

## *Spiroplasma ixodetis* sp. nov., a New Species from *Ixodes pacificus* Ticks Collected in Oregon

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**Eight strains of mollicutes were isolated from pooled suspensions prepared from western black-legged ticks (*Ixodes pacificus*) collected in Oregon. Morphologic examination by electron and dark-field microscopic techniques showed that each strain consisted of a mixture of motile, tightly coiled helical cells, small coccoid cells with diameters ranging from 300 to 500 nm, and pleomorphic, straight or branched filamentous forms. All cellular forms were surrounded by a single cytoplasmic membrane, and there was no evidence of a cell wall. The organisms were filterable and fastidious in their growth requirements. The optimum temperature for growth was 30°C, but multiplication occurred at temperatures ranging from 23 to 32°C. The strains catabolized glucose but did not hydrolyze arginine or urea. The genome size of strain Y32<sup>T</sup> (T = type strain) was 2,220 kbp, and the DNA base composition (guanine-plus-cytosine content) of this organism was 25 ± 1 mol%. The eight isolates were serologically related to each other but were not related to 37 other type or representative strains belonging to the genus *Spiroplasma*. Strain Y32 (= ATCC 33835) is the type strain of *Spiroplasma ixodetis* sp. nov.**

Spiroplasmas (class *Mollicutes*) are helical, motile, wall-less prokaryotes that are associated with a variety of insects, other arthropods, and some plant hosts (11, 38, 51). Although members of the genus *Spiroplasma* are usually commensal organisms in their arthropod hosts, several are pathogenic for insects and plants (11, 12, 24, 29, 30).

The occurrence of spiroplasmas in hematophagous arthropods was first demonstrated in 1976 when two serologically distinct helical mollicutes (represented by strains SMCA and 277F) were identified in rabbit ticks (*Haemaphysalis leporispalustris*) (4, 41). Strain SMCA and two related strains were eventually characterized as *Spiroplasma mirum* (39). Strain 277F is serologically and genomically related to *Spiroplasma citri* and was assigned to subgroup I-4 in an interim classification scheme for spiroplasmas (21, 36, 44). Subsequently, spiroplasmas have been identified in blood-sucking members of the Diptera, including horseflies (*Tabanus* spp.), deerflies (*Chrysops* spp.), and mosquitoes (*Aedes* spp., *Culex* spp., etc.). The current status of these organisms has been summarized in several recent reviews or reports (1, 10, 15, 18, 19, 38, 45).

We described additional isolations of tick-associated spiroplasmas in 1981 (37), including primary isolation of seven helical mollicutes from triturates of the western black-legged tick, *Ixodes pacificus*. While these organisms were isolated directly in SP-4 spiroplasma culture medium (43), a later report showed that most of the same isolates could be cultivated from stored triturates in several continuously maintained

cultured tick cell lines (53). An extensive serologic comparison of the strains isolated from *I. pacificus* showed that these organisms are closely related to each other but distinct from other previously described spiroplasmas. Strain Y32<sup>T</sup> (T = type strain) was selected as a representative of the group and was eventually designated a group VI strain in the interim classification system mentioned above (21, 36, 48). Some general properties of group VI spiroplasmas have been described previously in other reports (28, 32, 36, 49).

In this paper, we describe additional taxonomic features of the isolates obtained from *I. pacificus* and propose that they should be assigned to a new species in the genus *Spiroplasma*.

### MATERIALS AND METHODS

**Spiroplasma strains.** Approximately 600 adult *I. pacificus* ticks that had been collected in southern Oregon between December 1979 and July 1980 were divided into about 30 pools (representing male, female, and mixed-sex collections). The techniques used to prepare whole-tick suspensions for culture and the media used for primary isolation have been described previously (37). Seven of the pools (designated pools Y29, Y30, Y32, Y39, Y43, Y49, and Y107) yielded spiroplasma isolates, usually after incubation for 20 to 25 days at 30°C. The eighth pool (Y85) was positive only after cultures were incubated for 2 months and three or four blind passages. Isolates were obtained from both male and female tick pools, and all spiroplasmas isolated in culture were purified by conventional triple-cloning techniques (33). The strain selected as the representative of the group, strain Y32<sup>T</sup>, has been deposited in the American Type Culture Collection as strain ATCC 33835<sup>T</sup>.

The 37 other spiroplasmas used in our serologic analysis of Y32<sup>T</sup>, including members of previously described *Spiroplasma* species or currently recognized serogroups, are listed in Table 1. These strains were maintained as individual reference collections in the Beltsville, Frederick, or Stony Brook laboratory.

**Culture medium and cultivation techniques.** All primary isolations of spiroplasmas from ixodid ticks were made in SP-4 broth (43) at 30°C, and most characterization tests also were performed in this medium. This broth formulation also contained 500 U of penicillin per ml. Solid SP-4 medium was prepared by adding 2.25% Noble agar (so-called "hard agar"); we used this formulation rather than the conventional 0.8% agar formulation used for nonhelical mollicutes. Agar plates were incubated at 30°C either under aerobic conditions or in jars containing an anaerobic hydrogen GasPak system (BBL Microbiology Systems, Cockeysville, Md.).

