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Enumeration and Selective Isolation of Rumen Spirochetes

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Enumeration by means of light microscopy showed that from 0.4×10^8 to 2.0×10^8 spirochetes were present per ml of bovine rumen fluid. Viable cell counts yielded slightly lower values, ranging from 0.1×10^8 to 1.2×10^8 spirochetes per ml of rumen fluid. The antibiotic rifampin, which served as a selective agent for rumen spirochetes, was added to agar media used in the estimation of viable spirochete numbers in rumen fluid. Morphologically diverse spirochetes were isolated from rumen fluid by means of a procedure involving the use of rifampin as a selective agent in agar media. The strains isolated represented seven morphological types of spirochetes differing in cell size, cell coiling pattern, and number of periplasmic fibrils per cell. Electron microscopy showed that the number of periplasmic fibrils present in the different morphological types of rumen spirochetes ranged from 2 to more than 20 per cell. The results of this study indicate that the bovine rumen is a highly favorable environment for a number of morphologically diverse spirochetes.

It has been known for many years that spirochetes are present among the microorganisms that inhabit the rumen (3, 8). However, only a limited amount of information is available on the population densities of spirochetes in the rumen and on the extent of morphological and physiological diversity that exists among these bacteria.

In the studies described in this article we have determined population densities of spirochetes in bovine rumen fluid. Furthermore, we developed a selective procedure for the isolation of rumen spirochetes and used it to obtain morphologically different strains of these microorganisms from rumen fluid. The ultrastructure of the isolates was examined by means of electron microscopy. Our main intent was to obtain information important for the interpretation of the ecological properties of spirochetes that inhabit the rumen.

MATERIALS AND METHODS

Culture conditions and media. All media were prereduced, and all cultures were incubated anaerobically in CO_2 atmosphere. Methods used for preparation of prereduced media and for anaerobic culturing were described by Hungate (10). Incubation temperature was 39°C.

Medium RFC, used for viable cell counts and for the isolation of spirochetes, contained: clarified rumen fluid, 30 ml; salts solution A, 20 ml; salts solution B, 20 ml; resazurin (0.1%, wt/vol), 0.1 ml; distilled water, 19 ml; L-cysteine $HCl \cdot H_2O$, 0.1 g; Difco Noble agar (for agar deeps), 0.7 g. Salts solution A contained (grams per 1,000 ml of distilled water): $CaCl_2$, 0.45; $MgSO_4$, 0.45. Salts solution B contained (grams per 1,000 ml of distilled water): KH_2PO_4, 2.25; K_2HPO_4, 2.25; NaCl, 4.5; $(NH_4)_2SO_4$, 4.5. The medium was heated under a CO_2 atmosphere until the resazurin indicator became colorless and the agar, when present, was dissolved. Then, the solution was distributed into anaerobic culture tubes (18 by 142 mm; Bellco Glass Inc.; 8.9 ml of solution per tube) as the tubes were being flushed with CO_2. The tubes were sealed with green Neoprene stoppers and were autoclaved. Subsequently, 1 ml of a filter-sterilized $NaHCO_3$ solution (5%, wt/vol) and 0.1 ml of a filter-sterilized cellobiose solution (10%, wt/vol) were added to each tube. The final pH of the complete medium was 6.7 to 7.0. Rifampin, added to medium RFC in some experiments, was dissolved (10 μg/ml) in the 5% $NaHCO_3$ solution that served as a component of this medium. A liquid medium used to grow spirochetes and other rumen bacteria was identical in composition to RFC medium, except that the agar was omitted and the final cellobiose concentration was 0.2 g/100 ml.

Rumen fluid used in culture media was processed as follows. Rumen fluid, freshly collected from cows maintained on a diet of corn silage, was filtered through two double thicknesses of cheesecloth, bottled, and stored at −25°C. Before use, the frozen rumen fluid was thawed and then clarified by centrifuging it three times at $4,100 \times g$ for 20 min (5°C) and discarding the sediments.

Strain 606, a large treponeme described by Ziolecki (16), did not grow in RFC medium and was cultured in a pectin-containing medium similar to that described by Tomerska (14).

Rumen fluid samples. Rumen fluid samples for bacterial counts were obtained from six fistulated cows housed at the University of Massachusetts Dairy Fa-

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cility, South Deerfield, Mass. and at the Cornell University Dairy Facility, Ithaca, N.Y. The three University of Massachusetts animals (Holstein breed) had been maintained on a mixed diet, but were fed corn silage during the time this investigation was conducted. Two of the Cornell University animals (Holstein breed; cows D and E, Table 1) were fed a diet of timothy hay, while the other (Ayrshire breed; cow F, Table 1) was on a grain diet.

