

## Characterization of Some *Actinomyces*-Like Isolates from Human Clinical Specimens: Reclassification of *Actinomyces suis* (Soltys and Spratling) as *Actinobaculum suis* comb. nov. and Description of *Actinobaculum schaalii* sp. nov.

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**Five strains of a hitherto unknown *Actinomyces*-like bacterium were isolated from human clinical sources, including blood cultures. Biochemical and chemotaxonomic characterization indicated that the strains were distinct from previously described *Actinomyces* and *Arcanobacterium* species. A comparative 16S rRNA gene sequence analysis demonstrated that the undescribed strains constitute a new subline within the *Actinomyces-Arcanobacterium* species complex. The closest known relative of the isolates was found to be *Actinomyces suis*, although a 16S rRNA sequence divergence value of approximately 6% clearly demonstrated that the unknown bacterium represents a distinct species. Based on the results of the present and earlier phylogenetic investigations, it is proposed that *Actinomyces suis* should be reclassified in a new genus, the genus *Actinobaculum*, as *Actinobaculum suis* comb. nov. In addition, a new species, *Actinobaculum schaalii*, is proposed for the *Actinomyces*-like bacterium from human sources. The type strain of *Actinobaculum schaalii* is CCUG 27420.**

The genus *Actinomyces* is a heterogeneous group of anaerobic and facultatively anaerobic, asporogenous, gram-positive, non-acid-fast, rod-shaped organisms which generally show various degrees of branching (9, 10). Nearly all of the currently recognized *Actinomyces* species occur as inhabitants of mucosal surfaces of humans and other homiothermic animals. Some *Actinomyces* species (e.g., *Actinomyces bovis* and *Actinomyces israelii*) have long been recognized as pathogens of humans and animals (9, 10). Several new *Actinomyces* species associated with disease in humans have been described in recent years (e.g., *Actinomyces bernardiae* [4], *Actinomyces europae* [5], *Actinomyces neuui* [3], *Actinomyces turicensis* [14], and *Actinomyces radingae* [14]), and *Actinomyces*-like organisms are attracting increasing interest as opportunistic pathogens. In this article we report the phenotypic and phylogenetic characterization of five strains of a hitherto unknown *Actinomyces*-like bacterium from human sources. Based on the taxonomic results presented below, a new species, *Actinobaculum schaalii*, is described.

Five human clinical isolates (CCUG 19698, CCUG 29359B, CCUG 27420<sup>T</sup>, CCUG 32296, and CCUG 36567) were referred to the Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden, for identification. Strains CCUG 27420<sup>T</sup> and CCUG 36567 were isolated from human blood, whereas CCUG 19698, CCUG 29359B, and CCUG 32296 originated from human urine. All of the strains were cultured on 5% horse blood agar (Columbia base; Difco, Detroit, Mich.) at 37°C in a 5% CO<sub>2</sub> atmosphere. The isolates were biochemically characterized by using the API ZYM (enzymatic profiling), API coryne, API rapid ID 32 STREP, and API rapid ID 32A systems according to the instructions of the manufacturer (API bioMérieux, Marcy l'Etoile, France). For polyacrylamide

