Characterization of Spiroplasmas by Polyacrylamide Gel Analysis of their Proteins and Enzymes

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Following the first successful cultivation of the mycoplasma associated with citrus stubborn disease (Saglio *et al.*, 1971; Fudl-allah *et al.*, 1972) its recognition as a new genus and species, *Spiroplasma citri* (Bové *et al.*, 1973; Saglio *et al.*, 1973; Cole *et al.*, 1973*a* and *b*), and its role as the causal agent of stubborn disease (Markham and Townsend, 1974), important new developments have occurred. The host range of *S. citri* has been considerably extended beyond rutaceous plants, and spiroplasmas other than *S. citri* have been identified and cultured.

Calavan and Oldfield (1979) have reviewed the symptomatology of herbaceous plants naturally or experimentally infected with *S. citri.* The corn stunt spiroplasma (CSS) was eventually cultured in 1975 and shown to be the causal agent of corn stunt disease (Chen and Liao, 1975; Williamson and Whitcomb, 1975). The CSS does not seem to be restricted to maize, since it could be experimentally transmitted to various dicotyledonous plants (Markham *et al.*, 1977). Spiroplasmas have also been reported from *Opuntia tuna monstrosa* (Kondo *et al.*, 1976) and Bermuda grass, *Cynodon dactylon* (L.) Pers., (Chen *et al.*, 1977). There are even claims that spiroplasmas could be cultured from plants with aster yellows symptoms (Kondo *et al.*, 1977), western-X disease or pear decline (Raju and Nyland, 1978). Spiroplasmas have also been found on, and cultured from, the surface of a number of flowers (Davis, 1978*a, b, c,* 1979; McCoy *et al.*, 1979).

Three leafhopper species are vectors of *S. citri* in California: *Circulifer (Neoaliturus) tenellus, Scaphytopius nitridus* and *S. acutus delongi* (Calavan and Oldfield, 1979). In Morocco, *S. citri* has been isolated and cultured from the leafhopper *Neoaliturus (Circulifer) haematoceps.* Six other species of leafhoppers, captured in stubborn-affected sweet orange orchards, were found positive for the presence of *S. citri* by ELISA (enzyme-linked immunosorbent assay) using *S. citri* specific immunoglobulins (Bové *et al.*, 1979; Saillard *et al.*, 1980). Spiroplasmas occur in natural populations of the xylem feeding leafhopper (sharpshooter) *Oncometopia nigricans* (McCoy *et al.*, 1978) and of the green leaf bug *Trigonotylus ruficornis* from which two antigenically different organisms have been obtained (Lei *et al.*, 1979).

Spiroplasmas pathogenic for the honey bee (*Apis mellifera ligustica),* and present in the hemolymph at high concentrations, have been discovered by Clark (1977). Certain spiroplasmas isolated from the surface of flowers were found pathogenic for the honey bee (Davis, 1979). Conversely, the honey bee carries spiroplasmas which are related, by a number of techniques, to some flower spiroplasmas (Bordeaux group, unpublished).

Three more spiroplasmas have been identified, two of which were originally described as "spirochetes." The *Drosophila*-associated "sex ratio organism," shown by Williamson and Whitcomb (1974) to be a spiroplasma, is the only one not yet cultured, and the agent 277F, isolated from rabbit ticks, was shown by Brinton and Burgdorfer (1976) to be a helical, motile mycoplasma. The third spiroplasma, also derived from the rabbit tick, is the suckling mouse cataract agent (SMCA) which was believed to be a virus until

1974, when its real nature was recognized by Tully *et al.* (1976). The SMCA, as well as the closely related GT48 spiroplasma, were cultured *in vitro* and their pathogenicity in chick embryos and in suckling mice and rats established (Tully *et al.*, 1977).

It thus appears that the spiroplasmas are quite ubiquitous and that beside *S. citri,* a number of different types exist. When a spiroplasma has been isolated and cultured from a given source, it must be characterized. There are serological techniques such as the deformation test (Williamson *et al.*, 1978) or ELISA (Saillard *et al.*, 1980); however, they require specific antisera. This paper illustrates how the analysis of spiroplasma proteins by an improved polyacrylamide gel electrophoresis technique contributes to the characterization and identification of spiroplasmas.

MATERIALS AND METHODS

Mycoplasma isolates and growth conditions. Spiroplasmas isolated from flowers (BNR 1 from tulip tree and OBMG from magnolia tree) and honey bee (KC3 from Hawaii and BC3 from Maryland) were kindly made available to us by T. B. Clark, bee laboratory, Beltsville, Maryland, USA (see also Clark, 1978). Other mycoplasmas or spiroplasmas used in this work were described previously (Mouches *et al.*, 1979). All mycoplasmas or spiroplasmas were triple cloned, except the spiroplasma isolates 12P, P50, GII3, P41, P52 and P47 (fig. 3, tracks 7 to 12).

