Spiroplasma platyhelix sp. nov., a New Mollicute with Unusual Morphology and Genome Size from the Dragonfly *Pachydiplax longipennis*

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Spiroplasma strain PALS-1^T from the gut of the dragonfly *Pachydiplax longipennis* was shown to be distinct from other species, groups, and subgroups of the genus *Spiroplasma* as determined by reciprocal serological metabolism inhibition and deformation tests. However, this strain cross-reacted extensively with representatives of other groups when it was used as an antigen. Electron microscopy of cells of strain PALS-1^T revealed cells surrounded by a single cytoplasmic membrane. Light microscopy revealed helical cells that exhibited twisting motility rather than rotatory or flexing motility. Variations in the tightness of coiling were transmitted from one end of the helix to the other. The strain was resistant to penicillin, which confirmed that no cell wall was present. The organism grew well in M1D and SP-4 liquid media under either aerobic or anaerobic conditions. Growth also occurred in 1% serum fraction medium and in conventional horse serum medium. The optimum temperature for growth was 30°C, at which the doubling time was 6.4 h. Multiplication occurred at temperatures from 10 to 32°C. Strain PALS-1^T catabolized glucose and hydrolyzed arginine but not urea. The guanine-plus-cytosine content of the DNA was 29 ± 1 mol%. The genome size was 780 kbp, the smallest genome size in the genus *Spiroplasma*. Strain PALS-1 (= ATCC 51748) is designated the type strain of a new species, *Spiroplasma platyhelix*.

The genus *Spiroplasma* (18, 26) is a large, possibly immense genus of arthropod-associated helical wall-less procaryotes (8, 9). Most of these organisms are associated primarily with insects, in a variety of cycles (5–7, 9). In 1980, a group classification system was proposed for the genus *Spiroplasma* (11); later, criteria for recognition of groups were proposed (23). The number of recognized *Spiroplasma* groups recently increased from 25 to 34 as a result of descriptions of eight new strains of insect-related spiroplasmas (20, 23, 28). The majority of spiroplasma strains have been isolated from recent orders of insects (Paraneoptera [hemipterous insects] and Holometabola [insects with complete metamorphosis]), and a representative of only one primitive order (Odonata) has been found to carry a spiroplasma strain (8). This strain, designated PALS-1^T, was obtained from a dragonfly, *Pachydiplax longipennis* (Odonata: Libellulidae). In this paper, we present the results of a taxonomic study of strain PALS-1^T, organized on the basis of the proposed (10) minimal requirements for assignment of binomial names to mollicutes. As a result of this study, we designate strain PALS-1 (= ATCC 51748) (group XXVIII) the type strain of a new species, *Spiroplasma platyhelix*.

MATERIALS AND METHODS

Spiroplasma strains. The techniques used to isolate strain PALS-1^T from its dragonfly host have been described previously (13, 22). Strain PALS-1^T was isolated and cultivated from the gut of a *P. longipennis* adult of unknown sex

collected by R. B. Henegar on 14 July 1986 at Log Lodge Pond on the grounds of the Beltsville Agricultural Research Center in Beltsville, Md. Some of the genomic and serological features of the organism have been reported previously (23). Strain PALS-1^T was purified by conventional filtration-cloning techniques (15). Representative strains of all previously recognized groups and subgroups, including the type strains of recognized species, were employed in our comparative studies (17).

Culture medium and cultivation techniques. The culture from which strain PALS-1^T was derived was grown in primary culture in M1D liquid medium (21) at 30°C. After several early broth passages, the isolate was cloned (15). A triply cloned strain designated PALS-1^T was used in the characterization studies. Other media employed included SP-4 medium (21), serum fraction broth supplemented with 1% bovine serum fraction (16), and conventional horse serum medium (21). Solid formulations of these media were prepared by adding Noble agar (Difco Laboratories, Detroit, Mich.) to a final concentration of 2.25%. Agar cultures were incubated at 30°C either aerobically with 5% carbon dioxide (GasPak system; BBL Microbiology Systems, Cockeysville, Md.) or anaerobically (hydrogen GasPak system). Temperature requirements for growth were assessed by the method of Konai et al. (12).

