JOURNAL OF BACTERIOLOGY, Mar. 1979, p. 1406-1412
0021-9193/79/03-1406/07$02.00/0

Vol. 137, No. 3

Axial Filament Involvement in the Motility of *Leptospira interrogans*

DAVID B. BROMLEY* AND NYLES W. CHARON

Department of Microbiology, West Virginia University Medical Center, Morgantown, West Virginia 26506

Received for publication 26 December 1978

Motility mutants of *Leptospira interrogans* serovar *illini* were isolated and analyzed by dark-field and electron microscopy. Mutants were obtained by screening for small colonies after nitrosoguanidine treatment. One class of mutants did not have hook- or spiral-shaped ends. In addition, the axial filaments from these mutants were not coiled. An analysis of revertants of two of the mutants in this class indicated that the mutations were pleiotropic with respect to motility, hook- and spiral-shaped ends, and axial filament coiling. We conclude that the axial filaments and the hook- and spiral-shaped ends are involved in *L. interrogans* motility.

The ultrastructure of spirochetes has been extensively studied. These organisms have a helically shaped cell cylinder with a surrounding outer membrane sheath (see 5, 11, 12, 14, and 20 for recent reviews). Between the sheath and the cell cylinder are the axial filaments (also referred to as axial fibrils, flagella, and endoflagella). Each axial filament is subterminally attached to one end of the cell cylinder and extends towards the cell center. Although the axial filaments are both chemically and structurally similar to the flagella of rod-shaped bacteria, they differ from flagella as they have no contact with the ambient environment. In addition, there is no direct evidence that these structures are involved in spirochete motility.

In an attempt to determine the mechanism(s) of spirochete motility, our laboratory has concentrated its efforts on the aerobic spirochete *Leptospira interrogans* serovar *illini*. *L. interrogans* is easier to manipulate in the laboratory than most other spirochetes, and it is structurally one of the least complicated, i.e., it has only one axial filament attached at each end of the cell cylinder (5, 11, 12, 14, 20). *L. interrogans* differs from most other spirochetes in having hook-shaped ends (11, 13). Cells at rest or fixed cells have hook-shaped ends (2). In translating cells, the anterior end is spiral shaped, and the posterior end is hook shaped (2, 8, 13, 18).

The present study reports the isolation and partial characterization of nonmotile mutants of *L. interrogans* serovar *illini*. The results indicate that the axial filaments and the hook- and spiral-shaped ends are both involved in *L. interrogans* motility. In addition, the results are consistent with, and form a basis for, a model of *L. interrogans* motility (2).

(This work was presented in part at the annual meeting of the American Society for Microbiology [D. B. Bromley and N. W. Charon, Abstr. Annu. Meet. Am. Soc. Microbiol., J4, p. 182, 1977], and the Annual American Leptospirosis Research Conference held in Chicago, 1977.)

MATERIALS AND METHODS

Bacteria and media. *L. interrogans* serovar *illini* 3055 (referred to as serovar *illini*) was provided by R. C. Johnson of the University of Minnesota, Minneapolis. A clone of serovar *illini* was selected which consistently yielded colonies having a diameter of 8 to 10 mm on agar plates after 10 days of incubation. The progeny of this clone was used throughout this study.

The cells were maintained in the Tween-80 albumin medium described by Ellinghausen and McCullough and modified by Johnson and Harris (EMJH medium), except it did not contain glycerol (15). The basal medium (lacking glycerol) of Johnson and Harris was employed as diluent (15). EMJH agar plates contained 1% Noble agar (Difco Laboratories, Detroit, Mich.) in EMJH medium.

Culture conditions. All strains of serovar *illini* were maintained at 30°C on a rotary environmental shaker or on Leighton tube racks (7). Cell concentrations were monitored by nephelometry using a Coleman model 7 nephelometer (Coleman Instruments, Inc., Oak Brook, Ill.) and by plate count. Nephelometer readings have been previously correlated to total cell count (T. Auran, M. S. thesis, University of Minnesota, Minneapolis, 1968) and to viable counts (7). Agar plates were incubated at 30°C in a mixture of 97.5% air and 2.5% carbon dioxide (7). Colonies usually appeared within 7 days.