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TABLE 1. *Spiroplasma* strains and antisera used in our serologic analysis of strain Y32<sup>Ta</sup>

Group	Species and/or strain	Size of GI zone (mm) <sup>b</sup>	DF titer <sup>c</sup>	MI titer <sup>d</sup>
I-1	<i>S. citri</i> Maroc R8A2 <sup>T</sup>	16	2,560	>117,000
I-2	<i>S. melliferum</i> BC-3 <sup>T</sup>	12	10,240	>117,000
I-3	<i>S. kunkelii</i> E275 <sup>T</sup>	18	20,480	39,000
I-4	277F	14	5,120	39,000
I-5	LB-12	18	5,120	>117,000
I-6	<i>S. insolitum</i> M55 <sup>T</sup>	13	1,280	13,000
I-7	N525	11	10,240	39,000
I-8	<i>S. phoeniceum</i> <sup>T</sup>	17	10,240	>117,000
II	DW1	ND <sup>e</sup>	2,560	ND
III	<i>S. floricola</i> OBMG	6	10,240	13,000
IV	<i>S. apis</i> B31 <sup>T</sup>	5	1,280	13,000
V	<i>S. mirum</i> SMCA <sup>T</sup>	13	5,120	13,000
VI	Y32	16	2,560	4,374
VII	<i>S. monobiae</i> MQ-1 <sup>T</sup>	6	1,280	>117,000
VIII	EA-1	10	1,280	39,000
IX	<i>S. clarkii</i> CN-5 <sup>T</sup>	10	640	4,374
X	<i>S. culicicola</i> AES-1 <sup>T</sup>	8	640	4,374
XI	MQ-4	5	640	4,374
XII	DU-1	5	2,560	13,000
XIII	<i>S. sabaudiense</i> Ar-1343 <sup>T</sup>	8	2,560	13,000
XIV	EC-1	5	1,280	13,000
XV	I-25	8	1,280	>117,000
XVI	<i>S. cantharicola</i> CC-1 <sup>T</sup>	5	1,280	39,000
XVII	DF-1	10	5,120	39,000
XVIII	TN-1	6	1,280	>117,000
XIX	PUP-1	14	5,120	13,000
XX	LD-1	ND	5,120	ND
XXI	W115	15	1,280	4,374
XXII	<i>S. taiwanense</i> CT-1 <sup>T</sup>	8	1,280	13,000
XXIII	TG-1	7	2,560	39,000
XXIV	<i>S. chinense</i> CH <sup>T</sup>	4	640	1,458
Ungrouped	HYOS-1 (horsefly)	8	5,120	39,000
Ungrouped	BIUS-1 ( <i>Bidens</i> flowers)	7	10,240	13,000
Ungrouped	TABS-2 (horsefly)	7	640	39,000
Ungrouped	TALS-2 (horsefly)	7	320	1,458
Ungrouped	PLHS-1 (scorpion fly)	11	2,560	4,374
Ungrouped	TAUS-1 (horsefly)	7	5,120	13,000
Ungrouped	PALS-1 (dragonfly)	7	1,280	4,374

<sup>a</sup> Strain Y32<sup>T</sup> antigen was used in three serologic tests along with antiserum to each of the spiroplasma strains examined. Reciprocal tests against Y32<sup>T</sup> antiserum were also performed with each spiroplasma strain. We found that the group VI isolates were related, but strain Y32<sup>T</sup> was not serologically related to the strains belonging to groups I through V and VII through XXIV or to representatives of ungrouped spiroplasma clusters.

<sup>b</sup> Zones of homologous GI obtained with type-specific antisera to the organisms tested. We measured zones of GI around antiserum-saturated discs on agar plates containing colony growth. In homologous tests Y32<sup>T</sup> antigen produced a 16-mm zone of inhibition, while all other antisera produced zones that were 2 mm or less wide.

<sup>c</sup> Homologous DF test titers obtained with type-specific antisera to the organisms tested. We determined the reciprocal of the highest dilution of antiserum that deformed at least one-half of the spiroplasmas. Y32<sup>T</sup> antigen tested against homologous antiserum produced a DF titer of 1:2,560, while Y32<sup>T</sup> tests performed with all other antisera resulted in titers of 1:20 or less.

<sup>d</sup> Homologous MI test titers obtained with type-specific antisera to the organisms tested. We determined the reciprocal of the highest dilution of antiserum that resulted in GI of a standard broth inoculum. Y32<sup>T</sup> antigen tested against homologous antiserum produced an MI titer of 1:4,354, and Y32<sup>T</sup> antigen tested against all other antisera produced titers of 1:18 or less.

<sup>e</sup> ND, not done.

The temperature requirements for growth of the eight strains were determined by incubating broth cultures at various temperatures (10, 18, 23, 30, 32, and 37°C), and growth was recorded as the number of color-changing units (CCU) per milliliter observed over a 3-week period. Early passages of the eight strains were also grown in SP-4 broth medium lacking antibiotics for at least five consecutive passages. After each passage, the strains were plated onto conven-

tional blood agar plates and examined for evidence of reversion to bacterial-type colonies.

**Tests for biological and biochemical properties.** The procedures used to determine glucose fermentation (2), hydrolysis of arginine and urea (2), filtration characteristics in SP-4 broth (33), and hemadsorption (17) have been described previously.

**Morphological studies.** All eight spiroplasma isolates obtained from ticks were examined by dark-field microscopy (magnification,  $\times 1,250$ ), using SP-4 broth cultures that varied from the early logarithmic growth phase to the late stationary phase. Strain Y32<sup>T</sup> was studied electron microscopically by both negative staining and thin-section techniques. The procedures used for each of these techniques have been described previously (38).

**Sterol requirement.** Strain Y32<sup>T</sup> and other isolates obtained from ixodid ticks in this study had rather fastidious growth requirements, and little growth occurred in most medium formulations containing reduced amounts of serum. These circumstances made it difficult to measure sterol requirements by the conventional broth-protein yield method (26). However, we used a technique used previously for other fastidious spiroplasmas (*S. mirum*) (39). In this modified technique, growth responses (in CCU per milliliter) were measured in base serum-free broth alone and in base medium containing various quantities of fetal bovine serum or cholesterol. In addition, we also examined the susceptibility of ixodid spiroplasmas to 1.5% digitonin, which provided an indirect measurement of sterol requirements (16).