Morphological studies. Cells of strain PALS-1^T in the logarithmic phase were examined by dark-field microscopy at a magnification of ×1,250. For dark-field electronic flash photography, 500 µl of a mid-logarithmic-phase culture in M1D medium was centrifuged at 12,000 × g for 15 min at 4°C. The pellet was resuspended in 100 µl of the supernatant, and an equal volume of 3% glutaraldehyde in M1D medium was added dropwise. After 1 h of fixation, the suspension was examined and photographed without further treatment. A similar suspension of strain PALS-1^T fixed in 1.5% (final concentration) glutaraldehyde was prepared and used for electron microscopy of negatively stained specimens (24). Previously described procedures (24) were used for electron microscopic studies of the fine structure of strain PALS-1^T cells.

Sterol requirement. Sterol requirements for growth were determined by measuring the protein yields of strain PALS-1^T grown in serum-free broth supplemented with different amounts of cholesterol and by a modified broth culture dilution method (14, 16).

Tests for biological and biochemical properties. The procedures used to study glucose fermentation and hydrolysis of arginine and urea have been described previously (1). Filtration characteristics were determined in M1D broth by previously described techniques (15).

Serological tests. Antiserum to strain PALS-1^T was produced in rabbits as previously described (25). Hyperimmune antisera to all previously established *Spiroplasma* species and groups (17) were obtained from the reference collec-

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FIG. 1. Colonies of strain PALS-1^T on SP-4 medium supplemented with 2.25% Noble agar after 7 days of incubation at 30°C under aerobic conditions. Bar = 100 µm.

tions at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. These antisera and strain PALS-1^T were used in metabolism inhibition and deformation tests as previously described (25, 27).

Genomic analysis. The techniques used for extraction and purification of chromosomal DNA from strain PALS-1^T have been described previously (2). The genome size of strain PALS-1^T was determined as described by Carle et al. (4). The guanine-plus-cytosine content of purified strain PALS-1^T DNA was determined by the buoyant density and melting temperature methods (3). Purified DNA from *Spiroplasma citri* (genome size, 1,820 kbp; guanine-plus-cytosine content, 26 ± 1 mol%) was used as a reference in all procedures (4).

RESULTS AND DISCUSSION

Cultural and morphological properties. Strain PALS-1^T grew well in liquid M1D and SP-4 media and on solid SP-4 medium. The strain also grew in conventional mycoplasma media containing horse serum (Edward formulation) or 1% bovine serum fraction. Growth occurred at temperatures ranging from 10 to 32°C; optimum growth was observed at 30°C, at which the doubling time was 1.9 h. No growth was observed during 3 weeks of incubation in broth media at 5 or 37°C. Strain PALS-1^T grew on solid medium in agar plates incubated (aerobically) at 30°C. On 2.25% agar, strain PALS-1^T formed perfect fried-egg colonies with smooth edges; no satellites were observed (Fig. 1).

As determined by dark-field microscopy, logarithmic-phase cultures of strain PALS-1^T in M1D medium contained filaments that had barely visible helicity along most of their length and displayed very little rotatory or flexing motility. Organisms not attached to glass moved in a twisting fashion. Figure 2 shows glutaraldehyde-fixed filaments as revealed by dark-field microscopy; these cells displayed the low-pitch helical morphology of strain PALS-1^T spiroplasmas. A fascinating characteristic of these filaments, which is dramatically displayed by living spiroplasmas observed by dark-field microscopy, is the presence of a highly refractile segment (Fig. 2, arrow) that spontaneously appears at one end and moves along the length of each filament to the opposite end, at which point it appears to become unwound. When negatively stained specimens were examined by electron microscopy, this refractile segment was found to be comprised of three or four tightly wound helical

FIG. 2. Dark-field electronic flash photomicrograph of strain PALS-1^T cells fixed in 1.5% glutaraldehyde in M1D medium. The arrow indicates the light-refractive segment of tightly wound helices that travels along the length of the living cell. Bar = 5 µm.

turns (Fig. 3). Electron microscopy of thin sections of PALS-1^T cells embedded in plastic revealed that the cells possess a typical trilaminar membrane (Fig. 4). Figure 4 also shows that some cells contain viruslike particles in their cytoplasm.