Nitrosoguanidine mutagenesis. Cultures of logarithmic-phase cells at a concentration of 3.1×10^8 cells per ml were incubated in 34 ml of EMJH medium in a water-bath shaker at 30°C. Freshly prepared *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG, Aldrich Chemical Co., Milwaukee, Wis.) was added to the cells

at a final concentration of 50 μg/ml. Samples (4 ml) were withdrawn at 15-min intervals, diluted with 20 ml of cold (4°C) basal medium, and centrifuged at 12,000 × *g* for 20 min at 4°C. The pelleted cells were suspended in 4.0 ml of basal medium, serially diluted, and plated on EMJH agar. Motility mutants were isolated in a survival range of 5 to 10% of the NTG-treated cells.

Isolation of motility mutants and revertants. EMJH agar plates containing colonies of mutagenized cells were screened after 7 to 10 days of incubation for small, dense colonies (1 to 2 mm in diameter). For isolation and purification of mutants, small colonies were picked, grown in EMJH medium, and replated on EMJH agar plates. Small dense colonies were picked, regrown in EMJH medium, and assayed for motility.

Spontaneous motile revertants of nonmotile mutants were isolated in sealed EMJH agar plates as areas of diffuse growth emerging from dense mutant colonies after 21 to 38 days. Revertant clones are defined as those which possess wild-type colonial size (minimum 8 to 10 mm in diameter after 10 days of incubation). All such revertants were assayed for motility.

Dark-field microscopy and motility assays. Dark-field micrographs were made of cells fixed in 1% glutaraldehyde for 5 to 10 min at 20°C. Suspensions of fixed cells were combined with an equal volume of 2% methylcellulose (4,000 P, Fisher Scientific Co., Pittsburg, Pa.) and photographed at ×900 under dark-field illumination with an Ortholux microscope (E. Leitz Inc., Rockleigh, N.J.). Both translational and nontranslational motility of serovar *illini* were determined by examining cells from freshly inoculated cultures under a dark-field microscope at ×400 (2, 8).

Electron microscopy of whole cells. Cells to be examined with the electron microscope were subjected to a quick staining procedure. A 1-ml sample of cells was incubated with an equal volume of 2% sodium deoxycholate (DOC, Sigma Chemical Co., St. Louis, Mo.) for 2 to 5 min at 20°C. Samples from this mixture were dropped onto carbon-coated Formvar copper grids (300 mesh). The cells were allowed to adhere for 1 min, washed with 1 drop of 0.1 M sodium phosphate buffer, (pH 7.4), and stained with 1% sodium phosphotungstate. The grids were examined under either an RCA EMU-3G or a JEM T6-S electron microscope at accelerating voltages of 100 kV or 50 kV, respectively.

Axial filament isolation. Axial filaments were isolated by modifications of the procedures described by Bharier and Rittenberg and by Nauman et al. (3, 17). The cells were grown in aerated 500-ml cultures at 30°C to a density of 6.2×10^8 to 9.3×10^8 cells per ml, harvested by centrifugation at 12,000 × *g* for 20 min at 4°C, and suspended in 10 ml of 0.1 M sodium phosphate buffer (pH 7.4). This suspension was combined with an equal volume of 2% DOC and stirred on a magnetic stirrer at 20°C for 6 h. The resulting suspension was centrifuged as before for 20 min, and the supernatant fluid was retained. This fluid was centrifuged at 100,000 × *g* for 60 min at 4°C in a type 40 rotor in a Beckman L5-65 ultracentrifuge (Beckman Spinco, Palo Alto, Calif.), and the pellet containing the axial filaments was retained.

In some cases, the above crude axial filament preparation (CAP) was partially separated away from contaminating membrane material by density gradient centrifugation. The CAP was suspended in the fourth step of a four-step cesium chloride gradient (step 4—1.0 ml, 1.4 g/cm^3; step 3—1.0 ml, 1.30 g/cm^3; step 2—2.0 ml, 1.25 g/cm^3; step 1—1.0 ml, 1.0 g/cm^3). This gradient was centrifuged in a Beckman SW50.1 rotor at 100,000 × *g* for 18 to 24 h at 20°C. The axial filaments banded at a density of approximately 1.3 g/cm^3. The cesium chloride-prepared axial filaments were then diluted in 0.1 M sodium phosphate buffer (pH 7.4), and centrifuged at 100,000 × *g* for 60 min. The pellet containing the axial filaments was retained for electron microscopic examination.

