Serological Relatedness of Spiroplasmas Estimated by Enzyme-linked Immunosorbent Assay and Crossed Immunoelectrophoresis

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Enzyme-linked immunosorbent assay (ELISA) has been used to estimate the serological relatedness of spiroplasmas. The method was shown to be a sensitive and discriminating quantitative technique, with a minimum detection level of between 10^4 and 10^5 spiroplasmas. Antisera to *Spiroplasma citri* and corn stunt spiroplasma, and a monospecific antiserum to a purified membrane protein, spiralin, were used to compare spiroplasmas isolated from plant and insect hosts. Spiroplasma cell surface antigens were compared using intact cells as the antigens for ELISA, while the complete ranges of cell antigens were compared using lysed cells. Distinct serological groupings of spiroplasmas emerged, in accord with current schemes. Homology of some *Spiroplasma citri* and honeybee (BC3) spiroplasma antigens was confirmed by crossed immunoelectrophoresis. ELISA is discussed in relation to results from other serological typing procedures, and conditions for comparing isolates by tandem crossed immunoelectrophoresis are described.

INTRODUCTION

Despite the isolation of helical mycoplasmas from a diversity of plant and insect hosts, there is presently only one recognized species of *Spiroplasma* (*S. citri*) within the class Mollicutes although there is considerable support for the elevation of other isolates to species status. While comparison of spiroplasma DNA by guanine plus cytosine content, hybridization or restriction enzyme analysis (Bové & Saillard, 1979; Christiansen *et al.*, 1979) or comparison of cell proteins by one- or two-dimensional gel electrophoresis (Bové & Saillard, 1979; Mouches *et al.*, 1979) may be more precise methods for classification, serological typing is often more convenient. Recently, two serological comparisons of a large number of spiroplasma isolates (Davis *et al.*, 1979; Williamson *et al.*, 1979) indicated that at least four distinct serological groups of spiroplasmas exist. Two of the serological tests used by Davis *et al.* (1979) relied upon antigens solubilized in sodium dodecyl sulphate (SDS) and, while a number of proteins do retain some antigenicity in SDS (Kahane & Razin, 1969; Bjerrum *et al.*, 1975; Bhakdi *et al.*, 1976), the anionic detergent binds cooperatively to proteins and denatures them (Tanford & Reynolds, 1976), destroying a number of antigenic sites. Both Davis *et al.* (1979) and Williamson *et al.* (1979) employed the sensitive deformation test (Williamson *et al.*, 1978) which compares cell surface antigens. Enzyme-linked immunosorbent assay (ELISA) has been used to detect spiroplasmas (Clark *et al.*, 1978; Saillard *et al.*, 1978) and it was of interest to determine if this method, when quantified, was as sensitive and discriminating as the deformation test.

Spiralin is the major membrane protein of *S. citri* and is known to span the membrane and be antigenic (Wróblewski *et al.*, 1977a; Wróblewski, 1978). The role of spiralin in a comparison of cell surface antigens is therefore of great interest and was investigated by

ELISA using antiserum raised against purified spiralin. Two spiroplasmas, *S. citri* and the honeybee (BC3) spiroplasma, found to be related serologically were also compared by tandem crossed immunoelectrophoresis to estimate the number of common antigens.

METHODS

Organisms. *Spiroplasma citri* (Israel) strains SP-4 and SP-A (NCPBP 2565) were from M. J. Daniels and *S. citri* (Israel) non-motile variants ASP-1 (NCPBP 3095) and ASP-25 were from R. Townsend. *Spiroplasma citri* (Algeria) ALG, *S. citri* (Morocco) MH and *S. citri* (Morocco) R8A2 (ATCC 27556) were from J. M. Bové. Corn stunt spiroplasma (Jamaica) B969 was from S. Eden-Green and corn stunt spiroplasmas Rio Grande (E isolate) (ATCC 27954) and Mississippi E were obtained from R. F. Whitcomb. The honeybee (BC3) spiroplasma was from T. B. Clark. Tulip tree flower isolate SR-3 (ATCC 33095) was from R. E. Davis. Tulip tree flower isolate BNR-1 and tick isolates, suckling mouse cataract agent (SMCA) (ATCC 29335) and 277F (ATCC 29761), were from R. F. Whitcomb. The mycoplasma strains were *Acholeplasma laidlawii* NCTC 10116 and *Mycoplasma fermentans* NCTC 10117.

