Spiroplasma litorale sp. nov., from Tabanid Flies (Tabanidae: Diptera) in the Southeastern United States

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Spiroplasma strain TN-1T (= type strain), a strain serologically distinct from other spiroplasma species, groups, and subgroups, was isolated from the gut of a horsefly (Tabanus nigrovittatus) from a barrier island off the coast of North Carolina. Related strains were isolated from other Tabanus spp., T. atratus, T. americanus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor, from coastal Georgia. Cells of strain TN-1T in culture were helical and motile with an average of 5 to 10 helical turns per cell. Electron microscopic studies determined that the cells of strain TN-1T were surrounded by a single cytoplasmic membrane, and there was no evidence of a cell wall. The spiroplasma grew well in MID and SP-4 liquid media. Serum fraction (1%) medium and conventional horse serum medium also supported growth of strain TN-1T. Fried-egg colonies were not produced; instead, the strain produced small diffuse colonies that were surrounded by satellite growth. The optimum temperature for growth was 32°C, but multiplication was observed at temperatures from 10 to 41°C. The doubling time at the optimum temperature (32°C) was 1.6 h. No growth was observed at 5 or 43°C. Spiroplasma strain TN-1T passed through 220-nm filter pores but failed to pass through 100-nm filter pores. Strain TN-1T catabolized glucose but hydrolyzed neither arginine nor urea. The guanine-plus-cytosine content of the DNA was about 25 ± 1 mol%, and the genome size was 1,370 kbp. Based on results from this study and previously published data, strain TN-1T (= ATCC 43211) (group XVIII) is designated the type strain of a new spiroplasma species, Spiroplasma litorale.

In 1983, one of us (R.F.W.), while visiting North Carolina’s Ocracoke Island, observed that the booth of a campground attendant was besieged, as was the entire island, with innumerable individuals of a green-eyed horsefly, Tabanus nigrovittatus (4). The attendant graciously permitted us to collect a sample of flies, from which spiroplasma strain TN-1T (T = type strain) was derived. Isolates which have similar serological profiles or which exhibit partial serological cross-reactivity with strain TN-1T were subsequently isolated from other Tabanus spp., T. atratus, T. americanus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor collected from a barrier island off the coast of Georgia or from Bulloch County in Georgia’s coastal plain (6, 7, 22, 23). Strain TN-1T has been designated the representative strain of spiroplasma group XVIII (19). In this paper we describe the morphological, physiological, and biochemical properties of strain TN-1T (= ATCC 43211) and designate this strain the representative of a new spiroplasma species, the group XVIII species Spiroplasma litorale.

MATERIALS AND METHODS

Spiroplasma strains. Strain TN-1T was isolated by standard techniques (3, 4, 12) by one of us (T.B.C.) in MID medium from the gut of a female green-eyed horsefly (T. nigrovittatus) collected at Ocracoke, N.C. Serologically related isolates were also obtained from the guts of six other tabanid fly species, T. americanus, T. atratus, T. gladiator, T. lineola, T. sulcifrons, and T. zythicolor (6, 7, 23). Some of the genomic and serological features of the organism have been reported previously (2, 19). Representative strains of all previously recognized groups (16, 17, 19, 25, 26) and subgroups, including the type strains of all previously recognized species, were also employed.

Culture medium and cultivation techniques. Spiroplasma strain TN-1T was grown in MID liquid broth (20) at 30°C. The culture was filtered and cloned (14) in MID broth containing 500 U of penicillin per ml. A triply cloned strain was designated TN-1T and was subsequently used in characterization studies. Other culture media used in the study were SP-4, conventional 20% horse serum medium, and a 1% bovine serum fraction broth (20). Solid medium was prepared by adding 2.25% Noble agar (Difco Laboratories, Detroit, Mich.). Agar cultures were incubated at 30°C either aerobically or anaerobically in a hydrogen GasPak system (BBL Microbiology Systems, Cockeysville, Md.).

Temperature studies. The temperature requirements of cultures of strain TN-1T in the late logarithmic phase in MID broth were determined as previously described (10, 11). Growth was measured at temperatures of 5, 10, 15, 20, 25, 30, 32, 37, 41, and 43°C.

Filtration. Filtration characteristics were determined in MID broth by passing logarithmic-phase cultures through filters with 450-, 300-, 220-, and 100-nm pores. Each filtrate, as well as an unfiltered control, was subcultured in a series of 12 tubes by using 10-fold dilutions and was monitored for growth (14).

Morphological studies. Cells of strain TN-1T in MID broth cultures in the logarithmic phase were examined by dark-field microscopy by using a magnification of ×1,250. Electron microscopic examination of the membrane structure of strain TN-1T by standard methods (27) has been described previously (19).

Sterol requirements. Strain TN-1T was tested for a sterol requirement by two methods: the growth obtained in a serum-free broth formulation containing various concentrations of cholesterol was assessed, and the ability of the strain to show sustained growth in serum-free or serum-containing broth media (13, 15) was determined.

