

Characterization of *Actinomyces* Isolates from Infected Root Canals of Teeth: Description of *Actinomyces radidentis* sp. nov.

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Two strains of a previously undescribed *Actinomyces*-like bacterium were recovered in pure culture from infected root canals of teeth. Analysis by biochemical testing and polyacrylamide gel electrophoresis of whole-cell proteins indicated that the strains closely resembled each other phenotypically but were distinct from previously described *Actinomyces* and *Arcanobacterium* species. Comparative 16S rRNA gene-sequencing studies showed the bacterium to be a hitherto unknown subline within a group of *Actinomyces* species which includes *Actinomyces bovis*, the type species of the genus. Based on phylogenetic and phenotypic evidence, we propose that the unknown bacterium isolated from human clinical specimens be classified as *Actinomyces radidentis* sp. nov. The type strain of *Actinomyces radidentis* is CCUG 36733.

The genus *Actinomyces* embraces a heterogeneous group of anaerobic and facultatively anaerobic, asporogenous, gram-positive, non-acid-fast, rod-shaped organisms (10) many of which occur as inhabitants of mucosal surfaces, particularly the oral cavity, of humans and some other homeothermic animals. In addition, some *Actinomyces* species are long-established pathogens of humans and animals, and in recent years several new *Actinomyces* species and related taxa (e.g., *Arcanobacterium* and *Actinobaculum*) associated with human disease have been described (3, 4, 5, 7, 8, 13). At present, the identification of clinical isolates of *Actinomyces* and close relatives to the species level using conventional phenotypic methods can be very difficult, which has hampered knowledge of their relationships, natural habitats, prevalence, and pathogenicity. Much of this problem stems from poor test reproducibility, lack of discriminatory power of tests, and possible heterogeneity within some taxa. In recent years the implementation of molecular genetic techniques, in particular 16S rRNA gene sequencing, in concert with molecular chemical methods (e.g., whole-cell protein profiling) of analysis has resulted in much improved classification and species identification (3, 7, 8). As a result of these investigations, a plethora of new *Actinomyces* and related species have been described from human and animal sources. Despite this increase in the number of new *Actinomyces* species from humans, there is evidence that many isolates from clinical material do not correspond to described species and that the use of these more precise molecular diagnostic methodologies will inevitably result in the recognition of even more new taxa (6). In this article, we report the results of a polyphasic taxonomic study on two strains of an unusual *Actinomyces* species isolated in pure culture from infected root canals of teeth. Based on the presented findings, another new species of the genus *Actinomyces*, *Actinomyces radidentis*, is described.

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MATERIALS AND METHODS

Cultures and phenotypic characterization. Strains CCUG 36733^T and CCUG 42377 were isolated from infected root canals of human teeth. Strain CCUG 36733^T was isolated in 1996, from the upper right canine of an 80-year-old Swedish woman. The tooth had been treated by a dentist on earlier repeated occasions and finally had been root filled. Due to persistent symptoms, the patient was referred to a specialist in endodontics who removed the root filling and took a bacteriological sample from which strain CCUG 36733^T was the only organism recovered. The second strain, CCUG 42377, was isolated in 1999, from two upper incisors of a 25-year-old Swedish man. The teeth were treated by a dentist, but, due to persistent symptoms, permanent root filling was avoided during a 7-year period. The patient was finally referred to a specialist in endodontics, who filled the teeth. Three years later, a periapical abscess developed in this region. Penicillin was administered, and the root fillings were removed under aseptic conditions. Bacteriological analysis of samples from the two teeth revealed the growth of strain CCUG 42377 in pure culture. The unidentified isolates were cultured on Columbia agar (Difco, Detroit, Mich.) supplemented with 5% horse blood at 37°C in air. Forty-eight hours was needed for the development of colonies suitable for biochemical testing. The strains were characterized by using the API rapid ID32Strep, API Zym, and API Coryne systems according to the manufacturer's instructions (API bioMérieux, Marcy l'Etoile, France). Polyacrylamide gel electrophoretic analysis of whole-cell proteins was performed as described by Pot et al. (9). For the protein profiling, young cultures produced under optimal conditions were used. 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 a percentage similarity. Generated profiles were compared with a comprehensive database maintained by the Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden.

16S rRNA gene sequencing and phylogenetic analyses. The 16S rRNA genes of the two 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 (1). The resulting multiple-sequence alignment was corrected manually, and a distance matrix was calculated using the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (2). A phylogenetic tree was constructed by the neighbor-joining method with the program NEIGHBOR (2). The stability of the groupings was estimated by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE (2). Parsimony analysis was also performed using the same package (2).

