Mycoplasma-like Organisms from Plants with ‘Yellows’ Diseases Lack a Spiroplasma-specific Antigen

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An antigen which is specific to *Spiroplasma* and was unaffected by changes in cell morphology was used to demonstrate the presence of spiroplasmas in diseased plant material. The same antigen could not be detected in plants infected with eight different ‘yellows’ diseases with which non-cultivable mycoplasma-like organisms were associated.

INTRODUCTION

The characteristic feature of spiroplasmas which distinguishes them from all other cell wall-free prokaryotes is their helical morphology as seen in culture. However, they frequently lose this helical shape in the tissues of vector insects (Davis, 1979; Granados & Meehan, 1975; Townsend *et al.*, 1976; Whitcomb & Williamson, 1975) or infected plants (Davis *et al.*, 1981; Granados, 1969; Igwegbe & Calavan, 1970; Markham & Townsend, 1979; Townsend *et al.*, 1977). Instead they assume an apparently ovoid pleomorphic form which renders them indistinguishable from the mycoplasma-like organisms (MLOs) which are consistently associated with one hundred or so other ‘yellows’ plant diseases. Since these organisms have proved refractory to cultivation in cell-free media, it would be unwise to conclude that most yellows diseases are associated with mycoplasmas rather than spiroplasmas simply because profiles indicative of helices are not apparent in sections of diseased tissue. A more reliable diagnostic criterion is required which is stable and unique to spiroplasmas but independent of cell morphology and which can be detected in infected plants, without recourse to complex purification procedures or culture in cell-free media.

Spiroplasmas contain long flexuous fibrils which are composed of a single protein of mol. wt 55 000 (Townsend *et al.*, 1980a). Circumstantial evidence suggests that these form part of the mechanism which maintains cell shape and generates motility, although they also occur in a stable non-helical variant of *Spiroplasma citri*, ASP-1 (Townsend *et al.*, 1980b; Townsend *et al.*, 1977). Townsend & Archer (1983) have recently shown that this fibril protein is a *Spiroplasma*-specific antigen. Antibodies directed against SDS-denatured fibrils purified from the honeybee spiroplasma (BC3) recognized 55 000 mol. wt proteins in all the spiroplasmas examined by these authors, including ASP-1, but failed to react with any components in several *Mycoplasma* spp. or *Acholeplasma* spp. which were tested.

In the current study the effects of changes in spiroplasma morphology on the occurrence of fibril protein have been investigated. A method is described for the detection of specific antigens in diseased tissue and the results of screening for fibril protein antigen in plants infected with several different yellows diseases are reported.

METHODS

Spiroplasmas. The SP-A strain of *S. citri* (NCPFB 2526) (Markham *et al.*, 1974) and non-helical variants ASP-3 and ASP-12 were isolated in this laboratory. Honeybee spiroplasma BC3 was supplied by T. B. Clark, Bioenvironmental Bee Laboratory, Plant Protection Institute, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.

Cultures. Spiroplasmas were grown in SMC medium (100 ml) (Markham *et al.*, 1974) containing 10% (v/v) foetal calf serum and incubated at 32 °C. Cells were harvested after 36 h or 120 h by centrifugation (15000 g, 15 min, 4 °C). Spiroplasmas were isolated from plants as described previously (Markham *et al.*, 1974) except that duplicate 0.1 ml samples of diluted filtrate were mixed with SMC containing 0.7% agar at 48 °C and rapidly poured over the surface of 5 cm plates of SMC solidified with 1% agar. Resulting colonies were counted after 10–14 d incubation at 32 °C. This pour-plate method promoted better and more consistent growth of *S. citri* than direct plating (unpublished observation).

Infected material. Symptom-bearing tissue from Madagascar periwinkles (*Catharanthus roseus*) infected with five symptomatologically distinct yellows diseases originally occurring in apple (*Malus domestica*), cabbage (*Brassica oleracea*), clover (*Trifolium repens*), hydrangea (*Hydrangea macrophylla*) and primula (*Primula* sp.), as well as typical clover phyllody and European aster yellows, also in periwinkle (de Leeuw, 1977), was supplied by G. T. N. de Leeuw. The 'monstrose' variant of *Opuntia tuna* cactus (Casper *et al.*, 1970) and periwinkles infected with *S. citri* (SP-A) and corn stunt spiroplasma (Markham *et al.*, 1977) have been maintained for several years by grafting. *Dalbulus maidis* leafhoppers were used to transmit corn stunt spiroplasma (E275 Rio Grande ATCC 27954) to maize (*Zea mays*).

Electron microscopy. Small pieces of symptom-bearing shoot tissue were processed and examined as described previously (Markham *et al.*, 1974).

