Spiroplasma chrysopicola sp. nov., *Spiroplasma gladiatoris* sp. nov., *Spiroplasma helicoides* sp. nov., and *Spiroplasma tabanidicola* sp. nov., from Tabanid (Diptera: Tabanidae) Flies

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Four spiroplasma strains, DF-1^T, TG-1^T, TABS-2^T, and TAUS-1^T, all of which were isolated from deerflies or horseflies (Diptera: Tabanidae), were serologically distinct from previously described spiroplasma species, groups, and subgroups. Strain DF-1^T originated from a Maryland deerfly (*Chrysops* sp.); strain TG-1^T was isolated from a Maryland horsefly (*Tabanus gladiator*); strain TAUS-1^T originated from a member of the *Tabanus abdominalis-limbatae* complex of horseflies collected in Maryland; and strain TABS-2^T was isolated from a horsefly (*Tabanus abactor*) collected in Oklahoma. Cells of all of the strains appeared to be helical and motile when they were examined by dark-field microscopy. Cells of strain DF-1^T growing in MID medium were short helices with less than six turns; the helical cells of the other strains were long and usually had six or more turns. The short cells of strain DF-1^T passed through 450- and 300-nm filter pores with no reduction in titer, but the longer cells of the other strains were partially retained by 450-nm-pore-size filters. Electron microscopic examination of all of the strains revealed wall-less cells surrounded only by a single cytoplasmic membrane. All of the strains grew well in SP-4 liquid media and in conventional mycoplasma or MID media supplemented with horse or fetal bovine serum. Strains TABS-2^T, TAUS-1^T, and DF-1^T required serum or sterol for growth, but strain TG-1^T was able to grow in the absence of serum or sterol. The optimum temperatures for growth of the four strains varied from 30 to 32°C, and growth occurred at 10 to 37°C. All of the strains catabolized glucose but did not hydrolyze urea. Only strain DF-1^T hydrolyzed arginine. The guanine-plus-cytosine contents of the DNAs of the strains were: DF-1^T, 29 ± 1 mol%; TG-1^T, 26 ± 1 mol%; TABS-2^T, 27 ± 1 mol%; and TAUS-1^T, 26 ± 1 mol%. The genome sizes of strains DF-1^T and TAUS-1^T were 1,270 and 1,375 kbp, respectively. Strain DF-1 (= ATCC 43209), the representative of spiroplasma subgroup VIII-2, is designated the type strain of a new species, *Spiroplasma chrysopicola*. We also propose that strain TG-1^T (= ATCC 43525^T), the designated representative of group XXIII, should be placed in a new species, *Spiroplasma gladiatoris*. In addition, group XXXII spiroplasma strain TABS-2 (= ATCC 51746) is designated the type strain of *Spiroplasma helicoides* sp. nov., and group XXXIII representative strain TAUS-1 (= ATCC 51747) is designated the type strain of another new species, *Spiroplasma tabanidicola*.

In 1987, an ad hoc committee appointed by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* proposed criteria (24) for designating spiroplasma groups. Shortly thereafter, a revised group classification and current list of recognized species of the genus *Spiroplasma* appeared (17, 19, 32), which expanded the number of recognized groups to 23. Two additional species and eight newly proposed (34) group representatives have now increased the number of recognized *Spiroplasma* groups to 32 (group XVII is vacant) (18, 29, 30, 34). In addition, eight subgroups of group I and three subgroups each of groups VIII and XVI have been recognized (1, 2, 7).

Since the initial proposal of combined genomic and serological criteria for spiroplasma groups (11), these clusters have

been regarded as putative species, under the assumption that they represent groups of strains with little or no intergroup DNA-DNA homology. In all of the cases examined, the results of DNA-DNA hybridization studies support this view (32). Therefore, as the number of groups has expanded, groups have continued to represent clusters of organisms that have putative species status but have not been given formal epithets. Under certain circumstances, subgroups can be elevated to the species level (9). In this paper, we report the results of characterization studies that resulted in species names being assigned to three group representatives and one subgroup representative. We propose that the group XXIII spiroplasmas should be placed in a new species, *Spiroplasma gladiatoris*, whose type strain is strain TG-1 (= ATCC 43525). In addition, group XXXIII spiroplasmas are named *Spiroplasma tabanidicola* sp. nov., with type strain TAUS-1 (= ATCC 51747), and group XXXII spiroplasmas are named *Spiroplasma helicoides* sp. nov., with type strain TABS-2 (= ATCC 51746). We also designate subgroup VIII-2 *Spiroplasma chrysopicola* sp. nov.; the type strain of this species is DF-1 (= ATCC 43209).

