Note

Corynebacterium spheniscorum sp. nov.,
isolated from the cloacae of wild penguins

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Twenty unidentified Gram-positive, rod-shaped organisms were recovered from the cloacae of apparently healthy wild penguins (*Spheniscus magellanicus*) and subjected to a polyphasic taxonomic analysis. On the basis of cellular morphology and biochemical criteria, the isolates were tentatively assigned to the genus *Corynebacterium*, although the organisms did not appear to correspond to any recognized species. Lipid studies confirmed this generic placement, and comparative 16S rRNA gene sequencing showed that the unidentified organisms represent a hitherto unknown subline, associated with a small subcluster of species that includes *Corynebacterium diphtheriae* and its close relatives. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown isolates from penguins be classified in the genus *Corynebacterium*, as *Corynebacterium spheniscorum* sp. nov. The type strain is strain PG 39^T (=CCUG 45512^T =CECT 5986^T).

Currently, there is a paucity of information concerning the normal bacterial intestinal flora of wild birds (Bangert *et al.*, 1988a, b). Whilst Gram-negative bacteria and Gram-positive cocci are often identified to the species level, Gram-positive rod-shaped organisms such as bacilli, corynebacteria and lactobacilli are usually only identified to genus level. The genus *Corynebacterium* is a case in point. *Corynebacterium* spp. have been recovered at different frequencies from the intestinal and cloacal flora of different wild birds (Bangert *et al.*, 1988a, b; Bowman & Jacobson, 1980), and have occasionally been reported as causal agents of disease in birds (Fiennes, 1982). However, knowledge of the diversity of *Corynebacterium* species that reside within the intestine and cloaca of birds is exceedingly poor, because of limitations in the diagnostic methods used for species identification.

During the last decade, a very considerable number of novel corynebacterial species have been described, especially from human and animal sources (e.g. Collins *et al.*, 1999a, b, 2001a, b; Fernández-Garayzábal *et al.*, 1998; Funke *et al.*,

1998; Pascual *et al.*, 1998). Most of this new diversity has come to light because of the use of more consistent phenotypic methods in concert with molecular genetic diagnostic approaches, particularly 16S rRNA gene analysis. In this article, we have used phenotypic (including chemical markers) and molecular genetic methodologies to facilitate the characterization of some *Corynebacterium*-like organisms recovered from the cloacae of healthy wild penguins (*Spheniscus magellanicus*). On the basis of the findings presented, we propose a novel *Corynebacterium* species, *Corynebacterium spheniscorum* sp. nov.

During an investigation of the normal flora of the cloacae of healthy wild penguins living in a large rookery of Magellanic penguins in Peninsula Valdés (Argentina), 20 unidentified Gram-positive, rod-shaped organisms were recovered from different penguins. Samples from cloacae were collected with sterile swabs with transport medium and kept under refrigeration until being processed in the laboratory within 6 h. Strains were isolated on Columbia blood-agar plates (bioMérieux) and incubated for 24 h at 37 °C. The strains were characterized biochemically using the API Coryne (version 2.0), API 50 CH and API ZYM systems (bioMérieux) according to the manufacturer's instructions. The API 50 CH strips were examined at 24 and 48 h. The

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

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 45512^T is AJ429234.

Christie–Atkins–Munch–Petersen (CAMP) test with *Staphylococcus aureus* ATCC 25923 was performed according to standard procedures (Funke *et al.*, 1997). Lipophilic requirements were determined by growing the isolates in brain/heart infusion agar supplemented with 1 % Tween 80 and for comparison, in brain/heart infusion agar lacking lipid supplementation. Cell-wall murein was prepared by mechanical disruption of cells, and complete acid hydrolysates were 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). For 16S rRNA gene sequence analysis, a large fragment (around 1450 bases) of the 16S rRNA gene of the isolates was amplified by PCR and sequenced directly 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 novel isolates were determined by performing a database search. 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).

