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Corynebacterium caspium sp. nov., from a Caspian seal (*Phoca caspica*)

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A previously unknown Gram-positive, non-spore-forming, non-lipophilic, catalase-positive, irregular rod-shaped bacterium (M/106/00/5^T) was isolated, in mixed culture, from the penis of a Caspian seal (*Phoca caspica*). The strain was a facultative anaerobe that was able to grow at 22 and 42 °C. Comparative 16S rRNA gene sequencing showed that the organism formed a hitherto unknown subline within the genus *Corynebacterium*. Sequence divergence values of more than 5 % from other described *Corynebacterium* species, together with phenotypic differences, showed that the unidentified bacterium represents a previously unrecognized member of this genus. On the basis of phenotypic and phylogenetic considerations, it is proposed that the unknown bacterium isolated from a Caspian seal (strain M/106/00/5^T = CCUG 44566^T = CIP 107965^T) be classified as the type strain of a novel species of the genus *Corynebacterium*, *Corynebacterium caspium* sp. nov.

The genus *Corynebacterium* is one of the largest genera within the *Actinobacteria* and currently embraces over 50 species. Many of the species within the genus have come to light relatively recently due to the application of improved diagnostic methods to facilitate their identification and recognition (Funke *et al.*, 1997). Most of the newly described species have originated from human sources (e.g. Funke *et al.*, 1998; Renaud *et al.*, 2001; Shukla *et al.*, 2001; Sjödén *et al.*, 1998), and the corynebacterial flora of humans is now well established. Much less information is available on the species diversity of corynebacteria associated with animals, although it is clear that much new diversity remains to be discovered from these sources (e.g. Collins *et al.*, 1999, 2001a, b; Fernández-Garayzábal *et al.*, 1997, 1998; Goyache *et al.*, 2003). During the course of a study of bacteria isolated from sea mammals, we have characterized a novel *Corynebacterium*-like organism from a Caspian seal (*Phoca caspica*). On the basis of both phenotypic and molecular genetic criteria, we describe a hitherto unknown *Corynebacterium* species, *Corynebacterium caspium* sp. nov.

Strain M106/00/5^T was isolated from the penis of a Caspian seal, in mixed culture with *Atopobacter phocae* and *Streptococcus dysgalactiae* (serological group G). The strain was grown aerobically at 37 °C on Columbia agar (Oxoid) supplemented with 5 % sheep blood. It was characterized biochemically using the API Coryne and API ZYM systems according to the manufacturer's instructions (bioMérieux). The Christie–Atkins–Munch-Petersen (CAMP) test (with *Staphylococcus aureus*) was performed as described by von Graevenitz & Funke (1996). Cell-wall murein was prepared by mechanically disrupting the cells; complete acid hydrolysates were analysed as described by Schleifer & Kandler (1972). Long-chain cellular fatty acids were analysed using the MIDI microbial identification system. The presence of mycolic acids was investigated by GLC analysis of trimethylsilylated derivatives (TMS-MAME) (Klatte *et al.*, 1994). The 16S rRNA gene of the isolate was amplified by a PCR and directly sequenced using a *Taq* dye-deoxy terminator cycle-sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 377; Applied Biosystems). The closest known relatives of strain M106/00/5^T were determined by performing database searches in the GenBank/EMBL/DDBJ data libraries. The determined sequence and those of its nearest phylogenetic relatives were aligned using the program CLUSTAL W (Thompson *et al.*, 1994). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the program DNADIST (using the Kimura 2-correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed using the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The stability of the

Abbreviation: CAMP, Christie–Atkins–Munch-Petersen.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *C. caspium* sp. nov. M/106/00/5^T is AJ566641.

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groupings was estimated by bootstrap analysis (350 replications) using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The unknown isolate recovered from the penis of a Caspian seal stained Gram-positive, and, upon microscopic examination, appeared as irregular short rods. Cells were non-acid-fast and non-spore-forming. The strain was facultatively anaerobic, non-lipophilic, catalase-positive and oxidase-negative. Using the API Coryne system, positive results were obtained for acid production from glucose and D-ribose, urea hydrolysis and the production of pyrazinamidase. Using the API ZYM test gallery, activity was detected for esterase C-4, ester lipase C8 and trypsin. All other enzyme tests were negative using this kit. An examination of cell-wall murein acid hydrolysates of the strain revealed the presence of *meso*-diaminopimelic acid as the dibasic amino acid, which is consistent with membership of the genus *Corynebacterium*. The non-hydroxylated long-chain cellular fatty acids of the organism were found to be primarily of the straight-chain saturated and monounsaturated types. The major acids corresponded to $C_{14:0}$ (2·3 %), $C_{16:1}\omega9c$ (2·6 %), $C_{16:0}$ (48·4 %), $C_{18:1}\omega9c$ (43·4 %) and $C_{18:0}$ (3·3 %). TLC analysis of whole-cell methanolysates indicated that mycolic acids were not present in the unknown organism isolated from the seal. This result was confirmed by high-temperature GLC analysis. Despite the absence of mycolic acids, the presence of straight-chain saturated and monounsaturated long-chain cellular fatty acids and a cell-wall murein based on *meso*-diaminopimelic acid, together with morphological and biochemical properties, were strongly indicative that the unidentified bacterium was a member of the genus *Corynebacterium*. To investigate the taxonomic affinities of the unidentified strain in more detail, a large fragment (1351 bases) of its 16S rRNA gene sequence was determined. Sequence database searches revealed that the strain was most closely related to the *Actinobacteria*, with species of the genus *Corynebacterium* exhibiting the highest sequence similarities (data not shown). Treeing analysis confirmed the placement of the seal bacterium within the genus *Corynebacterium*, with the unknown organism forming a distinct subline with *Corynebacterium renale* as its nearest relative. Fig. 1 depicts the position of the unknown bacterium within a subset of *Corynebacterium* species.

