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New Infectious Spirochete Isolated from Short-Tailed Shrews and White-Footed Mice

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A spirochete with two periplasmic flagella was isolated from the blood or tissues of spleens and kidneys from short-tailed shrews (*Blarina brevicauda*) and white-footed mice (*Peromyscus leucopus*) in Connecticut and Minnesota. After inoculation, the shrew-mouse spirochete infected Swiss mice and Syrian hamsters. This spirochete is morphologically and serologically distinct from the species of *Treponema*, *Borrelia*, *Leptospira*, and *Spirochaeta* examined.

The development and improvement of media to grow borreliae (3, 17) and to culture *Borrelia burgdorferi* (7, 16) from tissues of kidneys, spleens, and other organs of rodents (1, 15) have enhanced our ability to isolate these bacteria from vertebrates. While studying the epizootiology of *B. burgdorferi*, we observed morphologically different spirochetes in the blood and tissues of *Blarina brevicauda* (short-tailed shrew) and *Peromyscus leucopus* (white-footed mouse). We report here the isolation and characterization of these shrew-mouse (SM) spirochetes.

MATERIALS AND METHODS

Rodents and shrews were captured in Sherman box traps from 11 sites within all eight counties of Connecticut from September 1984 through June 1985. White-footed mice were captured at Fort Snelling, Minn., from October through December 1985. Attempts to isolate spirochetes from five species of rodents and two species of shrews were made by inoculating blood or tissues of kidney and spleen into Barbour-Stoenner-Kelly (BSK) medium (3). Specifically, 1 or 2 drops of blood (ca. 0.1 ml) from the heart of each animal and a 1:10 dilution of each kidney and spleen, triturated in 7 ml of BSK medium, were inoculated separately into duplicate tubes of BSK medium containing 0.1% agarose (SeaKem LE; FMC Corp., Rockland, Maine) (15). L-Cysteine hydrochloride (0.023%), DL-dithiothreitol (0.015%), and superoxide dismutase (0.002%) were added to one of the duplicate tubes of culture medium. Inoculated media were kept at 31°C and examined for spirochetes at 1 to 4 weeks after inoculation.

For the ultrastructural studies, cells were washed twice in phosphate-buffered saline (pH 7.2) and concentrated by centrifugation at 35,000 × *g* for 30 min. Pellets were then fixed for 18 h at 4°C in 2.5% glutaraldehyde, containing 0.1% $CaCl_2$ and 1% sucrose, and buffered with 0.1 M sodium cacodylate (pH 7.2). Specimens were postfixed for 2 h at room temperature in 1% OsO_4, stained for 18 h at 4°C with 0.5% uranyl acetate in 70% ethanol, dehydrated through an ethanol and acetone series, and embedded in an LX-112/Araldite mixture. Thin sections were poststained with 5% methanolic uranyl acetate and Reynolds lead citrate and examined in a Zeiss EM-10 electron microscope at an accelerating voltage of 60 kV. Suspensions of washed but unfixed whole spirochetes were placed on Formvar-coated grids, negatively stained with 2% aqueous phosphotungstic acid (pH 7.0), and examined similarly.

The isolation of SM spirochete DNA and the determination of moles percent guanine plus cytosine (G+C) by the thermal denaturation method (22, 23) were conducted as described by Hyde and Johnson (12).

Antigenic relatedness of the SM spirochetes to species of *Borrelia*, *Treponema*, *Leptospira*, and *Spirochaeta* was determined by immunofluorescence (indirect fluorescent-antibody [IFA]) procedures modified from those of Philip et al. (26). *Borreliae* and the SM spirochetes were grown in 7 ml of BSK medium, and *Spirochaeta aurantia* was grown in the medium of Breznak and Canale-Parola (6). All cells were washed twice in phosphate-buffered saline (pH 7.2). The pellet was suspended in 0.5 ml of phosphate-buffered saline and in 0.5 ml of 5% yolk sac diluted in phosphate-buffered saline containing 0.01% sodium azide. Cells were applied as thin films to premarked microscope slides and allowed to dry before being fixed in acetone for 10 min.