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MATERIALS AND METHODS

Many of the techniques used in this study are standard techniques used for spiroplasma characterization and have been described previously (13, 21, 22). Departures from these techniques are described below.

Spiroplasma strains. All of the strains were isolated (14, 21, 22) from the guts of tabanid flies. Strain DF-1^T was isolated by T. B. Clark from a female deerfly (*Chrysops* sp.) collected at the Patuxent Wildlife Research Center, Laurel, Md. (Prince George's County), on 3 July 1983 by J. E. Lowry. Strain TG-1^T was isolated from a horsefly (*Tabanus gladiator*) collected at Bucktown, Md., on 16 June 1985. Strain TAUS-1^T was isolated by F. R. Green from the blood of a female member of the *Tabanus abdominalis-limbatae* complex that he had collected at the Beltsville Agricultural Research Center, Beltsville, Md. (Prince George's County), on 31 July 1986. Strain TABS-2^T was isolated from a horsefly (*Tabanus abactor*) collected near Ardmore, Okla., (Carter County), on 20 September 1987. Some of the genomic (4, 19) and serological (19, 28) features of the organisms have been reported previously. All of the strains were purified by conventional filtration-cloning techniques. Representative strains of all previously recognized groups and subgroups (18, 19, 29), including the type strains of previously recognized species (18, 34), were employed in various parts of this study.

Culture medium and cultivation techniques. Strain DF-1^T was grown in primary culture in SM-1 liquid medium (23) at 30°C. All other strains were isolated in M1D medium containing 500 U of penicillin per ml (23). In all cases, after several broth passages, the isolates were lyophilized and revived later for cloning. Cultures of all strains were incubated routinely at 30°C. Following filtration-cloning, triply cloned strains were selected and used in characterization studies. Other medium formulations used included SP-4 medium and serum fraction broth supplemented with 1% bovine serum fraction (23). Solid media were prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 2.25 or 3.0%. Agar cultures were incubated at 30°C either aerobically (with 5% carbon dioxide; GasPak system [BBL Microbiology Systems, Cockeysville, Md.]) or anaerobically (Hydrogen GasPak system). Temperature requirements for growth were assessed as described previously (12).

Morphological studies. Cells of all strains were grown in broth cultures to the logarithmic phase and were examined at a magnification of $\times 1,250$ by dark-field microscopy. For electron microscopic examination, the strains were grown in approximately 2 ml of broth and pelleted by centrifugation at 4°C. The pelleted cells were fixed for 2 h in 3% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, embedded in Epon, sectioned, and stained with 1% aqueous uranyl acetate and Reynold's lead citrate.

Sterol requirement. Sterol requirements for growth were determined by a modified test in which sustained growth was measured through 23 serial 10-fold dilutions in various media with or without animal serum (16). In addition, cholesterol requirements were also studied with a technique modified from the standard broth method. Spiroplasma strains were adapted to grow well in 1% bovine serum fraction medium; 1% inocula from this medium were added to serum-free media containing 1, 2, 5, 10, and 20 μ g of cholesterol per ml. The response to cholesterol was measured by microscopically counting spiroplasma cells and by titrating color-changing units per milliliter (15).

Tests for biological and biochemical properties. The procedures used to determine glucose fermentation, arginine and urea hydrolysis, and the ability of strains to pass through membrane filters with various pore sizes have been described previously (11).

Serological tests. Antisera to all strains were raised in rabbits as described previously (31, 33). Hyperimmune antisera to all previously established *Spiroplasma* species, groups, putative groups, and subgroups (18, 34) were obtained from reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. The strains and their antisera were tested reciprocally by performing metabolism inhibition and deformation tests by using previously described techniques (31, 33).

Genomic analysis. The techniques used to extract and purify mollicute chromosomal DNA, to determine guanine-plus-cytosine contents of purified DNAs (3), and to determine genome sizes by pulsed-field gel electrophoresis have been described previously (4).

