

# Phenotypic and Phylogenetic Characterization of a New *Corynebacterium* Species from Dogs: Description of *Corynebacterium auriscanis* sp. nov.

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**Six strains of a previously undescribed catalase-positive coryneform bacterium isolated from clinical specimens from dogs were characterized by phenotypic and molecular genetic methods. Biochemical and chemotaxonomic studies revealed that the unknown bacterium belonged to the genus *Corynebacterium* sensu stricto. Comparative 16S rRNA gene sequencing showed that the six strains were genealogically highly related and constitute a new subline within the genus *Corynebacterium*; this subline is close to but distinct from *C. falsenii*, *C. jeikeium*, and *C. urealyticum*. The unknown bacterium from dogs was distinguished from all currently validated *Corynebacterium* species by phenotypic tests including electrophoretic analysis of whole-cell proteins. On the basis of phylogenetic and phenotypic evidence, it is proposed that the unknown bacterium be classified as a new species, *Corynebacterium auriscanis*. The type strain of *C. auriscanis* is CCUG 39938<sup>T</sup>.**

The genus *Corynebacterium* contains many species which have long been recognized as pathogens of humans and/or animals. During the past two decades, the taxonomy of this important group of organisms has undergone dramatic change. In particular, the use of molecular chemical and molecular genetic methodologies has facilitated a much tighter circumscription of the genus, and the availability of comparative 16S rRNA gene sequence data combined with improved phenotypic data has resulted in much improved and more reliable species identification. These improvements in taxonomy and diagnostics, together with an increased interest in corynebacteria as opportunistic pathogens of humans, has resulted in the delineation of a plethora of new *Corynebacterium* species from human sources in recent years (see reference 14 for a review). Indeed, since 1990, over 20 new species that cause and/or that are associated with human disease have been described (see reference 14 for species identified prior to 1996 and previous reports for *C. confusum* [12], *C. coyleae* [13], *C. durum* [20], *C. falsenii* [24], *C. imitans* [8], *C. kroppenstedtii* [4], *C. lipophiloflavum* [9], *C. mucifaciens* [10], *C. singulare* [21], *C. sundsvallense* [3], *C. riegelii* [11], *C. thomssenii* [25]). The association of corynebacteria with animal disease has received much less attention, although there are indications (6, 7) that the implementation of improved diagnostic methods will result in a better understanding of the range of corynebacterial species involved in animal disease and in the recognition of new diversity. In this article we report on the polyphasic characterization (by use of phenotypic and molecular genetic techniques in concert) of six coryneform-like isolates that originated from clinical specimens from dogs. On the basis of the findings of this study, a new species, *Corynebacterium auriscanis*, is described.

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## MATERIALS AND METHODS

**Cultures and cultivation.** Three of the six strains examined were isolated from specimens that originated from dogs with ear infections: strain M598/96/1 was recovered in mixed culture with *Staphylococcus intermedius* and *Streptococcus canis* from a dog suffering from bilateral otitis; strain M135/96/2 was obtained along with a *Staphylococcus* sp. and a *Proteus* sp. from a dog suffering from chronic otitis externa with a purulent discharge; strain M1426/97/3 was also recovered in mixed culture (with *Malassezia pachydermatis*, *Streptococcus canis*, and *Staphylococcus intermedius*) from the ear of a dog suffering from chronic pustular otitis externa. Strain M2813/97/1 was the sole isolate obtained from a dog with a deep pyoderma, whereas strain M210/98/3 was recovered from a polymicrobial infection of a dog with an interdigital cyst. Isolate M2555/95/2 was obtained from a vaginal swab together with *Streptococcus canis* and *Staphylococcus intermedius*. Strains M598/96/1, M135/96/2, M1426/97/3, M2813/97/1, and M210/98/3 have been deposited in the Culture Collection of the University of Göteborg (CCUG) under accession nos. CCUG 39938<sup>T</sup>, CCUG 39940, CCUG 39941, CCUG 39784, and CCUG 39783, respectively. All strains were cultured on Columbia agar (Difco, Detroit, Mich.) supplemented with 5% defibrinated horse blood (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) at 37°C in air plus 5% CO<sub>2</sub>.

