Spiroplasma diminutum sp. nov., from *Culex annulus* Mosquitoes Collected in Taiwan

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Initially, strain CUAS-1^T (T = type strain), which was isolated from a frozen triturate of *Culex annulus* mosquitoes collected in Taiwan, was thought to be a member of *Spiroplasma* group VII. This placement was based on the *Spiroplasma* deformation test titer observed when strain CUAS-1^T *Spiroplasma* were tested with *Spiroplasma monobiae* MQ-1^T antiserum. The results of subsequent reciprocal *Spiroplasma* deformation, metabolism inhibition, and growth inhibition tests clearly revealed that strain CUAS-1^T is not serologically related to previously described *Spiroplasma* groups (groups I to XXIV) and thus is a representative of a new group, group XXV. Strain CUAS-1^T was characterized by using the minimal standards for mollicute species descriptions. During logarithmic-phase growth, strain CUAS-1^T cells are characteristically very short helices with 1.5 to 2 helical turns (1 to 2 μ m), highly motile, and bounded by a single trilaminar membrane and form granular colonies with satellites when the organism is grown aerobically on MID medium containing 1.6% agar. Growth in MID broth occurs at temperatures ranging from 10 to 37°C, and the optimum temperature is 30°C. Substrate utilization tests revealed that cholesterol is required for growth, that glucose is hydrolyzed, and that arginine is not hydrolyzed both in the presence and in the absence of glucose. The genome of strain CUAS-1^T is 1,080 kbp long, and the guanine-plus-cytosine content is 26 ± 1 mol%. On the basis of the results of our studies we propose that strain CUAS-1^T (group XXV) should be placed in a new species, *Spiroplasma diminutum*. Strain CUAS-1 (= ATCC 49235) is the type strain of *S. diminutum*.

The first mosquito *Spiroplasma* was isolated from a triturate of female salt marsh mosquitoes (*Aedes sollicitans*) (12). This organism was subsequently characterized and named *Spiroplasma culicicola* (8). Mosquitoes have proved to be a rich source of *Spiroplasma*, and the following two additional mosquito-derived *Spiroplasma* have been characterized: *Spiroplasma sabaudiense* (1) and *Spiroplasma taiwanense* (2). In addition, several *Spiroplasma* strains isolated from mosquitoes collected in France and the United States have recently been shown to be related to each other and to be members of three subgroups of group XVI (3). A previously existing collection of frozen mosquito triturates originally collected by one of us (L.R.) for a mosquito-borne virus survey in Southeast Asia (People's Republic of China, Japan, Taiwan) has been found to contain, in addition to *S. taiwanense*, several *Spiroplasma* strains that cannot be placed in previously described species. One of these triturates (triturate 250657) was derived from 83 *Culex annulus* adult female mosquitoes collected in Taishan, Taiwan, in 1980. The *Spiroplasma* found in this triturate (designated strain CUAS-1^T [T = type strain]) were studied because of their small size and rapid growth in broth medium,

traits shared with the mosquito-derived organism *Spiroplasma culicicola* AES-1^T. The results of initial one-way serological studies in which triply cloned strain CUAS-1^T was tested with antisera to members of the 23 previously recognized *Spiroplasma* groups and subgroups suggested that strain CUAS-1^T is serologically related to group VII (*Spiroplasma monobiae*) (23). However, a subsequent reciprocal serological analysis revealed that the one-way cross-reactions observed initially did not indicate that strain CUAS-1^T and the group VII *Spiroplasma* are related. Furthermore, the genome sizes of these two *Spiroplasma* are significantly different (940 kbp for *S. monobiae* MQ-1^T and 1,080 kbp for strain CUAS-1^T) (5), and the G+C contents are also significantly different (28 ± 1 mol% for strain MQ-1^T and 26 ± 1 mol% for strain CUAS-1^T).

In this paper we present data obtained in a taxonomic study of strain CUAS-1^T and propose that this strain should be given species status as the type strain of *Spiroplasma* group XXV.