Using IFA procedures, we tested this undescribed spirochete against (i) homologous Swiss mouse antiserum, (ii) Swiss mouse antiserum to *B. burgdorferi*, (iii) murine monoclonal antibody H9724 directed to the periplasmic flagellar protein unique to species of *Borellia* (4), and (iv) rabbit antisera to *Borrelia hermsii* (HS1 serotype), *Treponema pallidum*, and five *Leptospira interrogans* serovars. The latter were *canicola* (strain Moulton), *grippotyphosa* (strain SC4397), *hardjo* (strain Hardjoprajitno), *icterohaemorrhagiae* (strain CF-1), and *pomona* (strain MLS). In reciprocal IFA tests, cells of *Borrelia* sp., *T. pallidum*, *Leptospira* sp., and *S. aurantia*, prepared as described by Magnarelli et al. (21), were tested against Swiss mouse antiserum to the SM spirochete. All tests were performed with known positive and negative control sera.

Susceptibility of a Swiss mouse and Syrian hamsters to the SM spirochete was determined by inoculating ca. 3.4×10^7 spirochetes via tail vein and by intraperitoneal injection, respectively. Four weeks postinoculation of the Swiss mouse, triturated tissues of kidneys and spleens and blood from the heart were aseptically removed and inoculated into duplicate tubes of BSK medium containing agarose as described previously. Blood was drawn from the hearts of three hamsters 3 days after injection and inoculated into

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TABLE 1. Isolation of SM spirochetes from short-tailed shrews and white-footed mice captured in Connecticut and Minnesota, 1984 to 1985

Species	Specimen no.	Collection site	Date of collection	Isolation of spirochete[a] in:			
				Blood	Spleen	Left kidney	Right kidney
Shrew	124	West Haven, Conn.	10 Oct. 84	−	−	+	−
Mouse	2637	West Haven, Conn.	10 Oct. 84	+	+	+	+
Mouse	2663	East Haddam, Conn.	30 Oct. 84	−	−	−	+
Shrew	128	New Hartford, Conn.	31 Oct. 84	−	−	+	+
Shrew	129	New Hartford, Conn.	31 Oct. 84	−	+	+	+
Shrew	130	East Haddam, Conn.	14 March 85	−	+	−	−
Shrew	133	East Haddam, Conn.	27 June 85	−	+	−	+
Mouse	47	Fort Snelling, Minn.	12 Dec. 85	−	+	+	+

[a] Symbols: −, spirochete not isolated; +, spirochete isolated.

duplicate tubes of medium. Five hamsters were sacrificed 33 to 39 days after injection; blood was drawn, and the kidneys and spleens were aseptically removed, triturated, and inoculated into duplicate tubes of the medium, as noted above. Inoculated tubes were incubated at 31°C and were examined for spirochetes 2 to 4 weeks later. Uninoculated Swiss mice and hamsters were sacrificed and used as controls.

RESULTS

Spirochetes were cultured from all five short-tailed shrews examined and from 2 of 282 white-footed mice from Connecticut and from 1 of 47 white-footed mice captured in Minnesota. Infected mammals were captured in Connecticut in October 1984 and March and June 1985 from three geographically different areas (Table 1). No spirochetes were isolated from 10 *Microtus pennsylvanicus* (meadow vole), 2 *Tamias striatus* (Eastern chipmunk), 1 *Glaucomys volans* (Southern flying squirrel), 5 *Clethrionomys gapperi* (Gapper's red-backed mouse), and 1 *Sorex cinereus* (masked shrew).

Isolates from short-tailed shrews were made from tissues of spleens (n = 3), left kidneys (n = 3), and right kidneys (n = 3) (Table 1). Cultures from white-footed mice were made from blood (n = 1) and tissues of spleens (n = 2), left kidneys (n = 2), and right kidneys (n = 3). Spirochetes were detected by dark-field microscopy in cultures as early as 11 days after inoculation.

Electron microscopy of cultured SM spirochetes revealed a single, sheathed periplasmic flagellum (ca. 0.02 μm in diameter) extending subterminally from each blunt cell end and overlapping near the center of the cell (Fig. 1 through 4). The periplasmic flagella were positioned between the triple-layered outer membrane and the peptidoglycan layer (Fig. 1). Spirochetes measured 0.18 to 0.24 μm (n = 8) in diameter. Negatively stained organisms were 4.0 to 4.9 μm (n = 6) in length and had one or two waves (Fig. 5). Wavelength was 1.5 to 2.5 μm (n = 4).