The 20 unidentified isolates consisted of Gram-positive, non-motile, non-spore-forming, short, rod-shaped cells. When cultured aerobically on Columbia blood-agar plates, the isolates formed small (approx. 1–2 mm diameter after 24 h incubation at 37 °C) whitish colonies, which were circular, smooth, entire and non-haemolytic. The isolates were catalase-positive, grew slightly under anaerobic conditions, were non-lipophilic and displayed a positive CAMP reaction with *S. aureus* after 48 h. The isolates were biochemically homogeneous. None of the isolates hydrolysed aesculin, gelatin or urea, and none reduced nitrate. All strains produced acid from glucose, ribose, maltose, trehalose, D-fructose, D-mannose and *N*-acetylglucosamine, but did not produce acid from D- or L-xylose, mannitol, lactose, sucrose, glycerol, erythritol, D- or L-arabinose, adonitol, methyl β -xyloside, galactose, L-sorbose, rhamnose, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, 2- or 5-ketogluconate or glycogen. Nine of the isolates produced acid from dulcitol, whilst 11 strains did not. All of the strains gave positive reactions for ester lipase C8, esterase C4, pyrazinamidase and leucine arylamidase. No activity was detected for lipase C14, alkaline phosphatase, acid phosphatase, pyrrolidonyl arylamidase, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase, α -galactosidase, β -galactosidase, α -fucosidase, chymotrypsin, trypsin, valine arylamidase, cystine arylamidase or naphthol-AS-BI-phosphohydrolase. The penguin isolates displayed a numerical profile of 2000324 with the commercial API Coryne system,

which corresponded to a doubtful identification as *Gardnerella vaginalis* and *Corynebacterium jeikeium* (confidence levels of 38 and 27 %, respectively). However, the penguin isolates can be readily differentiated from *G. vaginalis* because the latter is catalase-negative. Unlike the penguin isolates, *C. jeikeium* is lipophilic, CAMP-test-negative, does not grow anaerobically and produces the enzyme alkaline phosphatase.

A representative strain, PG 39^T (=CCUG 45512^T), was subjected to cell wall and lipid analysis. An examination of wall murein acid hydrolysates revealed the presence of *meso*-diaminopimelic acid. The predominant non-hydroxylated cellular fatty acids of the penguin bacterium were 16:0 (22.6 %), 16:1 ω 9c (0.7 %), 18:0 (3 %), 18:1 ω 9c (71 %) and 19:0 (1.1 %). Tuberculostearic acid was not present. TLC analysis of whole-cell methanolsates and GLC analysis of trimethylsilylated derivatives revealed the presence of short-chain mycolic acids (predominant components 32:0, 7.5 %; 34:2, 4 %; 34:1, 24.3 %; 34:0, 10.5 %; 36:3, 3.2 %; 36:2, 25 %; 36:1, 16.3 %; 36:0, 5.4 %). These chemotaxonomic characteristics are consistent with the assignment of the isolates to the genus *Corynebacterium*.

To establish the phylogenetic position of the unknown isolates, their 16S rRNA gene sequences were determined by direct sequencing of *in vitro*-amplified rRNA gene products. The sequences of a large fragment (>1400 nt) from strains PG 5, PG 11, PG 35, PG 39^T, PG 69 and PG 135 were determined, and comparative analysis revealed 99.8–100 % 16S rRNA sequence similarity among the strains, thereby demonstrating high genealogical homogeneity. Sequence searches of GenBank revealed that the unknown isolates were, phylogenetically, members of the genus *Corynebacterium* (data not shown). Treeing analysis (employing the sequence of strain PG 39^T) demonstrated that the unidentified bacterium was phylogenetically distinct from all previously described *Corynebacterium* species. A tree depicting the phylogenetic relationship of the unidentified bacterium within the genus *Corynebacterium* is shown in Fig. 1. The unknown bacterium formed a distinct subline within the genus *Corynebacterium*, branching proximal to the base of a subcluster of species that includes *Corynebacterium diphtheriae* and its close relatives.

