***Spiroplasma poulsonii* sp. nov., a new species associated with male-lethality in *Drosophila willistoni*, a neotropical species of fruit fly**

David L. Williamson,^{1†} Bungo Sakaguchi,² Kevin J. Hackett,³
Robert F. Whitcomb,^{4‡} Joseph G. Tully,⁵ Patricia Carle,⁶ Joseph M. Bové,⁶
Jean R. Adams,³ Meghnad Konai^{3§} and Roberta B. Henegar³

Author for correspondence: David L. Williamson. Tel: +1 516 724 0897. Fax: +1 516 444 3947.
e-mail: dwmson@physiology.pnb.sunysb.edu

¹ Department of Anatomical Sciences, State University of New York, Stony Brook, NY 11794, USA

² Faculty of Agriculture, Kyushu University, Fukuoka, Japan

^{3,4} Insect Biocontrol Laboratory³ and Vegetable Laboratory⁴, US Department of Agriculture, Beltsville, MD 20705, USA

⁵ Mycoplasma Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Frederick Cancer Research Facility, Frederick, MD 21702, USA

⁶ Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique and Université de Bordeaux II, 33883 Villenave d'Ornon Cedex, France

Progenies from some wild-caught females of *Drosophila willistoni* and three other sibling species are entirely female. The proclivity for production of unisexual female progeny by these flies was named the sex ratio (SR) trait and was originally thought to be genetic. However, experiments in the laboratory of Donald F. Poulson in the early 1960s demonstrated that this 'trait' was vertically transmitted and infectious, in that it could be artificially transferred by injection from infected females to non-infected females. Motile, helical micro-organisms were observed in females showing the trait. In 1979, the SR organisms were designated as group II in the informal spiroplasma classification system. The organisms proved to be extremely fastidious, but were eventually cultivated in a very complex cell-free medium (H-2) after initial co-cultivation with insect cells. Cultivation in the H-2 medium and the subsequent availability of a triply cloned strain (DW-1^T) permitted comparative studies. Cells of strain DW-1^T were helical, motile filaments 200–250 nm in diameter and were bound by a single trilaminar membrane. Cells plated on 1.8% Noble agar formed small satellite-free colonies 60–70 µm in diameter with dense centres and uneven edges. The temperature range for growth was 26–30 °C; optimum growth occurred at 30 °C, with a doubling time in H-2 medium of 15.8 h. The strain passed through filters with 220 nm, but not 100 nm, pores. Reciprocal serological comparisons of strain DW-1^T with representatives of other spiroplasma groups showed an extensive pattern of one-way crossing when strain DW-1^T was used as antigen. However, variable, usually low-level reciprocal cross-reactions were observed between strain DW-1^T and representatives of group I sub-groups. The genome size of strain DW-1^T was 2040 kbp, as determined by PFGE. The G+C content was 26±1 mol%, as determined by buoyant density and melting point methods. The serological and molecular data indicate that strain DW-1^T is separated from group I representative strains sufficiently to justify retention of its group status. Continued group designation is also indicated by the ability of SR spiroplasmas to induce male lethality in *Drosophila*, their vertical transmissibility and their extremely fastidious growth requirements. Group II spiroplasmas, represented by strain DW-1^T (ATCC 43153^T), are designated *Spiroplasma poulsonii*.

Keywords: *Spiroplasma poulsonii* sp. nov., mollicute, sex ratio, *Drosophila willistoni*

[†]Present address: 4 Galahad Lane, Nesconset, NY 11767-2220, USA.

[‡]Present address: Carl Hayden Bee Research Center, USDA/ARS, 2000 E Allen Road, Tucson, AZ 85719, USA.

[§]Present address: 9125 Tymat Court, Laurel, MD 20723, USA.

Abbreviation: SR, sex ratio.

INTRODUCTION

A curious relationship between spiroplasmas and insects occurs in natural populations of four closely related neotropical species of *Drosophila*. In collections of flies of the *Drosophila willistoni* group from tropical South America and islands of the Antilles, single females were found whose progenies consisted of only daughters (Poulson, 1963). This trait, the sex ratio (SR) trait, is widely distributed among members of the *D. willistoni* species group: *D. willistoni* from Jamaica and Brazil; *Drosophila nebulosa* from Haiti and Brazil; *Drosophila equinoxialis* subsp. *equinoxialis* from Brazil and subsp. *caribbensis* from Puerto Rico, the Dominican Republic and Jamaica; and *Drosophila paulistorum* from Colombia, Brazil and the West Indies. Early studies of the SR trait conducted in the laboratory of Professor Donald F. Poulson at Yale University demonstrated that the SR trait in the *D. willistoni* species group was maternally (vertically) inherited and infectious, in that it could be artificially transferred by injection from infected females to non-infected females, who then transmitted the trait to their daughters (Malogolowkin & Poulson, 1957; Malogolowkin *et al.*, 1959; Sakaguchi & Poulson, 1963). An extensive review of the SR trait in *Drosophila* and the organisms that induce it was published in 1979 (Williamson & Poulson, 1979).

