Spiroplasma corruscae sp. nov., from a Firefly Beetle (Coleoptera: Lampyridae) and Tabanid Flies (Diptera: Tabanidae)

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Spiroplasma strain EC-1^T (T = type strain), which was isolated from the gut of a lampyrid beetle (*Ellychnia corrusca*) in Maryland, was serologically distinct from other *spiroplasma* species and groups. Similar strains were obtained from other *E. corrusca* specimens, and, later, numerous isolates of similar or partially related strains were obtained from several species of tabanid flies. Cells of strain EC-1^T were helical, motile filaments that were bound by a single cytoplasmic membrane, and there was no evidence of a cell wall. The cells were filterable through 220-nm-pore-size membrane filters but not through 100-nm-pore-size membrane filters. The organism was absolutely resistant to penicillin (1,000 U/ml) and required sterol for growth. Strain EC-1^T grew well in M1D and SP-4 liquid media and could be cultivated in the Edward formulation of conventional mycoplasma medium and in 1% serum fraction medium. Optimal growth occurred at 32°C (doubling time, 1.5 h). Strain EC-1^T multiplied at 10 to 41°C, but not at 5 or 43°C. This organism produced acid from glucose, but did not hydrolyze arginine or utilize urea. The guanine-plus-cytosine content of the DNA was determined to be 26.3 mol% by the melting temperature method and 27.0 mol% by the buoyant density method. As a result of our studies, strain EC-1 (= ATCC 43212) is designated the type strain of a new species, *Spiroplasma corruscae*.

Firefly beetles (Coleoptera: Lampyridae) are an extremely rich source of mollicutes (19). Several *Spiroplasma* species (5, 6, 17–19, 45) and *Mesoplasma* species (34) (formerly described as *Acholeplasma* species [1, 8, 19, 30, 35]) have been isolated from members of this insect family. Also, the first named *Entomoplasma* (28) species, *Entomoplasma ellychniae* (34) (formerly described as a mycoplasma [32]), was isolated (5, 19) in 1983 from the lampyrid beetle, *Ellychnia corrusca*. Subsequently, three additional serovar clusters containing organisms isolated from luminescent lampyrid species were elevated to species status as members of the genus *Mycoplasma* (42) and were later transferred to the genus *Entomoplasma* (28).

Ellychnia corruscae is a somewhat unusual lampyrid beetle in that it is nonluminescent. Beginning in late winter 1983, adults of this species were collected while they were emerging from their overwintering sites at the base of a white oak (*Quercus alba*) tree in Beltsville, Md., and their gut and hemocoel fluids were analyzed for mollicutes. Spiroplasmas were isolated from the gut contents of 9 of the 30 specimens examined and from the hemolymph of 2 specimens (16a, 17). Several serologically related spiroplasmas were subsequently obtained (5, 7, 17–19), including strain EC-1^T (T = type strain) and isolates EC-2, EC-7, EC-8, EC-9, and EC-10 from gut contents and strain EC-1a and isolate EC-4 from hemocoel contents. Strains and isolates related to strain EC-1^T proved to be serologically unlike members of previously described groups or subgroups.

Strain EC-1^T was designated the representative of *spiroplasma* group XIV in a revised classification (31) of *spiroplasma* groups.

The ecology of strain EC-1^T and its relatives has been studied extensively. Of particular significance is the occurrence of this *spiroplasma* in a wide range of deerflies and horseflies (7, 11, 12, 36, 38, 40, 41), important economic pests of livestock and humans. The occurrence of this *spiroplasma* in tabanid flies is being studied as a model for understanding *spiroplasma*-insect interactions.

In this paper we report the results of taxonomic studies of strain EC-1^T and related strains that satisfy proposed requirements for species descriptions for the class *Mollicutes* (20). Our results support designation of strain EC-1 (= ATCC 43212) as the type strain of a new species, *Spiroplasma corruscae*.