**Serological tests.** Antiserum to strain Y32<sup>T</sup> was prepared in rabbits by using previously described techniques (31). Disc growth inhibition (GI) tests in which we used Y32<sup>T</sup> antiserum and previously described *Spiroplasma* species or group representatives were carried out as previously described (13, 47), using SP-4 hard agar. The procedures used for reciprocal testing of Y32<sup>T</sup> antigen and antisera in spiroplasma metabolism inhibition (MI) and deformation (DF) tests have also been described previously (50, 52). The addition of complement (10% guinea pig serum) in the MI test was found to be inhibitory to all of the ixodid spiroplasma strains examined (49); therefore, complement was omitted from all MI tests in which these organisms were used.

**Genomic analysis.** Genome size was determined by pulsed-field gel electrophoresis of undigested linear genomic molecules and from the summed sizes of restricted DNA fragments. Genomic DNA embedded in LMP agarose (Geneline-Beckman, Palo Alto, Calif.) was prepared as described previously (6, 7, 25), except that the cellular pellet was resuspended in STE (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA). Restriction enzymes *Sma*I, *Apa*I, and *Sal*I were used as recommended by the manufacturer, and digestion preparations were incubated overnight. The restriction fragments were separated on a 1% LE agarose gel by using a transverse alternating field electrophoresis apparatus (Geneline-Beckman). The sizes of all separated fragments were evaluated by comparison with yeast chromosomal DNA fragments (Bio-Rad, Richmond, Calif.), which varied in size from 215 to 2,200 kbp, and with lambda phage DNA concatemers (New England Biolabs, Beverly, Mass.).

The guanine-plus-cytosine (G+C) content of purified DNA was determined by buoyant density, melting temperature, and high-performance liquid chromatography (HPLC) techniques (9). The methods used for extraction and purification of DNA from mollicutes have been described previously (8). Purified DNA from *S. citri* (base composition,  $26 \pm 1$  mol%) was used as a control.

## RESULTS AND DISCUSSION

**Cultural and morphologic properties.** All eight ixodid isolates grew in SP-4 broth but required prolonged incubation (20 to 25 days) for primary isolation. Following cloning and repeated broth passage, most strains began to exhibit evidence of carbohydrate fermentation after incubation at 30°C for 5 to 10 days. In early passages, little growth occurred in most other medium formulations used for spiroplasmas or mycoplasmas. After laboratory cultivation for 10 to 20 passages, some strains were able to grow on spiroplasma medium M1A or M1D (43). The organisms were very sensitive to changes in the quality of the fresh yeast extract or fetal bovine serum components in SP-4 broth, and strain Y32<sup>T</sup> eventually became a useful test organism for quality control of SP-4 medium. Likewise, reductions in the serum content severely reduced the amount of growth. Growth also occurred within a very narrow temperature range (23 to 32°C). After 21 days of incubation, optimum growth was observed at either 30°C ( $10^8$  CCU/ml) or 23°C ( $10^7$  CCU/ml), some growth was observed at 32°C ( $10^4$  CCU/ml), and no growth was apparent in cultures incubated at 10, 18, or 37°C. Agar colonies of the ixodid spiroplasmas were classical fried egg colonies on SP-4 hard agar when the organisms were incubated at 30°C under aerobic conditions (Fig. 1A). How-

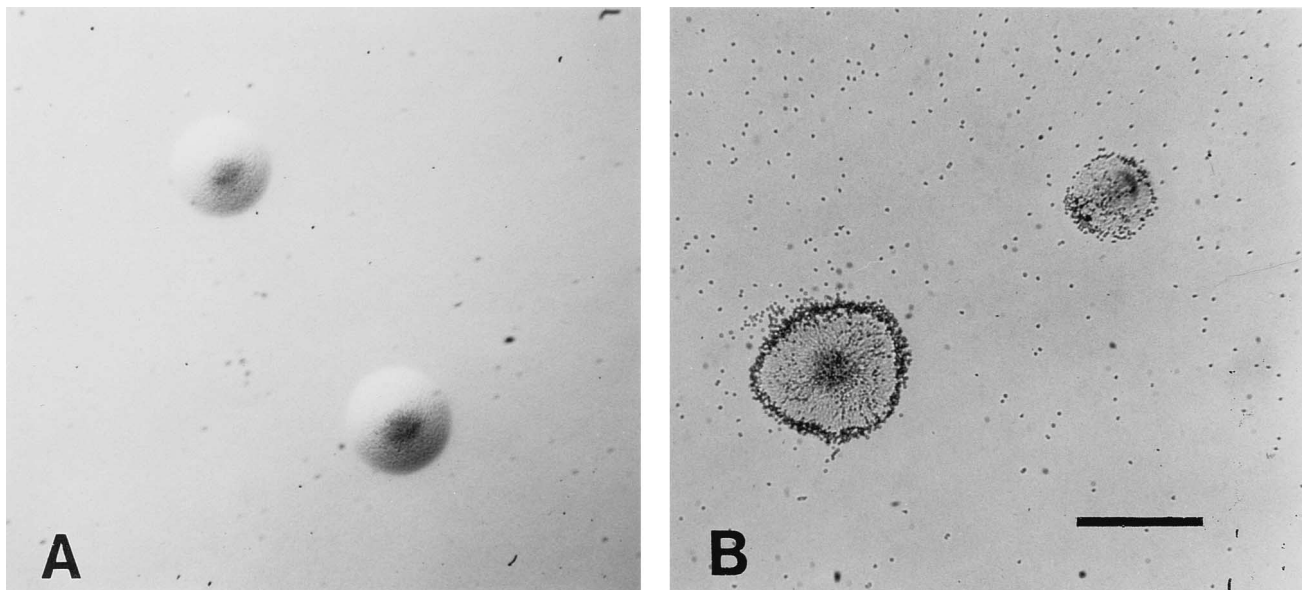


FIG. 1. Colony morphology and hemadsorption activity of spiroplasma strain Y32<sup>T</sup>. (A) Strain Y32<sup>T</sup> grown for 10 days on SP-4 hard agar at 30°C under aerobic conditions. (B) Strain Y32<sup>T</sup> agar colonies exhibiting positive hemadsorption of guinea pig erythrocytes. Bar = 100  $\mu$ m.

ever, a period of 10 to 14 days of incubation was usually required for satisfactory colony growth. No evidence of bacterial growth occurred on blood agar plates when spiroplasma isolates grown for five or more passages in SP-4 broth lacking antibiotics were plated onto this medium.