FIG. 3. Electron micrograph of a strain PALS-1^T cell fixed in 1.5% glutaraldehyde in M1D medium and negatively stained with 1% phosphotungstic acid (pH 7.4). The arrow indicates a segment of tightly wound helices. Bar = 1 µm.

FIG. 4. Electron micrograph of a sectioned and stained pellet of strain PALS-1^T cells. The section was stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The unlabeled arrows indicate the unit membrane. Intracellular viruslike particles (VLP) in a bulbous portion of one cell are indicated by arrows. Bar = 200 nm.

Sterol requirement. The response of strain PALS-1^T to cholesterol supplementation of serum-free SP-4 medium is shown in Table 1. No growth was apparent in base broth alone, but growth was enhanced when 5 or 10 µg of cholesterol per ml was included. Lower concentrations of cholesterol did not stimulate growth, and 20 µg/ml was inhibitory. As determined by the modified serial dilution method (data not shown), strain PALS-1^T grew through 23 passages in medium containing fetal bovine serum, but did not grow beyond a single 10-fold dilution in serum-free medium or serum-free medium supplemented

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

Medium	Cholesterol concn (µg/ml)	Amt of protein (mg/100 ml)
M1D (control)		0.63
Serum fraction (control)		0.51
Serum free	0	IG ^a
	1	IG
	2	IG
	5	3.04
	10	3.10
	20	0.03

^a IG, insufficient growth.

TABLE 2. Serological reactions and cross-reactions of strain PALS-1^T

Group	Strain	Titer in:			
		Spiroplasma deformation test		Metabolism inhibition test	
		Anti-serum	Antigen	Anti-serum	Antigen
I-3	E275 ^T	0 ^a	20 (20,480) ^b	0 ^c	486 (>117,000) ^b
III	23-6 ^T	0	20 (10,240)	0	0 (39,000)
VII	MQ-1 ^T	40	0 (1,240)	0	0 (117,000)
VIII-3	TAAS-1	0	0 (2,560)	0	54 (39,000)
XII	DU-1 ^T	0	0 (5,120)	0	54 (13,000)
XVI-2	CB-1	0	0 (2,560)	0	54 (39,000)
XVI-3	Ar 1357	0	0 (20,480)	0	18 (117,000)
XVIII	TN-1 ^T	40	0 (2,560)	0	486 (>117,000)
XIX	PUP-1 ^T	0	0 (10,240)	54	0 (>117,000)
XX	LD-1 ^T	0	40 (20,480)	162	1,458 (117,000)
XXI	W115	0	0 (40,960)	0	54 (13,000)
XXIV	CCH ^T	0	0 (640)	0	18 (39,000)
XXV	CUAS-1 ^T	80	40 (640)	0	18 (13,000)
XXVI	PLHS-1 ^T	0	0 (2,560)	162	54 (117,000)
XXVIII	PALS-1 ^T	1,280 ^d	1,280 ^d	4,374 ^d	4,374 ^d
XXX	BIUS-1	0	0 (10,240)	0	486 (39,000)
XXXII	TABS-2 ^T	0	0 (640)	0	54 (39,000)
XXXIII	TAUS-1 ^T	0	0 (5,120)	0	54 (39,000)
	All others	0	0	0	0

^a Reciprocal of the endpoint in the deformation test in which the antigen was tested with the homologous antiserum.

^b Homologous titer of the antiserum against which strain PALS-1^T cross-reacted when it was used as the antigen in heterologous tests.

^c Reciprocal of the endpoint in the metabolism inhibition test in which the antigen was tested with the homologous antiserum.

^d Homologous titer of strain PALS-1^T in the test system. The reaction, obtained once, is included for completeness.

with 0.04% Tween 80. The strain therefore requires serum or cholesterol (14, 16).