gel electrophoresis (PAGE) of whole-cell proteins and cellular fatty acid (CFA) analysis, the strains were grown on 5% horse blood agar (Columbia base) at 37°C in 5% CO<sub>2</sub>. The PAGE analysis was performed as described previously (8). For densitometric analysis, normalization, and interpretation of protein patterns a Pharmacia LKB-Ultro Scan XL instrument with Gel Scan XL software (Pharmacia, Uppsala, Sweden) and the Gelcompar GCW 3.0 software package (Applied Maths, Kortrijk, Belgium) were used. CFA analyses were conducted by preparing fatty acid methyl esters and analyzing them by high-resolution capillary gas chromatography with a model 5890A instrument (Hewlett-Packard, Avondale, Pa.) as described previously (12). A cell wall murein analysis was carried out by using the methods of Schleifer and Kandler (11) except that thin-layer chromatography on cellulose sheets (Merck, Darmstadt, Germany) was used instead of paper chromatography. The phylogenetic analysis performed was a comparative 16S rRNA gene sequence analysis. A large fragment of the 16S rRNA gene (corresponding to positions 30 to 1521 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR by using conserved primers close to the 3' and 5' ends of the gene. The PCR products were purified by using a Prep-A-Gene kit (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions and were directly sequenced by using a *Taq* DyeDeoxy 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 a database search with the program FASTA of the Genetics Computer Group package (1). The sequences of these organisms and those of other known related strains were retrieved from the EMBL and GenBank data libraries and were aligned with the newly determined sequences by using the program PILEUP (1). The resulting multiple-sequence alignment was corrected manually, and approximately 100 bases at the 5' end of the rRNA were omitted from further analyses because of alignment ambiguities. A distance matrix was calculated by

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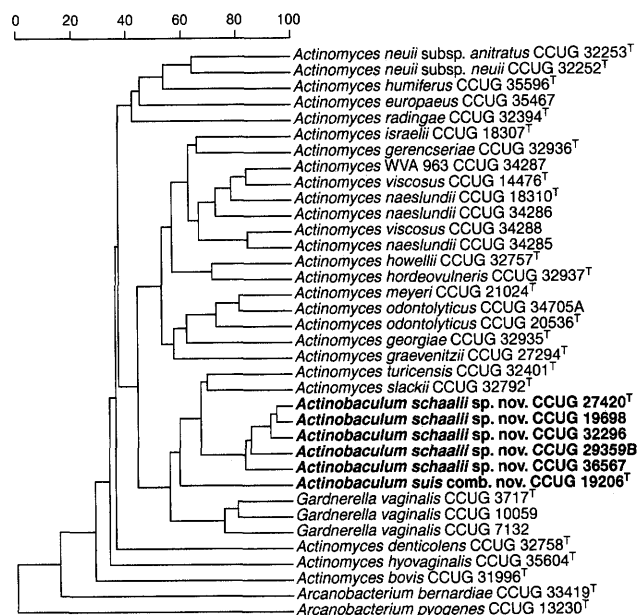


FIG. 1. Similarity dendrogram based on whole-cell protein patterns of *Actinobaculum schaalii* sp. nov. and related species. Levels of correlation were expressed as percentages of similarity for convenience.

using the programs PRETTY (1) 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 groups was estimated by performing a bootstrap analysis (500 replications) with the programs DNABOOT, DNADIST, NEIGHBOR, and CONSENSE (2).

The five human clinical isolates were gram-positive, nonmotile, non-spore-forming, non-acid-fast, straight to curved rods. Some cells exhibited branching. The strains were catalase negative and facultatively anaerobic. Acetate (the major component) and small amounts of succinate were produced as end products of metabolism in PYG broth. Relatively few sugars (viz., glucose, maltose, ribose, and D-xylose) were fermented. Some of the strains fermented L-arabinose, mannose, starch, sucrose, and trehalose. All of the isolates hydrolyzed hippurate, but alkaline phosphatase, arginine dihydrolase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\beta$ -mannosidase, gelatinase, and urease activities were not detected. None of the strains reduced nitrate to nitrite. The analysis of the CFA contents of the strains revealed that C<sub>10:0</sub> ( $1.5 \pm 0.2\%$  of the total CFA), C<sub>12:0</sub> ( $1.8 \pm 0.3\%$ ), C<sub>14:0</sub> ( $7.7 \pm 1\%$ ), C<sub>16:1w9c</sub> ( $1.1 \pm 0.2\%$ ), C<sub>16:1w7c</sub> ( $3.3 \pm 0.6\%$ ), C<sub>16:0</sub> ( $22.9 \pm 1.6\%$ ), C<sub>18:1w9c</sub> ( $34.8 \pm 1\%$ ), C<sub>18:0</sub> ( $14.4 \pm 0.9\%$ ), and C<sub>18:2w6,9c</sub> ( $6.6 \pm 1.2\%$ ) were the major acids. The cellular morphology, biochemical, and CFA characteristics of the unidentified clinical isolates resembled those of species of the genus *Actinomyces* and related taxa. The cell wall murein composition of strain CCUG 27420<sup>T</sup> was examined, and the cell wall murein type was found to be type A5 $\alpha$  (L-Lys-L-Lys-D-Glu). The same murein type is found in *Actinomyces neuii* and *Arcanobacterium haemolyticum*, and this fact reinforces the affinity of the new clinical isolates with the *Actinomyces-Arcanobacterium* complex of species. The whole-cell protein profiles of the five unknown strains were determined by sodium dodecyl sulfate-PAGE. A dendrogram derived from a numerical analysis of the protein patterns is shown in Fig. 1. The five strains grouped

