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Comparison of Strains of Gram-Negative, Anaerobic, Agar-Corroding Rods Isolated from Soft Tissue Infections in Cats and Dogs with Type Strains of *Bacteroides gracilis*, *Wolinella recta*, *Wolinella succinogenes*, and *Campylobacter concisus*

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A total of 64 strains of gram-negative, asaccharolytic, anaerobic, agar-corroding, rod-shaped bacteria from soft-tissue infections of cats and dogs were compared with other agar-corroding, anaerobic organisms isolated from human periodontal pockets (*Wolinella recta* ATCC 33238^T), bovine rumens (*Wolinella succinogenes* ATCC 29543^T), and gingival crevices of humans (*Bacteroides gracilis* ATCC 33236^T and *Bacteroides ureolyticus* NCTC 10941^T). *Campylobacter concisus* ATCC 33237^T (from human gingival crevices) which did not corrode agar but which biochemically resembled organisms in this group was also included in this study. Although the type strains of *W. recta*, *W. succinogenes*, and *B. gracilis* resembled the animal strains phenotypically and in DNA base ratios, none had bacterial protein patterns (as indicated by isoelectric focusing) identical with the animal strains studied. The animal strains could be divided into motile and nonmotile groups. The motile animal strains were similar biochemically but could be divided into three groups by isoelectric focusing of bacterial proteins. Some had cell wall ultrastructural features identical with *W. recta*; others had the smooth walls of conventional gram-negative organisms. One group of nonmotile animal strains closely resembled *B. gracilis* phenotypically, and they had the cell wall ultrastructure of conventional gram-negative bacteria as described previously (4). The other nonmotile group had cell wall ultrastructure like that of *W. recta*.

During 1978 to 1981, we isolated ca. 64 strains of anaerobic agar-corroding rods from soft tissue infections in dogs and cats. Subsequent investigation suggested that these organisms were unlike any previously described species. Descriptions of 38 of these strains were published subsequently (6), and 26 more strains have also been characterized similarly. For the initial publication, we had been reluctant to ascribe new genus or species names to these isolates. However, when that material was in press, descriptions were published of very similar organisms isolated from humans with periodontal disease (11). The description of *Wolinella recta* sp. nov. (11) closely resembles the motile animal strains that we have previously described (6), and the nonmotile animal strains of our initial study resemble *Bacteroides gracilis* isolated from human mouths (11).

Lai et al. (4) found that the cell wall of *W. recta* differed in ultrastructural appearance from other isolates they investigated, viz., *B. gracilis*, *Campylobacter concisus*, *Eikenella corrodens*, *Bacteroides ureolyticus*, and *Vibrio succinogenes*. During our initial investigations, we had studied the cellular protein patterns of *B. ureolyticus* NCTC 10941 as well as the motile and nonmotile corroding animal strains.

Because the basic descriptions of the corroding, animal strains (6) are similar to those of the human isolates (11), it was considered worthwhile to investigate the ultrastructural appearance of the cell wall of the dog and cat strains, as this may enable interpretation of the protein pattern obtained and thus lead to identification of these organisms to the species level. Subsequently, the type strains of *W. recta* ATCC 33238^T, *B. gracilis* ATCC 33236^T, and *C. concisus* ATCC 33237^T were obtained from A. C. R. Tanner (Forsyth Dental Center, Boston, Mass.) and L. V. Holdeman (Virginia Polytechnic Institute and State University, Blacksburg, Va.), and *Wolinella succinogenes* ATCC 29543^T was obtained from the American Type Culture Collection, Rockville, Md. The protein patterns and phenotypic characteristics of these organisms were compared with those of the animal strains.

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MATERIALS AND METHODS

The 64 animal strains were isolated from cats and dogs with either solitary, closed, subcutaneous abscesses or pyothorax. The organisms were part of a mixed flora of anaerobic and facultative bacteria found at these sites (7, 8). A total of 38 strains have been described in some detail previously (6). The type strain, *B. ureolyticus* NCTC 10941^T, was obtained from the National Collection of Type Cultures, Colindale, United Kingdom; *W. succinogens* ATCC 29543^T was obtained from American Type Culture Collection; and *W. recta* ATCC 33238^T, *B. gracilis* ATCC 33236^T, and *C. concisus* ATCC 33237^T were most generous gifts from A. C. R. Tanner and L. V. Holdeman.