In 1960, one of us (B. Sakaguchi), during a post-doctoral fellowship in Professor Poulson's laboratory, discovered the infectious nature of the SR trait. Using a glass micropipette, a small sample of haemolymph was aspirated from *D. willistoni* females producing unisexual (female) progenies and placed under a drop of crown immersion oil on a microscope slide and observed, first under ordinary light microscopy, by which nothing was visible, and subsequently under phase-contrast microscopy. Even though the optics of this microscope provided much less contrast than the optics of the dark-field microscopes currently used for spiroplasma observation, Sakaguchi was surprised to find that the haemolymph was teeming with small filamentous organisms. In freshly mounted preparations, waves of helicoidal movement could be seen to pass along the length of the filaments, giving them a regular spiral appearance. Fixation of such preparations over formaldehyde vapour, followed by various staining procedures, suggested that the organisms were similar in size and general aspect to spirochaetes in the genus *Treponema* (Poulson & Sakaguchi, 1961). Hence, the organisms were referred to as 'sex ratio spirochaetes', until they were shown (Williamson, 1969; Williamson & Whitcomb, 1974) by electron microscopic examination of fixed and negatively stained cells to be devoid of axial filaments and outer envelopes – structures characteristic of spirochaetes. Instead, the SR organisms proved to be structurally similar to spiroplasmas associated with corn stunt disease (Davis *et al.*, 1972; Chen & Liao, 1975; Williamson & Whitcomb, 1975) and stubborn disease of citrus (Cole *et al.*, 1973).

Prior to the establishment of a cloned isolate of the *D. willistoni* SR spiroplasma, studies on the organisms were performed using suspensions of spiroplasmas in physiological saline, which were prepared from small samples of haemolymph aspirated from *D. willistoni* flies carrying the SR trait. Microscopic enumeration of haemolymph dilutions indicated that haemolymph titres in *D. willistoni* were about $1 \times 10^{11} \mu\text{l}^{-1}$. Suspensions of organisms from fly haemolymph were used to produce an antiserum in rabbits, and to determine the G+C content of DNA from the uncloned organisms. Vigorous attempts were made to cultivate the SR spiroplasma, but even though certain medium formulations permitted them to be maintained for varying periods, none provided the necessary factors for growth (Williamson *et al.*, 1983). Finally, however, in 1986, Hackett and collaborators reported that they had successfully grown and cloned the *D. willistoni* SR spiroplasma (Hackett *et al.*, 1986). In these studies, the SR spiroplasma was first grown in co-culture with a lepidopteran cell line and subsequently in cell-free culture in a new medium, H-2 (Hackett *et al.*, 1986). Cultivation of the organisms permitted them to be cloned, and the DW-1^T strain was selected for further study. After injection of a suspension of strain DW-1^T spiroplasmas into *Drosophila pseudoobscura* Piñon Normal strain females, their progenies became entirely female (Williamson *et al.*, 1989). However, the cloned organisms had lost the ability to be vertically transmitted.

Reciprocal spiroplasma deformation and metabolism inhibition tests involving strain DW-1^T and representative strains of all other groups and sub-groups have now been completed. These tests showed strong patterns of one-way cross-reactions when strain DW-1^T was used as antigen (Williamson *et al.*, 1998). Some reciprocal crossing patterns between strain DW-1^T and group I spiroplasmas were observed, but the patterns were not as strong as most intragroup reactions among group I spiroplasmas. The phylogenetic relationship of strain DW-1^T to other spiroplasmas was clarified by the studies of Weisburg *et al.* (1989). These studies showed that group I and group II spiroplasmas were more closely related to each other than to other spiroplasma groups, but nonetheless had significant sequence dissimilarity. The status of strain DW-1^T as the representative strain of group II spiroplasmas is therefore justified on the basis of molecular and serological data. In addition, its unique biological characteristics – host specificity, pathogenicity for male progeny, transovarial transmission and fastidious growth requirements – also augur for its group status. In this report we present the results of characterization studies of strain DW-1^T in fulfillment of the minimal standards for new species descriptions for strains classified in the class *Mollicutes* (International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*, 1995). Strain DW-1^T is hereby designated as the type strain (ATCC 43153^T) of a new species, *Spiroplasma poulsonii*, named in

Fig. 1. Colony of strain DW-1^T on H-2 medium supplemented with 1.8% Noble agar after 32 d at 26 °C under aerobic conditions with 5% CO₂. Bar, 20 µm.

memory of Professor Donald F. Poulson, in whose laboratory at Yale University SR organisms were first studied.

