Actinomyces hordeovulneris sp. nov., an Agent of Canine Actinomycosis

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Of 30 *Actinomyces* strains isolated from infections in dogs, 15 were found to have galactose, glucose, and rhamnose in their cell wall carbohydrates, whereas the remainder had only galactose and glucose. No 6-deoxytalose was detected in any of our analyses. The biochemical characteristics of the rhamnose-positive strains deficient in 6-deoxytalose were similar to those of *Actinomyces viscosus* ATCC 19246 and ATCC 27045, a reference dog strain. In contrast, the 15 strains having only galactose and glucose in their wall carbohydrates had biochemical and serological characteristics unlike those of any previously accepted species of *Actinomyces*. The name *Actinomyces hordeovulneris* sp. nov. is proposed for this organism which is associated with thoracic, abdominal, or recurrent localized infections that sometimes are associated with awns of foxtails (*Hordeum* spp). The type strain of *A. hordeovulneris* is strain ATCC 35275 (= UCD 81-332-9).

Strains of *Actinomyces* are agents of abscesses or systemic infections in dogs. In the western United States these infections are often associated with awns of several members of the grass genus *Hordeum* (commonly called foxtails) (3, 14; E. L. Biberstein and S. Jang, unpublished data). The disease is common in California, probably because of the moist winters, dry hot summers, and abundance of foxtails. Each awn resembles a miniature badminton bird with a sharp tip and flaring hairs; each hair has many barbs that prevent retrograde movement. As an animal moves through the wild grass, foxtail awns become trapped in its coat, or they may be inhaled or swallowed. The tips of the awns penetrate skin or mucous membranes and are propelled forward through the tissue with any adjacent muscle contractions. They leave trails of inflammation and necrosis. *Actinomyces* and other oral or gastrointestinal flora may be introduced directly when the animal licks or bites a wound, or they may spread hematogenously to this necrotic focus. The migrating awns can penetrate brains, body cavities, or spinal columns and can elicit life-threatening infections.

Actinomyces viscosus was reported to be an agent of actinomycosis in dogs by Georg et al. (9). In an earlier report, Brion (4) described similar filamentous organisms from canine lesions, but the physiological studies of these agents were incomplete and no reference strains are available for comparisons. Davenport et al. (6) reported the isolation *A. viscosus* from six dogs, two with empyemas and four with abscesses; none of the infections was indicated as being associated with grass awns.

In this study, cell wall composition (2, 8, 15, 16), biochemical activities in miniaturized systems (19, 20), and serological tests (9, 10, 13, 22) were used to identify 30 catalase-positive *Actinomyces* strains isolated from dogs. We describe the characteristics of a new agent, for which we propose the name *Actinomyces hordeovulneris* sp. nov. in

recognition of its frequent clinical association with migrating foxtail awns.

MATERIALS AND METHODS

Strains. Hamster, human, and canine reference strains of *A. viscosus* (strains ATCC 15987^T [T = type strain], ATCC 19246, and ATCC 27045) were obtained from the American Type Culture Collection, Rockville, Md., and were used for comparisons. The canine isolates were obtained during the last 4 years from 28 dogs treated at the University of California Veterinary Medicine Teaching Hospital. Older isolates were stored in skim milk at -20°C. Other organisms used as controls occasionally were *Nocardia asteroides* UCD 81-1-5 and *Streptomyces griseus*. All strains were maintained on Trypticase soy agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 5% whole bovine blood and were transferred triweekly.

Oxygen requirements. The oxygen requirement of strain UCD 80-19-4 was determined at the Mycotic Diseases Division, Centers for Disease Control, by using the technique described by Slack (21). Plate cultures were incubated in candle extinction jars.