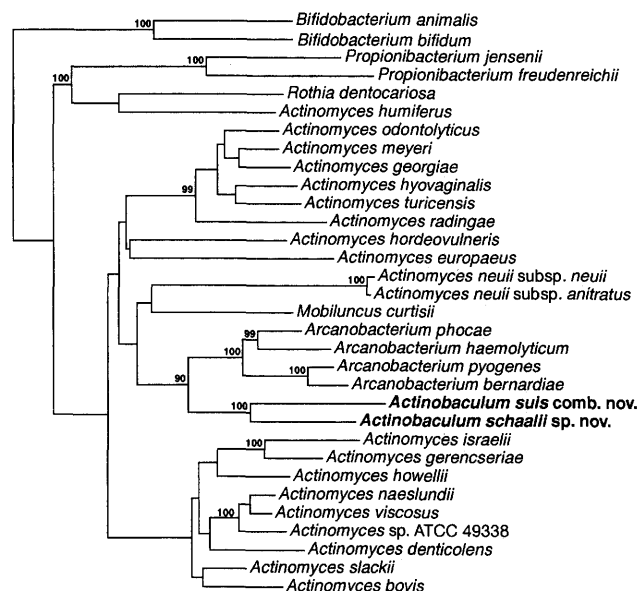


FIG. 2. Unrooted tree showing the phylogenetic relationships of *Actinobaculum schaalii* sp. nov., members of the genus *Actinomyces*, and some other taxa. The tree, which was constructed by using the neighbor-joining method, was based on a comparison of approximately 1,320 nucleotides. Bootstrap values, expressed as percentages of 500 replications, are indicated at branch points.

together and formed a distinct branch. These data demonstrate that the unknown strains represent a phenotypically relatively homogeneous group and that they are distinct from all of the *Actinomyces* and *Arcanobacterium* spp. examined. To establish the phylogenetic affinities of the clinical isolates, the 16S rRNA genes of four strains were amplified by PCR and sequenced. The sequences determined each consisted of >1,400 nucleotides. A comparative sequence analysis showed that the four strains were genealogically homogeneous, exhibiting 99.7 to 100% 16S rRNA similarity. Sequence searches of the EMBL and GenBank libraries revealed that the newly determined sequences were most closely related to sequences of species belonging to the genera *Actinomyces* and *Arcanobacterium*. The sequence of strain CCUG 27420<sup>T</sup> was subjected to a pairwise analysis with the sequences of *Actinomyces* and *Arcanobacterium* spp. and some other close relatives, and derived evolutionary distances were used to determine phylogenetic relationships. A tree depicting the phylogenetic affinities of strain CCUG 27420<sup>T</sup> is shown in Fig. 2, and the levels of sequence similarity of this strain with *Actinomyces* and *Arcanobacterium* spp. are shown in Table 1. The unidentified bacterium formed a distinct subline and displayed a relatively close affinity (approximately 94% 16S rRNA sequence similarity) with *Actinomyces suis*. Bootstrap resampling revealed that the association between the unknown bacterium and *Actinomyces suis* was statistically significant (bootstrap value, 100%). The next closest relatives of the unknown bacterium were *Arcanobacterium* spp., which exhibited approximately 91 to 92% 16S rRNA sequence similarity. Other *Actinomyces* spp. displayed substantially lower levels of relatedness (generally <90% sequence similarity) (Table 1).