The animal strains were isolated originally on sheep blood agar plates which had been stored under oxygen-free carbon dioxide and incubated at 37°C in a GasPak anaerobic system (BBL Microbiology Systems, Cockeysville, Md.). Organisms were subcultured onto brain heart infusion agar plates (2). Later, the type strains and animal strains were recovered from freeze-dried ampoules and maintained and subcultured on sheep blood agar plates (5% defibrinated sheep blood in blood agar base no. 2; Oxoid Ltd., Basingstoke, United Kingdom) to which 0.1% potassium nitrate and vitamin K, heme, and formate-fumarate (2) had been added. The other cultural conditions for growth and characterization of the animal strains and type strains have been described previ-

ously (6). Colonies were examined for colonial characteristics after a 5-day incubation. All media were supplemented with formate-fumarate. The basal media for fermentation studies were those of Holdeman et al. (2), and carbohydrate fermentation tests were performed in prereduced, anaerobically sterilized medium. Sugars tested were arabinose, cellobiose, esculin, fructose, glycogen, inositol, lactose, maltose, mannitol, raffinose, rhamnose, salicin, starch, sucrose, trehalose, and xylose. Carbohydrate fermentation was determined by measuring the pH of the culture with a carbohydrate after 5 days and comparing it with the pH of the basal medium. Fatty acids were extracted from cooked meat-carbohydrate, peptone-yeast extract (PY) (2), and PY-glucose, PY-threonine, PY-pyruvate, and PY-lactate and analyzed as described previously (5). Every day for 7 days after subculture from blood agar plates, cooked meat-carbohydrate cultures were examined for bacterial motility by use of hanging-drop preparations.

Nitrate reduction, catalase, esculin hydrolysis, and indole were detected as described by Holdeman et al (2), and the urease test was performed with the urea medium of Christensen (with and without formate-fumarate), by the Elek method and the methods of Holdeman et al. (2). Organisms were grown also in prereduced, anaerobically sterilized, nitrate reduction medium (grams per liter): tryptone (Difco Laboratories, Detroit, Mich.), 20; Na_2HPO_4, 2; glucose, 1; KNO_3, 1; and agar, 1 (pH 7.2), to which formate-fumarate and urea (2) had been added. Cultures were incubated for up to 10 days. Parallel cultures in which the rubber stopper had been replaced with a cotton wool bung were incubated in an anaerobic jar for a similar period before samples were tested for ammonia by addition of Nessler reagent, and pH determinations were made on other portions of the culture. *Proteus vulgaris* and *B. ureolyticus* NCTC 10941^T (3), were included as positive controls. Colonies picked from blood agar were tested for oxidase activity by using tetramethyl-*p*-phenylenediamine reagent and the dry filter paper method of Cruickshank (1).

Analysis of the bacterial proteins of 20 of the motile animal strains, 10 of the nonmotile strains, and the type strains were carried out as follows. For each strain, organisms harvested from one 90-mm sheep blood agar plate (sown for maximum all-over growth) were used as the innocula for two 40-ml tubes of prereduced, anaerobically sterilized, nitrate reduction medium to which formate-fumarate, vitamin K, and heme (2) had been added. They were incubated for 48 h at 37°C before being harvested. *C. concisus* ATCC 33237^T grew best when the rubber stopper had been replaced by cotton wool and incubation was completed in a jar in which the final oxygen concentration approached 6%. Organisms were harvested by centrifuging them (20,000 × *g* for 30 min); the pellets were drained completely of supernatant fluid, and the cells were weighed. Pellets were suspended in a quantity of distilled water equal to the wet weight of the cells. The cell suspension was sonicated in a Cup Horn by continuous sonication (sonicator model 375; Heats Systems Ultrasonics, Inc., Plainview, N.Y.) at full power output for 30 min. After sonication, the preparation was centrifuged again at 20,000 × *g* for 30 min, and the supernatant extract (120 to 130 mg of protein per 0.1 ml) was stored at −20°C.