The suckling mouse cataract agent (TS 221) was grown at 37°C in medium SP2 (Tully *et al.*, 1977), while all other organisms were cultured aerobically at 32°C in medium BSR (Bové *et al.*, 1978) buffered to pH 7.4 with 0.5 M HEPES (medium BSRH). The organisms were harvested at the end of the exponential phase of growth.

In some experiments, the cell proteins were labeled by adding a mixture of ^{14}C amino acids (NEN, ref. NEC-445) to the growth medium (25 μCi/ml).

One- and two-dimensional analysis of total spiroplasma cell proteins. Preparation of protein samples, one dimensional polyacrylamide gel electrophoresis in denaturing conditions in the presence of sodium dodecyl sulfate (SDS-PAGE), and two-dimensional polyacrylamide gel analysis were essentially as described previously (Mouches *et al.*, 1979). At the end of the electrophoretic run, proteins were detected in the slab gel by staining with coomassie brilliant blue, or by fluorography of the dried slab gel (Bonner and Laskey, 1974).

Electrophoretic analysis of enzymes from spiroplasmas. a.) Preparation of protein samples. Organisms were harvested from 500-ml cultures by centrifugation at 30,000 *g* for 30 minutes at 4°C. All further operations were carried out at 4°C. The pellet was resuspended in 20 ml of buffer A (0.05 M Tris-HCl pH 7.4, 0.001 M dithiothreitol, 0.001 M ethylenediamine tetracetic acid, disodium salt (EDTA)) and homogenized in a Dounce homogenizer. The homogenate was sonically disrupted with an MSE ultrasonic disintegrator operated at maximum power (60 watts) for six 15-second bursts, interspersed with 15-second intervals of cooling at 4°C. The preparation was centrifuged at 30,000 *g* for 30 minutes; the resulting supernatant was adjusted to 20 ml with buffer A and saturated with 8 g of ammonium sulfate. The precipitate was collected by centrifugation at 30,000 *g* for 30 minutes. The pellet was dissolved in 4 ml of buffer A containing 20 per cent (v/v) glycerol and dialyzed overnight against 5 liters of the same buffer.

The final protein extract (5 mg/ml) was stored at -20°C. Approximately 0.1-ml aliquots were used for electrophoresis. In some experiments, we have analyzed the soluble proteins from lemon leaves; these were extracted according to a procedure which will be described elsewhere (Protopapadakis *et al.*, personal communication).

b.) Polyacrylamide slab gel electrophoresis of enzymes. The vertical electrophoresis system used has been des-

cribed previously (Mouches *et al.*, 1979). The acrylamide slab gels (height, 16 cm; width, 34 cm; thickness, 0.3 cm) were prepared in molds made of glass plates and Teflon strips.

Linear 3 to 15 per cent acrylamide gradient slab gels were prepared from "heavy" and "light" solutions of acrylamide and N,N'-methylenbi-sacrylamide ("bisacrylamide"). The "light" solution contained in 0.52 M Tris-HCl pH 8.8 buffer, 3 per cent (w/v) acrylamide and 0.237 per cent (w/v) bisacrylamide; the "heavy" solution was made in the same buffer but contained 15 per cent (w/v) acrylamide, 0.0507 per cent (w/v) bisacrylamide and 6 per cent (w/v) sucrose. All acrylamide solutions were filtered on nitrocellulose filters (Millipore 0.45 μ) and submitted to vacuum before addition of the polymerization catalysts.

The linear gel gradients were prepared by mixing 50 ml of each acrylamide solution through a standard two-chambered linear gradient former. Mixing in the outlet chamber was obtained with a magnetic stirrer. A peristaltic pump was used to assure a uniform flow from the gradient former to the slab gel mold.

The following amounts of polymerisation catalysts (ammonium persulfate, AP, and N,N,N', N' . . . tetramethylene diamine, TMED) were added to 50 ml of the light and heavy acrylamide solutions immediately before the preparation of the gel gradient: 0.075 ml of 10 per cent (w/v) AP and 0.015 ml TMED for the light solution; 0.2 ml of the AP solution and 0.020 ml of TMED for the heavy solution.

Slab gels of uniform acrylamide concentration were also used. Such gels were prepared from a solution containing 7.5 per cent (w/v) acrylamide and 0.0507 per cent (w/v) bisacrylamide in 0.59 M Tris-HCl pH 8.8 buffer; 0.225 ml of 10 per cent ammonium persulfate solution and 0.045 ml of TMED were added to 100 ml of the acrylamide mixture.

A slot former made of Teflon, providing 21 1-cm-wide tracks, was placed into the unpolymerized gel mixture.