METHODS

Spiroplasma strains. Strain DW-1^T was cultivated (Hackett *et al.*, 1986) from a haemolymph suspension obtained from *D. pseudoobscura* (Piñon Normal strain) female flies artificially infected by injection of naturally infected Barbados-3 strain *D. willistoni* SR haemolymph. Briefly, 2.5 µl haemolymph from the infected females was collected by glass needle and transferred to 500 µl M1D medium. In *D. pseudoobscura*, SR organisms reach a titre of about $1 \times 10^{11} \mu\text{l}^{-1}$, so the initial cell titre in the primary culture was about $5 \times 10^{10} \mu\text{l}^{-1}$. An aliquot of this suspension was mixed with lepidopteran cell culture medium (DCCM) (Hackett & Lynn, 1995) and further aliquoted to microtitre wells containing embryo-derived cells from the cabbage looper (*Trichoplusia ni*) cell line (IPLB-TN-R³) (Rochford *et al.*, 1984). Primary isolation was accomplished in co-culture with *T. ni* cells initially growing in TNM-FH medium (Hackett *et al.*, 1986). Organisms growing in these cultures were eventually adapted to grow in the cell-free H-2 medium (Hackett *et al.*, 1986). Growth was evaluated by enumeration of helical cells in 3 or 5 µl samples under dark-field microscopy (Williamson, 1969). After nine co-culture passages, the SR organisms were adapted to grow in cell-free H-2 medium. At passage 20, the spiroplasmas were triply cloned by the limiting dilution method of Whitcomb & Hackett (1986). One triply cloned strain at passage 23 was selected and designated strain DW-1^T (ATCC 43153^T). A batch culture was prepared, aliquoted to ampules and lyophilized. Rehydrated cultures were used for the characterization studies reported here. Representatives of the

strains of all currently recognized groups and sub-groups, including the type strains of named species (Williamson *et al.*, 1998), were utilized for these comparative studies.

Culture medium and cultivation techniques. H-2 medium (Hackett *et al.*, 1986), a combination of 1 part DCCM (Hackett & Lynn, 1995), 2 parts M1D (Whitcomb, 1983) and 1 part modified Grace's Insect Medium (TNM-FH) (Hackett & Lynn, 1985), supplemented with 15% (v/v) foetal bovine serum and containing 800 U penicillin ml⁻¹, was used as the growth medium for all tests involving strain DW-1^T. Batch cultures of cells in this medium were prepared in 9 ml H-2 medium. About 10 d were required to reach maximum titre (about $1 \times 10^7 \text{ ml}^{-1}$, initially). To prepare solid H-2 medium, 8% (w/v) Noble agar (Difco) was added to the autoclavable fraction of M1D, microwaved to melt the agar, equilibrated at 56 °C and added to H-2 broth medium at 56 °C to produce a final agar concentration of 1.8%. Cultures of strain DW-1^T on solid media were incubated at 26 °C, either aerobically without added CO₂ or with 5% (v/v) CO₂ (Enhanced CO₂ Gaspak system, BBL Microbiology Systems) or anaerobically (Hydrogen and CO₂ Gaspak system). The SP-4 and M1D medium formulations were used for the cultivation of other spiroplasma strains. Temperature requirements for growth were determined by the method of Konai *et al.* (1996).

Morphological studies. The motility and gross morphology of strain DW-1^T cells were determined by observing them in 3–5 µl samples of broth cultures under dark-field illumination at $\times 800$ –1250. For electron microscopic studies of the fine structure of strain DW-1^T cells, previously published procedures (Williamson, 1983) were followed.

Sterol requirements. The sterol requirement for growth was evaluated by determining the protein yield of strain DW-1^T grown in serum-free H-2 broth supplemented with increasing amounts of foetal bovine serum following procedures previously described (Tully, 1983b).

Tests for biological and biochemical properties. The extremely fastidious nature of strain DW-1^T precluded adaptation in base broth media usually employed for the demonstration of glucose fermentation and hydrolysis of arginine and urea (Aluotto *et al.*, 1970). The filtration characteristics were measured in H-2 broth by procedures previously reported (Tully, 1983a).

Serological tests. Cell pellets of organisms were prepared by growing bulk cultures in 500 ml H-2 media. Homologous antiserum against strain DW-1^T was produced in rabbits as previously described (Williamson *et al.*, 1979). Hyperimmune antisera against all established *Spiroplasma* species, groups and sub-groups (Williamson *et al.*, 1998) were obtained from reference collections at the USDA Beltsville Agricultural Research Center, Beltsville, MD, USA and the National Institute of Allergy and Infectious Diseases laboratory, Frederick, MD, USA. These antisera and their respective antigens were tested against strain DW-1^T in reciprocal deformation and metabolism inhibition tests (Williamson *et al.*, 1978, 1979).