MATERIALS AND METHODS

Spiroplasma strains. Techniques for isolating spiroplasmas from insect guts and hemocoels have been described previously (17). Strain EC-1^T was isolated and cultivated by T. B. Clark from the gut of an adult firefly beetle (*Ellychnia corrusca* [Coleoptera: Lampyridae]) (5, 7). Other strains and isolates obtained from this beetle species (5, 19) and from tabanid flies (7, 11, 12, 17, 22, 23, 38, 40) were characterized serologically. Representative strains (16, 28, 29, 33, 43) of previously recognized groups and subgroups, including type strains of previously recognized species (29, 33, 43), were cultivated for comparative purposes.

Culture media and cultivation techniques. Strain EC-1^T was grown in primary culture (24) and was subsequently cloned (26) in SM-1 liquid medium (37) at 30°C. Other media used in this study included M1D medium (37), SP-4 medium (37), the Edward formulation of conventional mycoplasma medium (HSI medium) containing 20% horse serum (10, 37), and serum-fraction broth supplemented with 1% bovine serum fraction (27). Solid formulations of these media were prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 2.25%. Cultures on solid media were incubated aerobically and anaerobically by using a BBL Anaerobic GasPak system (Becton Dickinson and Co., Gaithersburg, Md.) containing 25 to 35% H₂, 4 to 7% CO₂, and <1% O₂.

Temperature requirements for growth were determined by preparing 10-fold dilutions of strain EC-1^T in M1D broth and incubating the dilution series at 5, 10,

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15, 20, 25, 30, 32, 37, 41, and 43°C. Growth of the organisms was determined by observing indicator changes in the media and by examining the cultures microscopically during a 45-day observation period. Doubling times at each temperature were estimated by observing the time required for medium acidification and fitting the temperature-growth curve to a logistic growth equation (21).

Morphological studies. Cells of strain EC-1^T from cultures in the logarithmic phase were examined by dark-field microscopy (magnification, $\times 1,250$). Electron microscopic techniques for spiroplasmas have been described previously (9).

Sterol requirement. Sterol requirements for growth were determined by a standard broth culture method (27).

Tests for biological, biochemical, and serological properties. Procedures for determining carbohydrate fermentation (1), arginine hydrolysis and urea utilization (1), hemadsorption (14), and film and spot production (13) have been described previously. Filtration characteristics (26) were determined by using cultures in M1D broth.

Spiroplasma deformation (DF) (46) and metabolism inhibition (MI) (44) tests were used to compare strains serologically with all previously described spiroplasma groups and subgroups. Hyperimmune antisera to all previously established *Spiroplasma* species and existing and putative groups and subgroups (29) were obtained from reference collections at the U. S. Agricultural Research Center in Beltsville, Md., and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. In the DF test (46), a specific homologous antiserum deformed spiroplasma cells into spheroidal forms, which could be readily identified. Quantitative relationships among various serum dilutions were determined by examining, by dark-field microscopy, samples containing serum dilutions and a measured number of organisms, which was kept constant in each test. For the MI test (44), diluted antisera and complement were added to an SP-4 broth culture containing phenol red as a pH indicator, and the preparation was incubated at 30°C. The MI titer was the reciprocal of the final antiserum dilution in which there was no change in the pH indicator.

Genomic analysis. Extraction and purification of DNA from mollicutes were performed as described previously (2). Estimates of the guanine-plus-cytosine (G+C) content of purified DNA of strain EC-1^T were obtained by two procedures, the buoyant density and melting temperature procedures (3).

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain EC-1^T grew rapidly in liquid SM-1, M1D, and SP-4 media. Dark-field microscopy of logarithmic-phase cultures of strain EC-1^T revealed helical filamentous cells with six or more turns. The cells were delimited by only a membrane, and there was no evidence of a cell wall in electron micrographs (31). Strain EC-1^T also grew (more slowly) in the Edward formulation of conventional mycoplasma medium containing horse serum or in 1% bovine serum fraction medium. Growth occurred at temperatures ranging from 10 to 41°C, but not at 5 or 43°C; optimal growth was observed at 32°C. The doubling times at 10, 15, 20, 25, 30, 32, and 37°C were 33.6, 13.9, 5.5, 3.1, 1.9, 1.5, and 5.2 h, respectively. Strain EC-1^T grew on solid medium containing 2.25% Noble agar incubated at 30°C. Aerobically, colonies grew slowly and were slightly diffuse. Anaerobically, colonies grew faster, and, although they were discrete, there were two types, rough colonies and dew drop (slightly fried-egg) colonies (Fig. 1).