A strain from a short-tailed shrew (CT11616) was recovered from a Swiss mouse and from Syrian hamsters that were experimentally infected. Spirochetes were isolated from spleen tissues of the Swiss mouse 4 weeks postinjection. From hamsters, they were cultured from blood and kidney tissues 3 days and 33 to 39 days after injection, respectively. None of the inoculated animals showed signs of illness. Spirochetes were not isolated from uninoculated Syrian hamsters and Swiss mice.

Short tailed shrew (CT11616) and white-footed mouse (CT11397, MN47) isolates reacted at titers of 1:64 to 1:256 in homologous and heterologous IFA tests (Table 2). The two Connecticut and one Minnesota isolates were nonreactive with the *Borrelia*-specific monoclonal antibody H9724 and with antisera against *B. burgdorferi*, *B. hermsii*, *T. pallidum*, and five *L. interrogans* serovars. Antisera against these shrew and mouse isolates did not react with antigens of *B. burgdorferi*, *B. hermsii*, *T. pallidum*, *S. aurantia*, or five serovars of *L. interrogans*.

The mole percent G+C for the SM spirochete (MN47) was 36.7%.

DISCUSSION

Although we isolated this spirochete from both wild-caught short-tailed shrews and white-footed mice, this bacterium appears to be more prevalent in the former. Isolates were made from all five short-tailed shrews tested, whereas less than 1% of the white-footed mice were infected in Connecticut. Although we did not isolate this spirochete from a masked shrew and four other species of wild-caught rodents, its ability to infect laboratory animals leads us to believe that it may have a relatively wide host range. We are unaware of spirochetes of this type previously being cultured from shrews of the genus *Blarina* or mice of the genus *Peromyscus*. The white-footed mouse is a reservoir for *B. burgdorferi* (1, 2, 5, 19, 20). Since the SM spirochete was isolated from small animals captured in widely separated geographical areas, Connecticut and Minnesota, we believe it may be widespread in the United States.

Because we obtained SM spirochetes from the blood of only one of the infected wild-caught animals, spirochetemia may exist for brief periods or may develop intermittently as it apparently occurs in *B. burgdorferi* infections (9, 15). Although we isolated spirochetes from kidney tissues of hamsters 39 days after inoculation, cultures from blood were obtained only at 3 days postinfection.

The SM spirochete (strains CT11616, CT11397, and MN47) did not react with antisera to leptospires, borreliae, or a treponeme or with a murine monoclonal antibody to the genus *Borrelia*. Thus, there appears to be little antigenic relatedness between our new isolates and other spirochetes known to cause infections in mammals. In addition, antiserum to the SM spirochete was nonreactive with *S. aurantia*, a free-living spirochete with two periplasmic flagella (8).

Morphologically, the single periplasmic flagellum which arises from each cell end distinguishes this spirochete from borreliae and pathogenic treponemes, which have two or more periplasmic flagella per cell end (11). It further differs from the borreliae by the presence of a sheath around the periplasmic flagellum and by its smaller cell diameter. Growth in BSK medium suggests that its nutritional characteristics may be similar to those of borreliae. The absence of a "hook" at the end of the cell, its overlapping periplasmic

