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# *Spiroplasma leucomae* sp. nov., isolated in Poland from white satin moth (*Leucoma salicis* L.) larvae

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*Spiroplasma* sp. strain SMA<sup>T</sup>, isolated in Poland from white satin moth larvae, *Leucoma salicis* L. (Lepidoptera: Lymantriidae), 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 single cell membrane (lacking a cell wall). Growth of strain SMA<sup>T</sup> occurred in M1D medium at 30 °C. Strain SMA<sup>T</sup> catabolized both glucose and arginine, but did not hydrolyse urea. The G+C content of the DNA was 24 ± 1 mol% as determined by melting temperature analysis. Serological analysis revealed a very weak cross-reactivity (positive reaction only up to a 1 : 80 dilution) with two *Spiroplasma* strains, 277F (*Spiroplasma* sp. group I-4) and LB-12 (*Spiroplasma* sp. group I-5). Strain SMA<sup>T</sup> (= ATCC BAA-521<sup>T</sup> = NBRC 100392<sup>T</sup>) is designated the type strain of a novel species, *Spiroplasma leucomae* sp. nov. (class *Mollicutes*: order *Entomoplasmatales*: family *Spiroplasmataceae*).

*Spiroplasma* infection among white satin moth [*Leucoma* (= *Stilpnotia*) *salicis* L.] larvae was first discovered in 1986, which was the first record of spiroplasma infection in the insect order Lepidoptera (Lipa *et al.*, 1988). Previous spiroplasma infections have been recorded in members of the insect orders Hymenoptera, Hemiptera, Coleoptera and Diptera and in ticks of the family Ixodidae (Hackett *et al.*, 1990). Strain SMA<sup>T</sup> was isolated from a fifth instar larval satin moth collected in Poland (Lipa *et al.*, 1988). Satin moth larvae were collected from poplar trees in 1986 and 1987, and data recorded a 43 % natural infection from the 1986 collections and a range of 8·3–20 % infection in the 1987 collection (Lipa *et al.*, 1988). Among laboratory-reared satin moth larvae, the infection level was higher, 55 % among 41 examined larvae, indicating that horizontal transmission of the spiroplasmas was a means to spread the infection among members of a satin moth larvae population. As this is the first natural spiroplasma infection recorded in a lepidopteran, characterization of strain SMA<sup>T</sup> was performed.

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). In

this paper, we report the results of taxonomic studies of strain SMA<sup>T</sup> that satisfy the requirements for species descriptions for the class *Mollicutes* (International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes*, 1995). Results support the designation of strain SMA<sup>T</sup> as the type strain of a novel species.

## Isolation and cultivation

Techniques for isolating spiroplasmas from insect guts and haemocoel have been described previously (Hackett & Clark, 1989; Rose *et al.*, 1993). Strain SMA<sup>T</sup> was isolated and cultivated from the haemolymph of a larval satin moth (*L. salicis*) collected in Poland (Lipa *et al.*, 1988). Primary isolation of strain SMA<sup>T</sup> was performed in a culture of M1D medium (Whitcomb, 1983) and in a co-culture of M1D medium with insect tissue cells (1 : 1) (Konai *et al.*, 1996b). For comparative purposes, strain SMA<sup>T</sup> was adapted to serum fraction medium (SFM; Smith *et al.*, 1954), to which 10 % glucose was added. Modifications were made to SFM by replacing Difco PPLO serum fraction with horse serum. Other media used for cultivation purposes included DCCM (Hackett *et al.*, 1996), H-2 (Konai *et al.*, 1996b) and a co-culture of M1D/H-2 (1 : 1) (Konai *et al.*, 1996b). Isolates of strain SMA<sup>T</sup> were grown at an optimal temperature of 30 °C (Konai *et al.*, 1996a) both aerobically and anaerobically in a BBL anaerobic GasPak system (Becton Dickinson). Growth of strain SMA<sup>T</sup> was determined by observing indicator changes in the medium (M1D) and by examining the cultures microscopically during a weekly observatory period. Initial inoculations of strain SMA<sup>T</sup> into M1D and

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Abbreviations: DF test, deformation test; MI test, metabolism inhibition test.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SMA<sup>T</sup> is DQ101278.

