A Fibril Protein Antigen Specific to *Spiroplasma*

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An antiserum, raised in rabbits against honey-bee spiroplasma (BC3) fibril protein monomer, mol. wt 55000, excised from SDS–polyacrylamide gels, had precipitating activity against SDS-denatured fibril protein but not against the native protein. This antiserum was used to probe spiroplasma cell proteins separated by SDS–PAGE and blotted on to nitrocellulose filters. Antigens of mol. wt 55000 were identified in 13 different spiroplasmas, including a non-helical strain of *Spiroplasma citri*, representing five different sero-groups. The antiserum did not react with proteins in any of the *Mycoplasma* spp. or *Acholeplasma* spp. tested. Two-dimensional immunoelectrophoresis revealed variations in the amount of antigen in different isolates from the same sero-group. Peptide mapping by limited proteolysis showed that the fibril protein was highly conserved within a sero-group but that there was some heterogeneity between groups. All fibril proteins yielded common peptides recognised by the antiserum.

INTRODUCTION

Helical morphology and rotary motility are properties which distinguish members of the family *Spiroplasmataceae* (genus *Spiroplasma*) from other families of the Class Mollicutes. The reliability of morphology as a taxonomic criterion has been called into question by the isolation of non-helical and non-motile spiroplasma strains (Townsend *et al.*, 1977). We have attempted to identify alternative properties characteristic of *Spiroplasma*.

Several different spiroplasmas release long flexuous fibrils 3–4 nm in diameter when cell lysis is induced by osmotic shock or non-ionic detergents (Stalheim *et al.*, 1978; Williamson, 1974; Williamson & Whitcomb, 1974). Fibrils purified from the honey-bee spiroplasma (BC3) are composed of a single protein of mol. wt 55000 unrelated to any bacterial proteins (Townsend *et al.*, 1980*a*). The function of these structures is unresolved but they may play a role in maintaining the helical morphology and rotary motility of these cell wall-less prokaryotes (Townsend *et al.*, 1980*a*). Similar fibrils have been observed in a non-helical strain of *Spiroplasma citri* (Townsend *et al.*, 1980*b*). However, this organism lacks a single membrane-associated protein which may also be important in determining cell shape (Townsend *et al.*, 1977).

We have examined the distribution of fibril protein among spiroplasmas from five different sero-groups (Junca *et al.*, 1980) including a non-helical isolate of *S. citri* (Townsend *et al.*, 1977). We have also looked for the presence of fibril protein in several *Mycoplasma* spp. and *Acholeplasma* spp. which have been reported to contain fibrillar structures (Göbel *et al.*, 1981; Meng & Pfister, 1980) or 'actin-like' proteins (Ghosh *et al.*, 1978; Kahane & Muhlrud, 1977; Neimark, 1977).

METHODS

Organisms. The following cloned cultures were used: sero-group I-1, *S. citri* SP-A (NCPFB 2565), R8A2 (ATCC 27556) and the non-helical isolate ASP-1 (NCPFB 3095); sero-group I-2, honey-bee spiroplasma BC3, obtained from T. B. Clark, Bioenvironmental Bee Laboratory, Plant Protection Institute, U.S. Department of

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Agriculture, Beltsville, Maryland, U.S.A.; sero-group I-3, corn stunt spiroplasma E275 Rio Grande (ATCC 27954); sero-group I-4, tick spiroplasma 277F, obtained from R. F. Whitcomb, Insect Pathology Laboratory, Plant Protection Institute, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.; the other sero-group I spiroplasma, *Cocos* isolate (ATCC 33287); sero-group III, flower spiroplasma BNR1, obtained from R. F. Whitcomb, and *Spiroplasma floricola* 23-6A (ATCC 29989); sero-group IV, flower spiroplasma SR3 (ATCC 33095); sero-group V, tick spiroplasma SMCA (S221), obtained from R. F. Whitcomb; the unassigned spiroplasma, BREVI (ATCC 33474); *Mycoplasma gallisepticum* (NCTC 10115); *Mycoplasma pneumoniae* (NCTC 10119); *Mycoplasma orale* (NCTC 10112); *Acholeplasma axanthum* (NCTC 10138); *Acholeplasma granularum* (NCTC 10128); *Acholeplasma laidlawii* (NCTC 10116) and *Acholeplasma oculi* (NCTC 10150).