Genome analysis. The techniques for extraction and purification of chromosomal DNA from strain DW-1^T have been described previously (Carle *et al.*, 1983a). The DNA G+C composition of strain DW-1^T was determined by buoyant density and melting temperature following the procedures of Carle *et al.* (1983b). G+C determinations were performed first on pelleted uncloned organisms and

later on pelleted organisms of strain DW-1^T. The genome size of strain DW-1^T was determined by PFGE, employing techniques described earlier (Carle *et al.*, 1995). Purified DNA from *Spiroplasma citri* (genome size 1820 kbp; G+C content 26 ± 1 mol%) was used as a reference in all procedures (Carle *et al.*, 1995).

RESULTS AND DISCUSSION

Cultural and morphological properties

Strain DW-1^T grew to titres of 10^7 – 10^8 organisms ml⁻¹ in H-2 medium. All attempts to adapt strain DW-1^T in other media commonly employed for spiroplasma cultivation (Whitcomb, 1983) were unsuccessful: organisms failed to grow beyond the first 10-fold dilution when inoculated into M1D or SP-4 media or into basal broth (BSR) medium containing either 10% foetal bovine serum or 1% (v/v) bovine serum fraction. The doubling time for strain DW-1^T growing in H-2 medium was 15.8 h at 30 °C, its optimum temperature for growth. This is one of the longest doubling times for spiroplasmas in culture. Strain DW-1^T grew on solid medium on 1.8% Noble agar plates incubated aerobically with 5% CO₂ at 26 °C. Colonies were first evident 32 d after inoculation; fully developed colonies were small (60–70 µm in diameter) with dense centres and uneven edges (Fig. 1); no satellite colonies were observed.

Dark-field microscopy of broth cultures of strain DW-1^T showed motile helical filaments with four or more turns. Electron microscopy of thin sections of pellets of uncloned organisms from insect haemolymph (Williamson & Whitcomb, 1974) and of strain DW-1^T (Fig. 2) revealed the presence of filamentous cells 200–250 nm in diameter bounded by a single trilaminar membrane.

Sterol requirement

The amounts of protein contained in pellets of strain DW-1^T inoculated into H-2 medium supplemented with 15, 10 and 2% foetal bovine serum were 0.52, 0.54 and 0.22 mg per 100 ml, respectively. No protein production or colour change (see below) was observed in media containing 0% foetal bovine serum, and no spiroplasma cells were found in 50 fields examined under dark-field microscopy (5 µl culture sample). These results indicate that serum or sterol is required for growth of strain DW-1^T.

Biochemical and biological properties

Growth of strain DW-1^T in H-2 medium, which contains 0.025% (v/v) phenol red as a pH indicator, is accompanied by a colour shift from red-orange to yellow as the number of cells increases, indicating that acid is produced from one or more of the various substrates contained in the medium. A subsequent shift back to red-orange colour, as is observed in

Fig. 2. Electron micrograph of a sectioned and stained pellet of strain DW-1^T cells. The section was stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The arrow indicates the unit membrane. Bar, 200 nm.

cultures of strains that utilize arginine, has not been observed. Filtration of strain DW-1^T broth cultures with a cell titre of 1×10^7 colour-changing units (CCUs) ml⁻¹ through 450, 300 and 220 nm pore-size membrane filters resulted in cell titres of 10^7 , 10^6 and 10^6 CCUs ml⁻¹, respectively. There were no viable cells in the 100 nm pore-size membrane filtrate. The end point of the unfiltered dilution series was reached after 70 d culture.

Serological tests

The results of reciprocal spiroplasma deformation (DF) and metabolism inhibition (MI) tests of strain DW-1^T versus representatives of the currently recognized strains of the groups and sub-groups of spiroplasmas were published in a recent revision of the group classification of the genus *Spiroplasma* (Williamson *et al.*, 1998). Serological data pertaining to strain DW-1^T are presented in Table 1. In the DF test, when strain DW-1^T was used as an antigen, titres of 20–640 were obtained with the antisera to group I representatives; four sera to other spiroplasma groups cross-reacted at low levels. Serum directed against strain DW-1^T reacted with antigens from six of the group I representatives, with a maximum titre of 80, and with PUP-1^T (group XIX). The titre in the homologous reaction was 1280. In the MI test, when strain DW-1^T was used as antigen, titres of 162–39000 were observed against group I antisera. However, some reactivity was observed with almost all spiroplasma antisera. Outside group I, a titre of 1458 was

Table 1. Serological reactivities of the group II spiroplasma strain DW-1^T

Antigen and antisera of the strains were tested in all heterologous combinations against all known and putative groups and sub-groups. All cross-reactions were negative (—) except where shown. Values in the 'Antigen' columns are reciprocals of the end points when antiserum to DW-1^T was used against the designated antigen. Values in 'Antiserum' columns are reciprocals of end points when DW-1^T was used as antigen against heterologous antiserum.