Electron-microscopy of axial filaments. The CAP- or the cesium chloride-purified axial filaments were dropped onto carbon-coated Formvar copper grids (300 mesh) and stained with 1% sodium phosphotungstate. To control for artifacts which could affect axial filament shape, extremes in pH and the use of certain electron-opaque stains were avoided (16, 22).

RESULTS

Colonial and microscopic morphology. The colonies of serovar *illini* were diffuse, subsurface, and entire, and were 8 to 10 mm in diameter after 10 days of incubation of EMJH agar (Fig. 1a). The continued incubation of the plate resulted in further growth in the size of the colonies. When viewed by dark-field microscopy, fixed cells were seen to possess hook-shaped ends (Fig. 1b), and motile cells were seen to have hook- and/or spiral-shaped ends.

The gross morphology and ultrastructure of serovar *illini* is illustrated in Fig. 2a. An outer membrane sheath surrounds the entire cell cylinder. Between the sheath and the protoplasmic cylinder lie two subterminally inserted, antiparallel axial filaments. As others have observed, the axial filaments neither met nor overlapped (4, 12). Examination of the axial filaments revealed that they were extensively coiled. This coiling was found in all types of preparations examined including cells partially disrupted by DOC, CAPs, and axial filaments isolated from cesium chloride gradients (Fig. 2b). The results confirm the findings of others who have reported and noted the marked coiling property of *L. interrogans* axial filaments (1, 4, 12, 17, 19, 20).

Isolation of motility mutants. NTG treatment of serovar *illini* resulted in the isolation of small dense colonies which failed to spread on EMJH agar. Approximately 475 small colonies were picked after NTG treatment, and 20 of these consistently formed small colonies on subculture. All 20 mutant isolates were completely deficient in translational motility, and some were also deficient in nontranslational motility. All 20

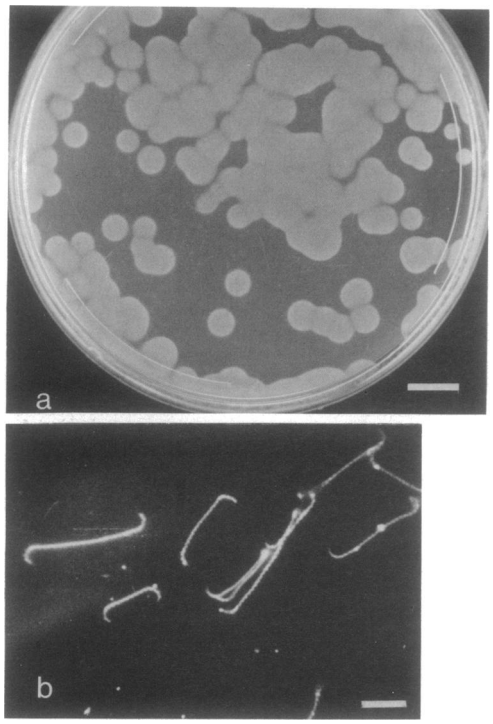


FIG. 1. *(a) Colonies of wild-type serovar illini on EMJH agar at 10 days of incubation. Note apparent spreading growth of colonies. Bar represents 1 cm. (b) Dark-field micrograph of wild-type serovar illini illustrating the hook-shaped ends and helical cell morphology. Bar represents 10 μm.*

mutant isolates possessed axial filaments as determined by electron microscopy.

Four of the 20 motility mutants had lost their hook- and spiral-shaped ends (Fig. 3a). These linear mutants were further characterized (Table 1). All four linear mutants completely lacked both translational and nontranslational motility. Their colony size was 1 to 2 mm after 10 days of incubation on EMJH agar. Although these four mutants had lost their hook- and spiral-shaped ends, they retained their body helices (as represented by DB115, Fig. 3a). In addition, the appearance of the cell cylinder and the outer membrane sheath of the mutants was identical to that of the wild type. However, the axial filaments from these strains were altered. Neither axial filaments attached to partially disrupted mutant cells, nor CAP, nor cesium chloride-purified axial filaments from the mutants displayed the extensive coiling property of wild-type axial filaments (as represented by the CAP preparation of DB115, Fig. 3b). The axial filaments from these mutants had no defined shape. These results suggest that the coiling of the axial filaments and the hook- and spiral-shaped ends of the organisms are both involved in *L. interrogans* motility.