Nucleotide sequence accession number. The 16S rRNA gene sequence of strain CCUG 36733^T has been deposited in GenBank under accession number AJ251986.

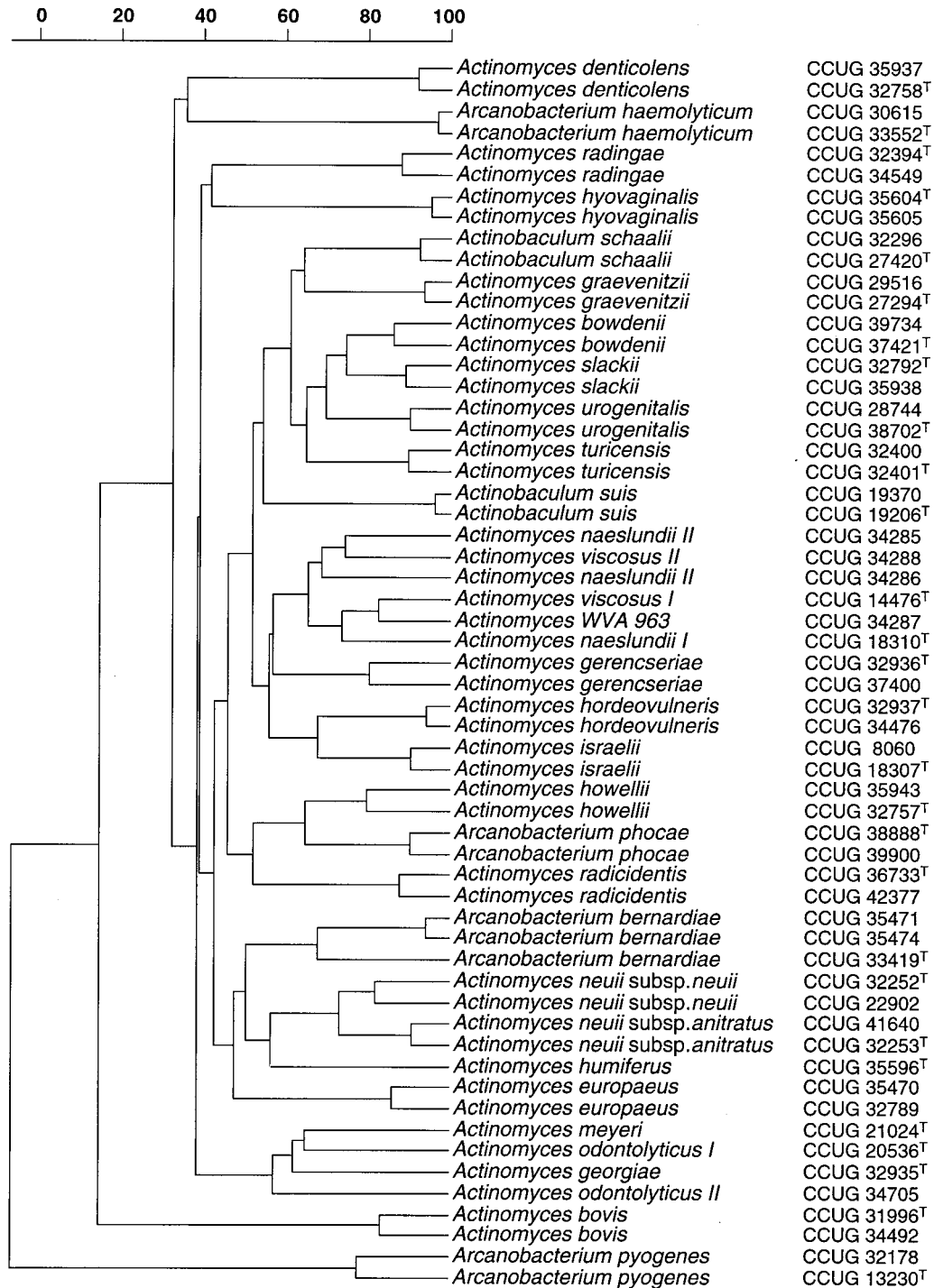


FIG. 1. Similarity dendrogram based on whole-cell protein patterns of *A. radidentis* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

RESULTS AND DISCUSSION

The two isolates consisted of gram-positive coccoid-shaped cells which were non-acid fast and non-spore forming. The strains were catalase positive and grew on Columbia blood agar under aerobic and anaerobic conditions. Using the commercial biochemical kits, both strains were unidentified. Phenotypically they closely resembled each other, producing acid from glucose, maltose, mannitol, melibiose, melezitose, lactose,

