

Analysis of Spiroplasma Proteins: Contribution to the Taxonomy of Group IV Spiroplasmas and the Characterization of Spiroplasma Protein Antigens

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Spiroplasma strains of group IV were compared by two-dimensional protein analyses on polyacrylamide gels. Although considerable diversity was evident, the assemblages studied were less heterogeneous than the known strains of group I.

Two electrophoretic techniques were used to identify spiroplasma proteins that had been used to immunize rabbits. These included monoclonal antibodies prepared against *Spiroplasma citri*. In the first technique, protein antigens were purified by immunoaffinity chromatography, then identified with SDS-PAGE. In the second technique, spiroplasma proteins were first separated by SDS-PAGE, then antigens were identified by antibody binding to blot-transferred proteins.

Finally, two-dimensional protein electrophoresis has been used as a source of immunogens to characterize monospecific antibodies against individual *S. citri* proteins.

INTRODUCTION

Previous work has shown that one- and two-dimensional analysis of proteins on polyacrylamide gels can be used conveniently to characterize and identify newly isolated spiroplasmas [1,2,3].

We have used this approach to compare the protein maps of several spiroplasma strains of group IV [4]. Results of the *S. citri* cluster of spiroplasma strains (group I) will be presented elsewhere in these proceedings [5].

In addition, we shall illustrate how protein analysis on polyacrylamide gels can be used for the identification of spiroplasma protein antigens and the characterization of monospecific antibodies against individual spiroplasma proteins.

RESULTS

Contribution of Protein Analysis to the Taxonomy of Group IV Spiroplasmas

Spiroplasma strains of group IV have been isolated in many different regions from insects and flower surfaces [6,7]. Recently, more than fifty nearly identical strains of this group have been isolated in southwestern France from honeybees showing symptoms of a May disease-like affection [8]. Two of the strains (B31 and

B39) selected from these fifty nearly identical isolates have been shown to be pathogenic to honeybees by injection or feeding [8].

Spiroplasma strains F12 and F16 have been cultured from the surface of flowers (*Leontodum autumnalis* and *Erica cinerea*, respectively) growing near the May disease-affected apiaries.

The protein map of spiroplasma B31, which we have chosen as a reference strain, has been compared to those of strains B39, F12, F16, and various other group IV spiroplasma strains as indicated in Table 1. The results of such comparisons are conveniently expressed in terms of percentages of co-migrating and homologous proteins [1,3] between the reference strain (B31) and any one of the other strains studied.

Table 1 shows that spiroplasma strains B39 and F12 share approximately 90 percent co-migrating proteins with the B31 reference strain. This is typical of strains of the same species and shows that the "flower" strain F12 is nearly identical to the honeybee strain B39. "Flower" spiroplasma F16 has slightly less co-migrating proteins than strain F12 but more than all other flower or insect isolates, for which the percentages vary from 49 to 66 percent.

In contrast to the large amount of co-migrating proteins in group IV, the honeybee strains of group I-2 and the corn stunt spiroplasma (group I-3) have, respectively, 45 percent and 28 percent co-migrating proteins with *S. citri* (group I-1) [5]. These figures indicate that the group IV spiroplasmas, although heterogeneous, do not consist of an extremely diverse assemblage such as that observed in group I.

Identification of Spiroplasma Protein Antigens by Immunoaffinity Chromatography (IAC)

¹⁴C labeled *S. citri* proteins were obtained by growing the organisms in the presence of ¹⁴C amino acids and solubilized in a urea solubilizing buffer described

TABLE 1
Analysis of Group IV Spiroplasma Strains by Two-Dimensional Protein Analysis

Strains	Host	Geographic Origin	Percentage of co-migrating (C) and homologous (H) proteins shared by honeybee spiroplasma B31 and other group IV spiroplasma strains	
			C	H
B31	Honeybee <i>Apis mellifera</i> ^a	(France)	100	0
B39	Honeybee	(France)	88	4
F12	Flower <i>Leontodum autumnalis</i>	(France)	91	5
F16	Flower <i>Erica cinerea</i>	(France)	73	7
F4	Flower <i>Chichorium intybus</i>	(France)	66	7
F25	Flower <i>Helminthia echioides</i>	(Corsica)	57	14
F1	Flower <i>Helminthia echioides</i>	(France)	55	7
F2	Flower <i>Ammi majus</i>	(France)	55	12
B13	Honeybee <i>Apis mellifera</i>	(Morocco)	60	9
L89	Spittlebug <i>Neophilaenus sp</i>	(Corsica)	49	8
PPS1	Flower		52	11
W13	Flower		53	7

