Journal of Bacteriology, Sept. 1990, p. 5494–5496
0021-9193/90/095494-03$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 172, No. 9

Antigenic Relatedness between the Spiralins of *Spiroplasma citri* and *Spiroplasma melliferum*

AHMED ZAARIA, CATHERINE FONTENELLE, MICHEL LE HENAFF, AND HENRI WRÓBLEWSKI*

Laboratoire d'Immunochimie des Membranes Bactériennes, Université de Rennes I, Centre National de la Recherche Scientifique, Unité de Recherche Associée no. 256, Campus de Beaulieu, 35042 Rennes Cédex, France

Received 21 March 1990/Accepted 13 June 1990

Four spiralins were compared by rocket immunoelectrophoresis, quantitative immunoblotting techniques, and the spiroplasma deformation test with the use of antispiralin (polyclonal) monospecific antibodies. This investigation revealed that the spiralins of *Spiroplasma citri* and *S. melliferum* are antigenically related and that probably no more than two epitopes simultaneously saturable with antibodies are shared by the two proteins. One at least of these epitopes is accessible to antibodies on the spiroplasma cell surface.

Spiroplasmas are mollicutes (trivial name, mycoplasmas) characterized by a helical morphology and a peculiar type of motility combining rotations and flexings (4, 5). A 26-kilodalton acylated polypeptide called spiralin (18, 19) was isolated from the plasma membrane of four strains of *Spiroplasma citri* (18, 20), the causal agent of the citrus stubborn disease (4), and one strain of *S. melliferum* (20). The actual function of this protein is still unknown. However, since it accounts for more than 20% of the membrane total protein mass and is permanently synthesized by the cells, even after many subcultures in vitro, one may hypothesize that spiralin has an important function in the spiroplasma cell (18). Surprisingly, despite a high similarity between the amino acid compositions of *S. citri* and *S. melliferum* spiralins (20), these two proteins do not cross-react in two-dimensional immunoelectrophoresis (1, 2, 20). To explain this discrepancy, we compared the spiralins of two strains of *S. citri* and two strains of *S. melliferum* by rocket immunoelectrophoresis, immunoblotting techniques, and the spiroplasma cell deformation test.

S. citri R8A2, *S. citri* C189, *S. melliferum* B88, and *S. melliferum* BC3 were grown as previously described (20). Cells were harvested by centrifugation at 15,000 × *g* for 15 min at 4°C, dispersed into 50 mM Tris hydrochloride buffer (pH 7.5), and disrupted by ultrasonication at 20 kHz twice for 1 min each at 0°C. The membranes were sedimented by centrifugation at 38,000 × *g* for 1 h at 4°C, washed with 50 mM Tris hydrochloride buffer (pH 8.0), and depleted of the bulk of extrinsic proteins (17). Spiralin was purified under nondenaturing conditions from *S. citri* C189 and *S. melliferum* B88 by agarose suspension electrophoresis in the presence of sodium deoxycholate (18, 19). Antibodies against purified spiralins were raised in rabbits as described previously (20), and rocket immunoelectrophoresis was done by the method of Laurell (11) in 1% agarose gels in Veronal buffer (pH 8.6) (I = 0.03) containing 13 mM sodium deoxycholate or 0.5% SB_{12} (9).

After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (10), membrane polypeptides were electrophoretically transferred onto a nitrocellulose membrane (13) in 25 mM Tris–190 mM glycine buffer (pH 8.3) containing 20% methanol (vol/vol). The electrophoresis was run for 3 h at 100 V and 4°C. The transferred polypeptides were immunolabeled as described previously (7). The primary antibodies were rabbit antimembrane or antispiralin sera (dilution 1:1,000). The secondary antibodies were goat immunoglobulin Gs labeled with peroxidase and directed against rabbit immunoglobulin Gs (dilution 1:5,000). 4-Chloro-1-naphthol was used as the enzyme substrate (7). To quantify the results, the nitrocellulose membranes were scanned by reflexion densitometry and the absorbances were converted to percent cross-reactivity. The data were normalized against the homologous reactions and corrected for the amount of loaded protein. Linearity was observed when absorbances were plotted versus amounts of spiralin in the range from 0 to 1 μg. Dot blotting (6) was performed on nitrocellulose membranes with a vacuum filtration manifold. Spiralin was selectively extracted with 0.1 M SB_{12} (3) in 50 mM Tris hydrochloride buffer (pH 8.0). Samples (50 μl containing 2 μg of spiralin) were applied to the membrane to allow protein adsorption. Labeling of spiralin and quantification of the results were done as described above.