Reversion studies of mutants. Conceivably, the nonmotile linear mutants could be nonmotile for reasons other than a mutation (or mutations) which results in noncoiled axial filaments or loss of the hook- and spiral-shaped ends. Thus, because NTG is known to produce numerous closely linked mutations (10), secondary mutations at genes other than those involved in axial filament coiling and hook- and spiral-shaped ends could be responsible for nonmotility. Genetic recombination has not been demonstrated in spirochetes. Accordingly, the isolation of axial filament mutations away from possible secondary mutations is currently not possible. This leaves us with the alternative of examining revertants to motility. If all motile revertants regain the wild-type coiled axial filaments and hook- and spiral-shaped ends, then the results would indicate that secondary mutations were not responsible for the loss in motility.

Full revertants to motility were selected for that colonial property of motile cells, i.e., the ability to demonstrate a colonial size at least equivalent in diameter to the wild type after 10 days of incubation on EMJH agar. Ten independently isolated, spontaneously occurring revertants of each of the nonmotile linear mutants of DB115 and DB290 were obtained. These 20 revertants were characterized with respect to motility, coiling of the axial filaments, and hook- and spiral-shaped ends (Table 1). All revertants produced colonies equivalent in size to the wild type, and all regained translational and nontranslational motility. The axial filaments from the 20 revertants were found to be extensively coiled (as represented by R29, Fig. 4a). In addition, the hook- and spiral-shaped ends were also regained in all 20 revertants. Fixed cells had hook-shaped ends (as represented by R29 in Fig. 4b). These results indicate that secondary mutations were not responsible for the loss of motility in DB115 and DB290.

DISCUSSION

The results reported in this communication indicate that the axial filaments are involved in *L. interrogans* motility. Four nonmotile mutants were obtained which did not have hook- and spiral-shaped ends. The axial filaments from these mutants were not coiled. Revertant analysis of two of the linear mutants indicated that the mutations were pleiotropic, i.e., the loss of motility, hook- and spiral-shaped ends, and coiled filaments occurred together.

We propose that the linear mutants have mu-

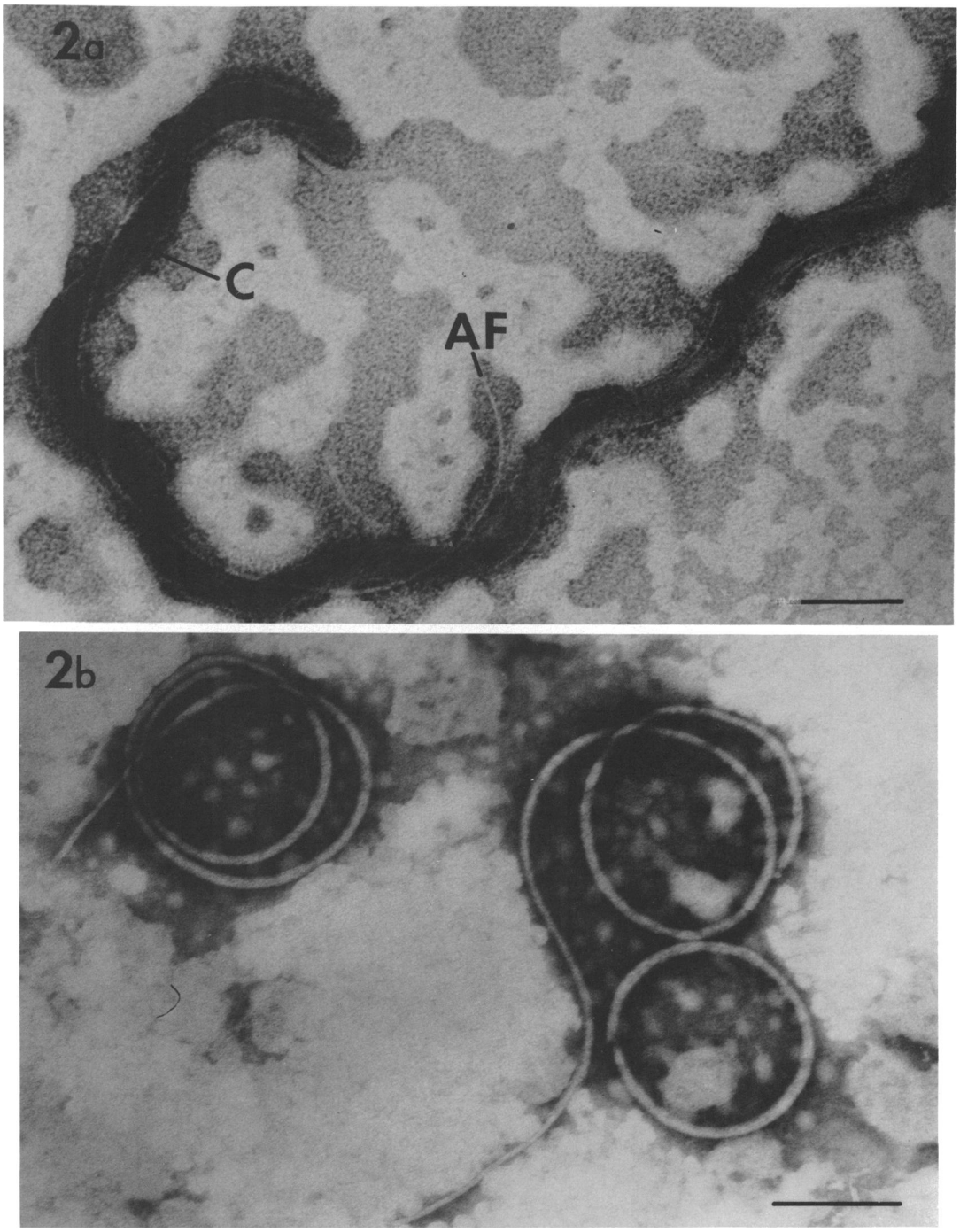
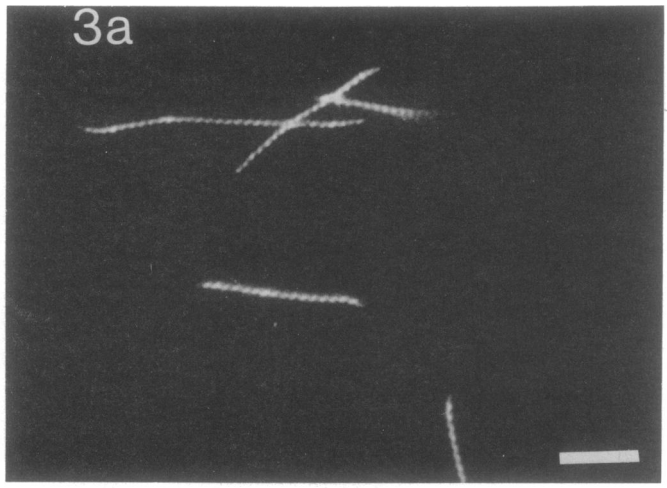


