Isolation and Characterization of a Non-Helical Strain of *Spiroplasma citri*

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SUMMARY

A non-helical strain of *Spiroplasma citri* from little-leaf diseased oranges was subcultured over 40 times in different media without producing helical cells. It was non-motile and produced colonies of a characteristic morphology on soft agar plates. Serology, DNA hybridization and toxin production confirmed that it was a strain of *S. citri* but its membrane protein pattern after polyacrylamide gel electrophoresis differed from those of other *S. citri* strains in that one band was absent. The organism caused symptoms in plants identical to those produced by a pathogenic helical strain of *S. citri*.

INTRODUCTION

Spiroplasmas differ from other Mollicutes in shape and motility. All those isolated are typically helical during at least part of their growth phase and show rotary or 'screw'-like motility as well as flexing movements. The helical shape is apparently maintained without the assistance of a rigid cell wall or axial filaments (Cole *et al.*, 1973).

Spiroplasma citri was isolated and characterized by Saglio *et al.* (1973) and is the leaf-hopper-transmitted agent of little-leaf disease of citrus (Markham *et al.*, 1974). We have isolated a pathogenic strain of *S. citri*, which lacks the characteristic helical morphology, from diseased citrus material.

METHODS

Isolation and culture. A non-helical spiroplasma and six normal strains of *S. citri* were isolated from 'lop-sided' fruits of little-leaf diseased sweet orange trees by the method of Daniels *et al.* (1973). Isolates were subcultured twice in complete sorbitol medium (SMC; Saglio *et al.*, 1973) without arginine and tryptone, before 0.1 ml samples were plated on SMC solidified with 1 % (w/v) agar (Oxoid no. 1). After incubation for 7 days at 32 °C, colonies showing unusual morphology were picked off into SMC medium. One of these isolates was cloned three times and designated ASP-1. All cultures were maintained in SMC at 32 °C. Characterized strains of *S. citri* R8-A2 (type strain) and C189 were kindly supplied by Dr P. Saglio, and the pathogenic strain SP-A was isolated in this laboratory (Daniels *et al.*, 1973).

Media and incubation conditions. The colony and cell morphologies of strains SP-A and ASP-1 were compared under different conditions. Plates of SMC solidified with 0.75 % and 1 % (w/v) agar were spread with 0.1 ml of diluted spiroplasma culture and incubated at 32 °C for 7 days in air or in N₂/CO₂ (95:5, v/v). Cultures in SMC were incubated at 25, 28,

32 and 37 °C. The osmolarity of SMC was altered by varying the concentration of sorbitol up to maximum of 20 % (w/v). Sera tested included 20 % (v/v) horse serum (Gibco-Biocult, Paisely, Renfrewshire), 20 % (v/v) horse serum no. 3 (Wellcome Reagents), 20 % (v/v) donor horse serum (Sera Lab., Crawley, Sussex), 10 % (v/v) foetal calf serum (Gibco-Biocult), 10 % (v/v) fresh chicken serum and 20 % (v/v) horse serum from which globulins had been removed by ammonium sulphate precipitation. All sera were heated at 60 °C for 30 min before use and were tested against both strains in SMC and on SMC with 0.75 % agar.

Light and electron microscopy. Spiroplasma morphology was examined by dark field optics, and colonies, unstained or stained with Dienes' stain (Scriba, 1968), were examined with a stereo dissecting microscope. Cultures in the exponential phase of growth were fixed by adding an equal volume of 7 % (w/v) glutaraldehyde in SMC (Cole, Tully & Popkin, 1974) and negatively stained with saturated uranyl acetate for electron microscopy. Examinations for the presence of virus particles in strain ASP-1 were performed by Dr R. M. Cole, National Institutes of Health, Bethesda, Maryland, U.S.A. Sections of diseased plant tissue were prepared and examined as described by Markham *et al.* (1974).

Motility. Molten 0.5 % agar (Oxoid no. 2) at 40 °C was added to an equal volume of exponentially growing culture. Movement of the organisms through the agar was observed by dark field microscopy.

