Spiroplasma turonicum sp. nov. from Haematopota horse flies (Diptera: Tabanidae) in France

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Strain Tab4cT, a helical prokaryote that was isolated from the body of a Haematopota sp. fly collected in Champchevrier, Indre-et-Loire, Touraine, France, was found to be a member of the class Mollicutes. The cells of strain Tab4cT were small, motile helices that were devoid of a cell wall. The organism passed through filters with mean pore diameters as small as 0.20 mm. Strain Tab4cT grew rapidly in liquid SP-4 medium at both 30 and 37 °C. The organism fermented glucose but did not hydrolyse arginine or urea, and did not require serum for growth. In preliminary electrophoretic analyses, the cell protein patterns of strain Tab4cT were distinct from those of 14 other spiroplasmas found in mosquitoes, deer flies and horse flies from Europe and the Far-East. In reciprocal metabolism inhibition and deformation serological tests, employing antigens and antisera representative of spiroplasma groups I–XXXIII (including all sub-groups), plus ungrouped strains BARC 1901 and BARC 2649, no serological relationship with Tab4cT was found. The G+C content of the DNA of strain Tab4cT was about 25.2 mol% and its genome size was 1.305 kbp. It is proposed that spiroplasma strain Tab4cT be assigned to group XVII (presently vacant) and that strain (ATCC 7002717) is the type strain of a new species, Spiroplasma turonicum.

Keywords: Spiroplasma turonicum sp. nov., Mollicutes, Diptera

INTRODUCTION

A large number of spiroplasmas have been isolated from horse flies and deer flies in both the USA (5–8, 11, 22–25) and France (4, 9, 13, 14).

In general, tabanid spiroplasmas exhibit a considerable degree of biodiversity within the genus Spiroplasma, class Mollicutes, and recently a number of North American tabanid spiroplasmas were described as new species (7, 11, 23, 24). This was accompanied by the definition of new groups and sub-groups (22) and required a complete revision of the group classification of spiroplasmas (28). Moreover, several other strains, including some isolated from France (13, 14), remain to be fully characterized. On the basis of previous attempts to classify members of the genus Spiroplasma, serological analysis has been a prominent criterion (28). The validity for a number of proposed species designations has been confirmed by DNA–DNA hybridization data and 16S rDNA sequence analysis (28). However, until sequence data are available for representatives of all spiroplasma groups and sub-groups, phenotypic characters, including serological data, will provide the most logical basis for construction of polyphasic classifications.

Abbreviations: CCU, colour-changing units; DF, deformation; MI, metabolism inhibition; TEM, transmission electron microscopy.
During the summer of 1991, horse flies of the genus *Haematopota*, probably *H. pluvialis*, were collected in the field from Indre-et-Loire, Touraine, France, and yielded three spiroplasma isolates (20). One of these, strain Tab4c, proved to be unrelated to spiroplasmas belonging to previously described groups. In this paper, we present the results of studies that fulfill the proposed minimal criteria (10) for description of new species of the class *Mollicutes*. Therefore, we propose that strain Tab4c (= ATCC 700271T) be recognized as the type strain of a new spiroplasma species, *Spiroplasma turonicum*, with the organism designated as group XVII in the currently recognized grouping scheme (18, 28).

**METHODS**

**Isolation of Tab4c**. Strain Tab4c was isolated from a triturate of a single *Haematopota* fly, probably *Haematopota pluvialis*, collected on 12 August 1991 at Champchevrier, Indre-et-Loire, Touraine, France (46°21' N, 0°25' E), during a field survey that produced two other isolates from 13 *Haematopota* flies (20). All three isolates originated from the triturated flies and not from external washings (14). Strain Tab4c was selected for more complete characterization.

After filtration through a 0.45 μm membrane, primary isolation of strain Tab4c was achieved in M1D medium (21) (containing 500 U penicillin G ml⁻¹) at 28 °C. The strain was then cloned in triplicate by plating onto solid SP-4 medium (21) before morphological, biochemical and serological characterization.

**Morphological studies**. Cell morphology was studied by dark-field microscopy and by transmission electron microscopy (TEM) of negatively stained cells from a 24-48 h M1D broth culture. Membrane structure of Tab4c was also determined by TEM using standard procedures (26). Briefly, Tab4c cells were fixed for 3 h in 3% glutaraldehyde that was added directly to the culture, pelleted by centrifugation at 16000 g, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, and embedded in Epon-Araldite. Sections were stained with 2% aqueous uranyl acetate and Reynolds's lead citrate, and then examined and photographed using a 1000 S JEOL microscope. The morphology of Tab4c colonies on solid SP-4 medium containing 3% Noble agar (Difco Laboratories) was observed and photographed, using phase and scanning electron microscopy.