FIG. 2. *(a) Transmission electron micrograph of one end of wild-type serovar illini after partial disruption with DOC illustrating the helical cell cylinder (C) and axial filament (AF). The outer membrane sheath was removed by DOC in this preparation. Bar represents 0.28 μm. (b) Cesium chloride preparation of axial filaments from wild-type serovar illini illustrating coiled axial filament. Bar represents 0.17 μm.*



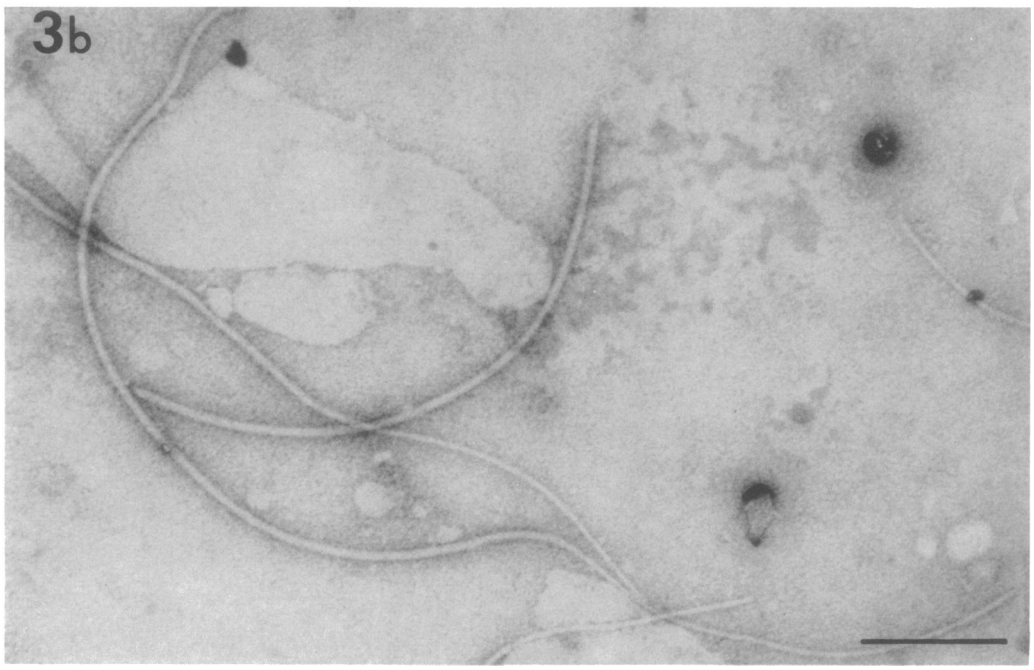
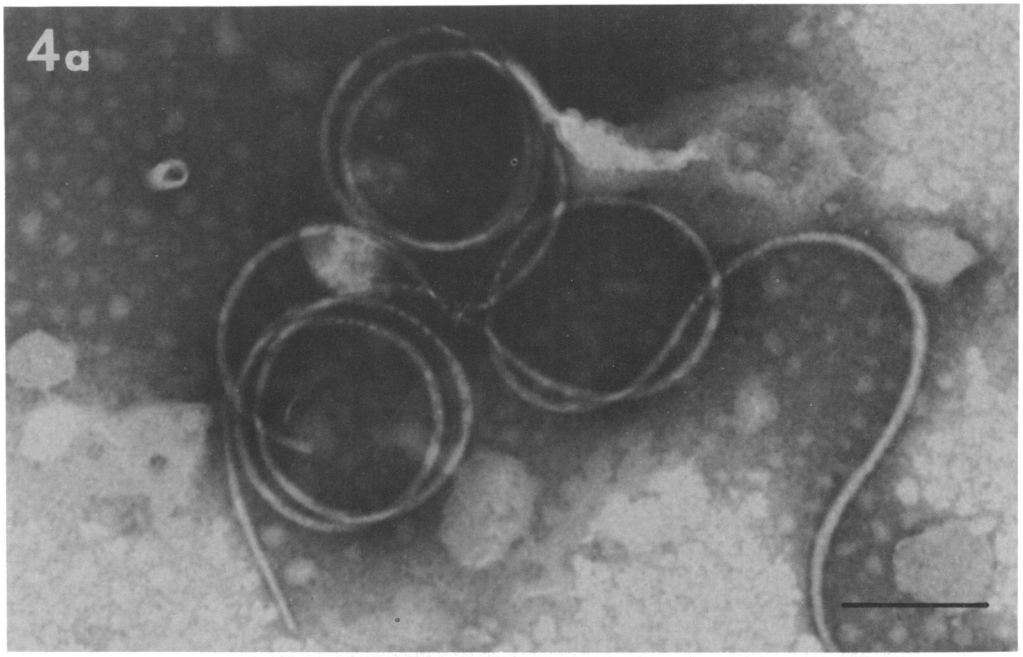


FIG. 3. *(a) Dark-field micrograph of nonmotile linear mutant DB115. Note non-hook-shaped ends. Bar represents 10 μm. (b) Crude axial filament preparation of DB115 illustrating lack of coiling. Bar represents 0.25 μm.*

TABLE 1. *Characteristics of wild-type, mutant, and revertant serovar illini*

Strain and description	Colony size (mm)[a]	Translational and nontranslational motility	Cell morphology	Axial filament morphology
Wild type	8–10	+	Hook and spiral-shaped ends	Coiled
Mutants DB115, DB218, DB290, DB340	1–2	–	Linear	Noncoiled
Revertants of DB115 (total of 10)	8–12	+	Hook and spiral-shaped ends	Coiled
Revertants of DB290 (total of 10)	10–12	+	Hook and spiral-shaped ends	Coiled

[a] At 10 days of incubation on EMJH agar.