Gel electrophoresis. Spiroplasmas were harvested by centrifugation at 20000 *g* for 30 min at 4 °C from 100 ml cultures in SMC with 10 % (v/v) horse serum, and membranes were prepared by a procedure similar to that described by Razin *et al.* (1973). After solubilization in a mixture of 2 % (w/v) sodium dodecyl sulphate (SDS), 2 % (v/v) 2-mercaptoethanol and 20 % (w/v) sucrose, and heating for 3 min at 100 °C, samples of these preparations were run in 7.5 % (w/v) polyacrylamide-SDS gels (Hull, 1971) which were then stained and photographed (Daniels & Meddins, 1973).

Growth. Strains SP-A and ASP-1 were grown in 5 ml portions of SMC supplemented with [2-¹⁴C]thymidine (57 mCi mmol⁻¹, about 0.1 µCi ml⁻¹). Samples (0.1 ml) were removed at various times after inoculation. Growth was measured by colony-forming units (c.f.u.) and by the quantity of [¹⁴C]thymidine incorporated (Daniels, 1969).

Serology. Antiserum to *S. citri* SP-A was produced as described previously (Markham *et al.*, 1974). Spiroplasmas were compared by metabolic inhibition tests (Taylor-Robinson *et al.*, 1966) and growth inhibition tests (Clyde, 1964). *Mycoplasma mycoides* subsp. *capri* and *Acholeplasma laidlawii* were included as negative controls.

DNA hybridization. DNA was prepared from (500 ml) cultures of spiroplasmas and *M. mycoides* by the method of Marmur (1961). Radioactive DNA from strain SP-A was obtained after growth in 500 ml SMC supplemented with [methyl-³H]thymidine (46 Ci mmol⁻¹, about 1 µCi ml⁻¹). Homologies were assessed by hybridizing 2 µg of denatured, radioactively-labelled DNA from strain SP-A with 50 µg of denatured, unlabelled DNA fixed on filters in the presence of 50 % (v/v) formamide (Saglio *et al.*, 1974). After 36 h incubation at 37 °C, the filters were washed and dried and the fixed radioactivity was determined by liquid scintillation counting.

Toxin production. *Spiroplasma citri* toxin was partially purified from the cell-free supernatant fluids of SP-A and ASP-1 cultures and then tested on pieces cut from broad bean leaves (unpublished).

Pathogenicity tests. Leafhoppers were reared, injected and maintained as reported previously (Markham *et al.*, 1974). (i) 120 *Euscelis plebejus* leafhoppers were injected with strain SP-A and 120 with strain ASP-1. Insects were caged on white clover for 3 weeks, and the survivors were then transferred in groups of 6 or 12 to 12 broad bean seedlings for

Fig. 1

Fig. 2

Fig. 1. Stained colonies of *S. citri* strains (a) SP-A and (b) ASP-I on SMC medium solidified with 0.75 % agar. Bar markers represent 0.01 mm.

Fig. 2. Negatively stained organisms from exponential phase cultures of *S. citri* strains (a) SP-A and (b) ASP-I. Bar markers represent 0.5 μ m.

2 weeks. (ii) 300 *Euscelidius variegatus* insects were injected with strain ASP-I, caged on rye-grass for 3 weeks and then transferred in groups of 8 to 21 broad bean plants.

RESULTS

Colony morphology. Six isolates produced colonies which were poorly defined 'fried eggs' less than 0.1 mm diam., slightly brown, granular and surrounded by tiny satellite colonies, typical of the SP-A strain of *S. citri* (Fig. 1a). However, about 1 % of the colonies from one batch of diseased oranges were atypical well-defined 'fried eggs' about 0.05 mm diam., transparent, smooth and without satellite colonies (Fig. 1b). This morphology was maintained throughout subculture and cloning and was typical of strain ASP-I. The differences in colony morphology between strains SP-A and ASP-I were most apparent on plates containing 0.75 % agar. No reversion of the ASP-I colony type to the SP-A colony form was observed in N_2/CO_2 or on any of the media used. Both organisms produced the largest colonies on agar containing 10 % (v/v) foetal calf serum or 10 % (v/v) chicken serum and the smallest colonies with 20 % (v/v) globulin-free horse serum.