**Biochemical and biological properties**. Ability to metabolize glucose, arginine and urea was tested in heart infusion broth (Difco) according to standard methods (1); *Spiroplasma taiwanense* CT-1T (Glu +; Arg -) and *Spiroplasma sabaudiense* Ar 1343T (Glu +; Arg +) were used as controls. Sterol and fatty acid requirements were determined according to the techniques of Rose et al. (16). Filtration characteristics were determined by measuring the ability of individual organisms in young broth cultures to pass through a series of sterile membrane filters with graded porosities (0.45, 0.30 and 0.20 μm). The syringe filters (cellulose acetate for 0.45 and 0.30 μm and polyethersulfone filters for 0.20 μm) were pre-washed with 5 ml SP-4 broth. Using minimum hand pressure, about 3 ml of the culture was passed through each filter. The control broth culture and the individual filtrates were assayed in a series of 10-fold serial dilutions in SP-4 broth incubated at 30 °C for 7 d. At this time, the vials showing a positive colour change in the medium pH (red to yellow), were recorded as the number of colour-changing units (CCU) ml⁻¹ in control broth or filtrate.

**Temperature requirements**. The growth of strain Tab4c at various temperatures was determined by inoculating an exponential-phase culture into 25 ml SP-4 medium, giving a final concentration of about 5 x 10⁸ spiroplasma cells ml⁻¹. This seeded medium was divided into seven tubes, and the individual tubes were incubated at 15, 20, 25, 30, 35, 37 or 40 °C. After 2 d, each broth culture that showed acid colour shift of the pH indicator was examined by dark-field microscopy for the presence of motile helical forms.

**Serological tests**. Hyperimmune antisera to all established groups and sub-groups of the genus *Spiroplasma* were taken from the reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases, Frederick, MD, USA.

Mouse immune ascitic fluids were prepared against strain Tab4c and several other tabanid and mosquito spiroplasmas isolated in France, according to a previously described protocol (9). Reciprocal deformation and metabolism inhibition tests (27) were carried out for comparison of Tab4c antigen and antibody with antigens and antibodies of representatives of the 35 spiroplasma groups, and nine additional sub-groups (18).

**Proteins and genomic analysis**. One-dimensional gel electrophoresis (SDS-PAGE) of total proteins of strain Tab4c and other spiroplasmas was performed as described previously (9). The methods used for DNA extraction, determination of the G+C content and the genome have also been published previously (2, 3).

**RESULTS AND DISCUSSION**

**Isolation of strain Tab4c**

Tab4c was isolated from the filtered supernatant of a single fly triturate but not from external washings of the same insect. Medium pH indicator change occurred within 5 d and growth was confirmed by dark-field microscopy. The isolate showed turbidity and pH change to acid within 24 h in SP-4 medium (containing 500 U penicillin G ml⁻¹) at both 30 and 37 °C, and exhibited the typical helical morphology and motility of spiroplasmas. On solid SP-4 medium, strain Tab4c exhibited 'cauliflower-like' colonies (Fig. 1a) very similar to colonies formed by other motile spiroplasmas (23–25). The classical 'fried-egg' colony morphology observed with non-motile mollicute was never seen in Tab4c.

**Electron microscopy**

Helical morphology was confirmed by negative staining; measurements of cells by this technique were: length 3.15–5.75 μm, width 104–149 nm, and helix-turn 0–56 μm. Frequently, one of the extremities was blunt whereas the other appeared cone-shaped. In thin sections, cells exhibited a marked pleomorphism but were delimited only by an unit membrane, an ultrastructural finding typical for spiroplasmas (Fig. 1b).
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Fig. 1. (a) Colony morphology of strain Tab4cT grown for 9 d on SP-4 medium containing 3% Noble agar under aerobic conditions; bar, 80 μm. (b) Electron micrograph of a thin section of a cell pellet of strain Tab4cT; bar, 50 nm.

Biochemical and biological properties

Tab4cT fermented glucose but did not hydrolyse arginine or urea. The strain was maintained through 23 consecutive 10-fold serial passages in media with and without a serum supplement, indicating no serum requirement for growth. Filtration studies indicated that a 24 h SP-4 broth culture of Tab4cT contained approximately 10⁹ CCU ml⁻¹. The following CCU ml⁻¹ were recorded for filtrates obtained after passage of this broth culture through membrane filters with porosities of 0.45, 0.30 and 0.20 μm, respectively: 10⁹, 10⁸ and 10⁷.

Strain Tab4cT grew at temperatures ranging from 25 to 37 °C, with an optimal growth at 30 °C. No growth was observed at 15, 20 or 40 °C.