^aWith May disease symptoms

previously [1]; 0.015 ml aliquots were dispersed in 0.5 ml of NET-T buffer (0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris HCl, pH 7.4, 0.05 percent (w/v) Triton \times 100); insoluble material was removed by a ten-minute centrifugation in an Eppendorf microfuge ("antigen preparation"). Columns were prepared in an Eppendorf micropipet tip plugged with cotton glass, using 0.020 ml of protein A-Sepharose suspended in NET-T. After washing the column with 0.5 ml NET-T, 0.025 ml antiserum was deposited on the protein A-Sepharose bed, followed by 1.0 ml NET-T. The antigen preparation was then added to the immunoglobulin-coated protein A-Sepharose column and allowed to flow through the column. The column was subsequently washed with 5 ml NET-T followed by 0.2 ml of the same buffer without Triton. The antigens and immunoglobulins (Igs) bound to the protein A-Sepharose were eluted by washing the column with 0.25 ml of SDS solubilizing buffer [1], then directly submitted to SDS-PAGE. Coomassie blue staining of the gel allowed detection of the Igs bound to the column, whereas the ^{14}C protein antigens retained by the Igs on the column were specifically detected upon fluorography of the gel [1].

In the experiments of Fig. 1, rabbit anti *S. citri* strain R8A2 antibodies [9] coated on the protein A-Sepharose column retained a number of *S. citri* (R8A2) ^{14}C -proteins ("homologous" reaction: Fig. 1A, track 6). Some *S. citri* (R8A2) proteins were also retained when anti BC3 honeybee spiroplasma Igs were used ("heterologous" reaction: Fig. 1A, track 4).

When the IAC was carried out with non-immune serum, spiroplasma proteins were normally not retained (Fig. 1B, track 9). However, in one case where the Igs from a non-immunized rabbit was used, one *S. citri* protein was retained (Fig. 1B, track 4).

Mouse monoclonal antibodies (MCAs) have been prepared by McGarrity et al. against *S. citri* strain R8A2 using techniques described here and elsewhere [10,11]. Specific MCA secreting hybridoma clones giving positive ELISA with *S. citri* antigens only (*S. citri* "specific MCAs") were selected. Non-specific MCAs secreting clones were also obtained that gave positive ELISA with *S. citri* (group I-1), and with spiroplasmas from other groups (I-2, I-3, I-4, III, and IV). In IAC, all the "*S. citri* specific MCAs" tested so far retained one single *S. citri* protein, namely, protein 39 or spiralin [12] (Fig. 1B, tracks 5,6,7). None of the *S. citri* proteins were retained by the one clone of non-specific MCAs tested (Fig. 1B, track 8).

IAC has also been used to identify proteins from spiroplasmas sharing immunological relatedness to various eukaryotic structural proteins. We have shown [13] that *S. citri* synthesizes a 45,000-dalton protein which is specifically recognized by rabbit antibodies against rabbit actin (Fig. 1A, track 2). The same antibodies also recognized a 45,000-dalton protein of *M. mycoides* (PG50). Using IAC, no serological relatedness between one of the *S. citri* proteins and eukaryotic tubulin, tropomyosin, or myosin has been demonstrated (results not shown).

Identification of Spiroplasma Protein Antigens by Antibody Binding to Blot-Transferred Proteins

The proteins of *S. citri* strains R8A2 and P511 were separated by SDS-PAGE (Fig. 2). Fig. 2A shows the electrophoretic protein profiles obtained upon Coomassie blue staining of two tracks of the slab gel. The spiroplasma proteins separated on parallel tracks were transferred from the polyacrylamide gel to a nitrocellulose sheet by the blotting procedure described by Bowen et al. [14].