Analysis of whole cell carbohydrates. Cells were grown in two unvented 50-ml blood culture bottles containing Trypticase soy broth with CO₂ and without sodium polyanetholsulfonate (BBL Microbiology Systems, Cockeysville, Md.). The inoculum was a suspension of cells from a 48-h blood agar plate in 3 ml of sterile fetal calf serum. The suspension was adjusted to a turbidity of a McFarland no. 3 or 4 tube, and 2.5 ml was added to each 50-ml unvented bottle with a syringe. After incubation at 37°C for 48 to 72 h, 0.5 ml of 37% formaldehyde was added to each bottle. Then 18 h later, the cells were harvested by centrifugation at 3,000 × g for 15 min and were washed twice with distilled water. The method of Stanek and Roberts (23) was used for analysis of whole cell carbohydrates, with the modifications described below. A 1-g portion of wet packed cells was acidified with 1.5 ml of 1 N H₂SO₄ and held in a sealed tube for 2 h in a boiling water

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bath. The pH of the hydrolysate was adjusted to 5.2 to 5.5 by dropwise addition of saturated barium hydroxide, and the precipitate was removed by centrifugation. The supernatant was evaporated to dryness, and the residue was dissolved in 0.3 ml of distilled water. Samples were analyzed for carbohydrates by thin-layer chromatography as described by Stanek and Roberts (23), except that 3 μ l was applied in 1- μ l increments with intervening drying periods of 5 min. In some experiments the amount spotted onto the chromatogram was increased to 6 μ l. Sugar residues of some strains were also analyzed by gas-liquid chromatography as described previously by Beaman et al. (1).

Amino acid analysis of cell walls. Cell walls were partially purified from 0.5 g of packed wet cells by treatment with 10% trichloroacetic acid followed by treatment with trypsin, as described by Schleifer and Kandler (18), except that the suspension in trichloroacetic acid was adjusted to the turbidity of a McFarland no. 4 tube. The two-dimensional thin-layer chromatography method of Harper and Davis (11) was used with type 6064 Chromagram cellulose sheets (Eastman Kodak Co., Rochester, N.Y.) without fluorescent indicator and a thin-layer chromatography chamber (model 13259 Eastman Kodak Chromagram Developing Apparatus). Lysine, ornithine, aspartic acid, and diaminopimelic acid standards obtained from Sigma Chemical Co., St. Louis, Mo., were included in each analysis. Isopropanol-acetic acid-water (75:10:15, vol/vol) was used ascending, and methanol-pyridine-10 N hydrochloric acid-water (64:8:2:14, vol/vol) was used ascending in the second direction.

Biochemical tests. The tests for catalase and starch hydrolysis were performed by the technique described by Slack (21); the test for cytochrome oxidase activity was performed by the method of Deibel and Evans (7); and the tests for urease activity and nitrate reduction were performed as described by Schofield and Schaal (20). The carbohydrate fermentation tests were done by using the Schofield-Schaal modifications of the Minitek system (19, 20). Commercially available esculin disks (MiniTek) were inoculated at the same time. Fermentation products were determined by using the methods of Holdeman and Moore (12), except that we used a 3-ml samples of Trypticase soy broth cultures supplemented with 5% fetal calf serum instead of the recommended culture medium.

Serological studies. Fluorescein isothiocyanate-conjugated antisera specific for the following organisms were tested against strains UCD 80-19-4, UCD 81-154-4, and UCD 81-345-981: *A. viscosus* serotype 1 strain WVU 745^T (= ATCC 15987^T); *A. viscosus* serotype 2 strains WVU 371 (= ATCC 19246) and WVU 398B; *Actinomyces israelii* serotype 1 strains WVU 46 (= ATCC 12102), WVU 390, and WVU 30 (= ATCC 10048); *A. israelii* serotype 2 strain WVU 307 (= ATCC 29322); *Actinomyces bovis* serotypes 1 and 2; *Actinomyces naeslundii* serotypes 1, 2, and 3; *Actinomyces odontolyticus* serotypes 1 and 2; *Actinomyces suis* (Franke); *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*); *Rothia dentocariosa* serotypes 1, 2, and 3; and *Rothia*-like strain WVU 1556. Reactions at titers of 1:5, 1:10, 1:20, 1:40, and 1:80 were compared with homologous titers.

Antibiotic susceptibility. The minimal inhibitory concentrations of various antibiotics were determined by the agar dilution technique, using 72-h cultures in mycoplasma broth (without inhibitors; Difco) supplemented with 5% fetal calf serum and 5% yeast extract (freshly prepared from a 25% aqueous slurry of active dry yeast) adjusted to the turbidity of a McFarland no. 0.5 tube as inocula and Mueller-Hinton agar plates supplemented with 5% fetal calf serum as the test

medium. The minimal inhibitory concentrations were compared with the minimal inhibitory concentrations for *Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* and were corrected for the influence of CO₂ on the test.