The acrylamide was allowed to polymerize at room temperature for at least 2 hr. Then, the slot former was removed, and the mold containing the slab gel was clamped onto the electrophoresis apparatus. Each buffer reservoir was filled with 1 liter of nondenaturing electrophoresis buffer (0.025 M Tris base, 0.0192 M glycine; final pH: 8.8).

Before loading the protein samples, gels were prerun 1 hr. at 100 volts. Electrophoresis of the proteins was carried out at 100 volts for 16 hr. Electrophoresis and preelectrophoresis were performed at 4° C.

After electrophoretic separation, the slab was removed and appropriately stained to detect a given enzyme activity. Peroxidases were detected according to Gove and Hoyle (1975), amylases according to Doane (1964), catalases according to Shaw and Prasard (1970), and isoesterases according to Beranek (1974).

RESULTS AND INTERPRETATION

One-dimensional polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE). When spiroplasma proteins are analyzed, more than 60 protein bands can be detected by using the high-resolution PAGE system previously described (Mouches *et al.*, 1978 and 1979). The proteins can easily be visualized in the slab gel by staining with coomassie blue.

The various mycoplasmas have distinct protein patterns (fig. 1.). *Spiroplasma citri* isolates from different origins have the same overall pattern but show small differences between various isolates (fig. 1, tracks 1 and 3). This is also shown in fig. 2, where two isolates of *Spiroplasma citri*, SC-R8A2 (track 3) and SC-Iran (track 5), were compared with the corn stunt agent (CSS, track 4) and the suckling mouse cataract agent (SMCA, tracks 1 and 2). Although the two *S. citri* profiles (tracks 3 and 5) are not identical, they are clearly similar. All *S. citri* isolates studied so far show similar profiles. Neither the CSS (track

4) nor the SMCA (tracks 1 and 2) yielded such profiles, although the CSS profile contained a number of bands whose intensity and relative mobilities were reminiscent of analogous bands in the *S. citri* profiles. One-dimensional SDS-PAGE technique can be used conveniently to characterize spiroplasma isolates (fig. 3). In that experiment, we analyzed the proteins of known isolates of *S. citri* (fig. 3, tracks 1 to 6) with spiroplasmas newly isolated from different types of material in the Tadla area of Morocco: 1) aborted seeds of stubborn-suspicious clementines (track 7); 2) four different wilted periwinkles (*Vinca rosea* L.) from an experimental periwinkle bed established close to a stubborn-affected Valencia orange orchard (Bové *et al.*, 1979; Nhami *et al.*, 1980) (tracks 8, 10, 11 and 12); and 3) *haematoceps* leafhoppers from the same periwinkle bed (track 9). The protein patterns of all the newly isolated spiroplasmas are characteristic of *S. citri* (fig. 3). These data and those from ELISA tests (Saillard *et al.*, 1980) establish that these spiroplasmas belong to the species *S. citri*. On the basis of their protein patterns, the four periwinkle isolates and the leafhopper isolate appear to be identical and very similar to the clementine isolate. Tracks 4 and 5 correspond to *S. citri* isolates obtained from two trees in the same orchard. One (track 4) is the well-known isolate SC-R8A2 (ATCC 27556) from tree No. 2 in row 8, severely affected by stubborn, and the other (track 5) from tree No. 10 in row 7 mildly affected by the disease. These two *S. citri* isolates are not identical (fig. 3), suggesting that more work along these lines could eventually lead to the biochemical characterization of severe and mild strains of the stubborn agent.

In fig. 2 and 3, the zones indicated by SP contain poorly resolved proteins which are serum proteins from the growth media and are adsorbed on the spiroplasma cells. Adsorption of these proteins can be partially avoided by growing the organisms in well-buffered media and collecting the cells at the middle of the exponential phase of growth. For critical work, masking of the spiroplasma proteins by proteins from the media can be avoided by growing the organisms in media containing ^{14}C-labeled amino acids, where only the newly synthesized spiroplasma proteins, and not media proteins, became labeled. After PAGE, the labeled proteins can be detected by the fluorography version of autoradiography. We used this technique to compare a *S. citri* isolate (SC-R8A2, track 6) with a rabbit tick spiroplasma (277 F, track 1), two spiroplasmas from the surface of flowers (BNR 1, track 2 and OBMG, track 3), and two spiroplasmas pathogenic to honey bee (KC 3, track 4 and BC 3, track 5) (fig. 4). The protein patterns of the two honey bee spiroplasmas are nearly identical and closely related to the *S. citri* pattern. This close relationship is also seen by ELISA (Saillard *et al.*, 1980) and by DNA-DNA hybridization (84 per cent homology, Degorce-Dumas, unpublished results). The two flower spiroplasmas give almost identical protein patterns, but this pattern is clearly different from all other spiroplasma protein profiles, again in agreement with data from ELISA and DNA-DNA hybridization.