Protein was determined by the method of Lowry et al. (12), with serum albumin as the standard. Specific titration of spiralin within complex protein mixtures was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and scanning densitometry after staining with Coomassie brilliant blue R-250, with purified spiralin as the standard.

When the four spiralins were analyzed by rocket immunoelectrophoresis with monospecific (polyclonal) antibodies (Fig. 1), the only cross-reactions that could be observed were those between proteins of the same spiroplasma species. Specifically, cross-reactions were obtained between spiralins of *S. citri* C189 and *S. citri* R8A2 on the one hand and between spiralins of *S. melliferum* B88 and *S. melliferum* BC3 on the other hand (Table 1). The results obtained in Western blotting (immunoblotting) with monospecific (polyclonal) antibodies (Fig. 2) clearly show that the antibodies used in the present study were actually monospecific since a single band was detected per strain out of more than 50 polypeptides present in each membrane. The strongest labels were obtained in homologous reactions and in heterologous reactions involving strains of the same species. However, labeling was also observed in heterologous reactions between strains of different species, which is evidence of shared epitopes in *S. citri* and *S. melliferum* spiralins. Quantitative analysis of the blots showed (Table 1) that the two spiralins of strains C189 and R8A2 of *S. citri* exhibited a

* Corresponding author.

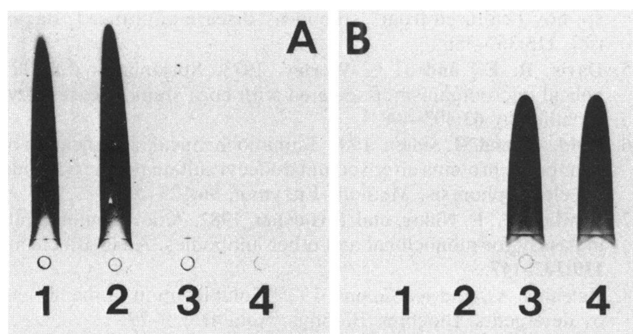

FIG. 1. Analysis of spiroplasma membranes by rocket immunoelectrophoresis. Antigens: well 1, *S. citri* C189; well 2, *S. citri* R8A2; well 3, *S. melliferum* B88; and well 4, *S. melliferum* BC3. The different wells contained 15 μg (gel A) or 5 μg (gel B) of proteins extracted with 0.1 M SB_{12} and 0.24 M 2-mercaptoethanol from membranes depleted of extrinsic proteins. Antibodies: gel A, anti-*S. citri* C189 membrane serum; gel B, anti-*S. melliferum* B88 membrane serum. Both sera were diluted 20 times in the gels. Electrophoresis was performed at room temperature under 50 V for 18 h in agarose gels (7 by 7 by 0.1 cm). The immunoprecipitates were stained with Coomassie brilliant blue R-250. The rockets corresponding to wells 1 and 2 in gel A and to wells 3 and 4 in gel B are spiralin immunoprecipitates.

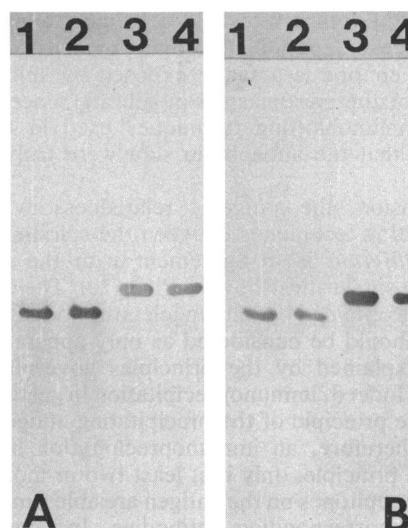

FIG. 2. Comparison of spiralins by Western blotting. The wells of the polyacrylamide gels were loaded with 20-μl samples of membrane proteins solubilized with 1% sodium dodecyl sulfate in the presence of 0.24 M 2-mercaptoethanol and containing 1 μg of spiralin: well 1, *S. citri* R8A2; well 2, *S. citri* C189; well 3, *S. melliferum* B88; and well 4, *S. melliferum* BC3. Electrophoresis was performed at 85 V for 10 h. Anode is at bottom on the figure. Specific labeling of spiralins was done with a serum containing antibodies directed against spiralin from *S. citri* C189 (blot A) and a serum containing antibodies directed against spiralin from *S. melliferum* B88 (blot B). The dilution of both sera was 1:1,000 in 1% serum albumin. Only one band, corresponding to spiralin, was visible in each lane.