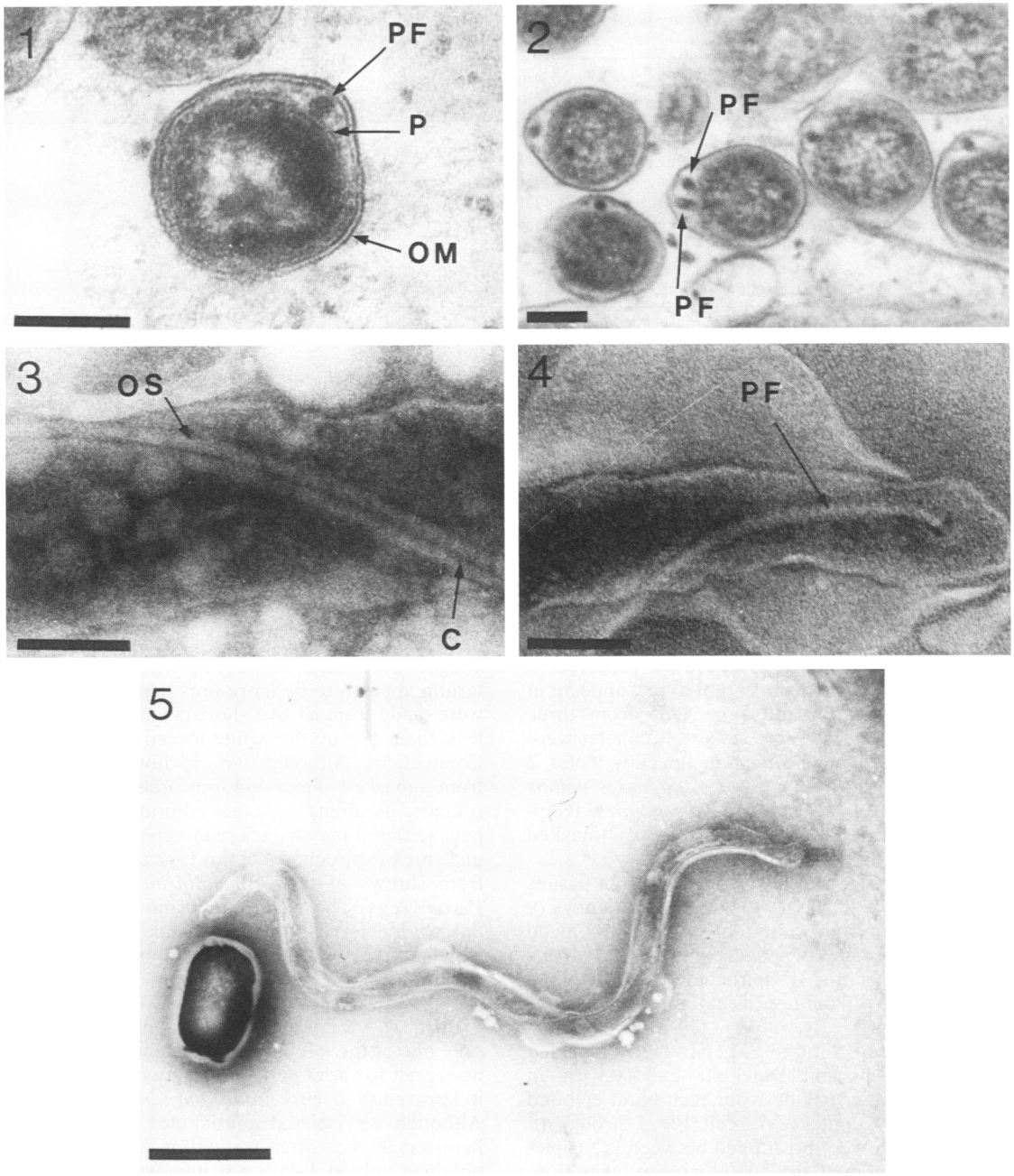

FIG. 1. Cross-section of SM spirochete with single periplasmic flagellum (PF) positioned between the triple-layered outer membrane (OM) and the peptidoglycan layer (P). Bar, 0.1 μm.

FIG. 2. Cross-section of SM spirochete showing two periplasmic flagella (PF). This cell was sectioned near the center where the periplasmic flagella, originating at opposite ends, overlap. Bar, 0.1 μm.

FIG. 3. Periplasmic flagellum (PF) showing outer sheath (OS) and central core (C). Unfixed and negatively stained. Bar, 0.1 μm.

FIG. 4. Blunt end of SM spirochete showing subterminal insertion of the periplasmic flagellum (PF). Unfixed and negatively stained. Bar, 0.1 μm.

FIG. 5. Unfixed, negatively stained micrograph of whole SM spirochete showing number of cell waves. Bar, 1.0 μm.

flagella, and its relatively short length, wider diameter, and longer wavelength differentiate it from the leptospires (14). The two remaining genera of spirochetes, *Spirochaeta* and *Cristispira*, are free-living and are not known to be infectious to vertebrates.