Culture. All spiroplasmas were grown in medium M1A (Jones *et al.*, 1977) supplemented with 10% (v/v) 10 × CMRL tissue culture medium (GIBCO, Irvine, U.K.), instead of Schneider's *Drosophila* medium, and 10% (v/v) heat-inactivated foetal calf serum. Mycoplasmas and acholeplasmas were grown in a standard mycoplasma medium (Hayflick, 1965). *Mycoplasma pneumoniae* was provided as a washed cell suspension by M. Sillis, Mycoplasma Reference Laboratory, Bowthorpe Road, Norwich, U.K.

Sample preparation. Cells were harvested from 100 ml cultures by centrifugation (20000 g for 20 min at 4 °C), washed in 10 mM-Tris [which was adjusted to pH 7.4 with HCl and contained 7% (w/v) sorbitol] and finally resuspended in 1 ml water. The cells were disrupted with a sonic probe (Dawe type 7530A, London, U.K.) and stored at -20 °C.

Triton X-100 extracts were made by adding 100 µg total cell protein to 5 ml detergent (1%, w/v) and centrifuging at 75000 g for 45 min at 4 °C. The detergent-insoluble material was washed in 10 mM-Tris/HCl buffer and freeze-dried.

Protein assay. The protein content of cell suspensions and purified fibril preparations was determined by the Lowry method or, after solubilization in SDS, by Coomassie brilliant blue binding (Zaman & Verwilghen, 1979).

PAGE. Samples of BC3 spiroplasma fibril protein, purified as described previously (Townsend *et al.*, 1980a), or total cell proteins were freeze-dried and resuspended in SDS/2-mercaptoethanol sample buffer (Laemmli, 1970). Samples were electrophoresed in slabs of polyacrylamide 15 × 12 cm in size and 0.15 cm thick, prepared according to Laemmli (1970) but containing either 15% (w/v) acrylamide or 7.5–25% linear gradients of acrylamide (Covey & Hull, 1981). A potential difference of 100–150 V was applied until the bromophenol blue marker dye, incorporated into the sample, had reached the end of the gel. Gels were stained with Coomassie brilliant blue (Daniels & Meddins, 1973).

Partial enzymic proteolysis. Proteins insoluble in Triton were separated on 15% polyacrylamide gels which were stained for 30 min in Coomassie brilliant blue and de-stained rapidly (1 h) in several changes of 15% (v/v) methanol and 5% (v/v) acetic acid. Bands corresponding to molecular weights of 55000 were excised and processed according to the method of Cleveland *et al.* (1977). Digestion with *Staphylococcus aureus* V8 protease (0.25 µg per gel slice) occurred in the stacking gel and the digestion products were separated by subsequent electrophoresis through a 15–30% linear gradient acrylamide gel. Gels were silver-stained using the method described by Oakley *et al.* (1980).

Preparation of antiserum. Fibrils were partially purified from BC3 cells harvested from 2 l culture as previously described (Townsend *et al.*, 1980a) but omitting the salt treatment before isopycnic centrifugation. Fibrils, equivalent to about 2 mg protein, were solubilized in sample buffer and loaded across the entire width of two 15% acrylamide slab gels. After electrophoresis, slices of gel were stained to locate the 55000 mol. wt polypeptide. The corresponding unstained strips of gel were excised, washed in 10 mM-Tris/HCl buffer pH 7.0 for 1 h and then homogenized in 3 ml Freund's complete adjuvant and stored at -20 °C. The total sample was divided into four 1.5 ml samples which were injected subcutaneously into a New Zealand white rabbit at bi-weekly intervals. The animal was bled weekly after the last injection. The activity of the antiserum was monitored by double immunodiffusion (Crowle, 1961) against purified fibril protein or BC3 cells solubilized in SDS (1%, w/v). Samples showing precipitating activity were pooled for use in these experiments.