Dark-field microscopic examination of broth cultures of the eight ixodid isolates at various phases of growth revealed a unique mixture of predominantly filamentous forms, some of which were straight and some of which were branched or had Y-shaped elements. A few helical and coccoid forms were usually found in each preparation, but the number of helices was always much less than the number of other morphologic forms. In older cultures, we observed large cellular clumps, each of which contained numerous motile filaments at the periphery of the clump. Most of the motility was expressed as flexional but not translational movement. In several instances, we observed peripheral filaments that changed rapidly from helical cells to relaxed straight filaments.

Fixed cells were examined by negative staining and electron microscopy, and these preparations showed that many small cells were tightly coiled helical filaments (Fig. 2A). It seemed likely that these small helical cells were interpreted as small straight filaments when they were examined by dark-field microscopy at a lower magnification. Thin sections of fixed cells revealed primarily coccoid cells (diameter, 300 to 500 nm) or curved forms (37) (Fig. 2B). All cells observed by electron microscopy were surrounded only by a single membrane and lacked cell walls (37). However, some sectioned cells also had a unique 8-nm-thick structure that was under the plasmalemma in the cell (Fig. 2B); the function of this structure is not known. Like many other *Spiroplasma* species and unclassified strains (38, 51), intracellular virus-like particles were observed in strain Y32<sup>T</sup> (Fig. 2C).

**Sterol requirement.** The requirement for cholesterol or serum by strain Y32<sup>T</sup> was established by a modified sterol test (Table 2). Growth enhancement was apparent when 10 to 20  $\mu$ g of cholesterol per ml was incorporated into the serum-free base medium or when 5 to 15% fetal bovine serum was added. The inhibition zone for digitonin susceptibility was 9 mm wide,

which indirectly indicated that sterol was required (16, 34). In addition, strain Y32<sup>T</sup> was also included in a recently described study in which Rose et al. determined the requirements of mollicutes for serum or polyoxyethylene sorbitan (Tween 80) (27). In this study, sustained growth of Y32<sup>T</sup> through 23 serial dilutions occurred only in media containing serum.

**Biochemical and biological properties.** All eight ixodid isolates catabolized glucose. Repeated attempts to demonstrate arginine hydrolysis, even in the presence of glucose, were uniformly negative. Likewise, tests for urea hydrolysis were negative. Although colonies of all strains hemadsorbed guinea pig erythrocytes (Fig. 1B), we did not observe hemadsorption with sheep, bovine, rat, rabbit, monkey, or human type O erythrocytes. Unfiltered broth cultures of Y32<sup>T</sup> had a titer of  $10^8$  CCU/ml. Filtration through membranes with average pore diameters of 450, 300, 220, and 100 nm yielded titers of  $10^7$ ,  $10^7$ ,  $10^4$ , and  $10^1$  CCU/ml, respectively.

**Serological tests.** GI, DF, and MI tests were performed with the eight ixodid spiroplasmas and type-specific antiserum to strain Y32<sup>T</sup> prepared in rabbits. The results of these tests (data not shown) confirmed that all eight ixodid tick-derived strains were closely related. We also performed reciprocal GI, MI, and DF tests, in which we used Y32<sup>T</sup> antigen and antisera versus many antigens and type-specific antisera to previously described *Spiroplasma* species (designated group representatives) or currently ungrouped strains (Table 1). Table 1 shows the homologous values obtained with the various type-specific antisera in each of the serologic tests, which were determined to establish the potency of the test sera. In heterologous tests of Y32<sup>T</sup> antigen or antisera versus other *Spiroplasma* species, groups, or putative species, GI zones of 2 mm or less, DF titers of 1:20 or less, and MI titers of 1:18 or less were observed. Our findings clearly established that strain Y32<sup>T</sup> is not significantly serologically related to previously described *Spiroplasma* species or groups.

**Genome size and DNA base composition.** DNA renaturation kinetics were used in early attempts to determine the genome sizes of the ixodid spiroplasmas. The results of such tests were difficult to interpret since at least three renaturation curves