DNA isolation and determination of base contents. Deoxyribonucleic acids (DNAs) of *A. viscosus* ATCC 15987^T and strain UCD 81-332-9^T were isolated by a procedure generously shared with us before publication (I. Yu et al., manuscript in preparation). Briefly, all solutions, glass, and plastic ware were sterilized. Cells were grown for 24 h in 1 liter of brain heart infusion broth supplemented with 5% fetal calf serum. After 2 ml of diethylpyrocarbonate (Calbiochem-Behring, La Jolla, Calif.) was added, the cells were harvested by centrifugation, washed with 0.02 M 2-amino-2-(hydroxymethyl)-1,3-propanediol buffer (pH 7.8) containing 0.01 M ethylenediaminetetraacetic acid and 0.44 M sucrose (sucrose buffer), and suspended in 10 ml of 1% Sarkosyl-sucrose buffer. The cells were mixed with an equal volume of acid-cleaned and neutralized glass beads (diameter, 0.45 mm) and shaken for 5 min under intermittent CO₂ in a homogenizer (Braun, Melsungen, Federal Republic of Germany). The suspension was decanted and combined with one washing of the glass beads with 1% Sarkosyl-sucrose buffer. Sodium perchlorate and 30% Sarkosyl-sucrose buffer were added to final concentrations of 1 M and 1%, respectively. The DNA was deproteinized once with chloroform-isoamyl alcohol (24:1), precipitated overnight in 3 volumes of absolute ethanol, and harvested by centrifugation at 7,700 $\times$ g. The pellet was dissolved in 0.15 M NaCl-0.15 M trisodium citrate (pH 7.0 $\pm$ 0.2) and treated for 6 h at 25°C with 100 μ g of amylase derived from *Bacillus licheniformis* per ml and 100 μ g of ribonuclease (type X1A; nuclease-free) per ml, both previously heated at 90°C; this was followed by treatment with 100 μ g of proteinase K (type XI; Sigma) per ml for 4 h at room temperature. The DNA was deproteinized a second time with chloroform-isoamyl alcohol, precipitated with ethanol as described above, collected by centrifugation, and dissolved in 0.003 M sodium phosphate buffer. DNA base contents (guanine-plus-cytosine contents) were determined from the buoyant densities in cesium chloride gradients by the method of Schildkraut et al. (17). *Micrococcus lysodeikticus* and *E. coli* K-12 DNAs were used as internal standards for the gradients.

RESULTS AND DISCUSSION

Physiological characters have been used for many years to identify members of the *Actinomycetaceae*; however, the recommended carbohydrate fermentation (21) and end product analysis (12) tests were not applicable with the canine strains due to inadequate growth. The modified Minitek procedures described by Schofield and Schaal (19, 20) gave reproducible results in repeated tests. Some investigators consider carbohydrate fermentation tests of limited value in the delineation of species except when a large battery of tests is performed (16). Nevertheless, the high degree of correlation which we observed among the cell wall carbohydrates, colony type, and fermentation pattern of each dog isolate suggests otherwise.

A total of 15 isolates and reference strain ATCC 27045 lacked 6-deoxytalose in their cell wall carbohydrates, but had other cell wall and biochemical characteristics similar to those of *A. viscosus* ATCC 19246. The absence of 6-deoxytalose was inferred by comparisons with chromatograms obtained from *A. viscosus* ATCC 15987^T and ATCC

FIG. 1. (A) Morphology of strain UCD 81-332-9^T cells in Gram stains prepared from 48-h blood agar cultures. $\times 1,340$. (B) Strain UCD 81-332-9^T cells grown for 48 h in brain heart infusion broth supplemented with 15% fetal calf serum. Diphtheroid cells were observed infrequently. The arrow indicates a swollen filament terminus where spheroplast-like bodies occasionally develop. $\times 162$. (C) Colonies of strain UCD 81-332-9^T grown for 48 h on 5% bovine blood agar plates. $\times 12.5$.

19246 and probably accounts for the one-way cross-reaction observed by Georg et al. (9) between strain ATCC 19246 antisera and strain ATCC 27045 (A1231) antigen. Infections containing this kind of *A. viscosus* were usually of a localized nature and responded well to ampicillin administration.

