Isolation of *Corynebacterium falsenii* and description of *Corynebacterium aquilae* sp. nov., from eagles

J. F. Fernández-Garayzábal,¹ R. Egido,¹ A. I. Vela,¹ V. Briones,¹ M. D. Collins,² A. Mateos,¹ R. A. Hutson,² L. Domínguez¹ and J. Goyache¹

Correspondence

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

¹Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

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

Biochemical, molecular chemical and molecular genetic studies were performed on seven unidentified Gram-positive, rod-shaped organisms recovered from eagles. The strains were provisionally identified as *Corynebacterium jeikeium* with the commercial API Coryne system, but they were able to grow under anaerobic conditions and were non-lipophilic. Comparative 16S rRNA gene sequencing studies demonstrated that the isolates belonged phylogenetically to the genus *Corynebacterium*. Three strains were identified genotypically as *Corynebacterium falsenii*; the remaining four strains corresponded to a hitherto unknown lineage within the genus *Corynebacterium*, associated with a small subcluster of species that included *Corynebacterium diphtheriae* and its close relatives. The unknown bacterial strains were readily distinguished from these and other species of the genus by biochemical tests. Based on both phenotypic and phylogenetic evidence, it is proposed that the unknown bacterial strains from eagles should be classified as *Corynebacterium aquilae* sp. nov. (type strain is S-613^T = CECT 5993^T = CCUG 46511^T).

Corynebacteria represent a major group of diphtheroid organisms within the Gram-positive, high-G+C content *Actinobacteria*. The genus *Corynebacterium* has undergone considerable expansion in the past decade and, at the time of writing, over 50 species are recognized. Corynebacteria have been isolated from a wide range of environments (e.g. dairy products, soil, sewage, sediments, aquatic sources and animals) but the vast majority of novel species described in recent years have originated from human clinical sources (e.g. Funke *et al.*, 1997, 1998; Sjöden *et al.*, 1998; Collins *et al.*, 1999a; Renaud *et al.*, 2001), due to increasing concern about their potential pathogenic significance (Lagrou *et al.*, 1998; Riegel, 1998). Corynebacteria also occur as part of the indigenous flora of animals other than man, but presently there is little information on the nature of these corynebacterial species and their host distribution. However, there are indications from the implementation of improved molecular taxonomic methodologies that much new corynebacterial species diversity remains to be discovered from animal sources (e.g. Fernández-Garayzábal *et al.*, 1997, 1998; Pascual *et al.*, 1998; Collins *et al.*, 1999b,

2001a, b). In the course of a study of the normal flora of the respiratory tract of eagles, we have characterized seven *Corynebacterium*-like organisms by using phenotypic, molecular chemical and molecular genetic methods. Based on the results of this study, we report the isolation of *Corynebacterium falsenii* from birds for the first time and describe a hitherto unknown *Corynebacterium* species, *Corynebacterium aquilae* sp. nov.

Bacterial strains S-102 and S-107 were isolated from the mouth of an adult and a juvenile Spanish Imperial eagle (*Aquila adalberti*), respectively. Five strains were isolated from Golden eagles (*Aquila chrysaetos*), three (S-613^T, S-622 and S-623) from choanae and two (S-672 and S-676) from trachea. Eagles were housed in a rehabilitation centre located in the region of Castilla-La Mancha, Spain. All strains were isolated on Columbia sheep-blood agar plates (bioMérieux) and incubated for 48 h at 37 °C under aerobic and anaerobic (AnaeroGen; Oxoid) conditions. The strains were characterized biochemically by 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 read after up to 7 days incubation at 37 °C. The CAMP (Christie–Atkins–Munch–Petersen) test with *Staphylococcus aureus* ATCC 25923 was performed according to standard procedures (Funke *et al.*, 1997). Lipophilic requirements were determined by growing

Published online ahead of print on 13 January 2003 as DOI 10.1099/ijs.0.02533-0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CECT 5993^T is AJ496733.

the isolates in brain-heart infusion agar supplemented with 1% Tween 80, in comparison with brain-heart infusion agar that lacked lipid supplementation. Cell-wall murein was prepared by mechanical disruption of cells and analysis of complete acid hydrolysates, 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 (approx. 1450 bases) of the 16S rRNA gene of the isolates was amplified by PCR and sequenced directly by using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit and a model 373A automatic DNA sequencer (both from Applied Biosystems). The closest known relatives of the new 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) by using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

