Spiroplasma monobiae sp. nov. from the Vespidae Wasp *Monobia quadridens* (Hymenoptera: Vespidae)

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Spiroplasma strain MQ-1^T (T = type strain) from the hemolymph of the vespidae wasp *Monobia quadridens* differed serologically from other *Spiroplasma* species, groups, and subgroups. Cells of strain MQ-1^T were helical and motile and possessed a single cytoplasmic membrane, with no evidence of a cell wall. The organism grew in conventional mycoplasma medium, in serum fraction, SM-1, M1D, and SP-4 liquid media, and on SP-4 solid medium in either aerobic or anaerobic environments. The optimum temperature for growth was 32°C, but multiplication occurred over a wide temperature range (10 to 37°C). The doubling time at 32°C in M1D medium was 1.9 h. Strain MQ-1^T catabolized glucose but hydrolyzed neither arginine nor urea. Previous work showed that strain MQ-1^T has a unique methylase, previously known only in eucaryotes. Also, strain MQ-1^T induces production of tumor necrosis factor in bone marrow macrophages. The guanine-plus-cytosine content of the DNA was 28 ± 1 mol%. The genome size of strain MQ-1^T was 940 kb (627 MDa); a similar strain, MQ-8, had a genome size of 985 kb (657 MDa). Strain MQ-1^T and its allies have the smallest genomes known in the genus *Spiroplasma*. Strain MQ-1 (= ATCC 33825) is designated the type strain of a new species, *Spiroplasma monobiae*.

The genus *Spiroplasma* (36), originally thought to consist of plant pathogens (22), was later shown, largely through the efforts of T. B. Clark and his associates (7, 15), to be associated with arthropods. As the immense diversity of this genus unfolded, numerous interesting properties emerged. For example, group I organisms (2) were the only *Spiroplasmas* found to be associated with plant disease or with plant-sucking bugs. Yet, the subgroup I-2 *Spiroplasma*, *Spiroplasma melliferum* (8), which was closely related to the plant *Spiroplasmas*, was primarily associated with bees. One subgroup of this cluster (subgroup I-6), which appears to occur in the insect-plant surface habitat (15), had a guanine-plus-cytosine (G+C) content that was higher than the G+C contents of its cohort subgroups (5). Another *Spiroplasma* (subgroup I-4), isolated only once, appeared to be associated with ticks. Group II *Spiroplasmas* (34) produced sex ratio abnormalities in *Drosophila* flies. Group III *Spiroplasmas* (9) (*Spiroplasma floricola*) proved to be associated with magnoliaceous flowers and their beetle and fly visitors and to have idiosyncratic metabolic pathways (6) that were readily studied because of the rapid growth of the species. Group IV appeared to be a widely distributed cluster of *Spiroplasmas*, some of whose members, including type strain B31 (*Spiroplasma apis*), were pathogenic (19, 20) for honeybees (*Apis mellifera*). Groups V (*Spiroplasma mirum* [30]) and VI (29) were associated with ticks. Thus, each of the first six established *Spiroplasma* groups appeared to possess unusual biological and/or ecological properties.

For some time, the group VII *Spiroplasmas*, strain MQ-1^T (T = type strain) and its allies, appeared to be exceptions to the emerging uniqueness of newly discovered strains. However, in 1985 Nur et al. (21) discovered that strain MQ-1^T had

a unique methylase, previously recognized only in eucaryotes. In addition, the membranes of strain MQ-1^T were shown to stimulate production of tumor necrosis factor by bone marrow macrophages (23). Furthermore, the genome sizes reported here for two group VII strains are the smallest genome sizes known for *Spiroplasmas*. Thus, group VII *Spiroplasmas* have joined predecessor groups in possession of unique features.

In this communication, we present the results of a taxonomic study of strain MQ-1^T, organized by the proposed (16) minimal requirements for assignment of binomial names to mollicutes. As a result of this study, we designate strain MQ-1 (= ATCC 33825) the type strain of a new species, *Spiroplasma monobiae*.

MATERIALS AND METHODS

***Spiroplasma* strains.** The techniques used for the isolation of *Spiroplasmas* from insects have been described previously (18). Strain MQ-1^T was isolated and cultivated by T. B. Clark from the hemolymph of an adult *Monobia quadridens* wasp collected in Maryland. Some genomic and serologic features of the organism were reported earlier (5, 28). Strain MQ-1^T was purified by conventional filtration-cloning techniques (26).