RESULTS AND DISCUSSION

Cultural and morphological properties. All of the strains grew well in M1D and SP-4 media. The strains also grew in conventional mycoplasma media containing horse serum (Edward formulation) and in 1% bovine serum fraction medium. Strain TG-1^T adapted readily to the latter medium, but the other strains required 5 to 20 passages in mixtures of serum fraction medium and M1D medium before they adapted. All strains were resistant to 500 U of penicillin per ml. The temperature growth requirements have been described previously (12). Strain DF-1^T grew at temperatures between 10 and 32°C,

and the optimum temperature was 30°C; the doubling time at the optimum temperature was 6.4 h, and the doubling times at 10, 15, 20, 25, 30, and 32°C were 105, 52.7, 23.4, 8.5, 6.4, and 8.2 h, respectively. Strain TG-1^T grew at temperatures between 15 and 37°C, and the optimum temperature was 30 to 32°C; the doubling time at the optimum temperature was 4.1 h, and the doubling times at 15, 20, 25, 30, 32, and 37°C were 43.3, 11.1, 5.8, 4.1, 4.1, and 10.9 h, respectively. Strain TABS-2^T grew at temperatures between 20 and 37°C, and the optimum temperature was 32°C; the doubling time at the optimum temperature was 3.0 h, and the doubling times at 20, 25, 30, 32, and 37°C were 13.9, 6.0, 3.1, 3.0, and 15.5 h, respectively. Strain TAUS-1^T grew at temperatures between 10 and 37°C, and the optimum temperature was 30°C; the doubling time at the optimum temperature was 3.7 h, and the doubling times at 15, 20, 25, 30, 32, and 37°C were 64.3, 14.4, 14.3, 3.7, 5.2, and 10.6 h, respectively. No strain grew during 3 weeks of incubation in broth media at 5 or 43°C.

The colonial morphologies of the spiroplasma strains on "hard" agar (media containing 2.25 or 3.0% Noble agar) are shown in Fig. 1. Strain DF-1^T growing aerobically on solid SP-4 medium containing 2.25% Noble agar formed colonies that had dense centers, smooth edges, and no satellites (Fig. 1A). Colonies of strain TG-1^T on SP-4 medium containing 3% Noble agar were granular with dense centers and had no satellites (Fig. 1A). Colonies of strain TABS-2^T growing on SP-4 medium containing 3% Noble agar were uneven and granular and had dense centers, irregular edges, and no satellites (Fig. 1C). The colonies formed by strain TAUS-1^T growing on SP-4 medium containing 2.25% Noble agar had smooth edges, were perfectly umbonate, and did not have satellites (Fig. 1D).

Morphological studies. Logarithmic-phase cultures of strain DF-1^T in M1D medium that were examined by dark-field microscopy contained numerous short motile filaments with less than six turns. As previously reported (19), electron microscopy revealed that cells of this organism were filamentous and had no evidence of a cell wall; representative cells were surrounded by a single cytoplasmic membrane. The cells of strains TG-1^T, TAUS-1^T, and TABS-2^T were long and had more than six turns. These helical cells were surrounded by typical cytoplasmic membranes, and there was no evidence of a cell wall (Fig. 2).

Sterol requirement. In the sustained growth test performed to determine sterol requirements, strains DF-1^T, TABS-2^T, and TAUS-1^T all grew well in serum-containing media through 23 serial 10-fold dilutions, but were unable to grow beyond 2 to 5 serial 10-fold dilutions in serum-free medium, whether or not it was supplemented with 0.04% polyoxyethylene sorbitan (Tween 80). On the other hand, strain TG-1^T did not have a sterol requirement since it grew through more than 30 10-fold serial dilutions in the serum-free broth formulation. This strain, however, did not grow in serum-free medium supplemented with polyoxyethylene sorbitan. In an initial report describing this sterol test method (16), Rose et al. suggested that strain TG-1^T required sterol. However, in our study, the morphology of this strain was monitored by dark-field microscopy, and the organism was subcultured at appropriate intervals in fresh serum-free medium, which provided conditions for prolonged growth of the organism in the absence of serum. The responses of strains TG-1^T, TABS-2^T, and TAUS-1^T to additions of cholesterol to the base medium in one series of experiments are shown in Table 1. Limited growth occurred in base broth alone that was inoculated with a 1:100 or 1:1,000 inoculum from serum fraction medium. Growth occurred in the presence of serum fraction in base media inoculated with 10% inocula, which permitted carryover of serum fraction from the