M1D/TC media involved weekly passes due to the slow growth of the organism. Other media were used for comparative growth purposes: DCCM, H-2, SFM supplemented with 10 % glucose and M1D/H-2. Slower growth rates were observed in all of these media. After a 1-month adaptation in M1D medium (containing 500 U penicillin  $\text{ml}^{-1}$ ), strain SMA<sup>T</sup> grew rapidly, requiring daily passes. Strain SMA<sup>T</sup> was readily filterable through 450 nm and 220 nm pore-size filters. A 100 nm pore-size membrane filtrate was free of viable cells. Strain SMA<sup>T</sup> was triply cloned in M1D medium (Whitcomb, 1983) by conventional filter-cloning procedures (Tully, 1983a) and designated strain SMA<sup>T</sup>. 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 statically in M1D medium at 30 °C.

### Morphological studies

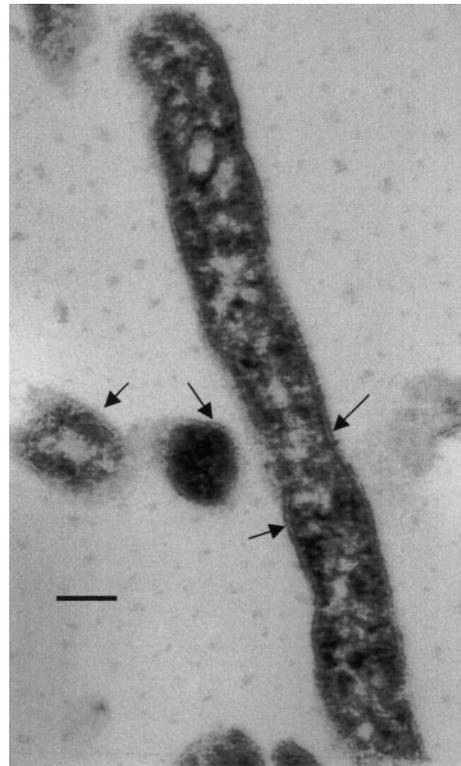
Dark-field microscopy (magnification  $\times 1000$ ) of strain SMA<sup>T</sup> observed during the exponential phase revealed helical cells with four or more turns. Strain SMA<sup>T</sup> was highly motile and formed clumps after 3 days in the same culture.

For TEM, 20 ml exponential-phase culture of strain SMA<sup>T</sup> was pelleted and fixed for 2 h in 3 % glutaraldehyde, post-fixed in 1 % osmium tetroxide 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 Reynold's lead citrate prior to visualization. As determined by TEM (Fig. 1), cells were filamentous, approximately 150 nm in diameter and surrounded by a single cytoplasmic membrane.

### Serological tests

Strain SMA<sup>T</sup> was tested serologically against hyperimmune antisera of each of the 40 different spiroplasmas that represent established type strains and subgroup reference strains (Williamson *et al.*, 1998) using the deformation (DF) test (Williamson *et al.*, 1978) and the metabolism inhibition (MI) test (Williamson & Whitcomb, 1983). Positive reactions for the DF test were confirmed by the presence of grape-like clusters on the spiroplasma, indicative of a reaction between the cell membrane and the antiserum. The presence of normal, helical/spiral-shaped spiroplasmas indicated no reaction to the antiserum (Williamson *et al.*, 1978). *Spiroplasma syrphidicola* EA-1<sup>T</sup> and *Spiroplasma citri* R8A2<sup>T</sup> were also tested against their own antiserum as controls. This procedure was performed twice. Antiserum to strain SMA<sup>T</sup> was produced in rabbits as described previously (Williamson *et al.*, 1978) and used in reciprocal DF tests against strains whose antisera had a positive reaction against strain SMA<sup>T</sup>.