MATERIALS AND METHODS

Origins of strains. Strain CUAS-1^T was isolated by one of us (L.R.) from a frozen triturate of 83 adult female *C. annulus* mosquitoes collected on 5 September 1980, in Taishan, Taiwan. In 1986, this triturate was thawed and inoculated into SP-4 broth. When the culture was found to consist of helical filaments, a 10-fold dilution series in SP-4 medium was shipped to the State University of New York at Stony Brook for cloning and serological studies; this culture was designated *Spiroplasma* Sp18. At the fifth passage following isolation, *Spiro-*

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TABLE 1. Sterol requirement of strain CUAS-1^T

Sterol(s)	Titer of spiroplasmas (CCU/ml) after incubation at 30°C on:					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
None	N ^a	N	N	N	N	N
Albumin (0.5%) + palmitic acid (10 µg/ml)	N	N	N	N	N	N
Albumin (0.5%) + palmitic acid (10 µg/ml) + Tween 80 (0.01%)	N	N	N	N	N	N
Cholesterol (1 µg/ml)	10 ³	10 ⁶	10 ⁷	10 ⁷	10 ⁷	
Cholesterol (5 µg/ml)	10 ⁴	10 ⁷	10 ⁹	10 ⁹	10 ⁹	
Cholesterol (10 µg/ml)	10 ⁵	10 ⁷	10 ⁸	10 ⁸	10 ⁸	
Cholesterol (20 µg/ml)	10 ⁵	10 ⁷	10 ⁸	10 ⁸	10 ⁸	
Bovine serum fraction (1%)	10 ⁴	10 ⁷	10 ⁷	10 ⁷	10 ⁷	

^a N, no growth.

plasma Sp18 was filter cloned three times by using previously described procedures (13). At the end of the third cloning, a single colony was selected and designated strain CUAS-1^T. A 25-ml batch culture was prepared, and portions of this culture were transferred to sterile microtubes for storage at -70°C; the remainder was transferred to ampoules for lyophilization. A second triturate (triturate 250648) from 100 female adult *Culex tritaeniorhynchus* mosquitoes, also collected in Taishan, Taiwan, on 5 September 1980, contained spiroplasma Sp19, which was morphologically similar to strain CUAS-1^T. After filter cloning three times, this strain was designated strain CT-4, and it was subsequently shown to be serologically identical to strain CUAS-1^T.

Culture media and cultivation procedures. Both MID medium and SP-4 medium (19) were used for broth cultures. Solid MID and SP-4 media were prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 1.6 or 2.25%. Broth and agar cultures were grown aerobically at either room temperature (23°C) or 30°C.

Source of other spiroplasmas. Spiroplasmas obtained from frozen (-70°C) batch cultures representing strains of the 23 previously recognized groups and the subgroups of groups I, VIII, and XVI and eight new strains that represented putative new groups (15, 16, 20, 23) were used in various procedures.

Filtration studies. A fresh culture of strain CUAS-1^T in MID broth lacking penicillin was passed through a series of membrane filters with pore diameters of 450, 300, 220, and 100 nm by using a syringe and minimum hand pressure. A 50-µl portion of each filtrate was then diluted (10-fold serial dilutions) in 450 µl of MID medium lacking penicillin. One dilution series was used as a control. All of the tubes were incubated at room temperature. After 4 days, the tubes were examined for growth by identifying the last tube in which the color had changed. Cultures of strain CUAS-1^T were maintained in medium without penicillin G for more than five consecutive passages without reversion to bacterial forms.

Temperature requirements. Temperature requirements were determined by inoculating 900 µl of MID medium lacking penicillin with 100 µl of a spiroplasma culture. Then 10-fold dilutions were prepared in six different series, and each of these series was incubated at one of seven temperatures (10, 18, 22, 26, 30, 37, and 42°C). Growth was assessed by monitoring the number of color-changing units (CCU) during a 7-day period. Each of the dilution series was examined at its endpoint by dark-field microscopy.

Sterol requirements. The growth response to cholesterol was determined by first preparing BSR (18) medium adjusted to pH 7.4. Seven individual flasks containing this medium without bovine serum were prepared and subsequently supplemented with albumin, palmitic acid, Tween 80, cholesterol, and 1% Bacto PPLO Serum Fraction (Difco) (Table 1). Each of these preparations was distributed to tubes and incubated at 30°C. Growth responses were assessed by monitoring the number of CCU per milliliter (18).

Biochemical tests. Tests to determine the utilization of glucose, arginine, and urea were performed by using previously described procedures (4). All tubes were incubated at room temperature (21 to 23°C). The ability to utilize a substrate was evaluated by observing the color change of the pH indicator (phenol red) and by dark-field examination.

