

Actinomyces cardiffensis sp. nov. from Human Clinical Sources

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Eight strains of a previously undescribed catalase-negative *Actinomyces*-like bacterium were recovered from human clinical specimens. The morphological and biochemical characteristics of the isolates were consistent with their assignment to the genus *Actinomyces*, but they did not appear to correspond to any recognized species. 16S rRNA gene sequence analysis showed the organisms represent a hitherto unknown species within the genus *Actinomyces* related to, albeit distinct from, a group of species which includes *Actinomyces turicensis* and close relatives. Based on biochemical and molecular genetic evidence, it is proposed that the unknown isolates from human clinical sources be classified as a new species, *Actinomyces cardiffensis* sp. nov. The type strain of *Actinomyces cardiffensis* is CCUG 44997^T.

The genus *Actinomyces* embraces a heterogeneous group of anaerobic and aerotolerant, non-spore-forming, non-acid-fast, gram-positive rods with high G+C DNA content (14). Many *Actinomyces* species primarily belong to the facultative anaerobic indigenous microflora of human and animal mucous membranes, particularly those of the oral cavity. Some members of the genus are known to cause classical actinomycosis and may also be found in polymicrobial infections, while others are significant in periodontal disease (14, 15). The genus *Actinomyces* has undergone considerable expansion in recent years, with a plethora of new species defined, especially from human sources. Recently described *Actinomyces* species, including *Actinomyces funkei* (9), *Actinomyces europaeus* (4), *Actinomyces graevenitzi* (13), *Actinomyces neuii* subsp. *anitrat* and *neuii* (5), *Actinomyces radingae* (17), *Actinomyces radicidentis* (1), *Actinomyces turicensis* (17), and *Actinomyces urogenitalis* (12), have been isolated from clinical specimens, where they occur as contaminants and/or represent possible opportunistic pathogens. Despite this increase in the number of recognized species, it is clear that knowledge of the habitats, clinical prevalence, and pathogenic potential of many *Actinomyces* and related organisms is inadequate, and there are indications (6, 7) that much new diversity remains to be discovered from human sources. During the course of study of clinical isolates of *Actinomyces*, we have characterized a hitherto unknown *Actinomyces*-like species. Based on both phenotypic and phylogenetic evidence, we propose yet another new species of the genus *Actinomyces*, namely, *Actinomyces cardiffensis*.

MATERIALS AND METHODS

Cultures and phenotypic characterization. All eight strains were isolated in the United Kingdom and were presumptively identified as *Actinomyces* spp. and were referred to the Public Health Laboratory Service Anaerobe Reference Unit, Cardiff, United Kingdom, for confirmation of identity. Strain R10394^T

(CCUG 44997^T) was recovered in 1993 from an intrauterine contraceptive device (IUCD) which was in situ for 7 years in a 35-year-old female in Great Yarmouth, United Kingdom. Strains R13895 (CCUG 45110) and R7770 (CCUG 46084) were also recovered from IUCDs of 26- and 37-year-old females in Farnborough, Hampshire, United Kingdom (2000), and Bury St. Edmunds, West Suffolk, United Kingdom (1994), respectively. The latter patient was described as having the IUCD in situ for a considerable period of time, and *Actinomyces*-like organisms were seen in her cervical smear. Strain R5999 (CCUG 45108) was recovered from pus from temporal, large parietal, small parietal, and ear abscesses of a 32-year-old male at 4 weeks postmastoidectomy (Cardiff, United Kingdom, 1993). Concomitant organisms in each of the four abscesses were *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Bacteroides ureolyticus*, *Porphyromonas endodontalis*, *Porphyromonas levii*, *Prevotella oris*, *Peptostreptococcus micros*, *Peptostreptococcus* sp., *Arcanobacterium bernardiae*, *Eggerthella lenta*, and a microaerophilic *Streptococcus* sp. strain R11374 (CCUG 45109) was recovered from pleural fluid of a 74-year-old male with glaucoma, shortness of breath, and wheezing (Burton upon Trent, East Staffordshire, United Kingdom, 1997). Strain R5571 (CCUG 44995) was recovered from pus from an actinomycotic jaw abscess of a 78-year-old female (Hereford, United Kingdom, 1993). Strain R2037 (CCUG 46083) was recovered from a pericolic abscess, discovered upon resectioning of a colon carcinoma of a 71-year-old female (Lincoln, Lincolnshire, United Kingdom, 1985). Strain R9463 (CCUG 46085) was recovered from a right antral washout of a 48-year-old female with sinusitis (Carlisle, Cumbria, United Kingdom, 1996). Volatile and nonvolatile end products of glucose metabolism were detected by gas-liquid chromatography (8). For biochemical testing, the strains were cultured on Columbia agar (Difco, Detroit, Mich.) supplemented with 5% horse blood at 37°C, incubated anaerobically. The strains were biochemically characterized using both conventional tests (10) and the commercially available API rapid ID32A, API rapid ID32Strep, and API Coryne systems according to the manufacturer's instructions (API BioMérieux, Marcy l'Etoile, France).

