruminis* showed only 96.0% similarity. The results of the treeing analysis together with sequence divergence values of 3% or more from other members of the genus therefore demonstrate unequivocally that the two organisms from a tortoise and a cat represent distinct species. Biochemically, the unknown organisms differ from each other and from all recognized members of the genus *Corynebacterium*. Therefore, based on phylogenetic and phenotypic evidence, we propose that the unknown tortoise and cat bacteria be classified in the genus *Corynebacterium*, as *Corynebacterium testudinoris* sp. nov. and *Corynebacterium felinum* sp. nov., respectively. Tests that are useful in distinguishing *C. testudinoris* and *C. felinum* from their closest phylogenetic relatives are shown in Table 1.

Description of *Corynebacterium testudinoris* sp. nov.

Corynebacterium testudinoris (tes.tu.din.o'ris. L. n. *testudo* tortoise, L. gen. neut. n. *oris* of the mouth, N. L. gen. neut. n. *testudinoris* of the mouth of a tortoise).

Cells are Gram-positive, non-motile, non-spore-forming diphtheroids. Yellow pigmented. Facultatively anaerobic and catalase-positive. Non-lipophilic. Acid is produced from glucose, maltose, ribose and sucrose but not from glycogen, lactose, mannitol or D-xylose. Activity is detected for acid phosphatase (weak), esterase C-4 (weak), ester lipase C-8 (weak), β -glucosidase and leucine arylamidase. No activity is detected for alkaline phosphatase, cystine arylamidase, chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, lipase C14, α -mannosidase, valine arylamidase,

pyrazinamidase, phosphoamidase, pyrrolidonyl arylamidase, urease or trypsin. α -Glucosidase may or may not be produced. Aesculin is hydrolysed but gelatin is not. Nitrate is reduced. Cell wall contains *meso*-diaminopimelic acid. Long-chain fatty acids are of the straight-chain saturated and monounsaturated types, with C_{16:0} and C_{18:1} ω 9c predominating. Tuberculo-stearic acid is not present. Mycolic acids are present (C30 to C36). Isolated from necrotic lesions in mouth of a tortoise. Habitat is unknown. The type strain is M935/96/4^T, which has been deposited in the Culture Collection of the University of Göteborg as strain CCUG 41823^T and in the Collection of Bacterial Strains of Institut Pasteur as CIP 106763^T.

Description of *Corynebacterium felinum* sp. nov.

Corynebacterium felinum (fe.li'num. L. neut. adj. *felinum* pertaining to cats).

Cells are Gram-positive, non-motile, non-spore-forming diphtheroids. Facultatively anaerobic and catalase-positive. Non-haemolytic. Acid is produced from glucose, maltose and ribose but not from lactose, mannitol, sucrose, *N*-acetyl- β -glucosamine or D-xylose. Activity is detected for α -glucosidase, pyrazinamidase, pyrrolidonyl arylamidase and leucine arylamidase. No activity is observed for alkaline phosphatase, acid phosphatase, chymotrypsin, ester lipase C-8, esterase C-4, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, α -fucosidase, lipase C14, α -mannosidase, phosphoamidase, valine arylamidase or trypsin. Aesculin, urea and gelatin are not hydrolysed and nitrate is not reduced. Cell wall contains *meso*-diaminopimelic acid. Long-chain fatty acids are of the straight-chain saturated and monounsaturated types. Tuberculo-stearic acid is not present. Mycolic acids are present (C32 to C36). MK-8(H₂) is the predominant menaquinone. Isolated from a dead Scottish wild cat (*Felis sylvestris*). Habitat is unknown. The type strain is M714/95/5^T, which has been deposited in the Culture Collection of the University of Göteborg as strain CCUG 39943^T.

and in the Collection of Bacterial Strains of Institut Pasteur as CIP 106740^T.

Acknowledgements

We are grateful to Professor Hans Trüper (University of Bonn, Germany) for help in coining the species epithets. SAC Veterinary Science Division receives support from the Scottish Executive Rural Affairs Department.

References

- Collins, M. D., Hoyles, L., Lawson, P. A., Falsen, E., Robson, R. L. & Foster, G. (1999). Phenotypic and phylogenetic characterization of a new *Corynebacterium* species from dogs: description of *Corynebacterium auriscanis* sp. nov. *J Clin Microbiol* **37**, 3443–3447.
- Felsenstein, J. (1989). PHYLIP – Phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.
- Fernández-Garayzábal, J. F., Collins, M. D., Hutson, R. A., Fernández, E., Monasterio, R., Marco, J. & Domínguez, L. (1997). *Corynebacterium mastitidis* sp. nov., isolated from milk of sheep with subclinical mastitis. *Int J Syst Bacteriol* **47**, 1082–1085.
- Fernández-Garayzábal, J. F., Collins, M. D., Hutson, R. A., Gonzalez, I., Fernández, E. & Domínguez, L. (1998). *Corynebacterium camporealensis* sp. nov., associated with subclinical mastitis in sheep. *Int J Syst Bacteriol* **48**, 463–468.
- Funke, G., Von Graevenitz, A., Clarridge, J. E., III & Bernard, K. A. (1997). Clinical microbiology of coryneform bacteria. *Clin Microbiol Rev* **10**, 125–159.
- Funke, G., Lawson, P. A. & Collins, M. D. (1998). *Corynebacterium riegelii* sp. nov., an unusual species isolated from female patients with urinary tract infections. *J Clin Microbiol* **36**, 624–627.
- Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.
- Klatte, S., Kroppenstedt, R. M. & Rainey, F. A. (1994). *Rhodococcus opacus* sp. nov., an unusual nutritionally versatile *Rhodococcus* species. *Syst Appl Microbiol* **14**, 355–360.
- Riegel, P., Ruimy, R., de Briel, D., Prevost, G., Jehl, F., Bimet, F., Christen, R. & Monteil, H. (1995). *Corynebacterium argentoratense* sp. nov., from the human throat. *Int J Syst Bacteriol* **45**, 533–537.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.