DF tests showed that strain SMA<sup>T</sup> exhibited only weak serological reactivity with four representatives from group I: I-3 (*Spiroplasma kunkelii* E275<sup>T</sup>) at 1 : 20, I-4 (*Spiroplasma*



**Fig. 1.** Transmission electron micrograph of cells of *S. leucomae* strain SMA<sup>T</sup>. Arrows indicate the cell membrane. Bar, 0.1  $\mu\text{m}$ .

sp. strain 277F) at 1 : 80, I-5 (*Spiroplasma* sp. strain LB-12) at 1 : 80 and I-6 (*Spiroplasma insolitum* M55<sup>T</sup>) at 1 : 20. All other reactions were negative. The homologous titre for *S. citri* R8A2<sup>T</sup> was 2560 (Hackett *et al.*, 1996). The homologous titre for strain SMA<sup>T</sup> was 5120. The MI test results were determined by colour change, or lack thereof, and were performed twice. There were no positive reactions with any of the strains tested in the MI test.

The slight serological cross-reactions with group I spiroplasmas are interesting, as initial observations suggested that infection of satin moth larvae with strain SMA<sup>T</sup> increased insect mortality (J. J. Lipa, unpublished data). Additional tests are planned to determine the degree (if any) of pathogenicity of strain SMA<sup>T</sup> to several lymantriids including the gypsy moth (*Lymantria dispar*).

### Biochemical analysis

Procedures for determining carbohydrate fermentation, arginine hydrolysis and urea utilization have been described previously (Aluotto *et al.*, 1970). Strain SMA<sup>T</sup> was adapted to a minimal medium, SFM supplemented with 1 % bovine serum fraction (Tully, 1983b) and 10 % glucose. After weaning, strain SMA<sup>T</sup> was used to inoculate five tubes for biochemical testing as follows: tube 1 with SFM, tube 2 with SFM and 10 % glucose, tube 3 with SFM and 10 % glucose

and 21 % arginine, tube 4 with SFM and 21 % arginine and tube 5 with SFM and 1 % urea (Aluotto *et al.*, 1970). Dark-field microscopy, as well as colour change, was used to identify growth of the cultures. A positive test showed growth in ten subsequent dilutions of the original tube and each positive test was repeated three times to ensure reproducibility. After adaptation to SFM supplemented with 10 % glucose, strain SMA<sup>T</sup> was able to grow in media containing glucose, glucose and arginine, and arginine alone, indicating the abilities to ferment glucose and catabolize arginine. No growth was observed in SFM and urea, therefore urea hydrolysis was not observed.

### Genome analysis

Strain SMA<sup>T</sup> 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 for a final sodium concentration of 0.195 M. This sample was analysed (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 and *Escherichia coli* DNA in 1 × SSC buffer was used as a control. The G + C base content of strain SMA<sup>T</sup> genomic DNA was measured as 21.56, 25.12 and 25.29 mol% (mean 24 ± 1 mol%).

### Phylogenetic analysis

ATCC accession numbers and GenBank accession numbers for 16S rRNA gene sequences used in this study are indicated