Rumen samples were collected at random times, i.e., without consideration of the animals' feeding times. Rumen fluid was sampled by making depressions in the matted plant material occupying the dorsal region of the rumen and collecting the liquid that filled the depressions during ruminal contractions. In this article, liquid collected as described above is referred to as "rumen fluid." Immediately after collection, rumen fluid samples were added to culture tubes (25 by 142 mm). The tubes, approximately 3/4 full, were tigthly stoppered and placed in an ice bath for transport to the laboratory. Within 30 min of sampling, the air atmosphere in the tubes was replaced with O_2-free CO_2, and thereafter the samples were always maintained in CO_2 atmosphere at 5°C. The rumen fluid samples were used for viable cell counts immediately upon their arrival in the laboratory (approximately 30 min after the time of collection). Direct counts by light microscopy were performed within 24 h from the time of sample collection. During this 24-h period there were no detectable changes in the numbers of spirochetes observed microscopically in rumen fluid samples maintained in CO_2 atmosphere at 5°C. Stoppered culture tubes containing rumen fluid samples were inverted three to four times to mix the rumen contents just before direct and viable cell counts were made.

Direct counts. Direct counts of spirochetes in rumen fluid were performed as follows. A 5-μl volume of rumen fluid was placed on a microscope slide and covered with a glass cover slip (22 by 22 mm; Corning no. 1). The cover slip was pressed gently so that the rumen fluid spread and filled the space between cover slip and slide. The average number of spirochetes per microscope field in these wet-mount preparations was determined using a phase-contrast microscope at ×1,250 magnification. Spirochete cells in 25 microscope fields were counted for each wet-mount preparation. The spirochetes were recognized according to criteria discussed in the Results section. Spirochetes in a minimum of three wet-mount preparations were counted per rumen fluid sample (75 microscope fields). Spirochete population densities (spirochetes per milliliter of rumen fluid) were determined using a standard curve relating the number of spirochetes per microscope field to the number of spirochetes per milliliter. The standard curve was obtained by counting the number of spirochetes per microscope field in wet mounts prepared from dilutions of a spirochete culture (strain RUS-1, isolated from rumen fluid). The number of spirochetes per milliliter in each culture dilution was known, having been determined by using a Petroff-Hausser counting chamber.

Viable cell counts. Numbers of viable spirochetes and other bacteria were determined by means of a procedure involving inoculation of rumen fluid (serial dilutions) into test tubes partially filled with RFC agar medium. In many experiments, rifampin (3-[4-methylpiperazinyl iminomethyl]-rifamycin SV) was included (1 μg/ml, final concentration) in the medium as described above. After 36 to 48 h of incubation at 39°C, the colonies that had developed in these agar medium deeps were counted. Spirochete colonies in the agar medium deeps were readily recognizable because they were spherical and increased in size by diffusing characteristically through the agar medium. Thus, different spirochete colonies resembled in appearance a "cotton ball," a "transparent bubble," or "veil-like growth with a denser center." Spirochete colonies were usually white, except for cotton-ball colonies 48 h old or older, which occasionally were yellow.

The colonies with the morphologies described above did not always consist of spirochetes. Cells from representative colonies that developed from each sample were examined microscopically, and adjustments were made in each viable count to correct for colonies resembling those formed by spirochetes, but consisting of other bacteria. During the course of these investigations, we examined, by phase-contrast microscopy, cells from 63 spherical colonies (with the appearance of spirochete colonies) growing in rifampin-containing RFC agar medium deeps inoculated with different rumen fluid samples. Approximately 8% of those colonies consisted of bacteria other than spirochetes.

Numbers of viable spirochetes in the rumen fluid samples were estimated by using agar medium deeps each containing from 25 to 100 spirochete colonies.

Isolation of rumen spirochetes. Rumen spirochetes were selectively isolated as follows. Rumen fluid samples were serially diluted into RFC agar medium deeps containing rifampin (1 μg/ml, final concentration). After incubation at 39°C, samples of spherical colonies typical of spirochetes (described above) were removed from the agar deeps by stabbing a Pasteur pipette through each selected colony. Growth in the agar plug removed with the pipette was examined by phase-contrast microscopy. If spirochetes were present, a portion of the sample was transferred to a deep of melted (45°C) RFC agar medium, which was then mixed (CO_2 atmosphere) using a Vortex mixer. This suspension was used to make serial dilutions into RFC agar medium deeps. Cells from spirochete colonies that developed in the RFC agar medium deeps were cloned (three successive transfers in RFC agar medium deeps).