For isoelectric focusing studies, a Multiphor apparatus (no. 2112-301; LKB Instruments Inc., Rockville, Md.) was used in conjunction with a direct current power supply (no. 2103; LKB). The Isogel agarose isoelectric focusing (Marine Colloids, Rockland, Maine) technique was used for preparation and processing of 1-mm-thick gels. Paper electrofocusing electrode strips (no. 2117-106; LKB) were soaked in 1 M H_3PO_4 for the anode and in 1 M NaOH for the cathode and drained of excess solution before careful positioning on the gel surface. A Thermomix II (B. Braun Melsunger AG, West Germany) was used to circulate a melting ice-water solution through the glass cooling plate at the rate of 21 liters/m to control the temperature during the run. Bacterial extracts (5 μl each) were placed into the template slits on the surface of the gel. Samples (1 μl) of Isogel (Marine Colloids) pI markers (pure defined isoelectric point proteins) were included in the runs to determine the pH of bacterial proteins. Confirmation of four of the pH positions was made by using carbonic anhydrase (no. C-2273; Sigma Chemical Co., St. Louis, Mo.) for pH 6.1, β-lactoglobulin and bovine milk (no. L-4756; Sigma) for pH 5.4 and pH 5.5, and ovalbumin (no. A-7642; Sigma) for pH 4.8. When direct comparison of organisms was to be made, the organisms were all run on the same plate. pH markers were included also at regular intervals on the plate to enable pH markers to abut the strains to be compared. This enabled accurate pH determination of individual protein bands and accurate comparison of the strains under investigation. Samples were run on the plates of 10 mA for 20 min. The template was removed, and the plate was run 30 min longer at 25 W. The final voltage was stable at 1,020 V. At the conclusion of the run, the gel was treated as detailed in the Isogel technique notes, using Kenacid blue R (no. 44245; BDH) as the protein stain.

Organisms for ultrastructural investigation were grown anaerobically for 24 h on sterile filter membranes (diameter, 47 mm). Cells were scraped for the filter disks with a bent glass rod and suspended in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Cells were pelleted by centrifugation at 1,000 × *g* for 15 min and fixed for 2 h in 2% glutaraldehyde in cacodylate buffer. The cells were postfixed in 2% uranyl acetate, dehydrated through a graded series of ethanol solutions, embedded, sectioned, and examined in a transmission electron microscope. Bacteria grown in cooked meat-glucose (7) plus formate-fumarate were examined for flagella after they were stained with 2% phosphotungstic acid.

RESULTS AND DISCUSSION

All organisms studied were anaerobic, gram-negative, rod-shaped bacteria. All the animal strains were straight rods (0.5 to 1.0 by 3.0 μm in size) or filaments (up to 13 μm in length), although *W. succinogenes* ATCC 29543^T and *C. concisus* ATCC 33237^T had predominantly curved forms in young cultures. All organisms studied were asaccharolytic and produced only small amounts of fatty acids (mainly acetic and succinic acids), and type and animal strains were identified in biochemical tests as reported elsewhere (6, 11). The major differences found between the dog and cat strains and the type strains studied are presented in Table 1. All animal strains differed from all type strains studied by being weakly oxidase positive and by liquefying gelatin. Animal strains fell into motile and nonmotile groups (Table 1). Thin-section, ultrastructural examination of the cell walls of these organisms subdivided them into two groups: those which resembled the morphology of *W. recta* ATCC 33238^T and those which had the conventional, smooth, trilaminar membranes common to gram-negative bacteria. Thus, on ultrastructural and morphological grounds, the strains could be formed into four groups: (i) motile bacteria with *W. recta*-like cell walls, Veterinary Pathology and Bacteriology (VPB) 3435 and VPB 3365; (ii) motile bacteria with smooth walls,

TABLE 1. Comparison of motile and nonmotile strains of anaerobic, corroding rods isolated from cats and dogs with type strains of *B. ureolyticus*, *B. gracilis*, *W. recta*, *W. succinogenes*, and *C. concisus*

Bacterial strain	Characteristic[a]					
	Flagellum	Corroding colonies	Urease production	Oxidase production	Gelatin liquefaction	DNA guanosine-plus-cytosine ratio
Strains isolated from animals						
Motile	+	+	−	+	+	35–47
Nonmotile	−	+	−	+	+	39–41
Type strains						
B. ureolyticus NCTC 10941^T	−	+	+	+	+	29.28
B. gracilis ATCC 33236^T	−	+	−	−	−	44–46[b]
W. recta ATCC 33238^T	+	+	−	−	−	42–46[b]
W. succinogenes ATCC 29543^T	+	+	−	−	−	46–49[b]
C. concisus ATCC 33237^T	+	−	−	−	−	34–38[b]

[a] All strains were rod shaped in cell morphology. The DNA guanosine-plus-cytosine ratios were measured in molar percents.
[b] These values are from Tanner et al. (11).

VPB 3381; (iii) nonmotile bacteria with *W. recta*-like walls, VPB 3351; and (iv) nonmotile bacteria with smooth walls, VPB 3423.

Each strain described was represented by a distinct band pattern on isoelectric focusing of bacterial proteins (Fig. 1), although regions of band patterns were similar among the motile animal strains and *W. succinogenes* ATCC 29543^T and *W. recta* 33238^T.