D-raffinose, ribose, sucrose, and trehalose. Neither of the isolates produced acid from D-arabitol, L-arabinose, cyclodextrin, glycogen, pullulan, sorbitol, or tagatose. Alanine phenylalanine proline arylamidase, acid phosphatase, α -glucosidase, β -glucosidase, α -galactosidase, β -galactosidase, β -galacturonidase, leucine arylamidase, and pyrazinamidase were produced by the isolates, but tests for arginine dihydrolase, alkaline phosphatase, α -fucosidase, β -glucuronidase, glycol tryptophane arylami-

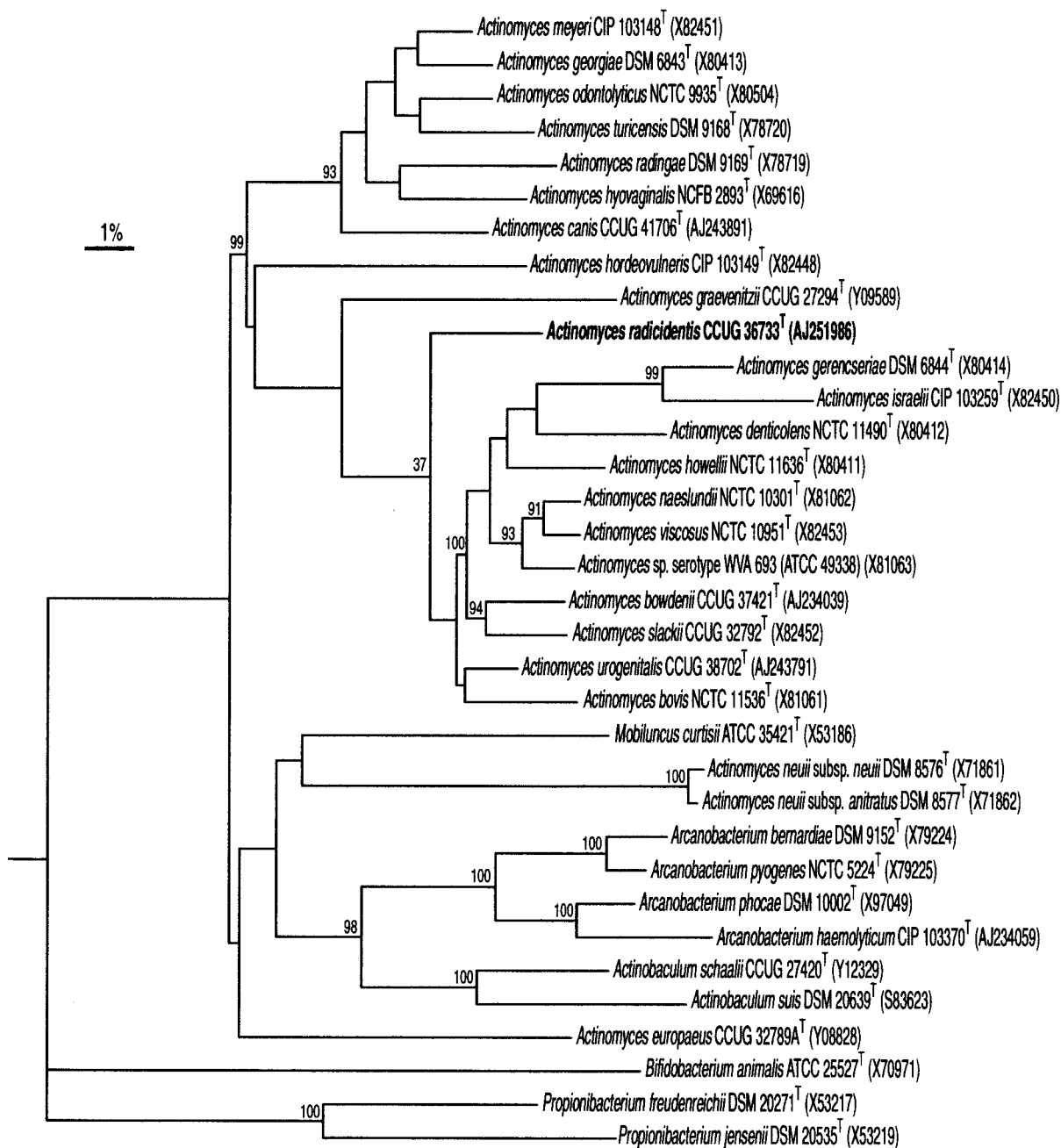


FIG. 2. Unrooted tree showing the phylogenetic relationships of *A. radidentis* sp. nov and some other high-G+C content gram-positive bacteria. The tree constructed using the neighbor-joining method was based on a comparison of approximately 1,327 nucleotides. Bootstrap values, expressed as percentages of 500 replications, are given at branching points. Bar, 1% sequence divergence.