Serological properties

In deformation (DF) and metabolism inhibition (MI) tests, antigen of strain Tab4cT did not react with any of antisera prepared against representative strains of established groups, sub-groups or species of Spiroplasma (18). In reciprocal DF tests, using mouse ascitic fluid prepared against Tab4cT antigen, DF cross-reactions were observed with strain TABS-2 and PLHS-1, but at low dilution, 1:40 and 1:20, respectively. In reciprocal MI tests, one-way cross-reactions were observed with the following strains as antigens tested against a 1:4 stock dilution of anti-Tab4cT mouse ascitic fluid: SMCA (1:24), Ar-1343 (1:216), PLHS-1 (1:72), PALS-1 (1:24) and BARC 2649 (1:24). The homologous MI titre of anti-Tab4cT mouse ascitic fluid was 1:648. Accordingly, these one-way reactions were considered to be insignificant and non-specific in nature. Strain Tab4cT is therefore serologically distinct and is eligible for assignment to the previously vacant spiroplasma group XVII.

Proteins and genomic properties

SDS-PAGE of Tab4cT total proteins revealed about 40 different bands, with an electrophoretic profile that is different from the profiles of S. sabaudiense, S. taiwanense, Spiroplasma floricola and 11 Tabanid spiroplasmas isolated from France and serologically related to an existing group (either VIII, XIV, XXIII, HYOS-1, TABS-1 or TAAS-1). An example of such electrophoretic comparison is presented in Fig. 2. The G + C content of the DNA of Tab4cT was 25 ± 1 mol % (28). Its genome size was 1305 kbp.

Habitat, biological properties and medical importance

We are unaware of any pathogenic effect of Tab4cT spiroplasma on its Haematopota host. The fact that the culture from surface rinsing the fly was negative and the triturate of the whole fly was positive, clearly indicates that strain Tab4cT originated from the tissues of the fly or the gut contents. Nothing is known about
the natural biological cycle of spiroplasmas of the Tab4cT cluster, as is also the case with other tabanid spiroplasmas.

In Touraine, the province in France from which Haematopota flies were collected, H. pluvialis Linné is the only Haematopota species to be regularly found during the summer, and it is thus probable that this species is the usual host of spiroplasmas related to strain Tab4cT. Moreover, H. pluvialis is very abundant in this area, as in many other places in France (15).

In the medical and veterinary fields, H. pluvialis is known as a potentially dangerous vector of pathogens for cattle, pig, and, eventually, man. It has been found naturally infected with both Bacillus anthracis and Francisella tularensis (12), the aetiologic agents of anthrax and tularemia, respectively. This fly is also able to transmit experimentally a number of other pathogenic bacteria, such as Anaplasma marginale, Brucella melitensis, Brucella abortus, Brucella suis and Erysipelothrix rhusiopathiae (12). However, we have no knowledge about a possible effect of Tab4cT on the vector competence of Haematopota flies for these different pathogenic agents.

On the other hand, the capability of Tab4cT to multiply at a fast rate at 37 °C, the corporeal temperature of man and other mammals, renders this spiroplasma unsuitable a priori for use in the biological control of tabanids or other haematophagous arthropods.

The properties described herein for strain Tab4cT fulfil proposed criteria (10) for species of the class Mollicutes, including absence of cell wall, filterability, and penicillin resistance. The helicity and motility of strain Tab4cT and its inability to utilize urea place the organism in the family Spiroplasmataceae (19). Finally, serological comparison of Tab4cT with other Spiroplasma species demonstrates the uniqueness of the new insect strain. We therefore propose the designation of Spiroplasma turonicum for this organism. The taxonomic description below summarizes the properties of the organism.

**Description of Spiroplasma turonicum sp. nov.**

*Spiroplasma turonicum* (tu.ro.ni.cum. L. neut. adj. *turonicum* of Touraine, the province in France from which the organism was first isolated).

Cells are motile helical filaments, 100–150 nm in diameter, with a helix-turn of 0.56 nm, and lack a true cell wall. Colonies on solid SP-4 medium containing 3% Noble agar exhibit a ‘cauliflower-like’ appearance, never appearing as ‘fried eggs’. Chemo-organotroph. Acid is produced from glucose. Does not hydrolyse arginine or urea. Cholesterol or serum not required for growth. Growth was observed from 25 to 37 °C, with optimal growth at 30 °C. Serologically distinct from previously described Spiroplasma species. Isolated from a Haematopota fly. The G + C content of the DNA is 25 ± 1 mol%, as determined by buoyant density. The genome size is 1305 kbp. The type strain is Tab4cT (= ATCC 700271T).

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**References**