Microscopy. A Zeiss GFL phase-contrast microscope was used for light microscopy observations and for direct cell counts. Photomicrographs were taken through this same microscope equipped with a Leitz Mikas camera attachment and a series M1 Leica camera body. Images were recorded on Kodak Panatomic-X film.

Spirochetes to be examined by electron microscopy were harvested from cultures in the exponential phase of growth. Cell suspensions were prepared by centrifuging 5 ml of culture, removing 4.5 ml of supernatant fluid, and resuspending the cell pellet in the remaining 0.5 ml of culture medium. A drop of this suspension was placed on a 300-mesh, collodion-coated, carbon-reinforced copper grid. After 90 s the drop was drawn off by gently touching it with tissue paper. Cells re-

maining on the grid were negatively stained by placing a drop of phosphotungstic acid (1.0%, wt/vol; pH 6.5) on the grid. After 10 s the liquid was removed from the grid with tissue paper. Specimens were examined in a Philips EM 200 electron microscope equipped with a 30-μm objective aperture and operating at 60 kV. Images were recorded on 35-mm Kodak Fine Grain Release Positive Film 5302.

Rifampin sensitivity assay. Rumen bacteria tested for rifampin sensitivity included strains isolated in our laboratory (*Bacteroides succinogenes* CAO and spirochete strains RHO-2, 8C, 6D, 6A, and RUS-1); strains supplied by R. B. Hespell, Department of Dairy Science, University of Illinois, Champaign-Urbana, Ill. (*Succinivibrio dextrinosolvens* 24, *Streptococcus* sp., and spirochete strains 606, $B_2$5, JZ-2, and JZ-3); and strains provided by M. J. Wolin, Division of Laboratories and Research, New York State Department of Health, Albany, N.Y. (*Ruminococcus albus* 7).

Except for spirochete strain 606, all bacteria were grown in RFC liquid medium and then inoculated by stabbing into RFC agar medium deeps with or without added rifampin (1 μg/ml, final concentration). Pectinolytic spirochete 606 was grown in a pectin-containing medium (see above) and was tested for rifampin sensitivity in the same medium. Bacterial strains were considered sensitive to rifampin if their growth was inhibited (determined by visual examination) in rifampin-containing medium as compared to growth in the same medium lacking rifampin.

RESULTS

Enumeration of spirochetes. In direct enumerations by light microscopy, only helical bacteria that had gross morphology typical of spirochetes (7) were counted (Fig. 1). Helical cells that were motile were counted only if their movements were characteristic of spirochetes (5). The accuracy of these criteria for recognizing spirochetes was tested as follows. Helical bacteria that had the gross morphology of spirochetes, as determined by light microscopy, were isolated from rumen fluid. Twelve helical isolates were examined by electron microscopy. Included among these isolates was every morphological type of helical bacterium regarded as a spirochete during light microscopy examination of rumen fluid. It was found that all 12 isolates had ultrastructural features typical of spirochetes, i.e., periplasmic (axial) fibrils wound around the protoplasmic cylinder, and an outer sheath. These results indicated that spirochetes in rumen fluid could be recognized by light microscopy observations with reasonable accuracy.

From 0.4×10^8 to 2×10^8 spirochetes per ml were present in samples of rumen fluid, as determined by light microscopy enumerations (Table 1). Each rumen fluid sample examined contained from three to five morphologically diverse types of spirochetes, differing in cell size and in cell coiling.

When rumen fluid was inoculated (serial dilutions) into RFC agar medium, many bacterial colonies developed during incubation, but usually the ratio of the number of spirochete colonies to the total number of bacterial colonies was low. The spirochete colonies observed were too few for an accurate enumeration of viable spirochetes (Table 1).

Previous observations had shown that the growth of a strain of spirochetes we had isolated from rumen fluid was not inhibited by rifampin (1 μg/ml). In view of this finding, rifampin (1 μg/ml, final concentration) was added to RFC agar medium and the medium was inoculated with rumen fluid (serial dilutions) to determine whether this antibiotic served as a selective agent for rumen spirochetes. The total number of colonies that developed during incubation of the cultures was 5- to 10-fold lower as compared to control cultures to which no rifampin had been added (Table 1). More importantly, the ratio of the number of spirochete colonies to the total number of colonies was markedly greater than in the absence of rifampin (Table 1). In rifampin-containing medium, the number of spirochete colonies was as great as 1/3 of the total number of colonies that developed. Inasmuch as these results showed that rifampin served as a selective agent for rumen spirochetes, this antibiotic was added routinely to media used in the estimation of viable spirochete numbers in rumen fluid.