dase, lipase C₁₄, α-mannosidase, β-mannosidase, N-acetyl-β-glucosaminidase, chymotrypsin, and trypsin were negative. Variable reactions were observed for urease production and nitrate reduction. The biochemical reactions of the isolates were consistent with their assignment to the genus *Actinomyces*. To assess the phenotypic resemblance of the two isolates to each other and to reference *Actinomyces* species, a comparative analysis of whole-cell protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) was performed. The two isolates clustered together and formed a distinct group with a within-group correlation level of 85%. The nearest taxa

to the unknown isolates on the protein-profiling analysis corresponded to *Actinomyces howellii* and *Arcanobacterium phocae*, although this association was loose, joining the aforementioned cluster at a correlation level of approximately 50% (Fig. 1). The PAGE results therefore confirmed that the two unidentified strains represent a phenotypically homogeneous group of organisms and that they are distinct from all *Actinomyces* species and close relatives described to date. To ascertain the phylogenetic relationships of the clinical isolates, their 16S rRNA genes were sequenced and subjected to a comparative analysis. The almost complete gene sequences (>1,400

nucleotides) of the two strains were determined, and pairwise analysis showed these to be almost identical (99.8% sequence similarity). Sequence database searches confirmed that the unknown bacterium was most closely related to species of the genus *Actinomyces* (results not shown). Highest sequence relatedness was shown with *Actinomyces bovis* (95.6% sequence similarity), *Actinomyces bowdenii* (95%), *Actinomyces naeslundii* (95%), *Actinomyces viscosus* (95.2%), and *Actinomyces slackii* (96.8%). The results of neighbor-joining analysis are shown in Fig. 2 and confirmed the association of the unknown clinical bacterium (as exemplified by strain CCUG 36733^T) with *A. bovis* and related species, with the unknown organism branching at the periphery of the *A. bovis* rRNA cluster.

It is now recognized that the genus *Actinomyces* is not monophyletic and consists of several rRNA lineages worthy of separate generic status (7, 8). It is evident from the present 16S rRNA study that the novel bacterium reported here forms a distinct subline branching proximal to the base of a cluster of species which includes the type species *A. bovis*; therefore, it can be regarded as an authentic *Actinomyces* species. Sequence divergence values of 3.2% to 9.0% with other members of the *A. bovis* cluster are clearly indicative of a new species. Although there is no precise correlation between percentage 16S rRNA divergence values and species delineation, it is recognized that organisms that differ by $\geq 3\%$ do not belong to the same species (11). The observed $>3\%$ sequence divergence between the unknown clinical isolates and currently described *Actinomyces* species is therefore consistent with separate species status. Support for the separateness of the unknown bacterium from infected teeth also comes from phenotypic evidence. The unknown bacterium can be biochemically readily distinguished from all other described catalase-positive or catalase-variable *Actinomyces* species (Table 1). Similarly the two clinical isolates formed a very distinct cluster upon PAGE analysis of whole-cell proteins, which was far removed from all members of the *Actinomyces* species group (Fig. 1). It is now firmly established that this molecular chemical approach is extremely reliable for comparing closely related strains and shows excellent correlation with DNA-DNA pairing (12). Furthermore, PAGE protein profiling has been used with great success in *Actinomyces* systematics (see, e.g., references 3 and 7). The presented PAGE protein profiling results unequivocally demonstrate that the two unknown isolates represent a separate species from all described *Actinomyces* species. Therefore, based on the distinct phenotypic characteristics of the unknown bacterium and the use of molecular chemical and molecular genetic evidence in concert, we conclude that the two clinical isolates merit classification as a new species of the genus *Actinomyces*, for which we propose the name *A. radidentis*. It seems likely that *A. radidentis* may be part of the normal indigenous oral microflora of humans. The recovery of this bacterium in pure culture from infected root canals of teeth indicates that it is possibly a hitherto unrecognized facultative pathogen. We consider that the formal description of this new species will facilitate its identification in the clinical laboratory, thereby permitting a future evaluation of its distribution and clinical significance.

