Spiroplasma montanense sp. nov., from *Hybomitra* Horseflies at Northern Latitudes in North America

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Spiroplasma strain HYOS-1^T was isolated from a tabanid fly, *Hybomitra opaca*. The organism was serologically distinct from other *spiroplasma* species, groups, and subgroups and was recently designated the representative of *spiroplasma* group XXXI. The cells of strain HYOS-1^T, as determined by light microscopy, were long motile helices. Electron microscopic examination revealed wall-less cells delimited by a single membrane. The cells passed through 450- and 300-nm filter pores with a 10-fold reduction in titer, but failed to pass through 100-nm pores. Strain HYOS-1^T grew very well in most conventional medium formulations for *spiroplasmas* or other mollicutes. The organism grew at temperatures ranging from 5 to 41°C, and the optimum temperature was 32°C. The doubling time at the optimum temperature was 0.7 h, one of the shortest values obtained for members of the genus *Spiroplasma*. The strain catabolized glucose and hydrolyzed arginine but not urea. Growth of the organism was stimulated by cholesterol and serum, but the strain was nevertheless able to grow in the absence of sterols or serum. The guanine-plus-cytosine content of the DNA was about 28 ± 1 mol%, and the genome size was 1,225 kbp. On the basis of the experimental results reported here and previously reported data, group XXXI strain HYOS-1 (= ATCC 51745) is designated the type strain of a new species, *Spiroplasma montanense*.

Thirteen *spiroplasma* groups, putative groups, or subgroups are associated with tabanid flies (5, 8–10, 34, 36). The following species or unnamed strains appear to occur primarily in tabanid hosts: some group IV strains (*Spiroplasma apis*); representatives of three group VIII subgroups (11), including subgroup VIII-1 strain EA-1^T (*Spiroplasma syrphidicola*) (35), subgroup VIII-2 strain DF-1^T (*Spiroplasma chrysopicola*) (34), and subgroup VIII-3 strain TAAS-1 (= ATCC 51123); strains related to EC-1^T (group XIV; *Spiroplasma corruscae*) (14); strain TN-1^T (group XVIII; *Spiroplasma litorale*) (19); strain TG-1^T (group XXIII; *Spiroplasma gladiatoris*) (34); group XX-VII strain TALS-2 (37); strain TABS-2^T (group XXXII; *Spiroplasma helicoides*) (34); strain TAUS-1^T (group XXXIII; *Spiroplasma tabanidicola*) (34); and representatives of two putative groups, strains B1901 and B2649. In this paper we describe strain HYOS-1^T, a representative of group XXXI of *spiroplasmas* found in tabanid flies, including several species of the genus *Hybomitra*. *S. litorale* was the first tabanid *spiroplasma* shown to have a geographically limited distribution; this organism is confined essentially to the southeast Atlantic coastal plain in the United States (19). Strain HYOS-1^T is also geographically limited since it has been isolated only from northern latitudes. Our characterization studies established that strain HYOS-1 can be considered the type strain of a new species. In this paper we designate strain HYOS-1 (= ATCC 51745) the type strain of *Spiroplasma montanense* sp. nov.

MATERIALS AND METHODS

Spiroplasma strains. Strain HYOS-1^T was isolated by previously described techniques (5, 21) from the gut of a tabanid fly (*Hybomitra opaca*) collected in Livingston, Mont., by R. F. Whitcomb and E. A. Clark. Strain HYOS-1^T was purified by conventional filtration-cloning techniques (25). Representative strains of all previously recognized *spiroplasma* groups and subgroups (28–30, 37, 40), including the type strains of all previously recognized species, were also employed in this study.