Comparative 16S rRNA gene sequencing clearly demonstrates that the unknown bacterium from penguins represents a distinct species within the genus *Corynebacterium*. Phylogenetically, the novel bacterium is loosely associated with a group of species that includes *C. diphtheriae*, the type species of the genus. Bootstrap resampling, however, shows that the association of the penguin bacterium with this subcluster of species is not statistically significant, and it is evident from the treeing analysis that the organism does not exhibit a particularly significant affinity with any recognized species. In terms of sequence similarities, the unidentified bacterium displayed highest relatedness to *Corynebacterium argentoratense* (95.7 %), *Corynebacterium felinum* (95.6 %), *Corynebacterium ulcerans* (95.3 %),

Fig. 1. Unrooted tree, based on 16S rRNA, showing the phylogenetic relationships of *Corynebacterium spheniscorum* sp. nov. Bootstrap values (expressed as percentages of 500 replications) are given at branching points. The tree was rooted using outgroup sequences (not shown) from *Gordonia amarae* DSM 43392^T (X80635), *Tsukamurella paurometabola* DSM 20162^T (X53207), *Nocardia asteroides* DSM 43005 (X53205) and *Rhodococcus erythropolis* NCIMB 11148 (X76691). Bar, 1 % sequence divergence.

Description of *Corynebacterium spheniscorum* sp. nov.

Table 1. Characteristics that differentiate *C. spheniscorum* from *C. argentoratense* and other CAMP-positive *Corynebacterium* spp.

Characteristic	1	2	3	4	5	6	7	8	9
Nitrate	—	—	—	V	—	+	—	—	—
Urease	—	—	—	V	+	—	—	—	—
Aesculin	—	—	—	V	—	—	—	—	—
Pyrazinamidase	+	+	+	+	+	+	+	+	W
Alkaline phosphatase	—	V	+	V	—	+	+	+	+
Acid production from:									
Maltose	+	—	—	V	—	—	—	—	+
Sucrose	—	—	—	+	—	V	—	—	W
Xylose	—	—	—	V	—	—	—	—	—
Other characteristics*	—	A, B	C	D	—	—	B	B	E

Cells are Gram-positive, non-spore-forming, non-motile rods. Colonies are whitish, circular, smooth, entire and approximately 1–2 mm diameter on Columbia blood agar after 24 h incubation at 37 °C. Colonies are non-haemolytic. Facultatively anaerobic; grows slightly under anaerobic conditions. Non-lipophilic, and CAMP-positive with *S. aureus*. Aesculin, gelatin and urea are not hydrolysed. Nitrate is not reduced. Acid is produced from glucose, ribose, D-fructose, D-mannose, trehalose, *N*-acetyl β -glucosamine and maltose, but not from D-xylose, L-xylose, mannitol, lactose, rhamnose, galactose, adonitol, inositol, D- or L-arabinose, sucrose, melibiose, melezitose or glycogen. Acidification of dulcitol is variable. Activity for ester lipase C8, esterase C4, pyrazinamidase and leucine arylamidase (weak) is detected. Alkaline and acid phosphatase, pyrrolidonyl arylamidase, α -glucosidase, β -glucosidase, β -glucuronidase, α -galactosidase, β -galactosidase, α -mannosidase, α -fucosidase, chymotrypsin, trypsin, valine arylamidase, cystine arylamidase and naphthol-AS-BI-phosphohydrolase are not produced. Cell wall contains *meso*-diaminopimelic acid. Long-chain fatty acids are of the straight-chain saturated and monounsaturated types, with C16:0 and C18:1 ω 9c predominating. Tuberculostearic acid is not present. Mycolic acids are present (C32–C36). Isolated from the cloacae of apparently healthy penguins (*Spheniscus magellanicus*). The type strain is strain PG 39^T (=CCUG 45512^T =CECT 5986^T).

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