Amplified 16S rDNA restriction analysis. Amplified 16S rDNA restriction analyses were performed using *Hae*III and *Hpa*II as described previously (6, 7).

16S rRNA gene sequencing and phylogenetic analyses. The 16S rRNA genes of the eight isolates were amplified by PCR and directly sequenced using a *Taq* dye-deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) 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 libraries and aligned with the newly determined sequences using the program PILEUP (2). The resulting multiple sequence alignment was corrected manually, and a distance matrix was calculated using the programs PRETTY and DNADIST (using Kimura 2-correction) (3). A phylogenetic tree was constructed according to the neighbor-joining method with the program NEIGHBOR (3). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE (3).

Protein profiling. Polyacrylamide gel electrophoresis analysis of whole-cell proteins was performed as described by Pot et al. (11). For densitometric anal-

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ysis, normalization, and interpretation of protein patterns, the GCW 3.0 software package (Applied Maths) was used. The similarity between all pairs of traces was expressed by the Pearson product moment correlation coefficient, which was converted for convenience to percent similarity.

Nucleotide sequence accession number. The GenBank accession number for the 16S rRNA sequence of CCUG 44997^T is AJ421779.

RESULTS AND DISCUSSION

The eight isolates consisted of slender, straight or curved, pleomorphic, gram-positive rod-shaped cells. Beaded and branched filamentous forms occurred. Cells were non-acid-fast, non-spore-forming, and catalase negative. Strains were facultatively anaerobic but grew better under anaerobic conditions. After anaerobic incubation for 48 h, colonies on Fastidious Anaerobe Agar (LabM; Bury, Lancashire, United Kingdom) with 5% horse blood were pinpoint, convex, smooth, entire-edged, opaque cream to pink, and nonhemolytic. Principal end products of glucose metabolism were small amounts of acetic acid and large amounts of lactic and succinic acids. When subjected to conventional biochemical testing (10), the isolates formed acid from glucose and sucrose but not from amygdalin, L-arabinose, cellobiose, lactose, mannitol, mannose, D-raffinose, salicin, trehalose, or D-xylose. The level of acid production from D-ribose was variable. The isolates failed to hydrolyze esculin and starch and were lipase and lecithinase negative. They were indole negative. Based upon these findings, the unknown isolates resembled *Actinomyces odontolyticus* but were differentiated from this species by the fermentation of lactose, dark red pigment, and growth in air, which are characteristic of the latter. Using the commercial biochemical kits, the strains were unidentified. When the API Coryne system was used, the eight strains produced acid from glucose and sucrose and displayed α -glucosidase activity. Acid was produced from ribose by five of the eight strains, while seven of the eight strains formed acid from maltose. Nitrate reduction was variable, with two of the eight strains testing positive. None of the isolates produced acid from glycogen, lactose, mannitol, or D-xylose, and activity for alkaline phosphatase, catalase, β -galactosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, pyrazinamidase, pyrolydonyl arylamidase, and urease was not detected. None of the strains hydrolyzed esculin or gelatin. A code of 0/1 0 1 0 1/3 2 1 was generated. When the API rapid 32S system was used, all eight strains showed activity for alanine phenylalanine proline arylamidase and three of the eight strains produced acid from sucrose. However, none of the strains produced acid from D-arabitol, L-arabinose, cyclodextrin, glycogen, maltose, mannitol, melibiose, melezitose, methyl- β -D-glucopyranoside, lactose, pullulan, D-raffinose, D-ribose, sorbitol, tagatose, or trehalose and none displayed activity for arginine dihydrolase, alkaline phosphatase, β -glucosidase, α -galactosidase, β -galactosidase, β -glucuronidase, glycyl tryptophan arylamidase, β -mannosidase, N-acetyl- β -glucosaminidase, pyroglutamic acid arylamidase, or urease, using the rapid ID 32S kit. When the API rapid ID32A system was used, all eight strains displayed activity for alanine arylamidase, arginine arylamidase, α -glucosidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucylglycine arylamidase, proline arylamidase, phenylalanine arylamidase, serine arylamidase, and tyrosine arylamidase. None of the strains displayed activity for alkaline phosphatase, α -arabinosidase, argi-