Culture medium and cultivation techniques. The isolate from which strain HYOS-1^T was derived was grown in primary culture in M1D liquid medium containing 500 U of penicillin per ml (33) at 30°C. After several early broth passages, the isolate was lyophilized. For characterization, the dried culture was revived and cloned (25) in M1D broth at 30°C. Strain HYOS-1^T also grew well in other medium formulations, including SP-4 medium, conventional mycoplasma medium containing bovine or horse serum, and 1% bovine serum fraction medium (32). Solid medium was prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 2.25%. Agar cultures were incubated at room temperature (21°C) and at 30°C either aerobically or anaerobically in a GasPak system (BBL Microbiology Systems, Cockeysville, Md.). Temperature requirements for growth were assessed as described previously, with growth measured at temperatures ranging from 5 to 43°C (18, 19).

Morphological studies. Cells of strain HYOS-1^T cultivated in M1D broth to the logarithmic phase were examined by dark-field microscopy at a magnification of ×1,250. An electron microscopic examination of the membrane structure of strain HYOS-1^T was performed by using previously described techniques (38).

Tests for biological and biochemical properties. The procedures used to study carbohydrate fermentation and arginine and urea hydrolysis have been described previously (1). Filtration characteristics were determined in M1D broth by passing logarithmic-phase cultures through filters with 450-, 300-, 220-, and 100-nm pores. Each filtrate, as well as an unfiltered control, was subcultured in a series of 12 tubes by using 10-fold dilutions and was monitored to determine growth at 30°C (25).

Sterol requirement. Sterol requirements for growth were determined by two methods. The cell protein yields of strain HYOS-1^T grown in serum-free broth media containing various concentrations of cholesterol were determined, and the ability of the organism to continue to grow in various serum-free or serum-containing medium formulations was also studied (24, 26).

Serological tests. Antiserum to strain HYOS-1^T was produced in rabbits as described previously (41). Hyperimmune antisera to all previously established

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FIG. 1. Colonies of strain HYOS-1^T on SP-4 medium supplemented with 2.25% Noble agar after 3 days of incubation at room temperature (21°C) under aerobic conditions. Bar = 50 μ m.

Spiroplasma species, groups, putative groups, and subgroups (28–30, 37, 40) were obtained from the reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. Strain HYOS-1^T, the antisera, and the other strains were tested reciprocally by performing metabolism inhibition and deformation tests as previously described (39, 41). Reciprocal crosses in both of these tests are required to reliably establish serological relationships among spiroplasmas.

Genomic analysis. The techniques used for extraction and purification of mollicute chromosomal DNA have been described previously (3). The guanine-plus-cytosine content of purified DNA of strain HYOS-1^T was determined by the buoyant density method (4). The genome size was determined by pulsed-field gel electrophoresis, as described previously (3).

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain HYOS-1^T grew well in M1D and SP-4 media, in conventional mycoplasma media containing horse serum (Edward formulation) or fetal bovine serum, and in bovine serum fraction medium. The organism grew in the presence of 500 U of penicillin per ml. Strain HYOS-1^T grew at temperatures between 5 and 41°C, and optimum growth occurred at 32°C (17, 18). The doubling time at the optimum temperature in M1D broth was 0.7 h. Perceptible but nonquantifiable growth occurred at 41°C. Colonies of strain HYOS-1^T on solid SP-4 medium supplemented with 2.25% agar at room temperature under aerobic conditions appeared to be granular and to have dense centers, irregular margins, and numerous small satellites (Fig. 1). "Fried-egg" colonies were never observed.

Logarithmic-phase cultures of strain HYOS-1^T in M1D medium were examined by dark-field microscopy, and these cultures contained numerous long motile filaments. The cells of the organism sedimented from broth cultures and examined by electron microscopy were filamentous with no evidence of a cell wall (Fig. 2); instead, they were surrounded by a single cytoplasmic membrane.