FIG. 1. Colonies of four tabanid-derived spiroplasmas. (A) Strain DF-1^T, passage 3, on SP-4 medium with 2.25% Noble agar. The organism was grown for 6 days aerobically at 30°C. (B) Strain TG-1^T, passage 6, on SP-4 medium with 3% Noble agar. The organism was grown for 9 days anaerobically at 30°C. (C) Strain TABS-2^T, passage 41, on SP-4 medium with 3% Noble agar. The organism was grown for 8 days anaerobically at 30°C. (D) Strain TAUS-1^T, passage 23, on SP-4 medium with 2.25% Noble agar. The organism was grown for 9 days aerobically at 30°C. Bars = 50 μ m.

inoculating media. Growth also occurred in media that were supplemented with 1 to 20 μ g of cholesterol per ml; lower concentrations did not stimulate growth as much as higher concentrations.

Biochemical and biological properties. All of the strains produced acid from glucose, but none hydrolyzed urea. Strain DF-1^T, but not the other strains, hydrolyzed arginine. Passage of broth cultures of strain DF-1^T through membrane filters with 450- or 300-nm pores failed to reduce the viable cell titer; the organism did not pass through 100-nm filter pores. Passage of broth cultures of strain TG-1^T through membrane filters with 450-, 300-, and 220-nm pores reduced the viable cell titers by 1, 2, and 3 log₁₀ units, respectively. Passage of broth cultures of strain TABS-2^T through membrane filters with 450-, 300-, and 220-nm pores reduced the viable cell titers by 2, 0, and 1 log₁₀ units, respectively. Passage of broth cultures of strain TAUS-1^T through membrane filters with 450-nm pores re-

duced the titer 10-fold; passage through 220-nm-pore-size filters reduced the titer 100-fold. All strains failed to pass through filters with 100-nm pores.

Serological tests. The results of metabolism inhibition and spiroplasma deformation tests performed with antisera directed against previously described spiroplasma species, groups, subgroups, and putative species indicated that strains TG-1^T, TAUS-1^T, and TABS-2^T are serologically unrelated to other *Spiroplasma* representatives or type strains (Table 2) (18, 34). Low-level (1:40) reciprocal deformation cross-reactions were observed with strain TG-1^T and strain I-25 (group XV). Other reciprocal deformation and metabolism inhibition cross-reactions (Table 2) were observed only with strain DF-1^T and representatives of other group VIII subgroups, including strain EA-1^T (*Spiroplasma syrphidicola*; subgroup VIII-1) and strain TAAS-1 (subgroup VIII-3) (7, 25, 34). We have recently reported (25) that strain B1357, a representative of a large as-

FIG. 2. Electron micrograph of sectioned and stained cells of strain TABS-2^T (A) and strain TAUS-1^T (B). Sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The arrows indicate the unit membrane. Bar = 100 nm.

semblage of isolates obtained from *Tabanus lineola*, is partially related to all three group VIII subgroups, but cannot be classified in any of them.

Justification for elevation of subgroup VIII-2. There are now three clusters of spiroplasma subgroups. Group I is subdivided into eight subgroups (2, 32, 34), and group XVI is subdivided into three subgroups (1). In 1993, Gasparich et al. (7) subdivided group VIII into three subgroups. Initially, strain DF-1^T fit the criteria used to classify spiroplasma strains into groups (24) and was classified as a member of group XVII (17). However, a large array of spiroplasma isolates obtained from dipterous insects, including horseflies and deerflies (Diptera: Tabanidae), have been found to cross-react significantly with both group VIII strain EA-1^T (27) and strain DF-1^T. One of the group VIII strains, TAAS-1, which was isolated from a horsefly, was studied in detail (5). Strains EA-1^T, DF-1^T, and TAAS-1 were found to be serologically interreactive (7, 29). Strains EA-1^T, DF-1^T, and TAAS-1 had similar G+C contents (30 ± 1 mol%). Strains DF-1^T and EA-1^T exhibited levels of DNA-DNA homology of 33 to 48%, while strain TAAS-1 exhibited levels of homology of 42 and 67% with strains DF-1^T and EA-1^T, respectively. These data confirm that the levels of genomic relatedness of strain DF-1^T to other group VIII strains are low enough to justify designation of this strain as a member of a new species, in accordance with current concepts of microbial species identification (10). Strain DF-1^T appears to be confined to deerflies; thus, it exhibits the ecological specialization expected of a subgroup elevated to species status (8).