Biochemical and biological properties. Strain EC-1^T produced acid from glucose, but did not hydrolyze arginine or utilize urea (31). This organism did not produce a film and spot reaction, and colonies on an agar-containing medium did not hemadsorb guinea pig erythrocytes. Strain EC-1^T passed readily through 450- and 300-nm-pore-size membrane filters. Passage through 220-nm-pore-size filters reduced the viable cell titer 10-fold to 10^{-9} color-changing units per ml; a 100-nm-pore-size membrane filtrate was free of viable cells.

Serological studies. Strain EC-1^T, which had homologous titers of 1:2,560 in the spiroplasma DF test and 1:13,000 in the MI test (31), exhibited weak serological reactivity with representatives of 12 other spiroplasma groups or subgroups (Table 1). None of the reactions was reciprocal, and only two reactions, the reactions of strain EC-1^T antiserum with group VII type strain MQ-1 and with ungrouped strain PLHS-1, occurred in both DF and MI tests. Strain EC-1^T is therefore serologically unrelated to all previously described species and existing

FIG. 1. Colonies of strain EC-1^T on HSI solid medium containing 2.25% Noble agar after 4 days of incubation at 30°C under anaerobic conditions. Bar = 100 μ m.

or putative groups or subgroups. Many tabanid spiroplasma isolates have proven to be closely related to strain EC-1^T. These isolates include isolates TS-1 (DF titer, 1:2,560), TS-2 (DF titer, 1:1,280), TS-2B (DF titer, 1:1,280), and TC-1 (DF titer, 1:1,280). The DF titers of tabanid isolates with strain EC-1^T antiserum have been reported previously (15, 40) to vary from 1:320 to 1:5,120, and there is evidence of biogeographical influence.

Sterol requirement. Strain EC-1^T responded positively to cholesterol supplementation of serum-free SP-4 medium (Table 2). This organism failed to grow in base broth alone, but grew when 1 to 20 μ g of cholesterol per ml was included.

DNA base composition. The G+C base composition of the DNA of strain EC-1^T was 27 ± 1 mol% as determined by the buoyant density method and 26.3 ± 1 mol% as determined by the melting temperature method (31).

Habitat. Although a search of other lampyrid and beetle species did not yield additional group XIV isolates (19), serologically similar isolates and some partially related isolates were obtained from many species of horseflies and deerflies (Diptera: Tabanidae) from Maryland. These isolates included

TABLE 1. Serological reactions of strain EC-1^T

Group	Strain	Spiroplasma DF test titer		MI test titer	
		Antiserum	Antigen	Antiserum	Antigen
I-5	LB-12	40 ^a	0 ^b	0 ^c	0 ^d
IV	B31	40	0	0	0
VII	MQ-1	40	0	54	0
IX	CN-5	40	0	0	0
XI	MQ-4	40	0	0	0
XIV	EC-1 ^T	2,560 ^e	2,560 ^e	39,000 ^e	39,000 ^e
XX	LD-1	40	0	0	0
XXII	CT-1	160	0	0	0
XXIII	TG-1	0	40 (20,480) ^f	0	0
NG ^g	HYOS-1	20	0	0	0
NG	PLHS-1	80	0	162	0
NG	TALS-2	0	160 (320)	0	0
NG	TIUS-1	0	0	54	0

^a Reciprocal of the end point in the DF test in which the antigen indicated was tested against strain EC-1^T antiserum.

^b Reciprocal of the end point in the DF test in which the antiserum indicated was tested against strain EC-1^T antigen.