nine dihydrolase, α -fucosidase, α -galactosidase, β -galactosidase, β -galactosidase 6-phosphate, β -glucosidase, β -glucuronidase, glutamic acid decarboxylase, glutamylglutamic acid arylamidase, N-acetyl- β -glucosaminidase, pyroglutamic acid, arylamidase, or urease. Some strains produced acid from mannose (one of eight) and raffinose (two of eight), and three of eight reduced nitrate. None of the isolates produced indole. In this test system, a code of 0 4 0 0/2/4 0/1 7 3 7 0 5 was generated. The cellular morphology and biochemical reactions of the isolates were consistent with their tentative assignment to the genus *Actinomyces*, but the strains did not appear to correspond to any recognized species of this genus. To investigate the genetic relatedness of the isolates, amplified 16S rDNA restriction analyses was performed. All eight strains produced identical 16S rDNA restriction profiles with *Hae*III and *Hpa*II (profile, 001/016), indicating that the strains were highly related to each other. To ascertain the phylogenetic relationships of the unknown organisms, their almost complete 16S rRNA gene sequences (>1,400 nucleotides) were determined. Pairwise analysis showed that the organisms were genetically highly related to each other, displaying >99.5% sequence relatedness. These data, in conjunction with the high phenotypic resemblance of the clinical isolates, strongly support their assignment to a single species. Sequence database searches confirmed that the unknown isolates were most closely related to species of the genus *Actinomyces*. The highest sequence similarity values were seen with *Actinomyces* species and phylogenetically related organisms. Other *Actinomyces* species showed substantially lower levels of relatedness (data not shown). Tree analysis further demonstrated the placement of the unidentified bacterium (as exemplified by strain R10394^T = CCUG 44997^T) within the genus *Actinomyces*, with the novel bacterium displaying a specific association to a small subcluster of species which included *Actinomyces funkei*, *Actinomyces radingae*, *Actinomyces turicensis*, and their close phylogenetic relatives (Fig. 1). The results of comparative analysis of whole-cell protein profiles of the unidentified strains and reference species of the genus *Actinomyces* and related genera are shown in Fig. 2. The eight clinical isolates formed a distinct group (greater than 70% intragroup similarity) that was distinct from all other recognized species. *Actinomyces funkei* displayed the closest similarity to the unidentified clinical group, joining the latter at about 55% similarity (Fig. 2). Other species displayed much lower levels of similarity.

In recent years, numerous new *Actinomyces* species have been isolated from human and animal sources. Most of these new species have come to light as a result of increased interest by clinical microbiologists in the possible role of such organisms as opportunistic pathogens and due to the implementation of improved molecular identification methodologies that permit their differentiation from recognized species. It is evident from the results of the present investigation that the unidentified gram-positive, catalase-negative, rod-shaped organisms represent another hitherto undescribed *Actinomyces* species from clinical sources. The novel bacterium forms a distinct subline within a subcluster of species within the *Actinomyces* genus, which includes *Actinomyces turicensis*, *Actinomyces funkei*, *Actinomyces radingae*, *Actinomyces canis*, *Actinomyces georgiae*, *Actinomyces hyovaginalis*, *Actinomyces meyeri*, *Actinomyces odontolyticus*, and *Actinomyces suimastitidis*. Boot-