Culture media and cultivation techniques. Strain MQ-1^T was grown in a primary culture in SM-1 liquid medium (32) at 30°C. After three to five early broth passages, the isolate was lyophilized. For characterization, the dried culture was revived and passaged twice at 30°C before cloning in SM-1 broth. Following filtration-cloning (26), a triply cloned strain was designated MQ-1^T and was used in characterization studies. Other culture media used included (32) M1D and SP-4 media, conventional mycoplasma medium (10), and serum fraction broth supplemented with 1% bovine serum

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fraction (27). A solid formulation of each of these media was prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to final concentrations of 0.8 to 2.25%. Agar cultures were incubated at 30°C, either aerobically with or without 5% carbon dioxide (GasPak System [BBL Microbiology Systems, Cockeysville, Md.]) or anaerobically (hydrogen GasPak system [BBL]).

Temperature requirements for growth were assessed by preparing 10-fold dilutions of strain MQ-1^T in M1D broth. One series of dilutions was incubated at each of nine temperatures (5, 10, 15, 20, 25, 30, 32, 37, and 41°C). *Spiroplasma* growth was assessed by recording medium indicator change and/or by microscopic enumeration of helical filaments in the cultures. Each series was incubated for 40 days. The doubling times at each temperature were computed by fitting the temperature-time curves to a logistic growth equation (17).

Morphological studies. Cells of strain MQ-1^T in broth cultures in logarithmic phase were examined by dark-field microscopy (magnification, $\times 1,250$). For electron microscopic examination, the strain was grown in 20 ml of broth and pelleted by centrifugation. 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 requirements. Sterol requirements for growth were assayed by a standard broth culture method (25).

Tests for biological and biochemical properties. The procedures used for demonstration of carbohydrate fermentation (1) and arginine and urea hydrolysis (1), the hemadsorption assay (12), and the film and spot reaction (11) have been

described earlier. Filtration characteristics were measured in M1D broth by techniques reported previously (26).

Serological tests. Antiserum to strain MQ-1^T was raised in rabbits as previously described (37). Hyperimmune antisera to all established *Spiroplasma* species and groups were obtained from the reference collections at the Agricultural Research Center in Beltsville, Md., and the National Institute of Allergy and Infectious Diseases in Frederick, Md. These antisera and strain MQ-1^T were tested in a standard disc growth inhibition test (33). Metabolism inhibition and deformation tests were employed as previously described (35, 37).

Genomic analysis. Techniques for extraction and purification of chromosomal DNA from mollicutes have been described previously (3). The G+C content of purified strain MQ-1^T DNA was determined by buoyant density, melting temperature, and high-performance liquid chromatography methods (4). Purified DNA from *Spiroplasma citri* (genome size, approximately 1,186 MDa; G+C content, 26 ± 1 mol%) was used as a reference. The genome size was determined by pulsed-field gel electrophoresis as described by Grau et al. (13).

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain MQ-1^T grew well in liquid SM-1, M1D, and SP-4 media and on SP-4 solid medium. The strain also grew in conventional mycoplasma medium or bovine serum fraction. Growth occurred over a temperature range of 10 to 37°C; optimum growth was observed at 32°C, with a doubling time of 1.9 h. Doubling times at 10, 15, 20, 25, 30, and 37°C were 80.2, 15.6, 6.3, 3.4,

FIG. 1. Colonies of strain MQ-1^T on SP-4 agar medium containing 2.25% agar after 3 days of incubation at 30°C under aerobic conditions.

FIG. 2. Electron micrograph of a sectioned and stained cell pellet of strain MQ-1^T. Sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The arrows indicate the unit membrane.

2.4, and 20.2 h, respectively. Colonies of strain MQ-1^T on SP-4 agar medium containing 2.25% agar (Fig. 1) were diffuse; colonies having fried-egg morphology were never observed. Growth of strain MQ-1^T on solid medium was observed on agar plates incubated aerobically at 30°C or when the plates were placed in GasPak systems with enhanced carbon dioxide atmospheres.

Logarithmic-phase cultures of strain MQ-1^T examined by dark-field microscopy contained numerous long, helical, motile filaments. The cells of the organism examined by electron microscopy were filamentous and were surrounded by a single cytoplasmic membrane (Fig. 2), with no evidence of a cell wall.