The G+C content of the SM spirochete, 36.7 mol%, differs from those values of *Borrelia* sp. (27.3 to 30.5; 12, 18), *T. pallidum* (52.0 to 53.7; 24), *Treponema hyodysenteriae* (25.7 to 25.9; 25), and *Spirochaeta* sp. (50.5 to 62; 8). Although the G+C content of *Leptospira* sp., 35 to 40 mol% (10), is similar to that of the SM spirochete, the SM spirochete and *Leptospira* sp. differ antigenically and morphologically as described above. Also, the G+C content of some nonpathogenic *Treponema* spp. such as *T. denticola* (37 to 38 mol%), *T. minutum* (37 mol%), *T. succinifaciens* (36 mol%), *T. phagedensis* (38 to 39 mol%), and *T. bryantii* (36 ± 1 mol%)

TABLE 2. Homologous and heterologous reciprocal titers of SM spirochetes isolated from a short-tailed shrew and white-footed mice in IFA tests

Antiserum to:	Antigen												
								L. interrogans serovars					
	CT11616	CT11397	MN47	*B. burgdorferi*	*B. hermsii*	*T. pallidum*	*S. aurantia*	*canicola*	*icterohaemorrhagiae*	*pomona*	*grippotyphosa*	*hardjo*
CT11616[a]	128	128	64	0	0	0	0	0	0	0	0	0
CT11397[b]	256	128	128	0	0	0	0	0	0	0	0	0
MN47[c]	128	ND[d]	128	0	ND	ND	0	ND	ND	ND	ND	ND
H9724[e]	0	0	0	1,024	ND	ND	ND	ND	ND	ND	ND	ND
B. burgdorferi	0	0	ND	1,024	ND	ND	ND	ND	ND	ND	ND	ND
B. hermsii	0	0	ND	ND	16,384	ND	ND	ND	ND	ND	ND	ND
T. pallidum	0	0	ND	ND	ND	16,384	ND	ND	ND	ND	ND	ND
L. interrogans serovars												
canicola	0	0	ND	ND	ND	ND	ND	16,384	ND	ND	ND	ND
icterohaemorrhagiae	0	0	ND	ND	ND	ND	ND	ND	16,384	ND	ND	ND
pomona	0	0	ND	ND	ND	ND	ND	ND	ND	16,384	ND	ND
grippotyphosa	0	0	ND	ND	ND	ND	ND	ND	ND	ND	16,384	ND
hardjo	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	16,384

[a] SM spirochete isolated from a short-tailed shrew from Connecticut.
[b] SM spirochete isolated from a white-footed mouse from Connecticut.
[c] SM spirochete isolated from a white-footed mouse from Minnesota.
[d] ND, Not done. Results of cross-reactivity studies have been published (21), with exception of SM antiserum and antigens.
[e] Monoclonal antibody specific to the genus *Borrelia* (4).

were similar to that of the SM spirochete, but these treponemes differ morphologically (27). Possibly, the SM spirochete represents a new genus of spirochetes. Additional studies are needed to clarify its taxonomic position within the *Spirochaetales* (13).