The results of both biochemical and whole-cell protein PAGE analyses showed that the five new clinical strains represent a homogeneous group of organisms and constitute a new species. Phylogenetically, the unknown bacterium forms a distinct subline within the *Actinomyces-Arcanobacterium* group

TABLE 1. Similarity values based on 16S rRNA sequences of some *Actinomyces* spp., *Arcanobacterium* spp., and *Actinobaculum schaalii* sp. nov.

Taxon (EMBL accession no.) <sup>a</sup>	% Sequence similarity with <i>Actinobaculum schaalii</i> sp. nov.
<i>Actinobaculum suis</i> (S83623).....	94.2
<i>Actinomyces bovis</i> (X81061).....	88.7
<i>Actinomyces denticolens</i> (X80412).....	89.4
<i>Actinomyces europae</i> (Y08828).....	88.4
<i>Actinomyces georgiae</i> (X80413).....	89.1
<i>Actinomyces gerencseriae</i> (X80414).....	88.3
<i>Actinomyces hordeovulneris</i> (X82448).....	90.2
<i>Actinomyces howellii</i> (X80411).....	89.4
<i>Actinomyces hyovaginalis</i> (X69616).....	89.9
<i>Actinomyces humiferus</i> (X82449).....	87.8
<i>Actinomyces israelii</i> (X82450).....	87.7
<i>Actinomyces meyeri</i> (X82451).....	89.6
<i>Actinomyces naeslundii</i> (X81062).....	89.7
<i>Actinomyces neuii</i> subsp. <i>anitratus</i> (X71862).....	88.8
<i>Actinomyces neuii</i> subsp. <i>neuii</i> (X71861).....	88.7
<i>Actinomyces odontolyticus</i> (X80504).....	90.0
<i>Actinomyces radingae</i> (X78719).....	88.6
<i>Actinomyces slackii</i> (X82452).....	90.4
<i>Actinomyces turicensis</i> (X78720).....	89.8
<i>Actinomyces viscosus</i> (X82453).....	89.6
<i>Arcanobacterium bernardiae</i> (X79224).....	91.5
<i>Arcanobacterium haemolyticum</i> (X73952).....	91.0
<i>Arcanobacterium phocae</i> (X97049).....	91.8
<i>Arcanobacterium pyogenes</i> (X79225).....	91.8

<sup>a</sup> The numbers in parentheses are EMBL 16S rRNA nucleotide sequence accession numbers.

of species (see reference 7) and exhibits a statistically significant relationship with *Actinomyces suis*, an organism associated with cystitis and pyelonephritis in pigs (13). The affinity between the new unidentified human bacterium and *Actinomyces suis* was also evident from the whole-cell protein profile analysis results. Levels of 16S rRNA sequence divergence of >6% between the unidentified new strains and *Actinomyces suis*, however, clearly demonstrated that the bacterium from humans represents a previously unrecognized species. Although the novel bacterium phylogenetically resembles *Actinomyces suis*, it can be readily distinguished from the latter taxon by its production of acid from glucose and D-xylose, by its lack of production of acid from glycogen, and by its lack of production of  $\beta$ -glucuronidase and urease. The unknown new bacterium also differs from *Actinomyces suis* in its cell wall murein structure. Based on its phenotypic and phylogenetic distinctiveness, the unidentified new bacterium from humans clearly merits a new species. The generic assignment of this bacterium is, however, problematic. It has long been recognized that the genus *Actinomyces* is phenotypically very diverse (9, 10). Furthermore, recent comparative 16S rRNA sequencing studies (7) have demonstrated that the genus *Actinomyces* is not monophyletic and that members of several validated non-*Actinomyces* genera (e.g., the genera *Arcanobacterium* and *Mobiluncus*) are intermixed with *Actinomyces* species. The genus *Actinomyces* is clearly in urgent need of taxonomic revision, with several species groups and lineages representing the nuclei of new genera. As pointed out by Pascual et al. (7), *Actinomyces suis* is one such organism. This species displays approximately 10 to 14% 16S rRNA sequence divergence with the *Actinomyces bovis* cluster of species (sensu Pascual et al. [7]) and exhibits a somewhat closer affinity with *Arcanobacterium* species (approximately 8 to 11% sequence divergence). Both sequence diver-