Biochemical and physiological properties. The ability of strain TN-1T to utilize glucose, arginine, and urea was assessed by previously described methods (1, 30). Serological tests. Antiserum to strain TN-1T was raised in rabbits as previously described (29). Hyperimmune antiserum to all previously established groups, putative groups, and subgroups (16, 17, 19, 25, 26) of Spiroplasma species were taken from reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, Md. These antisera and spiroplasma TN-1T were tested reciprocally in both metabolism inhibition (28) and spiroplasma deformation tests (28, 29).
was included in the serum-free broth. In experiments to test TN-lT grew well in M1D and SP-4 broth media containing 500 organism did not replicate at 5°C, it was able to retain its supplemented with 2.25% Noble agar grown under aerobic con-
ditions were granular with dense centers, uneven margins, and
multiple satellites (Fig. 1). Logarithmic-phase cultures of strain TN-1 in M1D and SP-4 media examined by dark-field micros-
omy revealed filamentous cells with no evidence of a
copy contained numerous helical motile filaments. Electron
microscopy revealed filamentous cells with no evidence of a
cell wall (19). Representative cells were about 200 to 300 nm in
diameter and were surrounded by a single cytoplasmic mem-
brane.
Passage of broth cultures of strain TN-1 through 450- and
300-nm-pore-size membrane filters did not reduce the viable
cell titer of a nonfiltered control broth (10^9 color-changing
units/ml). The titer of a filtrate obtained after passage through a
220-nm-pore-size membrane filter was reduced about 100-
fold (to 10^7 color-changing units/ml); a 220-nm-pore-size membrane filter was reduced about 100-
appearance in control base broth devoid of any supplement, but
no evidence of arginine or urea hydrolysis was observed.
Serological tests. Metabolism inhibition and deformation
tests performed with antisera prepared against known and
putative Spiroplasma groups and subgroups indicated that strain TN-1 was not related serologically to representatives of
previously established groups or species or to any of the eight
representatives of putative new groups tested. No reciprocal
crosses were observed in either test. One-way crosses were
observed with 11 groups or putative groups when antisera
against strain TN-1 was reacted with heterologous antigen
(Table 2). When strain TN-1 antigen was tested against heterologous sera, a low-level one-way cross-reaction was ob-
served with strain PALS-1 (an ungrouped representative of a
putative new group) in deformation tests. No other cross-
reactions were observed.
Genomic analysis. The guanine-plus-cytosine content of purified DNA of
strain TN-1 and the genome size of the organism have been reported previously
(2, 17, 19, 25).

RESULTS AND DISCUSSION

Cultural and morphological properties. Spiroplasma strain TN-1 grew well in M1D and SP-4 broth media containing 500
U of penicillin per ml. The strain also grew in conventional
mycoplasma media containing horse serum or bovine serum
fraction. Growth occurred over a temperature range of 10 to
41°C; optimum growth was observed at 32°C. The doubling
time at the optimum temperature was 1.6 h. Although the
organism did not replicate at 5°C, it was able to retain its
helical morphology and remained viable at this temperature.
Clark and colleagues (4) reported an optimum temperature of
35°C for strain TN-1 and noted that this organism grew at
37°C, but a complete range of temperatures was not tested.
Colonies of spiroplasma strain TN-1 on SP-4 medium sup-
plemented with 2.25% Noble agar grown under aerobic con-
ditions were granular with dense centers, uneven margins, and
multiple satellites (Fig. 1). Logarithmic-phase cultures of strain
TN-1 in M1D and SP-4 media examined by dark-field micros-
copy contained numerous helical motile filaments. Electron
microscopy revealed filamentous cells with no evidence of a
cell wall (19). Representative cells were about 200 to 300 nm in
diameter and were surrounded by a single cytoplasmic mem-
brane.

Habitat. The genus Spiroplasma offers a rich opportunity to
study the ecology of microorganisms associated with insects (3,
8). Strain TN-1 described here was isolated from the gut of the
horsefly T. nigrovittatus from a barrier island, Ocracoke
Island, N.C. Strain TN-1 has not been studied as an insect
pathogen. Hundreds of spiroplasma cultures have been ob-
tained from tabanids collected on the coastal plains from Flor-
da to Nova Scotia and in the Rocky Mountains from Montana
to New Mexico. Other sites of isolation include Vermont and
Texas. The most extensive survey was performed from 1987 to
1994 in Bulloch County, Ga., some 80 km from the Atlantic
Ocean. Cultures closely related serologically to strain TN-1
were isolated from six other Tabanus species (4–7, 22, 23). Four
isolates were obtained from T. americanus from Bulloch
County, Ga., and Johnston County, N.C.; eight cultures were
obtained from T. gladiator from Bulloch County, Ga.; nine
isolates were obtained from T. lineola from Bulloch and Cam-
den Counties, Ga.; one isolate was obtained from T. sulcifrons
from Bulloch County, Ga.; and one isolate was obtained from
T. zythicolor from Camden County, Ga. A closely related

