Spiroplasma atrichopogonis sp. nov., from a ceratopogonid biting midge

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Spiroplasma sp. strain GNAT3597^T was isolated from the biting midge genus *Atrichopogon* (Diptera: Ceratopogonidae). It was serologically distinct from other *Spiroplasma* species, groups or subgroups. Dark-field microscopy of the cells revealed the classical helical shape and subsequent transmission electron microscopy revealed cells surrounded by only a cell membrane (i.e. lacking a cell wall). Growth of strain GNAT3597^T occurred in M1D medium at 30 °C. Strain GNAT3597^T catabolized both glucose and arginine, but did not hydrolyse urea. The DNA G + C content of strain GNAT3597^T was 29 ± 1 mol%. Only one strain, SMCA^T (*Spiroplasma mirum*), is serologically related to strain GNAT3597^T, although the relationship is weak (positive reaction to only a 1 : 80 dilution). It is therefore proposed that strain GNAT3597^T (= ATCC BAA-520^T = NBRC 100390^T) represents a novel species, *Spiroplasma atrichopogonis* sp. nov. (class *Mollicutes*: order *Entomoplasmatales*: family *Spiroplasmataceae*).

INTRODUCTION

Spiroplasmas are host-specific microbes of the class *Mollicutes* (Tully *et al.*, 1993, 1987; Williamson *et al.*, 1989). They most commonly reside in the lumen of the gut of insects as commensal organisms, but can also invade into the haemocoel and/or move to different organs (e.g. salivary glands and ovaries) (Hackett & Clark, 1989). A few spiroplasmas are vectored to the sieve tubes of plants by their insect hosts causing detrimental effects to the plant's health (e.g. citrus stubborn disease and corn stunt disease). Since the first spiroplasma was cultivated in 1973 (Saglio *et al.*, 1973), more than 1000 spiroplasma isolates have been obtained (Hackett, 1990; Hackett & Clark, 1989; French *et al.*, 1990). Of these isolates, several have been shown to be pathogens to their insect hosts, whereas others are commensals (Hackett & Clark, 1989; Clark, 1982; Clark *et al.*, 1984). In the order Diptera, spiroplasmas have been isolated from fruit flies (Drosophilidae) (Williamson *et al.*, 1999), several genera of mosquitoes (Culicidae) (Chastel & Humphery-Smith, 1991), a hover fly (Syrphidae) (Whitcomb *et al.*, 1996), and from several genera of deer and horse flies (Tabanidae) (Whitcomb *et al.*, 1997). The success of isolations from dipteran hosts led to the investigation of biting and predaceous midges (*Ceratopogonidae*) as spiroplasma hosts.

Strain GNAT3597^T was isolated from biting midges from Salisbury, MD (Wicomico County), USA. The insects were collected by light trap or by sweeping insect nets on flowers and other vegetation. The isolation and initial cultivation efforts have been described previously (Frana *et al.*, 2001).

Several characteristics are common amongst all spiroplasmas, including the classic spiral/helical shape, lack of a cell wall, glucose fermentation and a growth temperature requirement of about 30 °C (Tully & Whitcomb, 1991). Classification of spiroplasmas provides useful information concerning species relationships. Information obtained from the characterization tests can then be added to a growing database of all species in the genus *Spiroplasma*.

The classification of spiroplasmas requires several morphological, biochemical and genetic tests as prescribed by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (1995). Cultivation of spiroplasmas can be achieved in various media, with the best growth typically occurring in M1D medium (Whitcomb, 1983). Identification methods for spiroplasmas include simple microscopy, usually under a dark-field microscope to show the classical helical shape, and determination of the lack of a cell wall by transmission electron microscopy. Biochemical, serological and genetic tests (G + C content analysis) are also components of the classification of spiroplasmas.

In this report, the results of taxonomic studies of strain GNAT3597^T that satisfy the requirements for descriptions of species in the class *Mollicutes* (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995) are reported. Results support the designation of strain GNAT3597^T (= ATCC BAA-520^T = NBRC 100390^T) as the type strain of a novel species, *Spiroplasma atrichopogonis* sp. nov.