gence and tree topology considerations clearly demonstrated that *Actinomyces suis* warrants a new genus. In the present study the unknown new bacterium from clinical sources exhibited a close (approximately 6% sequence divergence) and statistically significant (bootstrap value, 100%) phylogenetic association with *Actinomyces suis*. Therefore, based on the results of the previous study (7) and this study, we propose that *Actinomyces suis* and the unknown new bacterium from humans should be classified in a new genus, the genus *Actinobaculum*, as *Actinobaculum suis* comb. nov. and *Actinobaculum schaalii* sp. nov., respectively. Characteristics which distinguish *Actinobaculum suis* and *Actinobaculum schaalii* from each other and from related *Actinomyces* and *Arcanobacterium* species are shown in Table 2.

**Description of *Actinobaculum* gen. nov.** *Actinobaculum* (Acti.no.ba'cu.lum. Gr. n. *actis*, *actinis*, ray; L. neut. n. *baculum*, rod, stick; M. L. neut. n. *Actinobaculum*, ray stick). On blood agar cells are straight to slightly curved rods, some of which exhibit branching. Hemolysis is not observed. Cells are gram positive, not acid fast, and nonmotile and do not form spores. Anaerobic or facultatively anaerobic and catalase negative. Acid may or may not be produced from glucose. Acid is produced from maltose but is not produced from lactose, mannitol, melezitose, melibiose, raffinose, or sorbitol. The major end product of glucose and/or maltose metabolism is acetate. Esculin and gelatin are not hydrolyzed. Nitrate is not reduced to nitrite. Acetoin is not produced. The cell wall murein type is type A5 $\alpha$  (L-Lys-L-Ala-Lys-D-Glu or L-Lys-Lys-D-Glu). The major long-chain CFA are straight-chain saturated and mono-unsaturated fatty acids. The DNA base composition ranges from 55 to 57 mol% G+C. The type species of the genus is *Actinobaculum suis*. The genus *Actinobaculum* is a member of the high-G+C-content branch of the gram-positive bacteria, in which it forms a distinct phylogenetic lineage. The closest phylogenetic relative of the genus *Actinobaculum* is the genus *Arcanobacterium*.