Genome sizes and DNA base compositions. The genome size of strain DF-1^T has been reported to be 1,270 kbp (4). The genome size of strain TAUS-1^T was determined to be 1,375 kbp by pulsed-field gel electrophoresis (4). The genome sizes of strains TG-1^T and TABS-2^T have not been determined yet. The base composition (guanine-plus-cytosine content) of the DNA of strain DF-1^T, as determined (7, 19) by the buoyant density, melting temperature, and high-performance liquid chromatography methods, was 29 ± 1 mol%. The base compositions of strains TG-1^T, TAUS-1^T, and TABS-2^T, as determined by the buoyant density method, were 26 ± 1 , 27 ± 1 , and 26 ± 1 mol%, respectively.

Habitats. All of the strains described here were isolated from the abdominal viscera of adult female tabanids. The relative specificity of carriage of these and related strains can be assessed by examining the results of comparative studies in which hundreds of spiroplasma cultures were obtained (6) from tabanids collected on coastal plains from Florida to Nova

TABLE 1. Growth responses of strains TG-1^T, TABS-2^T, and TAUS-1^T to cholesterol^a

Strain	Supplement added to serum-free base medium	Cholesterol concn (μg/ml)	Growth response (color-changing units/ml) on:	
			Day 2	Day 3
TG-1 ^T	Serum fraction	0	8	7
	None	0	6	5
	Cholesterol	1	6	7
		2	6	6
		5	6	7
		10	6	8
		20	6	8
TABS-2 ^T	Serum fraction	0	9	4
	None	0	4	4
	Cholesterol	1	6	4
		2	6	4
		5	6	6
		10	8	6
		20	9	7
TAUS-1 ^T	Serum fraction	0	8	3
	None	0	4	3
	Cholesterol	1	6	5
		2	6	5
		5	6	5
		10	6	5
		20	8	5

^a All media were inoculated with 10^6 color-changing units of a logarithmic-phase culture of the strain in base medium supplemented with serum fraction. The growth responses were determined 2 and 3 days after inoculation. Phenol red pH indicator indicated that there was significant acid production in cultures containing 10^8 or 10^9 color-changing units/ml.

TABLE 2. Serological reactivities of the four new spiroplasma species^a

Strain	Challenge organism		Titer in:			
			Deformation test		Metabolism inhibition test	
	Group	Strain	Antigen ^b	Antiserum ^c	Antigen ^d	Antiserum ^e
DF-1 ^T (group VIII-2)	I-1	R8A2 ^T	n ^f	n	n	54
	I-2	E275 ^T	n	n	n	54
	II	DW-1	n	n	n	1,458
	VIII-1	EA-1 ^T	n	80	54	486
	VIII-2	DF-1 ^T	10,240 ^g	10,240	117,000	117,000
	VIII-3	TAAS-1	160	80	486	54
	XVI-3	Ar-1357	n	n	54	n
	XIX	PUP-1 ^T	n	40	n	n
	XX	LD-1 ^T	n	n	n	54
	XXVI	PLHS-1	n	n	n	162
	XXXIII	TAUS-1 ^T	n	n	54	n
TG-1 ^T (group XXIII)	II	DW-1	n	n	n	54
	XIV	EC-1 ^T	n	40	n	n
	XV	I-25	40	n	n	n
	XIX	PUP-1 ^T	n	40	n	n
	XX	LD-1 ^T	n	n	n	486
	XXII	CT-1 ^T	40	n	n	n
	XXIII	TG-1 ^T	20,480 ^g	20,480	117,000	117,000
	XXVI	PLHS-1	n	160	n	54
TABS-2 ^T (group XXXII)	I-2	E275 ^T	n	40	n	n
	I-8	P40 ^T	n	n	n	162
	XI	MQ-4 ^T	n	n	n	54
	XIX	PUP-1 ^T	n	40	n	162
	XX	LD-1 ^T	n	n	n	54
	XXVI	PLHS-1	n	n	n	54
	XXVIII	PALS-1	n	n	n	54
	XXXI	HYOS-1	n	40	n	n
	XXXII	TABS-2 ^T	640	640	39,000	39,000
TAUS-1 ^T (group XXXIII)	I-8	P40	n	n	n	54
	VIII-2	DF-1	n	n	n	54
	XIX	PUP-1 ^T	n	n	n	486
	XX	LD-1 ^T	n	n	n	162
	XXI	W115	n	n	n	54
	XXVI	PLHS-1	n	n	n	162
	XXVIII	PALS-1	n	n	n	54
	XXXIII	TAUS-1 ^T	5,120	5,120	39,000	39,000

^a Antigens and antisera of the strains were tested in all combinations against all known and putative groups and subgroups. All cross-reactions except those shown were negative.