**Phenotypic characterization.** The strains were biochemically characterized by using the API Coryne system (API bioMérieux, Marcy l'Etoile, France). Enzyme reactions were read after 24 h of incubation at 37°C, whereas acid production from carbohydrates was observed after 48 h. Further enzyme reactions were studied by means of the API ZYM system. Polyacrylamide gel electrophoretic (PAGE) analysis of whole-cell proteins was performed as described by Pot et al. (19). For densitometric analysis and normalization and interpretation of protein patterns, the GelCompar 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 percent similarity value. Cell wall murein was prepared by mechanical disruption of cells with a Braun homogenizer, and complete acid hydrolysates (4 M HCl) were analyzed as described by Schleifer and Kandler (23). Fatty acid methyl esters were prepared and analyzed as described by Kampfer and Kropfenstedt (16). Mycolic acid composition was determined by the methods of Minnikin et al. (18) and Klatt et al. (17). The G+C content of DNA was determined by thermal denaturation (15).

**Phylogenetic characterization.** Phylogenetic analysis was conducted by 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene was amplified by PCR with universal primers pA and pH\* (2), close to the 3' and 5' ends of the gene, respectively, and was directly sequenced with 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 (RDP) Libraries and

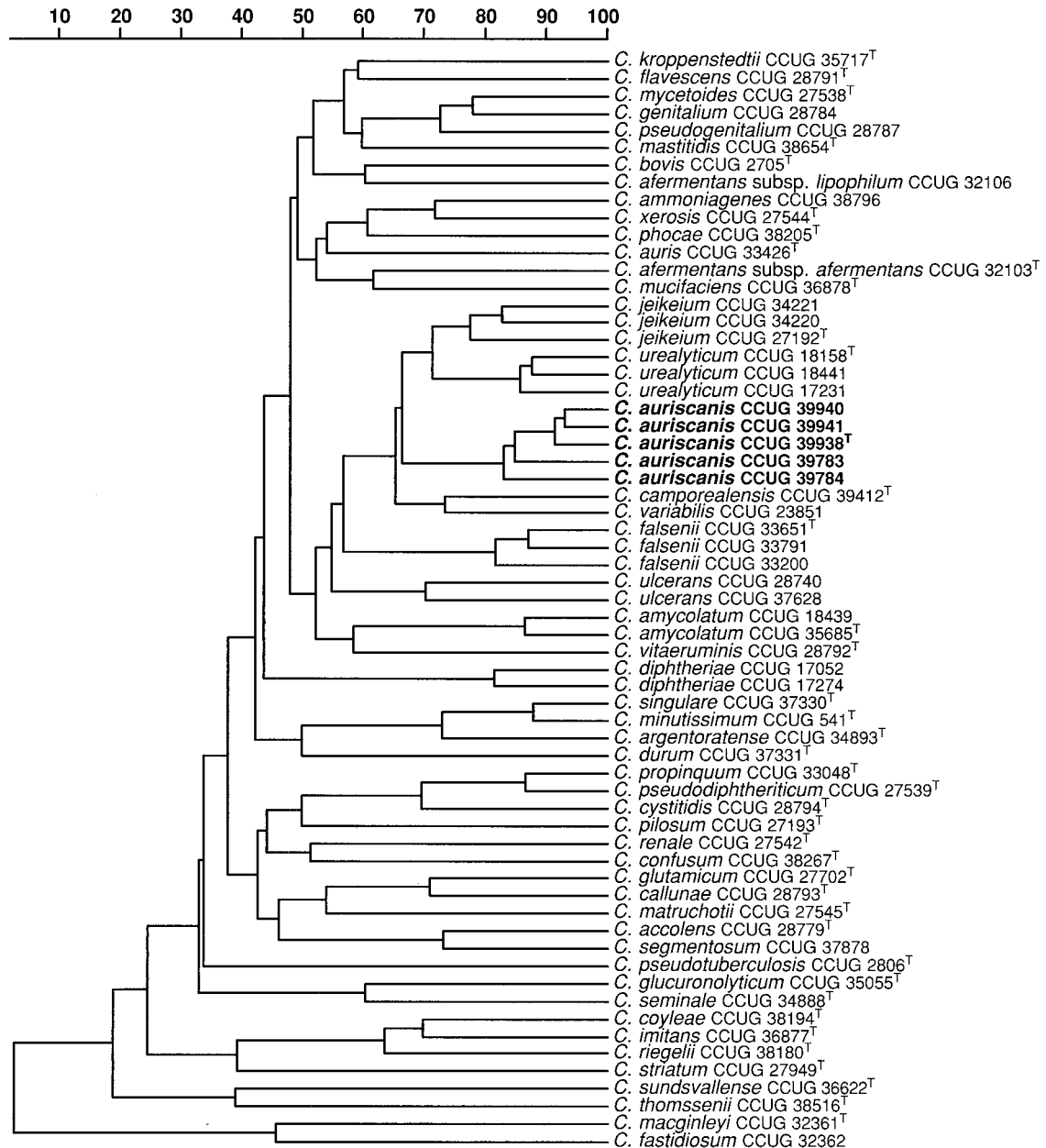


FIG. 1. Similarity dendrogram based on whole-cell protein pattern of *C. auriscanis* sp. nov. and related species. Levels of correlation are expressed as percentages of similarity for convenience.