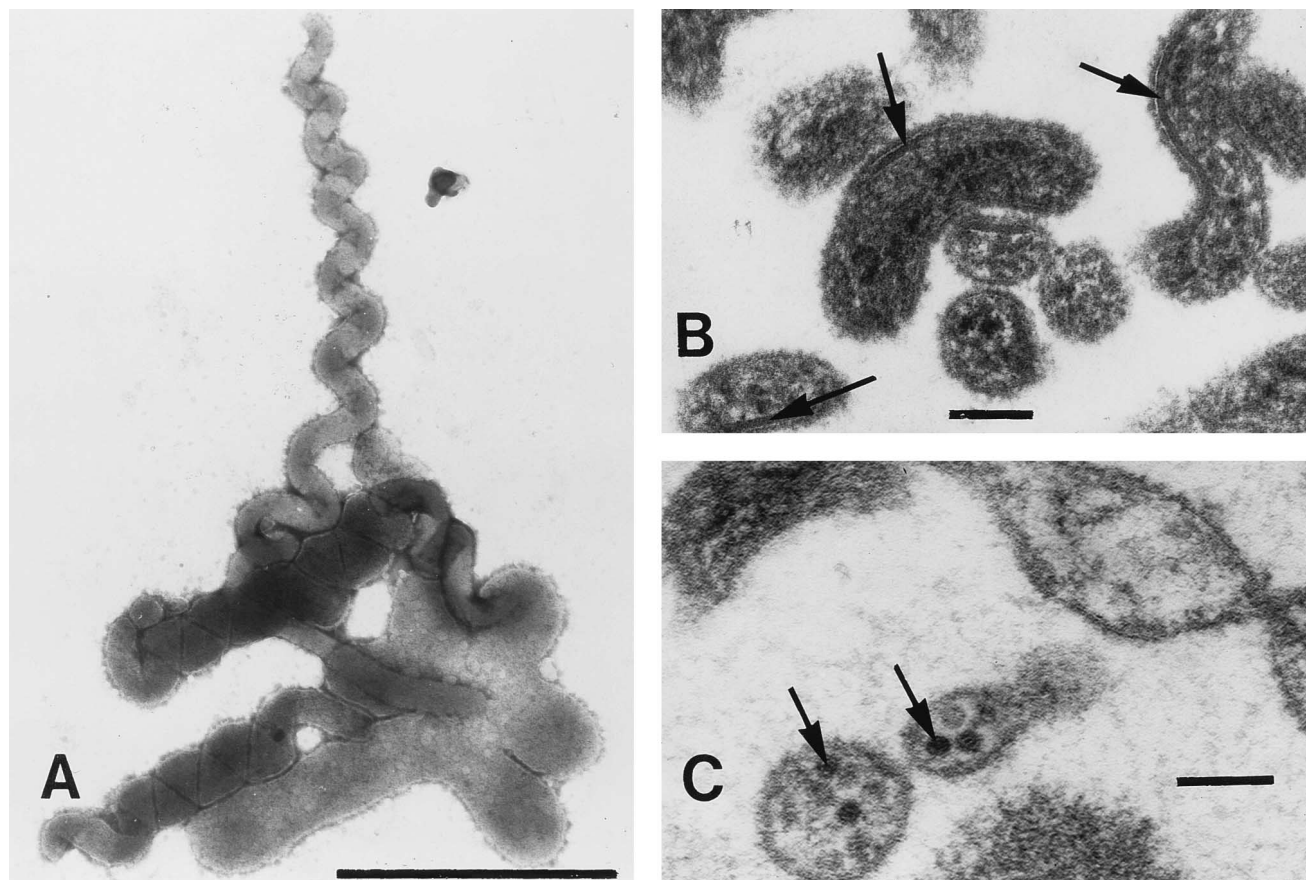


FIG. 2. Ultrastructural morphology of strain Y32<sup>T</sup>. (A) Negatively stained spiroplasma strain Y32<sup>T</sup> cells, showing that the cells are pleomorphic. Bar = 1  $\mu$ m. (B) Thin section of a strain Y32<sup>T</sup> cell pellet. The arrows indicate the 8-nm-thick subplasmalemmal structure that appears to be limited to one side of the organism. Bar = 100 nm. (C) Thin section of a strain Y32<sup>T</sup> cell pellet. The arrows indicate intracellular virus-like particles. Bar = 100 nm.

were observed consistently when purified DNA from each of the organisms was analyzed. Pulsed-field gel electrophoresis provided a much more accurate procedure for measuring the sizes of large genomes. Undigested linear genomic material from Y32<sup>T</sup> was found to be 2,220 kbp long. Genome sizes of 2,225, 2,240, and 2,265 kbp were obtained for strain Y32<sup>T</sup> when

we added the sizes of the restriction fragments obtained after digestion with enzymes *Sma*I, *Apa*I, and *Sal*I, respectively. Thus, the genome of Y32<sup>T</sup> is close to 2,220 kbp long; this is the largest genome size that has been obtained so far for any mollicute (6). The DNA base composition values determined by the three techniques mentioned above indicated that the G+C content was  $25 \pm 1$  mol%.

Although strain Y32<sup>T</sup> and its allies appeared to be true spiroplasmas, the occurrence of many nonhelical forms in young broth cultures and the unusual genome size raised the possibility that these organisms might belong to a new taxon. However, information obtained in a recent phylogenetic study of 45 mollicutes supported the hypothesis that Y32<sup>T</sup> and other members of the genus *Spiroplasma* are related (42). In a comparison of 16S rRNA sequences, strain Y32<sup>T</sup> clustered with a group of nine other spiroplasmas.

**Habitat.** The eight serologically related spiroplasmas isolated from *I. pacificus* are the only helical mollicutes that have been found in ticks other than the mollicutes associated with the rabbit tick (*H. leporispalustris*). A limited number of other ticks, including members of both the Ixodidae (hard ticks) and the Argasidae (soft ticks), have been examined for spiroplasma, with negative results (40). In general, the real extent of spiroplasma occurrence in ticks is unknown, since few epidemiologic surveys have been performed. *I. pacificus* is an important pest of humans, domestic stock, and game animals. This species occurs along the western seaboard of North

TABLE 2. Sterol requirement of spiroplasma strain Y32<sup>T</sup>

Addition to base medium	Concn	No. of organisms (CCU/ml) after incubation at 30°C for:		
		7 days	14 days	21 days
None <sup>a</sup>		10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>1</sup>
None <sup>b</sup>		10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>1</sup>
Cholesterol <sup>c</sup>	1 $\mu$ g/ml	10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>1</sup>
	5 $\mu$ g/ml	10 <sup>1</sup>	10 <sup>1</sup>	10 <sup>1</sup>
	10 $\mu$ g/ml	10 <sup>1</sup>	10 <sup>3</sup>	10 <sup>3</sup>
	20 $\mu$ g/ml	10 <sup>1</sup>	10 <sup>3</sup>	10 <sup>3</sup>
	Fetal bovine serum <sup>c</sup>	0.5%	10 <sup>1</sup>	10 <sup>2</sup>
	5%	10 <sup>1</sup>	10 <sup>3</sup>	10 <sup>3</sup>
	10%	10 <sup>1</sup>	10 <sup>3</sup>	10 <sup>5</sup>
	15%	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>5</sup>
	20%	10 <sup>2</sup>	10 <sup>2</sup>	10 <sup>3</sup>

<sup>a</sup> Serum-free base medium alone.