The protein profiles of *S. citri* as visualized by protein staining (fig. 3) or fluorography after *in vivo* labeling of the proteins with ^{14}C amino-acids (fig. 4) differ in band intensity. This difference results from the fact that staining reflects total proteins and fluorography, the specific activity of the labeled proteins. If the gel to be examined by fluorography is first stained, then a protein profile of the type in fig. 3 is obtained.

Finally, it should be noted that some variations can be observed from one gel to another between the protein profiles of the same spiroplasma isolates. Such variations are not observed when the proteins of a given isolate are analyzed on several tracks of the same gel. Therefore, protein controls of known spiroplasmas and protein samples from spiroplasmas to be characterized should always be run side by side on the same gel slab.

Enzyme detection after one-dimensional PAGE in the absence of SDS. For further characterization and comparison of various spiroplasmas, we also used the technique of enzyme detection after PAGE. The soluble spiroplasma proteins were obtained by osmotic lysis of the spiroplasma cells, and centrifugation of the lysate. The soluble proteins in the supernatant were concentrated by ammonium sulfate precipitation, dialyzed and subjected to PAGE in the absence of SDS (non-denaturing gels).

We analyzed the soluble proteins from *S. citri* (strain SC-R8A2, track 1), *Acholeplasma laidlawii* (strain PG 8, track 2) and leaves from *lemon* (tracks 3) (fig. 5). After electrophoresis, the slab gel was divided into four identical strips, each strip containing the three protein tracks. Each strip was stained for a given enzyme activity: peroxidase (strip A), amylase (strip B), acid phosphatase (strip C), and catalase (strip D). All four enzyme activities were detected among the proteins purified from lemon leaves (tracks 3). No peroxidase activity (strip A) was found in *S. citri* (track 1 A), nor in *A. laidlawii* (track 2A). One band with acid phosphatase activity (strip C) was present in *A. laidlawii* extracts (track C 2) but not in *S. citri* proteins (track C 1). In contrast, amylases (strip B) and catalases (strip D) were found in *A. laidlawii* (B 2, D 2) and *S. citri* protein extracts (B 1, D 1).

Amylase activities of SC-Iran, SC R8A2, KC3, BNR-1, and SMCA spiroplasmas were analyzed in comparison with those of *A. laidlawii* and lemon leaves. All five spiroplasma protein extracts were found to contain amylase and the electrophoretic mobility was identical for all five spiroplasmas, but different from amylase activities in *A. laidlawii*. Under these conditions, amylase activity did not differentiate among the above spiroplasmas.

Esterases of the same five spiroplasmas were analyzed in comparison with those of *A. laidlawii*. The esterase patterns were found to be different not only when the comparison was between *A. laidlawii* and the spiroplasmas, but also between some of the spiroplasmas themselves (fig. 6). The esterase pattern of *S. citri* (tracks 1 and 5) was different from that of the SMCA (track 4), as well as that of the flower spiroplasma (BNR 1). However, the main esterase band of *S. citri* (tracks 1 and 5) and of the honey bee spiroplasma (KC 3, track 3), as well as some minor bands, have identical electrophoretic mobilities. These results confirm again the close relationship between *S. citri* and the honey bee spiroplasma mentioned above.

Two-dimensional polyacrylamide gel analysis of spiroplasma proteins. We have shown previously (Mouches *et al.*, 1979) that relationships between spiroplasmas can be better studied by two- rather than by one-dimensional protein analysis. In the two-dimensional work (O'Farrell, 1975), the proteins are first separated according to their isoelectric point by electrofocusing in a cylindrical polyacrylamide gel. They are then separated in the second dimension according to their molecular weight by using slab PAGE in the presence of SDS. When proteins from two geographically different *S. citri* isolates are mapped by this technique, more than 150 polypeptides with migrational properties identical from one isolate to the other (comigrating proteins) can be detected between the two fluorograms. The number of comigrating proteins between two different types of spiroplasmas, such as *S. citri* and the CSS, is much smaller than in the case of any two *S. citri* isolates. However, in the case of *S. citri* and the CSS, many proteins have migrational properties which differ only slightly from one spiroplasma to the other (homologous proteins) (Mouches *et al.*, 1979). These results clearly indicate a definite relationship between *S. citri* and the CSS, which is better demonstrated by protein mapping than by one-dimensional electrophoresis. The protein maps obtained for the two flower spiroplasmas (OBMG and BNR 1) are highly similar and show more than 100 comigrating proteins