Protein blotting and immunodetection of antigens. Proteins solubilized in SDS sample buffer and separated by PAGE were blotted on to S & S nitrocellulose membrane filters (0.45 µm pore diameter; Anderman & Co., East Molesey, U.K.) by diffusion in the presence of 4 M-urea (Bowen *et al.*, 1980). The pattern of transferred proteins was revealed using Coomassie brilliant blue stain followed by rapid de-staining in 50% methanol and 5% acetic acid to avoid damaging the nitrocellulose filters. The double antibody technique used to detect bound antigens was based on that of Towbin *et al.* (1979). Dried blots were incubated for 2 h in 20 mM-Tris/HCl buffer pH 7.4 containing 0.9% (w/v) sodium chloride and 1% (w/v) bovine serum albumin (BSA; fraction V) to block non-specific binding sites. The primary antiserum or pre-immune serum was then added at a concentration of 1:1000 and the blots were incubated overnight. After washing five times with 200 ml Tris-buffered saline without BSA the blots were incubated for 2 h in Tris-buffered saline with BSA containing a 1:2000 concentration of goat antiserum raised against rabbit IgG and conjugated to horse-radish peroxidase (Miles, Slough, U.K.). After further washing, the bound conjugate was developed by incubating in 10 mM-Tris/HCl pH 7.2 containing 0.01%

(v/v) hydrogen peroxide and 0.05% (w/v) 4-chloro-1-naphthol (Hawkes *et al.*, 1982). Areas to which antibodies had bound were identified by development of a blue coloured product which was insoluble in aqueous solutions and light-stable. All incubations were performed at room temperature.

Immunoelectrophoresis. Two-dimensional immunoelectrophoresis using proteins separated by SDS-PAGE was performed as described previously (Archer & Townsend, 1981). Comparisons were made using stained first dimension gels which had not been electrophoresed in the second dimension.

RESULTS

Double diffusion tests

Serum from rabbits immunized with SDS-denatured fibril protein formed strong precipitin lines against antigen or BC3 cells solubilized in SDS but failed to react with sonicated preparations of purified fibrils, BC3 cells or 0.1% SDS.

Immunodetection of antigens on protein blots

A comparison of stained blots of BC3 proteins and corresponding gels stained before and after blotting showed that most of the detectable material had been transferred to the nitrocellulose filters. All the major proteins appeared to transfer quantitatively irrespective of molecular weight except one of 25000 which was selectively retained within the gel (Fig. 1*a*).

Blots of total BC3 proteins probed with antiserum raised against BC3 fibril protein showed large quantities of antigen located at a position equivalent to a molecular weight of 55000 (Fig. 1*b*, track 3). Two or three minor antigens of lower molecular weight were also detected, in particular one of mol. wt 25000. Similarly, probed blots of all the spiroplasmas detailed in Methods, including the non-helical isolate, ASP-1, also revealed a major antigen of mol. wt 55000 (Fig. 1*b*). The amount of antigen detected varied between spiroplasmas. The SR-3 spiroplasma (Fig. 1*b*, track 8) and the BREVI (Fig. 1*b*, track 10) isolates, loaded at a concentration of 5 µg total cell protein, produced relatively faint bands which became quite clear if the loading was increased fivefold (Fig. 1*c*). Some spiroplasmas contained small amounts of antigenic material of mol. wt 30000–40000 but nearly all bound antibodies to a protein, in the range 23000–25000, which was present in particularly large amounts in the corn stunt spiroplasma. Some smearing of antigenic material was associated with those strains which normally produce low cell yields in culture and was attributed to interference with solubilization of cellular proteins by the higher proportion of precipitated medium components (Townsend & Daniels, 1981). Non-specific binding of globulins or spiroplasma peroxidase activity was not observed when duplicate blots were probed with pre-immune serum.

