Corynebacterium ciconiae sp. nov., isolated from the trachea of black storks (*Ciconia nigra*)

J. F. Fernández-Garayzábal,¹ A. I. Vela,¹ R. Egido,¹ R. A. Hutson,² M. P. Lanzarot,³ M. Fernández-García³ and M. D. Collins²

Correspondence

J. F. Fernández-Garayzábal
garayzab@vet.ucm.es

¹Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

²School of Food Biosciences, University of Reading, Reading RG6 6AP, UK

³Gesnatura S.L., Avda. Brasil, 4, 28020 Madrid, Spain

Eight unidentified Gram-positive, rod-shaped organisms were recovered from the tracheas of apparently healthy black storks (*Ciconia nigra*) and subjected to a polyphasic taxonomic analysis. Based on cellular morphology and biochemical criteria the isolates were tentatively assigned to the genus *Corynebacterium*, although three of the organisms did not appear to correspond to any recognized species. Comparative 16S rRNA gene sequencing studies demonstrated that all of the isolates were phylogenetically members of the genus *Corynebacterium*. Five strains were genotypically identified as representing *Corynebacterium falsenii*, whereas the remaining three strains represented a hitherto unknown subline, associated with a small subcluster of species that includes *Corynebacterium mastitidis* and its close relatives. On the basis of phenotypic and phylogenetic evidence, it is proposed that the unknown isolates from black storks represent a novel species within the genus *Corynebacterium*, for which the *Corynebacterium ciconiae* sp. nov. is proposed. The type strain is CECT 5779^T (=BS13^T=CCUG 47525^T).

The genus *Corynebacterium* currently comprises over 50 recognized species, many of which have been described during the last decade. The recognition of large numbers of novel species in recent years has been possible by the use of improved taxonomic methods, such as chemotaxonomic and molecular-based approaches, especially 16S rRNA gene sequencing. Many of the novel *Corynebacterium* species have been isolated from human (e.g. Funke *et al.*, 1997a, c, 1998; Sjöden *et al.*, 1998; Collins *et al.*, 1999b; Renaud *et al.*, 2001) or animal (e.g. Fernández-Garayzábal *et al.*, 1998; Pascual *et al.*, 1998; Collins *et al.*, 1999a, 2001a, b) clinical sources. However, corynebacteria also occur as part of the indigenous flora of animals, although there is little information on the diversity of these corynebacterial species or their host distribution. Several novel *Corynebacterium* species have been described that may represent inhabitants of the natural microflora of different mucosal surfaces in different animals (Fernández-Garayzábal *et al.*, 2003; Goyache *et al.*, 2003a, b). Here, we have used phenotypic (including chemical markers) and molecular genetic methodologies to characterize several *Corynebacterium*-like organisms recovered from the tracheas of wild nestling

black storks (*Ciconia nigra*). Based on these findings, we propose a novel *Corynebacterium* species, *Corynebacterium ciconiae* sp. nov.

During an investigation of the normal flora of the tracheas of healthy nestling black storks, eight unidentified Gram-positive, rod-shaped organisms were recovered from different animals. Samples from tracheas 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 biochemically characterized using the API Coryne (version 2.0), API ZYM and API 50CH systems (bioMérieux) according to the manufacturer's instructions. Incubation of the API 50CH strips was extended up to 72 h, with examination every 24 h. Lipophilic requirements and the CAMP test with *Staphylococcus aureus* ATCC 25923 were determined according to standard procedures (Funke *et al.*, 1997b). 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 directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic

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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 eight 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 white (strains BS17, BS26, BS28, BS48 and BS60) or creamy (strains BS2, BS8 and BS13^T), small (approximately 1–2 mm diameter, after 24 h incubation at 37 °C) colonies, which were circular, convex, dry and non-haemolytic. All isolates were catalase-positive, grew under anaerobic conditions, were non-lipophilic and did not display a positive CAMP reaction with *S. aureus* after 48 h. The isolates did not hydrolyse either gelatin or aesculin, and they did not reduce nitrate. Three strains did not hydrolyse urea (BS2, BS8 and BS13^T). All strains produced acid from glucose and ribose, but not from D-xylose, mannitol, lactose, sucrose or glycogen. In addition, isolates BS2, BS8 and BS13^T produced acid from maltose. All of the strains gave positive reactions for alkaline phosphatase and pyrazinamidase, but no activity was detected for pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase, α -glucosidase and *N*-acetyl- β -glucosaminidase. Two different numerical profiles were obtained with the commercial API Coryne system, 2101304 (strains BS17, BS26, BS28, BS48 and BS60) and 2100324 (BS2, BS8 and BS13^T), which correspond to good and excellent identification within the genus *Corynebacterium*, respectively. The API Coryne numerical profile 2101304 has also been reported for *Corynebacterium falsenii* (Sjödén *et al.*, 1998) and the numerical profile 2100324 for *Corynebacterium imitans* and *C. falsenii* (Funke *et al.*, 1997a; Fernández-Garayzábal *et al.*, 2003), but the black stork isolates can be easily differentiated from these species based on additional biochemical tests (Table 1). Three isolates (BS2, BS8 and BS13^T) were biochemically characterized further using the API 50CH and API ZYM systems. All produced acid from glycerol, D-fructose, D-mannose and *N*-acetylglucosamine. None of the strains produced acid from erythritol, L-arabinose, L-xylose, adonitol, methyl β -xyloside, galactose, sorbose, L-rhamnose, dulcitol, 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-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. Two isolates (BS2 and BS8) produced acid from D-arabinose, L-fucose and gentiobiose, one isolate (BS8) produced acid from starch and another (isolate BS2) formed acid from trehalose. All eight strains gave positive reactions for acid phosphatase, esterase C4, ester lipase C8, lipase C14, naphthol-AS-BI-phosphohydrolase, β -glucosidase and leucine arylamidase. No activity was

detected for α -mannosidase, α -galactosidase, α -fucosidase, chymotrypsin, trypsin, valine arylamidase or cystine arylamidase. The morphological and biochemical characteristics of both groups of strains were consistent with their provisional assignment to the genus *Corynebacterium*.

To establish the phylogenetic position of the two groups of strains, 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) of representative strains of each group (BS2, BS13^T, BS26 and BS48) and approximately 1000 nt of the other strains were determined. Sequence searches of GenBank revealed that the unknown isolates were phylogenetically representative of members of the genus *Corynebacterium* (data not shown). The isolates from black storks formed two separate phylogenetic groups consistent with their phenotypic division, with strains displaying 99.8–100% 16S rRNA gene sequence similarity within their respective groups. The 16S rRNA gene sequence of strains BS26 and BS48 displayed 99.1% similarity to the sequence of *C. falsenii* CCUG 33651^T. The genotypic identification of isolates BS17, BS26, BS28, BS48 and BS60 as *C. falsenii* is consistent with the overall results of their phenotypic characteristics. Note that *C. falsenii* has also been isolated from normal flora of the trachea of healthy eagles (Fernández-Garayzábal *et al.*, 2003). These results suggest that this species may represent a normal inhabitant of the natural microflora of the higher respiratory tract of birds.

The second group of strains, as exemplified by strains BS2, BS8 and BS13^T, was phylogenetically distinct from all previously recognized *Corynebacterium* species. A tree depicting the phylogenetic relationships of this unidentified bacterium (based on the 16S rRNA gene sequence of strain BS13^T) within the genus *Corynebacterium* is shown in Fig. 1. The unknown organism formed a distinct subline within the genus *Corynebacterium*, branching proximal to the base of a subcluster of species, that includes *Corynebacterium propinquum* and *Corynebacterium pseudodiphtheriticum* as its close relatives. Bootstrap resampling, however, showed that the association of the black stork bacterium with this subcluster of species is not statistically significant and, from the tree construction analysis, it is evident that the unknown organism does not exhibit a significant affinity with any recognized species. The unidentified bacterium displayed highest sequence similarities to *Corynebacterium mastitidis* CECT 4843^T (94.4%), *Corynebacterium durum* ICB G15036^T (92.0%), *Corynebacterium matruchotii* CIP 81.82^T (93.3%), *C. propinquum* CIP 103792^T (94.1%) and *C. pseudodiphtheriticum* CIP 103420^T (94.5%), with other species showing lower levels of sequence similarity. However, sequence divergence values of >3% with recognized corynebacterial species show unequivocally that the black stork bacterium represents a hitherto unknown species (Stackebrandt & Goebel, 1994). The cellular lipid composition of the unknown black stork bacterium was also examined, as these compounds