Growth of organisms. All spiroplasmas, apart from corn stunt organisms and tick isolates, were grown at 32 °C in medium containing 2.1 % (w/v) PPLO broth (Difco), 7 % (w/v) sorbitol, 0.1 % (w/v) glucose, 0.1 % (w/v) sucrose, 0.1 % (w/v) fructose and 10 % (v/v) foetal calf serum (Sera-Lab, Sussex). The foetal calf serum was dialysed against 0.1 M-KCl, held at 56 °C for 1 h and filtered (0.45 µm pore size filter) before use. Corn stunt spiroplasmas were grown at 29 °C in the same medium supplemented with 0.01 % (w/v) 2-oxoglutaric acid. Tick isolates (277F and SMCA) were grown at 32 °C in SP-4 medium (Tully *et al.*, 1977) containing 10 % (v/v) foetal calf serum. Other mycoplasmas were grown at 37 °C in the unsupplemented medium. Growth was detected by microscopic observation under dark field optics and cell numbers were estimated by counting cells in a micro-haemocytometer slide (Hawksley, London). Cell counts of helical organisms in actively growing cultures by this method agreed very well with estimates of viable cells made by serial dilutions of cultures and counting colonies grown on solid media.

Antisera. Antiserum to *S. citri* strain SP-A was a gift from R. Townsend. Antiserum to corn stunt spiroplasma (Rio Grande) was a gift from S. Eden-Green (ex. J. G. Tully). Antiserum to purified spiralin (purified from *S. citri* strain C189) was a gift from H. Wróblewski. Washed cell suspensions were used as antigens for three, once-weekly, intravenous injections into rabbits, and Freund's incomplete adjuvant was added for subcutaneous injection, administered once. Sera with (homologous) deformation titres of over 10000 were collected about 6 weeks after the initial challenge.

Enzyme-linked immunosorbent assay (ELISA). Purification of γ -globulins, their conjugation to alkaline phosphatase, and the essential details of the ELISA method were carried out as described by Clark & Adams (1977). γ -Globulins at 10 and 1 µg ml⁻¹ were used to coat the wells of the microtitre plates (Sterilin) and incubated for 4 h at 32 °C. After washing (Clark & Adams, 1977), the test sample was incubated in the wells for 18 h at 4 °C. The wells were then washed, and alkaline phosphatase conjugated to γ -globulins was added at dilutions of 400, 800 and 3200 (Clark & Adams, 1977) and held at 37 °C for 6 h before washing once more; enzyme substrate (*p*-nitrophenyl phosphate, Sigma) was then added at 0.6 mg ml⁻¹. A range of antigen concentrations was used for each determination, usually over a 1000-fold dilution range. Plates containing the conjugate and enzyme substrate were incubated at either 4 or 25 °C, and usually for 18 h, during which time the colour production was linear with all but the highest levels of bound conjugate, when shorter reaction times were required.

Intact cell antigens were cultures diluted in phosphate-buffered saline (PBS; 0.02 M-sodium phosphate pH 7.4, 0.15 M-NaCl) containing 0.05 % (w/v) Tween and 2 % (w/v) polyvinylpyrrolidone. Little cell lysis was apparent in this solution by microscopic examination. Broken cell antigens were dilutions of cultures disrupted ultrasonically using a Dawe Soniprobe type 7530A (Dawe Instruments, London).

Homologous antigens were included on each test plate as a standard, as were blank wells lacking any antigen.

Crossed immunoelectrophoresis. The method of crossed immunoelectrophoresis has been described by Clarke & Freeman (1967). The procedure used here differs in detail from this and other methods and is described below. Antigens were prepared by harvesting organisms in the exponential phase of growth by centrifuging at 15000 *g* for 15 min. They were washed once in PBS containing 7 % sorbitol, resuspended in 2 M-glycerol and incubated at 30 °C for 15 min. The cell suspension was then injected into ice-cold water whereupon the cells lysed and membranes were harvested by centrifuging at 30000 *g* for 30 min. This glycerol loading procedure facilitates complete lysis of mycoplasmas (Rottem *et al.*, 1968), and although not necessary for lysis of *S. citri* it was found to be a satisfactory method for lysing the osmotically more resistant bee (BC3) spiroplasma. Membranes were washed twice in 20 mM-Tris/HCl, pH 7.5, and then solubilized in

50 mM-Tris/citric acid, pH 8.6, (TC buffer) containing either 2 % (v/v) Triton X-100 or 5 % (w/v) sodium deoxycholate. The final protein concentration was 2 mg ml⁻¹. The mixture was sonicated briefly and then centrifuged at 30000 g for 30 min. The supernatant obtained was used as the antigen preparation in crossed immunoelectrophoresis.