^c Reciprocal of the end point in the MI test in which the antigen indicated was tested against strain EC-1^T antiserum.

^d Reciprocal of the end point in the MI test in which the antiserum indicated was tested against strain EC-1^T antigen.

^e Homologous titer of strain EC-1^T in the test system used.

^f The values in parentheses are the homologous titers of antisera against which strain EC-1^T cross-reacted when it was used as the antigen in heterologous tests.

^g NG, not grouped.

TABLE 2. Growth response of strain EC-1^T to cholesterol^a

Supplement(s) added to serum-free base medium	Cholesterol concn (μg/ml)	Amt of protein (mg/100 ml)
None	0	IG ^b
Bovine serum fraction (1%)	0	2.80
Albumin (1%) and palmitic acid (10 μg/ml)	0	0.26
Albumin (1%), Tween 80 (0.1%), and palmitic acid (10 μg/ml)	0	0.28
	1	1.10
	5	1.55
	10	2.23
	20	1.84

^a All flasks received an inoculum from 1% bovine serum fraction broth, were incubated at 30°C, and were harvested by centrifugation after 2 days.

^b IG, insufficient growth for detection.

strain TATS-1 from *Tabanus atratus* (gut contents); strain TC-1 from *Tabanus calens* (gut contents); isolates TG-1 (gut contents) and TG-2 (hemocoel contents) from *Tabanus gladiator*; strain TS-1 (gut contents) and isolates TS-2 (gut contents) and TS-2B (hemocoel contents) from *Tabanus sulcifrons*; and isolates from the gut contents and blood of three *Tabanus sulcifrons* specimens (Insect Biocontrol Laboratory accession numbers 00153, 00161, and 00187) (7, 17, 40). Isolates were also obtained from tabanid species from Georgia north to Maryland, including *Chlorotabanus crepuscularis*, *Tabanus lineola*, *Tabanus melanocerus*, *Tabanus molestus*, *Tabanus nigripes*, *Tabanus petiolatus*, and *Tabanus trimaculatus* (11, 12, 38, 40), as well as from insects from South Dakota (*Tabanus sulcifrons* [40]) and France (*Chrysops viduatus*, *Hybomitra bimaculata*, and *Tabanus bromius*) (22, 23). Spiroplasmas sometimes occurred in mixed infections in tabanids (38, 39), whereas spiroplasmas, mesoplasmas, and entomoplasmas occurred in mixed infections in the beetle *Ellychnia corruscae* (17, 19). *Entomoplasma ellychniae* (32) appears to be regularly associated with *Ellychnia corruscae*. Compared with other groups of insects (17, 18), a very high proportion of tabanid specimens was infected with spiroplasmas, with the frequencies of infection commonly exceeding 50% (11). In one estimate of the number of group XIV spiroplasmas in tabanid gut viscera, a titer of 10⁵ cells was obtained, which is typical for estimates of the titers of other spiroplasmas in tabanid guts (11).

Several hypotheses have been offered to explain the occurrence of group XIV spiroplasmas in both tabanid flies and firefly beetles. One of these hypotheses (17) involves possible transmission of spiroplasmas on flower surfaces, which may be visited by adult tabanids and lampyrids. The existence of a spiroplasma transmission cycle involving exposed host plant surfaces is supported by evidence which shows that (i) the spiroplasma persists for up to 30 days on leaf surfaces (36), (ii) transmission is affected by weather, particularly rainfall (11), and (iii) spiroplasmas are transmitted from infected *Ellychnia corruscae* adults to tabanid flies at feeding sources under laboratory conditions (36).

