Corynebacterium sphenisci sp. nov., isolated from wild penguins

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Six unidentified Gram-positive, rod-shaped organisms recovered from the cloacae of apparently healthy wild penguins were characterized by phenotypic and molecular taxonomic methods. Chemotaxonomic investigations revealed the presence of a cell wall based on *meso*-diaminopimelic acid and long-chain cellular fatty acids of the straight-chain saturated and monounsaturated types, consistent with the genus *Corynebacterium*. Corynomycolic acids, which are characteristic of the genus, were also detected, albeit in small amounts. Comparative 16S rRNA gene sequencing studies showed that the unidentified organisms were phylogenetically related to corynebacteria and represent a novel subline associated with a small subcluster of species that includes *Corynebacterium xerosis*, *Corynebacterium amycolatum* and *Corynebacterium freneyi*. The unknown isolates were readily distinguished from their closest phylogenetic relatives and all other *Corynebacterium* species with validly published names by using a combination of biochemical and chemotaxonomic criteria. Based on both phenotypic and 16S rRNA gene sequence considerations, it is proposed that the unknown isolates recovered from penguins be classified as a novel species in the genus *Corynebacterium*, *Corynebacterium sphenisci* sp. nov. The type strain is CECT 5990^T (= CCUG 46398^T).

The genus *Corynebacterium* has undergone considerable expansion in recent years and now represents one of the largest genera within the *Actinobacteria*. Currently, the genus accommodates in excess of 50 species, more than 30 of which have been described during the last decade. In the past, the recognition of novel corynebacteria was problematic due to limitations in the taxonomic tests (primarily morphological and biochemical) used to identify these organisms. However, in recent years, the implementation of molecular identification methods (in particular 16S rRNA gene sequencing) in concert with improved phenotypic approaches has greatly facilitated the discovery of novel species diversity. The great majority of the novel species that have been described in recent years have originated from human (e.g. Collins *et al.*, 1999a; Funke *et al.*, 1997a, 1998; Renaud *et al.*, 2001; Sjöden *et al.*, 1998) and, to a lesser extent, animal sources (e.g. Fernández-Garayzábal *et al.*, 1997, 1998; Pascual *et al.*, 1998; Collins *et al.*, 1999b,

2001a,b). Despite this rapid expansion, it is clear that much corynebacterial diversity from human and animal sources remains to be discovered (e.g. Tanner *et al.*, 1999). In the course of an on-going study of unusual *Actinobacteria* from wild animals, we have characterized a hitherto unknown *Corynebacterium* species from magellanic penguins (*Spheniscus magellanicus*). Based on the results of a polyphasic taxonomic study, a novel *Corynebacterium* species, *Corynebacterium sphenisci* sp. nov., is described.

Six unidentified Gram-positive, rod-shaped organisms were recovered from the cloacae of healthy wild penguins. Samples from cloacae were collected with sterile swabs with transport medium and kept under refrigeration until being processed in laboratory within 6 h. Strains were isolated on Columbia blood-agar plates (bioMérieux) and incubated for 24 h at 37 °C under aerobic and anaerobic conditions. The unknown isolates were recovered from the cloacal samples together with *Staphylococcus sciuri*. The unidentified strains were grown on Columbia blood-agar plates at 37 °C under aerobic conditions and were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CECT 5990^T is AJ440964.