Cell morphology. Exponential phase cultures of strain ASP-I contained flexuous filaments, many of which were branched and tangled into large clumps. No helical cells were observed during more than 40 subcultures. Fixed, negatively stained preparations showed that the organisms had similar dimensions to SP-A spiroplasmas (about 3 to 8 μ m $\times$ 0.2 μ m) (Fig. 2a) but were not helical (Fig. 2b). Three viruses, SV-C1, SV-C2 and SV-C3 (Cole *et al.*, 1974), which are normally associated with helical forms of *S. citri*, including strain SP-A, were identified in strain ASP-I. Complete extracellular particles of SV-C1 and SV-C3 and incomplete intracellular particles of SV-C2 or SV-C3 were present in cultures examined by

Fig. 3. Electrophoretic patterns of partially purified *S. citri* membrane proteins: 1, SP-A; 2, R8-A2; 3, C189; 4, ASP-I. The position of the pattern variation is marked by the arrows.

Dr R. M. Cole. Subsequently we observed occasional complete extracellular particles of SV-C2.

Both strains grew best at 32 °C and progressively less well at 30, 28 and 25 °C. After 48 h incubation at 32 °C both strains produced sufficient acid to turn the phenol red indicator yellow and the pH of the medium ultimately fell below 5. Both strains grew well in media containing 0 to 10 % (w/v) sorbitol but poorly with 20 % (w/v) sorbitol, though varying the osmolality of the medium had no effect on the morphology of strain ASP-I. None of the sera tested caused strain ASP-I to become helical, but medium containing 20 % (v/v) of certain batches of horse serum no. 3 (Wellcome Reagents) did cause strain SP-A to produce distorted cells which often lacked a definite spiral. The same serum caused both strains to clump. Neither strain grew well in media containing 20 % (v/v) globulin-free horse serum.

Motility. In soft agar, strain SP-A showed characteristic rotational motion and flexing of the organisms. Filaments of strain ASP-I produced erratic twitching movements only.

Gel electrophoresis. The membrane protein patterns of strains SP-A, R8-A2, C189 and ASP-I were identical, qualitatively, except that one band was absent in gels of ASP-I (Fig. 3).

Growth curves. Cultures of strains SP-A and ASP-I grew at similar rates when measured by [¹⁴C]thymidine incorporation (Fig. 4), but ASP-I apparently had a longer lag phase when measured by c.f.u. (Fig. 5). Both organisms produced peak titres of more than 1×10^9 c.f.u. ml⁻¹.

Serological reactions. Antiserum to *S. citri* SP-A was equally inhibitory to *S. citri* SP-A and ASP-I but not to *M. mycoides* or *A. laidlawii* (Table 1).

Fig. 4

Fig. 5

Fig. 4. Growth curves of *S. citri* strains SP-A (●) and ASP-1 (○) in SMC medium at 32 °C, as measured by incorporation of [2-¹⁴C]thymidine (0.1 μCi ml⁻¹).

Fig. 5. Growth curves of *S. citri* strains SP-A (●) and ASP-1 (○) in SMC medium at 32 °C, as measured by colony-forming units.

Table 1. *Inhibitory activity of antiserum to S. citri SP-A on spiroplasmas and mycoplasmas*

Metabolic inhibition titres are expressed as the highest dilution of antiserum which permitted acid production by cultures in SMC medium after 4 days incubation at 32 °C for spiroplasmas and after 3 days incubation at 37 °C for mycoplasmas.

Growth inhibition results are expressed as the diameter of the zone of growth inhibition surrounding a 6 mm diam. filter paper disc impregnated with 20 μl antiserum after 7 days incubation at 32 °C for spiroplasmas and after 4 days incubation at 37 °C for mycoplasmas.