<sup>b</sup> Base medium supplemented with 0.5% fatty acid-poor albumin, 0.01% Tween 80, and 10  $\mu$ g of palmitic acid per ml.

<sup>c</sup> All medium preparations contained the supplements described in footnote b.

America from British Columbia to Mexico, usually west of the Cascade and Sierra Nevada mountain ranges, and has been found in several sites west of the Wasatch Range in Utah (14).

At least 55 vertebrate species have been reported to be hosts for adult, larval, or nymphal forms of *I. pacificus* (3); this tick has been recovered most frequently from the western fence lizard (*Sceloporus occidentalis*) (22, 23), other reptiles, and rodent or avian species. The optimum growth temperature for the Y32<sup>T</sup> group of spiroplasmas (30 to 32°C) suggests that reptiles or other poikilotherms might be more important reservoirs of the organisms than mammals or avian hosts. It is not known whether spiroplasma occurrence is related to the developmental stages of the ticks (i.e., transovarial and transstadial transmission) or whether vertebrates play an essential role in maintaining spiroplasmas in ticks. Thus, the spiroplasmas in *I. pacificus* ticks might well represent primary spiroplasma infections in reptile, rodent, or avian hosts rather than natural arthropod infections. It should also be noted that *I. pacificus* is one of the principal reservoirs and transmission vectors for *Borrelia burgdorferi* (5), an important disease agent in humans. Since *Borrelia* and *Spiroplasma* species are not easily differentiated by dark-field microscopy, assessments to determine the occurrence of either species in *Ixodes* tick suspensions should not be based on direct morphologic observations. However, the presence of spiroplasmas in *I. pacificus* ticks, which have been described as "vicious human-biters" (23), and in a variety of blood-feeding tabanids, deerflies, and mosquitoes suggests that these organisms may have the potential to be agents of human disease. The apparent inability of the spiroplasmas described in this paper to grow well at 37°C (in contrast to *S. mirum* strains [39]) argues against this possibility. However, descriptions of the biologic and serologic properties of microbial agents such as the Y32<sup>T</sup> group may provide the basis for a useful detection system if in the future tick-derived diseases of unknown etiology appear.

The properties of strain Y32<sup>T</sup> described in this paper fulfill the criteria for species descriptions of members of the class *Mollicutes* (20, 35). The cells of this organism lack a cell wall, are filterable, fail to revert to walled bacteria when they are grown in antibiotic media, are resistant to penicillin, and produce typical fried egg colonies on solid media. The growth requirement for sterol or serum, the optimum growth temperature of 30°C, helicity, and the close phylogenetic relationships to other spiroplasmas (as determined by 16S rRNA comparisons) place this organism in the order *Entomoplasmatales*, the family *Spiroplasmataceae*, and the genus *Spiroplasma* (35, 38, 46). The lack of serological relatedness of strain Y32<sup>T</sup> to other *Spiroplasma* species demonstrates that this strain is a member of a previously unrecognized species in the genus. We propose the name *Spiroplasma ixodetis* for strain Y32<sup>T</sup>.

The taxonomic description below summarizes the properties of the species.

**Description of *Spiroplasma ixodetis*.** *Spiroplasma ixodetis* (ix.o.de'tis M.L. gen. n. *ixodetis*, of *Ixodes*, the genus name of *I. pacificus* ticks, from which the organism was first isolated). Cells consist of various mixtures of coccoid forms (diameter, 300 to 500 nm), straight and branched filamentous cells, and helical organisms. Cells lack a cell wall. Motile. Colonies on solid medium containing 2.25% Noble agar usually have the appearance of fried eggs. Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea. Agar colonies hemadsorb guinea pig erythrocytes but not erythrocytes of other mammalian species.

Cholesterol or serum is required for growth.

The temperature range for growth is 23 to 32°C; optimum growth occurs at 30°C.

Serologically distinct from previously described *Spiroplasma* species.

Isolated from macerated tissue suspensions prepared from pooled adult *I. pacificus* ticks (Ixodidae) collected in Oregon.

Pathogenicity for plants or insects has not been determined. Organisms exhibit growth and cytoadherence to various tick and insect cells in culture, without obvious cytopathogenic effects.

The average genome size is 2,220 kbp. The average G+C content of the DNA is 25.0 mol%, as determined by buoyant density, melting temperature, and HPLC techniques.

The type strain is Y32 (= ATCC 33835).