The remaining 15 isolates lacked rhamnose and 6-deoxytalose in their cell wall carbohydrates and had biochemical and serological characteristics that were distinct from those of previously described species of *Actinomyces*. For these strains we propose the name *Actinomyces hordeovulneris* sp. nov. (hor. de. o. vul' ne. ris. L. n. *Hordeum* genus of grass; L. n. *vulnus* wound, injury; N. L. adj. *hordeovulneris* *Hordeum* injury). A description of this new species is given below.

***Actinomyces hordeovulneris* sp. nov.** Cells are pleomorphic rods and filaments that are 0.5 to 1.0 μ m in diameter and usually exhibit a noticeable degree of branching on blood agar (Fig. 1A). In broth supplemented with 15% fetal calf serum, the filaments are long and extensively branched and have swollen termini (Fig. 1B), where spheroplast-like bodies occasionally develop (data not shown). Dissociation to diphtheroid cells is incomplete in broth cultures.

Surface colonies on bovine blood agar are white, agar adherent, and molar toothed (data not shown, but similar to *A. israelii* [16]), with a tendency to shift to a white, conical, domed, buttery, less adherent type (Fig. 1C) upon laboratory passage. Diameters are 0.5 to 1.0 mm after 48 h at 37°C and 2 mm after 3 days. On clear media (Trypticase soy or brain heart infusion agar plates supplemented with 10 to 20% fetal calf serum) microcolonies after 24 h of incubation at 37°C are regularly filamentous and similar to those of *A. israelii* (16). Young colonies are more white and raised and have smaller diameters than those of the 6-deoxytalose-negative *A. viscosus* strains isolated from dogs.

Growth is weak in unvented or vented Trypticase soy broth cultures unless they are supplemented with serum; it is flocculant with $\geq 5\%$ fetal calf serum. All strains are facultatively anaerobic, but require CO₂ for growth. Acetic acid, lactic acid, and major amounts of succinic acid are detectable upon analysis of 3-ml samples withdrawn from Trypticase soy broth cultures supplemented with 5% fetal calf serum. All strains have weak to moderate catalase activity and are negative in the benzidine test (7). Most strains, including the type strain, are nonhemolytic on bovine blood agar, but a few exhibit weak activity which becomes noticeable after 7 days. Acetylmethyl carbinol and urease are not produced, and nitrates are not reduced. Esculin is hydrolyzed.

All strains produce acid from cellobiose, glucose, lactose,

maltose, trehalose, and xylose. Acid is produced from mannose, melibiose, and raffinose, but production is weak by some strains other than the type strain. All strains fail to produce acid from arabinose, glycerol, mannitol, meso-inositol, rhamnose, and ribose.

Peptidoglycans of all strains contain lysine, alanine, and glutamic acid. Ornithine is present in the walls of most strains. The wall carbohydrates include glucose and galactose. Rhamnose and 6-deoxytalose are absent.

As shown in Table 1, all three strains tested cross-reacted with antisera against *A. israelii* serotype 1 strain ATCC 12102 and *A. viscosus* serotype 1 strain ATCC 15987^T but in titers much lower than homologous levels. The reactions of these dog strains with *A. israelii* serotype 1 antisera exceeded those with *A. viscosus* ATCC 15987^T. No serological reactions were observed with antisera against *A. viscosus* serotype 2 strain ATCC 19246, *A. israelii* serotype 2 strain ATCC 29322, *A. bovis* serotypes 1 and 2, *A. naeslundii* serotypes 1, 2, and 3, *A. odontolyticus* serotypes 1 and 2, *A. suis*, *A. pyogenes* (formerly *C. pyogenes*), *R. dentocariosa* serotypes 1, 2, and 3, and *Rothia*-like strain WVU 1556.

Five of five *A. hordeovulneris* strains tested were susceptible to ≤ 0.25 μ g of penicillin G per ml and ≤ 0.25 μ g of cephalothin per ml, and all were inhibited by 1 μ g of chloramphenicol per ml and 1 μ g of trimethoprim sulfa per ml. Strain UCD 81-332-9^T had a tetracycline minimal inhibitory concentration of 4 μ g/ml, whereas the other four strains tested were susceptible to 0.5 or 1.0 μ g of tetracycline per ml.

