***Arcanobacterium pluranimalium* sp. nov., isolated from porpoise and deer**

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Two strains of a previously undescribed *Arcanobacterium*-like bacterium were isolated from a dead harbour porpoise and a dead fallow deer. Biochemical testing and PAGE analysis of whole-cell proteins indicated that the strains were phenotypically closely related to each other and distinct from previously described *Actinomyces* and *Arcanobacterium* species. Comparative 16S rRNA gene sequencing studies showed the bacterium to be a hitherto unknown subspecies within the genus *Arcanobacterium*. Based on phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium be classified as *Arcanobacterium pluranimalium* sp. nov. The type strain of *Arcanobacterium pluranimalium* is CCUG 42575^T (= CIP 106442^T).

Keywords: taxonomy, phylogeny, *Arcanobacterium pluranimalium*, 16S rRNA

INTRODUCTION

The genus *Arcanobacterium* (Collins *et al.*, 1982) was created to accommodate the species formerly designated *Corynebacterium haemolyticum*. Initially, the genus consisted of the single species *Arcanobacterium haemolyticum*. However, recent phylogenetic studies have resulted in the reclassification of *Actinomyces bernardiae* and *Actinomyces pyogenes* as *Arcanobacterium bernardiae* and *Arcanobacterium pyogenes*, respectively (Pascual *et al.*, 1997). In addition, a fourth member of the genus, *Arcanobacterium phocae*, isolated from seals, has been described (Pascual *et al.*, 1997). The aforementioned *Arcanobacterium* species have all been recovered from human and/or animal sources and some are recognized pathogens. *Arcanobacterium haemolyticum* is associated primarily with pharyngitis and with mixed wound infections, whereas *Arcanobacterium bernardiae* has been recovered from a variety of human clinical specimens (e.g. abscesses, blood; see Funke *et al.*, 1995). *Arcanobacterium pyogenes* is a well-established cause of pyogenic infections in animals and is also known to cause human disease, albeit rarely (Funke *et al.*, 1995). *Arcano-*

bacterium phocae has been isolated from seals but is of unknown pathological significance (Pascual *et al.*, 1997). During the course of a study of taxonomically problematic *Actinobacteria* from wild animals, two *Arcanobacterium*-like organisms, one from a dead harbour seal and the second from a dead fallow deer, were characterized. Based on the results of a polyphasic taxonomic study, yet another new species of the genus *Arcanobacterium*, *Arcanobacterium pluranimalium*, is described.

METHODS

Strains. Strain M430/94/2^T (= CCUG 42575^T) was isolated from the spleen of a dead harbour porpoise. Strain CCUG 41729 was recovered from a lung abscess of a dead fallow deer. The unidentified *Arcanobacterium*-like isolates were cultivated on Columbia agar (Difco) supplemented with 5% horse blood at 37 °C, in air plus 5% CO₂.

Phenotypic characterization. The strains were biochemically characterized by using the API rapid ID32 Strep and API CORYNE systems according to the manufacturer's instructions (API bioMérieux). The type strains and other reference strains used in the investigation were all maintained by the Culture Collection of the University of Göteborg, Sweden. All tests were performed in duplicate.

SDS-PAGE of whole-cell protein. To assess the phenotypic resemblance of the two isolates to each other and to reference

The GenBank accession number for the 16S rRNA gene sequence of strain CCUG 42575^T is AJ250959.

Fig. 1. Similarity dendrogram based on whole-cell protein patterns of *Arcanobacterium pluranimalium* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

Arcanobacterium species, a comparative analysis of whole-cell protein profiles by SDS-PAGE was performed. PAGE analysis of whole-cell proteins was performed as described previously (Pot *et al.*, 1994; Vandamme *et al.*, 1998). For densitometric analysis, normalization and interpretation of protein patterns, the GCW 3.0 software package (Applied Maths, Kortrijk, Belgium) was used. The similarity between all pairs of traces was expressed by the Pearson product-moment correlation coefficient converted for convenience to percentage similarity.