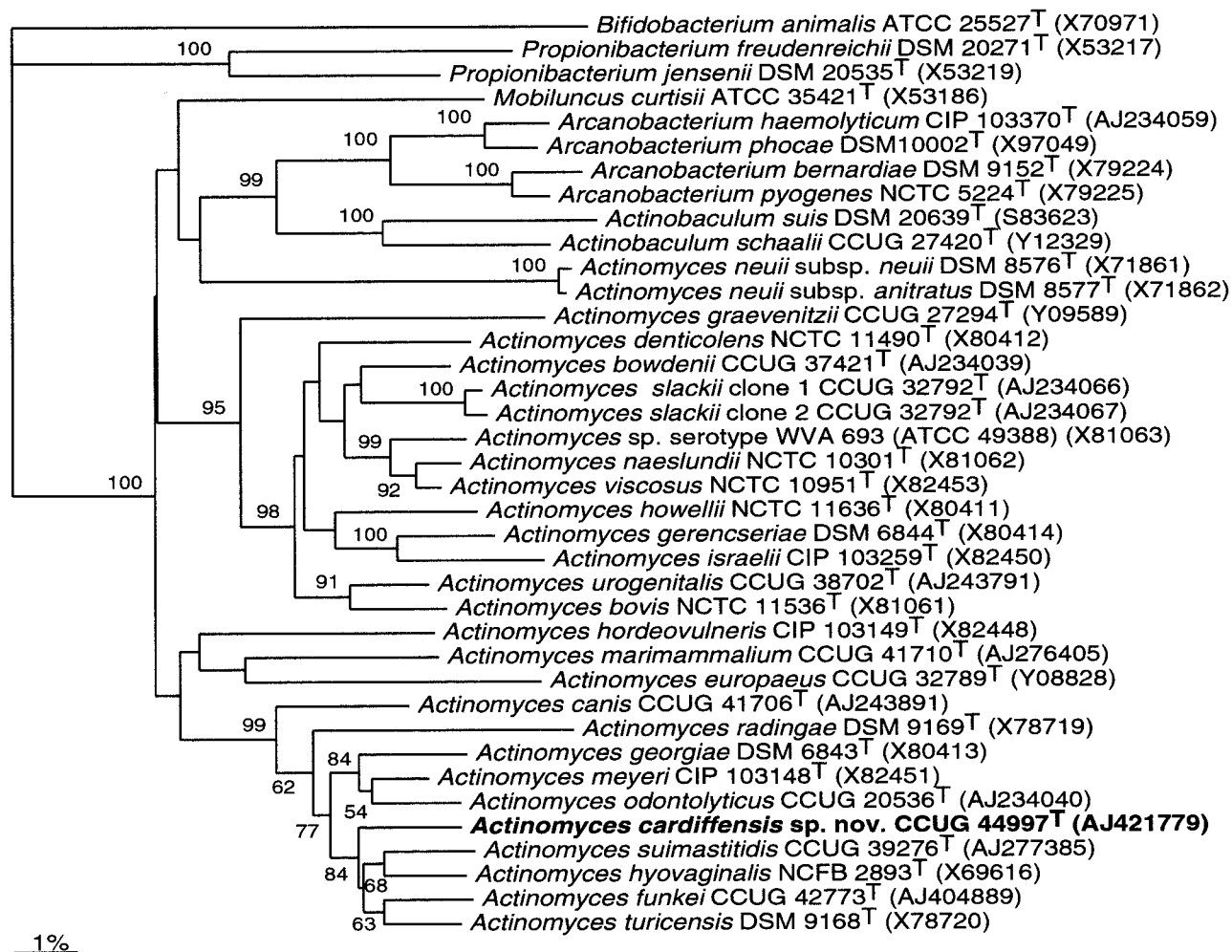


FIG. 1. Unrooted tree showing the phylogenetic relationships of *Actinomyces cardiffensis* sp. nov. (boldface). The tree, constructed using the neighbor-joining method, was based on a comparison of approximately 1,327 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branch points.

strap resampling, however, showed that the novel bacterium did not possess a particularly significant affinity with any member of this subcluster. Sequence divergence values of 3 to 6% with the aforementioned species further reinforced the distinctiveness of the clinical isolates. Although there is no precise correlation between percent 16S rRNA sequence divergence values and species delineation, it is now universally recognized that organisms displaying values close to 3% or more do not belong to the same species (16). The observed >3% sequence divergence between the unidentified clinical isolates and all presently defined *Actinomyces* species is therefore consistent with separate species status. The separateness of the unknown bacterium is also supported by phenotypic evidence. Protein profiling showed that the clinical isolates were phenotypically closely related to each other and were quite distinct from other species within the *A. turicensis*-*A. radingae* rRNA subcluster. The biochemical profile of the novel bacterium also readily distinguishes it from all presently described *Actinomyces* species. Therefore, on the basis of the distinct phenotypic characteristics of the unidentified rod-shaped bacterium and mo-

lecular chemical and molecular genetic evidence, we think the clinical isolates warrant classification as a new species of the *Actinomyces* genus, for which the name *Actinomyces cardiffensis* sp. nov. is proposed. Tests that are useful in distinguishing *Actinomyces cardiffensis* from its closest relatives are shown in Table 1. We believe that the formal description of this new species, together with biochemical tests for its identification, will facilitate its recognition in the clinical laboratory, thereby permitting a future evaluation of its distribution, clinical prevalence, and significance.