METHODS

Isolation and cultivation. Spiroplasmas were isolated from a pooled sample of two species of biting midges, *Atrichopogon levis* and *Atrichopogon geminus*, and initially cultured in R2 medium and incubated at 30 °C (Frana *et al.*, 2001). The culture was subsequently triple-cloned in M1D medium using filtration cloning procedures as described previously (Tully, 1983a) and designated strain GNAT3597^T. Representative strains of previously recognized groups and subgroups, including the type strains of previously recognized species (Williamson *et al.*, 1998), were cultivated for comparative purposes. All spiroplasmas were grown at 30 °C.

Morphological studies. Cells from exponential cultures of strain GNAT3597^T were examined by dark-field microscopy (magnification $\times 1000$) and by the physical colour change of the medium (from red to yellow). For electron microscopy, 20 ml exponential cultures of strain GNAT3597^T were pelleted and fixed for 2 h in 3% glutaraldehyde, post-fixed by a 1% osmium tetroxide treatment for 3 h, and then dehydrated in acetone and embedded in Spurr's Embedding Medium (Williamson, 1983). Sections were stained with 2% aqueous uranyl acetate and Reynolds' lead citrate prior to visualization.

Deformation test. Strain GNAT3597^T was tested serologically against the hyperimmune antisera of 40 different species of spiroplasmas that represent established type strains (Williamson *et al.*, 1998) using the deformation test as described previously (Williamson *et al.*, 1978). Positive reactions were confirmed by the presence of grape-like clusters on the spiroplasma, indicative of a reaction between the cell membrane and the antisera. The presence of normal, helical/spiral-shaped spiroplasmas indicated no reaction to the antiserum (Williamson *et al.*, 1978). *Spiroplasma syphidicola* (strain EA-1^T) and *Spiroplasma citri* (strain R8A2^T) were also tested against their own antiserum as controls. This procedure was performed twice. Antiserum to strain GNAT3597^T was produced in rabbits as described previously (Williamson *et al.*, 1978). This antiserum was then used in reciprocal deformation tests against strains whose antisera had a positive reaction against strain GNAT3597^T.

Metabolism inhibition test. Strain GNAT3597^T was serologically tested against the hyperimmune antisera of the most closely related insect species (Williamson & Whitcomb, 1983). Colour change, or lack of, was noted to determine if growth was established in each of the microtitre plate wells. This procedure was performed twice.

Biochemical tests. Strain GNAT3597^T was weaned from M1D medium onto a minimal medium [serum fraction (SF) broth supplemented with 1% bovine serum fraction (Tully, 1983b)] by sequential passage from 3:1 (M1D:SF) to 1:1 to 1:3 and finally into SF plus 10% glucose. After weaning, strain GNAT3597^T was used to inoculate five tubes for biochemical testing as follows: tube 1, SF; tube 2, SF and 10% glucose; tube 3, SF, 10% glucose and 21% arginine; tube 4, SF and 21% arginine; and tube 5, SF and 1% urea (Aluotto *et al.*, 1970). Dark-field microscopy, as well as colour change, was used to identify growth of the cultures. For the arginine samples, the colour change was from red to yellow and then back to red. Multiple tests were performed to ensure results and repeatability.

G+C content analysis. Strain GNAT3597^T was grown in a large culture (100 ml) and DNA was extracted as described previously (Gasparich *et al.*, 1993). The DNA was diluted in $1 \times$ SSC buffer to give a final sodium concentration of 0.195 M. This sample was used (with a $1 \times$ SSC buffer blank) in a Cary Varian Thermal Spectrophotometer equipped with software to determine the melting temperature and the G+C content was determined (Carle *et al.*, 1983). This process was repeated several times; *Escherichia coli* DNA in $1 \times$ SSC buffer was used as a control to verify the results.