Sample preparation. Samples (100 mg) of symptom-bearing or healthy shoot and flower tissue from periwinkles, or pieces of maize leaf showing symptoms of severe chlorotic striping typical of corn stunt disease, were homogenized in 10 ml 50-mM-Tris (which was adjusted to pH 7.8 with 5 M-HCl and contained 60 mM-KCl and 6 mM-2-mercaptoethanol). Homogenates were clarified by centrifugation (1000 g, 5 min, 20 °C) and Triton X-100 was added to a final concentration of 1% (w/v). The solution was centrifuged (70000 g, 30 min, 20 °C) to bring down detergent-insoluble material, which includes spiroplasma fibrils (Townsend *et al.*, 1980a). The resulting pellets were extracted with detergent, centrifuged and finally taken up in SDS sample buffer (100 µl) (Laemmli, 1970) and heated at 98 °C for 5 min.

Harvested cells were washed in 25 ml 10 mM-Tris/HCl pH 7.8 (containing 5%, w/v, sorbitol to prevent cell lysis) and then centrifuged (15000 g, 15 min, 4 °C). The pelleted cells were then resuspended in 1 ml water with the aid of a sonic probe (Dawe type 7530A, London, U.K.) and then solubilized in an equal volume of 2 × SDS sample buffer. Protein determinations were made by a modified Coomassie brilliant blue binding assay (Zaman & Verwilghen, 1979).

Fibril protein was prepared from BC3 cells as described previously (Townsend *et al.*, 1980a) and dilutions containing between 500 µg ml⁻¹ and 5 ng ml⁻¹ were made in SDS sample buffer.

Electrophoretic separation, blotting and immunodetection of antigens. Samples of SDS-solubilized plant material (equivalent to 40 mg fresh tissue) or cell extracts (equivalent to 5 µg total cell protein) or samples containing between 5 µg and 0.05 ng purified fibril protein were electrophoresed in SDS-polyacrylamide linear gradient gels (7.5–25%, w/v, acrylamide). Separated proteins were then blotted on to nitrocellulose filters by diffusion in the presence of 4 M-urea (Bowen *et al.*, 1980). Antigens bound to the filters were detected by a double-antibody probe technique (Towbin *et al.*, 1979). Briefly, filters were probed first with antiserum raised against the SDS-denatured BC3 fibril protein (Townsend & Archer, 1983) (1:1000) and secondly with goat antiserum raised against rabbit IgG and conjugated to horse-radish peroxidase (Miles, Slough, U.K.) (1:2000). Bound conjugate was developed with the substrate 4-chloro-1-naphthol which gives rise to a blue coloured insoluble product (Hawkes *et al.*, 1982). Molecular weights were calculated with reference to standards of known molecular weight blotted to nitrocellulose and stained with Coomassie brilliant blue. Full details of this technique have been published recently (Townsend & Archer, 1983).

RESULTS

Morphology of spiroplasmas in culture

Cultures of *S. citri* (SP-A) contained numerous helical motile cells at the completion of the exponential phase of growth (36 h). If incubation was continued all the morphological changes associated with ageing spiroplasma cultures occurred (Townsend *et al.*, 1980b). After 120 h, all the cells had lost their helical shape and the filaments had begun to constrict into chains of small spherical bodies. These changes were accompanied by a decline in viability from more than 10⁹ c.f.u. ml⁻¹ at the end of exponential growth to less than 10⁴ c.f.u. ml⁻¹. Isolates ASP-3 and ASP-12 grew as short branching, non-helical filaments similar in appearance to cells of strain ASP-1 (Townsend *et al.*, 1980b; Townsend *et al.*, 1977).

Fig. 1. Blot showing the occurrence of antigens in plant extracts and BC3 spiroplasma cells. Gels were loaded with detergent-insoluble extracts equivalent to 40 mg fresh tissue from (track 1) healthy periwinkle and (tracks 2–10) periwinkles infected with 2, *S. citri*; 3, corn stunt; 4, clover phyllody; 5, clover disease; 6, cabbage disease; 7, European aster yellows; 8, primula disease; 9, hydrangea disease and 10, apple disease. Track 11 contains 125 ng BC3 cell protein. Smearing in track 2 is probably due to incomplete dissociation of the Triton X-100–protein complexes (Townsend *et al.*, 1980a).

Electron microscopy of infected plants

Representative samples from each of the eight yellows diseases contained numerous membrane-bound structures resembling mycoplasmas within the phloem sieve elements. Most of these structures were apparently spherical or ovoid but filamentous forms were present in some sections, particularly those from periwinkles infected with cabbage and hydrangea diseases. Sometimes these filaments terminated in chains of spherical bodies resembling structures which occurred in aged spiroplasma cultures (Townsend *et al.*, 1980b). The phloem sieve elements of the monstrose cactus were packed with ovoid MLOs, some of which were nearly 2 μ m in diameter, but no filaments or helical cells were detected.

Detection of fibril antigen in cultured spiroplasmas

Less than 0.5 ng purified fibril protein produced a detectable band at a position corresponding to a mol. wt of 55 000 on probed and developed blots. Blots of total SP-A proteins (36 h) showed the presence of a major antigen of the same molecular weight and another of mol. wt 23 000. The same proteins were present on blots derived from non-helical strains ASP-3 and ASP-12 and SP-A after 120 h incubation.