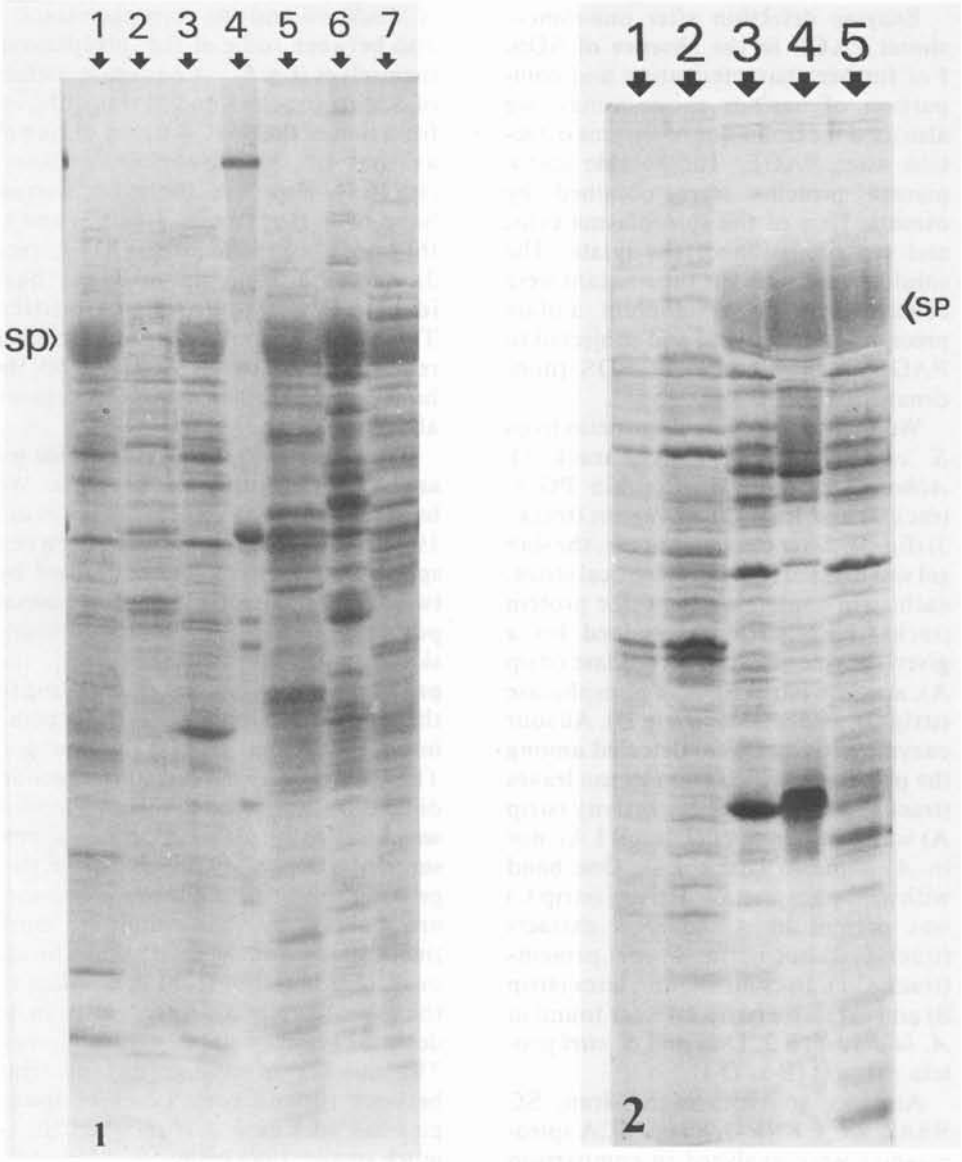

Fig. 1. Coomassie blue-stained proteins from various mollicutes after one-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). *Spiroplasma citri* (SC-Iran), track 1; suckling mouse cataract agent, track 2; *S. citri* (SC-R8A2, ATCC 27556), track 3; muscle myosin (molecular weight, 210,000 d) and muscle actin (molecular weight, 45,000 d), track 4; *Acholeplasma axanthum*, track 5; *Mycoplasma* sp. (PG 50), track 6; *Acholeplasma laidlawii*, track 7. SP = serum proteins.

Fig. 2. Coomassie blue-stained proteins from various spiroplasmas separated by one-dimensional SDS-PAGE. Suckling mouse cataract agent, tracks 1 and 2; *S. citri* (SC-R8A2), track 3; corn stunt spiroplasma, track 4; *S. citri* (SC-Iran), track 5. SP = serum proteins.