characterized biochemically by using the API CORYNE (version 2.0), API 50 CH and API ZYM systems according to the manufacturer's instructions (bioMérieux). Incubation of the API 50 CH strips was extended up to 72 h. The CAMP test with *Staphylococcus aureus* ATCC 25923 was carried out according to standard procedures (Funke *et al.*, 1997b). Lipophilic requirements were determined by growing the isolates on brain heart infusion agar supplemented with 1% Tween 80 in comparison with 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 phylogenetic analysis, a large fragment 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 groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The unidentified rod-shaped organisms from penguins stained Gram-positive and displayed a coryneform morphology. The isolates were non-motile and did not form endospores. They grew on sheep-blood agar at 37 °C, forming small (approx. 1–2 mm diameter after 48 h incubation), whitish, low-convex, dry and rough colonies that were non-haemolytic. The isolates were catalase-positive, non-lipophilic and CAMP-test-negative. Using commercially available API systems, the isolates failed to hydrolyse aesculin, gelatin or urea. All of the strains reduced nitrate and produced acid from glucose, maltose, galactose, D-fructose, D-mannose and trehalose, but not from xylose, ribose, mannitol, lactose, sucrose or glycogen. Activity for pyrazinamidase, esterase C4, ester lipase C8, lipase C14, leucine arylamidase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase and naphthol-AS-BI phosphohydrolase was observed. No activity was detected for alkaline phosphatase, pyrrolidonyl arylamidase, α -glucosidase, β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -galactosidase, β -galactosidase, α -fucosidase or trypsin. The morphological and biochemical characteristics of the isolates were consistent with their tentative assignment to the genus *Corynebacterium*. The unknown strains gave a numerical profile with the API CORYNE system of 3000124 that corresponds to a good identification of *Corynebacterium striatum*/*Corynebacterium amycolatum*. However, the isolates were readily differentiated from the latter two species by their inability to produce acid from sucrose and to produce alkaline

phosphatase. By contrast, *C. striatum* and *C. amycolatum* are positive for both of these tests (Martínez-Martínez *et al.*, 1995; Funke *et al.*, 1996). Additionally, *C. amycolatum* produces acid from ribose, while the unidentified isolates are negative for this test (Funke *et al.*, 1996). Analysis of the amino acid composition of the cell-wall murein of a representative isolate (strain CECT 5990^T) revealed meso-diaminopimelic acid as the dibasic acid, which reinforces its provisional assignment to the genus *Corynebacterium*. The major long-chain cellular fatty acids of the organism were C_{12:0} (3%), C_{14:0} (4%), C_{16:0} (28%), C_{18:0} (25%), C_{18:1 ω 9c} (28%), C_{18:1 ω 7c} (1%) and C_{20:0} (2%). Small

Fig. 1. Unrooted tree based on 16S rRNA gene sequences showing the phylogenetic relationships of *Corynebacterium sphenisci* 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^T (X76691). Bar, 1% sequence divergence.

amounts of short-chain mycolic acids (C₂₂–C₃₂) were also detected. The wall murein and lipid composition of the penguin bacterium strongly resembled those of corynebacteria.

To ascertain the phylogenetic position of the unknown isolates, 16S rRNA gene sequencing was performed. For strain CECT 5990^T, the almost complete sequence was determined (>1400 nt), whereas approximately 1000 nucleotides were determined for each of the other isolates. Comparative sequence analysis revealed 99.9–100 % sequence similarity between the strains, thereby demonstrating their high genealogical relatedness. Sequence searches of EMBL/GenBank databases confirmed that the unknown strains were members of the genus *Corynebacterium*, with other actinomycete taxa showing lower levels of relatedness (data not shown). Treeing analysis demonstrated the placement of the unidentified bacterium (as exemplified by strain CECT 5990^T) within the genus *Corynebacterium*. A tree constructed using the neighbour-joining method depicting the phylogenetic position of the unidentified bacterium is shown in Fig. 1 and shows unequivocally that it represents a distinct subline associated with a small group of species that includes *C. amycolatum*, *Corynebacterium xerosis* and *Corynebacterium freneyi* (respectively exhibiting 96.2, 97.0 and 96.3 % sequence similarity to the penguin bacterium).