Detection of fibril antigen in diseased plants

Duplicate samples from two periwinkles graft-infected 4 weeks previously with *S. citri* contained significant amounts of 55 000 mol. wt antigen (Fig. 1, track 2). Several minor bands were also visible including one of mol. wt 23 000. Some of these were also detected in extracts from healthy plants (Fig. 1, track 1) and showed non-specific binding of rabbit globulins on blots incubated with pre-immune serum. No antigens of mol. wt 55 000 or 23 000 were detected in healthy plants. Extracts from periwinkles (Fig. 1, track 3) and two maize plants infected with corn stunt spiroplasmas also contained 55 000 mol. wt antigen. No 55 000 mol. wt antigen was detected in any samples of symptom-bearing tissue (two samples per plant) from pairs of periwinkles infected with any of the other diseases or from two monstrose cacti.

Table 1. *Isolation of colony-forming units from a periwinkle following graft infection with S. citri*Plant material was maintained at a mean temperature of $30 \pm 5^\circ\text{C}$.

No. of c.f.u. (g leaf tissue) ⁻¹		
Time after grafting (d):		
11	25	39
$< 1.0 \times 10^3$	1.6×10^8	7.5×10^6
$< 1.0 \times 10^3$	6.9×10^8	3.1×10^6
1.9×10^4	1.0×10^8	6.0×10^6

Isolation of spiroplasmas and detection of fibril antigen

Table 1 shows the numbers of c.f.u. isolated from a single periwinkle plant at various time intervals after grafting on three branches with *S. citri*. After 11 d, 55000 mol. wt antigen was just detectable in the only sample which contained quantifiable numbers of c.f.u. Thereafter, 55000 mol. wt material was detected in all samples. There was a marked decrease in c.f.u. titre between day 25 and day 39, by which time symptoms had become severe, but there was little difference in the amount of 55000 mol. wt material that was detected in samples taken on days 25 and 39.

DISCUSSION

The unstable morphology of spiroplasmas and the variety of filamentous and pleomorphic forms assumed by non-cultivable MLOs in plants clearly demonstrates the problems associated with the use of morphology as a diagnostic or taxonomic criterion. However, fibril protein was found to be a stable antigenic marker for *Spiroplasma*, which was not affected by changes in cell morphology.

Viable counting suggested that concentrations of spiroplasmas as low as 2×10^4 cells per g of infected tissue could be detected. However, tissue containing less than 6×10^6 spiroplasmas per g is unlikely to yield sufficient fibril protein to be resolved by this technique. This disparity probably reflects the loss of viable organisms during the extraction procedure. Changes in the morphology or impaired motility have been shown to result in losses of up to 99% of viable spiroplasmas during passage through 0.22 μm pore diameter membrane filters (Saglio, 1979).

Furthermore, since the antigenic character and concentration of fibril protein were not significantly affected by loss of cell viability, it is possible that many of the pleomorphic mycoplasma-like structures present in the phloem sieve elements of plants showing severe *S. citri* symptoms may no longer be viable.

The antiserum also reacted with proteins of 23000 mol. wt from cultured cells and spiroplasma-infected plants. These proteins are thought to be related to spiralin which is the major *S. citri* membrane antigen (Archer & Townsend, 1981).

It has been reported that a spiroplasma serologically related to *S. citri* was isolated from a monstrose variant of *O. tuna* (Kondo *et al.*, 1976). The cacti used in the present study showed the same symptoms but did not contain detectable quantities of fibril protein. Although repeated attempts to culture spiroplasmas from these plants have been unsuccessful (unpublished results) it is possible that they are dually infected with a non-cultivable MLO and *S. citri* which is present in very low concentrations. A disease complex of *S. citri* and an MLO associated with virescence (greening of the flowers) of *C. roseus* has recently been described (Oldfield, 1982). Dually infected plants showed typical virescence, symptoms of *S. citri* infection were markedly suppressed and the numbers of spiroplasmas present were significantly lower than in appropriate controls (Kloepper *et al.*, 1982).

The sensitivity with which fibril protein could be detected by the method described here is comparable to that achieved using enzyme-linked immunosorbent assay (ELISA) of non-denatured spiroplasma antigens (Archer & Best, 1980). Unlike ELISA, this method is not quantitative and is unsuitable for multiple assays. But, it can provide qualitative information on the occurrence and relative concentrations of a number of different antigens in a complex

mixture depending only on a difference in molecular weight. No fibril protein was detected in plants infected with any of the other yellows diseases although they contained numerous MLOs. It has been estimated that the phloem sap of a plant infected with a typical yellows disease contains as many as 10^{13} MLOs ml^{-1} (Daniels *et al.*, 1982). This represents about 10^{10} MLOs per g of *C. roseus* tissue which is well within the detection limits of the method described here, even allowing for antigenic diversity among proteins from different spiroplasmas (Townsend & Archer, 1983). Therefore it is very unlikely that the non-cultivable MLOs associated with the majority of yellows diseases are related to *Spiroplasma*.

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