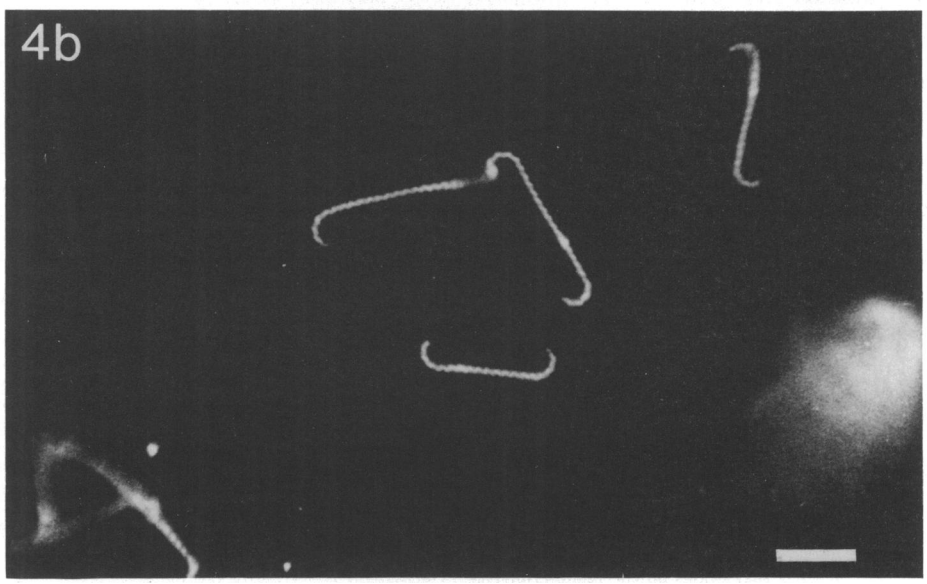


FIG. 4. *(a) Cesium chloride preparation of axial filaments from R29. Bar represents 0.17 μm. (b) Dark-field micrograph of motile revertant R29 derived from DB115. Note hook-shaped ends. Bar represents 10 μm.*

tations in their axial filament genes. Moreover, these mutations are believed to result in not only altered axial filaments, but also in the loss of motility, and in the loss of the hook- and spiral-shaped ends. We make this proposal for two reasons. First, it is consistent with, and in part forms a basis for, a recent model of *L. interrogans* motility (2). Berg et al. (2) proposed that the wild-type axial filaments are more rigid than the protoplasmic cell cylinder. Rotary motors at the base of the axial filaments are believed to drive the axial filaments in rotational motion in a manner analogous to flagella of rod-shaped bacteria (2). Depending on the direction of rotation of the motors and the three-dimensional shape of the axial filaments, the ends of the cell change shape. A spiral wave propagated by rotation of the anterior axial filament is be-

lieved to propel *L. interrogans* forward in liquid medium. Thus, according to this model, the failure of the linear mutants to be motile is not only consistent with their inability to generate the anterior spiral wave, but also with the failure of the axial filaments to have a rigid and defined structure.

Second, preliminary evidence with certain other motility mutants indicates that the axial filaments determine the shape of the cell ends. These partially motile mutants have partially hook-shaped ends. Although the axial filaments from these mutants are not as extensively coiled as wild-type axial filaments, they have a defined partially coiled shape (D. B. Bromley and N. Charon, unpublished results).

The results reported in this communication do not necessarily indicate a causal relationship between the structure of the axial filaments and both the cell shape and motility. For example, we may have obtained mutants in a regulatory gene which control the expression of a number of structural genes: one gene may be involved in motility, another in axial filament coiling, and the third in hook and spiral end formation. To determine if the above proposal is correct, i.e., that mutations in the axial filament genes are responsible for the loss in motility and the loss in hook- and spiral-shaped ends, it will be necessary to analyze the axial filament proteins from wild-type, mutant, and revertant strains.

It should be noted that similar linear motility mutants of *L. interrogans* have been obtained by others (9, 21, 23, 24). Simpson and White reported differences in ultrastructure between linear and hook-shaped serovars (21). We were unable to detect such differences between our linear mutants and wild-type serovar *illini*. Because Simpson and White compared differences between serovars rather than isolates of the same isogenic strain, their results could possibly be attributed to genetic background differences rather than mutations in specific genes.

ACKNOWLEDGMENTS

We thank H. Berg, A. Campbell, H. Thompson, H. Voelz, and D. Yelton for their suggestions on the manuscript and their encouragement.

This research was supported by Public Health Service grant number 1-R23-DEO4645 from the National Institute for Dental Research, by Biomedical Research Support Grant number 5 S01 RR05433, and by the West Virginia Medical Corporation.

