Spiroplasma cantharicola sp. nov., from Cantharid Beetles (Coleoptera: Cantharidae)

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Spiroplasma strain CC-1^T, isolated from the gut of the soldier beetle *Cantharis carolinus*, was serologically distinct from other *spiroplasma* species, groups, and subgroups. Cells of strain CC-1^T were shown by light microscopy to be helical, motile filaments. Electron microscopy showed that the cells were bounded by a single cytoplasmic membrane, with no evidence of a cell wall. The organism was insensitive to penicillin. Strain CC-1^T grew well in SM-1, MID, and SP-4 liquid media under aerobic or anaerobic conditions. The strain also grew in 1% serum fraction medium. Optimal growth occurred at 32°C, with a doubling time of 2.6 h, but the strain multiplied at temperatures of 10 to 37°C. Strain CC-1^T produced acid from glucose but hydrolyzed neither arginine nor urea. The guanine-plus-cytosine (G+C) content of the DNA was 26 ± 1 mol%. Other uncloned isolates from *C. carolinus* exhibited similar or identical serological patterns. On the basis of the data presented here, strain CC-1^T (= ATCC 43207), previously proposed as the representative strain of subgroup XVI-1, is designated the type strain of a new species, *Spiroplasma cantharicola*.

In 1982, adults of the soldier beetle, *Cantharis carolinus* (Coleoptera: Cantharidae), were found to harbor *spiroplasmas* in the gut but not in the hemolymph. Strain CC-1^T, derived from one of five isolates, was chosen as a representative of the cluster, which was designated group XVI (33) and, more recently, subgroup XVI-1 (1, 4). This group, however, eventually turned out to be complex and to contain organisms not only from beetles but from mosquitoes and flowers also. Abalain-Colloc and colleagues (1, 4) characterized group XVI *spiroplasmas* by serology, DNA-DNA homology, and polyacrylamide gel electrophoresis, and proposed recognition of three subgroups. In this classification, the cluster of *spiroplasma* strains and isolates from *C. carolinus* was designated subgroup XVI-1. Chastel and his colleagues, in 1985, discovered (11) a large assemblage of serologically diverse *spiroplasma* strains in mosquitoes in France that partially cross-reacted with strain CC-1^T. These *spiroplasmas* were later designated subgroup XVI-3 (1, 4). In 1983, an apparently unrelated strain (CB-1) had been isolated from the gut of a *Cantharis bilineatus* adult. Although minor reciprocal cross-reactivities between the two strains were observed when the strains were compared by metabolism inhibition, deformation, and growth inhibition serology, the two strains were at first regarded (unpublished data) as representatives of separate *spiroplasma* groups. However, studies of Shaikh et al. (28) on *spiroplasma* carriage by mosquitoes in Alabama revealed a strain, AEF-2, that showed substantial cross-reactivity between strains CC-1^T and CB-1, indicating that strains CC-1^T and CB-1 belonged to a single *spiroplasma* group. Strain CB-1 was subsequently assigned to subgroup XVI-2 (1, 4). In this paper, we describe taxonomic studies on strain CC-1^T (subgroup XVI-1 of

Abalain-Colloc et al. [1, 4]) that satisfy requirements for descriptions of species of the class *Mollicutes* (23). Strain CC-1^T is designated herein as the representative of a new species of the genus *Spiroplasma*.

MATERIALS AND METHODS

***Spiroplasma* strains.** Techniques for isolation of *spiroplasmas* from insects have been described previously (26). The CC-1^T strain was isolated and cultivated by T. B. Clark on 9 June 1982 from the gut of an adult soldier beetle, *C. carolinus*. Other related strains were isolated in June 1983. Some of the genomic and serological features of strain CC-1^T have been reported earlier (33).

Strain CC-1 was purified by conventional filtration-cloning techniques (30). Representative strains of the 24 recognized groups (19, 33) and eight subgroups, including type strains of recognized species (33, 36), were cultivated for comparative studies.