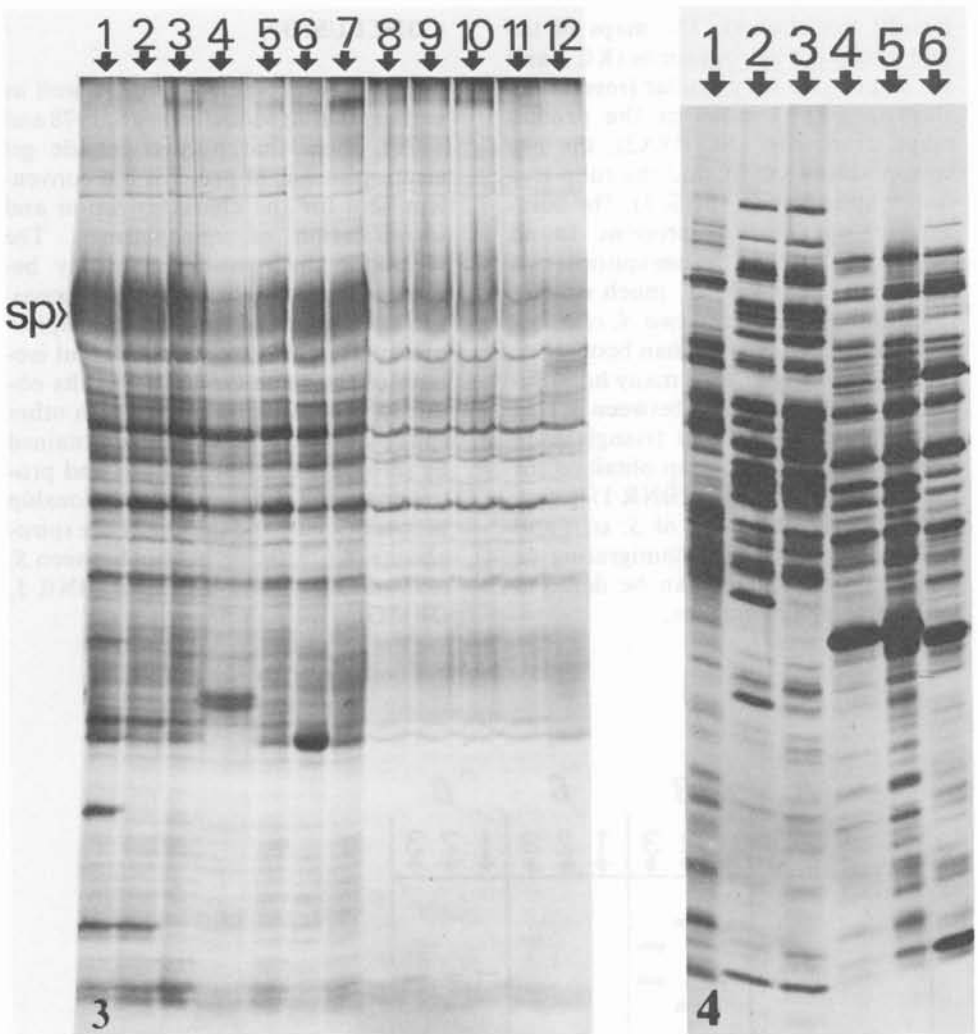

Fig. 3. Coomassie blue-stained proteins from various isolates of *Spiroplasma citri* separated by one-dimensional SDS-PAGE. SC-Iran, from sweet orange, track 1; SC-Israel from periwinkle, track 2; SC-Boufarik (Algeria) from sweet orange, track 3; (SC-R8A2) (ATCC 27556) (Morocco) from sweet orange, track 4; SC-R7A10 from sweet orange, track 5; SC-MH (Morocco) from sweet orange, track 6; spiroplasma 12P from seeds of Clementine mandarin, track 7; spiroplasma P50 from periwinkle, track 8; spiroplasma GII 3 from *Neoaliturus haematoceps* (Mulsant et Rey), track 9; spiroplasma P41 from periwinkle, track 10; spiroplasma P52 from periwinkle, track 11; spiroplasma P47 from periwinkle, track 12. Isolates analyzed on tracks 5 to 12 were from the Tadla region in Morocco. SP = serum proteins.

Fig. 4. Fluorography of *in vitro* ^{14}C-labeled proteins from various spiroplasmas after separation by SDS-PAGE. Spiroplasma from rabbit tick (277 F), track 1; spiroplasma from tulip tree flower (BNR 1), track 2; spiroplasma from magnolia tree flower (OBMG), track 3; spiroplasma from honey bee from Hawaii (KC-3), track 4; spiroplasma from honey bee from Maryland (BC 3), track 5; *S. citri* (SC-R8A2), track 6.

(results not shown). The maps of the two honey bee spiroplasmas (KC 3 and BC 3) are also very similar (results not shown). Fig. 7 indicates the protein maps of *S. citri* (SC-R8A2), the bee spiroplasma (KC 3), and the tulip tree flower spiroplasma (BNR 1). The number of comigrating proteins found between *S. citri* and the bee spiroplasma (fig. 7, dark arrows) is much smaller than in the case of any two *S. citri* isolates, but much greater than between *S. citri* and the CSS. Also, many homologous proteins are found between *S. citri* and KC 3 (fig. 7, open triangles). In contrast, the protein map obtained for the flower spiroplasma (BNR 1) is very different from the map of *S. citri* and KC 3 and only a few comigrating or homologous proteins can be detected with these two organisms.