First dimension electrophoresis using 20 µg protein antigen was performed in 1 % (w/v) agarose (low electroendosmosis; Miles Laboratories, Stoke Poges) gels in TC buffer (electrode buffer) containing either 0.5 % (v/v) Triton X-100 or 0.5 % (w/v) sodium deoxycholate. Gels were cooled by circulating water at about 15 °C and run at 7.5 V cm⁻¹ for either 1.5 h (Triton X-100) or 1 h (deoxycholate). The second dimension antibody-containing gel was run at 2.5 V cm⁻¹ for 18 h. Tandem crossed immunoelectrophoresis and immunoelectrophoresis with an intermediate gel have been described (Bjerrum & Bøg-Hansen, 1976).

After electrophoresis, gels were pressed under filter paper, soaked in 0.1 M-NaCl for 15 min, pressed again and dried. Staining was for 5 min in 0.5 % (w/v) Coomassie brilliant blue R (in ethanol/acetic acid/water; 9:2:9, by vol.) and the gels were then destained in the ethanol/acetic acid/water mixture.

Polyacrylamide gel electrophoresis. Separation of cell proteins solubilized in 1 % (w/v) sodium dodecyl sulphate (SDS) was by one-dimensional polyacrylamide (12 %, w/v, acrylamide) slab gel electrophoresis in the presence of 0.1 % SDS (Archer *et al.*, 1978). Staining was by the method of Fairbanks *et al.* (1971). Protein molecular weight standards were run on the same gel slabs as cell protein samples. The standards used were β-galactosidase (molecular weight 116000), phosphorylase a (94000), bovine serum albumin (67000), aldolase (40000), concanavalin A (26000) and myoglobin (17200). Solubilized proteins of *S. citri* R8A2 and tulip tree spiroplasma (BNR-1) were provided by M. A. Stephens.

Protein estimation. Protein content of cell and membrane samples was estimated by the method of Lowry, using bovine serum albumin as the standard.

RESULTS

Quantification of ELISA

Using intact spiroplasmas as the antigen together with the homologous antiserum the ELISA reaction was quantified with respect to cell number (and cell protein content) and reaction time with a variety of combinations of γ-globulin and conjugate concentrations present in each plate. Using a cell concentration of 10⁶ ml⁻¹ (sample volume 0.2 ml) and a conjugate dilution of 800 the enzyme reaction was followed over a period of 21 h (Fig. 1), for two different γ-globulin well-coating concentrations (10 and 1 µg ml⁻¹) and two different temperatures of incubation (25 and 4 °C). Clearly, under all these conditions the reaction was linear with time and proceeded faster at 25 than 4 °C. However, higher blank readings occurred at 25 °C so the lower temperature was preferred as long as sufficient antigen was available. There was also less chance of bacterial contamination in the wells at the lower temperature, although this problem has not been encountered. Coating the wells with more γ-globulin increased the reaction rate, as would be expected, although there was not a proportional increase when the γ-globulin concentration was increased from either 0.1 to 1 µg ml⁻¹ or from 1 to 10 µg ml⁻¹.

Similarly, higher conjugate concentrations gave an increased, if not proportional, colour production (Fig. 2). There was sufficient alkaline phosphatase substrate in the reaction conditions described by Clark & Adams (1977) to reach very high absorbances (measured as A₄₀₅ after dilution in the appropriate buffer). Between 10⁵ and 10⁶ cells ml⁻¹ gave a suitable absorbance without dilution under these conditions; although 10⁴ cells ml⁻¹ could be detected, the blank reaction was appreciable in comparison at such low antigen concentrations, and so 10⁵ cells ml⁻¹ was considered to be the operational minimum. This means that 2 × 10⁴ cells (or about 16 ng cell protein) were required per reaction well in the microtitre plates. This amount of protein per well is comparable with the minimum detection levels of plant viruses by ELISA (Clark & Adams, 1977). The cell concentration (10⁵ cells ml⁻¹) is of the same order as that required for the sensitive spiroplasma deformation test (Williamson *et al.*, 1978).

Fig. 1

Fig. 2

Fig. 1. Development of colour in ELISA. *Spiroplasma citri* antigens were used with microtitre plates coated with γ -globulins against *S. citri*. Alkaline phosphatase- γ -globulin conjugate was an 800-fold dilution; the cell concentration was 10^6 ml $^{-1}$. $\circ$, 10 μ g γ -globulin ml $^{-1}$ 25 °C; $\bullet$, 1 μ g γ -globulin ml $^{-1}$ 25 °C; $\square$, 10 μ g γ -globulin ml $^{-1}$ 4 °C; $\blacksquare$, 1 μ g γ -globulin ml $^{-1}$ 4 °C.