Culture medium and cultivation techniques. An isolate from which strain CC-1^T was derived was grown in primary culture in SM-1 liquid medium (34) at 30°C. After five passages, the isolate was lyophilized. For characterization, the culture was recovered from ampoules, passaged twice at 30°C, and cloned (30). Other culture media used included MID broth (34) and serum fraction broth with 1% bovine serum fraction (32). Solid formulations of these media were prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to achieve a final agar concentration of 0.8 to 2.25%, wt/vol. Cultures on solid medium were incubated at 30°C, either aerobically with or without 5% carbon dioxide (GasPak system [BBL Microbiology Systems, Cockeysville, Md.]) or anaerobically with 95% H₂-5% CO₂ (hydrogen GasPak system [BBL]).

Temperature requirements for growth were determined by

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FIG. 1. Colonies of strain CC-1^T on SP-4 agar medium (2.25% Noble agar) after 3 days of incubation at 30°C in an anaerobic environment.

preparing 10-fold dilutions of strain CC-1^T cultures in late logarithmic growth phase in M1D broth. Dilution series were incubated at 5, 10, 15, 20, 25, 30, 32, 37 and 41°C. Growth of organisms was determined by observing indicator change of the media and by microscopic examination of the cultures during a 40-day observation period. The doubling times at each temperature were determined by observing the time required for medium acidification and fitting the curve to a logistic growth equation (24).

Morphological studies. Cells of strain CC-1^T in logarithmic phase were examined by dark-field microscopy (magnification, $\times 1,250$). For electron microscopy, strain CC-1^T was grown in approximately 20 ml of M1D broth. 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 standard broth culture method (31).

Tests for biological and biochemical properties. Procedures for determination of carbohydrate fermentation (5), arginine and urea hydrolysis (5), hemadsorption (18), and film and spot production (17) have been described earlier. Filtration characteristics were measured in M1D broth by techniques reported previously (30).

Serological tests. Antiserum to strain CC-1^T was produced in rabbits as previously described (38). Hyperimmune anti-

sera to all established *Spiroplasma* species and groups were taken from reference collections at the Beltsville Agricultural Research Center or the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. Metabolism inhibition and deformation tests were performed as previously described (37).

Genomic analysis. Extraction and purification of DNAs were performed as previously described (6). Estimates of the G+C content of purified DNA of strain CC-1^T were obtained by buoyant-density, melting-temperature, and high-performance liquid chromatography techniques (7). Purified DNA from *Spiroplasma citri* (genome size, 1,186 MDa; G+C content, 26 ± 1 mol%) was used as a reference.

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain CC-1^T grew well in SM-1, M1D, SP-4, and conventional mycoplasma fluid media and aerobically or anaerobically on SP-4 solid medium. In all gaseous environments, it produced diffuse or rough colonies on SP-4 agar (Fig. 1), with some satellite growth. Fried egg colonies were never observed. The strain also grew in versions of the conventional Edward formulation of mycoplasma medium containing either horse serum (15) or bovine serum fraction (32). Growth occurred at temperatures ranging from 10 to 37°C; optimal growth was observed at 30°C. In broth media incubated at 5 or 41°C, no

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

Supplement(s) ^a	Cholesterol concn (μg/ml)	Amt of protein (mg/100 ml)
1% Serum fraction (control)	0	2.64 ^b
None	0	0.19
1% Albumin, 0.01% Tween 80, and 10 μg of palmitic acid per ml	0	0.38
	1	0.79
	5	1.24
	10	1.82
	20	1.96

^a Added to serum-free base medium.^b Yield from a 100-ml volume of broth after incubation at 30°C for 2 days.

growth was recorded. The doubling times at 10, 15, 20, 25, 30, 32, and 37°C were 63.6, 18.3, 6.8, 3.5, 3.1, 2.6, and 14.4 h, respectively. The optimum is therefore 32°C. Dark-field microscopy of logarithmic-phase cultures of strain CC-1^T showed numerous helical, motile filaments. Electron microscopy showed filamentous cells with no evidence of a cell wall (33). Typical cells were surrounded by a single cytoplasmic membrane.

Sterol requirement. Growth of strain CC-1^T was enhanced by cholesterol supplementation of serum-free SP-4 medium (Table 1). The strain failed to grow in base broth alone, but growth was increasingly enhanced when 1 to 20 μg of cholesterol per ml was included.

