Anaerobiospirillum, a New Genus of Spiral-Shaped Bacteria

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Three strains of an anaerobic spiral-shaped bacterium that possessed tufts of bipolar flagella were isolated from beagle dogs. The organisms are gram negative and saccharolytic and produce mainly acetic and succinic acids from glucose. The cells of these strains do not contain spores or axial fibrils. The isolates do not fit any described genus or species of anaerobic spiral-shaped bacteria. The generic name *Anaerobiospirillum* gen. nov. is proposed for the isolates; at present, the genus contains only one species, the type species *Anaerobiospirillum succiniciproducens* sp. nov. *A. succiniciproducens* strain S411 (= ATCC 29305) is designated as the type strain of the species. The guanine plus cytosine content of the deoxyribonucleic acid of the type strain is 44 mol%.

Spiral-shaped bacteria have been detected in rats (9, 10), mice (9, 15, 19), cows (5), cats (13, 19), dogs (14, 18, 20), pigs (11), monkeys (22), and humans (17, 21). They can colonize suckling mice and remain associated with the animal well into its adulthood (8). Large populations of spiral-shaped microbes have been found in the crypts of Lieberkühn in rats and in a layer adjacent to the colonic and cecal epithelium of mice (9, 10, 19). Occasionally, spiral-shaped microbes can be cultivated from the animals listed above, but the isolates often either are not identified or die on subculture (19). The notable exceptions are butyri-vibrios, succinivibrios, and members of the family *Spirochaetaceae* isolated from pigs and cows (4, 5, 11).

Consequently, when a spiral-shaped bacterium was isolated during a study of the bacterial flora of dogs raised in a "locked environment" (3), we decided to characterize the isolate and compare it to members of known genera.

MATERIALS AND METHODS

Animals. Nine purebred male beagles were obtained from a closed colony at the Argonne National Laboratory, Argonne, Ill. Seven of the dogs were housed and maintained in a "locked environment" in germfree dog isolators described elsewhere (3). Two of the dogs were housed in the regular dog-holding facilities at the University of Wisconsin. All dogs were fed a steam-sterilized diet (Purina Dog Meal, Ralston Purina Co., St. Louis, Mo.) and water ad libitum. Four dogs were removed from the isolators 1 month prior to the time they were killed by an overdose of phenobarbital. Two of the four dogs were fed the sterilized diet and the other two were fed the same diet, but unsterilized, ad libitum while they were housed in the regular dog-holding facilities at the University of Wisconsin. All four dogs were given nonsterilized water ad libitum.

Microbial isolation techniques. Dry, prereduced, sterile cotton swabs were used to culture the nose and throat. Fresh fecal matter was removed from the dog's rectum, while under mild anesthesia, with a sterile stainless steel spatula. Culture swabs and fecal matter were immediately placed into tubes of a prereduced transport broth (2), which were gassed out with oxygen-free CO₂ to reduce the oxygen level that was introduced when the tubes were opened to admit the samples. These tubes were transported to the laboratory within 3 h and processed in the glove box as described below. The transport tubes with fecal material or swabs were then put into an anaerobic glove box (1) through a rapid-entry port where serial 10-fold dilutions were made in prereduced transport broth. At that time, 0.1 ml of each dilution (10⁻¹ to 10⁻⁹) was plated onto prereduced A II agar (1), and the plates were incubated at 37°C in a Plexiglas incubator especially designed for the glove box (UW Machine Shop, Madison, Wis.).

Samples of tissue from stomachs, ilea, ceca, and colons were obtained by clamping the tissue with hemostats, cutting out about 1 cm² from the clamped-off portion of the intestine, and quickly placing the excised tissue in prereduced transport broth (2). Ileal and colonic tissues, used for culture and microscopy, were removed from the region of the bowel about 20 cm proximal and distal to the cecum while the stomach tissue was removed from the cardiac region. The tissues, after they were placed in the glove box, were homogenized in a blender, diluted, and plated as described above.