Colony counts in rifampin-containing medium indicated that the number of viable spirochetes in rumen fluid ranged from 0.14×10^8 to 1.2×10^8 cells per ml (Table 1). These values are in general agreement with those obtained by direct enumeration by light microscopy, but are consistently somewhat lower. The differences may be due to failure of some spirochetes to grow in the enumeration medium used, either because they were inhibited by rifampin or because the medium was not otherwise suitable. Furthermore, spirochetes forming atypical colonies would not have been detected by our viable count procedures, and some of the spirochetal cells counted microscopically may have been dead.

Selective isolation of spirochetes. By using rifampin (1 μg/ml, final concentration) as a selective agent in RFC agar medium, it was possible to isolate readily a variety of morphologically diverse spirochetes from rumen fluid. Addition of rifampin to the medium was not necessary for the isolation of some small spirochetes (0.2 to 0.3 by 3 to 8 μm) from rumen fluid. However, the addition of rifampin greatly facilitated the isolation of these spirochetes because, in the presence of the antibiotic, the number of

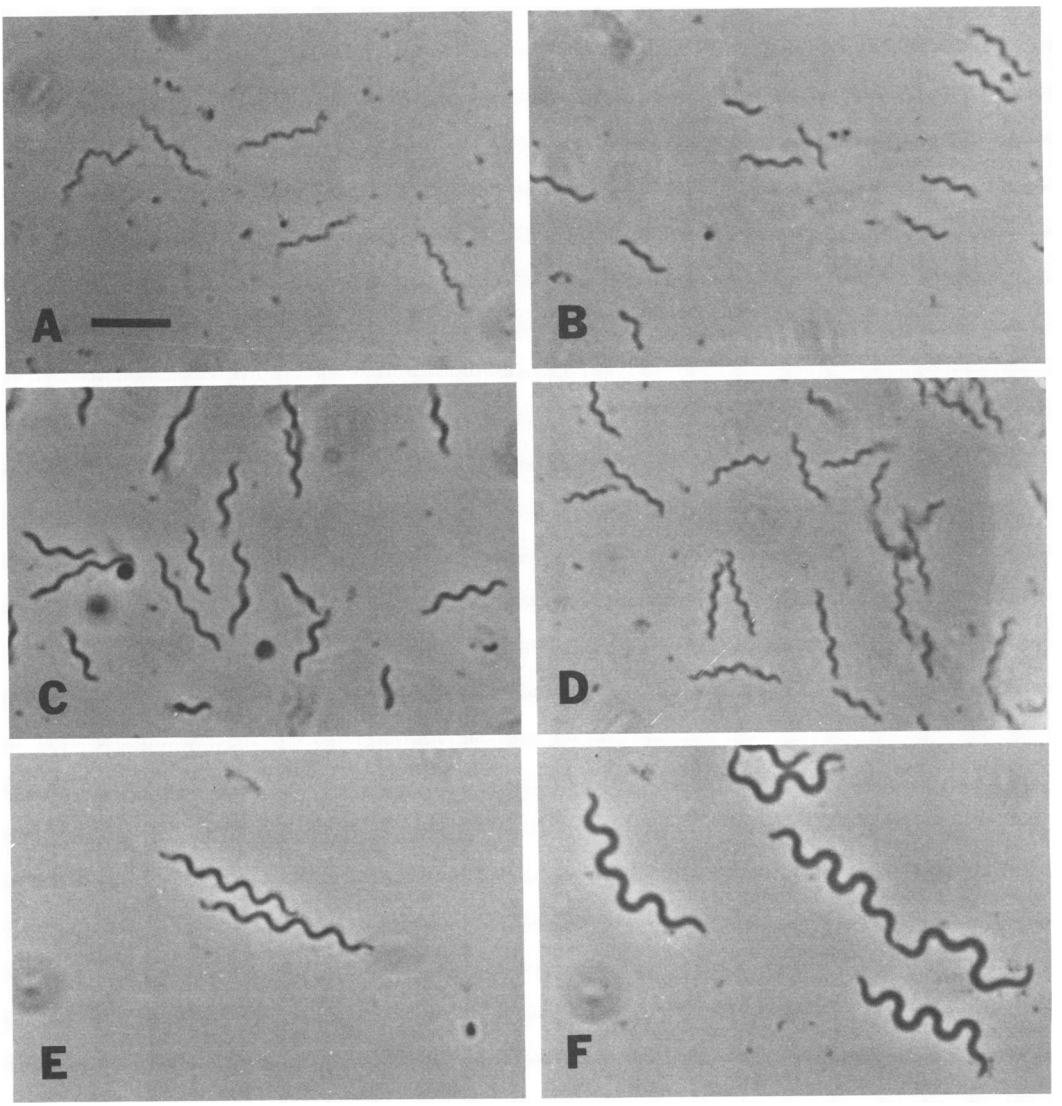


FIG. 1. *(A–F) Phase-contrast photomicrographs of various morphological types of rumen spirochetes (wet-mount preparations). (A) Type 1 (strain 4H); (B) type 2 (strain 8G); (C) type 3 (strain 6M); (D) type 5 (strain 8H); (E) type 6 (strain CA); (F) type 7 (strain DA). Type 4 resembled type 3 (Fig. 1C) in gross morphology, but the cells had six to eight periplasmic fibrils (Table 2, Fig. 4). All spirochetes were grown in liquid medium RFC at 39°C and were photographed in the exponential growth phase. All micrographs are at the same magnification. Bar = 5 μm.*

spirochete colonies was markedly greater in relation to the number of other colonies.

Some of the large spirochetes (e.g., strains CA and DA, described below) could be isolated only when rifampin was present in the medium. Apparently, in the absence of rifampin, growth of the large spirochetes was either prevented or obscured by the growth of other bacteria.