The antiserum did not recognize any antigens in any of the *Mycoplasma* spp. or *Acholeplasma* spp. examined, even when the loading was increased to 50 µg of total cell protein (Fig. 1*b*, tracks 11–16).

Immunoelectrophoresis

A single rocket was formed in the antiserum-containing slab, located directly over the fibril protein band in the first-dimension gel at a position equivalent to a molecular weight of 55000 (Fig. 2*a*). Rockets of similar shape were produced in the same position relative to gels of BC3 whole cell proteins (Fig. 2*b*). No other rockets were visible and no stainable material remained in the first-dimension gel. The absence of precipitated 25000 mol. wt material almost certainly reflects the reduced sensitivity of this technique compared to the immunodetection of antigens on protein blots. Spiroplasmas from sero-groups I and V produced sharp rockets of varying dimensions at the 55000 mol. wt position (Fig. 2*c*). After corrections for differences in protein loading, the quantities of precipitated antigen were determined on the basis of the area below each rocket relative to those produced by known concentrations of purified fibril protein (Table 1). Only the corn stunt spiroplasma gave rise to an additional rocket at the 25000 mol. wt position. Group III and group IV spiroplasmas only produced rockets when a total of 200 µg cell protein was loaded and the concentration of antiserum doubled. Even then the rockets stained too diffusely to be measured accurately (Fig. 2*d*).

Fig. 1. (a) Polyacrylamide gel, stained after blotting of SDS soluble BC3 whole cell proteins to nitrocellulose filters (cf. Fig. 1b, track 3). (b) Immunodetection of antigens bound to filters. Samples (5 µg) of SDS-solubilized total cell protein were separated by electrophoresis in a linear gradient polyacrylamide gel and blotted on to a nitrocellulose filter which was then probed with antiserum raised against SDS-denatured BC3 fibril monomer, mol. wt 55000, and developed as described. The tracks were loaded as follows: track 1, SP-A; track 2, ASP-1; track 3, BC3; track 4, E275; track 5, 277F; track 6, *Cocos* isolate; track 7, BNR1; track 8, SR-3; track 9, SMCA; track 10, BREVI; track 11, *M. gallisepticum*; track 12, *M. hominis*; track 13, *M. pneumoniae*; track 14, *A. axanthum*; track 15, *A. oculi*; and track 16, *A. laidlawii*. (c) As Fig. 1b but loaded with 25 µg SDS-solubilized total cell protein from: track 1, SR-3; track 2, BREVI.

Fig. 2. Immuno-electrophoresis of SDS-solubilized antigens. The lower gels were polyacrylamide gels loaded at the right-hand side as viewed. (a) Purified BC3 fibril protein, mol. wt 55000. (b-d) SDS-solubilized whole cell proteins from: (b) BC3 (50 µg), (c) SMCA (100 µg), and (d) SR-3 (200 µg). Agarose second-dimension gels in deoxycholate contained antiserum against SDS-denatured BC3 fibril monomer.

Fig. 3. (a) Electrophoretic separation of peptides generated by limited proteolysis with *S. aureus* V8 protease of gel slices containing 55000 mol. wt material from: track 1, purified BC3 fibrils (without enzyme); track 2, purified BC3 fibrils. Tracks 3-6, Triton-insoluble proteins from: track 3, BC3; track 4, SP-A; track 5, BNR1; and track 6, SR-3. Track 7 contained enzyme only. Asterisks indicate the three peptides in each digest with similar staining properties. (b) Peptides from V8 digests were blotted on to a nitrocellulose filter which was then probed with antiserum against SDS-denatured BC3 fibril protein monomer to detect peptides binding antibodies. Digests were as follows: track 1, purified BC3 fibril protein (no enzyme); track 2, purified BC3 fibril protein; track 3, BC3; track 4, BNR1; track 5, SR-3 and track 6, SMCA.