CONCLUSION

The data presented here, as well as earlier results (Mouches *et al.*, 1978 and 1979), show that polyacrylamide gel electrophoresis of proteins is a convenient tool for the characterization and identification of spiroplasmas. The technique distinguishes not only between different types of spiroplasmas, such as *S. citri* and the corn stunt spiroplasma, but also between different isolates of the same type. The results obtained in comparing *S. citri* with other spiroplasmas agree with those obtained by serology. Enzyme analysis and protein mapping show a close relationship between *S. citri* and the honey bee spiroplasma (KC 3, BC 3), but not between *S. citri* and the flower organism (BNR 1, OBMG).

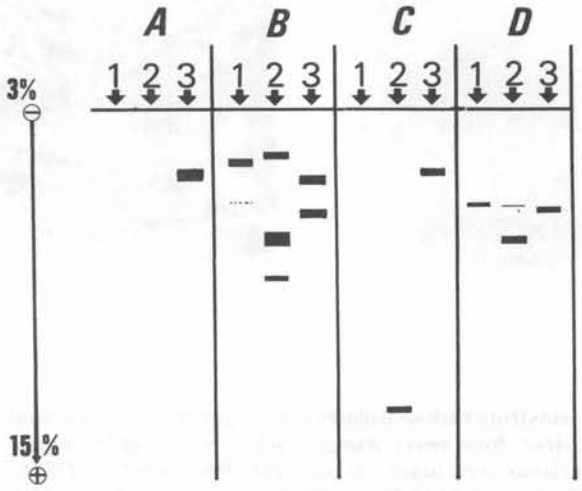

Fig. 5. Detection of enzymes isolated from *S. citri* (SC-R8A2), track 1; from *Acholeplasma laidlawii*, track 2, and from Adamopoulou lemon, track 3 by PAGE. After electrophoresis, the slab gel was divided into four strips. Each strip was stained for detection of specific enzyme activity — A, peroxidases; B, amylases; C, acid phosphatases; and D, catalases.

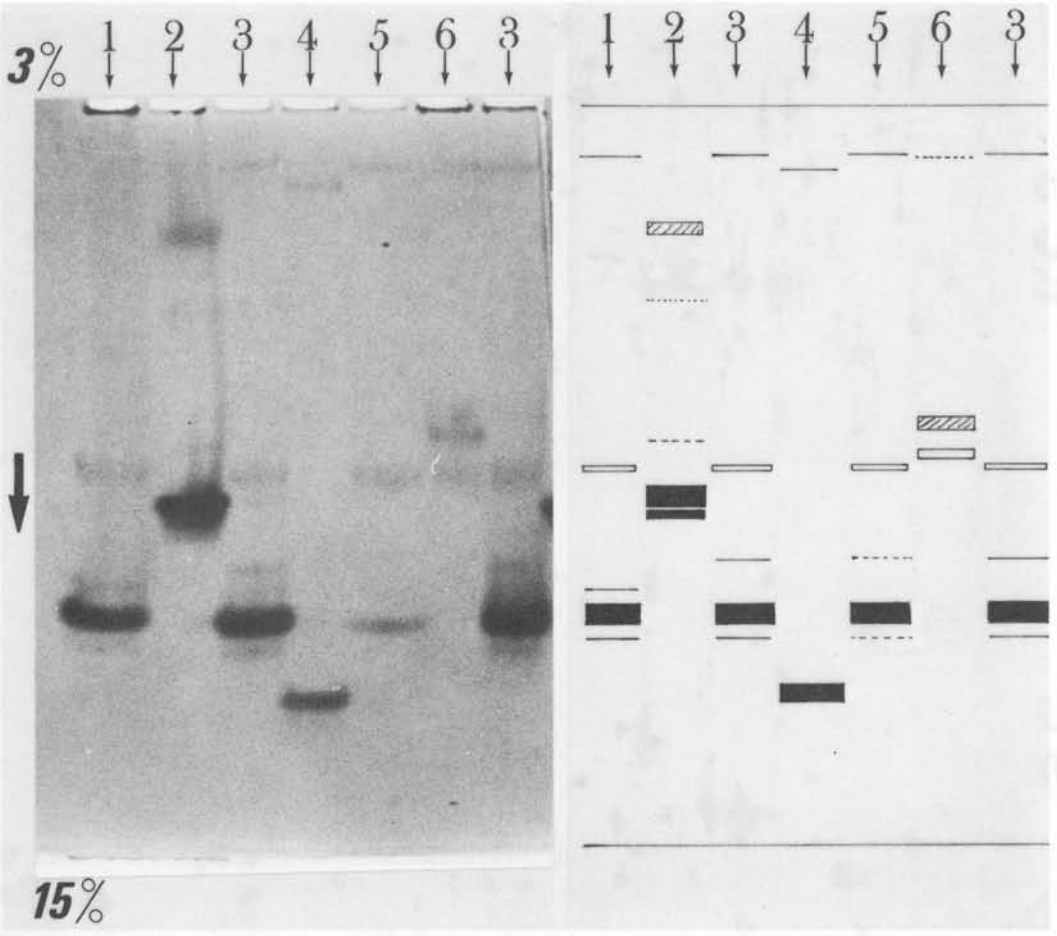

Fig. 6. Comparison of esterases: from *S. citri* (SC-R8A2), track 1; from *A. laidlawii*, track 2; from a honey bee spiroplasma (KC 3), track 3; from the suckling mouse cataract agent, track 4; from *S. citri* (SC-Iran), track 5; and from a tulip tree flower spiroplasma (BNR 1), track 6 by PAGE.