Sterol requirement. The growth responses of strain HYOS-1^T (as measured by total cell protein) when cholesterol was added to serum-free SP-4 medium are shown in Table 1. The results of this test indicated that growth of the strain was stimulated by cholesterol. However, in the test described by Rose et al. (24), in which the ability of strain HYOS-1^T to continue to grow in serum-free or serum-containing media was examined, the organism grew through 23 serial 10-fold dilutions in serum-free medium devoid of sterols. Addition of 0.04% polyoxyethylene sorbitan (Tween 80) to the serum-free

FIG. 2. Electron micrograph of a thin section of pelleted cells of strain HYOS-1^T. The arrows indicate the single trilaminar plasma membrane. Bar = 100 nm.

formulation did not support sustained growth of strain HYOS-1^T. The latter findings indicate that strain HYOS-1^T does not have a growth requirement for cholesterol. The strain therefore resembles several other spiroplasmas (24) in its ability to grow in sterol-free media.

Biochemical and biological properties. Strain HYOS-1^T produced acid from glucose and hydrolyzed arginine, but was negative for urea hydrolysis. Passage of broth cultures of strain HYOS-1^T through 450- and 300-nm-pore-size membrane filters reduced the viable cell titer 10-fold. No cells passed through 100-nm-pore-size filters.

Serological tests. The results of metabolism inhibition and spiroplasma deformation tests performed with antisera prepared to known spiroplasma species, groups, and subgroups (Table 2) indicated that strain HYOS-1^T was serologically unrelated to representatives of previously established *Spiroplasma* groups or species. Low-level heterologous one-way cross-reactions were observed with several groups, but such reactions are not significant for assessing serological relationships.

Genome size and DNA base composition. The genome size of strain HYOS-1^T was 1,225 kbp (3), and the base composition (guanine-plus-cytosine content) of the DNA was 28 ± 1 mol%.

TABLE 1. Growth response of strain HYOS-1^T to cholesterol

Medium	Cholesterol concn (μ g/ml)	Amt of protein (mg/100 ml) ^a
M1D medium	0 (control)	3.08
Base medium with no supplement	0	0.02
Base medium with serum fraction	0 (control)	3.16
Base medium with cholesterol	1	0.31
	2	0.72
	5	2.16
	10	2.92
	20	2.50

^a The amount of cell protein was determined from a cell pellet obtained from 100 ml of each culture medium.

TABLE 2. Serological reactions and cross-reactions of strain HYOS-1^T in deformation and metabolism inhibition serological tests^a

Group	Strain	Titer in:			
		Spiroplasma deformation test		Metabolism inhibition test	
		Antiserum	Antigen	Antiserum	Antigen
I-4	277F	0 ^b	0 ^c	486 ^d	0 ^e
I-5	LB-12	0	0	18	0
I-8	P40 ^T	20	0	0	0
II	DW-1	0	0	54	0
IV	B31 ^T	40	0	0	0
VII	MQ-1 ^T	0	0	18	0
VIII-3	TAAS-1	40	0	0	0
XIV	EC-1 ^T	0	20	0	0
XVI-1	CC-1 ^T	0	20 (2,560) ^f	0	0
XVI-2	CB-1	0	0	0	18 (39,000) ^f
XIX	PUP-1 ^T	0	0	54	0
XX	LD-1 ^T	0	0	486	0
XXIII	TG-1 ^T	20	0	0	0
XXV	CUAS-1 ^T	40	0	0	0
XXVI	PLHS-1 ^T	40	0	162	0
XXXI	HYOS-1 ^T	5,120 ^g	5,120	117,000 ^g	117,000
XXXII	TABS-2 ^T	0	40 (640) ^f	0	0

^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 antiserum against strain HYOS-1^T.

^c Reciprocal of the endpoint in a deformation test in which the antiserum was tested against strain HYOS-1^T antigen.

^d Reciprocal of the endpoint in a metabolism inhibition test in which the antigen was tested against antiserum against strain HYOS-1^T.

^e Reciprocal of the endpoint in a metabolism inhibition test in which the antiserum was tested against strain HYOS-1^T antigen.

^f The value in parentheses is the reciprocal of the endpoint obtained with the homologous antiserum against which strain HYOS-1^T antigen was tested.