The seven strains consisted of Gram-positive, non-motile, non-spore-forming, catalase-positive rods. Colonies were non-haemolytic and non-lipophilic and the CAMP test result was negative. All strains produced acid from glucose and ribose but not from xylose, mannitol, lactose or sucrose. None of the strains reduced nitrate and they did not hydrolyse aesculin, gelatin or urea. Reactions for pyrazinamidase and alkaline phosphatase were positive, but no activity was detected for pyrrolidonyl arylamidase, β -glucuronidase, β -glucosidase, α -glucosidase or *N*-acetyl- β -glucosaminidase. Three strains (S-107, S-672 and S-676) produced acid from maltose, whereas the other four strains did not. The former three strains grew on Columbia sheep-blood agar as whitish, circular, smooth colonies that developed a yellowish pigment after 48–72 h incubation. This pigmentation is produced by only a few non-lipophilic *Corynebacterium* species (Funke *et al.*, 1997; Sjöden *et al.*, 1998). Two different numerical profiles were obtained with the commercial API Coryne system: 2100304 (strains S-107, S-613^T, S-622 and S-623) and 2100324 (S-102, S-672 and S-676), which correspond to a good identification of *Corynebacterium jeikeium* and an excellent identification within the genus *Corynebacterium*, respectively. However, *C. jeikeium* is lipophilic and does not grow anaerobically (Funke *et al.*, 1997), whereas the seven eagle isolates were able to grow under anaerobic conditions and were non-lipophilic.

The eagle isolates were further characterized biochemically by using the API 50 CH and API ZYM systems. All strains produced acid from D-fructose, D-mannose, glycerol, ribose, *N*-acetylglucosamine and galactose. None of the strains produced acid from maltose, trehalose, D-xylose, L-xylose, mannitol, lactose, sucrose, erythritol, D-arabinose, L-arabinose, adonitol, methyl β -xyloside,

L-sorbose, rhamnose, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, amygdalin, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose, xylitol, β -gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate or glycogen. All strains gave positive reactions for alkaline phosphatase, ester lipase C8, esterase C4, naphthol-AS-BI-phosphohydrolase, acid phosphatase and leucine arylamidase. No activity was detected for trypsin, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, α -fucosidase, α -galactosidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, valine arylamidase or cystine arylamidase. Strains S-102, S-672 and S-676 gave a positive reaction for lipase C14 and were negative for chymotrypsin. On the other hand, strains S-107, S-613^T, S-622 and S-623 gave a positive reaction for chymotrypsin and were negative for lipase C14. Morphological and biochemical characteristics of both groups of strains were consistent with their provisional assignment to the genus *Corynebacterium*.

In order to investigate the phylogenetic relationships of the two groups of strains, their 16S rRNA gene sequences were determined by direct sequencing of *in vitro*-amplified rRNA gene products. The almost-complete 16S rRNA gene sequences (>1400 nt) of two representative strains of each group (S-676, S-672, S-622 and S-613^T) and approximately 1000 nt of those of the other three strains were determined and subjected to comparative analysis. The isolates from eagles formed two separate phylogenetic groups that were consistent with their phenotypic division, with >99.5% 16S rRNA sequence similarity within groups. Sequence searches of GenBank revealed that the unknown isolates belonged phylogenetically to the genus *Corynebacterium* (data not shown). Treeing analysis clearly demonstrated that the two groups of unidentified strains were phylogenetically separate from each other. The 16S rRNA gene sequences of strains S-672 and S-676 displayed 99.9% similarity with *C. falsenii* CCUG 33651^T, whereas the second group of strains was phylogenetically distinct from all previously described *Corynebacterium* species. The genotypic identification of isolates S-102, S-672 and S-676 as *C. falsenii* is consistent with their phenotypic characteristics, although the three eagle strains failed to produce acid from glycogen and did not hydrolyse urea with the API Coryne system. However, the aforementioned strains did hydrolyse urea using Christensen's urea medium, which also agrees with previous results of studies on *C. falsenii* (Sjöden *et al.*, 1998). *C. falsenii* has been isolated from different, normally sterile body sites in humans (Sjöden *et al.*, 1998), but to our knowledge this is the first isolation of this species from birds.