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cholesterol concn (µg/ml)</th>
<th>Amt of protein (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1D</td>
<td>0 (Control)</td>
<td>3.06¹</td>
</tr>
<tr>
<td>Serum-free base medium with 1% serum fraction</td>
<td>0 (Control)</td>
<td>2.28</td>
</tr>
<tr>
<td>Serum-free base medium alone</td>
<td>0</td>
<td>0.06</td>
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<tr>
<td>Serum-free base medium with cholesterol</td>
<td>1</td>
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<tr>
<td></td>
<td>2</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.21</td>
</tr>
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</table>

¹ Amount of total cell protein obtained from a cell pellet cultivated from 100 ml of each serum control medium or serum-free base medium supplemented with cholesterol.
strain, TAAS-2, was isolated from Tabanus atratus from Cumberland Island, Camden County, Ga. A large number of isolates were obtained from T. lineola collected in Florida, Georgia, North Carolina, and Maryland. Overall, the spiroplasma infection rate of these flies was about 50% (7). However, a low prevalence of isolates related to TN-1T was indicated by the fact that only 3.9% (10 of 256) of the isolates obtained from T. lineola reacted to TN-1T antiserum. Although other spiroplasmas were isolated from T. lineola collected during May and June, isolates related to strain TN-1T were obtained from flies collected from July to November, thus indicating possible seasonality in carriage by tabanids. Many tabanids belonging to the genera Chrysops and Hybomitra were collected with the Tabanus hosts of TN-1T, but no strains related to TN-1T have been detected in cultures derived from members of these horsefly genera. 

Since mixed infections of different spiroplasmas are common (21), strain TN-1T may be frequently overgrown by members of other Spiroplasma groups in vivo or in vitro. The occurrence of spiroplasmas in narrow habitats in insects (tabanid fly viscera) has suggested that these microorganisms could be coexisting with other unclassified spiroplasma strains that represent putative new groups demonstrated the uniqueness of the new insect strain. We therefore propose the name Spiroplasma litori for this organism.

The taxonomic description below summarizes the properties of the organism.

**Description of Spiroplasma litori sp. nov.** Spiroplasma litori (litori. L. neut. adj. litorale, of the shore or coastal area).

Cells are filamentous, helical, and motile, vary from 200 to 300 nm in diameter, and lack true cell walls. Colonies on solid medium containing 2.25% Noble agar are granular with dense centers, uneven margins, and multiple satellites and never have a fried-egg appearance.

Chemoorganotroph. Acid is produced from glucose. Hydrolyzes neither arginine nor urea.

Cholesterol or serum is required for growth.

The temperature range for growth is 10 to 41°C, with optimum growth occurring at 32°C. The doubling time in M1D medium at the optimum temperature is 1.6 h.

Seroologically distinct from previously established Spiroplasma species. Isolated from the gut of T. nigrovittatus (Diptera: Tabanidae). Pathogenicity for insects has not been determined.

The guanine-plus-cytosine content of the DNA is 25 ± 1 mol%, as determined by the buoyant density method. The genome size is 1,370 kbp.

The type strain is ATCC 43211.

**ACKNOWLEDGMENT**

We thank Jeffrey Buller, College of Liberal Arts and Sciences, Georgia Southern University, Statesboro, for his advice concerning the Latin name for this spiroplasma.

**REFERENCES**


### TABLE 2. Serological reactions and cross-reactions of strain TN-1T in deformation and metabolism inhibition serological tests a

<table>
<thead>
<tr>
<th>Group</th>
<th>Strain</th>
<th>Spiroplasma deformation test</th>
<th>Metabolism inhibition test</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Antiserum</td>
<td>Antigen</td>
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<tr>
<td>I-8</td>
<td>P40</td>
<td>0 a</td>
<td>0 a</td>
</tr>
<tr>
<td>II</td>
<td>DW-1</td>
<td>0 b</td>
<td>0 b</td>
</tr>
<tr>
<td>IV</td>
<td>B31</td>
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<tr>
<td>VII</td>
<td>MQ-1</td>
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<tr>
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<td>EA-1</td>
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<tr>
<td>IX</td>
<td>CN-5</td>
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<td>TN-1T</td>
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<tr>
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<td>BIUS-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XXVI</td>
<td>PLHS-1</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>XXVIII</td>
<td>PALS-1</td>
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<td>0</td>
</tr>
<tr>
<td>All others</td>
<td>All others</td>
<td>&lt;20</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

a Strain TN-1T antigen and antisera were tested in all heterologous combinations against representatives of all known and putative groups and subgroups. All cross-reacts were negative except those shown. All cross-reactions observed were in one direction only, demonstrating the serological uniqueness of strain TN-1T.

b Reciprocal of the endpoint in a deformation test in which the antigen was tested against the homologous antiserum and vice versa.

c Reciprocal of the endpoint in a metabolism inhibition test in which the antigen was tested against the homologous antiserum and vice versa.

The value in parentheses is the homologous titer for antisera against which strain TN-1T cross-reacted when it was used as the antigen in a heterologous test.