The strains isolated represented seven morphological types of spirochetes differing in gross morphology, cell size, cell coiling pattern, and number of periplasmic fibrils per cell (Table 2, Fig. 1 to 6). In deeps of RFC agar, type 1 spirochetes (Table 2) formed spherical, almost transparent, bubble-like colonies that increased greatly in size during incubation. Types 2 to 7 (Table 2) formed spherical, cotton ball-like, or veil-like colonies with diffusing edges.

Resistance to rifampin. Nine strains of rumen spirochetes, not previously exposed to ri-

fampin, were tested for sensitivity to this antibiotic. All strains were resistant to rifampin (1 μg/ml, final concentration) under the assay conditions used. The rumen bacterium *Succinivibrio dextrinosolvens* 24 was also resistant. In contrast, the growth of other rumen bacteria, such as *Ruminococcus albus* 7, *Bacteroides succinogenes* CAO, and a species of *Streptococcus*, was inhibited by rifampin under the same test conditions. These results, and the diversity of

TABLE 1. *Enumeration of spirochetes present in rumen fluid*[a]

Cow	Sample	Microscopy counts, spirochetes (×10⁷)	Viable cell counts[b]			
			Total no. of bacteria (×10⁷)		No. of spirochetes (×10⁷)	
			Medium RFC	Medium RFC (+rif)	Medium RFC	Medium RFC (+rif)
A	1	5.5	38	6.5	NC[c]	2.3
	2	7.4	216	21.0	NC	3.3
B	1	5.7	86	6.5	6.0	2.4
	2	17.0	230	25.0	NC	4.2
C	1	ND[d]	106	14.0	NC	4.0
	2	20.0	213	30.0	NC	12.0
	3	8.7	110	17.0	NC	5.3
D	1	7.8	34	7.6	NC	2.0
E	1	5.4	60	6.4	NC	2.2
F	1	3.7	90	4.8	NC	1.4

[a] Numerical values (microscopy counts and viable cell counts) indicate cells or colony-forming units per milliliter of rumen fluid. Cows A, B, and C were from the University of Massachusetts Dairy Facility. Cows D, E, and F were from the Cornell University Dairy Facility.

[b] Colony counts of rumen fluid serially diluted into RFC agar medium deeps with (+rif) or without addition of rifampin (1 μg/ml, final concentration).

[c] NC, Not counted. Colonies were too few for an accurate estimation of the number of spirochetes.

[d] ND, Not determined.

TABLE 2. *Morphological types of rumen spirochetes*[a]