high degree of similarity (80 ± 5%) and that the similarity between the spiralins of the two *S. melliferum* strains was still higher (94 ± 4%). With a range of 36 ± 4% to 47 ± 6%, the interspecies similarities were lower but nonetheless indicative of a high degree of relatedness. When analyzed by the dot-blotting technique with antispiralin antibodies (data not shown), the samples gave results similar to those obtained by Western blotting, except that the corresponding cross-reactivities were higher. Indeed, antigenic similarities of 92 ± 4% between spiralins of *S. citri* strains and 96 ± 6% between spiralins of *S. melliferum* strains were recorded (Table 1). The interspecies similarities ranged from 54 ± 4 to 59 ± 3%, indicating a degree of relatedness still higher than

TABLE 1. Antigenic properties of *S. citri* and *S. melliferum* spiralins

Properties	*S. citri*		*S. melliferum*	
	C189	R8A2	B88	BC3
Reactivity with antibodies against *S. citri* C189 spiralin				
Rocket immunoelectrophoresis[a]	+	+	−	−
Western blotting (%)[b]	100	80 ± 5	45 ± 2	36 ± 4
Dot blotting (%)[b]	100	92 ± 4	58 ± 6	58 ± 5
Cell deformation test[c]	320	320	40	40
Reactivity with antibodies against *S. melliferum* B88 spiralin				
Crossed immunoelectrophoresis[a]	−	−	+	+
Western blotting (%)[b]	46 ± 7	47 ± 6	100	94 ± 4
Dot blotting (%)[b]	54 ± 4	59 ± 3	100	96 ± 6
Cell deformation test[c]	40	40	160	160

[a] +, Formation of an immunoprecipitate; −, absence of immunoprecipitate.
[b] 100% is, by definition, the value attributed to the homologous reactions.
[c] Reciprocal of endpoint dilution at which at least one-half of the initially helical spiroplasma cells were deformed.

shown in Western blotting. The higher similarities recorded in dot-blotting are probably due to the fact that this technique was used under nondenaturing conditions, while Western blotting was performed after treatment of spiralin with sodium dodecyl sulfate, a highly denaturing surfactant (8). Though some degree of renaturation may occur during protein electrotransfer onto nitrocellulose membranes, it is highly improbable that full renaturation could be achieved under such conditions (14).

We have reported in a previous work that antispiralin antibodies play a prominent role (15) in the antibody deformation test of spiroplasmas (16). It was also shown that a monospecific serum containing antibodies directed against *S. citri* spiralin was capable of deforming and inhibiting the growth of *S. melliferum* cells, but with a lower activity. Two hypotheses were proposed to explain this observation: (i) the presence of shared epitopes in *S. citri* and *S. melliferum* spiralins and (ii) the presence in the antispiralin sera of antibodies directed against traces of highly immunogenic proteins contaminating spiralin preparations and common to both *S. citri* and *S. melliferum*. In the present study, the two antispiralin sera exhibited the same titer with a threshold deforming dilution of 1/320 in homologous reactions (Table 1). The same result was obtained in intraspecies cross-deformations, i.e., when *S. citri* R8A2 cells were treated with antibodies directed against spiralin of *S. citri* C189 and *S. melliferum* BC3 cells were treated with antibodies against spiralin of *S. melliferum* B88. In interspecies deformation tests (i.e., treatment of the two *S. citri* strains with antibodies directed against spiralin of *S. melliferum* B88 and treatment of the two *S. melliferum* strains with antibodies directed against spiralin of *S. citri* C189), cell deformations

were observed with a threshold deforming dilution of 1/40 (Table 1). These experiments provide evidence that at least one shared epitope is actually exposed by spiralin on the outer face of the spiroplasmal membrane since the highly sensitive immunoblotting techniques used in the present work show that the antispiralin sera were truly monospecific.