Sterol requirement. The response of strain MQ-1^T to cholesterol supplementation of serum-free SP-4 medium is shown in Table 1. No growth was apparent in base broth, but growth was observed when 5 to 20 μg of cholesterol per ml was included.

Biochemical and biological properties. Strain MQ-1^T produced acid from glucose, but no evidence of arginine or urea hydrolysis was observed. Strain MQ-1^T produced the film and spot reaction, but colonies of the organism on an agar medium did not hemadsorb guinea pig erythrocytes. Passage of broth cultures of strain MQ-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 the broth culture

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

Supplement(s) added to serum-free base medium	Cholesterol concn (μg/ml)	Amt of protein (mg/100 ml)
17% fetal bovine serum (control)	0	2.80
None	0	IG ^a
1% albumin, 0.01% Tween 80, and 10 μg of palmitic acid per ml	0	IG
	1	IG
	5	0.23
	10	0.90
	20	1.98

^a IG, insufficient growth for detection.

filtrates obtained after passage through 300- and 220-nm-pore-size membrane filters was reduced to 10⁶ color-changing units per ml; the 100-nm-pore-size membrane filtrate was free of viable cells.

Serological tests. Metabolism inhibition and spiroplasma deformation tests (28) indicated that strain MQ-1^T was not related serologically to representatives of established groups (14, 28) or species of the genus *Spiroplasma*.

Genome size and DNA base composition. The genome size of strain MQ-1^T, as determined by pulsed-field gel electrophoresis, was 940 kb (627 MDa), and the genome size of strain MQ-8 was 985 kb (657 MDa). Thus, strain MQ-1^T has the smallest genome of all of the spiroplasmas examined so far. The G+C base composition of the DNA of strain MQ-1^T was 28 ± 1 mol%.

Habitat. Strain MQ-1^T and isolate MQ-7 were isolated from hemolymph samples from male vespid wasps (*M. quadridens*). Two isolates (MQ-8 and MQ-9) serologically identical to strain MQ-1^T were isolated from the guts of wasps; most mollicutes cultivated from insects are isolated from guts (7, 8, 15, 36). Some mollicutes that reside in insect hemolymph, such as *S. melliferum* (8) and *S. apis* (19), reduce the longevity of the host. Whereas *S. melliferum* titers in bee hemolymph reach 10¹⁰ organisms per ml, strain MQ-1^T had a titer of 7 × 10⁷ organisms per ml in the wasps (15). Because the wasp host of strain MQ-1^T is a predator, it is possible that the natural maintenance of this spiroplasma involves prey insects. The pathogenicity of strain MQ-1^T for *M. quadridens* or other insects has not been determined.

The properties described here for strain MQ-1^T fulfill the proposed criteria (16) for species of the class *Mollicutes*, including the 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 requirement for sterol or serum, the inability to utilize urea, helicity, and motility place this organism in the family *Spiroplasmataceae* (24). Molecular phylogenetic studies have shown that strain MQ-1^T belongs to a cluster of insect-associated spiroplasma species (31). Finally, serologic comparison of strain MQ-1^T with other *Spiroplasma* species and with other unclassified spiroplasma strains that probably represent putative species demonstrated the uniqueness of this new insect strain. On the basis of the relationship of the spiroplasma to its host, we propose the name *Spiroplasma monobiae* for this organism. The taxonomic description below summarizes the properties of the organism.

***Spiroplasma monobiae* sp. nov.** *Spiroplasma monobiae* (mon. o' bi. ae. M.L. n. *Monobia*, a genus of vespid wasps; M.L. gen. n. *monobiae*, of the genus *Monobia*, from which the organism was isolated). Cells are helical, motile filaments. Cells lack true cell walls. Colonies on solid medium

containing 2.25% Noble agar are diffuse, never with a fried-egg appearance.

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

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

Cholesterol or serum required for growth.

Temperature range for growth, 10 to 37°C; optimum growth occurs at 32°C with a doubling time of 1.9 h.

Serologically distinct from other established *Spiroplasma* species. Isolated from the hemolymph of an adult vespid wasp (*M. quadridens*). Pathogenicity for insects not determined.

The G+C content of the DNA is 28 ± 1 mol%. The genome size is 940 kb (627 MDa).

The type strain is MQ-1 (= ATCC 33825).

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