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

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**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 & Humphrey-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 GNAT3597T 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 GNAT3597T 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 GNAT3597T (= ATCC BAA-520T = NBRC 100390T) 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 GNAT3597T. 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 GNAT3597T were examined by dark-field microscopy (magnification ×1000) and by the physical colour change of the medium (from red to yellow). For electron microscopy, 20 ml exponential cultures of strain GNAT3597T 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 GNAT3597T 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-1T) and *Spiroplasma citri* (strain R8A2T) were also tested against their own antisera as controls. This procedure was performed twice. Antiserum to strain GNAT3597T was produced in rabbits as described previously (Williamson et al., 1978). This antisera was then used in reciprocal deformation tests against strains whose antisera had a positive reaction against strain GNAT3597T.

Metabolism inhibition test. Strain GNAT3597T was serologically tested against the hyperimmune antiserum 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 GNAT3597T 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 GNAT3597T 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 (Aliotti 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 GNAT3597T 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× SSC buffer to give a final sodium concentration of 0.195 M. This sample was used (with a 1× 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× SSC buffer was used as a control to verify the results.

RESULTS AND DISCUSSION

Characterization of *Spiroplasma* sp. strain GNAT3597T 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 GNAT3597T grew well in M1D medium containing 500 U penicillin ml⁻¹. Exponential-phase cultures of strain GNAT3597T 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 GNAT3597T was unrelated serologically to representatives of previously established *Spiroplasma* groups or subgroups. There were no detectable serological reactions of strain GNAT3597T with any of the type strain antisera in the metabolism inhibition assays. The serological reactions of strain GNAT3597T 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 GNAT3597T antisera against the seven strains indicated in Table 1 gave no detectable reaction at 20-fold dilution. Homologous titres for strains R8A2T, TAAS-1 and SMCA T (controls) were all 2560, which is what has been
Table 1. Serological reactions of strain GNAT3597T in the deformation test (DF) against the seven most closely related species.

<table>
<thead>
<tr>
<th>Antisera group and strain</th>
<th>Spiroplasma strain host organism (order: family)</th>
<th>Titre in DF test</th>
</tr>
</thead>
<tbody>
<tr>
<td>V, SMCA(^T)</td>
<td>Parasitiformes: Isodidae</td>
<td>160</td>
</tr>
<tr>
<td>VIII-2, DF-1(^T)</td>
<td>Diptera: Tabanidae</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>VIII-3, TAAS-1</td>
<td>Diptera: Tabanidae</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>X, AES-1(^T)</td>
<td>Diptera: Culicidae</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>XVI-3, Ar-1357</td>
<td>Diptera: Culicidae</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>XXIII, TG-1(^T)</td>
<td>Diptera: Tabanidae</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>XXXII, TABS-2(^T)</td>
<td>Diptera: Tabanidae</td>
<td>&lt; 20</td>
</tr>
</tbody>
</table>

ACKNOWLEDGEMENTS

This work was supported in part by the NSF REU grant number 9732442 and by the Towson University College of Science and Mathematics Committee on Undergraduate Research. We also gratefully acknowledge support from the Henson School of Science & Technology and the Department of Biological Sciences at Salisbury University.

REFERENCES


referred previously (Hackett et al., 1996). The homologous titre for strain GNAT3597T was also 2560.

Biochemical and biological properties and G+C content

After subsequent weanings into SF and glucose, strain GNAT3597T 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 GNAT3597T 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 GNAT3597T 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. atrichopogonis 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 GNAT3597T (=ATCC BAA-520T = NBRC 100390T), 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.