Upon transfer, the nitrocellulose sheet was dried for 30 minutes at 32°C, then

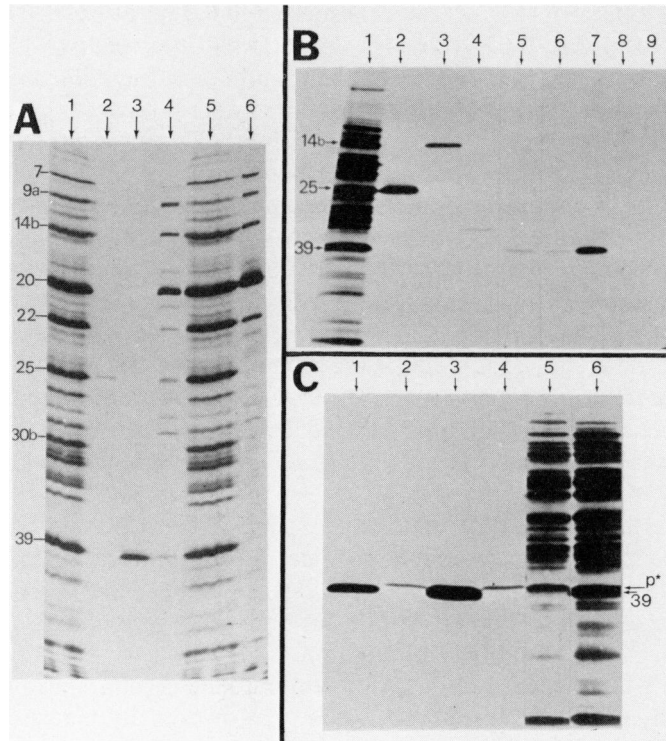


FIG. 1. SDS-PAGE of *S. citri* proteins retained by immunoaffinity chromatography. **A.** Proteins of *S. citri*-R8A2 retained by Igs against: rabbit actin (2), protein 39 (3), spiroplasma BC3 membranes (4), *S. citri*-R8A2 membranes (6). (*S. citri*-R8A2 proteins: 1 and 5). **B.** Proteins of *S. citri*-R8A2 retained by guinea pig Igs against protein 25 (2) or protein 14b (3), by mice MCAs (sc29: 5, sc21: 6, scl2: 7, sp211: 8), or by Igs from rabbit non-immune sera (4 and 9). (*S. citri*-R8A2 proteins: 1). **C.** IAC was carried out using: (a) *S. citri* specific monoclonal antibodies with proteins from *S. citri*-R8A2 (1), or *S. citri*-A3 (2) as antigens; (b) rabbit Igs against protein 39 with proteins from *S. citri*-R8A2 (3) or *S. citri*-A3 (4) as antigens. (*S. citri*-A3 proteins: 5; *S. citri*-R8A2 proteins: 6).

soaked for 30 minutes at room temperature in TBS buffer (0.2 M NaCl, 0.05 M Tris HCl, pH 7.4) containing 3 percent (w/v) bovine serum albumin (BSA) and 5 percent (w/v) horse serum. It was further incubated for four hours at 20°C with 50 μ l of rabbit antiserum prepared against *S. citri* strain R8A2, diluted in 2.5 ml of TBS containing 3 percent BSA and 5 percent horse serum. The sheet was then washed for 40 minutes in three changes of TBS containing 0.2 percent (w/v) Triton \times 100 and 0.2 percent (w/v) SDS, followed by two changes of TBS alone.

The electrophoretic transfer was further incubated for two hours with horseradish peroxidase-conjugated sheep antibodies against rabbit immunoglobulins and used at 1:800 dilutions in TBS containing 3 percent BSA and 5 percent horse serum. After another washing cycle, the sheet was soaked in a solution made by adding 30 mg of 4-chloro-1-naphthol dissolved previously in 10 ml of pure methanol, 50 ml TBS, and 0.02 ml 30 percent H₂O₂.

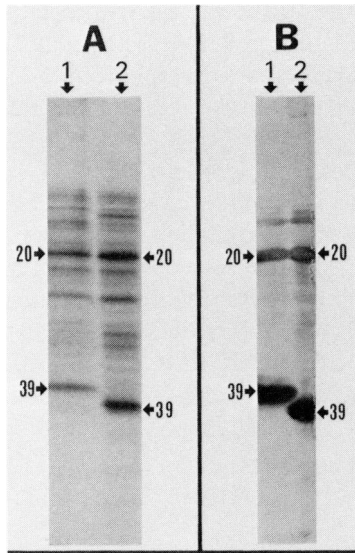


FIG. 2. Blot transfer of *S. citri* proteins after their separation by SDS-PAGE. **A.** Coomassie blue staining of proteins from *S. citri* strains R8A2 (1) and P511 (2) upon SDS-PAGE. **B.** Immunological identification of *S. citri* antigens after blot transfer by using a rabbit antiserum against *S. citri* membranes. The proteins transferred were respectively from *S. citri* strains R8A2 (1) and P511 (2). About 50 μ g of spiroplasma proteins solubilized in "SDS-solubilizing buffer" were first subjected to SDS-PAGE. The SDS-PAGE system consists of a 12 cm long separating gel containing an exponential acrylamide gradient (15–20 percent) with an upper stacking gel (2).