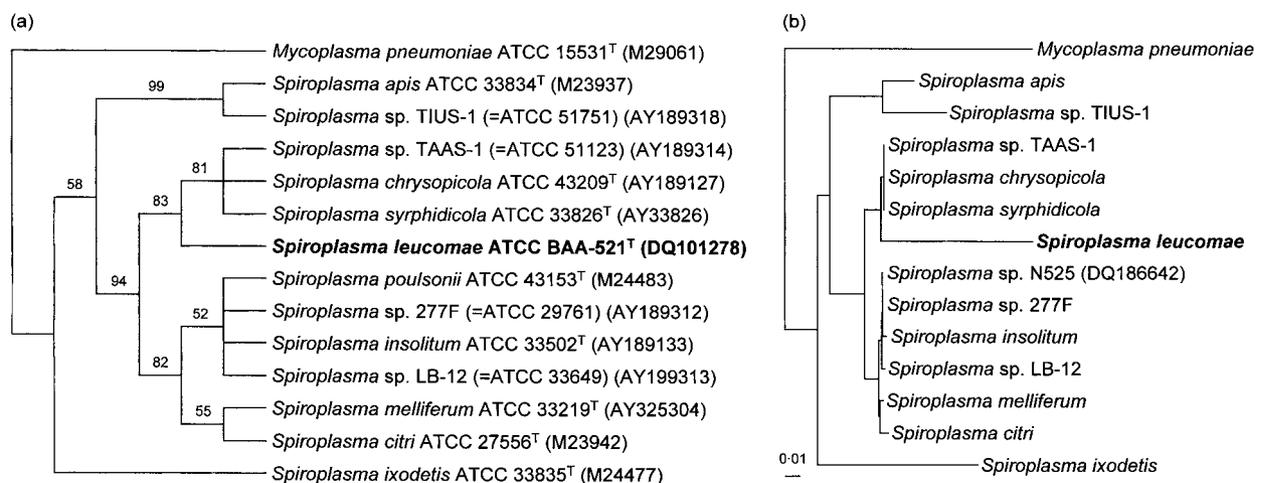
in Fig. 2(a). Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and then aligned manually in MACCLADE (Maddison & Maddison, 1992). A 1000 replicate bootstrap analysis was performed using a heuristic search and the tree bisection reconnection parsimony algorithm from PAUP (Fig. 2a) and neighbour-joining analysis using the Jukes–Cantor model for substitutions (Fig. 2b) (version 4.0b; Swofford, 1998). In all analyses, strain SMA<sup>T</sup> consistently grouped with the group VIII subgroups.

This is the first taxonomic and diagnostic description of a spiroplasma isolated from a lepidopteran larva. The white satin moth (*L. salicis*) is recognized as an invasive alien species and a major pest that defoliates mainly poplar trees (*Populus* spp.) but is spreading damage to other trees like trembling aspen, black cottonwood and sometimes willow (Humphreys, 1996). Isolation and characterization of strain SMA<sup>T</sup> may contribute to attempts to establish biological control of satin moth larvae, which will be useful in efforts to curb deforestation.

As all the tests needed for the characterization of a novel spiroplasma species, as required by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Mollicutes*, have been fulfilled, we propose a novel species *Spiroplasma leucomae* sp. nov.

### Description of *Spiroplasma leucomae* sp. nov.

*Spiroplasma leucomae* [leu.co'mae. N.L. gen. fem. n. *leucomae* of *Leucoma*, systematic genus name of the white



**Fig. 2.** Phylogenetic trees showing the position of *S. leucomae* strain SMA<sup>T</sup> among representatives of the genera *Spiroplasma* and *Mycoplasma*. The 16S rRNA gene sequence of *S. leucomae* strain SMA<sup>T</sup> was included in a dataset described by Gasparich *et al.* (2004). *Mycoplasma pneumoniae* ATCC 15531<sup>T</sup> was used as an outgroup strain. (a) The tree shows a bootstrap parsimony analysis of the dataset, which was resampled 1000 times. Bootstrap percentage values are given at nodes. ATCC accession numbers are shown next to the organism names and GenBank accession numbers for the 16S rRNA gene sequences used are given in parentheses. (b) Tree constructed by neighbour-joining analysis with the Jukes–Cantor substitution model. Bar, 0.01 substitutions per site. Strain and sequence details are shown in (a) with the exception of strain N525 [not included in (a)].

satin moth (Lepidoptera: Lymantriidae), the source of the type strain].

Cells are filamentous, helical and motile, and are approximately 150 nm in diameter. They pass freely through filters with 450 and 220 nm pores, but do not pass through filters with 100 nm pores. The cells lack true cell walls. Chemoorganotrophic. 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 was isolated from a fifth instar white satin moth (*Leucoma salicis*) larva. Pathogenicity for these moth larvae is not known. The G+C content of the DNA is  $24 \pm 1$  mol% as determined by the melting temperature method.

The type strain is strain SMA<sup>T</sup> (= ATCC BAA-521<sup>T</sup> = NBRC 100392<sup>T</sup>).

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