Description of *A. radidentis* sp. nov. *Actinomyces radidentis* (L. n. *radix* root, L. gen. masc. n. *dentis* of the tooth, L. gen. masc. n. *radidentis* of the root of the tooth) cells are coccoid, stain gram positive, and are non-acid fast and nonmotile, facultatively anaerobic, and catalase positive. Using API systems, acid is produced from D-glucose, maltose, mannitol, melibiose, melezitose, methyl- β -D-glucopyranoside, lactose, D-raffinose, ribose, sucrose, and trehalose. Acid is not produced from D-arabitol, L-arabinose, cyclodextrin, glycogen, pullulan, sorbitol,

TABLE 1. Characteristics^a useful in differentiating *A. radidentis* from other catalase-positive or catalase-variable *Actinomyces* species^b

Species	Hydrolysis of:										Fermentation of:										Production of:			
	Catalase	Nitrate reduction	Aesculin	Gelatin	Urea	D-Ara	L-Ara	Glu	Man	Suc	D-Xyl	Mel	Gly	Pull	Rib	Tre	PAL	PYZ	β -GLU	β -NAG				
<i>Actinomyces radidentis</i>	+	V	+	W	V	-	-	+	+	+	+	-	-	-	+	-	+	+	+	-				
<i>Actinomyces bowdenii</i>	+	+	+	-	-	-	+	+	V	+	+	+	+	+	+	V (-)	V	+	+	-				
<i>Actinomyces canis</i>	+	+	+	-	-	-	+	+	V	+	+	+	+	+	+	-	+	+	+	+				
<i>Actinomyces hordovulneris</i>	+	+	+	W	-	-	+	+	+	+	+	+	+	+	+	-	V	+	+	+				
<i>Actinomyces howellii</i>	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
<i>Actinomyces naeslundii</i> (serotype I)	V (-)	+	V (+)	V (-)	W	-	V (+)	V (-)	V (+)	V (-)	V (-)	-	-	-	+	+	+	+	+	-				
<i>Actinomyces naeslundii</i> (serotype II)	V (-)	+	V	-	W	-	+	+	+	V (+)	+	-	-	-	+	+	+	+	+	-				
<i>Actinomyces neuii</i> subsp. <i>anitratu</i> s	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-				
<i>Actinomyces neuii</i> subsp. <i>neui</i>	+	+	-	-	-	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-				
<i>Actinomyces slackii</i>	+	+	+	V	V (-)	-	+	+	V	+	+	-	-	-	+	+	+	+	+	-				
<i>Actinomyces viscosus</i> (serotype I)	+	+	+	-	V	-	+	+	+	+	+	-	-	-	+	+	+	+	+	-				
<i>Actinomyces viscosus</i> (serotype II)	+	+	+	-	V	-	+	+	+	+	+	-	-	-	+	+	+	+	+	-				
<i>Arcanobacterium phocae</i>	V (+)	-	-	-	-	-	+	V (+)	+	V (-)	V (+)	+	+	+	+	+	+	+	+	-				

^a Tests performed using commercial API systems, as described in the text. +, positive; -, negative; W, weak reaction; V, variable; V (+), most strains positive; V (-), most strains negative.
^b D-Ara, D-arabitol; L-Ara, L-arabinose; Glu, glucose; Man, mannitol; Mel, melezitose; Suc, sucrose; D-Xyl, D-xylose; Gly, glycogen; Pull, pullulan; Rib, ribose; Tre, trehalose.
^c PAL, alkaline phosphatase; PYZ, pyrazinamidase; β -GLU, β -glucosidase; β -NAG, β -N-acetylglucosaminidase.

or tagatose. Esculin and gelatin (weak) are hydrolyzed, but hippurate is not. Alanine phenylalanine proline arylamidase, acid phosphatase, α -galactosidase, β -galactosidase, β -galacturonidase, α -glucosidase, β -glucosidase, leucine arylamidase, and pyrazinamidase are produced. Arginine dihydrolase, alkaline phosphatase, cystine arylamidase, α -fucosidase, ester lipase C₈, β -glucuronidase, glycyl tryptophane arylamidase, α -mannosidase, β -mannosidase, *N*-acetyl- β -glucosaminidase, valine arylamidase, chymotrypsin, trypsin, and pyroglutamic acid arylamidase are not produced. Activities for urease are variable. Acetoin production is weakly positive. Nitrate reduction is variable. Organisms have been isolated from infected root canals of teeth. Habitat is not known. The type strain is CCUG 36733 (= CIP 106352^T).

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