were aligned with the newly determined sequence by using the program PILEUP. The resulting multiple sequence alignment was corrected manually, and a distance matrix was calculated by using the programs PRETTY and DNADIST (with the Kimura-2 correction parameter) (5). A phylogenetic tree was constructed by the neighbor-joining method (22) with the program NEIGHBOR (5), and the stability of the groupings was estimated by bootstrap analysis (500 replications) with the programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE (5).

**Nucleotide sequence accession number.** The 16S rRNA gene sequence of strain CCUG 39938<sup>T</sup> has been deposited in GenBank under accession no. AJ23851.

## RESULTS AND DISCUSSION

The six isolates obtained from clinical specimens from dogs consisted of gram-positive, nonmotile, short, club-shaped rods

which occurred as single cells, in pairs, or in small clusters. The strains were catalase positive and did not require lipid supplementation for optimal growth. The isolates produced acid from glucose but not from the other carbohydrates examined. They hydrolyzed hippurate but not gelatin, starch, or urea. Esculin hydrolysis was variable. The strains displayed acid phosphatase, alkaline phosphatase, ester lipase C8 (weak reaction), esterase C4 (weak reaction), leucine arylamidase, phosphoamidase, and pyrrolidonyl arylamidase activity. They were pyrazinamidase negative and did not reduce nitrate to nitrite. On the basis of their cellular morphologies and biochemical profiling, the isolates from dogs resembled corynebacteria, although their reactions did not conform to those for any currently recognized species. To ascertain whether or not the unidenti-