Both phenotypic and molecular phylogenetic data show unequivocally that the unidentified bacterium is a member of the genus *Corynebacterium sensu stricto*. Phylogenetically, the bacterium displays a specific affinity with *C. renale*, a species associated with genitourinary infections in animals, especially cattle (Collins & Cummins, 1986). The clustering of the seal bacterium with *C. renale* was supported by a bootstrap resampling value of 71 % (Fig. 1). However, a sequence divergence of 5·3 % (based on a comparison of 1351 bases) between the unknown organism and *C. renale* clearly demonstrated that these taxa represent quite

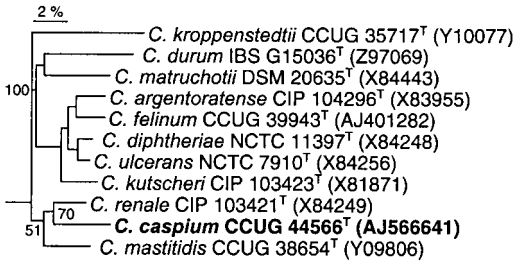

Fig. 1. Neighbour-joining tree, based on a comparison of ~1265 bases, showing the relationship between *C. caspium* sp. nov. and some closely related members of the genus *Corynebacterium*. Bootstrap values (shown at the nodes) are expressed as percentages of 350 replications.

different species. It is now recognized that a 16S rRNA gene sequence divergence value of 3 % or more between organisms indicates that they represent different genomic species (Stackebrandt & Goebel, 1994). Biochemically, the unidentified bacterium closely resembles *C. renale*; however, strains of the latter species can be readily distinguished from the novel seal organism by the production of β-glucuronidase. The seal bacterium also differs from *C. renale* by producing a negative CAMP reaction and by the absence of mycolic acids. From the treeing analysis, the next nearest relative of the seal bacterium corresponds to *Corynebacterium mastitidis*, an organism originally isolated from mastitic sheep (Fernández-Garayzábal *et al.*, 1997). The clustering of *C. mastitidis* with the aforementioned pair of species was statistically significant (bootstrap resampling value of 96 %). However, the unknown bacterium isolated from the seal and *C. mastitidis* displayed over 5·5 % 16S rRNA gene sequence divergence, showing that these taxa are only distantly related. Phenotypically, the seal bacterium can be readily distinguished from *C. mastitidis* in forming acid from a variety of sugars, by its negative alkaline phosphatase reaction and by the absence of mycolic acids. Hence, on the basis of both phenotypic and phylogenetic evidence, we consider that the unidentified bacterium isolated from a seal merits classification as a novel species of the genus *Corynebacterium*, for which the name *Corynebacterium caspium* sp. nov. is proposed. Some tests that are useful in distinguishing *C. caspium* from its nearest relatives are listed in Table 1.

Description of *Corynebacterium caspium* sp. nov.

Corynebacterium caspium (cas.pi′um. L. neut. adj. *caspium* belonging to the Caspian Sea, referring to the isolation of the type strain from a Caspian seal).

Cells are Gram-positive, non-spore-forming, irregular-shaped tapered rods and clubs. Colonies were 0·5 mm in diameter after 24 h, increasing to 2 mm at 72 h, and were circular, convex, entire, opaque and dull and can be moved

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Table 1. Characteristics that are useful in distinguishing *C. caspium* sp. nov. from its nearest phylogenetic relatives

Species: 1, *C. caspium* sp. nov.; 2, *C. mastitidis*; 3, *Corynebacterium matruchotii*; 4, *Corynebacterium durum*; 5, *C. renale*. +, Positive; −, negative; V, variable. Tests are from the API Coryne test system (bioMérieux).

Characteristic	1	2	3	4	5
Acid from:					
Glucose	+	V	+	+	+
Maltose	−	V	+	+	−
Mannitol	−	−	−	+	−
Ribose	+	V	+	+	+
Sucrose	−	V	V	+	−
Production of:					
β-Glucuronidase	−	−	−	−	+
Alkaline phosphatase	−	+	−	−	−
Pyrrolidonyl arylamidase	−	V	+	−	−
Urease	+	+	−	V	+
Hydrolysis of aesculin	−	V	V	+	−
Nitrate reduction	−	−	+	+	V
CAMP reaction	−	−	−	−	+
Corynemycolic acids	−	+	+	+	+

across the plate whilst retaining their integrity. Facultatively anaerobic, catalase-positive and oxidase-negative. Non-haemolytic. Non-lipophilic. CAMP-negative. Acid is produced from D-glucose and D-ribose, but not from glycogen, lactose, maltose, mannitol, sucrose or D-xylose. Urea is hydrolysed but aesculin and gelatin are not. Activity is detected for esterase C-4, ester lipase C8, pyrazinamidase and trypsin. No activity is detected for acid phosphatase, alkaline phosphatase, chymotrypsin, cysteine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, lipase C14, leucine arylamidase, α-mannosidase, pyrrolidonyl arylamidase, phosphoamidase or valine arylamidase. Nitrate is not reduced. Cell-wall murein is based on *meso*-diaminopimelic acid. Corynemycolic acids are not present. The long-chain cellular acids are of the straight-chain saturated and monounsaturated types, with $C_{16:0}$ and $C_{18:1}\omega 9c$ predominating; tuberculostearic acid is not present.

The type strain, M/106/00/5^T (=CCUG 44566^T=CIP 107965^T), was isolated from a Caspian seal. Habitat is not known.

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