Group	Strain	Deformation		Metabolism inhibition	
		Antigen	Antiserum	Antigen	Antiserum
I-1	Maroc R8A2 ^T	20	20	162	—
I-2	BC-3 ^T	160	—	486	54
I-3	E275 ^T	160	20	486	—
I-4	277F	160	80	4374	—
I-5	LB-12	160	—	39000	—
I-6	M55 ^T	320	20	4374	—
I-7	N525	320	40	39000	—
I-8	P40 ^T	640	20	1458	54
II	DW-1 ^T	1280*	1280*	> 117000*	> 117000*
III	23-6 ^T	—	—	18	—
IV	B31 ^T	20	—	54	—
VI	Y32 ^T	—	—	18	—
VII	MQ-1 ^T	—	—	54	—
VIII-1	EA-1 ^T	—	—	54	—
VIII-2	DF-1 ^T	—	—	1458	—
IX	CN-5 ^T	—	—	162	—
X	AES-1 ^T	—	—	486	—
XII	DU-1 ^T	—	—	18	—
XIII	Ar-1343 ^T	—	—	54	—
XV	I-25	—	—	54	—
XVI-1	CC-1 ^T	20	—	—	—
XVI-2	CB-1	—	—	54	—
XVI-3	Ar-1357	—	—	54	—
XVIII	TN-1 ^T	40	—	18	—
XIX	PUP-1 ^T	—	40	18	—
XX	LD-1 ^T	—	—	54	18
XXI	W115	—	—	18	—
XXII	CT-1 ^T	—	—	18	—
XXIII	TG-1 ^T	—	—	54	—
XXIV	CCH ^T	—	—	18	—
XXV	CUAS-1 ^T	20	—	18	—
XXVI	PLHS-1 ^T	—	—	—	54
XXIX	TIUS-1	—	—	18	—
XXX	BIUS-1	—	—	18	—
XXXI	HYOS-1 ^T	—	—	54	—
XXXII	TABS-2 ^T	—	—	18	—
XXXIII	TAUS-1 ^T	—	—	18	—
XXXIV	BARC 1901	—	—	486	—
Ungrouped	BARC 2649	—	—	1458	—

* Homologous titres of strain DW-1^T in the respective test system. The reaction, obtained only once, is listed in each column for completeness.

obtained when strain DW-1^T reacted with serum against strain DF-1^T (sub-group VIII-2; *Spiroplasma chrysopicola*) and with serum against ungrouped strain BARC 2649. When strain DW-1^T was represented as antiserum, growth of only two group I strains (I-2 and

I-8) was inhibited by antiserum directed against strain DW-1^T. The homologous titre in the MI test was > 117000. When the DF and MI tests are considered together, only one set of reactions, those with sub-group I-8 (*Spiroplasma phoeniceum*) showed complete

reciprocal cross-reactivity, and then only at low titre. This profile of serological cross-reactivity is considerably weaker than that observed among the sub-groups of group I (Whitcomb *et al.*, 1982), suggesting a more distant link between group I sub-groups and strain DW-1^T than among the group I sub-groups.

Genome size and DNA base composition

The base composition (G+C content) of strain DW-1^T was 26 ± 1 mol%. The same value was obtained from analysis of DNA from uncloned organisms. The undigested linear DNA of strain DW-1^T was 2040 kbp, as determined by PFGE.

Habitat and pathogenicity

Haemolymph is the normal habitat of the *D. willistoni* SR spiroplasma, where it grows to titres of approximately 10^{11} organisms μl^{-1} . The ability of strain DW-1^T to grow in haemolymph, to eliminate male progeny from injected females and to be vertically transmitted to the subsequent generation was tested (Williamson *et al.*, 1989) by injection of *D. pseudoobscura* females with a suspension of strain DW-1^T spiroplasmas at the sixteenth passage after triple cloning (39 total passages). In these studies, strain DW-1^T was capable of growing in the haemolymph and causing the elimination of male progeny, but had lost the ability to be vertically transmitted.