Media. A II agar and broth media were prereduced in the glove box for 48 to 72 h prior to use (1). Transport broth and dilution medium were composed of a solution of Trypticase soy broth without glucose but with Na₂CO₃ and cysteine-HCl (2). Tubed transport-broth dilution medium was prepared, sterilized in screw-capped tubes, put into an anaerobic glove box (80% N₂, 10% H₂, 10% CO₂) via an airlock (2), and allowed to reduce, with screw caps loose, for at least 48 h prior to use. After 48 h, the screw caps were replaced with sterile rubber stoppers and, for the transport tubes (Kontes, 2.2 by

14 cm), screw caps were used to tighten down the rubber stoppers. The latter tubes were used to acquire specimens from dogs in germfree isolators. A II medium, a complex nonselective agar medium (1), commercially prepared blood agar (Gibco), and PYG broth were placed in the glove-box atmosphere for 48 h prior to use. PYG broth (12) was the medium in which the spiral-shaped microbe was grown to determine both the moles percent guanine plus cytosine (G+C) content of the deoxyribonucleic acid (DNA) and the volatile and nonvolatile fatty acids produced from glucose.

Biochemicals. Volatile and nonvolatile fatty acids produced in PYG broth were identified with a gas chromatograph (Dohrman, Anabac, Mt. View, Calif.) (12). Other biochemical tests were done by a modification of the Minitex procedure (BBL, Cockeysville, Md.) as follows: the Minitex broth was prerduced in the glove box as previously described, and the broth was inoculated with a loopful of bacteria transferred from an A II or blood agar plate. Other media, such as litmus milk, chopped-meat glucose, gelatin, and egg-yolk agar, were inoculated with bacteria grown on A II or blood agar plates. All biochemical tests were carried out at 35° to 37°C in the glove box.

Microscopy. Gram-stained bacteria and bacteria observed by phase optics were viewed with a Zeiss Universal microscope (C. Zeiss, Federal Republic of Germany). In addition, pieces of colonic and cecal epithelium were sectioned with a cryostat, stained with a tissue Gram stain as previously described (7), and examined with light and phase optics.

Cultures incubated for 24 h at 37°C in PYG broth were centrifuged and fixed in cold (4°C) 2.5% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.2) for transmission electron microscopy. The bacteria were either prepared for phosphotungstic acid staining or embedded in Spurr medium for thin sectioning as described previously (9). All specimens were examined with a Hitachi HU-8 electron microscope.

For scanning electron microscopy (SEM) of dog cecal and colonic epithelium, tissue pieces were cut from the dogs, frozen, fixed, dehydrated, critical-point dried, and attached to stubs by a previously described method (7). Specimens were examined in a JEOL JEM U3 scanning electron microscope at 20 kV.

G+C content of DNA. The G+C content of the DNA was determined by the thermal melting point (T_m) of the DNA (15). *Escherichia coli* B DNA was used as a standard.

Culture preservation. Cultures were grown in a prerduced enriched milk medium in the anaerobic chamber for 18 to 48 h. The milk medium (10 ml/tube) was made from 52 g of litmus milk (Difco) and 375 ml of distilled water. The screw caps of the test tube cultures were tightened in the chamber, and the tubes were removed, immediately placed in liquid nitrogen for about 2 to 3 min, and then placed into a -70°C freezer. The isolates were also freeze-dried, as follows. A small piece of blood agar (about 2 by 5 mm) containing the cells on its surface was cut from the plates and put into a sterile vial. One milliliter of sterile, prerduced, enriched milk me-

dium was added, the vial was sealed with a rubber stopper (9-mm [outer diameter] stoppers were made by cutting no. 9 stoppers with a stopper borer), and the milk medium and agar were mixed on a Vortex Genie mixer (Scientific Products, Evanston, Ill.) for 15 s in the glove box. The vials were removed from the glove box and placed in liquid nitrogen for about 2 to 3 min, and the contents in the vials were then freeze-dried with a freeze-drying apparatus (New Brunswick Scientific, New Brunswick, N.J.).

RESULTS

Spiral-shaped bacteria were observed by phase microscopy of wet mounts prepared from three throat swabs and two fecal samples from five different dogs. The spiral-shaped bacteria were also detected in seven of nine dogs by phase microscopy of cecal homogenates or of cecal and colonic tissue sections and in four of nine dogs by SEM of cecal and colonic tissues (Fig. 1). Two of the nine dogs consistently showed no spiral-shaped organisms in throat swabs, fecal samples, or tissue preparations while housed in the locked environment or in the conventional dog-holding facility. Spiral-shaped organisms were cultivated on A II agar from five of the nine dogs. To obtain pure cultures of the spiral-shaped organism, a large number (over 50) of individual colonies from the A II agar plates had to be picked and examined because the colonial morphology of the spiral-shaped bacteria was not easily distinguishable from those of the other bacterial colonies present on the plate. Total counts of spiral-shaped organisms varied from undetectable to 10^5 to 10^7 per ml or per g (dry weight) as determined by direct microscope counts and by colony counts, respectively. Total counts of all anaerobes varied in each dog, but usually ranged from 10^8 to 10^{10} per g of dry weight of tissue and contents of large bowel. Strain S411 was obtained from the throat and strains S10 and S39 were obtained from a cecal homogenate.

