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Characterization of Some *Actinomyces*-Like Isolates from Human Clinical Sources: Description of *Varibaculum cambriensis* gen. nov., sp. nov.

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Fifteen strains of an anaerobic, catalase-negative, gram-positive diphtheroid-shaped bacterium recovered from human sources were characterized by phenotypic and molecular chemical and molecular genetic methods. The unidentified bacterium showed some resemblance to *Actinomyces* species and related taxa, but biochemical testing, polyacrylamide gel electrophoresis analysis of whole-cell proteins, and amplified 16S ribosomal DNA restriction analysis indicated the strains were distinct from all currently named *Actinomyces* species and related taxa. Comparative 16S rRNA gene sequencing studies showed that the bacterium represents a hitherto-unknown phylogenetic line that is related to but distinct from *Actinomyces*, *Actinobaculum*, *Arcanobacterium*, and *Mobiluncus*. We propose, on the basis of phenotypic and phylogenetic evidence, that the unknown bacterium from human clinical specimens should be classified as a new genus and species, *Varibaculum cambriensis* gen. nov., sp. nov. The type strain of *Varibaculum cambriensis* sp. nov. is CCUG 44998^T = CIP 107344^T.

The gram-positive, non-acid-fast, asporogenous, rod-shaped organisms of the genus *Actinomyces* and related taxa (e.g., *Actinobaculum*, *Arcanobacterium*, and *Mobiluncus*) comprise a phylogenetically heterogeneous group of organisms within the high G+C content *Actinobacteria* (18, 19). In recent years, *Actinomyces* and related organisms have attracted the attention of clinical microbiologists, mainly because of a growing awareness of their presence in clinical specimens and because of their possible association with disease (7, 9). Many *Actinomyces* species and related organisms belong to the facultative anaerobic indigenous microflora of human and animal mucous membranes, and some species are known to cause classical actinomycosis and may also be found in polymicrobial infections, whereas others are significant in periodontal disease (18, 19). Knowledge of the interrelationships among *Actinomyces* and related organisms has improved greatly in the past decade, and the use of improved phenotypic and molecular diagnostic methods of analysis has not only resulted in more reliable species identification but has also facilitated the recognition of many new taxa. Recently described *Actinomyces* and related organisms from clinical specimens include *Actinomyces funkei* (12), *Actinomyces europaeus* (4), *Actinomyces graevenitzii* (15), *Actinomyces neuii* subspp. *anitratus* and *neuii* (6), *Actinomyces radingae* (22), *Actinomyces radicidentis* (2), *Actinomyces turicensis* (22), *Actinomyces urogenitalis* (14), *Actinobaculum schaalii* (11), and *Arcanobacterium bernardiae* (5) where they occur as contaminants and/or represent possible opportunistic human pathogens. Despite the plethora of new species defined from human sources in recent years, it is clear that information on the habitats, clinical prevalence, and pathogenic potential of many *Actinomyces* and related organisms is inadequate, and there are indications (8, 9) that much new diversity still remains to be discovered. During the course of an investigation into taxonomically problematic *Actinobacteria* from clinical specimens we characterized a novel organism that represents a hitherto-unknown genus related to, but distinct from, *Actinomyces* and its near relatives.

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MATERIALS AND METHODS

Cultures and phenotypic characterization. Fifteen strains were analyzed, ten of which originated in polymicrobial infections and were isolated in the United Kingdom. These 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 R5231 (CCUG [Culture Collection of the University of Göteborg] 44992) was isolated from a cerebral abscess of a 6-year-old girl in London 1993. The patient had a long history of ear and mastoid problems prior to emergency admission for surgical drainage of the abscess, from which "mixed anaerobes" were isolated. Initially, the patient responded well to therapy with benzylpenicillin, cefotaxime, and metronidazole, but she was readmitted with seizures some 3 weeks later. Pus drained from the reformed cerebral abscess yielded *Peptostreptococcus anaerobius*, *Peptoniphilus asaccharolyticus*, *Porphyromonas endodontalis*, *Prevotella corporis*, *Prevotella oralis*, *Propionibacterium* sp., *Bifidobacterium* sp., and strain R5231, originally reported as *Actinomyces meyeri* (1). Treatment with metronidazole and clindamycin resulted in a good recovery. Strain R12359 (CCUG 44998^T) was isolated from a postauricular abscess of a 27-year-old male (London, 1998), strain R13662 (CCUG 45111) was isolated from a large ischiorectal abscess of a 24-year-old female (Merthyr Tydfil, 1999), and strain R13224 was isolated from a submandibular abscess of a 20-year-old male (Farnborough, 1999). Three strains were isolated from breast abscesses: R3521 (CCUG 45113) from a female of unknown age (Nottingham, 1989); R5619, which was isolated with mixed obligate anaerobes from a 29-year-old male (London, 1993); and R16017, which was isolated with an unidentified diphtheroid from a 49-year-old female (Merthyr Tydfil, Wales, 2001). Strains R11462 (CCUG 45112) and R16101 were isolated from intrauterine contraceptive devices (IUCDs) from a