The *A. hordeovulneris* strains were isolated from cases of pleuritis, peritonitis, visceral abscesses, septic arthritis, and recurrent localized infections often occurring secondary to tissue-wandering foxtailawns. Three animals died despite ampicillin treatment. A second drug, usually tribrissen, was added to or substituted for ampicillin to achieve favorable responses in other cases due to *A. hordeovulneris*. Occasionally, *A. viscosus* and *A. hordeovulneris* were present in the same sample. The unfavorable responses to ampicillin may be related to a marked tendency to produce L-forms spontaneously with coincident uptake of calcium (A. M. Buchanan and J. L. Scott, submitted for publication).

The guanine-plus-cytosine content of the DNA of *A. hordeovulneris* UCD 81-332-9^T (= ATCC 35275^T) was 67 mol%, compared with 69 mol% for *A. viscosus* ATCC 15987^T. This value for strain ATCC 15987^T is in agreement with the data of Coykendall and Munzenmaier (5), whose calculation was based on the thermal denaturation temperature.

Strain ATCC 35275 (= UCD 81-332-9) is the type strain of

TABLE 1. Fluorescent antibody reactions of canine strains with *A. israelii* and *A. viscosus* antisera

Antiserum ^a	Titer against strain:			Homologous titer ^b
	UCD 80-19-4	UCD 81-154-4	UCD 81-345-981	
<i>A. israelii</i> serotype 1 strains				
WVU 46 (= ATCC 12103)	1:40	1:40	1:40	1:640
WVU 390	1:20	<1:20	1:20	1:160
WVU 30 (= ATCC 10048)	<1:20	1:20	<1:20	1:320
<i>A. israelii</i> serotype 2 strain WVU 307 (= ATCC 29322)	<1:640	<1:640	<1:640	1:1,280
<i>A. viscosus</i> serotype 1 strain WVU 745 ^T (= ATCC 15987 ^T) (hamster)	1:10	<1:5	1:5	1:80
<i>A. viscosus</i> serotype 2 strains				
WVU 371 (= ATCC 19246) (human)	<1:10	<1:10	<1:10	1:40
WVU 398B (human)	<1:10	<1:10	<1:10	1:160

^a Fluorescein-to-protein ratios for antisera to strains *A. israelii* WVU 46 and WVU 307 were 11 and 13 μ g/mg, respectively; ratios for *A. israelii* WVU 30 and WVU 390 were not available. The values for antisera to *A. viscosus* WVU 745^T, WVU 371, and WVU 398B were 36, 22, and 24 μ g/mg, respectively.

^b Homologous titers of the antisera with *A. israelii* and *A. viscosus* cells.

TABLE 2. Characteristics useful for distinguishing *A. hordeovulneris* from other fermentative actinomycetes^a

Taxon	Catalase	NO ³ reduction	Acid produced from:					Xylose
			Cellobiose	Glycerol	m-Inositol	Ribose	Trehalose	
<i>A. hordeovulneris</i>	100 ^b	0	100	0	0	0	100	100
<i>A. viscosus</i> ATCC 15987 ^T	+	+	—	+	+	—	—	—
<i>A. viscosus</i> ATCC 19246	+	+	—	—	+	+	+	—
<i>A. viscosus</i> dog strains ^c	100	100	0	100	100	100	100	0
<i>A. israelii</i>	0	59	87	8	91	100	83	93
<i>A. naeslundii</i>	0	100	35	18	94	35	100	35
<i>R. dentocariosa</i>	100	100	0	14	0	0	0	35
<i>Bacterionema matruchotii</i>	100	100	0	0	0	78	0	0

^a The characteristics of *A. hordeovulneris*, *A. viscosus* ATCC 15987^T and ATCC 19246, and the 6-deoxytalose-negative *A. viscosus* strains isolated from dogs are based on data obtained in this study. The data of Schofield and Schaal (18, 19) were used for *A. israelii*, *A. naeslundii*, *R. dentocariosa*, and *Bacterionema matruchotii*.

^b The values are (number of strains positive divided by number tested) × 100.

^c These strains were the rhamnose-positive, 6-deoxytalose-negative strains identified in this study as *A. viscosus* and *A. viscosus* strain ATCC 27045.

A. hordeovulneris. Characteristics that are useful in distinguishing this new *Actinomyces* species from other fermentative actinomycetes are given in Table 2.

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