**Description of *Actinobaculum suis* (Wegienek and Reddy) comb. nov.** *Actinobaculum suis* (*Eubacterium suis* Wegienek and Reddy 1982; "*Corynebacterium suis*" Soltys and Spratling 1957) (su'is. L. gen. n. *suis*, of a hog). The description below is based on that given in reference 13. Slender, nonmotile, pleomorphic rods that are 1 to 3 by 0.5  $\mu$ m and are arranged singly, in pairs (cells are often at an angle to each other or in palisades), or in small clusters. Gram positive, but rather easily decolorized, especially in old cultures. Not acid fast and nonsporulating; does not survive heating at 80°C for 10 min. Capsules are not observed by capsule staining; however, a fringed-like outer coat external to the cell wall is seen in thin-section electron micrographs. Colonies on anaerobic blood agar plates are 0.5 to 3.0 mm in diameter after 48 h, white, circular, and granular and have entire to slightly irregular margins. Colonies often have slightly raised centers, which gives them a fried-egg appearance. After 1 week, colonies are 3 to 5 mm in diameter and flatter. Growth is barely discernible after incubation for 7 days under 6% CO<sub>2</sub> or air. Peptone-yeast extract-starch broth supports excellent growth. The optimal pH is 7 to 8; no growth occurs at pH 5.0 or less. The optimal temperature is 37°C; the temperature range for growth is 30 to 43°C; and no growth occurs at 22 to 23°C. Anaerobic. Metabolism is strictly fermentative. Maltose, starch, and glycogen are fermented. Acetate, ethanol, and formate are the main products from maltose fermentation. Adonitol, amygdalin, arabinose, cellobiose, dulcitol, erythritol, esculin, fructose, galactose, glucose, glycerol, inositol, inulin, lactose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, lactate, pyruvate, and threonine are not fermented. Strongly

TABLE 2. Characteristics that differentiate *Actinobaculum schaalii* from *Actinomyces* and *Arcanobacterium* species and subspecies<sup>a</sup>

Species or subspecies	Nitrate reduction	Urease activity	Esculin hydrolysis	Acid produced from mannitol	Acid produced from xylose	$\beta$ -Galactosidase	$\alpha$ -Glucosidase	<i>N</i> -Acetyl- $\beta$ -glucosaminidase	Beta-hemolysis	Murein type
<i>Actinomyces bovis</i>	- <sup>b</sup>	-	V	-	-	-	-	+	V	ND
<i>Actinomyces denticolens</i>	+	ND	+	V	-	+	+	-	ND	ND
<i>Actinomyces georgiae</i>	V	-	+	V	+	ND	ND	ND	-	A5 $\beta$ (L-Orn-Lys-D-Glu)
<i>Actinomyces gerencseriae</i>	V	-	V	V	+	ND	ND	ND	-	A5 $\beta$ (L-Orn-Lys-D-Glu)
<i>Actinomyces israelii</i>	V	-	+	V	+	+	+	-	-	A5 $\beta$ (L-Orn-Lys-D-Glu)
<i>Actinomyces meyeri</i>	-	V	-	-	+	-	+	-	-	ND
<i>Actinomyces naeslundii</i>	V	+	+	-	V	V	V	-	-	ND
<i>Actinomyces neuü</i>	+	-	-	+	+	+	+	V	-	A5 $\alpha$ (L-Lys-Lys-D-Glu)
subsp. <i>neuü</i>										
<i>Actinomyces neuü</i>	-	-	-	+	+	+	+	V	-	A5 $\alpha$ (L-Lys-Lys-D-Glu)
subsp. <i>anitratus</i>										
<i>Actinomyces odontolyticus</i>	+	-	V	-	V	-	-	-	-	ND
<i>Actinomyces radingae</i>	-	-	+	V	+	+	+	+	-(w)	A5 $\alpha$ (L-Lys [L-Orn]-Lys-D-Glu)
<i>Actinomyces turicensis</i>	-	-	-	V	+	-	+	-	-(w)	A5 $\beta$ (L-Orn-L-Lys-D-Glu)
<i>Actinomyces viscosus</i>	+	V	V	-	V	V	V	-	-	ND
<i>Actinobaculum schaalii</i>	-	-	-	-	+	-	+	-	-	A5 $\alpha$ (L-Lys-Lys-D-Glu)
<i>Actinobaculum suis</i>	-	+	-	-	-	+	ND	ND	-	A5 $\alpha$ (L-Lys-L-Ala-Lys-D-Glu)
<i>Arcanobacterium bernardiae</i>	-	-	-	-	-	-	+	-	V	A5 $\alpha$ (L-Lys-L-Ala-Lys-D-Glu)
<i>Arcanobacterium haemolyticum</i>	-	-	-	-	-	+	ND	+	+	A5 $\alpha$ (L-Lys-Lys-D-Glu)
<i>Arcanobacterium pyogenes</i>	-	-	-	V	+	V (w)	-	+	+	A5 $\alpha$ (L-Lys-L-Ala-Lys-D-Glu)
<i>Arcanobacterium phocae</i>	-	-	-	V	-	+	(w)	+	(s)	ND

<sup>a</sup> Data from references 4 through 7, 9, 10, 13, and 14.