Properties described herein for strain DW-1^T fulfil the proposed criteria (International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes*, 1995) for species of the class *Mollicutes*, including the absence of a cell wall, filterability, the lack of reversion to walled bacteria when grown in antibiotic-free media, and penicillin resistance. The helicity and motility of strain DW-1^T place it in the order *Entomoplasmatales* and the family *Spiroplasmataceae* (Tully *et al.*, 1993). The results of the serological comparison of strain DW-1^T with other *Spiroplasma* species and groups indicate that strain DW-1^T shares antigens with group I sub-group strains. Most of the cross-reactions involving these antigens were much stronger when strain DW-1^T served as antigen. Strain DW-1^T, when used as antigen, also cross-reacted with antisera directed against representatives of many other spiroplasma groups. The property of extensive cross-reaction with many anti-spiroplasma sera has also been observed with *Spiroplasma lamproyridicola* (Stevens *et al.*, 1997), *Spiroplasma alleghenense* (Adams *et al.*, 1997) and, to a somewhat lesser degree, *Spiroplasma platyhelix* (Williamson *et al.*, 1997). Levels of cross-reaction greater than the intergroup reactions between groups I and II occur among the sub-groups of group I, but have not prevented the designation of separate species for sub-groups I-1 (*S. citri*; Saglio *et al.*, 1973); sub-group I-2 (*Spiroplasma melliferum*; Clark *et al.*, 1985); sub-group I-3 (*Spiroplasma kunkelii*; Whitcomb *et al.*,

1986); sub-group I-6 (*Spiroplasma insolitum*; Hackett *et al.*, 1993); and sub-group I-8 (*S. phoeniceum*; Saillard *et al.*, 1987). Therefore, minor serological cross-reactivity is not a barrier to separate species designation, even for spiroplasma sub-groups. In the case of the group I sub-groups, species designations were supported by DNA-DNA homology data. In the case of the group II strain DW-1^T, separation from the group I spiroplasmas is indicated by the 16S rRNA gene sequencing data (GenBank M24483) of Weisburg *et al.* (1989), which showed substantial sequence dissimilarity between group I and group II strains. This divergence has recently been recalculated (Hurst *et al.*, 1999) and found to be about 2.5%. Not only the molecular data, but biological data, including the extremely fastidious growth requirements, host restriction, male lethality and vertical transmissibility of uncloned organisms, indicate that the group II designation of strain DW-1^T should be preserved to reflect its unique status. As a result of the characterization studies described herein, the name *Spiroplasma poulsonii* is proposed for the group II representative, strain DW-1^T. The taxonomic description below summarizes the properties of this new species.

Description of *Spiroplasma poulsonii* sp. nov.

Spiroplasma poulsonii (poul.son'i.i. M.L. n. *poulsonii* of Poulson, named in memory of Donald F. Poulson, Professor of Biology at Yale University, in whose laboratory this spiroplasma was discovered and studied intensively).

Cells are motile helical filaments 200–250 nm in diameter. The membrane-bound cells lack cell walls. Cells plated on 1.8% Noble agar form small satellite-free colonies 60–70 μm in diameter that have dense centres and uneven edges. Chemo-organotroph. Acid is produced during growth in H-2 medium. The temperature range for growth is 26–30 °C, with optimum growth at 30 °C. The doubling time at the optimum temperature in H-2 medium is 15.8 h. Serologically distinct from other established *Spiroplasma* species. Isolated from the haemolymph of *Drosophila pseudoobscura* females infected by haemolymph transfer of the Barbados-3 strain of *Drosophila willistoni* SR organism. Pathogenicity (lethality to male progeny) confirmed by injection into *Drosophila pseudoobscura*. Vertical transmissibility lost after cultivation and cloning. The G+C content of the DNA is 26 ± 1 mol%, as determined by buoyant density and melting point methods. The genome size is 2040 kbp, as determined by PFGE. The GenBank accession number for the 16S rRNA gene sequence is M24483. The type strain is DW-1^T (=ATCC 43153^T).

REFERENCES

- Adams, J. R., Whitcomb, R. F., Tully, J. G. & 7 other authors (1997). *Spiroplasma alleghenense* sp. nov., a new species from the scorpion fly *Panorpa helena* (Mecoptera: Panorpidae). *Int J Syst Bacteriol* **47**, 759–762.