33-year-old (Farnborough, 1997) and a 42-year-old (Oxford, 2001), respectively, and strain R4898 was from a high vaginal swab of a female of unknown age with an IUCD in situ (London, 1992). The remaining five strains came from Scabdinavia: four from Sweden and one strain from Norway. The Scandinavian strains were submitted to the CCUG collection for identification. The sources of these were as follows: strains CCUG 20976 and CCUG 20977 were from an abscess of a 28-year-old female in Skövde in 1987, strain CCUG 37510 was from a case of hidradenitis in a 37-year-old male (Stockholm, 1997), strain CCUG 46939 was from a fistula of a 57-year-old female (Kalmar, 2002), and strain CCUG 34258 was from a cheek abscess (Tromsö, 1995). The unidentified isolates were cultured anaerobically on Columbia agar (Difco, Detroit, Mich.) supplemented with 5% horse blood at 37°C for 48 h. The strains were characterized by using conventional tests (16) and the commercially available API Rapid ID 32Strep, API Zym, and API Coryne systems according to the manufacturer's instructions (API bioMérieux, Marcy l'Étoile, France). Volatile and nonvolatile end products of glucose metabolism were examined by gas-liquid chromatography (10).

DNA base composition determination. For the determination of G+C content, DNA was isolated after disruption of cells by using a French pressure cell and purified by hydoxyapatite. The mol% G+C content was determined by high-pressure liquid chromatography after digestion of DNA with P1 nuclease and alkaline phosphatase as described by Mesbah et al. (13).

Amplified 16S rDNA restriction analysis. Amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) were performed by using *Hae*III and *Hpa*II as described previously (8, 9).

16S rRNA gene sequencing and phylogenetic analyses. The 16S rRNA genes of five isolates were amplified by PCR and directly sequenced by 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 GenBank/EMBL database searches. These sequences and those of other known related strains were retrieved from GenBank/EMBL and aligned with the newly determined sequences by using the program CLUSTALW (21). The resulting multiple sequence alignment was corrected manually and a distance matrix was calculated by using DNADIST (using the Kimura-2 correction parameter) (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 (1000 replications) by using the programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE (3).

Protein profiling. Polyacrylamide gel electrophoresis (PAGE) analysis of whole-cell proteins was performed as described by Pot et al. (17). For densitometric analysis, 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 converted for convenience to a percentage similarity.

GenBank accession number. The 16S rRNA gene sequence of strain CCUG 44998^T has been deposited in GenBank under accession number AJ491326.