The results of the polyphasic taxonomic analysis clearly show that the unknown bacteria isolated from the cloacae of penguins represents a hitherto unrecognized species within the genus *Corynebacterium*. Phylogenetically, the bacterium forms a novel subline closely associated with *C. amycolatum*, *C. xerosis* and *C. freneyi*. Bootstrap resampling revealed the affinity between the unknown penguin bacterium and the aforementioned cluster of species to be statistically

significant (value 99 %), which, together with sequence divergence values of 3 % or greater, demonstrates unequivocally that the isolates represent a distinct *Corynebacterium* species. The unknown bacterium can also be easily distinguished phenotypically from all other *Corynebacterium* species described to date. Therefore, based on both phenotypic and phylogenetic evidence, we propose that the bacteria from penguins be assigned to the genus *Corynebacterium*, as *Corynebacterium sphenisci* sp. nov. Tests that serve to distinguish *C. sphenisci* from its closest phylogenetic relatives are shown in Table 1.

Description of *Corynebacterium sphenisci* sp. nov.

Corynebacterium sphenisci (sphe.nis'ci. N.L. masc. gen. n. *sphenisci* of *Spheniscus*, a systematic genus of penguins, indicating the source of the type strain).

Cells stain Gram-positive and consist of non-motile, non-spore-forming rods. Colonies are whitish, low-convex, dry and rough and approximately 1–2 mm in diameter after 48 h incubation at 37 °C on sheep-blood agar. Facultatively anaerobic. Non-haemolytic, non-lipophilic and CAMP-negative. Catalase-positive and oxidase-negative. Nitrates are reduced. Acid is produced from glucose, maltose, galactose, fructose, mannose and trehalose, but not from ribose, sucrose, glycogen, xylose, mannitol, lactose, erythritol, D-arabinose, L-arabinose, adonitol, galactose, L-sorbose, rhamnose, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, dulcitol, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose, xylitol, β -gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, N-acetylglucosamine, 2-ketogluconate, 5-ketogluconate or glycerol. Gelatin, urea and aesculin are not hydrolysed. Activity for pyrazinamidase,

Table 1. Characteristics that differentiate *Corynebacterium sphenisci* sp. nov. from its closest phylogenetic relatives and from *C. striatum*

Characteristics are scored as: +, positive; –, negative; V, variable; S, susceptible; R, resistant; ND, not determined.

Characteristic	<i>C. sphenisci</i>	<i>C. xerosis</i>	<i>C. amycolatum</i>	<i>C. freneyi</i>	<i>C. striatum</i>
Fermentation of glucose at 42 °C	+	–	+	+	+
Growth at 20 °C	+	+	–	+	+
Susceptibility to O/129	S	S	Mostly R	S	ND
Nitrate reduction	+	V	V ^a , + ^{b*}	V	+
Acid production from:					
Sucrose	–	+	V ^a , + ^b	+	V ^a , + ^c
Ribose	–	+	+ ^b	+	–
5-Ketogluconate	–	+	–	ND	ND
Activity for:					
Alkaline phosphatase	–	+	+	+	+
α -Glucosidase	–	+	–	+	+
Valine arylamidase	+	–	–	–	ND
Chymotrypsin	+	–	–	–	ND

*Sources of data taken from other studies are indicated as: a, Funke *et al.* (1997b); b, Funke *et al.* (1996); c, Martínez-Martínez *et al.* (1995).

esterase C4, ester lipase C8, lipase C14, leucine arylamidase, valine arylamidase, cystine arylamidase, chymotrypsin, acid phosphatase and naphthol-AS-BI phosphohydrolase is detected. No activity is detected for alkaline phosphatase, pyrrolidonyl arylamidase, α -glucosidase, β -glucosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -galactosidase, β -galactosidase, α -fucosidase or trypsin. Cell-wall murein is based on *meso*-diaminopimelic acid. Long-chain cellular fatty acids are primarily of the straight-chain saturated and monounsaturated types, with C_{16:0}, C_{18:0} and C_{18:1 ω 9c} as the major components. Tuberculostearic acid is not present. Corynomycolic acids are produced, but in small amounts.

The type strain, CECT 5990^T (=CCUG 46398^T), was isolated from the cloaca of an apparently healthy wild magellanic penguin (*Spheniscus magellanicus*).

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