- Aluotto, B. B., Wittler, R. G., Williams, C. O. & Faber, J. E. (1970). Standardized bacteriologic techniques for characterization of *Mycoplasma* species. *Int J Syst Bacteriol* **20**, 35–58.
- Carle, P., Saillard, C. & Bové, J. M. (1983a). DNA extraction and purification. *Methods Mycoplasmol* **1**, 295–299.
- Carle, P., Saillard, C. & Bové, J. M. (1983b). Determination of guanine plus cytosine content of DNA. *Methods Mycoplasmol* **1**, 301–308.
- Carle, P., Laigret, F., Tully, J. G. & Bové, J. M. (1995). Heterogeneity of genome sizes within the genus *Spiroplasma*. *Int J Syst Bacteriol* **45**, 178–181.
- Chen, T. A. & Liao, C. H. (1975). Corn stunt spiroplasma: isolation, cultivation and proof of pathogenicity. *Science* **188**, 1015–1017.
- Clark, T. B., Whitcomb, R. F., Tully, J. G. & 8 other authors (1985). *Spiroplasma melliferum*, a new species from the honeybee (*Apis mellifera*). *Int J Syst Bacteriol* **35**, 296–308.
- Cole, R. M., Tully, J. G., Popkin, T. J. & Bové, J. M. (1973). Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasma citri* gen. nov., sp. nov.) cultured from 'stubborn' disease of citrus. *J Bacteriol* **115**, 367–386.
- Davis, R. E., Whitcomb, R. F., Chen, T. A. & Granados, R. R. (1972). Current status of the aetiology of corn stunt disease. In *Pathogenic Mycoplasmas* (Ciba Foundation Symposium), pp. 205–225. Edited by K. Elliott & J. Birch. Amsterdam: Associated Scientific Publishers.
- Hackett, K. J. & Lynn, D. E. (1985). Cell-assisted growth of a fastidious spiroplasma. *Science* **230**, 825–827.
- Hackett, K. J. & Lynn, D. E. (1995). Insect cell culture approaches in cultivating spiroplasmas. In *Molecular and Diagnostic Procedures in Mycoplasma*, vol. 1, pp. 55–64. Edited by S. Razin & J. G. Tully. San Diego, CA: Academic Press.
- Hackett, K. J., Lynn, D. E., Williamson, D. L., Ginsberg, A. & Whitcomb, R. F. (1986). Cultivation of the *Drosophila* sex ratio spiroplasma. *Science* **232**, 1253–1255.
- Hackett, K. J., Whitcomb, R. F., Tully, J. G. & 9 other authors (1993). *Spiroplasma insolitum* sp. nov., a new species of group I spiroplasma with an unusual DNA base composition. *Int J Syst Bacteriol* **43**, 272–277.
- Hurst, G. D. D., Schulenberg, J. H. G., Majerus, T. M. O., Bertrand, D., Zakharov, I. A., Baungaard, J., Volkl, W., Stouthamer, R. & Majerus, M. E. N. (1999). Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Mol Biol* **8**, 133–140.
- International Committee on Systematic Bacteriology Subcommittee on the taxonomy of *Mollicutes* (1995). Revised minimum standards for descriptions of new species of the class *Mollicutes* (Division *Tenericutes*). *Int J Syst Bacteriol* **45**, 605–612.
- Konai, M., Clark, E. A., Camp, M., Koch, A. L. & Whitcomb, R. F. (1996). Temperature ranges, growth optima, and growth rates of *Spiroplasma* (*Spiroplasmataceae*, class *Mollicutes*) species. *Curr Microbiol* **32**, 314–319.
- Malogolowkin, C. & Poulson, D. F. (1957). Infective transfer of maternally inherited abnormal sex-ratio in *Drosophila willistoni*. *Science* **126**, 32.
- Malogolowkin, C., Poulson, D. F. & Wright, E. Y. (1959). Experimental transfer of maternally inherited abnormal sex-ratio in *Drosophila willistoni*. *Genetics* **44**, 59–74.
- Poulson, D. F. (1963). Cytoplasmic inheritance and hereditary infections in *Drosophila*. In *Methodology in Basic Genetics*, Appendix III, pp. 404–424. Edited by W. J. Burdette. San Francisco, CA: Holden Day.
- Poulson, D. F. & Sakaguchi, B. (1961). Nature of "sex-ratio" agent in *Drosophila*. *Science* **133**, 1489–1490.
- Rochford, R., Dougherty, E. M. & Lynn, D. E. (1984). Establishment of a cell line from embryos of the cabbage looper, *Trichoplusia ni* (Hubner). *In Vitro* **20**, 823–825.
- Saglio, P., L'Hospital, M., Lafliche, D., Dupont, G., Bové, J. M., Tully, J. G. & Freundt, E. A. (1973). *Spiroplasma citri* gen. and sp. n.: a mycoplasma-like organism associated with "stubborn" disease of citrus. *Int J Syst Bacteriol* **23**, 191–204.
- Saillard, C., Vignault, J. C., Bové, J. M. & 8 other authors (1987). *Spiroplasma phoeniceum* sp. nov., a new plant-pathogenic species from Syria. *Int J Syst Bacteriol* **37**, 106–115.
- Sakaguchi, B. & Poulson, D. F. (1963). Interspecific transfer of the "sex-ratio" condition from *Drosophila willistoni* to *D. melanogaster*. *Genetics* **48**, 841–861.
- Stevens, C., Tang, A. Y., Jenkins, E. & 12 other authors (1997). *Spiroplasma lampyridicola* sp. nov., from the firefly beetle *Photuris pennsylvanicus*. *Int J Syst Bacteriol* **47**, 709–712.
- Tully, J. G. (1983a). Cloning and filtration techniques for mycoplasmas. *Methods Mycoplasmol* **1**, 173–177.
- Tully, J. G. (1983b). Tests for digitonin sensitivity and sterol requirement. *Methods Mycoplasmol* **1**, 355–362.
- Tully, J. G., Bové, J. M., Laigret, F. & Whitcomb, R. F. (1993). Revised taxonomy of the class *Mollicutes*: proposed elevation of a monophyletic cluster of arthropod-associated mollicutes to ordinal rank (*Entomoplasmatales* ord. nov.), with provision for familial rank to separate species with nonhelical morphology (*Entomoplasmataceae* fam. nov.) from helical species (*Spiroplasmataceae*), and emended descriptions of the order *Mycoplasmatales*, family *Mycoplasmataceae*. *Int J Syst Bacteriol* **43**, 378–385.
- Weisburg, W. G., Tully, J. G., Rose, D. L. & 9 other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.
- Whitcomb, R. F. (1983). Culture media for spiroplasmas. *Methods Mycoplasmol* **1**, 147–158.
- Whitcomb, R. F. & Hackett, K. J. (1986). Cloning by limiting dilution in liquid media: an improved alternative for cloning mollicute species. *Program and Abstracts of the 6th International Congress of the International Organization for Mycoplasma*, Birmingham, AL, 26–31 August 1986, p. 167.
- Whitcomb, R. F., Tully, J. G., Clark, T. B., Williamson, D. L. & Bové, J. M. (1982). Revised serological classification of spiroplasmas, new provisional groups, and recommendations for serotyping isolates. *Curr Microbiol* **7**, 291–296.
- Whitcomb, R. F., Chen, T. A., Williamson, D. L. & 7 other authors (1986). *Spiroplasma kunkelii*, sp. nov.: characterization of the etiological agent of corn stunt disease. *Int J Syst Bacteriol* **36**, 170–178.
- Williamson, D. L. (1969). The sex ratio spirochete in *Drosophila robusta*. *Jpn J Genet* **44**, Suppl. 1, 36–41.
- Williamson, D. L. (1983). Specialized electron microscopic techniques for spiroplasmas in plant and insect tissues. *Methods Mycoplasmol* **1**, 71–76.
- Williamson, D. L. & Poulson, D. F. (1979). Sex ratio organisms (spiroplasmas) of *Drosophila*. In *The Mycoplasmas*, vol. 3, pp. 175–208. Edited by R. F. Whitcomb & J. G. Tully. New York: Academic Press.
- Williamson, D. L. & Whitcomb, R. F. (1974). Helical, wall-free