Phylogenetic analysis. The 16S rRNA genes of the two isolates were amplified by PCR and directly sequenced using a *Taq* dideoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest known relatives of the new isolates were determined by performing database searches. These sequences and those of other known related strains were retrieved from the GenBank or Ribosomal Database Project (RDP) databases and aligned with the newly determined sequences using the program PILEUP (Devereux *et al.*, 1984). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura two-correction parameter) (Felsenstein, 1989). 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 SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

RESULTS AND DISCUSSION

The two isolates consisted of Gram-positive, straight to slightly curved, non-branching, slender rods which were non-acid-fast and non-spore-forming. The strains were catalase-positive and α -haemolytic on blood agar. Phenotypically, they closely resembled each other producing acid from D-glucose and D-ribose. Neither strain produced acid from D-arabitol, L-arabinose, cyclodextrin, glycogen, pullulan, sorbitol, mannitol, melibiose, melezitose, lactose, D-raffinose, sucrose, tagatose, trehalose or D-xylose. Results for acid production from maltose differed between the different API systems employed. Using the API CORYNE kit, acid was produced with this substrate. However, with the API rapid ID32 Strep system, this substrate was not fermented. Both isolates gave positive reactions for alanine phenylalanine proline arylamidase, pyroglutamic acid arylamidase and β -glucuronidase, but they were negative for arginine dihydrolase, alkaline

Fig. 2. Unrooted tree showing the phylogenetic relationships of *Arcanobacterium pluranimalium* sp. nov. and some other high-G+C-content Gram-positive bacteria. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1327 nt. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Scale bar, 1% sequence divergence.

phosphatase, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -galacturonidase, *N*-acetyl- β -glucosamine, β -mannosidase and urease. Variable reactions were observed for pyrazinamidase and glycyl tryptophan arylamidase. Both isolates hydrolysed aesculin (weak reaction), gelatin and hippurate and gave a negative Voges–Proskauer test. They did not reduce nitrate to nitrite. The cellular morphology and biochemical reactions of the isolates were consistent with their assignment to the genus *Arcanobacterium*, although they did not appear to correspond to any described species of the genus. An examination of the cell wall murein of strain CCUG 42575^T revealed a murein structure L-Lys-Lys-D-Glu (type A5 α) (nomenclature of Schleifer & Kandler, 1972) consistent with *Arcanobacterium haemolyticum*, the type species of the genus. To our knowledge, the only other described species containing this murein type corresponds to *Actinomyces europaeus* and *Actinomyces neuii* (Funke *et al.*, 1997). The G+C content of strain CCUG 42575^T was 57 mol %. This value is within the published range (approx. 48–66 mol %) for the genus *Arcanobacterium* (Collins *et al.*, 1982; Funke *et al.*, 1995).

A numerical analysis of the whole-cell protein patterns of the two strains of *Arcanobacterium pluranimalium*

together with the reference strains of *Arcanobacterium* is shown in Fig. 1. The two isolates clustered together and formed a distinct group with a correlation level of 85%. *Actinomyces neuii* subsp. *antitratus* and subsp. *neuii* were the nearest species to the unknown isolates on PAGE analysis, joining the latter group at a correlation of about 55% (Fig. 1). The PAGE results confirmed that the two unidentified strains represent a phenotypically homogeneous group of organisms and indicated that they are distinct from all *Arcanobacterium* and *Actinomyces* species described to date.

To ascertain the phylogenetic relationships of the unknown isolates, their 16S rRNA genes were sequenced and subjected to a comparative analysis. The almost complete gene sequences (> 1500 nt) of the two strains were determined and pairwise analysis showed these to be identical (100% sequence similarity in 1420 bases compared). Sequence database searches confirmed that the unknown bacterium was most closely related to species of the genus *Arcanobacterium* (range 93.9–96.5%). Highest sequence relatedness was shown with *Arcanobacterium phocae* (96.5% similarity). The results of neighbour-joining analysis are shown in Fig. 2 and confirmed the association of the unknown clinical bacterium (as exemplified by strain CCUG 42575^T) with the genus *Arcanobacterium*.