Of the five band types studied, colony morphology was the only feature with which band type could be correlated. As stated elsewhere (6), and as shown in Table 1 and by isoelectric focusing patterns (not illustrated), none of the animals strains resembled *B. ureolyticus* NCTC 10941^T. Within group (i), organisms represented by VPB 3435 closely resembled *W. recta* ATCC 33238^T in colony morphology (11); by isoelectric focusing, band sharing was common (Fig. 1). Organisms represented by VPB 3365 (which had a distinct band pattern) had colonies 3 to 4 mm in diameter, were umbonate, and were cream-colored with a matt surface and a corroding edge. Group (ii) organisms had biochemical and cell wall features similar to those of *W. succinogenes* ATCC 29543^T but isoelectric focusing band patterns and cellular morphology which were different from those of *W. succinogenes*. Colonies of these organisms were 1 to 2 mm in diameter, translucent, and dome-shaped with a corroding edge. The colony mass was not depressed below the agar surface, as was that of *W. succinogenes* ATCC 29543^T.

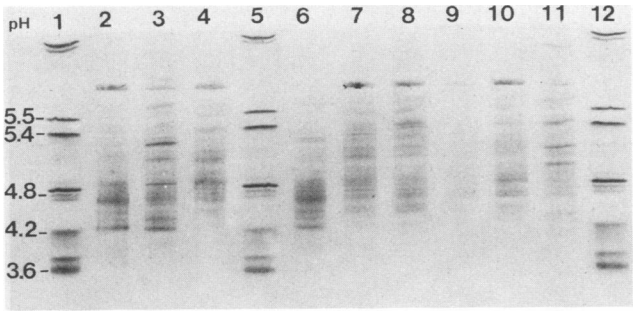

FIG. 1. Isoelectric focusing agarose gel patterns of ATCC type strains and groups of corroding *Bacteroides* organisms isolated from cats and dogs. Lanes 1, 5, and 12, Isogel pI markers (with pH values indicated); lane 2, VPB 3423; lane 3, VPB 3351; lane 4, VPB 3381; lane 6, VPB 3365; lane 7, VPB 3435; lane 8, *W. recta* ATCC 33238^T; lane 9, *W. succinogenes* ATCC 29543^T; lane 10, *B. gracilis* ATCC 33236^T; lane 11, *C. concisus* ATCC 33237^T.

Group (iii) organisms had ultrastructural features of *W. recta* 33238^T but were nonmotile and differed from *W. recta* 33238^T in colony morphology. Colonies of this group, as represented by VPB 3351, were 1 to 2 mm in diameter, orange-pink umbonate colonies with a shiny mucoid center. A 1- to 2-mm corroding edge surrounded the colony. Isoelectric focusing band patterns, also, differed from those of *W. recta*. Despite extensive and intensive measures to try to observe motility in these organisms, we were unable to do so. Group (iv) organisms, represented by VPB 3423, resembled *B. gracilis* ATCC 33236^T (11) in biochemical and cell wall morphology, although their isoelectric focusing pattern and colonial morphology were different from the type strain. Colonies of these organisms were 1 to 2 mm in diameter, dome-shaped, orange-pink, and were surrounded by a 0.5-mm-wide corroding edge. No group resembled *C. concisus*. Despite that these corroding organisms produced noncorroding variants (11) and despite the observation that the colony morphology of some of the larger, swarming colonies changes with age, it seems from this work that it may be possible to recognize different corroding animal species by observation of colony morphology. This possibility is strengthened by the association of isoelectric focusing patterns of bacterial proteins with colony morphology characteristics. However, if some of the animal strains, such as group (i) organisms, have colony morphology similar to those of type strains but differ in isoelectric focusing patterns, one must consider that the colony morphology may not be a reflection of the band differences seen. Also, analysis of bacterial proteins by isoelectric focusing may be too sensitive, i.e., producing too much division for this group of organisms. The technique has been used most successfully to compare asaccharolytic, pigmented *Bacteroides* spp., and analysis of similar extracts by polyacrylamide gel electrophoresis has had extensive application (10). Despite the apparent sensitivity of isoelectric focusing, cell wall ultrastructures do not produce dominant band sharing in isoelectric focusing patterns. This is not surprising, as the preparation used is unlikely to contain significant amounts of cell wall proteins (9). It seems, however, as illustrated by group (iii) organisms that it may not be helpful within corroding organisms to use cell wall ultrastructure to identify *W. recta* to the species level.

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