Morphology. Strain CUAS-1^T cells were routinely examined by dark-field microscopy. For electron microscopy, strain CUAS-1^T cells were fixed for 3 h in 3% (final concentration) glutaraldehyde that was added directly to the culture, pelleted by centrifugation at 16,000 × g, postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, and embedded in Epon-araldite; sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate.

Serological tests. Hyperimmune serum against strain CUAS-1^T was prepared in rabbits as described previously (11). Antisera were heat inactivated at 56°C for 30 min, membrane filtered (pore size, 450 nm), and stored at -20°C. Disc growth inhibition (GI) tests in which we used undiluted CUAS-1^T antiserum and representative spiroplasma strains on SP-4 hard agar were performed as described

previously (22). Reciprocal deformation (DF) and metabolism inhibition (MI) tests were performed by using previously described procedures (24, 26).

Genomic analysis. The genome size of strain CUAS-1^T was determined by pulsed-field gel electrophoresis as described by Carle et al. (5). The guanine-plus-cytosine (G+C) content of purified DNA was determined by the buoyant density, melting temperature, and high-performance liquid chromatography methods (6). The procedures used to extract and purify strain CUAS-1^T DNA were the procedure described by Carle et al. (6). Purified DNA from *Spiroplasma citri* R8A2 was used as a control (G+C content, 26 ± 1 mol%; genome size, 1,820 kbp).

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain CUAS-1^T grew very rapidly and to very high titers in both MID medium and SP-4 medium. For example, after 24 h the titer of a strain CUAS-1^T batch culture in MID broth growing at room temperature (21 to 23°C), which was prepared by using a 0.1% inoculum from the first passage after triple cloning, was 10¹⁰ CCU/ml. During logarithmic growth strain CUAS-1^T cells were short filaments (length, 1 to 2 µm) having one to three helical turns. They were highly motile, and exhibited rapid rolling or tumbling movements that made it difficult to discern their helical morphology when samples of broth cultures were observed by dark-field microscopy. Suspending living strain CUAS-1^T cells in methylcellulose (final concentration, 0.4%) allowed us to determine the typical cell length by dark-field microscopy (Fig. 1A). Electron microscopy of thin sections of CUAS-1^T cells embedded in plastic revealed that they had a typical trilaminar membrane (Fig. 1B).

Strain CUAS-1^T growing aerobically on solid MID medium containing 1.6% Noble agar formed colonies with dense centers, granular perimeters, and nondistinct edges, as well as small satellite colonies (Fig. 1C). Colony growth occurred on or just below the surface.

The numbers of CCUs per milliliter after filtration through membrane filters having average pore sizes of 450, 300, and 220 nm were 2 × 10¹⁰, 2 × 10⁹, and 2 × 10⁸, respectively. The organisms did not pass through membrane filters having an average pore size of 100 nm.

Biochemical properties. Strain CUAS-1^T fermented glucose but did not hydrolyze arginine and urea.

Sterol requirements. Strain CUAS-1^T did not grow in basal medium without serum or in basal medium supplemented with albumin, palmitic acid, and Tween 80 (Table 1). However, addition of 1% serum fraction to the basal medium or addition of 1 to 20 µg of cholesterol per ml to the basal medium containing fatty acid supplements significantly stimulated growth. In a study in which mollicutes were tested for growth responses to Tween 80, Rose et al. (10) showed that strain CUAS-1^T grew only when the medium contained sterol (serum).

Serological tests. A list containing the strains used in the serological studies and the results of some homologous GI, DF, and MI tests have been published previously (16). In addition, the MI titers of group II strain DW-1, group XX strain LD-1, and ungrouped strain TIUS-1 were 39,000, 117,000, and 39,000, respectively, and strain TIUS-1 produced a 9-mm inhibition zone in the GI test. The results of homologous serological tests performed with CUAS-1^T were as follows: size of GI zone, 5 mm; DF titer, 5,120; and MI titer, 13,000. In GI tests performed with strain CUAS-1^T and heterologous antisera produced against representative strains, none of the antisera inhibited the growth of strain CUAS-1^T. Likewise, in reciprocal GI tests, strain CUAS-1^T antiserum did not inhibit the growth of any of the spiroplasma strains that were representatives of previously described or putative groups or subgroups.