^b Reciprocal of the endpoint in a deformation test in which the antigen was tested against a heterologous antiserum.

^c Reciprocal of the endpoint in a deformation test in which the antiserum was tested against a heterologous antigen.

^d Reciprocal of the endpoint in a metabolism inhibition test in which the antigen was tested against a heterologous antiserum.

^e Reciprocal of the endpoint in a metabolism inhibition test in which the antiserum was tested against a heterologous antigen.

^f n, negative.

^g Homologous titer of the strain in the test system. The reaction, obtained only once, is listed in each column for completeness.

Scotia and in the Rocky Mountains from Montana to New Mexico. Other collection sites were located in Vermont and Texas. The most extensive survey was made during 1987 to 1995 at four sites in Bulloch County, Georgia. We used one-way serological deformation tests (28) to identify more than 400 cultures isolated from tabanids in these comparative studies.

Strain DF-1^T was isolated from a deerfly (*Chrysops* sp.) from Prince George's County, Maryland. Thirteen related strains were isolated from six *Chrysops* spp. from Bulloch County, Georgia, and Yellowstone National Park, Wyoming. Most group VIII spiroplasmas (7) react to some degree with antiserum directed against strain DF-1^T. However, no isolates identified as *S. chrysopicola* were obtained from many tabanids belonging to other horsefly genera that were collected with the

Chrysops spp. There is therefore an apparent natural exclusive relationship between strains referable to *S. chrysopicola* and deerflies (*Chrysops* spp.). Strains TG-1^T and TAUS-1^T were isolated from tabanid flies from Maryland, and strain TABS-2^T was isolated from a tabanid fly from Texas. Isolates closely related to these strains have been isolated from insects collected in Georgia, Florida, and North Carolina. These three tabanid spiroplasmas therefore appear to have southern and southeastern United States distributions, since they have not appeared in collections from Wyoming, Vermont, Connecticut, and Nova Scotia, Canada.

Isolates related to strain TG-1^T were commonly carried by horseflies in the southeastern United States (26). These organisms were recovered from individual females of *Chlorotabanus crepuscularis*, from one *Chrysops vittatus* specimen, and from

more than 100 individuals belonging to 11 *Tabanus* species. The largest number of cultures referable to *S. gladiatoris* came from *Tabanus americanus*, *T. gladiator*, and *Tabanus lineola*. Most of these isolates came from Bulloch County, Georgia. *S. gladiatoris* often occurs in mixed infections with other spiroplasma species.

Isolates related to strain TAUS-1^T were recovered only from members of the horsefly subfamily Tabaninae. These isolates were identified by deformation tests in one culture from *Leucotabanus annulatus* and in 10 cultures from *Hybomitra difficilis*. Isolates related to strain TAUS-1^T were frequently recovered from *T. gladiator* and *T. lineola*. Cultures of *S. tabanidicola* from *T. lineola* generally gave deformation endpoints of 1:640 or 1:1,280, compared to a homologous titer of 5,120. This spiroplasma species was also isolated from *Tabanus atratus*, *Tabanus imitans*, and *Tabanus subsimilis*. The majority of the TAUS-1^T isolations were from insects from Bulloch County, Georgia, but there were also isolations from tabanids captured in Johnston County, North Carolina, Carteret County, North Carolina, and Alachua County, Florida.

Strain TABS-2^T was isolated from *T. abactor* from Carter County, Oklahoma. Related isolates were obtained from *H. difficilis*; these strains represented about 40% of the isolations from this species, whose flight activity is restricted to April in Bulloch County, Georgia. Strains related to TABS-2^T were also isolated from three horseflies (*T. americanus*, *T. gladiator*, and *T. lineola*) whose flight activities range from late spring through early fall. However, the isolates related to strain TABS-2^T comprised only a small fraction of the isolations from these *Tabanus* spp. In all cases, the significance of antigenic variation among the type strains and related isolates of tabanid spiroplasmas has yet to be determined.