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#### REFERENCES

- Abalain-Colloc, M. L., D. L. Williamson, P. Carle, J. H. Abalain, F. Bonnet, J. G. Tully, M. Konai, R. F. Whitcomb, J. M. Bové, and C. Chastel. 1993. Division of group XVI spiroplasmas into subgroups. *Int. J. Syst. Bacteriol.* 43:342-346.
- Aluotto, B. B., R. G. Wittler, C. O. Williams, and J. E. Faber. 1970. Standardized bacteriologic techniques for characterization of *Mycoplasma* species. *Int. J. Syst. Bacteriol.* 20:35-58.
- Arthur, D. R., and K. R. Snow. 1968. *Ixodes pacificus* Cooley and Kohls, 1943: its life-history and occurrence. *Parasitology* 58:893-906.
- Brinton, L. P., and W. Burgdorfer. 1976. Cellular and subcellular organization of the 277F agent, a spiroplasma from the rabbit tick *Haemaphysalis leporispalustris* (Acari: Ixodidae). *Int. J. Syst. Bacteriol.* 26:554-560.
- Burgdorfer, W., R. S. Lane, A. G. Barbour, R. A. Gresbrink, and J. R. Anderson. 1985. The western black-legged tick, *Ixodes pacificus*: a vector of *Borrelia burgdorferi*. *Am. J. Trop. Med. Hyg.* 34:925-930.
- Carle, P., F. Laigret, J. M. Bové, and J. G. Tully. Heterogeneity of genome sizes within the genus *Spiroplasma*. Submitted for publication.
- Carle, P., D. L. Rose, J. G. Tully, and J. M. Bové. 1992. The genome size of spiroplasmas and other mollicutes. *IOM Lett.* 2:263.
- Carle, P., C. Saillard, and J. M. Bové. 1983. DNA extraction and purification. *Methods Mycoplasmol.* 1:295-299.
- Carle, P., C. Saillard, and J. M. Bové. 1983. Determination of guanine plus cytosine content of DNA. *Methods Mycoplasmol.* 1:301-308.
- Chastel, C., and I. Humphrey-Smith. 1991. Mosquito spiroplasmas, p. 149-206. *In* K. F. Harris (ed.), *Advances in disease vector research*, vol. 7. Springer-Verlag, New York.
- Clark, T. B. 1983. Spiroplasmas: diversity of arthropod reservoirs and host-parasite relationships. *Science* 217:57-59.
- Clark, T. B., R. F. Whitcomb, J. G. Tully, C. Mouches, C. Saillard, J. M. Bové, H. Wróblewski, P. Carle, D. L. Rose, R. B. Henegar, and D. L. Williamson. 1985. *Spiroplasma melliferum*, a new species from the honeybee (*Apis mellifera*). *Int. J. Syst. Bacteriol.* 35:296-308.
- Clyde, W. A., Jr. 1983. Growth inhibition tests. *Methods Mycoplasmol.* 1:405-410.
- Easton, E. R., J. E. Keirans, R. A. Gresbrink, and C. M. Clifford. 1977. The distribution in Oregon of *Ixodes pacificus*, *Dermacentor andersoni*, and *Dermacentor occidentalis*, with a note on *Dermacentor variabilis* (Acarina: Ixodidae). *J. Med. Entomol.* 13:501-506.
- French, F. E., R. F. Whitcomb, J. G. Tully, K. J. Hackett, E. A. Clark, R. B. Henegar, A. G. Wagner, and D. L. Rose. 1990. Tabanid spiroplasmas of the southeast USA: new groups, and correlation with host life history strategy. *Zentralbl. Bakteriologie, Suppl.* 20:919-921.
- Freundt, E. A., B. E. Andrews, H. Ernø, M. Kunze, and F. T. Black. 1973. The sensitivity of *Mycoplasma* to sodium-polyanethanol-sulfonate and digitonin. *Zentralbl. Bakteriologie, Parasitenkd. Infektionskr. Hyg.* 225:104-112.
- Gardella, R. S., and R. A. Del Giudice. 1983. Hemagglutination, hemadsorption, and hemolysis. *Methods Mycoplasmol.* 1:379-384.
- Gasparich, G. E., C. Saillard, E. A. Clark, M. Konai, F. E. French, J. G. Tully, K. J. Hackett, and R. F. Whitcomb. 1993. Serologic and genomic relatedness of group VIII and group XVII spiroplasmas and subdivision of spiroplasma group VIII into subgroups. *Int. J. Syst. Bacteriol.* 43:338-341.
- Hackett, K. J., and T. B. Clark. 1989. Ecology of spiroplasmas, p. 113-200. *In* R. F. Whitcomb and J. G. Tully (ed.), *The mycoplasmas*, vol. 5. Academic Press, New York.
- International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*. 1979. Proposal of minimal standards for descriptions of new species of the class *Mollicutes*. *Int. J. Syst. Bacteriol.* 29:172-180.
- Junca, P., C. Saillard, J. G. Tully, O. Garcia-Jurado, J. R. Degorce-Dumas,