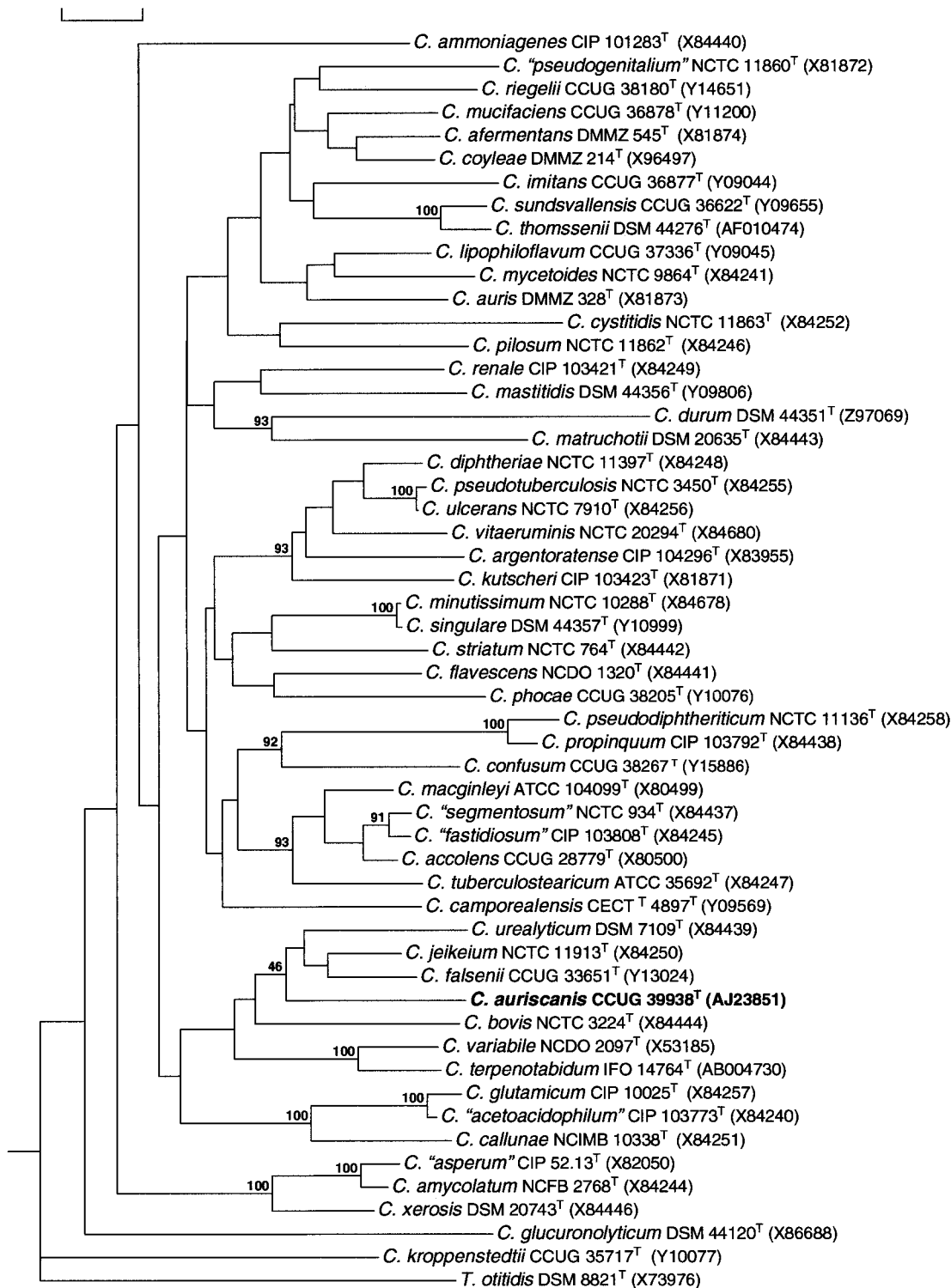


FIG. 2. Unrooted tree showing the phylogenetic relationships of *C. auriscanis* sp. nov. and members of the genus *Corynebacterium*. The tree was constructed by the neighbor-joining method and was based on a comparison of approximately 1,320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Bar, 1% sequence divergence.

fied bacterium was a member of the genus *Corynebacterium*, the cell wall murein structure and lipid composition of a representative strain, strain CCUG 39938<sup>T</sup>, were determined. Analysis of the cell wall amino acid composition revealed

*meso*-diaminopimelic acid (*meso*-Dpm) as the dibasic acid, together with alanine and glutamic acid, consistent with a type A1  $\gamma$  directly cross-linked murein. Thin-layer chromatographic analysis of whole-organism acid methanolsates demonstrated

TABLE 1. Characteristics useful in differentiating *C. auriscanis* from some other nonlipophilic *Corynebacterium* species<sup>a</sup>