Fig. 2. Quantification in ELISA. *Spiroplasma citri* antigens were used with γ -globulins against *S. citri*. Plates coated with 10 μ g γ -globulin ml $^{-1}$ were developed for 18 h at 25 °C. Conjugate dilutions: $\circ$, 400; $\bullet$, 800; $\square$, 3200.

Spiroplasma relatedness estimated by ELISA

Results are expressed as percentage homology, i.e. $(A_{He}/A_{Ho}) \times (N_{Ho}/N_{He}) \times 100$, where N is the cell number, and A is the absorbance at 405 nm *minus* the blank value, for heterologous (He) and homologous (Ho) reactions under identical conditions of γ -globulin and conjugate concentrations. Although homologies can be estimated by reference to standard curves of homologous reactions, it is better to make comparisons with a standard on the same plate to minimize plate variation.

Table 1 contains the values calculated for the homologies of various intact spiroplasmas when tested against antisera to *S. citri* corn-stunt spiroplasma and purified spiralin. None of the *S. citri* isolates could be distinguished by ELISA and so strain SP-4 was arbitrarily given a value of 100 % homology with antisera against *S. citri* and spiralin. *Spiroplasma citri* and corn stunt spiroplasmas were clearly related albeit to a small degree, by their cell surface antigens. Of the other spiroplasmas only isolate BC3 gave a clear positive reaction with antiserum to *S. citri* and only the tick isolate 277F reacted slightly with antiserum to corn stunt spiroplasma. Reactions with antiserum to spiralin were comparable with those with antiserum to *S. citri*, consistent with spiralin being a major cell surface antigen which may differ antigenically between isolates, although this is not the only possible explanation. For instance, the spiralin may be masked to different extents in different organisms and, indeed, antiserum raised against any other purified surface antigen might give similar results.

When lysed cell antigens were compared by ELISA an altered pattern of relatedness emerged (Table 2). The homologous reaction with broken cells was about threefold higher than with intact cells. In comparison with the broken cell antigen homologous reaction, isolate BC3 and corn stunt spiroplasma were clearly related to *S. citri* and, while both tulip tree spiroplasmas (BNR-1 and SR-3) also showed relatedness to *S. citri*, only 277F and not SMCA of the tick isolates showed some homology.

Table 1. *Percentage homology of spiroplasmas compared by ELISA using intact cells as antigens*

Results are compared with those for strain SP-4 (against antiserum to *S. citri* and spiralin) and strain Rio Grande (against corn stunt spiroplasma), taken as 100 %. Results are the average of 10 determinations; errors were less than 20 % for high homologies, but 50 % for homologies less than 1 %.

Whole cell antigen	Percentage homology		
	<i>S. citri</i>	Antiserum: Corn-stunt spiroplasma	Spiralin
<i>S. citri</i> SP-4	(100)	0.5	(100)
<i>S. citri</i> SP-A	109	ND	109
<i>S. citri</i> ASP-1	90	ND	89
<i>S. citri</i> ASP-25	107	ND	90
<i>S. citri</i> R8A2	76	ND	115
<i>S. citri</i> ALG	102	ND	93
<i>S. citri</i> MH	72	ND	77
Corn stunt Rio Grande	0.3	(100)	0.3
Corn stunt Miss E	< 0.1	131	< 0.1
Corn stunt B969	< 0.1	84	< 0.1
Honeybee BC3	0.2	< 0.1	< 0.1
Tulip tree BNR-1	< 0.1	< 0.1	< 0.1
Tulip tree SR-3	< 0.1	< 0.1	< 0.1
Tick SMCA	< 0.1	< 0.1	< 0.1
Tick 277F	< 0.1	5.1	< 0.1
<i>Acholeplasma laidlawii</i>	< 0.1	< 0.1	ND
<i>Mycoplasma fermentans</i>	< 0.1	< 0.1	ND

ND, Not determined.

Table 2. *Percentage homology of spiroplasmas compared by ELISA using broken cells as antigens*

Results are compared with those for *S. citri* and corn stunt spiroplasma against their homologous antisera, taken as 100 %. Errors as in Table 1.