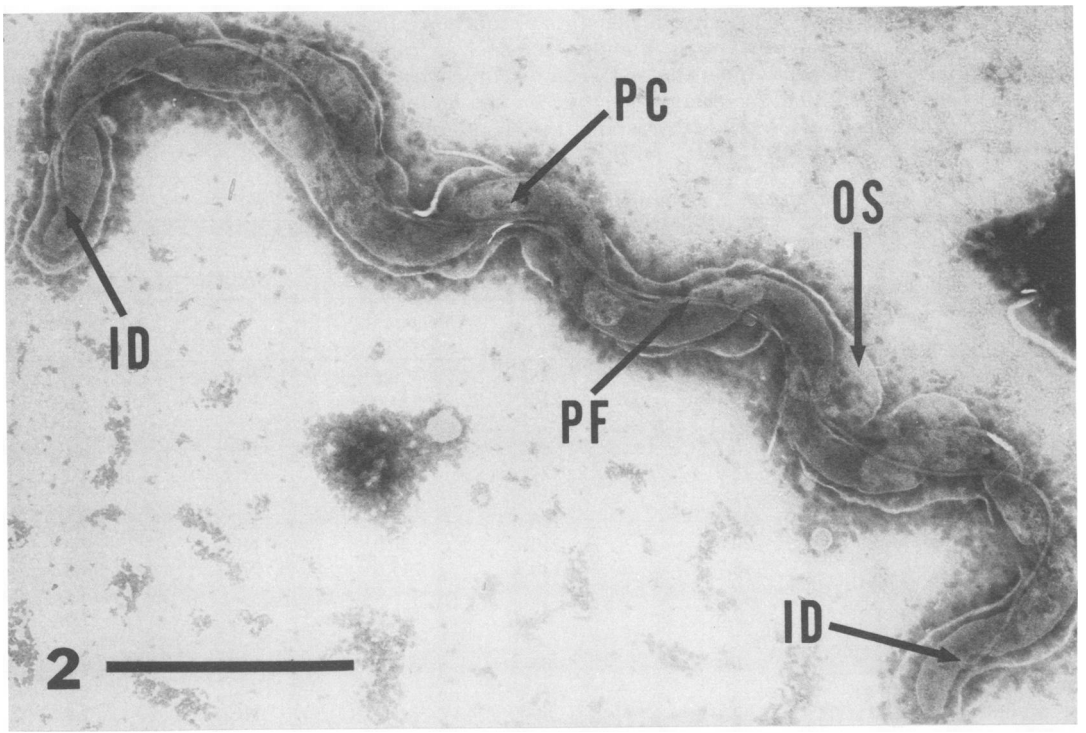
Type	Representative strains	Fig.[b]	Cell size (μm)	Periplasmic fibrils per cell[c]	Cell coiling
1	4H	1A, 2	0.1–0.2 × 5–8	2	Irregular
2	8G	1B	0.2–0.3 × 3–5	2	Loose, regular
3	6M	1C	0.3–0.4 × 5–8	2	Loose, regular
4	6A	4	0.3–0.4 × 5–8	6–8	Loose, regular
5	8H, RUS-1	1D, 3	0.2–0.3 × 5–8	2	Somewhat irregular, more coils per cell than types 3 and 4
6	CA	1E, 5	0.3–0.4 × 10–12	16–20	Large, regular coils
7	DA, 606	1F, 6	0.5–0.6 × 10–12	Bundle[d]	Large, regular coils

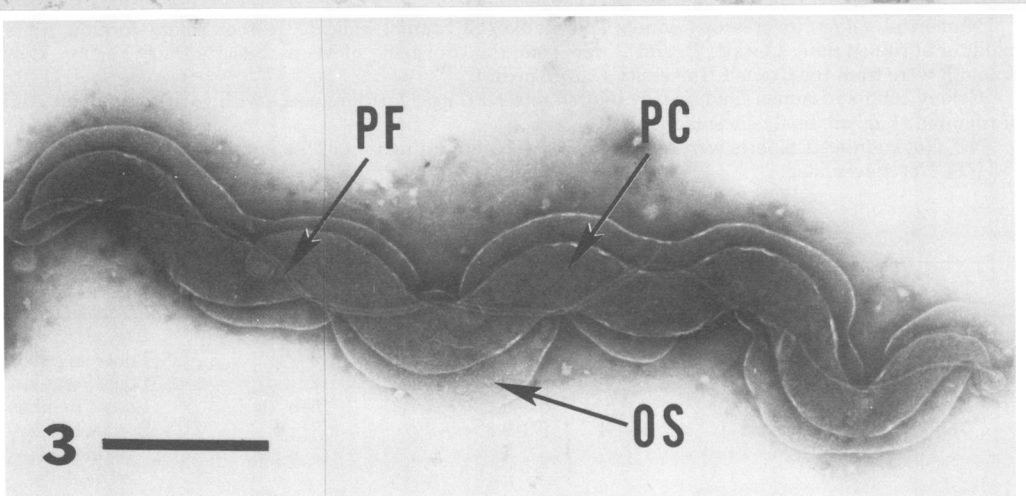
[a] The data refer to cells grown in RFC broth at 39°C and examined in the exponential growth phase.