Table 1. Tests useful for identifying *Arcanobacterium pluranimalium*

+, Positive; -, negative; +w, weak positive; -(+), a few strains positive; +(-), a few strains negative; v, variable.

Test	<i>Arcanobacterium bernardiae</i>	<i>Arcanobacterium haemolyticum</i>	<i>Arcanobacterium pluranimalium</i>	<i>Arcanobacterium phocae</i>	<i>Actinomyces pyogenes</i>
Acid from:					
Lactose	-	+	-	v	+
Glycogen	+	-	-	+	v
D-Ribose	+	-	+	+	+
D-Xylose	-	-	-	v	+
Hydrolysis of:					
Aesculin	-	-	+w	-	-
Gelatin	-	-	+	-	+
Hippurate	-	-	+	-	+(-)
Production of:					
Catalase	-	-(+)	+	v	-(+)
α -Galactosidase	-	-	-	+w	-
β -Galactosidase	-	-	-	+	+
β -Galacturonidase	-	+	-	+	+
β -Glucuronidase	-	-(+)	+	-	+
Pyroglutamic acid arylamidase	-	-(+)	+	-	-

It is apparent from both phylogenetic and phenotypic evidence that the two unidentified isolates recovered from a dead harbour seal and a dead fallow deer represent a hitherto unknown *Arcanobacterium* species. Both sequence divergence and treeing analysis show that the unidentified bacterium represents a distinct subline within the genus *Arcanobacterium*. The nearest relative of the unknown organisms corresponds to *Arcanobacterium phocae*, a species recovered from clinical specimens of common seals and grey seals (Pascual *et al.*, 1997). Although there is no precise correlation between the degree of 16S rRNA sequence divergence and species delineation, it is generally recognized that divergence values of 3% or more are significant (Stackebrandt & Goebel, 1994). The observed 3.5% divergence between the unknown bacterium and *Arcanobacterium phocae*, and even greater divergence values with other *Arcanobacterium* species, is consistent with separate species status. The separateness of the unknown bacterium was also demonstrated by PAGE analysis of whole-cell proteins and biochemical profiling. In particular, the unknown bacterium can be readily distinguished from all currently described *Arcanobacterium* species by the traits shown in Table 1. Therefore, based on both phylogenetic and phenotypic evidence, it is proposed that the unidentified isolates be classified in a new species of the genus *Arcanobacterium*, *Arcanobacterium pluranimalium*.

Description of *Arcanobacterium pluranimalium* sp. nov.

Arcanobacterium pluranimalium (plur.an.im.al'i.um. L. adj. *pluris* many; L. n. gen. pl. *animalium* from animals; M.L. gen. n. *pluranimalium* from many animals).

Cells are straight to slightly curved, non-branching, slender rods which stain Gram-positive, are non acid-fast and non-motile. Facultatively anaerobic and catalase-positive. Using API systems, acid is produced from D-glucose and D-ribose, but not from D-arabitol, L-arabinose, cyclodextrin, glycogen, pullulan, sorbitol, tagatose, mannitol, melibiose, melezitose, lactose, D-raffinose, sucrose, trehalose or D-xylose. Maltose is variable. Alanine phenylalanine proline arylamidase, pyroglutamic acid arylamidase and β -glucuronidase are detected, but not arginine dihydrolase, alkaline phosphatase, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -galacturonidase, *N*-acetyl- β -glucosamine, β -mannosidase or urease. Pyrazinamidase and glycyl tryptophan arylamidase may or may not be detected. Aesculin (weak reaction), gelatin and hippurate are hydrolysed. Acetoin is not produced. Nitrate is not reduced to nitrite. The cell wall contains a murein structure L-Lys-Lys-D-Glu (type A5 α). Isolated from a dead harbour porpoise and a dead fallow deer. Habitat is not known. The type strain is CCUG 42575^T. The G + C content of DNA of the type strain is 57 mol%.

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