Broken cell antigen	Percentage homology	
	<i>S. citri</i>	Antiserum: Corn stunt spiroplasma
<i>S. citri</i> SP-4	(100)	2.3
<i>S. citri</i> SP-4 (intact)	29	ND
Corn stunt Miss E	1.0	(100)
Honeybee BC3	11	1.0
Tulip tree BNR-1	1.2	< 0.1
Tulip tree SR-3	2.2	< 0.1
Tick SMCA	< 0.1	< 0.1
Tick 277F	3.2	1.3

ND, Not determined.

Polyacrylamide gel electrophoresis

Solubilized proteins of five selected spiroplasmas were separated in one-dimensional SDS-polyacrylamide gels to determine whether there were major differences in protein patterns (Fig. 3). (A more comprehensive survey of spiroplasma proteins separated by SDS-polyacrylamide gel electrophoresis is in progress; M. J. Daniels, M. A. Stephens, R. Townsend & J. Longland, personal communication.) *Spiroplasma citri* strains SP-4 and R8A2 and the honeybee (BC3) spiroplasma had very similar patterns, apart from the

Fig. 3. SDS-polyacrylamide gel electrophoresis. Conditions and molecular weight standards are described in Methods. A, Tulip tree (BNR-1) spiroplasma; B, tick (277F) spiroplasma; C, honeybee (BC3) spiroplasma; D, *S. citri* R8A2; E, *S. citri* SP-4.

mobility of the major band of apparent molecular weight between 23000 and 26000. The major protein of the tick spiroplasma 277F had an apparent molecular weight of 29000. Spiralin (from *S. citri*) is known to have a molecular weight of about 26000 (Wróblewski *et al.*, 1977a).

Crossed immunoelectrophoresis

Non-ionic detergents provide the best means of solubilizing membrane proteins for crossed immunoelectrophoresis. When membranes from strains SP-4 (Fig. 4a) and BC3 (Fig. 4b) were solubilized in Triton X-100 and electrophoresed into a gel containing anti-serum to *S. citri*, about 20 immunoprecipitation rockets were detected in the SP-4 gel and about 15 in the BC3 gel. Heterogeneity of one of the major densely staining precipitin rockets in the SP-4 gel is indicated by a double peak (arrowed in Fig. 4a), and in the tandem crossed immunoelectrophoretogram (SP-4 and BC3 preparations run together in a gel containing antiserum to *S. citri*) this has a counterpart in the BC3 membrane indicated by a continuous precipitin line (Fig. 4c). Although the pattern in Fig. 4(c) is complex, at least five, and probably more, common antigens can be distinguished.

Attempts were made to identify the precipitin peak due to spiralin in the crossed immunoelectrophoretograms by including an intermediate gel containing antiserum to spiralin

Fig. 4. Crossed immunoelectrophoresis. Antiserum to *S. citri* in each gel. Antigens: (a) Triton X-100-solubilized *S. citri* SP-4 membrane (arrow indicates a double rocket due to a heterogeneous antigen). (b) Triton X-100-solubilized honeybee (BC3) spiroplasma membrane. (c) Tandem crossed immunoelectrophoretogram: i, Triton X-100-solubilized BC3 membranes; ii, Triton X-100-solubilized *S. citri* membranes [arrows indicate some of the many common antigens (continuous precipitin rockets)]. (d) Sodium deoxycholate-solubilized *S. citri* membranes. (e) As for d, but with an intermediate gel containing antiserum to purified spiralin.

between the first dimension gel and the gel containing antiserum to *S. citri*. However, it was never possible to detect the presence of spiralin in Triton X-100-solubilized spiroplasma membranes in this way. Solubilization of SP-4 membranes in sodium deoxycholate instead of Triton X-100 was found to be an effective means of solubilizing antigens (Fig. 4d).

Deoxycholate-solubilized membranes gave fewer precipitin rockets, however, and were clearly less satisfactory than the Triton X-100 preparations. Sodium deoxycholate did solubilize spiralin, which was identified by the use of an intermediate gel containing antiserum to spiralin (Fig. 4e). When this system was applied to deoxycholate-solubilized BC3 membranes, no major antigen reacted with antiserum to spiralin. Wróblewski (1979) has similarly reported that Triton X-100 solubilizes spiralin very poorly compared with sodium deoxycholate.

DISCUSSION

The sensitivity of ELISA is comparable to that of the spiroplasma deformation test (Williamson *et al.*, 1978) in serological comparisons of spiroplasmas. While both tests are discriminating, low degrees of heterologous cross-reactions between different isolates do indicate some relatedness. Furthermore, neither of these tests, unlike the metabolism inhibition test, requires culture of the organisms in synthetic media. While the deformation test is more rapid, ELISA has the advantage that intact cells are not necessary, so that comparisons of cell surface antigens and lysed cell antigens may be made. Differences in percentage homology values between organisms must be due either to different amounts of common antigens (or altered availabilities for formation of an antigen/antibody complex) or to altered antigens such that the avidities of the antigen/antibody complexes vary.