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LITERATURE CITED

1. **Anderson, J. F., R. C. Johnson, L. A. Magnarelli, and F. W. Hyde.** 1985. Identification of endemic foci of Lyme disease: isolation of *Borrelia burgdorferi* from feral rodents and ticks (*Dermacentor variabilis*). J. Clin. Microbiol. **22**:36–38.
2. **Anderson, J. F., L. A. Magnarelli, W. Burgdorfer, and A. G. Barbour.** 1983. Spirochetes in *Ixodes dammini* and mammals from Connecticut. Am. J. Trop. Med. Hyg. **32**:818–824.
3. **Barbour, A. G.** 1984. Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. **57**:521–525.
4. **Barbour, A. G., S. F. Hayes, R. A. Heiland, M. E. Schrumph, and S. L. Tessier.** 1986. A *Borrelia*-specific monoclonal antibody binds to a flagellar epitope. Infect. Immun. **52**:549–554.
5. **Bosler, E. M., J. L. Coleman, J. L. Benach, D. A. Massey, J. P. Hanrahan, W. Burgdorfer, and A. G. Barbour.** 1983. Natural distribution of the *Ixodes dammini* spirochete. Science **220**: 321–322.
6. **Breznak, J. A., and E. Canale-Parola.** 1969. *Spirochaeta aurantia*, a pigmented, facultatively anaerobic spirochete. J. Bacteriol. **97**:386–395.
7. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease—a tick-borne spirochetosis? Science **216**:1317–1319.
8. **Canale-Parola, E.** 1981. Free-living anaerobic and facultatively anaerobic spirochetes: the genus *Spirochaeta*, p. 538–547. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed), The prokaryotes. Springer-Verlag, New York.
9. **Duray, P. H., and R. C. Johnson.** 1986. The histopathology of experimentally infected hamsters with the Lyme disease spirochete, *Borrelia burgdorferi*. Proc. Soc. Exp. Biol. Med. **181**:263–269.
10. **Haapala, D. K., M. Rogul, L. B. Evans, and A. D. Alexander.** 1969. Deoxyribonucleic acid base composition and homology studies of *Leptospira*. J. Bacteriol. **98**:421–428.
11. **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 Suppl. 255.
12. **Hyde, F. W., and R. C. Johnson.** 1984. Genetic relationship of Lyme disease spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp. J. Clin. Microbiol. **20**:151–154.
13. **Johnson, R. C.** 1976. Comparative spirochete physiology and cellular composition, p. 39–48. *In* R. C. Johnson (ed.), The biology of parasitic spirochetes. Academic Press, Inc., New York.
14. **Johnson, R. C., and S. Faine.** 1984. *Leptospira*, p. 62–70. *In* N. Krieg and J. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore.
15. **Johnson, R. C., N. Marek, and C. Kodner.** 1984. Infection of Syrian hamsters with Lyme disease spirochetes. J. Clin. Microbiol. **20**:1099–1101.
16. **Johnson, R. C., G. P. Schmid, F. W. Hyde, A. G. Steigerwalt, and D. J. Brenner.** 1984. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. Int. J. Syst. Bacteriol. **34**:496–497.
17. **Kelly, R.** 1971. Cultivation of *Borrelia hermsi*. Science **173**:443–444.
18. **Kelly, R. T.** 1984. *Borrelia*, p. 57–62. *In* N. Krieg and J. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams and Wilkins Co., Baltimore.
19. **Levine, J. F., M. L. Wilson, and A. Spielman.** 1985. Mice as reservoirs of the Lyme disease spirochete. Am. J. Trop. Med. Hyg. **34**:355–360.

20. **Loken, K. I., C. Wu, R. C. Johnson, and R. F. Bey.** 1985. Isolation of the Lyme disease spirochete from mammals in Minnesota. Proc. Soc. Exp. Biol. Med. **179**:300–302.
21. **Magnarelli, L. A., J. F. Anderson, C. S. Apperson, D. Fish, R. C. Johnson, and W. A. Chappell.** 1986. Spirochetes in ticks and antibodies to *Borrelia burgdorferi* in white-tailed deer from Connecticut, New York State, and North Carolina. J. Wild. Dis. **22**:178–188.
22. **Mandel, M., and J. Marmur.** 1968. Use of ultraviolet absorbance-temperature profile for determining the quanine plus cytosine content of DNA. Methods Enzymol. **12**:195–206.
23. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
24. **Miao, R., and A. H. Fieldsteel.** 1978. Genetics of *Treponema*: relationship between *Treponema pallidum* and five cultivatable treponemes. J. Bacteriol. **133**:101–107.
25. **Miao, R., A. H. Fieldsteel, and D. L. Harris.** 1978. Genetics of *Treponema*: characterization of *Treponema hyodysenteriae* and its relationship to *Treponema pallidum*. Infect. Immun. **22**:736–739.
26. **Philip, R. N., E. A. Casper, R. A. Ormsbee, M. G. Peacock, and W. Burgdorfer.** 1976. Microimmunofluorescence test for the serological study of Rocky Mountain spotted fever. J. Clin. Microbiol. **3**:51–61.
27. **Smibert, R. M.** 1984. *Treponema*, p. 49–57. *In* N. Krieg and J. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.