Morphology. Microscope examinations of Gram-stained cells and of wet mounts (phase microscopy) of both young (18-h-old) and older (24- to 48-h-old) cultures showed that the cells were gram negative and spiral shaped. The organism maintained these characters after 6 months of weekly and biweekly subculture on blood agar (Fig. 2). In PYG broth, however, straight rods and spherical forms were intermingled with the spiral-shaped cells (Fig. 3). As the PYG broth culture aged, the former became more numerous, and they comprised the major portion of the population after 24 to 48 h. Rods and spherical forms were also seen in smears obtained from isolated colonies on A II or blood agar plates, but their incidence was far

FIGS. 1-8

less than the incidence of these cells in PYG broth cultures. No refractile bodies (spores) were ever observed with phase-contrast microscopy.

Strains S411, S10, and S39 were examined for motility by phase-contrast microscopy. Strain S411 showed no motility on initial isolation from A II agar. However, S411 demonstrated motility after freeze-dried or frozen cultures of this strain were revived. Strains S10 and S39 have shown motility ever since they were isolated. All of the cultures of this organism grown on A II medium and blood agar plates and in PYG broth (less than 48 h of incubation) showed cells with a corkscrew-like motion. The rod forms of all three strains often were motile, although it was difficult to determine if they possessed a corkscrew-like motion. The circular forms of each strain rarely showed a clockwise and counterclockwise spinning motion.

Cells were 0.6 to 0.8 μm wide and 3.0 to 8.0 μm long. Occasionally, some cells reached a length of 20.0 μm . The helix diameter was 0.9 to 1.1 μm and the wavelength was about 1.3 to 1.7 μm . Phosphotungstic acid (PTA)-stained bacteria showed a tuft of flagella at both poles. Rod-shaped and circular forms were also observed in the PTA-stained preparations and in resin-embedded preparations (Fig. 4 and 5). In most cases, the rod form resembled the spiral form in its ultrastructural characteristics (Fig. 5). Thin sections of the organism showed that the cytoplasm was bound by a membrane which was also separated from the environment by another structure resembling a membrane (Fig. 6). The cell wall was difficult to discern, and it may have been juxtaposed to the outer layer of the membrane (Fig. 5). The circular forms showed a less electron-dense cytoplasm. The unsheathed flagella were found to extend from the cytoplasm through the membranes to the outside of the bacterium (Fig. 7 and 9). The

FIG. 9. Tuft of flagella on a spiral-shaped cell of strain S411. Note how some of the unsheathed flagella are matted together. Bar equals 0.1 μm . Magnification, $\times 65,700$.

flagella were 14 nm in diameter and varied in length from about 4 to 6 μm . The flagella were arranged in tufts (about 16 flagella per tuft) and often appeared matted together (Fig. 9). The flagellar wavelength was not determined because the flagella showed no consistent wavelength in our preparations. No axial fibrils were observed in either PTA-stained samples or in sectioned preparations.

Cultural characteristics. The strains were obligately anaerobic; they would not grow on blood agar plates incubated in air or in 5% CO_2 .

Strain S411 produced small, circular, convex, translucent colonies on both A II medium and blood agar incubated at 37°C in a glove box (Fig. 8). After 3 days of incubation, the colonies obtained a diameter of 0.5 to 1.0 mm. Strains S10 and S39 produced colonies similar to those of strain S411 except that the colonies of the former strains possessed a raised center or a

FIG. 1. Scanning electron micrograph of the bipolar tufts of flagella on a spiral-shaped organism adjacent to the colonic epithelium of a dog. Magnification, $\times 9,100$.

FIG. 2. Phase micrograph of cells of strain S39 scraped from a blood agar plate and suspended in PYG broth. Magnification, $\times 1,100$.

FIG. 3. Phase micrograph of the rod and spherical (arrows) forms of the spiral-shaped organism (strain S411) after 24 h of growth in PYG broth. Magnification, $\times 2,190$.