Both Davis *et al.* (1979) and Williamson *et al.* (1979) have proposed that the *S. citri* serological group should include subgroups of *S. citri*, honeybee spiroplasmas and corn stunt spiroplasmas. Williamson *et al.* (1979) has included the tick spiroplasma (277F) as a fourth subgroup. In all, at least four serologically distinct groups of spiroplasmas have been proposed and the ELISA results are consistent with this, but some reservations must be made. It is well documented that *S. citri* and the corn stunt spiroplasma are serologically related (Tully *et al.*, 1973). The honeybee (BC3) spiroplasma is also clearly related to *S. citri* as judged by ELISA, polyacrylamide gel electrophoresis of solubilized proteins and crossed immunoelectrophoresis.

The suckling mouse cataract agent (SMCA) had no cross-reaction by ELISA with either *S. citri* or the corn stunt spiroplasma and so should be considered serologically unrelated to the *S. citri* serological group, as indicated by Williamson *et al.* (1979). The relationships of the numerous flower isolates with other spiroplasmas is complex and they do not constitute one separate serological group (Davis, 1978; Davis *et al.*, 1979; Williamson *et al.*, 1979) although isolates OBMG (magnolia) and BNR-1 (tulip tree) are included together in a separate group by Williamson *et al.* (1979). Tulip tree isolates 23-6 and 27-31 are included together in a separate serological group by Davis *et al.* (1979) and tulip tree isolates SR-3 and SR-9 constitute yet another group. However, the groupings described by Davis *et al.* (1979) and Williamson *et al.* (1979) are not mutually exclusive and some flower isolates are related to *S. citri* (Davis *et al.*, 1979). By ELISA, neither isolate BNR-1 nor SR-3 had any cell surface antigens in common with either *S. citri* or corn stunt spiroplasma and yet both gave positive reactions with *S. citri* antiserum when lysed cells were used, indicating some serological relatedness. Curiously, Williamson *et al.* (1979) found some serological cross-reaction between the honeybee (BC3) spiroplasma and antiserum to BNR-1, and Davis *et al.* (1979) reported a low deformation titre for the reaction of SR-3 with antiserum to the honeybee (AS576) spiroplasma. Since the bee isolates BC3 and AS576 are known to be serologically related to *S. citri*, it is possible that tulip tree isolates BNR-1 and SR-3 may have a very low serological relatedness to *S. citri*. The extent of such a relatedness probably does not warrant their incorporation into the *S. citri* serological group and whether isolate 277F should be placed in a serological subgroup of *S. citri* is also a matter for debate which is best left to the Subcommittee on the Taxonomy of Mollicutes. Serological relatedness, although a convenient tool in spiroplasma classification, is clearly insufficiently precise to

indicate a relationship when different techniques give conflicting results and homologies are very low, so a more acceptable classification must await DNA:DNA pairing studies or two-dimensional electrophoretic separation of cell proteins.

Detection of individual protein antigens by crossed immunoelectrophoresis is very sensitive and has been applied to mycoplasmas (Thirkill & Kenny, 1974; Johansson & Hjertén, 1974; Johansson & Wróblewski, 1978). Non-ionic detergents are the preferred means of solubilizing membrane proteins for crossed immunoelectrophoresis because, as a rule, a high degree of solubilization occurs, the three-dimensional structure of the proteins is retained (Helenius & Simons, 1975), the antigen/antibody reaction is not disturbed and no charge is imparted to the proteins so that the first dimension electrophoretic separation is not affected. The non-ionic detergents Tween 20 and Triton X-100 have been used in studies with mycoplasma membranes as has the anionic detergent sodium deoxycholate which is relatively non-denaturing (Ne'eman *et al.*, 1972), but has the disadvantage that a negative charge is imparted to the protein antigens so that the separation in the first dimension is affected. Although Wróblewski *et al.* (1977*b*) preferred Triton X-100 to sodium deoxycholate for the solubilization of *A. laidlawii* membranes in crossed immunoelectrophoresis, neither detergent was as successful with *S. citri*. However, the results reported here show clearly that many more *S. citri* membrane protein antigens are solubilized by Triton X-100 than by deoxycholate. Tween 20, although affording excellent crossed immunoelectrophoretograms, does not appear to solubilize spiralin, the major membrane protein of *S. citri* (Wróblewski *et al.*, 1977*a*), whereas sodium deoxycholate does solubilize spiralin but fewer distinguishable precipitin rockets are formed. Immunoprecipitation of detergent-solubilized protein antigens and their detection either by two-dimensional gel electrophoresis (Archer, 1979) or crossed immunoelectrophoresis provides an excellent means of comparing antigens between mycoplasmas. The tandem crossed immunoelectrophoretogram of the honeybee (BC3) spiroplasma and *S. citri* proteins confirms that these two organisms share a number of membrane antigens.