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

Habitat. The single strain described here was isolated directly from the abdominal viscera of a tabanid fly (*H. opaca*) from Livingston, Mont. Isolates related to this strain have been obtained from four other *Hybomitra* spp. (*Hybomitra affinis*, *Hybomitra epistates*, *Hybomitra lasiophthalma*, and *Hybomitra nitidifrons*). All isolates obtained from *Hybomitra* spp. that are referable to *S. montanense* were derived from flies captured north of 38°30'N latitude. In addition to the original isolate, we have seven isolates from Nova Scotia, Canada, and one isolate from Vermont. Several strains from Connecticut tabanids (1a) have been found to be related to strain HYOS-1^T, and isolates have been obtained from deerflies (*Chrysops* sp.) in West Virginia (33).

A cluster of isolates from Georgia tabanids (*Hybomitra difficilis* and *Chrysops brimleyi*) which are active during the spring reacted significantly in one-way deformation tests with antisera against strain HYOS-1^T, but also reacted significantly with antisera against group IV strains B31^T and W13. Another cluster of Georgia isolates reacted significantly with strain HYOS-1^T and the group XIV strain *S. corruscae* EC-1^T. The relationship between strain HYOS-1^T and these southern strains should be studied further by performing reciprocal serological studies or, perhaps, by sequencing the 16S ribosomal DNA gene.

Spiroplasmas are especially interesting as models of the ecological interaction between microbes and insects (13). Strain HYOS-1^T has been used as a model organism by F. E. French's

group. A variety of tabanids have been infected by feeding them sugar water containing strain HYOS-1^T (2, 6–8, 15, 20, 23). The bacterium has been recovered after laboratory feeding from one *Hybomitra* species, three *Chrysops* species, and eight *Tabanus* species. The population sizes established in tabanid guts ranged from 10⁴ to 10⁶ color-changing units per gut (20). Horizontal transfer occurred from orally infected flies that were in the same glass jar (8). The longevity of tabanids in the laboratory was not affected by the presence of strain HYOS-1^T in the flies (6, 8). Injected *Tenebrio molitor* (Coleoptera: Tenebrionidae) mealworm pupae carried the infection through metamorphosis, and the strain could be cultured from the guts of adult beetles (7, 12). Strain HYOS-1^T survives for up to 180 days in air-dried droplets maintained at room temperature (22, 31). These experimental results provide important insights into the dynamics of spiroplasma maintenance in holometabolous insects.

Classification of strain HYOS-1^T. The properties of strain HYOS-1^T described below fulfill the following proposed criteria (16) for descriptions of species of the class *Mollicutes*: absence of a cell wall, filterability, lack of reversion to walled bacteria when the organism is grown in antibiotic-free media, and penicillin resistance. The growth requirements, the inability to utilize urea, and the helicity and motility of strain HYOS-1^T place the organism in the order *Entomoplasmatales* and the family *Spiroplasmataceae* (27). Finally, the results of a serological comparison of strain HYOS-1^T to other *Spiroplasma* species, groups, and subgroups demonstrated the uniqueness of this new insect strain. We therefore propose the name *Spiroplasma montanense* for this organism. The taxonomic description below summarizes the properties of this species.

Description of *Spiroplasma montanense* sp. nov. *Spiroplasma montanense* (mon.ta.nen'se. M.L. adj. *montanense*, pertaining to Montana, where the species was first isolated).

Cells are helical, motile filaments that lack a cell wall. Colonies on solid medium containing 2.25% Noble agar are granular and have dense centers, irregular margins, and numerous small satellites.

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

Cholesterol or serum is not required for growth.

The temperature range for growth is 5 to 41°C, and optimum growth occurs at 32°C; at 32°C the doubling time is 0.7 h.

Serologically distinct from previously established *Spiroplasma* species, groups, and subgroups. Isolated from the gut of the tabanid fly *H. opaca*. Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 28 ± 1 mol%, as determined by the buoyant density method. The genome size is approximately 1,225 kbp.

The type strain is HYOS-1 (= ATCC 51745).

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