RESULTS AND DISCUSSION

Characterization of *Spiroplasma* sp. strain GNAT3597^T was performed using the designated characterization tests as determined by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (1995).

Cultural and morphological properties

Strain GNAT3597^T grew well in M1D medium containing 500 U penicillin ml⁻¹. Exponential-phase cultures of strain GNAT3597^T in M1D medium examined by dark-field microscopy revealed helical, motile filaments with four to five turns per cell. Transmission electron microscope analysis revealed that cells were filamentous with no evidence of a cell wall (Fig. 1). Cells were about 150 nm in diameter and were surrounded by a single cytoplasmic membrane.

Serological tests

Metabolism inhibition and deformation tests indicated that strain GNAT3597^T was unrelated serologically to representatives of previously established *Spiroplasma* groups or subgroups. There were no detectable serological reactions of strain GNAT3597^T with any of the type strain antisera in the metabolism inhibition assays. The serological reactions of strain GNAT3597^T in the deformation test against strains isolated from related host organisms are summarized in Table 1. There was a slight reaction with strain SMCA^T up to 160-fold dilution. A reciprocal deformation test using strain GNAT3597^T antiserum against the seven strains indicated in Table 1 gave no detectable reaction at 20-fold dilution. Homologous titres for strains R8A2^T, TAAS-1 and SMCA^T (controls) were all 2560, which is what has been

Fig. 1. Transmission electron micrograph of cells of strain GNAT3597^T (arrows indicate cell membrane). Bar, 0.1 μ m.

Table 1. Serological reactions of strain GNAT3597^T in the deformation test (DF) against the seven most closely related species

Antisera <i>Spiroplasma</i> group and strain	<i>Spiroplasma</i> strain host organism (order: family)	Titre in DF test
V, SMCA ^T	Parasitiformes: Ixodidae	160
VIII-2, DF-1 ^T	Diptera: Tabanidae	<20
VIII-3, TAAS-1	Diptera: Tabanidae	<20
X, AES-1 ^T	Diptera: Culicidae	<20
XVI-3, Ar-1357	Diptera: Culicidae	<20
XXIII, TG-1 ^T	Diptera: Tabanidae	<20
XXXII, TABS-2 ^T	Diptera: Tabanidae	<20

reported previously (Hackett *et al.*, 1996). The homologous titre for strain GNAT3597^T was also 2560.

Biochemical and biological properties and G+C content

After subsequent weanings into SF and glucose, strain GNAT3597^T was able to grow in media containing glucose, glucose plus arginine and arginine alone, indicating that it is able to ferment glucose with the production of acid and catabolize arginine. No growth was observed in SF plus urea, indicating that urea is not hydrolysed. Strain GNAT3597^T passed readily through membrane filters with pore sizes of 450 and 220 nm. A 100 nm pore size membrane filtrate was free of viable cells.

The base composition (G + C content) of the DNA of strain GNAT3597^T was 28.79, 28.90 and 28.79 mol% (average 28.8 ± 1 mol%).

Description of *Spiroplasma atrichopogonis* sp. nov.

Spiroplasma atrichopogonis [a.tri.cho.po.go'nis. N.L. gen. n. *atrachopogonis* of *Atrichopogon*, systematic genus name of a biting midge (Diptera: Ceratopogonidae)].

Cells are filamentous, helical and motile and about 150 nm in diameter. They freely pass through filters with 450 and 220 nm pores, but do not pass through filters with 100 nm pores. The cells lack true cell walls. Chemo-organotrophic. Acid is produced from glucose and arginine is catabolized. Does not utilize urea. Serologically distinct from previously established *Spiroplasma* species, groups and subgroups.

The type strain is GNAT3597^T (=ATCC BAA-520^T = NBRC 100390^T), isolated from a pooled sample of two nearly identical species of biting midges (*Atrichopogon geminus* and *Atrichopogon levis*); the entire insects were ground up in media prior to incubation. Pathogenicity for these flies is not known. The G + C content of the DNA of the type strain is 28.8 ± 1 mol%, as determined by the melting-temperature method.

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