- C. Mouches, J. C. Vignault, R. Vogel, R. McCoy, R. F. Whitcomb, D. L. Williamson, J. Latrille, and J. M. Bové. 1980. Caractérisation de spiroplasmas isolés d'insectes et de fleurs de France continentale, de Corse et du Maroc. Proposition pour classification des spiroplasmas. C. R. Acad. Sci. Ser. D **290**:1209–1212.
22. Manweiler, S. A., R. S. Lane, W. M. Block, and M. L. Morrison. 1990. Survey of birds and lizards for ixodid tick (Acari) and spirochetal infection in northern California. J. Med. Entomol. **27**:1011–1015.
23. Manweiler, S. A., R. S. Lane, and C. H. Tempelis. 1992. The western fence lizard *Sceloporus occidentalis*: evidence of field exposure to *Borrelia burgdorferi* in relation to infestation by *Ixodes pacificus* (Acari: Ixodidae). Am. J. Trop. Med. Hyg. **47**:328–336.
24. Mouches, C., J. M. Bové, J. G. Tully, D. L. Rose, R. E. McCoy, P. Carle-Junca, M. Garnier, and C. Saillard. 1983. *Spiroplasma apis*, a new species from the honeybee (*Apis mellifera*). Ann. Microbiol. **134**:383–397.
25. Pyle, L. E., L. N. Corcoran, B. G. Cocks, A. D. Bergemann, J. C. Whitley, and L. R. Finch. 1988. Pulsed-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes. Nucleic Acids Res. **16**:6015–6025.
26. Razin, S., and J. G. Tully. 1970. Cholesterol requirement of mycoplasmas. J. Bacteriol. **102**:306–310.
27. Rose, D. L., J. G. Tully, J. M. Bové, and R. F. Whitcomb. 1993. A test for measuring growth responses of mollicutes to serum and polyoxyethylene sorbitan. Int. J. Syst. Bacteriol. **43**:527–532.
28. Rose, D. L., J. G. Tully, R. F. Whitcomb, D. L. Williamson, and J. M. Bové. 1983. Unique characteristics of the Y32 group of spiroplasmas (group VI) recovered from *Ixodes pacificus* ticks. Yale J. Biol. Med. **56**:844–845.
29. Saglio, P., M. Lhospital, D. Lafèche, G. Dupont, J. M. Bové, J. G. Tully, and E. A. Freundt. 1973. *Spiroplasma citri* gen. and sp. n.: a mycoplasma-like organism associated with "stubborn" disease of citrus. Int. J. Syst. Bacteriol. **23**:191–204.
30. Saillard, C., J. C. Vignault, J. M. Bové, A. Raie, J. G. Tully, D. L. Williamson, A. Fos, M. Garnier, A. Gadeau, P. Carle, and R. F. Whitcomb. 1987. *Spiroplasma phoeniceum* sp. nov., a new plant-pathogenic species from Syria. Int. J. Syst. Bacteriol. **37**:106–115.
31. Senterfit, L. B. 1983. Preparation of antigens and antisera. Methods Mycoplasmol. **1**:401–404.
32. Tully, J. G. 1983. Reflections on recovery of some fastidious mollicutes with implications of the changing host patterns of these organisms. Yale J. Biol. Med. **56**:799–813.
33. Tully, J. G. 1983. Cloning and filtration techniques for mycoplasmas. Methods Mycoplasmol. **1**:173–177.
34. Tully, J. G. 1983. Tests for digitonin sensitivity and sterol requirement. Methods Mycoplasmol. **1**:355–362.
35. Tully, J. G., J. M. Bové, F. Laigret, and R. F. Whitcomb. 1993. Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. Int. J. Syst. Bacteriol. **43**:378–385.
36. Tully, J. G., D. L. Rose, E. Clark, P. Carle, J. M. Bové, R. B. Henegar, R. F. Whitcomb, D. E. Colflesh, and D. L. Williamson. 1987. Revised group classification of the genus *Spiroplasma* (class *Mollicutes*), with proposed new groups XII to XXIII. Int. J. Syst. Bacteriol. **37**:357–364.
37. Tully, J. G., D. L. Rose, C. E. Yunker, J. Cory, R. F. Whitcomb, and D. L. Williamson. 1981. Helical mycoplasmas (spiroplasmas) from *Ixodes* ticks. Science **212**:1043–1045.
38. Tully, J. G., and R. F. Whitcomb. 1991. The genus *Spiroplasma*, p. 1960–1980. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, vol. 2, 2nd ed. Springer-Verlag, New York.
39. Tully, J. G., R. F. Whitcomb, D. L. Rose, and J. M. Bové. 1982. *Spiroplasma mirum*, a new species from the rabbit tick (*Haemaphysalis leporispalustris*). Int. J. Syst. Bacteriol. **32**:92–100.
40. Tully, J. G., R. F. Whitcomb, D. L. Rose, D. L. Williamson, and J. M. Bové. 1983. Characterization and taxonomic status of tick spiroplasmas: a review. Yale J. Biol. Med. **56**:599–603.
41. Tully, J. G., R. F. Whitcomb, D. L. Williamson, and H. F. Clark. 1976. Suckling mouse cataract agent is a helical wall-free prokaryote (spiroplasma) pathogenic for vertebrates. Nature (London) **259**:117–120.
42. Weisburg, W. G., J. G. Tully, D. L. Rose, J. P. Petzel, H. Oyaizu, D. Yang, L. Mandelco, J. Sechrest, T. G. Lawrence, J. Van Etten, J. Maniloff, and C. R. Woese. 1989. A phylogenetic analysis of the mycoplasmas: basis for their classification. J. Bacteriol. **171**:6455–6467.
43. Whitcomb, R. F. 1983. Culture media for spiroplasmas. Methods Mycoplasmol. **1**:147–158.
44. Whitcomb, R. F., J. M. Bové, T. A. Chen, J. G. Tully, and D. L. Williamson. 1987. Proposed criteria for an interim serogroup classification for members of the genus *Spiroplasma* (class *Mollicutes*). Int. J. Syst. Bacteriol. **37**:82–84.
45. Whitcomb, R. F., K. J. Hackett, J. G. Tully, E. A. Clark, F. E. French, R. B. Henegar, D. L. Rose, and A. Wagner. 1990. Tabanid spiroplasmas as a model for mollicute biogeography. Zentralbl. Hyg. Suppl. **20**:931–933.
46. Whitcomb, R. F., and J. G. Tully. 1984. Family III. *Spiroplasmataceae* Skripal 1983, 408. genus I. *Spiroplasma*, p. 781–787. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore.
47. Whitcomb, R. F., J. G. Tully, P. McCawley, and D. L. Rose. 1982. Application of the growth inhibition test to *Spiroplasma* taxonomy. Int. J. Syst. Bacteriol. **32**:387–394.
48. Whitcomb, R. F., J. G. Tully, D. L. Williamson, J. M. Bové, F. E. French, M. Konai, G. Gasparich, M. Abalain-Colloc, C. Saillard, C. Chastel, P. Carle, D. L. Rose, R. Henegar, E. A. Clark, and K. J. Hackett. 1992. Revised classification of spiroplasmas. IOM Lett. **2**:134.
49. Williamson, D. L., and J. G. Tully. 1983. Guinea pig complement inhibits growth of *Ixodes* tick-derived Y32 spiroplasmas. Yale J. Biol. Med. **56**:845–846.
50. Williamson, D. L., J. G. Tully, and R. F. Whitcomb. 1979. Serological relationships of spiroplasmas as shown by combined deformation and inhibition tests. Int. J. Syst. Bacteriol. **29**:345–351.
51. Williamson, D. L., J. G. Tully, and R. F. Whitcomb. 1989. The genus *Spiroplasma*, p. 71–111. In R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. 5. Academic Press, New York.
52. Williamson, D. L., R. F. Whitcomb, and J. G. Tully. 1978. The spiroplasma deformation test, a new serological method. Curr. Microbiol. **1**:203–207.
53. Yunker, C. E., J. G. Tully, and J. Cory. 1987. Arthropod cell lines in the isolation and propagation of tickborne spiroplasmas. Curr. Microbiol. **15**: 45–50.