In conclusion, the antigenic relatedness evidenced by immunoblotting techniques between the spiralins of *S. citri* and *S. melliferum* is in agreement with the amino acid composition similarities shown earlier (20). The discrepancy in the results obtained by immunoelectrophoresis and immunoblotting should be considered as only apparent and may easily be explained by the principles governing the two techniques. Indeed, immunoprecipitation in gel techniques is based on the principle of the precipitating antigen-antibody network. Therefore, an immunoprecipitation line may be obtained, in principle, only if at least two or more probably three distinct epitopes on the antigen are able simultaneously to bind the corresponding antibodies. In contrast, since immunoblotting techniques are based on antibody binding by an antigen immobilized on a solid support (e.g., a nitrocellulose membrane), a positive signal may be obtained in such a case by the fixation of an antibody by a single epitope. Therefore, the results obtained for spiralin by the two serological approaches may be reconciled if we assume that probably no more than two distinct epitopes capable of simultaneously binding antibodies are shared by *S. citri* and *S. melliferum* spiralins.

This work was supported by the Ministère de l'Education Nationale (Direction de la Recherche, Allocation Spécifique), the Région Bretagne, and the Langlois Foundation.

Thanks are due to Dennis Webb for critically reading the manuscript and to Marie-Madeleine Gaillard for typing the manuscript.

LITERATURE CITED

1. **Archer, D. B., and J. Best.** 1980. Serological relatedness of spiroplasmas estimated by enzyme-linked immunosorbent assay and crossed immunoelectrophoresis. J. Gen. Microbiol. **119:** 413–422.
2. **Archer, D. B., and R. Townsend.** 1981. Immunoelectrophoretic separation of spiroplasma antigens. J. Gen. Microbiol. **123:**61–68.
3. **Blanchard, A., G. Baffet, and H. Wróblewski.** 1985. Highly selective extraction of spiralin from the *Spiroplasma citri* cell membrane with alkyl-*N*-sulfobetaines. Biochimie **67:**1251–1256.
4. **Cole, R. M., J. G. Tully, T. J. Popkin, and J. M. Bové.** 1973. Morphology, ultrastructure, and bacteriophage infection of the helical mycoplasma-like organism (*Spiroplasma citri* gen. nov., sp. nov.) cultured from "stubborn" disease of citrus. J. Bacteriol. **115:**367–386.
5. **Davis, R. E., and J. F. Worley.** 1973. Spiroplasma: motile, helical microorganism associated with corn stunt disease. Phytopathology **63:**403–408.
6. **Haid, A., and M. Suissa.** 1983. Immunochemical identification of membrane proteins after sodium dodecyl sulfate polyacrylamide gel electrophoresis. Methods Enzymol. **96:**125–205.
7. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119:**142–147.
8. **Helenius, A., and K. Simons.** 1975. Solubilization of membrane by detergents. Biochim. Biophys. Acta **415:**29–79.
9. **Johansson, K.-E., and H. Wróblewski.** 1983. Characterization of membrane proteins by crossed immunoelectrophoresis. p. 257–267. *In* J. G. Tully and S. Razin (ed.), Methods in mycoplasmology, vol. 1. Academic Press, Inc., Orlando, Fla.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
11. **Laurell, C. B.** 1965. Antigen-antibody crossed immunoelectrophoresis. Anal. Biochem. **10:**358–361.
12. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
13. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
14. **Wedege, E., K. Bryn, and L. O. Froholm.** 1988. Restoration of antibody binding to blotted meningococcal outer membrane proteins using various detergents. J. Immunol. Methods **113:**51–59.
15. **Whitcomb, R. F., J. G. Tully, and H. Wróblewski.** 1983. Spiralin: major membrane protein specific for subgroup I-1 spiroplasmas. Curr. Microbiol. **9:**7–12.
16. **Williamson, D. L., R. F. Whitcomb, and J. G. Tully.** 1978. The spiroplasma deformation test, a new serological method. Curr. Microbiol. **1:**203–207.
17. **Wróblewski, H., R. Burlot, and D. Thomas.** 1981. Adsorption of proteins from the *Spiroplasma citri* cell membrane by magnesium lauroyl-sarcosinate crystals. Biochimie **63:**177–186.
18. **Wróblewski, H., K.-E. Johansson, and S. Hjertén.** 1977. Purification and characterization of spiralin, the main protein of the *Spiroplasma citri* membrane. Biochim. Biophys. Acta **465:**275–289.
19. **Wróblewski, H., S. Nyström, A. Blanchard, and Å Wieslander.** 1989. Topology and acylation of spiralin. J. Bacteriol. **171:**5039–5047.
20. **Wróblewski, H., D. Robic, D. Thomas, and A. Blanchard.** 1984. Comparison of the amino acid compositions and antigenic properties of spiralins purified from the plasma membranes of different spiroplasmas. Ann. Microbiol. (Paris) **135A:**73–82.