Strain	Metabolic inhibition titre	Growth inhibition zone (mm)
<i>S. citri</i> SP-A	1024	5.0
<i>S. citri</i> ASP-1	1024	4.5
<i>M. mycoides</i> (capri)	8	—
<i>A. laidlawii</i>	8	—

Table 2. *DNA-DNA hybridization among strains of S. citri and M. mycoides (capri)*

Radioactive DNA from strain SP-A was incubated with non-radioactive DNA, from various strains, immobilized on filters. After 36 h, the amount of radioactivity fixed on the filters was measured. The degree of hybridization is expressed as a percentage relative to the hybridization with DNA from strain SP-A itself (= 100).

DNA on filter	Fixed radioactivity (c.p.m.)	Percentage hybridization
Filter without DNA	472	—
<i>S. citri</i> SP-A	3966	100
<i>S. citri</i> R8-A2	4302	108
<i>S. citri</i> C189	3971	100
<i>S. citri</i> ASP-1	3919	99
<i>M. mycoides</i> (capri)	460	0

Fig. 6. Thick sections (150 to 200 nm) of the phloem cells in broad beans infected with *S. citri* strains (a) SP-A and (b) ASP-1. Bar markers represent 0.5 μ m.

DNA homology. DNA-DNA hybridization showed complete homology between the spiroplasmas but not with *M. mycoides* (Table 2).

Toxin production. Both strains produced a toxin which blackened pieces of broad bean leaves.

Pathogenicity. Three of six bean plants were infected with little-leaf disease by *Euscelis plebejus* insects injected with strain SP-A. Spiroplasmas were cultured from surviving insects and infected plants. Insects injected with strain ASP-1 infected two of six plants. *Euscelidius variegatus* infected with strain ASP-1 infected five of 21 bean plants. Only non-helical organisms were isolated from insects and plants. Bean plants infected with strain ASP-1 showed the same progression of symptoms as those infected by strain SP-A. Sections cut from SP-A-infected plants showed typical spiroplasmas in the phloem cells (Fig. 6a) but similar cells of ASP-1-infected plants contained straight filaments (Fig. 6b), many of which were branched.

DISCUSSION

Serology tests, DNA homology studies, electrophoretic analysis, the production of toxin and pathogenicity tests showed that ASP-1 was a strain of *S. citri* and not a contaminating filamentous mycoplasma.

Temporary changes in the morphology of *S. citri* have been noted before: ageing cultures of spiroplasmas contained non-helical cells which were often branched (Cole *et al.*, 1973). Possibly the morphology of different strains may change if their environment changes and indeed we have observed that some batches of horse serum cause changes in the cell and colony morphology of spiroplasmas. However, the absence of helical organisms in strain ASP-1 cultures under any of the conditions imposed, indicated that this strain was a non-helical variant of *S. citri*.

Despite its different morphology strain ASP-1 grew as well as strain SP-A. The apparent delayed start of exponential growth, as measured by c.f.u., may have been caused by the

clumping tendency of the non-helical organisms. *Acholeplasma laidlawii* infected with a virus produces atypical cratered colonies whereas when uninfected, it gives typical 'fried egg' colonies (Milne, Thompson & Taylor-Robinson, 1972). However, it seems unlikely that the smooth colonies produced by strain ASP-1 reflected the absence of viruses, since this strain contained all three spiroplasma viruses. The well-defined colony morphology appeared to be due to non-motile filaments being unable to form satellite micro-colonies, in contrast to normal spiroplasma colonies with their poorly defined edges and granular appearance caused by motile organisms moving away from the parent colony.

The absence of a membrane protein band in gel electrophoresis implied that at least one protein which might be of structural importance was either absent or bound less firmly into the membrane of strain ASP-1. Altered morphology and associated loss of motility, however, did not affect pathogenicity.

The variant may have arisen as a result of a single spontaneous mutation during the first two subcultures after primary isolation, but we have never observed similar mutations during a great many subcultures of other isolates. A non-helical form of *S. citri* may be present in some diseased trees together with the helical form. If this variant is found to occur in natural infections, it may be necessary to use criteria other than helical morphology for taxonomic purposes.

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