Organism	Nitrate reduction	Urease hydrolysis	Aesculin hydrolysis	Pyrazinamidase reaction	Alkaline phosphatase	Acid produced from:			CAMP reaction	Mycolic acid production
						Glucose	Maltose	Sucrose		
<i>C. auriscanis</i>	–	–	V	–	+	+	–	–	–	+
<i>C. confusum</i>	+	–	–	+	+	(+)	–	–	–	+
<i>C. falsenii</i>	–	(+)	–	W	+	(+)	(V)	–	–	+
<i>C. amycolatum</i>	V	V	–	+	+	+	V	V	–	–
<i>C. argentoratense</i>	–	–	–	+	V	+	–	–	–	+
<i>C. coyleae</i>	–	–	–	+	+	(+)	–	–	+	+
<i>C. diphtheriae</i>	V	–	–	–	–	+	+	–	–	+
<i>C. durum</i>	+	(V)	(V)	–	–	+	+	+	–	+
<i>C. glucuronolyticum</i>	V	V	V	+	V	+	V	+	+	+
<i>C. imitans</i>	–	–	–	W	+	+	+	W	+	+
<i>C. matruchotii</i>	+	+	+	+	–	+	+	+	–	+
<i>C. minutissimum</i>	–	–	–	+	+	+	+	V	–	+
<i>C. pseudotuberculosis</i>	–	+	–	–	V	+	+	V	REV	+
<i>C. striatum</i>	+	–	–	+	+	+	–	V	V	+
<i>C. ulcerans</i>	–	+	–	–	+	+	+	–	REV	+
<i>C. xerosis</i>	V	–	–	+	+	+	+	+	–	+

<sup>a</sup> Data are from references 1, 5, 6, 11, 12 and 13 and the present study. Symbols and abbreviations: parentheses, delayed reaction; W, weakly positive; V, variable; REV, reverse.

the presence of short-chain mycolic acids within the bacterium. High-temperature gas-liquid chromatographic analysis of trimethylsilylated derivatives revealed C<sub>28</sub> to C<sub>34</sub> mycolic acids, with C<sub>32:0</sub> predominating. Analysis of nonhydroxylated cellular fatty acids showed the presence of solely straight-chain saturated and monounsaturated types, with C<sub>16:0</sub> (37%) and C<sub>18:1ω9c</sub> (51%) as the major acids. Tuberculostearic acid was not present. These chemical findings unequivocally demonstrate that strain CCUG 39938<sup>T</sup> is a member of the genus *Corynebacterium*. The whole-cell protein profiles of five strains (strains CCUG 39938<sup>T</sup>, CCUG 39940, CCUG 39941, CCUG 39783, and CCUG 39784) were examined by sodium dodecyl sulfate-PAGE and were compared with those of known *Corynebacterium* species. A dendrogram derived from a numerical analysis of protein patterns is shown in Fig. 1. The isolates were found to be phenotypically highly related and formed a robust cluster (grouping at the correlation level of greater than 80%) which was separate from all other corynebacteria examined. To determine the phylogenetic relationships of the unknown bacterium from dogs, the almost complete 16S rRNA gene sequence (1,452 bases) of a representative strain (strain CCUG 39938<sup>T</sup>) was determined. Sequence searches of the GenBank and RDP libraries revealed that the newly determined sequence was phylogenetically highly related to those of species of the genus *Corynebacterium* (data not shown), with *C. falsenii*, *C. jeikeium*, and *C. urealyticum* displaying the highest level of sequence relatedness (97, 96.5, and 96.2% sequence similarity, respectively). A tree, constructed by the neighbor-joining method, that incorporates the unknown bacterium (as exemplified by strain CCUG 39938<sup>T</sup>) and all currently described *Corynebacterium* species, is shown in Fig. 2. The unidentified bacterium formed a distinct subline that was close to albeit distinct from *C. falsenii*, *C. jeikeium*, and *C. urealyticum*. Partial 16S rRNA gene sequencing (approximately 800 bases) was performed with the other five clinical isolates, and they were found to be highly related to strain CCUG 39938<sup>T</sup> (99.9 to 100% sequence similarity).