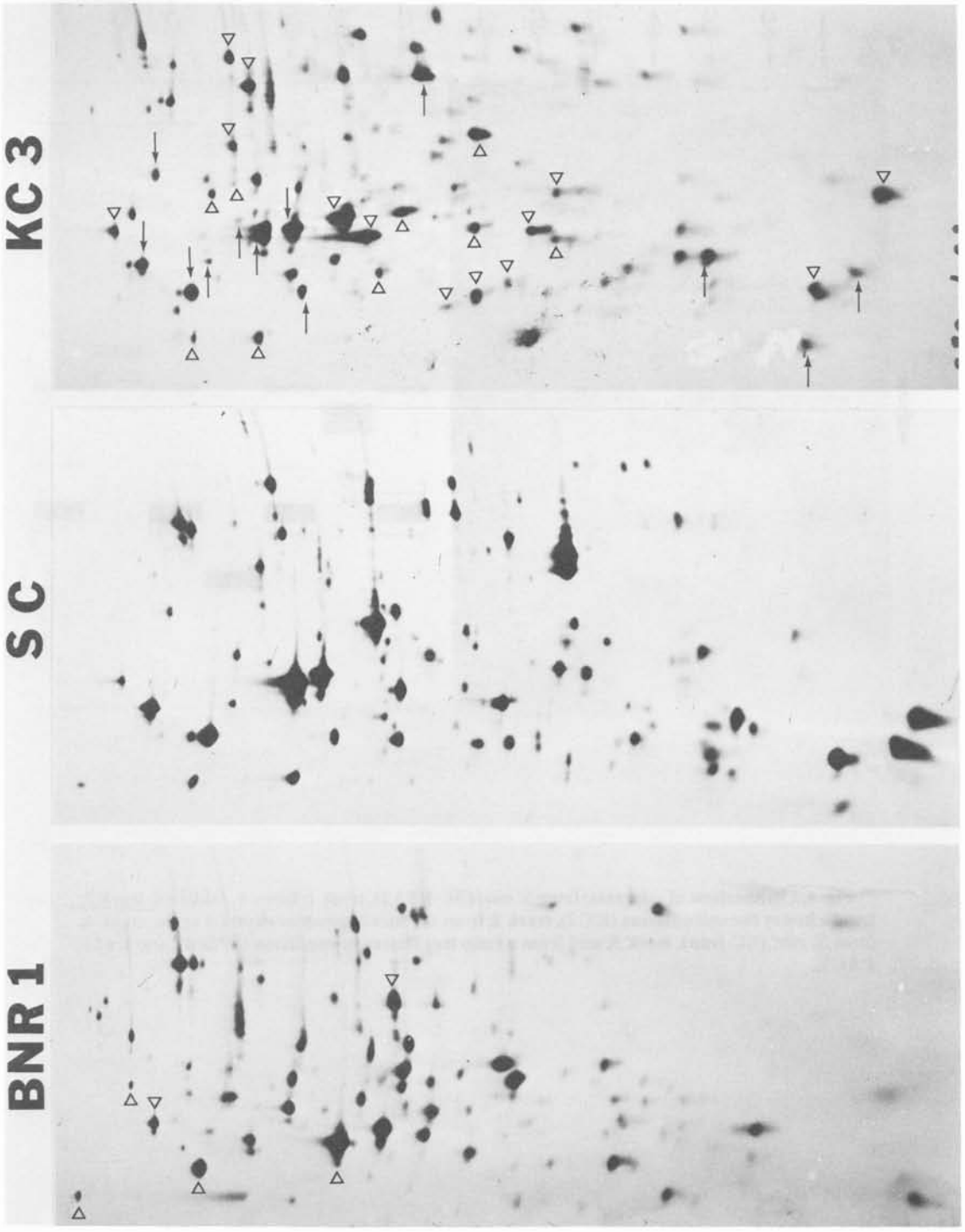

Fig. 7. Fluorography of proteins of three spiroplasmas separated by two-dimensional electrophoresis. SC = *S. citri* (SC-R82A). KC 3 = honey bee spiroplasma. BNR 1 = tulip tree flower spiroplasma.

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