A second hypothesis (17) is that larvae of members of the two groups, both of which are predaceous, may acquire group XIV spiroplasmas during foraging activities. However, there is no experimental evidence for this. Transmission among lampyrid or tabanid larvae has not been attempted. In limited sampling of lampyrid and tabanid larvae, group XIV spiroplasmas were not isolated. Nevertheless, other spiroplasmas have been isolated from lampyrid beetle larvae (19, 36), and entomoplas-

mas have been isolated from lampyrid pupae (36). And although strain EC-1^T injected into beetle pupae (*Tenebrio molitor* [Coleoptera: Tenebrionidae]) was not transmitted to firefly (*Photuris hebes*) larvae that fed on the pupae, the group XIX firefly spiroplasma was transmitted to larvae by this method (36).

Transmission among tabanid flies has also been investigated. The results of recent studies (11, 36) suggest that vertical transmission does not occur; tabanid spiroplasmas were not transmitted transovarially and were not found on fly surfaces. Horizontal fly-to-fly transmission has not been studied.

Overall, it appears that *Ellychnia corruscae* is the spiroplasma's overwintering host and that tabanid flies are critical in the spiroplasma's spring-to-fall distribution. This is consistent with the isolation of members of similar serovars and genovars of the spiroplasma from the guts of beetles exiting from overwintering sites in early March and April and from the guts and hemocoels of tabanid flies from June to September (15, 17). Higher-than-expected variability among serovars and genovars suggests that there are multiple host transmission cycles (15). The spiroplasma's tolerance of a wide range of temperatures (10 to 41°C [21]) may reflect a rather broad seasonality and variability of host transmission cycles.

Although group XIV spiroplasmas multiply at 37°C (the vertebrate body temperature), fly-to-fly transmission via vertebrate hosts is unlikely. There is no experimental evidence that group XIV spiroplasmas can multiply in vertebrates (4), and samples of animal sera in France were serologically negative for antibodies to these spiroplasmas (23). Nevertheless, since other tabanid spiroplasmas do multiply and persist in suckling mice (4), and since animal sera have been found to react positively to group XVI spiroplasmas (23), some of which are associated with blood-sucking mosquitoes, it may be premature to rule out a role for vertebrates in transmission.

The properties of strain EC-1^T described previously or reported in this paper fulfill proposed criteria (20) for descriptions of species of the class *Mollicutes*. Properties mandating assignment to this class include the absence of a cell wall, filterability, a lack of reversion to walled bacteria when the organism is grown in antibiotic-free media, and penicillin resistance. The sterol requirement of strain EC-1^T, its inability to utilize urea, and its helicity and motility place this organism in the family *Spiroplasmataceae* (25). Serological comparisons of strain EC-1^T with representatives of other *Spiroplasma* species and groups revealed that strain EC-1^T is a member of a distinct *Spiroplasma* species.

In the taxonomic description below the properties of the new organism are summarized.

Description of *Spiroplasma corruscae* sp. nov. *Spiroplasma corruscae* (cor' rus. cae. M. L. gen. n. *corruscae*, from *Ellychnia corruscae*, a firefly beetle [Coleoptera: Lampyridae], the original source of the organism). Cells are filamentous, helical, and motile, pass through filters with 450-, 300-, and 220-nm pores with a 10-fold loss of titer, and do not pass through filters with 100-nm pores. The cells lack true cell walls. Colonies on solid medium containing 2.25% Noble agar are slightly diffuse to discrete and generally without the characteristic fried-egg morphology.

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

Film and spot reaction negative. Does not hemadsorb guinea pig erythrocytes.

The sterol requirement is fulfilled by cholesterol.

Temperature range, 10 to 41°C; optimum temperature, 32°C. The doubling time at the optimum temperature is 1.5 h.

Serologically distinct from previously established *Spiroplasma* species, groups and subgroups.

Strain EC-1^T was isolated from gut of an adult lampyrid beetle (*Ellychnia corsusca*). Many additional isolates and strains have been isolated from the hemolymph of *Ellychnia corsusca* and from the gut contents and hemolymph of tabanid flies. Pathogenicity for insects is not known.

The G+C content of the DNA is 26.3 ± 1 mol% as determined by the melting temperature method and 27.0 ± 1 mol% as determined by the buoyant density method.

The type strain is strain EC-1 (= ATCC 43212).

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