RESULTS AND DISCUSSION

The isolates consisted of gram-positive, short, straight or curved diphtheroid-shaped rods, which were non-acid fast and non-spore-forming. After anaerobic incubation for 48 to 72 h on Columbia agar or Fastidious Anaerobe Agar (LabM, Bury, United Kingdom) with horse blood (5%), the colonies were pinpoint, convex, entire-edged, translucent white or gray, glistening, and nonhemolytic. Strains grew well or poorly in air plus 5% CO_2 and poorly or not at all in air. The strains were catalase negative and biochemically closely resembled each other. Thirteen strains were subjected to conventional biochemical testing, and all of these strains formed acid from D-glucose, sucrose, and D-ribose. Some strains also formed acid from fructose, mannitol, and xylose, but none produced acid from amygdalin, L-arabinose, cellobiose, lactose, mannose, D-raffinose, salicin, or trehalose. All of the strains were lecithinase and lipase negative, and none formed indole. None of the isolates hydolyzed esculin, gelatin, or starch, but all reduced nitrate to nitrite. With the API Zym system, all of the strains were α-glucosidase and leucine arylamidase positive; reactions for acid phosphatase, phosphoamidase, esterase C-4, and ester lipase C8 were either weakly positive or negative. All other tests in the API Zym system were negative. Using the API Coryne system, the isolates produced acid from D-glucose, maltose, and sucrose, and all were α-glucosidase positive. Some strains produced acid from D-ribose and D-xylose. None of the strains formed acid from glycogen, lactose, or mannitol, and activity for alkaline phosphatase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, pyrolidonyl arylamidase, pyrazinamidase, and urease was not detected. The numerical codes thus generated are listed in Table 1. In the manufacturer's database, these were identified as doubtful or unacceptable profiles for *Corynebacterium diphtheriae* var. *belfanti* or *Corynebacterium diphtheriae* var. *mitis*. However, the novel bacterium is clearly distinguished from these species by its negative catalase reaction, its ability to ferment sucrose, and its poor growth under aerobic conditions. Using the API Rapid ID 32Strep system, 7 of 10 strains formed acid from sucrose and 9 of 10 strains hydrolyzed hippurate. Some strains produced acid from maltose (4 of 10), D-ribose (3 of 10), and trehalose (2 of 10), and some showed activity for alanine phenylalanine proline arylamidase (6 of 10) and β-galactosidase (weak reactions, 2 of 10). All other tests in the API Rapid ID 32Strep system were negative. The numerical codes thus generated are listed in Table 1. In the manufacturer's database, all of the seven codes obtained were interpreted as good to excellent identifications of *Streptococcus acidominimus*. However, each strain of the novel bacterium produced one or more reactions that contradicted this identification, namely, negative alanine phenylalanine proline arylamidase, negative trehalose, positive ribose, and/or positive β-galactosidase reactions. Furthermore, cellular morphology clearly distinguishes the novel bacterium from *Streptococcus* spp. Commercial biochemical test systems such as the API Rapid ID 32Strep and Coryne kits have been used widely in the phenotypic differentiation of recently described novel bacteria, including *Actinomyces* spp. and related organisms (2, 4, 11, 12, 14, 15). These systems are very useful, standardized methods for the identification of many organisms from clinical sources. However, reliance upon the manufacturer's databases commonly leads to unacceptable or erroneous identifications due to the lack of regular updating of these databases. Clinical microbiologists should be aware of these shortcomings and should interpret results obtained in these systems with reference to the most recently published data and in conjunction with other findings such as cellular and colony morphology.

TABLE 1. Numerical codes for *Varibaculum cambriensis* obtained with the API Rapid ID 32Strep and Coryne test systems

Rapid ID32Strep code	Coryne code
00000010000	0010321
00000010100	0010721
00002010100	1010121
00102011100	1010321
00122011100	1410321
40002010000	1410721
40006010000	

The cellular morphology and biochemical reactions of the novel isolates somewhat resembled the genus *Actinomyces*, but