prokaryotes in *Drosophila*, leafhoppers and plants. *Colloq INSERM* **33**, 283–290.

Williamson, D. L. & Whitcomb, R. F. (1975). Plant mycoplasmas: a cultivable spiroplasma causes corn stunt disease. *Science* **188**, 1018–1020.

Williamson, D. L., Whitcomb, R. F. & Tully, J. G. (1978). The spiroplasma deformation test, a new serological method. *Curr Microbiol* **1**, 203–207.

Williamson, D. L., Tully, J. G. & Whitcomb, R. F. (1979). Serological relationships of spiroplasmas as shown by combined deformation and metabolism inhibition tests. *Int J Syst Bacteriol* **29**, 345–351.

Williamson, D. L., Steiner, T. & McGarrity, G. J. (1983). Spiro-

plasma taxonomy and identification of the sex ratio organisms: can they be cultivated? *Yale J Biol Med* **56**, 583–592.

Williamson, D. L., Hackett, K. J., Wagner, A. J. & Cohen, A. J. (1989). Pathogenicity of cultivated *Drosophila willistoni* spiroplasmas. *Curr Microbiol* **19**, 53–56.

Williamson, D. L., Adams, J. R., Whitcomb, R. F., Tully, J. G., Carle, P., Konai, M., Bové, J. M. & Henegar, R. B. (1997). *Spiroplasma platyhelix* sp. nov., a new mollicute with unusual morphology and genome size from the dragonfly *Pachydiplax longipennis*. *Int J Syst Bacteriol* **47**, 763–766.

Williamson, D. L., Whitcomb, R. F., Tully, J. G. & 10 other authors (1998). Revised group classification of the genus *Spiroplasma*. *Int J Syst Bacteriol* **48**, 1–12.