Table 1. Concentration of fibril antigen in different *spiroplasmas* as determined by immunoelectrophoresis

Sero-group	Strain	Fibril antigen concentration [mg (100 mg total cell protein) ⁻¹]
I-1	<i>S. citri</i> R8A2	0.8
I-2	<i>S. citri</i> SP-A	0.5
I-3	BC3	3.1
I-4	E275	1.3
I-4	277F	0.9
III	SMCA	1.2

Analysis of proteolysis products

Upon re-electrophoresis of gel slices containing undigested fibril protein, two additional peptides of mol. wt 28000 and 27000 appeared. It was assumed that these arose as a consequence of the acid hydrolysis of the monomer during primary staining. They proved partially resistant to digestion with V8 protease but all the remaining 55000 mol. wt material was digested and reproducibly yielded seven major peptides with molecular weights below 21000. We observed that each peptide stained with a different shade of colour. Digestion of the equivalent polypeptide excised from the separation of total BC3 Triton-insoluble proteins generated an identical set of fragments as did the digests of fibril protein from other group I *spiroplasmas* (Fig. 3a). Protein from the SMCA *spiroplasma* was cleaved to give reduced quantities of the same fragments and an additional product of mol. wt 23000. The peptide patterns generated by digestion of 55000 mol. wt protein from BNR1 and SR-3 were similar but were significantly different to that derived from the BC3 protein (Fig. 3a). However, all the digests contained at least three peptides which varied slightly in molecular weight but which stained the same colour

(Fig. 3a). In order to investigate the possibility that these peptides were derived from an antigenically conserved portion of the protein, duplicate digests were performed and the cleavage fragments blotted on to nitrocellulose filters which were then probed with antiserum to the SDS-solubilized BC3 fibril monomer or pre-immune serum.

Antibodies bound to all the V8 cleavage fragments from the BC3 and SMCA proteins including the extra 23000 mol. wt product. Antibody binding to BNR1 and SR-3 peptides was much weaker and at least one major fragment in each digest was unrecognized, but the three conserved peptides were identified (Fig. 3b). No peptides reacted with pre-immune serum.

DISCUSSION

By immunodetection of SDS-solubilized proteins blotted on to nitrocellulose filters we have demonstrated that representatives of four of the five recognized sero-groups within the family *Spiroplasmataceae* (Junca *et al.*, 1980), as well as the BREVI spiroplasma [which probably represents a new sero-group (R. E. Davis, personal communication)], contain a 55000 mol. wt protein antigenically related to the fibrils of the BC3 spiroplasma. We did not examine any representatives of sero-group II which comprises the non-cultivable *Drosophila* sex-ratio spiroplasmas. At least one member of this group has been shown to contain fibrils indistinguishable by electron microscopy from those observed in *S. citri* (Williamson, 1974). Since the non-helical isolate of *S. citri* contains fibril antigen, this protein can be used as a marker by which spiroplasmas exhibiting aberrant morphologies in culture may be correctly identified. The failure of the antiserum to react with any proteins in the *Mycoplasma* spp. or *Acholeplasma* spp. examined supports our previous assertion (Townsend *et al.*, 1980a) that spiroplasma fibrils are unrelated to 'actin-like' proteins isolated from mycoplasmas or to the fibrillar elements which occur in *M. pneumoniae*. These structures are almost certainly unique to *Spiroplasma*.

The antiserum also contained antibodies which recognized a 25000 mol. wt protein in BC3 which transferred with low efficiency to nitrocellulose. This protein is probably related to spiralin (Wróblewski *et al.*, 1977), the major membrane antigen in *S. citri* (Archer & Townsend, 1981). We have previously reported that large quantities of this protein co-purify with fibrils (Townsend *et al.*, 1980a). Purified spiralin can form dimers, even in SDS gels, which give rise to a second minor rocket in two-dimensional immunoelectrophoretic separations (Archer & Townsend, 1981). The dimer ran only slightly faster than fibril protein on SDS gels and may have been excised with the fibril protein. The BC3 spiralin is highly antigenic and only small quantities are required to elicit specific antibodies. Similar low molecular weight proteins were detected in all group I spiroplasmas except the *Cocos* isolate, as well as the BNR1 and SR3 spiroplasmas. This wide distribution of antigenically related proteins suggests they may have functional similarity. It has been proposed (Townsend *et al.*, 1980a) that spiralin may play a role in determining the cell shape of *S. citri*, possibly in association with fibrils and a 39000 mol. wt protein absent from the non-helical isolate ASP-1 (Townsend *et al.*, 1977).