Table 1. Characteristics that differentiate *Corynebacterium ciconiae* sp. nov. from its closest biochemical and phylogenetic relatives

Data for *Corynebacterium* species were obtained from the API Coryne system database and from Fernández-Garayzábal *et al.* (1997, 2003), Riegel *et al.* (1997a, b), Pascual *et al.* (1998), Funke *et al.* (1996, 1997a, b, c), Sjöden *et al.* (1998), Goyache *et al.* (2003a) and Yassin *et al.* (2002a, b). EST, Esterase C4; LIP, lipase C14; PA, acid phosphatase; PAL, alkaline phosphatase; β GLU, β -glucosidase; GLC, glucose; MAL, maltose; SUC, sucrose; GAL, galactose; GLY, glycerol; +, positive; –, negative; w, weakly positive; (+), delayed; v, variable; v⁺, most strains positive.

Species	Lipophilism	CAMP test	Nitrate reduction	Hydrolysis of urea	Production of:					Acid production from:					Other characteristics
					EST	LIP	PA	PAL	β GLU	GLC	MAL	SUC	GAL	GLY	
<i>C. ciconiae</i> sp. nov.	–	–	–	–	+	+	+	+	+	+	+	–	–	+	
<i>C. amycolatum</i>	–	–	+	–*	+	v ⁺	+	+	–	+	+	+	–	–	
<i>C. appendicis</i>	+	ND	–	+	–	–	–	+	–	+	+	–	ND	+	Ester lipase C8-, naphthol-AS-BI-phosphohydrolase- and leucine arylamidase-negative
<i>C. aquilae</i>	–	–	–	–	+	–	+	+	–	+	–	–	+	+	Chymotrypsin-positive
<i>C. coyleae</i>	ND	+	–	–	+	ND	+	+	–	+	–	–	–	–	5-Ketogluconate and cystine arylamidase-positive; <i>N</i> -acetyl- β -glucosamine-negative
<i>C. durum</i>	–	–	+	v	ND	ND	ND	–	ND	+	+	+	+	ND	Mannitol-positive; ribose-negative
<i>C. falsenii</i>	–	–	–	(+)	+	+	+	+	–	+	–	–	+	–†	
<i>C. imitans</i>	ND	+	–	–	+	–	+	+	–	+	+	w	–	–	Lactose-positive; <i>N</i> -acetylglucosamine- and leucine arylamidase-negative
<i>C. jeikeium</i>	+	–	–	–	ND	+	ND	+	ND	+	–*	–	ND	ND	
<i>C. mastitidis</i>	+	–	–	v	+	+	+	+	+	–	–	–	ND	ND	Ribose-negative; valine arylamidase- and cystine arylamidase-positive
<i>C. matruchotii</i>	–	–	+	–*	ND	ND	ND	–	ND	+	+	+	–	ND	
<i>C. minutissimum</i>	–	–	–	–	–	–	–	+	–	+	+	+	ND	–	Ribose-, mannose- and ester lipase C8-negative
<i>C. phocae</i>	ND	ND	–	v	–	–	+	+	–	+	+	v	–	–	D-Fructose- and α -glucosidase-positive
<i>C. propinquum</i>	–	–	+	–	ND	ND	ND	v	ND	–	–	–	ND	ND	
<i>C. pseudodiphtheriticum</i>	–	–	+	+	ND	ND	ND	v	ND	–	–	–	ND	ND	
<i>C. spheniscorum</i>	–	+	–	–	+	–	–	–	–	+	+	–	–	–	Naphthol-AS-BI-phosphohydrolase-negative
<i>C. striatum</i>	–	v	+	–	ND	ND	ND	+	ND	+	–*	+	v	ND	

*Variable, according to Funke *et al.* (1997b).