A tree constructed by using the neighbour-joining method, depicting the phylogenetic position of the second group of eagle isolates within the genus *Corynebacterium*, is shown in Fig. 1. The unknown group (as exemplified by strain S-613^T) formed a distinct lineage within the genus *Corynebacterium*, loosely associated with a small subcluster

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

of species that included *Corynebacterium diphtheriae*, the type species of the genus. However, bootstrap resampling showed that the unidentified eagle strains did not share a particularly close affinity with any member of this sub-cluster of species. Sequence similarity values confirmed the genotypic distinctiveness of this group of eagle isolates, as the highest values were shared with *Corynebacterium argenteratense* CIP 104296^T (96.4%), *Corynebacterium felinum* CCUG 39943^T (95.1%), *Corynebacterium ulcerans* NCTC 7910^T (95.6%), *Corynebacterium vitæruminis* DSM 20294^T (95.2%), *C. diphtheriae* NCTC 11397^T (95.1%), *Corynebacterium testudinoris* CCUG 41823^T (95.9%), *Corynebacterium kutscheri* CIP 103423^T (94.1%) and *Corynebacterium pseudotuberculosis* NCTC 3450^T (95.4%). Other corynebacterial species displayed substantially lower levels of similarity. To confirm the phylogenetic placement of the unknown eagle bacterial strains, cell-wall and lipid analyses were performed on a representative strain (S-613^T). Cell-wall murein analysis revealed the presence of *meso*-diaminopimelic acid, which is consistent with the definition of the genus *Corynebacterium*. Lipid analyses also reinforced the placement of the unknown strains within this genus, as short-chain mycolic acids (length C30–C36) and major amounts of straight-chain saturated (C_{14:0}, C_{16:0} and C_{18:0}) and monounsaturated (C_{16:1}ω9c and C_{18:1}ω9c) cellular fatty acids were detected. Thus it is clear, from both phenotypic and molecular genetic evidence, that the second group of eagle isolates represents a novel species within the genus *Corynebacterium*. Biochemically, the novel species can be readily distinguished from all currently recognized corynebacterial species. In addition, 16S rRNA gene treeing analysis and sequence divergence values of >3% between the unknown strains and all *Corynebacterium* species with validly published names show unequivocally that they represent a hitherto unrecognized species (Stackebrandt & Goebel, 1994). Therefore, based on both phylogenetic and phenotypic criteria, it is evident that the unidentified strains merit classification as a novel species of the genus *Corynebacterium*, for which the

name *Corynebacterium aquilae* sp. nov. is proposed. Tests that are useful for the differentiation of *C. aquilae* from its nearest phylogenetic relatives are shown in Table 1.

Description of *Corynebacterium aquilae* sp. nov.

Corynebacterium aquilae (a'qui.lae. N.L. gen. n. *aquilae* pertaining to the eagle, *Aquila*).

Cells are Gram-positive, non-motile, non-spore-forming rods. Colonies are whitish, low convex, dry, rough and 1–2 mm in diameter after 48 h incubation at 37 °C on sheep-blood agar. Facultatively anaerobic, catalase-positive and oxidase-negative. Non-haemolytic, CAMP-negative and non-lipophilic. Nitrate is not reduced. Acid is produced from D-glucose, D-fructose, D-mannose, glycerol, ribose, N-acetylglucosamine and galactose, but not from maltose, trehalose, D-xylose, L-xylose, mannitol, lactose, sucrose, erythritol, D-arabinose, L-arabinose, adonitol, methyl β-xyloside, L-sorbose, rhamnose, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose,

Table 1. Characteristics that differentiate *C. aquilae* sp. nov. from its nearest phylogenetic relatives

Taxa: 1, *C. aquilae* CECT 5993^T; 2, *C. argenteratense* CIP 104296^T; 3, *C. testudinoris* CCUG 41823^T; 4, *C. felinum* CCUG 39943^T; 5, *C. vitæruminis*; 6, *C. diphtheriae*; 7, *C. ulcerans* CCUG 2798^T; 8, *C. pseudotuberculosis*. Data for *C. vitæruminis*, *C. diphtheriae* and *C. pseudotuberculosis* are from the API Coryne system database and Collins *et al.* (2001b). +, Positive reaction; –, negative reaction; +w, weakly positive reaction; V, variable reaction; V+, most strains positive; V–, most strains negative; ND, not determined.