Biochemical and biological properties. Strain PALS-1^T produced acid from glucose and hydrolyzed arginine but not urea. Passage of broth cultures of strain PALS-1^T through 450-nm-pore-size membrane filters reduced the viable cell titer from 10⁹ to 10⁸ color-changing units/ml. The titer of a broth culture filtrate obtained after passage through a 300-nm-pore-size membrane filter was also reduced about 10-fold (to 10⁷ color-changing units/ml). Passage through 220-nm filter pores reduced the titer by 3 log₁₀ units. The 100-nm-pore-size membrane filtrate did not contain viable cells.

Serological tests. The results of metabolism inhibition and spiroplasma deformation tests (Table 2) indicated that strain PALS-1^T was not related serologically to representatives of previously established groups or species of the genus *Spiroplasma*. However, when this strain was used as an antigen, it exhibited a broad pattern of one-way cross-reactions in both metabolism inhibition and deformation tests, although some reciprocal cross-reactions did occur. For example, Table 2 shows the reciprocal metabolism inhibition test titers involving strains LD-1^T (group XX, *Spiroplasma leptinotarsa*) and PLHS-1^T (group XXVI) and the reciprocal deformation test titers for strain CUAS-1^T (group XXV, *Spiroplasma diminutum*). It should be noted that in each of these examples, the reciprocal cross-reactions occurred in either the deformation test or the metabolism inhibition test, but not in both.

Genome size and DNA base composition. The size of the undigested linear DNA of strain PALS-1^T was 780 kbp as determined by pulsed-field gel electrophoresis and 770 kbp as determined from the sum of the sizes of restriction fragments

after *Sst*II digestion (1a). The base composition (guanine-plus-cytosine content) of PALS-1^T DNA was 29 ± 1 mol%. Thus, the genome size of strain PALS-1^T is by far the smallest genome size in the genus *Spiroplasma*. The genome size and guanine-plus-cytosine content of strain PALS-1^T clearly distinguish this organism from the three strains with which it exhibited serological reciprocal cross-reactions, strains LD-1^T (genome size, 1,085 kbp; guanine-plus-cytosine content, 25 ± 1 mol%), PLHS-1^T (genome size, 1,465 kbp; guanine-plus-cytosine content, 31 ± 1 mol%), and CUAS-1^T (genome size, 1,080 kbp; guanine-plus-cytosine content, 26 ± 1 mol%).

Habitat. Strain PALS-1^T is not known to be pathogenic for insects. The single strain described here was isolated directly from the gut of a dragonfly (*P. longipennis*) collected in Beltsville, Md. Members of the insect order Odonata (dragonflies and damselflies) are predatory on other insects, and thus the spiroplasmas in the gut of the specimen collected at Log Lodge Pond may simply have been passively acquired from one of its recent repasts.

The properties described here for strain PALS-1^T fulfill proposed criteria (10) for species of the class *Mollicutes*, including the absence of a cell wall, filterability, the lack of reversion to walled bacteria when the organism is grown in antibiotic-free media, and penicillin resistance. The helicity and motility of strain PALS-1^T and its inability to utilize urea place this organism in the family *Spiroplasmataceae* (19). Finally, the results of the serological comparison of strain PALS-1^T with other *Spiroplasma* species demonstrate the uniqueness of this new insect strain. We therefore propose the name *Spiroplasma platyhelix* for this organism. The taxonomic description below summarizes the properties of the new species.

Description of *Spiroplasma platyhelix* sp. nov. *Spiroplasma platyhelix* (plat.ye'helix. Gr. adj. *platys*, flat; Gr. n. *helix*, a coil or spiral; M. L. fem. n. *platyhelix*, flat coil, referring to the flattened nature of the helical filament). Cells are flattened, helical, motile filaments that vary from 200 to 300 nm in diameter. The cells lack true cell walls. Cells plated on SP-4 solid medium containing 2.25% Noble agar form perfect fried-egg colonies with dense centers and smooth edges and without satellites.

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

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 32°C, and optimum growth occurs at 30°C. The doubling time at the optimum temperature in M1D medium is 6.4 h.

Serologically distinct from other established *Spiroplasma* species. Isolated from the gut of the dragonfly *P. longipennis*. Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 29 ± 1 mol%, as determined by the buoyant density method. The genome size is 780 kbp.

The type strain is PALS-1 (= ATCC 51748).

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