It is evident from the comparative 16S rRNA sequence analysis that the six isolates from clinical specimens from dogs are members of a hitherto unknown *Corynebacterium* species. Phylogenetically, the bacterium is closely related to *C. falsenii*, *C. jeikeium*, and *C. urealyticum*, although 16S rRNA divergence

values of approximately 3% clearly demonstrate that it merits separate species status. PAGE analysis of whole-cell proteins and biochemical profiling also showed that the bacterium from dogs is distinct from the aforementioned species and all other reference corynebacteria examined. In particular, the unknown bacterium could be readily distinguished from its closest phylogenetic relatives, *C. falsenii*, *C. jeikeium*, and *C. urealyticum*, by its production of pyrrolidonyl arylamidase and its failure to produce pyrazinamidase. Additionally, the bacterium differs markedly from *C. jeikeium* and *C. urealyticum* in that it is nonlipophilic, and it can be further distinguished from *C. falsenii* and *C. urealyticum* in that it does not hydrolyze urea. Tests which are useful in differentiating the unknown bacterium from dogs from other nonlipophilic *Corynebacterium* species are shown in Table 1. It is pertinent to note that the novel bacterium from dogs can also be readily distinguished from other nonlipophilic coryneform genera, such as *Arthrobacter*, *Aureobacterium*, *Cellulomonas*, *Curtobacterium*, *Exiguobacterium*, and *Microbacterium*, in that it possesses mycolic acids, predominantly straight-chain and monounsaturated cellular fatty acids, and meso-Dpm in its cell wall. By contrast, the coryneform taxa mentioned above invariably lack mycolic acids, possess high levels of methyl branched-chain cellular fatty acids, and contain cell walls based on lysine and/or ornithine (14). Therefore, on the basis of the results of the polyphasic taxonomic investigation, we propose that the bacterium from dogs be classified as a new species, *Corynebacterium auriscanis*.

**Description of *Corynebacterium auriscanis* sp. nov.** *Corynebacterium auriscanis* (au.ris.canis. L. fem. n. auris ear; L. masc. n. canis dog; M.L. gen. n. *auriscanis* of the ear of the dog).

Cells are gram positive, non-spore forming, and nonmotile. They are typically club-shaped rods, which appear as single cells, in pairs, or in clusters. The cells are nonfermentative and grow under aerobic conditions but not under anaerobic conditions. The cells are CAMP reaction negative. The cells are catalase positive and nonlipophilic. Acid is produced from glucose but not from glycogen, lactose, maltose, mannitol, sucrose, ribose, or D-xylose. Hippurate is hydrolyzed. Esculin hydrolysis is variable. Gelatin and starch are not hydrolyzed. Nitrate is not reduced, and the Voges-Proskauer test is negative. Acid phosphatase, alkaline phosphatase, ester lipase C8 (weak reaction), esterase C4 (weak reaction), leucine arylami-

dase, phosphoamidase, and pyrrolidonyl arylamidase activities are detected. *N*-Acetylglucosaminidase, chymotrypsin, cystine arylamidase,  $\alpha$ -fucosidase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucuronidase,  $\alpha$ -mannosidase, pyrazinamidase, trypsin, urease, and valine arylamidase activities are not detected. Lipase C14 activity is variable. The cell wall contains *meso*-Dpm as the dibasic amino acid. Short-chain mycolic acids ( $C_{28}$  to  $C_{34}$ ) are present, with  $C_{30:0}$ ,  $C_{32:0}$ , and  $C_{34:0}$  as the main components. The long-chain fatty acids are of the saturated straight-chain and monounsaturated types, with  $C_{16:0}$  and  $C_{18:\omega 9c}$  predominating. Tuberculostearic acid is not present. The G+C content of DNA is 61 mol%. The organism was isolated from clinical specimens from dogs. Its habitat is not known. The type strain is CCUG 39938<sup>T</sup>.

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