Characteristic	1	2*	3†	4‡	5	6	7§	8§
Nitrate reduction	–	–	+	–	+	V+	–	–
Hydrolysis of aesculin	–	–	+	–	+	–	–	–
Urease	–	–	–	–	+	–	+	+
Acid production from:								
Maltose	–	–	+	+	+	+	+	+
Sucrose	–	–	+	–	+	–	–	–
Activity of:								
Pyrazinamidase	+	+	–	+	+	–	–	–
Pyrrolidonyl arylamidase	–	–	–	+	+	–	–	–
Alkaline phosphatase	+	V–	–	–	V	–	+	ND
Acid phosphatase	+	V	+W	–	ND	ND	ND	+
α-Glucosidase	–	–	V	+	ND	+	+	V

**C. argenteratense* differs also from *C. aquilae* by its inability to produce acid from galactose and by exhibiting cystine arylamidase activity.

†*C. testudinoris* is α-chymotrypsin-negative and β-glucosidase-positive.

‡*C. felinum* is α-chymotrypsin-negative.

§*C. ulcerans* and *C. pseudotuberculosis* display reverse CAMP reactions.

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

xylitol, β -gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate or glycogen. Gelatin, urea and aesculin are not hydrolysed. Alkaline and acid phosphatases, ester lipase C8, esterase C4, naphthol-AS-BI-phosphohydrolase, leucine arylamidase and chymotrypsin activities are detected. No activity is detected for lipase C14, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase, α -galactosidase, β -galactosidase, α -fucosidase, N-acetyl- β -glucosaminidase, valine arylamidase, trypsin or cystine arylamidase. Cell wall contains meso-diaminopimelic acid. Mycolic acids (C₃₀–C₃₆) are present. Fatty acids are of the straight-chain saturated (C_{14:0}, C_{16:0} and C_{18:0}) and monounsaturated (C_{16:1} ω 9c and C_{18:1} ω 9c) types.

The type strain, S-613^T (=CECT 5993^T=CCUG 46511^T), was isolated from the choanae of the Golden eagle (*A. chrysaëtos*).

Acknowledgements

The authors thank N. Montero for her technical assistance.

References

- Collins, M. D., Bernard, K. A., Hutson, R. A., Sjöden, B., Nyberg, A. & Falsen, E. (1999a). *Corynebacterium sundsvallense* sp. nov., from human clinical specimens. *Int J Syst Bacteriol* **49**, 361–366.
- Collins, M. D., Hoyles, L., Lawson, P. A., Falsen, E., Robson, R. L. & Foster, G. (1999b). Phenotypic and phylogenetic characterization of a new *Corynebacterium* species from dogs: description of *Corynebacterium auriscanis* sp. nov. *J Clin Microbiol* **37**, 3443–3447.
- Collins, M. D., Hoyles, L., Foster, G., Sjöden, B. & Falsen, E. (2001a). *Corynebacterium capitovis* sp. nov., from a sheep. *Int J Syst Evol Microbiol* **51**, 857–860.
- Collins, M. D., Hoyles, L., Hutson, R. A., Foster, G. & Falsen, E. (2001b). *Corynebacterium testudinoris* sp. nov., from a tortoise, and *Corynebacterium felinum* sp. nov., from a Scottish wild cat. *Int J Syst Evol Microbiol* **51**, 1349–1352.
- 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, R. & 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., Osorio, C. R., Frei, R., Riegel, P. & Collins, M. D. (1998). *Corynebacterium confusum* sp. nov., isolated from human clinical specimens. *Int J Syst Bacteriol* **48**, 1291–1296.
- 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.
- Lagrou, K., Verhaegen, J., Janssens, M., Wauters, G. & Verbist, L. (1998). Prospective study of catalase-positive coryneform organisms in clinical specimens: identification, clinical relevance, and antibiotic susceptibility. *Diagn Microbiol Infect Dis* **30**, 7–15.
- Pascual, C., Foster, G., Alvarez, N. & Collins, M. D. (1998). *Corynebacterium phocae* sp. nov., isolated from the common seal (*Phoca vitulina*). *Int J Syst Bacteriol* **48**, 601–604.
- Renaud, F. N. R., Aubel, D., Riegel, P., Meugnier, H. & Bollet, C. (2001). *Corynebacterium freneyi* sp. nov., α -glucosidase-positive strains related to *Corynebacterium xerosis*. *Int J Syst Evol Microbiol* **51**, 1723–1728.
- Riegel, P. (1998). Bacteriological and clinical aspects of *Corynebacterium*. *Ann Biol Clin* **56**, 285–296 (in French).
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Sjöden, B., Funke, G., Izquierdo, A., Akervall, E. & Collins, M. D. (1998). Description of some coryneform bacteria isolated from human clinical specimens as *Corynebacterium falsenii* sp. nov. *Int J Syst Bacteriol* **48**, 69–74.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.