FIG. 4. PTA-stained preparation of spherical (arrow), rod, and spiral forms of strain S411. Note that all of the forms have flagella. Magnification, $\times 5,270$.

FIG. 5. Thin section of spherical (a), rod (b), and spiral (c) forms of strain S411. Note that the spherical form has a less electron-dense cytoplasm than has the rod or spiral form. Arrow shows the flagella on the spiral form. Magnification, $\times 13,600$.

FIG. 6. Outer membrane (double arrows) and inner membrane (single arrow) of a spiral-shaped cell of strain S411. Magnification, $\times 36,800$.

FIG. 7. Arrows indicate where the unsheathed flagella penetrate the outer membrane of a spiral-shaped cell of strain S411. Magnification, $\times 76,800$.

FIG. 8. Colonies of strain S411 on prereduced blood agar. Magnification, $\times 4$.

small button in the center. The strains grew well at 25°, 37°, and 40°C. However, growth at 25°C was much slower than that at 37° or 40°C. Growth did not occur below 18° or above 50°C. In unstirred tubes of PYG broth incubated at 37°C, the organism's generation time was calculated, by viable counts on blood agar, to be 2.5 h. The cells did not survive 80°C for 10 min.

Table 1 shows the results of the fermentation and biochemical tests.

Growth in PYG yielded acetic and succinic acids as the only major products detected by gas-liquid chromatography. All of the strains showed, however, a trace of lactic acid, and strains S10 and S39 showed a minor peak of formic acid; strain S411 showed no formic acid peak.

The strains cultivated in prereduced enriched milk medium survived a quick freeze in liquid nitrogen (2 to 3 min) followed by storage

at -70°C. If the frozen culture was quickly introduced into the glove box and if a few chips of the frozen suspension were removed and spread onto blood agar, the microbes grew well after suitable incubation. The tube containing the frozen culture could be quickly removed from the glove box before the suspension thawed, refrozen at -70°C, and used again. Strain S411 survived for over 6 months when stored at -70°C. The organism survived freeze-drying if the culture to be freeze-dried was prepared by suspending a small block of blood agar containing viable organisms in sterile, prereduced, enriched milk medium. Attempts to recover viable cells from freeze-dried preparations of cells suspended in sterile PYG broth or prereduced enriched milk medium were not successful.

The G+C content of the DNA of strain S411 was 44 mol%.

TABLE 1. Biochemical characteristics of strains of *Anaerobiospirillum succiniciproducens*^a

Tests	Reactions of strains		
	S411	S10	S39
Fermentation of:			
Adonitol	-	-	-
L-Arabinose	-	-	-
Cellobiose	-	-	-
Dulcitol	-	-	-
Esculin	-	-	-
Fructose	+	-	+
Galactose	-	-	-
Glucose	+	+	+
Glycerol	v	-	-
Inositol	v	-	-
Lactose	+	+	+
Maltose	v	-	+
Mannitol	-	-	-
Melibiose	v	-	-
Raffinose	v	+	+
Rhamnose	-	-	-
Salicin	-	-	-
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	v	-	-
D-Xylose	-	-	-
Catalase production	-	-	ND
Gelatin liquefaction	-	-	-
Indole production	-	-	-
Lecithinase production	v	-	-
Lipase production	-	-	-
Meat digestion	-	-	-
Milk curdling	v	-	-
Nitrate reduction	-	-	-
Starch hydrolysis	v	+	ND
Urease production	-	ND	ND

^a Symbols: +, Acid was produced or test was positive; -, no acid produced or test was negative; v, repeated tests gave variable results; ND, test not done.

DISCUSSION

The organism described above, a gram-negative, spiral-shaped anaerobe with bipolar flagellar tufts and no axial fibrils, does not correspond, to our knowledge, to the members of any of the previously described genera of bacteria. Its characteristics also do not permit it to be placed in any of the presently known families of bacteria. However, there may be some affiliation of this organism with the family *Spirillaceae* (6) because of its shape, flagellation, and G+C content. Furthermore, some species of spirilla produce spherical bodies (6), as does this organism. The principle differences that separate this organism from members of the family *Spirillaceae* are: no growth under aerobic or microaerophilic conditions, fermentation of carbohydrates in the absence of oxygen, and production of fatty acids easily detected by gas chromatography. Consequently, we feel that the microbe should not be placed in the family *Spirillaceae*.