Biochemical and biological properties. Strain CC-1^T produced acid from glucose, but no evidence of arginine or urea hydrolysis was observed. Strain CC-1^T produced the film and spot reaction. Colonies of the organism on an agar medium did not hemadsorb guinea pig erythrocytes. Passage of broth cultures of strain CC-1^T through 450-nm-pore-size membrane filters reduced the viable-cell titer 10-fold to 10⁹ color-changing units per ml. The titer of a broth culture filtrate obtained after consecutive passage through 300- and 220-nm membranes was reduced about 10-fold by each procedure, to titers of 10⁸ and 10⁷ color-changing units per ml, respectively; the 100-nm-pore-size membrane filtrate was free of viable cells.

Serological tests. Metabolism inhibition and spiroplasma deformation tests have demonstrated (19, 33) that strain CC-1^T is serologically unrelated to representatives of established *Spiroplasma* groups or species.

DNA base composition. The G+C base composition of the DNA of strain CC-1^T was 26 ± 1 mol% (8).

Habitat. Strain CC-1^T and serologically similar uncloned subgroup XVI-1 isolates (CC-2, CC-3, CC-4, and CC-6) were obtained from the guts of adult cantharid beetles. Strain CB-1 (subgroup XVI-2) was also obtained from a cantharid beetle. Studies by Chastel and colleagues in France (9–12) and Shaikh and colleagues in Alabama (28) show that mosquitoes are frequent hosts of group XVI spiroplasmas. However, the mosquito spiroplasmas were found (1, 4) to constitute a third subgroup (XVI-3). Members of this subgroup, because of their residence in insects that transmit arboviruses, may prove to be of special interest. In all, mosquitoes appear to carry a large number of spiroplasmas (2, 3, 9–12, 22, 28), and interest in spiroplasmas as possible agents for biological control of mosquito pests has been expressed (10, 21). Spiroplasmas may be frank pathogens of mosquitoes (21). It is also possible that genetic manipulation could produce a specific pathogen. On the other hand, the

wide occurrence of group XVI spiroplasmas in the insect world (13, 20), together with a possibility that they may be acquired from plant surfaces by the activities of adult insects (27), may present environmental concerns if the spiroplasmas are used for biological control. Much more needs to be learned about the biological specificity of spiroplasma serotypes of biting insects, including those occurring in tabanid flies (14, 16, 25, 35). If individual serotypes are host specific, they could be useful delivery systems of toxins produced by vectored genes. Although subgroup XVI-2 and XVI-3 spiroplasmas differ from those of strain CC-1^T by a substantial amount of DNA-DNA homology, their eventual taxonomic status remains undecided. The species name proposed herein applies only to subgroup XVI-1 organisms; members of other subgroups of group XVI can be described in terms of their group-subgroup classification.

The properties of strain CC-1^T described herein fulfill proposed criteria (23) for species of the class *Mollicutes*. Properties mandating assignment to this class include absence of a cell wall, filterability, and penicillin resistance. The requirement of strain CC-1^T for sterol, its inability to utilize urea, and its helical shape and motility place this strain in the family *Spiroplasmataceae* (29). Finally, serological comparison of strain CC-1^T with representatives of other *Spiroplasma* species and groups (19, 33) confirms its status as a distinct species.

Description of *Spiroplasma cantharicola* sp. nov. *Spiroplasma cantharicola* (can. thar. íco.la. Gr. kantharos, scarab beetle; the L.V. colo, to inhabit; M.L. n. cantharicola, an inhabitant of a family of beetles).

Cells filamentous, helical, motile, lacking true cell walls. Colonies on solid medium with 0.8% Noble agar diffuse, without fried egg morphology.

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

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

Sterol requirement met by cholesterol or serum.

Temperature range 10 to 37°C, optimum 32°C, with a doubling time of 2.6 h.

Serologically distinct from other established *Spiroplasma* species.

Isolated from gut of an adult cantharid beetle *C. carolinus*. Pathogenicity for insects not determined.

G+C content of DNA 26 ± 1 mol%, as determined by buoyant-density, melting-point, and high-performance liquid chromatography techniques.

The type strain is CC-1^T (= ATCC 43207).

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