<sup>b</sup> -, negative; +, positive; (w), weak; (s), slow; V, variable; ND, not determined.

urease positive. Does not produce catalase, indole, acetyl-methylcarbinol, hydrogen sulfide, lipase, or lecithinase; ammonia is not produced from peptone. Esculin and gelatin are not hydrolyzed. Meat and milk are not digested. Nitrates are not reduced. Major amounts of type *b* cytochrome and minor amounts of type *c* cytochrome are synthesized. The cell wall sugars are rhamnose and mannose. The cell wall murein type is type A5 $\alpha$  (L-Lys-L-Ala-Lys-D-Glu). The G+C content of the DNA is 55 mol%. Originally isolated from cases of cystitis and pyelonephritis and cases of metritis in pregnant sows. Not isolated from healthy sows but frequently recovered from urine and semen of apparently healthy boars. Sows can be infected artificially by intrarectal injection of live organisms plus 5% saponin. No demonstrable exotoxin is produced. The type strain of *Actinobaculum suis* is ATCC 33144 (= DSM 20639).

**Description of *Actinobaculum schaalii* sp. nov.** *Actinobaculum schaalii* (N. L. gen. n. *schaalii*, of Schaal, to honor Klaus P. Schaal, contemporary German microbiologist, for his contributions to actinomycete microbiology). Cells are straight to slightly curved rods, some of which exhibit branching. Cells are gram positive, not acid fast, and nonmotile and do not form spores. Nonhemolytic. A weak CAMP reaction occurs. Facultatively anaerobic and catalase negative. Acetate and succinate are the major end products of glucose fermentation. Acid is produced from glucose, maltose, ribose, and D-xylose. Some strains produce acid from L-arabinose, mannose, starch, sucrose, and trehalose. Acid is not produced from D-arabitol, N-acetyl- $\beta$ -glucosamine, cyclodextrin, glycogen, lactose, mannitol, melezitose, melibiose, methyl- $\beta$ -D-glucopyranoside, pullulan, raffinose, sorbitol, and D-tagatose. Hippurate is hydrolyzed. Esculin, gelatin, and urea are not hydrolyzed.  $\alpha$ -Glucosidase,

alanine-phenylalanine-proline arylamidase, and pyroglutamic acid arylamidase are produced. Alkaline phosphatase, arginine dihydrolase,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase, glycyl-tryptophan arylamidase, and  $\beta$ -mannosidase activities are not detected. Pyrazinamidase and pyrrolidonylarylamidase activities are shown by some strains. Nitrate is not reduced to nitrite. Acetoin is not produced. The cell wall murein type is type A5 $\alpha$  (L-Lys-Lys-D-Glu). The major cellular fatty acids are hexadecanoic, octadecanoic, and *cis*-delta-9-octadecenoic acids. The DNA base composition is 57 mol% G+C. Strains have been isolated from human blood and urine. The habitat is unknown but is probably the genital or urinary tract. The type strain of *Actinobaculum schaalii* is CCUG 27420. The source of CCUG 27420<sup>T</sup> was human blood (chronic pyelonephritis, 64-year-old male). The type strain has the characteristics of the species.

**Nucleotide sequence accession number.** The 16S rRNA gene sequence of strain CCUG 27420<sup>T</sup> has been deposited in the GenBank database under accession number Y12329.

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