Description of *Actinomyces cardiffensis* sp. nov. *Actinomyces cardiffensis* (car.dif.fen'sis, L. adj. *cardiffensis*, pertaining to Cardiff, a city in Wales) cells are pleomorphic, slender, straight-to-curved rods; beaded branching filaments occur. Cells stain gram positive, are non-acid-fast and nonmotile. On Fastidious Anaerobic Agar with 5% horse blood after 48 h of incubation, colonies are pinpoint, convex, smooth surfaced, entire-edged, and opaque cream to pinkish. Nonhemolytic. Facultatively anaerobic and catalase negative. Using conventional testing, acid is formed from glucose and sucrose but not

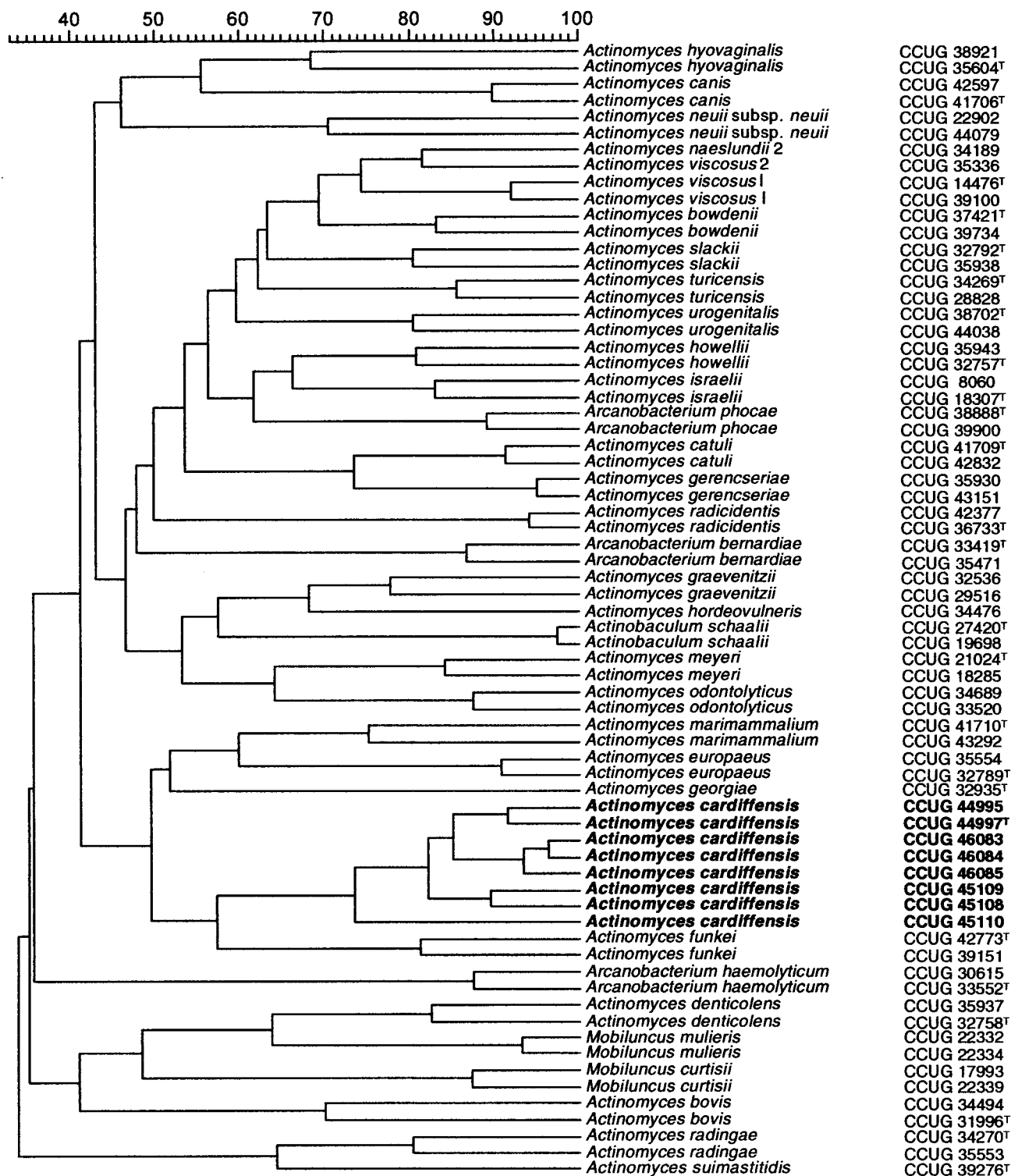


FIG. 2. Similarity dendrogram based on whole-cell protein patterns of *Actinomyces cardiffensis* sp. nov. (boldface) and its nearest relatives. Levels of correlation are expressed as percentages of similarity for convenience.

from amygdalin, L-arabinose, cellobiose, lactose, mannitol, mannose, D-raffinose, salicin, trehalose, or D-xylose. Acid production from D-ribose variable. Principal end products of glucose metabolism are lactic and succinic acids, together with

small amounts of acetic acid. Hydrolysis of esculin and starch is negative. Lipase and lecithinase are not produced. Using API systems, acid is produced from D-glucose but not from L-arabinose, D-arabitol, cyclodextrin, glycogen, lactose, manni-

TABLE 1. Results of tests used to distinguish *Actinomyces cardiffensis* sp. nov. from its nearest phylogenetic relatives^a

Test ^b	Result for species no. ^c :									
	1	2	3	4	5	6	7	8	9	10
Acid from:										
Lactose	-	+	-	+	-	+	+	V	-	-
Maltose	-	+	V	+	+	+	+	V	V	V
Melibiose	-	-	-	-	-	-	-	+	-	+
Pullulan	-	+	-	+	-	-	V	-	-	+
Ribose	-	+	V	+	+	-	-	V	V	+
Trehalose	-	-	-	+	-	-	-	V	-	-
Hydrolysis of hippurate	-	-	+	-	-	-	-	-	+	-
Production of:										