The quantity of fibril protein in BC3 cells, determined by two-dimensional immunoelectrophoresis, is in fairly close agreement with the estimate of 2% of total cell protein based on the yield of purified material (Townsend *et al.*, 1980a). The variations in antigen concentration between different group I spiroplasmas could be correlated with the amount of stainable 55000 mol. wt material resolved by SDS-PAGE (Daniels *et al.*, 1980). These findings are supported by direct electron microscopic observations which show that cells of the BC3 spiroplasma release about five times as many intact fibrils as cells of *S. citri* (SP-A) when lysed *in situ* (R. Townsend, unpublished observation). The variations in fibril protein concentration between different sero-group I organisms, and in particular the closely related BC3 and SP-A spiroplasmas, should provide a starting point for comparative studies to investigate the function of spiroplasma fibrils.

Both the BNR1 and SR-3 spiroplasmas contained much more 55000 mol. wt material than was indicated by the size of the rockets generated during immunoelectrophoresis. The unusual appearance of these rockets also indicated that there was antigenic heterogeneity between fibril

proteins from different sero-groups. Peptide mapping of V8 protease products, in conjunction with blotting and antibody probing, confirmed that the fibril protein is highly conserved amongst group I spiroplasmas and is closely related to the 55000 mol. wt protein in the group V spiroplasma, SMCA. Although corresponding proteins in spiroplasmas from other sero-groups exhibit variations in primary structure, the monomer molecular weight is not significantly altered. Furthermore, there is sufficient conservation of structure to maintain at least one recognition site for antibodies directed against BC3 fibril protein. The wide specificity of the antiserum was reflected in its capacity to react with all the peptides generated by proteolysis of the homologous BC3 antigen. This may be a general feature of antiserum prepared against SDS-denatured proteins and leads us to speculate that a similar antiserum raised against the native protein subunit might be more specific.

Silver staining proved to be a simple and relatively inexpensive alternative to the production of radioactively labelled substrates and the detection of peptide fragments by autoradiography as described by Cleveland *et al.* (1977). We found it to be about 50 times more sensitive than Coomassie brilliant blue staining. It also introduces an extra dimension of colour into the analysis of products which could probably be enhanced still further by using a recently described technique (Sammons *et al.*, 1981). The relationship between colour of staining and primary structure (Sammons *et al.*, 1981) enabled us to predict relationships between different peptides which were subsequently confirmed by antibody binding.

The characteristic helical morphology of cultured spiroplasmas is frequently lost in the tissues of insect and plant hosts (for review, see, Markham & Townsend, 1979) where they become indistinguishable from the non-cultivable mycoplasma-like organisms associated with 'yellows' diseases of plants. The presence of fibril protein is diagnostic for spiroplasmas and it can be detected at very low levels by the techniques described in this paper. In this way, the notion that other plant mycoplasma-like organisms are in fact non-cultivable spiroplasmas (Davis, 1974) can be tested directly. Also, unusual fibril structures composed of thin filaments 4–6 nm in diameter have been observed in the brains of animals infected with scrapie (Merz *et al.*, 1981) and electron microscopic evidence has been interpreted to indicate the presence of a spiroplasma in post-mortem tissue from a patient with Creutzfeld-Jakob disease (Bastian 1979; Bastian *et al.*, 1981). It should now be possible to investigate the implication of spiroplasma involvement in any clinical situation or pathogenesis in plants without culture of the agent.

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