The properties described here for strains DF-1^T, TG-1^T, TABS-2^T, and TAUS-1^T fulfill proposed criteria (8) for descriptions of species of the class *Mollicutes*, including the absence of a cell wall, filterability, and penicillin resistance. The helicity and motility of these strains and the inability of the strains to utilize urea place them (20) in the order *Entomoplasmatales* and the family *Spiroplasmataceae*. Finally, the results of serological comparisons of these strains, other *Spiroplasma* species, and group representatives confirm that they are members of separate *Spiroplasma* species. We therefore propose the designations below for these organisms.

Description of *Spiroplasma chrysopicola* sp. nov. *Spiroplasma chrysopicola* (chry.so.pi.'co.la. Gr. n. *Chrysops*, a genus of deerflies in the Tabanidae L. v. colo, to inhabit; N. L. adj. *chrysopicola*, inhabiting *Chrysops* spp.).

Cells are filamentous, helical, and motile and lack a cell wall. Colonies on solid medium containing 2.25% Noble agar have dense centers and smooth edges (a "fried-egg" appearance) and do not have satellites.

Chemoorganotroph. Acid is produced from glucose. Hydrolyzes arginine but not urea.

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 32°C. Optimum growth occurs at 30°C, and the doubling time at 30°C is 6.4 h.

Serologically distinct from other established *Spiroplasma* species and putative groups. Isolated from the gut of a deerfly (*Chrysops* sp.). Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 30 ± 1 mol%, as determined by the buoyant density method. The genome size is 1,270 kbp.

The type strain is DF-1 (= ATCC 43209).

Description of *Spiroplasma gladiatoris* sp. nov. *Spiroplasma gladiatoris* (gla.di.a'to.ris. M. L. gen. n. *gladiatoris*, reflecting

the initial isolation of the organism from the horsefly *T. gladiator*).

Cells are filamentous, helical, and motile and lack a cell wall.

Colonies on solid medium containing 3% Noble agar are granular with dense centers and diffuse edges, do not have satellites, and never have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea.

Cholesterol or serum is not required for growth.

The temperature range for growth is 15 to 37°C. Optimum growth occurs at 30 to 32°C, and the doubling time at 30 to 32°C is 4.1 h.

Serologically distinct from other established *Spiroplasma* species and putative groups. Isolated from the gut of a horsefly (*T. gladiator*). Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 26 ± 1 mol%, as determined by the buoyant density method. The genome size has not been determined.

The type strain is TG-1 (= ATCC 43525).

Description of *Spiroplasma tabanidicola* sp. nov. *Spiroplasma tabanidicola* (ta.ba.ni.di'co.la. M. L. n. Tabanidae, family name for horseflies; L. v. colo, to inhabit; M. L. n. *tabanidicola*, an inhabitant of horseflies).

Cells are filamentous, helical, and motile and lack a cell wall.

Colonies on solid medium containing 3% Noble agar are uneven and granular with dense centers and irregular edges, do not have satellites, and never have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea.

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 37°C. Optimum growth occurs at 30°C, and the doubling time at 30°C is 3.0 h.

Serologically distinct from other established *Spiroplasma* species and putative groups. Isolated from the gut of a horsefly belonging to the *Tabanus abdominalis-limbatae* complex. Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 26 ± 1 mol%, as determined by the buoyant density method. The genome size is 1,375 kbp.

The type strain is TAUS-1 (= ATCC 51747).

Description of *Spiroplasma helicoides* sp. nov. *Spiroplasma helicoides* (he.li.coid'es. Gr. n. *helix*, spiral, Gr. suff. -oides, like; G L. adj. *helicoides*, spiral-like).

Cells are filamentous, helical, and motile and lack a cell wall.

Colonies on solid medium containing 2.25% Noble agar have dense centers and smooth edges, do not have satellites, and have a perfect fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea.

Cholesterol or serum is required for growth.

The temperature range for growth is 20 to 37°C. Optimum growth occurs at 32°C, and the doubling time at 32°C is 3.0 h.

Serologically distinct from other established *Spiroplasma* species and putative groups. Isolated from the gut of a horsefly (*T. abactor*). Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 26 ± 1 mol%, as determined by the buoyant density method. The genome size has not been determined.

The type strain is TABS-2 (= ATCC 51746).

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