The possible functions of the major membrane protein of *S. citri*, spiralin, is an intriguing and as yet unresolved problem. There are major protein bands, differing somewhat in molecular weight, in the spiroplasmas examined by polyacrylamide gel electrophoresis but it is not known whether these proteins are spiralin analogues. The ELISA results clearly demonstrate antigenic differences between spiroplasmas using antiserum raised against purified spiralin. A precipitin rocket produced by deoxycholate-solubilized *S. citri* membranes was identified as being due to spiralin, but no major precipitin rocket was observed when deoxycholate-solubilized membranes of isolate BC3 were reacted with antiserum to spiralin. The major polypeptide of *S. citri* strain SP-4 separated by polyacrylamide gel electrophoresis had an estimated molecular weight of 23 500 and was identified as spiralin by the crossed immunoelectrophoresis method of Chua & Blomberg (1979) using a polyacrylamide gel as the first dimension separation (unpublished observation).

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REFERENCES

- ARCHER, D. B. (1979). Immunoprecipitation of Triton X-100-solubilized *Mycoplasma mycoides* proteins. *Journal of General Microbiology* **115**, 111–116.
- ARCHER, D. B., RODWELL, A. W. & RODWELL, E. S. (1978). The nature and location of *Acholeplasma laidlawii* membrane proteins investigated by two-dimensional gel electrophoresis. *Biochimica et biophysica acta* **513**, 268–283.
- BHAKDI, S., BJERRUM, O. J. & KNÜFERMANN, H. (1976). The major 'intrinsic' membrane protein of human erythrocytes. Preparative isolation and immunoelectrophoretic analyses. *Biochimica et biophysica acta* **446**, 419–431.
- BJERRUM, O. J. & BØG-HANSEN, T. C. (1976). The immunochemical approach to the characterization of membrane proteins. Human erythrocyte membrane proteins analysed as a model system. *Biochimica et biophysica acta* **455**, 66–89.