LITERATURE CITED

1. **Babudieri, B.** 1972. The axistyle of *Leptospira*, p. 93–114. *In* B. Babudieri (ed.), The fine morphology of spirochaetas. Leonardo Edizioni Scientifiche, Rome.
2. **Berg, H. C., D. B. Bromley, and N. W. Charon.** 1978. Leptospiral motility, p. 285–294. *In* R. Y. Stanier, H. J. Rogers, and J. B. Ward, (ed.), Relations between structure and function in the prokaryotic cell: 28th Symposium of the Society for General Microbiology. Cambridge University Press, Cambridge.
3. **Bharier, M. A., and S. C. Rittenberg.** 1971. Chemistry of axial filaments from *Treponema zuelzerae*. J. Bacteriol. **105**:422–429.
4. **Birch-Andersen A., K. Hovind-Hougen, and C. Borg-Petersen.** 1973. Electron microscopy of *Leptospira*. 1. *Leptospira* strain *Pomona*. Acta. Pathol. Microbiol. Scand. Sect. B **81**:665–676.
5. **Canale-Parola, E.** 1977. Physiology and evolution of spirochetes. Bacteriol. Rev. **41**:181–204.
6. **Chang, A., and S. Faine.** 1970. Electron-microscopic evidence for reactions of axial filaments of *Leptospira* with IgM and IgG antibodies. Bull. W.H.O. **43**:571–577.
7. **Charon, N. W., R. C. Johnson, and D. Peterson.** 1974. Amino acid biosynthesis in the spirochete *Leptospira:* evidence for a novel pathway of isoleucine biosynthesis. J. Bacteriol. **117**:203–211.
8. **Cox, P. J., and G. I. Twigg.** 1974. Leptospiral motility. Nature (London) **250**:260–261.
9. **Faine, S., and J. van der Hoeden.** 1964. Virulence-linked colonial and morphological variation in *Leptospira*. J. Bacteriol. **88**:1493–1496.
10. **Guerola, N., J. L. Ingraham, and E. Cerda-Olmedo.** 1971. Induction of closely linked multiple mutations by nitrosoguanidine. Nature (London) New Biol. **230**:122–125.
11. **Holt, S. C.** 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. **42**:114–160.
12. **Hovind-Hougen, K.** 1976. Determination by means of electron microscopy of morphological criteria of value for classification of some spirochetes, in particular treponemes. Acta. Pathol. Microbiol. Scand. Sect. B **255** (Suppl.):1–41.
13. **Jarosch, R.** 1967. Studien zur Bewegungsmechanik der Bakterien und Spirochäten des Hochmoores. Oesterr. Bot. Z. **114**:255–306.
14. **Johnson, R. C.** 1977. The spirochetes. Annu. Rev. Microbiol. **31**:89–106.
15. **Johnson, R. C., and V. G. Harris.** 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. J. Bacteriol. **94**:27–31.
16. **Kamiya, R., and S. Asakura.** 1976. Helical transformations of *Salmonella* flagella *in vitro*. J. Mol. Biol. **106**: 167–186.
17. **Nauman, R. K., S. C. Holt, and C. D. Cox.** 1969. Purification, ultrastructure, and composition of axial filaments from *Leptospira*. J. Bacteriol. **98**:264–280.
18. **Noguchi, H.** 1918. Morphological characteristics and nomenclature of *Leptospira* (Spirochaeta) *icterohaemorrhagiae* (Inada and Ido). J. Exp. Med. **27**:575–592.
19. **Ritchie, A. E.** 1972. A brief consideration of anatomic features of the spirochaetas as revealed by electron microscopy, p. 77–92. *In* Babudieri (ed.), The fine morphology of spirochaetas. Leonardo Edizioni Scientifiche, Rome.
20. **Ritchie, A. E.** 1976. Morphology of leptospires, p. 19–37. *In* R. C. Johnson (ed.), The biology of parasitic spirochetes. Academic Press Inc., New York.
21. **Simpson, C. F., and F. H. White.** 1964. Ultrastructural variations between hooked and nonhooked leptospires. J. Infect. Dis. **114**:69–74.
22. **Smith, R. W., and H. Koffler.** 1971. Bacterial flagella. Adv. Microbial Physiol. **6**:219–339.
23. **Tripathy, D. N., and L. E. Hanson.** 1971. Agar overlay method for growth of leptospires in solid medium. Am. J. Vet. Res. **32**:1125–1127.
24. **Tripathy, D. N., and L. E. Hanson.** 1972. Colonial and morphological variations of *Leptospira illini* strain 3055. Am. J. Vet. Res. **33**:1723–1727.