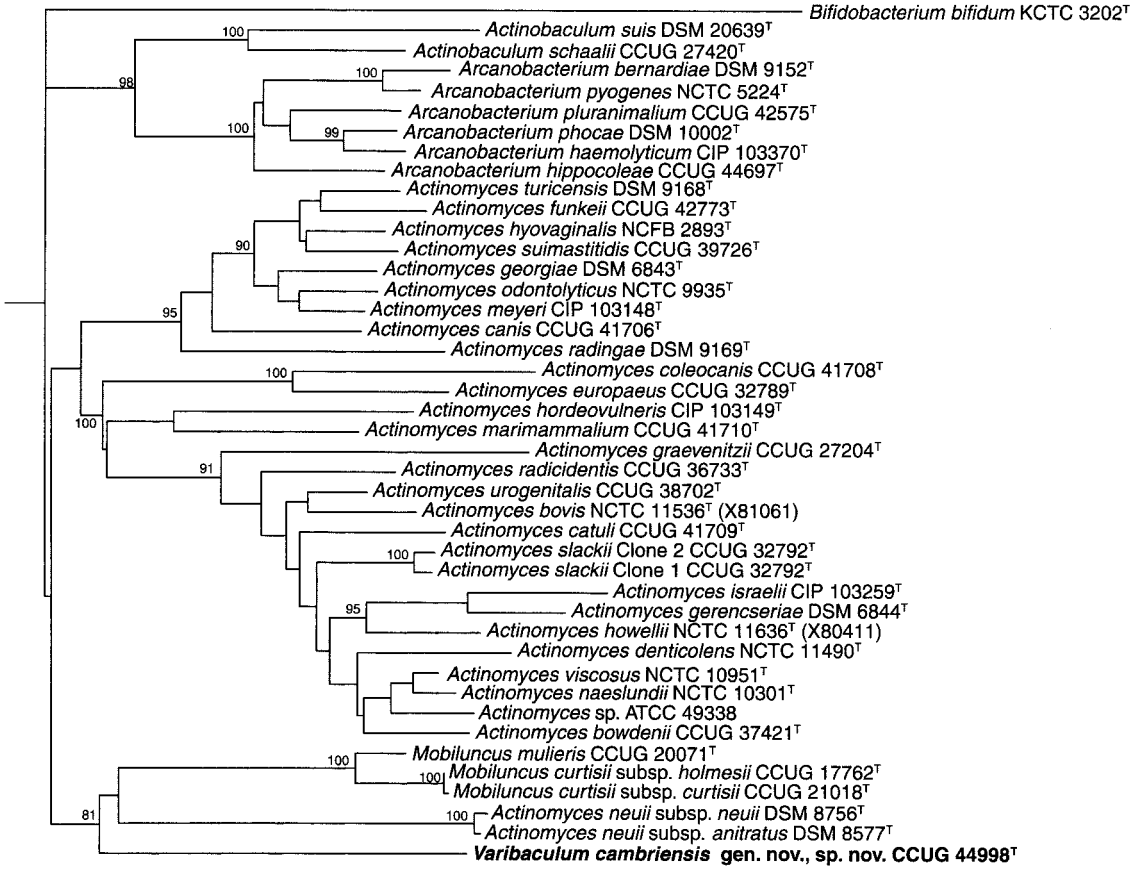

FIG. 1. Unrooted tree showing the phylogenetic relationships of *Varibaculum cambriensis* and its nearest relatives. The tree, constructed by using the neighbor-joining method, was based on a comparison of ca. 1,327 nucleotides. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching points.

the isolates did not appear to correspond to any recognized species of this genus or related taxa (e.g., *Arcanobacterium*, *Actinobaculum*, and *Mobiluncus*). To investigate the genetic relatedness of the isolates, ARDRA was performed. Two characteristic 16S rDNA restriction profiles, designated 035/023 and 034/014, were obtained that were distinct from those of recognized *Actinomyces*, *Arcanobacterium*, *Actinobaculum*, and *Mobiluncus* species. 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 strains were determined, and pairwise analysis showed these to be almost identical (99.1 to 100% sequence similarity). Sequence database searches showed that the unknown bacterium was most closely related to the high G+C gram-positive *Actinobacteria*, with highest sequence similarities shared with *Actinomyces*, *Arcanobacterium*, *Actinobaculum*, and *Mobiluncus* species. Treeing analysis confirmed the association of the unknown clinical bacterium (as exemplified by strain CCUG 44998^T) with the aforementioned taxa, with the unknown organism forming a relatively long and distinct line of descent among the *Actinomyces* and related taxa (Fig. 1). To assess the phenotypic homogeneity of the clinical isolates, PAGE of whole-cell proteins was performed, and their profiles were compared to each other and to reference *Actinomyces*, *Arcanobacterium*, *Actinobaculum*, and *Mobiluncus* species. The 15 isolates closely resembled each other, forming a distinct and robust group, which was distinct from all reference species examined (data not shown). A dendrogram from a subset of these analyses, showing the high affinity between the clinical isolates and their separateness from *Mobiluncus* species, is shown in Fig. 2.

It has become apparent from 16S rRNA gene sequencing that the genus *Actinomyces* is phylogenetically very heterogeneous and consists of several rRNA lines, some of which are equivalent in rank to other related genera such as *Arcanobacterium* and *Mobiluncus* (2, 14). It is evident from the present 16S rRNA analysis that the *Actinomyces*-like organisms from human clinical specimens represent a hitherto-unknown bacterium among the phylogenetic radiation of *Actinomyces* and related genera. Phylogenetically, the novel bacterium forms a long and deep line, which forms a loose association with *Actinomyces neuii* and species of the genus *Mobiluncus*. Bootstrap resampling, however, showed that the branching of the novel organism with *Actinomyces neuii* and the genus *Mobiluncus* was not statistically significant. Furthermore, sequence divergence values of ca. 10 to 12% with the aforementioned taxa unequivocally demonstrate the novel bacterium from human sources represents a distinct taxon, one worthy of a separate generic status. Phenotypically, the unknown bacterium can be readily identified and distinguished from all currently recognized *Ac-*