The spiral-shaped organism also shares characteristics with the genus *Succinivibrio* (5). Both are gram-negative, strictly anaerobic, curved rods that ferment carbohydrates with acetic and succinic acids as the major products of glucose fermentation. Furthermore, some *succinivibrios* and the spiral-shaped organism, when cultured on artificial media, may become straight or slightly curved; also, swollen forms can occasionally be seen. *Succinivibrios*, however, have monotrichous cells whereas the spiral-shaped organism has tufts of flagella at both poles (5). Although this is the main difference between our organism and *succinivibrios*, there are other differences: our organism ac-

tively ferments lactose and weakly hydrolyzes starch whereas none of the succinivibrios described to date ferments sucrose or hydrolyze starch. In addition, our organism does not ferment xylose, arabinose, or mannitol as do the presently described succinivibrios (5). Consequently, we feel that our isolate should not be classified as a member of the genus *Succinivibrio*.

We believe that our isolate represents a new genus, for which we propose the name *Anaerobiospirillum* (An.a.e.ro.bi.o.spi.ril'lum. Gr. pref. *an* not; Gr. n. *aer* air; Gr. n. *bios* life; M.L. dim. neut. n. *spirillum* a small spiral; M.L. neut. n. *Anaerobiospirillum* anaerobic small spiral).

A description of this genus follows. Gram-negative helical rods, 0.6 to 0.8 μm wide and 3.0 to 8.0 μm long, with rounded ends; some cells reach 20 μm in length. The helix diameter is 0.9 to 1.1 μm and the wavelength is about 1.3 to 1.7 μm . A tuft of flagella is present on both ends of the cells, which show a corkscrew-like motility. Cells usually occur singly. Rod-shaped and circular forms are found most frequently in broth cultures. Endospores are not produced. Carbohydrate metabolism is fermentative. Some strains may fail to ferment various carbohydrates; for example, S10 does not ferment fructose, but S411 and S39 do. In addition, S411 gives variable results with several carbohydrates. The major products from glucose metabolism are succinic and acetic acids, but traces of lactic and formic acids may also be formed. Strictly anaerobic. Found in throats and colons of dogs. The type species is *Anaerobiospirillum succiniciproducens* sp. nov.

A. succiniciproducens sp. nov. (suc.cin.i'ci.pro.du'cens. M.L.n. *acidum succinicum-succinic acid*; L. pres.part. *producens* producing; M.L. part. adj. *succiniciproducens* intended to mean producing succinic acid as a major fermentation product).

The description of the type species is the same as that for the genus. The type strain is *A. succiniciproducens* S411; a culture of this strain was deposited in American Type Culture Collection (ATCC) Rockville, Md., under the number 29305. The description of the type strain is the same as that of the species; the biochemical characteristics of the type strain are presented in Table 1.

Pindak et al. (17) described, on the basis of morphology, three types of spirochetes—borellias, treponemes, and double-contoured spirochetes—from the feces of beagles. Fifty-four beagles were examined, and about 80% had more than one type of spiral-shaped bacterium in their feces (4% of the dogs were free of spiral-

shaped bacteria). The types they described as borellias or treponemes resemble the spiral-shaped organisms we observed; we did not observe double-contoured spirochetes in the beagles we examined. Pindak et al. did not culture the organisms they observed. The organism we isolated resembles one of the microbes they designated as a treponeme (17).

Furthermore, our organism resembles a spirillum found in the gastric glands of cats (13). However, in contrast to this gastric spirillum, our organism has non-sheathed flagella, which are inserted into the plasma membrane region, and a less-tight helical coil. To our knowledge, these gastric spirilla have not been cultured or identified.

We observed spiral-shaped organisms in seven of the nine dogs we examined. The two dogs that did not possess these organisms were noted to be actively coprophagic. The recycling of fecal matter may have greatly influenced the colonization of spiral-shaped organisms in these two dogs and reduced their numbers to an undetectable level. Alternatively, these dogs may not have possessed the organisms before they were placed in the locked environment.

Although spiral-shaped bacteria can be detected in the normal biota of many mammalian species, including man, in many cases they have not been cultured or identified. We believe that the isolation and characterization of one of these organisms is the first step toward the understanding of the role of these bacteria in gastrointestinal ecosystems.

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REPRINT REQUESTS

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