[b] Numbers refer to figures in this article. The figures illustrate the morphology of the spirochetes.

[c] In cells with an even number of fibrils, one half of the fibrils were inserted near one end of the spirochetal cells and the other half were inserted near the opposite end. The fibrils inserted at opposite ends overlapped in the central region of the cell. Some cells of strain 6A (type 4) had seven fibrils per cell.

[d] Because the fibrils were present in a bundle, it was not possible to estimate their number accurately in the negatively stained preparations. The number of fibrils per cell appeared to be greater than 20.



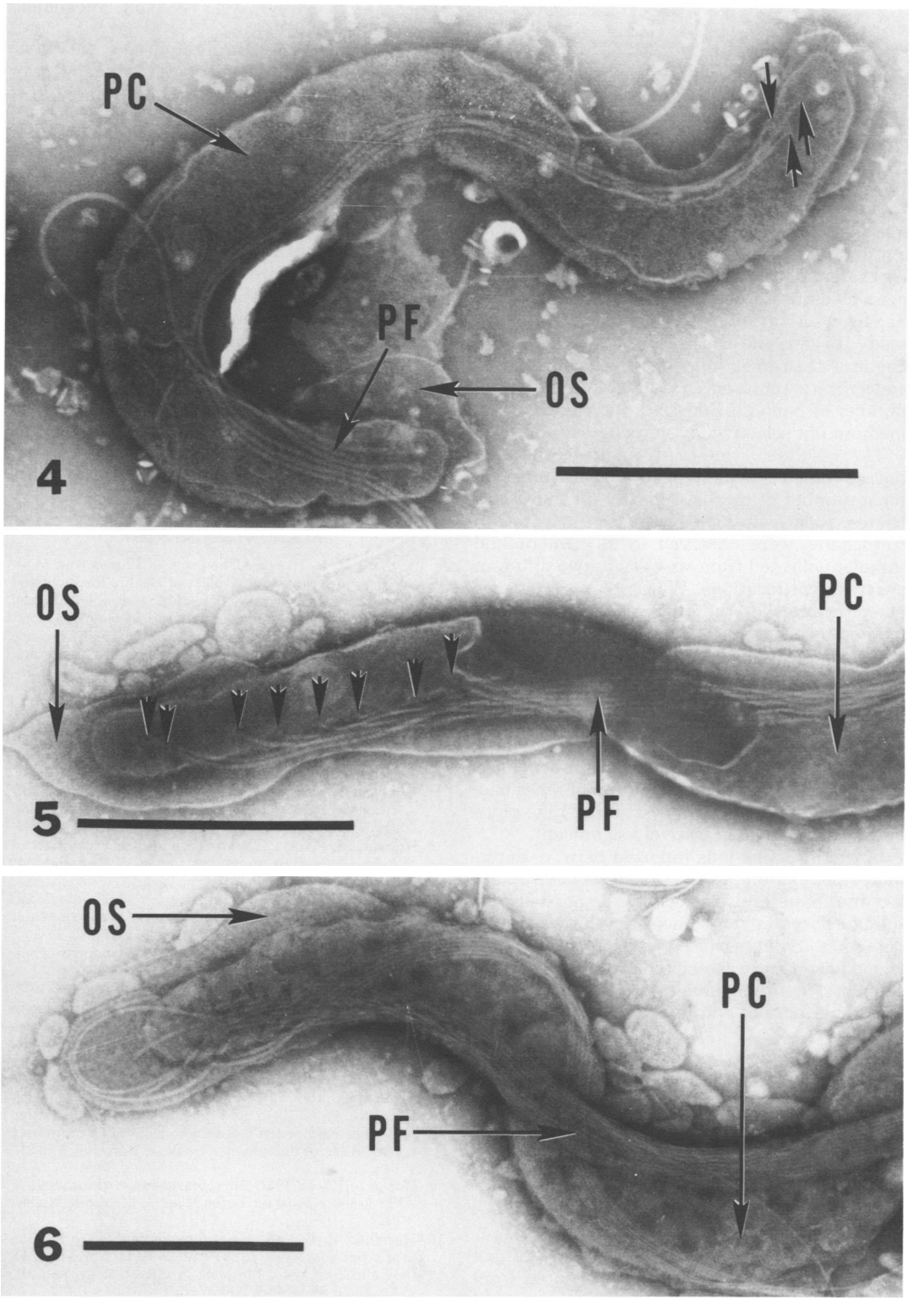


FIGS. 2 and 3. *Electron micrographs of small rumen spirochetes (Fig. 2: strain 4H, morphological type 1; Fig. 3: strain RUS-1, morphological type 5). Negatively stained preparations. In both strains, one periplasmic fibril (PF) originates at each end of the protoplasmic cylinder (PC). The two fibrils overlap in the central portion of the cell. The outer sheath (OS) appears to surround both PF and PC. Insertion disks (ID) anchoring the PF are visible (Fig. 2). Bars = 1 μm.*

spirochetes isolated by using rifampin-containing media, strongly suggest that resistance to this antibiotic is a general characteristic of rumen spirochetes.