- BJERRUM, O. J., BHAKDI, S., BØG-HANSEN, T. C., KNÜFFERMANN, H. & WALLACH, D. F. H. (1975). Quantitative immunoelectrophoresis of proteins in human erythrocyte membranes. Analysis of protein bands obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochimica et biophysica acta* **406**, 489-504.
- BOVÉ, J. M. & SAILLARD, C. (1979). Cell biology of spiroplasmas. In *The Mycoplasmas: III. Plant and Insect Mycoplasmas*, pp. 83-153. Edited by R. F. Whitcomb & J. G. Tully. London: Academic Press.
- CHRISTIANSEN, C., ASKAA, G., FREUNDT, E. A. & WHITCOMB, R. F. (1979). Nucleic acid hybridization experiments with *Spiroplasma citri* and the corn stunt and sucking mouse cataract spiroplasmas. *Current Microbiology* **2**, 323-326.
- CHUA, N.-H. & BLOMBERG, F. (1979). Immunochemical studies of thylakoid membrane polypeptides from spinach and *Chlamydomonas reinhardtii*. A modified procedure for crossed immunoelectrophoresis of dodecyl sulphate-protein complexes. *Journal of Biological Chemistry* **254**, 215-223.
- CLARK, M. F. & ADAMS, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483.
- CLARK, M. F., FLEGG, C. L., BAR-JOSEPH, M. & ROTTEM, S. (1978). The detection of *Spiroplasma citri* by enzyme-linked immunosorbent assay (ELISA). *Phytopathologische Zeitschrift* **92**, 332-337.
- CLARKE, H. G. M. & FREEMAN, T. A. (1967). A quantitative immunoelectrophoresis method (Laurell electrophoresis). In *Protides of the Biological Fluids*, vol. 14, pp. 503-509. Edited by H. Peeters. Amsterdam: Elsevier.
- DAVIS, R. E. (1978). Spiroplasma associated with flowers of the tulip tree (*Liriodendron tulipifera* L.). *Canadian Journal of Microbiology* **24**, 954-959.
- DAVIS, R. E., LEE, I.-M. & BASCIANO, L. K. (1979). Spiroplasmas: serological grouping of strains associated with plants and insects. *Canadian Journal of Microbiology* **25**, 861-866.
- FAIRBANKS, G., STECK, T. L. & WALLACH, D. F. H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606-2617.
- HELENIUS, A. & SIMONS, K. (1975). Solubilization of membranes by detergents. *Biochimica et biophysica acta* **415**, 29-79.
- JOHANSSON, K.-E. & HJERTÉN, S. (1974). Localization of the Tween 20-soluble membrane proteins of *Acholeplasma laidlawii* by crossed immunoelectrophoresis. *Journal of Molecular Biology* **86**, 341-348.
- JOHANSSON, K.-E. & WRÓBLEWSKI, H. (1978). Crossed immunoelectrophoresis, in the presence of Tween 20 or sodium deoxycholate, of purified membrane proteins from *Acholeplasma laidlawii*. *Journal of Bacteriology* **136**, 324-330.
- KAHANE, I. & RAZIN, S. (1969). Immunological analysis of mycoplasma membranes. *Journal of Bacteriology* **100**, 187-194.
- MOUCHES, C., VIGNAULT, J. C., TULLY, J. G., WHITCOMB, R. F. & BOVÉ, J. M. (1979). Characterization of spiroplasmas by one and two dimensional protein analysis on polyacrylamide gel slabs. *Current Microbiology* **2**, 69-74.
- NE'EMAN, Z., KAHANE, I., KOVARTOVSKY, J. & RAZIN, S. (1972). Characterization of the mycoplasma membrane proteins. III. Gel filtration and immunological characterization of *Acholeplasma laidlawii* membrane proteins. *Biochimica et biophysica acta* **266**, 255-268.
- ROTTEM, S., STEIN, O. & RAZIN, S. (1968). Reassembly of mycoplasma membranes disintegrated by detergents. *Archives of Biochemistry and Biophysics* **125**, 46-58.
- SAILLARD, C., DUNEZ, J., GARCIA-JURADO, O., NHAMI, A. & BOVÉ, J. M. (1978). Détection de *Spiroplasma citri* dans les agrumes et les pervenches par le technique immuno-enzymatique 'ELISA'. *Compte rendus hebdomadaire des séances de l'Académie des sciences* **286**, 1245-1248.
- TANFORD, C. & REYNOLDS, J. A. (1976). Characterization of membrane proteins in detergent solutions. *Biochimica et biophysica acta* **457**, 133-170.
- THIRKILL, C. E. & KENNY, G. E. (1974). Serological comparison of five arginine-utilizing *Mycoplasma* species by two-dimensional immunoelectrophoresis. *Infection and Immunity* **10**, 624-632.
- TULLY, J. G., WHITCOMB, R. F., BOVÉ, J. M. & SAGLIO, P. (1973). Plant mycoplasmas: serological relation between agents associated with citrus stubborn and corn stunt diseases. *Science* **182**, 827-829.
- TULLY, J. G., WHITCOMB, R. F., CLARK, H. F. & WILLIAMSON, D. L. (1977). Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* **195**, 892-894.
- WILLIAMSON, D. L., WHITCOMB, R. F. & TULLY, J. G. (1978). The spiroplasma deformation test, a new serological method. *Current Microbiology* **1**, 203-207.
- WILLIAMSON, D. L., TULLY, J. G. & WHITCOMB, R. F. (1979). Serological relationships of spiroplasmas as shown by combined deformation and metabolism inhibition tests. *International Journal of Systematic Bacteriology* **29**, 345-351.
- WRÓBLEWSKI, H. (1978). Spiraline: its topomolecular anatomy and its possible function in the *Spiroplasma citri* cell membrane. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene (Abteilung I, Originale A)* **241**, 179-180.
- WRÓBLEWSKI, H. (1979). Amphiphilic nature of spiraline, the major protein of the *Spiroplasma citri* cell membrane. *Journal of Bacteriology* **140**, 738-741.
- WRÓBLEWSKI, H., JOHANSSON, K.-E. & HJERTÉN, S. (1977a). Purification and characterization of spiraline, the main protein of the *Spiroplasma citri* membrane. *Biochimica et biophysica acta* **465**, 275-289.
- WRÓBLEWSKI, H., JOHANSSON, K.-E. & BURLOT, R. (1977b). Crossed immunoelectrophoresis of membrane proteins from *Acholeplasma laidlawii* and *Spiroplasma citri*. *International Journal of Systematic Bacteriology* **27**, 97-103.