FIG. 1. Strain CUAS-1^T. (A) Morphology of living strain CUAS-1^T cells (1 volume of culture suspended in 2 volumes of M1D medium containing 1.6% methylcellulose). Dark-field flash photograph showing typical short strain CUAS-1^T filaments. Bar = 5 μ m. (B) Electron micrograph of a thin section of a strain CUAS-1^T cell pellet. The arrows indicate the single trilaminar plasma membrane. Bar = 100 nm. (C) Colony morphology of strain CUAS-1^T grown for 6 days on M1D medium containing 1.6% Noble agar at 30°C under aerobic conditions. Bar = 100 μ m.

Table 2 shows the reactions of the spiroplasma strains that produced positive cross-reactions in DF and/or MI tests with strain CUAS-1^T and its specific antiserum. As shown in Table 2, when reciprocal DF tests were performed, we observed cross-reactions between strain CUAS-1^T and the group III organisms *Spiroplasma floricola* BNR1 and OBMG, but not between strain CUAS-1^T and strain 23-6^T. The DF test titers when strains OBMG and BNR1 were used as antigens with CUAS-1^T antiserum were 640, but the DF test titer was <20 when 23-6^T was used as the antigen. The DF test titers in the reciprocal tests, when strain CUAS-1^T antigen was used with the antisera of the three strains, were <20. Similar cross-reactions were observed with the group VII organism *S. monobiae* MQ-1^T. When MQ-1^T was used as the antigen with

CUAS-1^T antiserum, the DF test titer was 40, and in the reciprocal test the titer was 320. With the subgroup organism XVI-1 *Spiroplasma cantharicola* CC-1^T, the reciprocal MI test titer was 54, compared with homologous titers of 39,000 for strain CC-1^T and 13,000 for strain CUAS-1^T. Although reciprocal tests were performed, cross-reactions were observed in only one of the three serological tests. The titers of the cross-reactions were always much lower than the homologous titers and usually higher when CUAS-1^T antigen and heterologous antiserum were used. They were also erratic in that repeat tests produced different titers.

We also observed some one-way cross-reactions; for example, in the MI test performed with the group XI organism *Spiroplasma velicrescens* MQ-4^T as the antigen and strain

TABLE 2. Serological reactions of spiroplasma strains that exhibited positive cross-reactions in DF and MI tests performed with strain CUAS-1^T and its specific antiserum^a

Group	Strain	Homologous reactions		Heterologous reactions			
				DF titer		MI titer	
		DF titer ^b	MI titer ^c	AG direction ^d	AS direction ^e	AG direction ^d	AS direction ^e
III	<i>S. floricola</i> 23-6 ^T	2,560	13,000	<20	<20	<18	<18
	OBMG	5,120	13,000	<20	640	<18	<18
	BNR1	10,240	39,000	<20	640	<18	<18
VII	<i>S. monobiae</i> MQ-1 ^T	5,120	>117,000	40	320	<18	<18
XI	<i>S. velicrescens</i> MQ-4 ^T	640	4,374	<20	<20	1,458	<18
XVI-1	<i>S. cantharicola</i> CC-1 ^T	1,280	39,000	<20	<20	54	54
XVI-2	CB-1	1,280	39,000	80	<20	<18	<18
XVI-3	Ar-1357	1,280	>117,000	320	<20	<18	<18
Ungrouped	PLHS-1 (scorpionfly)	2,560	117,000	<20	<20	54	<18
Ungrouped	PALS-1 (dragonfly)	1,280	4,374	<20	<20	18	<18

^a We performed reciprocal tests with strain CUAS-1^T and sera and antigens of members of the 23 previously described groups, including all 11 subgroups that have been described, as well as eight strains representing ungrouped spiroplasma clusters.

^b Reciprocal of the highest antiserum dilution that deformed one-half the spiroplasmas in either homologous or heterologous tests. The homologous DF titer of strain CUAS-1^T was 5,120.

^c Reciprocal of the highest antiserum dilution that resulted in MI in either homologous or heterologous tests. The homologous MI titer of strain CUAS-1^T was 13,000.

^d Test in which we used the spiroplasma indicated as the antigen and antiserum to strain CUAS-1^T.

^e Test in which we used strain CUAS-1^T as the antigen and antiserum to the strain indicated.

CUAS-1^T antiserum, the titer was 1,458, and in the DF test performed with CUAS-1^T antiserum and subgroup XVI-2 and XVI-3 strains CB-1 and Ar-1357, the titers were 80 and 320, respectively. Such irregular cross-reactions are common in serological studies of spiroplasmas and may reflect some shared epitopes, but information concerning this possibility is lacking. The reciprocal tests for all other strains resulted in DF titers of <20 or MI titers of <18. Our serological data clearly demonstrate that strain CUAS-1^T is not serologically related to previously described *Spiroplasma* species or groups.