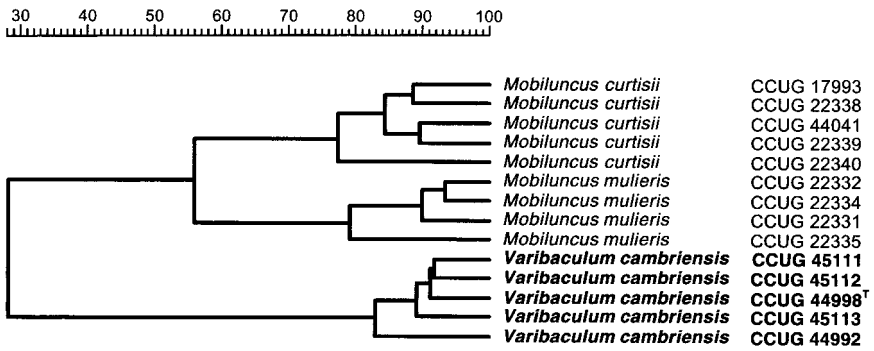

FIG. 2. Similarity dendrogram based on whole-cell protein patterns of *Varibaculum cambriensis* sp. nov. and *Mobiluncus* species. The levels of correlation are expressed as percentages of similarity for convenience.

tinomyces species and related taxa by using commercially available biochemical kits. Although the novel bacterium shows a phylogenetic association with *Actinomyces neuii* (6) and *Mobiluncus* species (20), it is phenotypically quite distinct from these taxa. Tests that are useful in distinguishing the novel bacterium from *Actinomyces neuii* include its negative reactions for catalase, pyrazinamidase, α-galactosidase, and β-galactosidase, whereas *Actinomyces neuii* gives positive reactions for these enzymes. Phenotypically, the novel bacterium differs from *Mobiluncus* species in being nonmotile. The unidentified bacterium can be differentiated by using the API Rapid ID 32Strep system from *Mobiluncus mulieris* by its ability to hydrolyze hippurate and can be distinguished from *Mobiluncus curtisii* by its failure to produce α-galactosidase, β-galactosidase, or arginine dihydrolase. Protein profiling further demonstrated the phenotypic coherence of the novel bacterium and its separateness from the two currently recognized *Mobiluncus* species. In addition, the novel bacterium can be distinguished from *Actinomyces* species by using a combination of biochemical tests. Its negative catalase reaction distinguishes the novel bacterium from the many catalase-positive species encountered in clinical specimens. Tests that are helpful in the routine laboratory to distinguish the unidentified bacterium from catalase-negative *Actinomyces* species with which it may be confused are shown in Table 2. Although clear differentiation from *Actinomyces turicensis* by using the tests indicated in Table 2 may sometimes be problematic, these species may be further distinguished by the ability of the unidentified bacterium to reduce nitrate to nitrite. Therefore, based on both phenotypic and molecular genetic evidence, we consider the 15 clinical isolates merit classification as a new genus and species, *Varibaculum cambriensis*, within the *Actinomyces* suprageneric grouping. To date, *Varibaculum cambriensis* has been isolated in the United Kingdom and Scandinavia from various human sites, including abscesses of the head and breast and IUCDs. The polymicrobial nature of these infections and a paucity of clinical information preclude ascertainment of its clinical significance at present. Its occurrence in facial abscesses and in an otogenic cerebral abscess, together with mixed anaerobes known to be of oral origin (1), suggests that the novel bacte-

TABLE 2. Tests that are useful in distinguishing *Varibaculum cambriensis* from catalase-negative *Actinomyces* species with which it may be confused