†Positive according to Fernández-Garayzábal *et al.* (2003).

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

have been shown to be taxonomically useful markers for the *Corynebacterium* group of organisms. Analysis of a representative strain, BS13^T (=CECT 5779^T), revealed the absence of corynomycolic acids. The absence of mycolic acids was confirmed by analysing concentrated extracts followed by high-temperature GLC. Examination of the long-chain cellular fatty acids of this strain showed the presence of straight-chain saturated C_{14:0} (0.6%), C_{15:0} (0.6%), C_{16:0} (36.2%), C_{17:0} (1.1%), C_{18:0} (18.7%), C_{20:0} (0.8%) and monounsaturated *cis*-9 C_{18:1} (42.2%), consistent with its phylogenetic assignment to the genus *Corynebacterium*.

It is evident from the presented findings that the Gram-positive, rod-shaped strains recovered from the trachea of black storks could be divided into two groups, one of which corresponded to *C. falsenii*. The second group of strains, although exhibiting overall cellular morphological and biochemical characteristics consistent with the genus *Corynebacterium*, did not appear to conform to any recognized species. Phylogenetic analysis based on 16S rRNA gene sequencing confirmed this provisional assignment and clearly demonstrated the unidentified black stork bacterium represents an unknown subline within the genus *Corynebacterium*. Despite its phylogenetic affinity with true corynebacteria, the black stork bacterium is somewhat unusual in not synthesizing corynomycolic acids. These α -alkyl- β -hydroxylated long-chain fatty acids (length 22–36 carbons) were considered to be highly characteristic of the genus *Corynebacterium*, but it is now recognized that several authentic species of this genus (e.g. *Corynebacterium amycolatum*, *Corynebacterium kroppenstedtii*, *Corynebacterium atypicum*) lack these unusual lipids. Biochemical data also provide support for distinctiveness of the black stork bacterium. Biochemically, the

unknown bacterium can easily be differentiated from the most closely phylogenetically related and other biochemically similar *Corynebacterium* species (Table 1). Based on both phenotypic and phylogenetic evidence, we consider the isolates from the trachea of nestling black storks merit classification as a novel species within the genus *Corynebacterium*, for which the name *Corynebacterium ciconiae* sp. nov. is proposed.

Description of *Corynebacterium ciconiae* sp. nov.

Corynebacterium ciconiae [ci.co'ni.æ. L. gen. fem. n. *ciconiae* of a (black) stork].

Cells are Gram-positive, non-spore-forming, non-motile rods. Colonies are creamy-white, circular, convex, dry and approximately 1–2 mm in diameter on Columbia blood agar after 24 h incubation at 37 °C. Colonies are non-haemolytic. Facultatively anaerobic. Non-lipophilic, and CAMP-negative with *S. aureus*. Aesculin, gelatin and urea are not hydrolysed. Nitrate is not reduced. Acid is produced from glucose, ribose, glycerol, D-fructose, D-mannose, *N*-acetyl- β -glucosamine and maltose, but not from D-xylose, mannitol, lactose, sucrose, glycogen, erythritol, L-arabinose, L-xylose, adonitol, methyl β -xyloside, galactose, sorbose, L-rhamnose, dulcitol, 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-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. Acidification of D-arabinose, L-fucose, trehalose, starch and gentiobiose is variable. Activity for alkaline and acid phosphatases, ester lipase C8, esterase C4, lipase C14, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, β -glucosidase and pyrazinamidase is detected. Pyrrolidonyl arylamidase, β -glucuronidase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -galactosidase, α -fucosidase, chymotrypsin, trypsin, valine arylamidase and cystine arylamidase are not produced. Mycolic acids are absent. Long-chain fatty acids are of the straight-chain saturated and monounsaturated types, with C_{16:0} and *cis*-9 C_{18:1} predominating.

The type strain, CECT 5779^T (=BS13^T=CCUG 47525^T), was isolated from the trachea of apparently healthy wild nestling black storks (*Ciconia nigra*).

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