The color reaction (conversion of a colorless soluble substrate to a blue insoluble product) was terminated after 20–30 minutes by washing with water. The blots were dried between filter paper.

As shown on Fig. 2B, the antibodies against *S. citri* recognized a number of *S. citri* proteins. Two proteins were particularly good antigens: protein 20 and protein 39 (spiralin). As described previously [1], proteins 20 and 39 are present in all *S. citri* strains tested. Protein 39 is specific of *S. citri*, but, according to the *S. citri* strain considered [1], it can have different electrophoretic mobilities as is the case for the two strains used in the experiment of Fig. 2.

Preparation and Characterization of Monospecific Antibodies Against Individual Spiroplasma Proteins

MCAs can provide monospecific antibodies against individual spiroplasma proteins (see above). However, monospecific antibodies can also be prepared by using individual proteins purified by preparative two-dimensional electrophoresis [2]. These antibodies are monospecific in that they are direct against a single protein. They differ from MCAs in that MCAs consist of a single antibody type against a single determinant site. Monospecific antibodies prepared against a single protein may contain different antibody types and/or antibodies directed against different determinant sites of a single protein. We have prepared antisera in guinea pigs against *S. citri* R8A2 proteins (P) 25, 14b, and 39, respectively (for nomenclature, see [1]).

P25 is the *S. citri* actin-like protein [13]. By IAC, P25 antibodies recognized not only P25 of *S. citri* (Fig. 1A, track 2) but also a homologous protein from all other spiroplasmas tested, a homologous protein of *M. mycoides* (PG50), and one from *U. urealyticum* (serotype V). Hence, P25 is a highly conserved protein, very probably common to all spiroplasmas and perhaps to all Mollicutes.

Antibodies against P14b recognized in IAC a homologous protein present in all spiroplasmas tested, but not in *M. mycoides*. Thus, P14b could be specific for spiroplasmas only.

Finally, antibodies to P39 recognized in IAC (Fig. 1A, track 3) a protein specific to all *S. citri* strains. When used in ELISA, antibodies against P39 detected only *S. citri* strains (subgroup I-1), but not the honeybee spiroplasma BC3 (subgroup I-2). Serological tests performed with complete immune serum always indicate relatedness between *S. citri* and BC3 [15].

The monospecific antibodies against protein P39 as well as one of the *S. citri* specific MCAs have been used to characterize a *S. citri* variant (A3) growing well at 37°C and deriving from *S. citri* strain R8A2 [16]. One- and two-dimensional protein analysis showed that the *S. citri*-A3 variant lacked P39 but possessed instead a new protein (P39) absent from *S. citri*-R8A2 and having a slightly higher molecular weight than P39. In IAC, the P39 protein of *S. citri*-A3 is retained by the monospecific antibodies against P39 and by the *S. citri* specific MCAs (Fig. 1C, tracks 4 and 2, respectively). Thus, although protein P39 of *S. citri*-A3 is serologically related to P39, it has a different mobility as demonstrated in two-dimensional SDS-PAGE analysis [16].

DISCUSSION

Two-dimensional protein analysis were used to compare several spiroplasma strains of group IV. The results indicate that even though the various spiroplasma strains belonging to this group show some heterogeneity, they are more homogeneous than group I.

Two different techniques have been used to identify spiroplasma protein antigens. In the first, protein antigens were first purified by immunoaffinity chromatography (IAC), then identified by SDS-PAGE. Since the spiroplasma proteins were radioactively labeled from growing the organisms in a medium containing ¹⁴C amino acids, spiroplasma protein antigens, but not proteins from the culture medium adsorbed on the organisms, were detected by this technique. This made possible the unambiguously immunological identification of an *S. citri* actin-like protein [13].

In the second technique, spiroplasma proteins were first separated by SDS-PAGE, then antigens were identified by antibody binding to blot-transferred proteins. For routine work, the blot transfer technique is easier to use than IAC. It also yields better results with proteins that are poorly soluble in the experimental conditions used in IAC. P39 from *S. citri* is such a protein. However, the technique does not distinguish genuine spiroplasma proteins from proteins from the culture medium that are adsorbed on the organisms.

One or the other of the two techniques can be used to identify, in various spiroplasma strains, protein antigens that have been used as immunogens. These include, for example, monoclonal antibodies or antibodies prepared against individual protein purified by preparative two-dimensional electrophoresis.

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