Test type	Test result[a]							
	V. combriensis	*A. europaeus*	*A. funkei*	*A. georgiae*	*A. meyeri*	*A. odontolyticus*	*A. radingae*	*A. turicensis*
Acid from:								
Lactose	−	−	+	+	+	V	V	−
Melibiose	−	V	−	−	−	−	+	−
Pullulan	−	V	−	+	−	V	−	−
Raffinose	−	−	−	−	−	−	+	−
Ribose	V	V	V	+	−	−	V	V
Sucrose	V	−	+	+	+	V	V	+
Trehalose	V	V	−	+	−	−	V	−
Methyl-BD	−	−	−	+	−	−	V	−
Hydrolysis of hippurate	+	−	+	−	−	−	−	+
Production of:								
α-Gal	−	+	−	−	−	−	+	−
β-Gal	V	+	V	+	+	V	+	−
PAL	−	−	+	−	−	−	−	−
APPA	V	+	+	+	+	+	+	+

[a] Tests were performed by using API Rapid ID 32Strep system. Results : +, positive; −, negative; V, variable.
[b] Methyl-BD, methyl-β-D-glucopyranoside; α-Gal, α-galactosidase; β-Gal, β-galactosidase; PAL, alkaline phosphatase; APPA, alanine phenylalanine proline arylamidase.

rium may occupy a similar habitat. We believe 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 *Varibaculum* gen. nov. *Varibaculum* (va.ri.ba′cu.lum. L. adj. *varus*, bent; L. neut. n. *baculum*, small rod; N.L. neut. n. *varibaculum*, small bent rod) cells consist of short, straight or curved diphtheroid rods which stain gram positive and are non-acid fast and nonmotile, nonhemolytic, anaerobic, and catalase negative. Acid is produced from glucose and some other sugars. Lactic and succinic acids are the major end products of glucose metabolism. Hippurate is hydrolyzed, but esculin, gelatin, and starch are not hydrolyzed. Acetoin is not produced. It is α-glucosidase and leucine arylamidase positive and arginine dihydrolase, pyrazinamidase, urease, and indole negative. Nitrate is reduced to nitrite by the majority of isolates. The G+C content of DNA is 51.7 mol%. The type species is *Varibaculum cambriensis*.

Description of *Varibaculum cambriensis* sp. nov. *Varibaculum cambriensis* (cam.bri.en′sis. L. adj. *cambriensis*, pertaining to cambria, the Latin name of Wales) cells consists of short, straight or curved diphtheroid rods which stain gram-positive and are non-acid-fast and nonmotile. Colonies after 48 h anaerobic incubation on Fastidious Anaerobic Agar with 5% horse blood are pinpoint, convex, entire edged, glistening, translucent white or gray. It is nonhemolytic, anaerobic, and catalase-negative. The end products of glucose metabolism are lactic and succinic acids, together with small amounts of acetic acid. Using conventional biochemical testing, acid is formed from D-glucose, sucrose, and D-ribose; acid may or may not be formed from fructose, mannitol, and xylose. Acid is not produced from amygdalin, L-arabinose, cellobiose, lactose, mannose, D-raffinose, salicin, or trehalose. It is lecithinase and lipase negative and indole negative. Esculin, gelatin, and starch are not hydrolyzed. Using API test systems, acid is produced from D-glucose, and most strains ferment sucrose. Some strains produce acid from maltose, D-ribose, trehalose and D-xylose. Acid is not produced from D-arabitol, L-arabinose, cyclodextrin, glycogen, mannitol, melibiose, melezitose, methyl-β-D-glucopyranoside, lactose, pullulan, D-raffinose, sorbitol, or tagatose. Hippurate is hydrolyzed by most strains, but esculin and gelatin are not hydrolyzed. Activity for α-glucosidase and leucine arylamidase are detected, and some strains display activity for alanine phenylalanine proline arylamidase and β-galactosidase (weak reaction). Activity for acid phosphatase, esterase C-4, ester lipase C8, and phosphoamidase is either weakly positive or negative. No activity is detected for arginine dihydrolase, alkaline phosphatase, chymotrypsin, α-fucosidase, α-galactosidase, β-glucosidase, β-glucuronidase, glycyl tryptophane arylamidase, lipase C14, α-mannosidase, β-mannosidase, *N*-acetyl-β-glucosaminidase, pyrolidonyl arylamidase, pyroglutamic acid arylamidase, pyrazinamidase, trypsin, valine arylamidase, or urease. Most strains reduce nitrate to nitrite. Acetoin is not produced. The G+C content of DNA is 51.7 mol%. It is isolated from human sources, including breast abscess, brain abscess, cheek abscess, submandibular abscess, postauricular abscess, ischiorectal abscess, and intrauterine contraceptive devices. The habitat is not known. The type strain is CCUG 44998^T = CIP 107344^T.

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