CAT	-	+	-	-	-	-	-	-	-	-
α-Gal	-	+	-	-	+	-	-	+	-	+
β-Gal	-	+	V	+	+	+	+	+	-	+
PAL	-	-	+	-	+	-	-	-	-	V

^a Tests performed using API rapid ID 32S system.
^b CAT, catalase; α-Gal, α-galactosidase; β-Gal, β-galactosidase; PAL, alkaline phosphatase.
^c Species are numbered as follows: 1, *A. cardiffensis*; 2, *A. canis*; 3, *A. funkei*; 4, *A. georgiae*; 5, *A. hyovaginalis*; 6, *A. meyeri*; 7, *A. odontolyticus*; 8, *A. radingae*; 9, *A. turicensis*; 10, *A. suimastitidis*. Symbols: +, positive; -, negative; V, variable.

tol, melibiose, melezitose, methyl-β-D-glucopyranoside, N-acetyl-β-glucosamine, pullulan, sorbitol, tagatose, trehalose, or D-xylose. Acid may or may not be formed from maltose, mannose, D-raffinose, D-ribose, and sucrose. Gelatin, esculin, and hippurate are not hydrolyzed. Alanine phenylalanine proline arylamidase, alanine arylamidase, arginine arylamidase, α-glucosidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucylglycine arylamidase, proline arylamidase, phenylalanine arylamidase, serine arylamidase, and tyrosine arylamidase are detected. No activity is detected for α-arabinosidase, arginine dihydrolase, alkaline phosphatase, α-fucosidase, α-galactosidase, β-galactosidase, β-galactosidase 6-phosphate, β-glucosidase, β-glucuronidase, glycyL tryptophan arylamidase, glutamic acid decarboxylase, glutamylglutamic acid arylamidase, N-acetyl-β-glucosaminidase, pyrazinamidase, pyroglutamic acid arylamidase, β-mannosidase, pyrrolydonyl arylamidase, or urease. Acetoin is not produced. Nitrate may or may not be reduced. Indole negative. The type strain is CCUG 44997^T = CIP 107323^T. Isolated from human clinical sources, including pleural fluid, brain, jaw, pericolic and ear abscesses, antrum, and IUCDs. Habitat is not known.

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