Genome size and DNA base composition. The genome size of the undigested linear DNA molecule of strain CUAS-1^T, as determined by pulsed-field gel electrophoresis, was 1,080 kbp. Thus, the strain CUAS-1^T genome is 13% larger than the genome of *S. monobiae* strain MQ-1^T (940 kbp). The G+C content of the DNA of strain CUAS-1^T was 26 ± 1 mol% compared with 28 ± 1 mol% for strain MQ-1^T.

Habitat. Mosquitoes are an excellent source of new spiroplasma isolates. Previously described mosquito-derived spiroplasma species include *S. culicicola* (group X) (from *Aedes sollicitans* collected in New Jersey) (8), *S. sabaudiense* (group XIII) (from *Aedes sticticus/vexans* collected in the French Alps) (1), and *S. taiwanense* (group XXII) (from *Culex tritaeniorhynchus* collected in Taiwan) (2). In addition, subgroups XVI-2 and XVI-3 contain several strains of spiroplasmas isolated from various species of mosquitoes collected in the French Alps and in Alabama. Strain CT-4, which is morphologically identical to strain CUAS-1^T and serologically homologous and produces almost the same polyacrylamide gel electrophoresis whole-cell protein and *EcoRI-HindIII* restriction enzyme band patterns (data not shown), was isolated from a triturate of female *C. tritaeniorhynchus* mosquitoes collected in Taiwan on the same day as the *C. annulus* females from which CUAS-1^T was derived. Despite the apparent potential, none of the mosquito-derived spiroplasma strains is known to be pathogenic to any vertebrate or to be associated with pathogenesis in its mosquito host. Mosquitoes are known to feed on flower nectaries, and nectaries may provide the medium through which horizontal transfer of spiroplasmas occurs among mosquitoes and other flower-visiting arthropods. The ecology of mosquito-associated spiroplasmas has been reviewed recently by Chastel and Humphery-Smith (7).

Many properties of strain CUAS-1^T, including the lack of a cell wall, the failure to revert to walled forms when it is grown in the absence of an antibiotic, penicillin resistance, the ability to pass through 220-nm-pore-size filters, and formation of dense-center colonies on solid medium, are all characteristics of members of the class *Mollicutes* (9, 14). The helical morphology of CUAS-1^T, its requirement for sterol or serum for growth, its optimum growth at 30°C, and the characteristics of its genome place this strain in the order *Entomoplasmatales*, the family *Spiroplasmataceae*, and the genus *Spiroplasma* (14, 17, 21, 25). Our serological studies, in which three standard procedures (GI, DF, and MI tests) were used, revealed that strain CUAS-1^T is not related to representative strains of previously described groups I through XXIV or subgroups or to any of seven strains that represent new putative groups. Therefore, strain CUAS-1^T was designated the representative strain of group XXV. The name *Spiroplasma diminutum* is proposed for strain CUAS-1^T.

The taxonomic description below summarizes the properties of this new species.

Description of *Spiroplasma diminutum* sp. nov. *Spiroplasma diminutum* (di.min.u'tum. L. verb *deminuere*, to make pieces, make smaller; L. neut. partic. perf. *diminutum*, made smaller, reflecting a smaller size). Cells are short (1- to 2- μ m) helical

filaments (diameter 100 to 200 nm) that appear to be rapidly moving irregularly spheroidal bodies when logarithmic-phase broth cultures are examined under dark-field illumination. Colonies on solid medium containing 1.6% Noble agar have dense centers, granular perimeters, and nondistinct edges with satellite colonies.

Chemoorganotroph. Acid is produced from glucose. Does not hydrolyze arginine or urea. Film and spot reaction positive. Does not hemadsorb guinea pig erythrocytes.

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 37°C; the optimum temperature is 30°C. Grows well in SP-4, M1A, or M1D medium.

Serologically distinct from previously described *Spiroplasma* species. Isolated from a frozen triturate of female *C. annulus* mosquitoes. Pathogenicity for insects has not been determined.

The G+C content of the DNA is 26 ± 1 mol%. The genome size is 1,080 kbp.

The type strain is strain CUAS-1 (= ATCC 49235).

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