DISCUSSION

It may be estimated that the individual morphological types of spirochetes observed in our investigations (Table 2, Fig. 1 to 6) are present in similar numbers in rumen fluid (i.e., approximately 10^7 viable cells of each type per ml), inasmuch as different types of spirochetes formed comparable numbers of colonies in culture tubes inoculated with the same serial dilu-



FIGS. 4–6. *Electron micrographs of a small spirochete (Fig. 4: strain 6A, morphological type 4) and of large spirochetes (Fig. 5: strain CA, morphological type 6; Fig. 6: strain DA, morphological type 7). Negatively stained preparations. The protoplasmic cylinder (PC), periplasmic fibrils (PF), and outer sheath (OS) are visible in each organism. Each of three PF is anchored to one end of the PC by an insertion disk in strain 6A (Fig. 4, small arrows). A row of insertion disks is visible in strain CA (Fig. 5, small arrows). Bars = 1 μm.*

tion of rumen fluid.

The population densities of spirochetes in rumen fluid that were determined in the present study are comparable to those of other bacteria which commonly occur in the rumen, i.e., *Bacteroides succinogenes* (4), *Streptococcus bovis* (9), and *Lactobacillus* sp. (11). Results similar to ours were reported by Bryant (3), who estimated population densities of spirochetes present in rumen fluid by means of direct counts and by examining the water of syneresis in tubes of agar medium cultures into which rumen fluid had been serially diluted as inoculum. In another study, Bryant and Burkey (4) found that spirochetes in the rumen fluid of a cow fed alfalfa hay concentrate represented 2 to 5% of the total number of bacteria that could be cultured in a medium not selective for spirochetes.

The results of this study indicate that the bovine rumen is a highly favorable environment for a number of morphologically diverse spirochetes. Relatively high population densities of spirochetes were observed in all rumen fluid samples collected from six cows in two different geographical locations. Widespread occurrence of spirochetes in the rumen has been documented by other investigators (3, 17). In view of these observations it may be concluded that spirochetes are common inhabitants of the bovine rumen and that those spirochetes usually present in this environment may be considered "true" rumen microorganisms. Furthermore, the occurrence of large populations of spirochetes is an indication that the contribution of these bacteria to the metabolic processes that take place in the rumen is quantitatively significant.

In the investigations reported here we determined population densities of spirochetes found free in rumen fluid. Spirochetes associated with solid surfaces in the rumen may have escaped detection. Solid surfaces in the rumen with which bacteria are associated include surfaces of cellulosic materials undergoing degradation (1, 6) and surfaces of epithelial cells lining the rumen (13).

Our work shows that rumen spirochetes, as a group, are more resistant to rifampin than many other rumen bacteria. This resistance to rifampin constituted the basis for the selective procedure used in isolating a diversity of rumen spirochetes. The addition of rifampin to growth media may facilitate or allow the isolation of spirochetes from natural environments other than the rumen. Indeed, preliminary work in our laboratory has indicated that not only rumen spirochetes, but also spirochetes isolated from the human gingival crevice, from the intestinal tract of a swine, and from natural bodies of water are resistant to rifampin added to their growth media (1 to 50 μg of rifampin per ml, final concentration). Thus, resistance to rifampin may be a general or widespread property among spirochetes.

Rifampin is a semisynthetic antibiotic of the rifamycin class. These antibiotics bind to bacterial ribonucleic acid polymerase, thus inhibiting ribonucleic acid transcription (15). It is possible that spirochetes are resistant to rifampin because this antibiotic cannot penetrate the outer sheath that envelops the spirochetal cell. The function of the outer sheath in spirochetes is largely unknown, but it has been suggested that it may act as a permeability barrier (2, 12).

Alternative or additional explanations for the resistance of spirochetes to rifampin may be: (i) detoxification of this antibiotic by enzymatic action, or (ii) presence in spirochetes of ribonucleic acid polymerases less sensitive to rifampin than ribonucleic acid polymerases of other bacteria.

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